Mild hypothermia protects against early brain injury in rats following subarachnoid hemorrhage via the TrkB/ERK/CREB signaling pathway

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Abstract. Subarachnoid hemorrhage (SAH) is a severe neurological disease, which is associated with a significant number of cases of premature mortality and disability worldwide. Mild hypothermia (MH) has been proposed as a potential therapeutic strategy to reduce neuronal injury following SAH. The present study aimed to investigate the mechanisms of MH's protective role in the process of SAH. The present study demonstrated that MH was able to protect against early brain injury in a rat model of SAH. Treating SAH rats with MH reduced the release of reactive oxygen species and prevented activation of apoptotic cascades. Furthermore, the protective effects of MH were shown to be mediated by enhanced activity of the tropomyosin receptor kinase B/extracellular signal-regulated kinases/cAMP response element binding protein (TrkB/ERK/CREB) pathway. Inhibition of TrkB/ERK/CREB activity using a small molecule inhibitor largely abolished the beneficial effects of MH in SAH rats. These results outline an endogenous mechanism underlying the neuroprotective effects of MH in SAH.

Introduction

Subarachnoid hemorrhage (SAH) is a severe neurological emergency, which accounts for 5% of all strokes (1). Although the diagnosis and treatment of SAH have improved in the past 20 years, SAH remains one of the most life-threatening acute neurological diseases, and is associated with a high rate of mortality and a poor prognosis. The primary cause of mortality in patients with SAH is brain injury that occurs during the early phase of SAH (within 48 h of SAH) (2), which is characterized by an initial sudden increase in intracranial pressure (ICP) and reduction in cerebral blood flow (CBF) (3). These complications trigger focal or global cerebral ischemia with various deleterious effects, including inflammation and neuronal cell death.

Due to the difficulties in predicting and preventing the occurrence of SAH, the management of SAH primarily focuses on protecting the brain from secondary damages during the acute and chronic phases that follow SAH. Mild hypothermia (MH) is well known for its powerful neuroprotective effects against neuronal injury following ischemia and traumatic brain injury, including stroke (4). Several mechanisms have been proposed to underlie the protective effects of MH. For example, MH has been reported to reduce mitochondrial dysfunction and per-ischemic production of reactive oxygen species (ROS) following ischemic disorders, which is believed to be one of the major causes of cell death and inflammation post-ischemia (5,6). Furthermore, MH treatment has been demonstrated to reduce global brain glucose and oxygen metabolic rates (7,8), which may help the brain to deal with energy failure, and prevent mitochondrial dysfunction and neuronal apoptosis. Other studies have suggested that MH treatment stabilizes the blood-brain barrier (9), reduces brain edema (10) and the release of excitatory amino acids (11,12), and attenuates inflammatory reactions (13) and lipid peroxidation (14).

Due to the well-documented protective effects of MH against ischemia and traumatic brain injury, MH has also been investigated as a potential therapeutic strategy for the treatment of SAH in humans and in animal models of SAH. Experimental studies in patients with SAH and animal models of SAH have suggested that MH treatment may improve ICP control (15), facilitate the resolution of cerebral vasospasm through modulation of blood flow velocity (16,17), and prevent neuronal damage and apoptosis caused by cerebral ischemia. However, little is currently known regarding the molecular mechanisms and signaling pathways associated with the effects of MH on protection against early brain injury following SAH.

Neurotrophic factors are required for the growth and survival of developing neurons and the maintenance of mature neurons. It has previously been reported that the tropomyosin receptor kinase B (TrkB)-mediated neurotrophic pathway has an important role in neuronal survival following ischemic

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stroke (18,19). Pharmacological activation of TrkB-cAMP response element binding protein (CREB) may ameliorate ischemic neuronal injury via the prevention of neuronal apoptosis, and therefore may improve functional recovery following stroke (18,19).

The present study aimed to determine the effects and mechanisms of MH on SAH development. The present study demonstrated that treatment with MH induced strong protective effects against neuronal injury in a rat model of SAH. Rats treated with MH exhibited a marked reduction in ROS production and caspase-3 activation following SAH. Furthermore, the TrkB/extracellular signal-regulated kinases (ERK)/CREB pathway mediated the protective effects of MH. Suppression of the TrkB/ERK/CREB pathway using an ERK inhibitor markedly abrogated the protective effects of MH in SAH rats. These findings indicated that activation of the TrkB/ERK/CREB pathway may be an essential mechanism underlying the protective effects of MH against early brain injury following SAH *in vivo*.

Materials and methods

Animal study. All experiments were approved by the Animal Care Committee at Harbin Medical University (Harbin, China). Male Wistar rats (weight, 200-250 g; n=45) were housed in a temperature and humidity-controlled environment under a 12:12-h light/dark cycle (light phase, 7:00 a.m.-7:00 p.m.), and were given *ad libitum* access to food and water. The rat brain tissues were surgically removed following overdose with 5% isoflurane.

Rat model of SAH. The SAH model was established in male rats as described previously (20-22). Briefly, the rats were anesthetized. A sharpened 3-0 monofilament was introduced into the right internal carotid artery through the external carotid artery until resistance was felt (10-12 mm from the common carotid bifurcation). The monofilament was subsequently pushed further to perforate the bifurcation of the internal carotid artery, and was then withdrawn immediately. In the sham group, the monofilament was inserted into the carotid artery; however, no perforation was performed. Following removal of the monofilament, the incision was closed. Endovascular occlusion by perforation lasted <5 min in each animal.

MH treatment. SAH was induced in 25 rats (except for 2 rats that had succumbed and the 3 which were excluded due to low weight, 30 rats purchased in total). Then, 5 rats were randomly selected and sacrificed at five different time points (0, 0.5, 4, 24 and 72 h) after induction.

A total of 20 rats (except for rats that had succumbed or did not qualify) were randomly divided into four groups: Sham, SAH, SAH + MH and SAH + MH + PD98059 groups. MH was conducted for 120 min as previously described (16), commencing 60 min after SAH. PD98059 (5 mg/kg/day dissolved in 0.2 ml dimethyl sulfoxide; New England Biolabs, Ipswich, MA, USA) was administered intravenously 0.5 h prior to SAH, in order to inhibit TrkB/ERK signaling as described previously (23). Mice were sacrificed 3 days after SAH for further evaluation.

Measurement of body weight and brain water content. The body weight and brain water content were measured on the third day following establishment of the SAH model. For brain water content, the brain tissues were removed, and the hemispheres were separated and weighed to assess their wet weight. After the wet weight of the brain tissues was quantified, the hemispheres were desiccated for 36 h at 110˚C, until the weight was constant. Hemispheric water content (%) was calculated as follows: (Wet weight - dried weight) / wet weight x 100%.

ROS detection. Intracellular ROS levels were determined in brain tissue homogenates using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Beijing, China) according to manufacturer's protocol. Briefly, the rat brain extracts were incubated with DCFH-DA at 37˚C in the dark for 30 min. The fluorescence intensity was then quantified using a multi-detection microplate reader at 485 nm excitation and 530 nm emission wavelengths.

Caspase-3 activity assay. Caspase-3 activity was determined using a CaspACE assay system (Promega Corporation, Madison, WI, USA) was conducted according to the manufacturer's protocol. Rat brain tissues were lysed on ice for 30 min and were centrifuged at 12,000 x *g* for 15 min at 4˚C. The levels of caspase-3 were expressed relative to the amounts in the control group. The caspase-3 activities in the supernatant were measured at 405 nm.

Western blot analysis. The rat brains were surgically collected and cut into pieces. Subsequently, the brain tissues were directly homogenized in ice-cold lysis buffer [62.5 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (w/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] for 30 min. The lysates were then centrifuged for 15 min at 12,000 x *g* at 4˚C, and the resulting supernatants were collected and boiled. Protein concentrations were measured in the extracts using a bichinchoninic acid assay. Protein samples (20-40 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then blocked overnight with 5% bovine serum albumin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in Tris-buffered saline containing 0.1% Tween-20 (TBST), and were incubated with antibodies against cleaved caspase-3 (cat. no. ab2302; 1:1,000; Abcam, Cambridge, UK), phosphorylated (p)-TrkB (sc-7987, 1: 500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), TrkB (cat. no. sc-377218; 1:1,000; Santa Cruz Biotechnology, Inc.), p-ERK1/2 (cat. no. sc-136521; 1:1,000; Santa Cruz Biotechnology, Inc.), ERK1/2 (cat. no. sc-292838; 1:1,000; Santa Cruz Biotechnology, Inc.) p-CREB (cat. no. sc-7978, 1:1,000; Santa Cruz Biotechnology, Inc.), CREB (cat. no. sc-377154; 1:1,000; Santa Cruz Biotechnology, Inc.) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. sc-47724; 1:1,000; Santa Cruz Biotechnology, Inc.) at 4˚C overnight, followed by washing with TBST. The membranes were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). The blots were detected using an Enhanced Chemiluminescence Plus

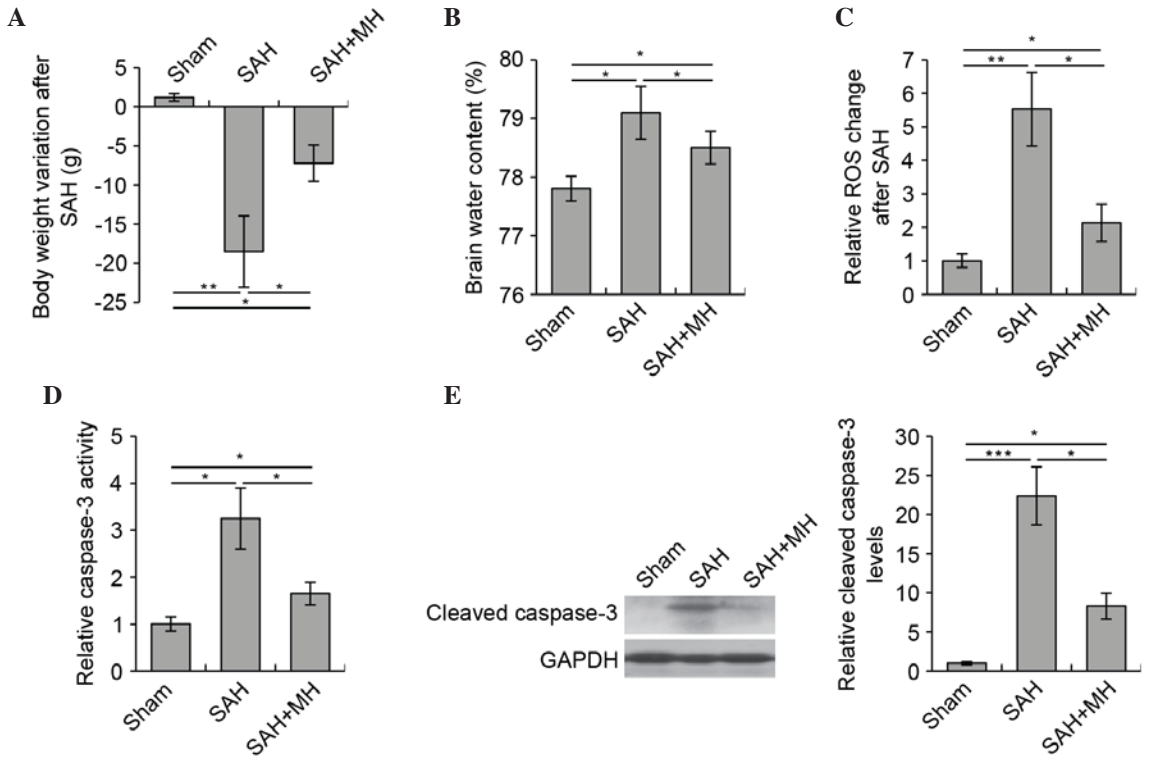

Figure 1. Mild hypothermia (MH) protects against early brain injury in a rat model of subarachnoid hemorrhage (SAH). (A) Body weight of rats in sham, SAH and SAH + MH groups 3 days after SAH. (B) Brain water content, (C) relative reactive oxygen species (ROS) release and (D) relative caspase-3 activity in sham, SAH and SAH + MH groups 3 days after SAH. (E) Western blot analysis of cleaved caspase-3 and quantification (right). *P<0.05; **P<0.01; ***P<0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

reagent kit (Wuhan Boster Biological Technology Co., Ltd.). GAPDH was used as a loading control. The bands were quantified using Image J version 1.37 software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining. The brain tissues were fixed in 4% paraformaldehyde, and were sliced using a cryostat. The sections (20 μm) were stored in anti-freeze solution (15% glucose and 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) at -20˚C and were used for immunofluorescence staining. Frozen sections were incubated overnight at 4˚C with primary antibodies against cleaved caspase-3 (cat. no. ab2302; 1:100; Abcam) and p-CREB (cat. no. sc-7987; 1:100; Santa Cruz Biotechnology, Inc.). The slices were then washed three times with phosphate-buffered saline and were incubated with the corresponding fluorescence dye-conjugated bovine anti-rabbit IgG-FITC (cat. no. sc-2365; 1:100; Santa Cruz Biotechnology, Inc.) and donkey anti-goat IgG-TR (cat. no. sc-2783; 1:100; Santa Cruz Biotechnology, Inc.) secondary antibodies for 2 h. After being washed and counterstained with 4',6-diamidino-2-phenylindole, immunofluorescence was observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. All data are presented as the mean ± standard error of the mean. Group differences were analyzed using one-way analysis of variance followed by Tukey's honest significant difference test. Statistical analyses were conducted using GraphPad Prism version 5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

MH protects against early brain injury in rats following SAH. To investigate the protective function of MH in SAH, a rat model of SAH was generated, and the rats were treated with MH 1 h after the model was established. Consistent with previous observations regarding this model (20-22), the induction of SAH reduced body weight and increased brain water content in the rats. These detrimental effects caused by SAH were significantly attenuated by MH, as indicated by the reduction in body weight loss (Fig. 1A) and brain water accumulation (Fig. 1B). Given that mitochondrial dysfunction and the activation of apoptotic cascades are key pathological events in early brain injury following SAH (24,25), the present study investigated whether MH was able to protect neurons from mitochondrial dysfunction and apoptosis in early brain injury. The results clearly demonstrated that MH improved mitochondrial function following SAH, as evidenced by the reduction in ROS production (Fig. 1C). Furthermore, the activation of caspase-3 was markedly reduced by MH (Fig. 1D and E). These data indicate that MH treatment in the early phase of SAH may reduce ROS release and neuronal apoptosis, thus improving the outcome of SAH *in vivo*.

MH promotes TrkB/ERK/CREB signaling. The present study aimed to determine the molecular mechanism underlying

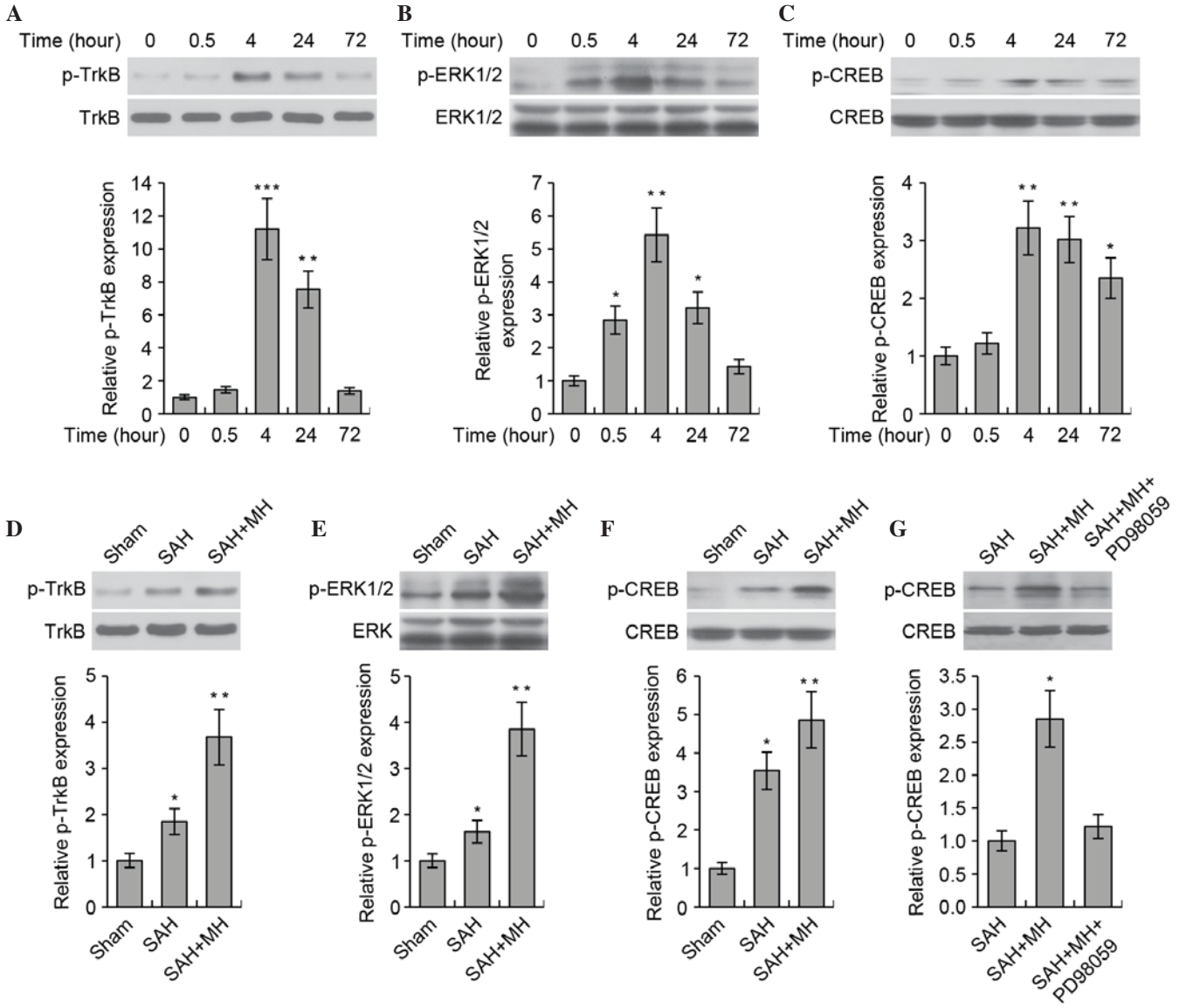

Figure 2. Mild hypothermia (MH) promotes activation of the tropomyosin receptor kinase B/extracellular signal-regulated kinases/cAMP response element binding protein (TrkB/ERK/CREB) pathway. Western blot analysis of (A) phosphorylated (p)-TrkB, (B) p-ERK1/2 and (C) p-CREB expression in rat brains following induction of subarachnoid hemorrhage (SAH), at various time points (0, 0.5, 4, 24 and 72 h). Western blot analysis of (D) p-TrkB, (E) p-ERK1/2 and (F) p-CREB expression in rat brains 72 h post-SAH induction, with or without MH treatment. (G) p-CREB expression in rat brains following SAH, and treatment with MH or MH + PD98059. *P<0.05; **P<0.01; ***P<0.001 vs. sham or 0 h group.

the protective effects of MH on SAH. The TrkB-mediated neurotrophic pathway has critical roles in neuronal survival and growth. Previous studies have demonstrated that MH was able to induce the expression of brain-derived neurotrophic factor (BDNF) in rat brains following cerebral ischemia; BDNF functions as a ligand of the TrkB receptor (26,27). Therefore, the present study hypothesized that MH would prevent neuronal injury via activation of the TrkB-mediated neurotrophic pathway. The activity of TrkB, and the downstream ERK1/2/CREB pathway, was analyzed at various time points (0, 0.5, 4, 24 and 72 h) following SAH. SAH markedly stimulated TrkB/ERK/CREB signaling (Fig. 2A-C). The levels of p-TrkB, p-ERK1/2 and p-CREB in the rat brain peaked at 4 h, and then decreased gradually. Treatment with MH markedly enhanced the phosphorylation of TrkB (Fig. 2D), ERK1/2 (Fig. 2E) and CREB (Fig. 2F) following SAH. Furthermore, MH-induced phosphorylation of CREB was shown to be dependent on the activation of TrkB and ERK, since inhibition of ERK using the small molecule inhibitor PD98059 markedly abrogated CREB phosphorylation (Fig. 2G). These data indicate that the TrkB/ERK/CREB signaling pathway may be involved in the progress of SAH, and that MH promotes activation of the TrkB/ERK/CREB pathway following SAH.

Inhibition of the TrkB/ERK/CREB signaling pathway reduces the protective effects of MH in SAH-induced early brain injury. To examine whether the TrkB/ERK/CREB signaling pathway is required for the protective effects of MH, the effects of MH on early brain injury were detected following inhibition of the TrkB/ERK/CREB pathway using PD98059. Notably, inhibition of the TrkB/ERK/CREB pathway almost completely abrogated the beneficial effects of MH on SAH. The reductions in body weight loss and brain water content were reversed by PD98059. There was no significant difference in body weight

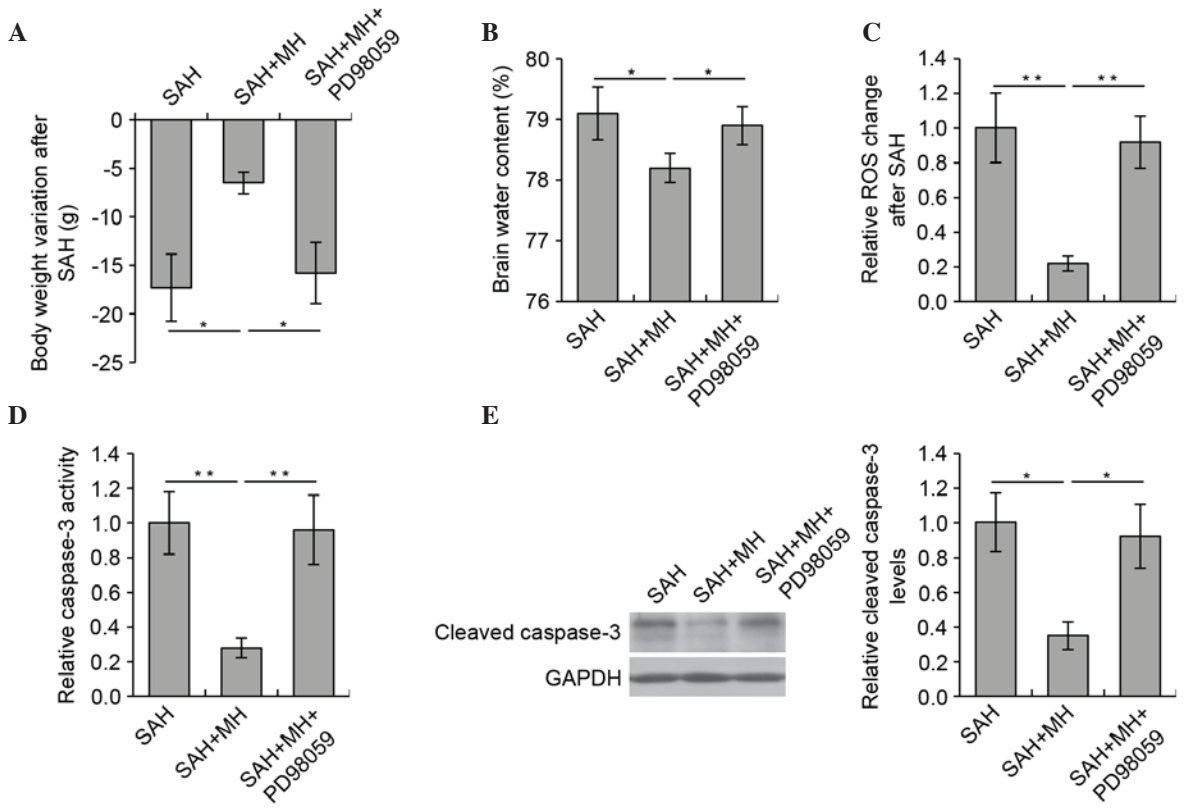

Figure 3. Inhibition of the tropomyosin receptor kinase B/extracellular signal-regulated kinases/cAMP response element binding protein (TrkB/ERK/CREB) pathway reduces the protective effects of mild hypothermia (MH) on early brain injury following subarachnoid hemorrhage (SAH). (A) Body weight of SAH rats treated with MH or MH + PD98059. (B) Brain water content, (C) relative reactive oxygen species (ROS) release and (D) relative caspase-3 activity in the brains of SAH rats treated with MH or MH + PD98059. (E) Western blot analysis of cleaved caspase-3 levels and quantification (right). *P<0.05; **P<0.01. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

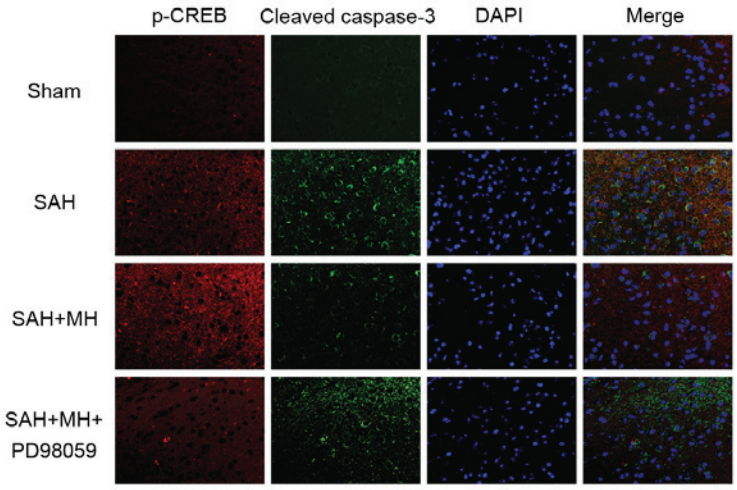

Figure 4. Immunohistochemical staining of phosphorylated-cAMP response element binding protein (p-CREB) and cleaved caspase-3. Immunofluorescent staining of p-CREB (red) and cleaved caspase-3 (green) in rat brain sections; 4',6-diamidino-2-phenylindole (DAPI; blue) was used for nuclear staining. Magnification, x 400. SAH, subarachnoid hemorrhage; MH, mild hypothermia.

(Fig. 3A) and brain water content (Fig. 3B) between the SAH and SAH + MH + PD98059 groups. Treatment with PD98059 also abrogated the effects of MH on the prevention of ROS production (Fig. 3C) and caspase-3 activation (Fig. 3D) following SAH.

To determine the role of CREB in SAH and MH-treated SAH, fluorescence staining of p-CREB and cleaved caspase-3 was performed on brain sections. SAH induced extensive cell apoptosis and promoted CREB phosphorylation (Fig. 4). In addition, MH increased the levels of p-CREB, and decreased the levels of cleaved caspase-3. These effects were abrogated by inhibition of TrkB/ERK signaling. These results indicate that MH may protect the brain from SAH-induced neuronal injury by activating the TrkB/ERK/CREB signaling pathway.

Discussion

MH has been revealed to be effective in minimizing neuronal damage and improving the functional outcome of SAH; however, the molecular mechanisms underlying the beneficial effects of MG remain unclear. Using a rat SAH perforation model, the present study demonstrated that MH was able to attenuate mitochondrial dysfunction and activation of apoptosis in the brain following the induction of SAH. These protective effects were mediated by enhanced activity of the TrkB/ERK/CREB pathway. These observations identify a potential mechanism by which MH protects rats from early brain damage following SAH.

Chronic post-SAH pathological consequences, characterized by delayed cerebral ischemia and vasospasm of the major cerebral arteries (3-7 days after SAH), have been extensively studied and treated; however, these efforts have not resulted in an effective treatment to prevent or ameliorate brain injury following SAH (28,29). The early brain injury that occurs within 48 h of SAH has gained more attention as a novel target for improving SAH patient outcome, since >60% of patients with SAH succumb due to early brain injury during the first 48 h after SAH (30,31). In addition, the majority of chronic secondary injuries are initiated by early brain injury. It has previously been indicated that mitochondrial dysfunction and extensive neuronal apoptosis are the key events associated with early brain injury following SAH (32). Following SAH-induced global ischemia, apoptosis has been observed in several regions of the brain, including the hippocampus, blood-brain barrier (BBB) and vasculature (24). Activation of apoptotic cascades may lead to severe pathological complications, including BBB disruption (33) and vasospasm (34). Therefore, anti-apoptosis may be considered a potential therapeutic intervention for the treatment of SAH.

MH is a neuroprotective approach that has been employed in various clinical scenarios, particularly in the treatment of ischemic stroke and traumatic brain injury. Based on the pathophysiological similarity between stroke and SAH, MH has been tested as a potential therapy for SAH in humans and animal models of SAH. Several studies have indicated that MH may reduce ICP and improve CBF in the early phase of SAH, and minimize the detrimental effects of delayed cerebral ischemia and vasospasm in the chronic secondary injuries following SAH, thus improving the functional outcome in patients with SAH (35,36). Although the exact molecular mechanisms underlying the protective effects of MH on SAH are largely unknown, it is generally believed that MH protects against neuronal damage via several mechanisms. The present study demonstrated that MH reduced ROS generation and activation of apoptotic cascades during the early phase following SAH. These results suggested that MH may reduce neuronal damage, at least partially, through improving mitochondrial function and promoting neuronal survival.

BDNF/TrkB signaling is critical for neuronal survival, morphogenesis and plasticity. It is well-known that activation of the TrkB receptor elicits various intracellular signaling pathways, including the mitogen-activated protein kinase/ERK pathway, the phosphoinositide 3-kinase pathway, and CREB transcription (37). All of these pathways have been reported to participate in the regulation of neuronal growth and survival (37). Beyond its physiological function, pharmaceutical activation of the TrkB pathway promotes neuronal survival following ischemic brain injury (19,38). The present study established a previously unappreciated link between MH and the neurotrophic pathway. MH enhanced activity of the TrkB/ERK/CREB pathway *in vivo* in a rat model of SAH. Notably, the TrkB/ERK/CREB pathway is essential for the neuroprotective effects of MH on SAH, since inhibition of this pathway using a small molecule inhibitor almost fully abolished the beneficial effects of MH. These results indicated a novel protective mechanism for MH in the context of early brain injury following SAH. Based on these findings, MH may reduce neuronal loss not only through inhibiting cell death activators (such as c-Jun N-terminal kinase) (39) but also through enhancing pro-survival signaling pathways. Notably, studies in other animal models of ischemic brain injury have suggested that treatment with MH could induce BDNF expression in the hippocampus (26,27), which may be a potential mechanism that explains how MH treatment activates the TrkB/ERK/CREB pathway. The phosphorylated TrkB, ERK and CREB were stumilated by SAH and reached peak at 4 h after hemorrhage. The levels of phosphorylated protein then decreased due to short half-lives. Downstream target genes were then elevated (40-42).

In conclusion, using a rat model of SAH, the present study demonstrated that MH ameliorates early brain injury through the prevention of mitochondrial dysfunction and inhibition of apoptotic cascades following SAH. The beneficial effects of MH are largely dependent on activation of the TrkB/ERK/CREB pathway. In the past few decades, early brain injury has evolved to be a promising therapeutic target for SAH. The present study indicated that MH is an effective strategy that may be used to reduce neuronal damage in the early phase of SAH. This mechanistic study of MH action in SAH revealed that the TrkB/ERK/CREB pathway may represent a novel therapeutic target for the intervention of early brain injury following SAH.

Acknowledgements

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