TAK1 inhibition attenuates early brain injury after SAH

TGFB-activated kinase 1 (TAK1) inhibition by 5Z-7-oxozeaenol attenuates early brain injury after experimental subarachnoid hemorrhage

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Key words: Brain; subarachnoid hemorrhage; apoptosis; MAPK; NF-kB; neuroprotection; TAK1; 5Z-7-oxozeaenol
Background: Role of TGFβ-activated kinase 1 (TAK1) in the pathogenesis of early brain injury after subarachnoid hemorrhage (SAH) has not been reported.

Results: TAK1 inhibition attenuates early brain injury and improves neurological deficits after SAH.

Conclusion: TAK1 inhibition exhibits neuroprotective effects possibly through anti-apoptotic function.

Significance: These results provide a novel target for SAH treatment.

ABSTRACT

Accumulating evidence suggests that activation of mitogen-activated protein kinases (MAPKs) and nuclear factor NF-κB exacerbates early brain injury (EBI) following subarachnoid hemorrhage (SAH) by provoking pro-apoptotic and pro-inflammatory cellular signaling. Here we evaluate the role of TGFβ-activated kinase 1 (TAK1), a critical regulator of the NF-κB and MAPK pathways, in the early brain injury following SAH. Although the expression level of TAK1 did not present significant alternation in the basal temporal lobe after SAH, the expression of phosphorylated TAK1 (Thr187, p-TAK1) showed substantial increase 24 h post SAH. Intracerebroventricular injection of a selective TAK1 inhibitor (10 min post-SAH), 5Z-7-oxozeaenol (OZ), significantly reduced the levels of TAK1 and p-TAK1 at 24 h post SAH. Involvement of MAPKs and NF-κB signaling pathways was revealed that OZ inhibited SAH-induced phosphorylation of p38 and JNK, the nuclear translocation of NF-κB p65, and degradation of IκBα. Further, OZ administration diminished the SAH-induced apoptosis and EBI. As a result, neurological deficits caused by SAH were reversed. Our findings suggest that TAK1 inhibition confers marked neuroprotection against EBI following SAH. Therefore, TAK1 might be a promising new molecular target for the treatment of SAH.
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TAK1 is a member of the mitogen-activated protein kinase (MAPK) kinase kinase family and was initially found to function in TGF-β-mediated MAPK activation (7). Previous study demonstrated that TGF-β is a fibrogenic factor involved in the etiology of post-SAH pathology and plays an important role in generating communicating hydrocephalus after SAH (8,9). Several other known stimuli of TAK1-signaling, including IL-1 β, TNF-α and TLR4 have also been shown to be activated and to play a detrimental role in SAH (10-12). The activated TAK1 in turn activates the NF-κB and MAPK pathways (13,14). Recent investigations also demonstrate that inhibition of TAK1 provides neuroprotection in ischemia and traumatic brain injury (15-17). Taking into account all this background, the present study aimed to evaluate whether TAK1 is activated after SAH. Moreover, the possible role of TAK1 in the regulation of SAH-induced neuronal apoptosis was also analyzed by means of TAK1 pharmacological modulation with its specific inhibitor 5Z-7-oxozeaenol (OZ).

EXPERIMENTAL PROCEDURES

Animals and subarachnoid hemorrhage model-
The male Sprague-Dawley rats weighing 300–350 g were used in this study. Rats were housed in a reversed 12-h light/12-h dark cycle controlled environment with free access to food and water. All procedures were approved by Nanjing University Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health (NIH).

Experimental SAH was performed as described previously in our laboratory and other laboratories (18-21). First, the rats were intraperitoneally anesthetized with 10% chloral hydrate (400 mg/kg body weight) and placed in a stereotaxic head frame. An insulin injection needle (BD Science) was tilted 45° in the sagittal plane, and placed 8 mm anterior to bregma in the midline, with the hole facing the right side. It was lowered until the tip reached the base of the skull, 2-3 mm anterior to the chiasma (approximately 10-12 mm from the brain surface) and retracted 0.5 mm. Loss of cerebrospinal fluid and bleeding from the midline vessels were prevented by plugging the burr hole with bone wax before inserting the needle. A total of 300 μl non-heparinized fresh autologous arterial blood was slowly injected into the prechiasmatic cistern for 3 min under aseptic technique. The heart rate was monitored and the rectal temperature was kept at 37 ± 0.5 °C by using physical cooling (ice bag) when required throughout experiments. Arterial blood samples were analyzed intermittently to maintain pO2, pCO2, and pH, parameters within normal physiological ranges. To maintain fluid balance, all rats were supplemented with 2 ml of 0.9% NaCl administered subcutaneously. After recovering from anesthesia, rats were returned to their cages with free-access food and water provided ad libitum. In the present study we observed that the basal temporal lobe was always stained with blood as described before. Therefore, the brain tissue adjacent to the clotted blood was used for analysis in our study (Fig. 1D).

Experimental design 1 - A total of 64 rats were used in this experiment. Of them, six rats with SAH were excluded later from the study because of little blood in prechiasmatic cistern but lots of blood clot in the frontal lobe instead, and ten SAH rats died before the intended sacrifice. The animals were randomly assigned to six groups post SAH: animals surviving SAH for 2 h (n=6), 6 h (n=6), 12 h (n=6), 24 h (n=9), 48 h (n=6) and 72 h (n=6). Control (n=9) animals underwent the
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Experimental design 2 - To determine the role of TAK1 in SAH, rats were randomly allocated into the following groups and survived 24 h: 1) Control group (n=18): Control animals were injected with 0.3 ml saline. 2) SAH group (n=12); 3) SAH+vehicle group (n=18): rats were subjected to SAH plus intracerebroventricular (ICV) administration of 5 μl DMSO; 4) SAH+OZ group (n=18): rats were subjected to SAH plus intracerebroventricular administration of the TAK1 inhibitor OZ (Tocris Bioscience; 25 μg 10 min post SAH). This dose was based on our prior published work using OZ in traumatic brain injury model (15). Coordinates for the injection placement were 1.0 mm posterior to bregma, 1.4 mm lateral to midline, and 4.4 mm below the skull surface and the injection duration was 10 min. Following the behavioral test, brain tissue of half number of rats was harvested for biochemical and histopathological analysis at 24 h post SAH. A total of 85 rats were used in this experiment (Seven rats with SAH were excluded later from the study for the same reason mentioned in experiment 1, twelve SAH rats died before the intended sacrifice).

The neurological scoring was performed with a separate cohort of rats at 24 h and 72 h after surgery. Finally, the rats were sacrificed for histological analysis. A total of 56 rats were used in this experiment (Four rats with SAH score of 0 were excluded from the study for low SAH grade, eight SAH rats died before the intended sacrificed).

Experimental design 3 - To determine whether administration of the TAK1 inhibitor OZ confers brain protection with a wide therapeutic window against SAH, OZ or vehicle was administered 4 h post SAH. Neurological scores were evaluated at 24 and 72 h post SAH, and histological analysis was performed at 72 h. A total of 30 rats were used in this experiment (Three rats with SAH score of 0 were excluded from the study for low SAH grade at 24 h post SAH, six SAH rats died before the intended sacrifice).

Biochemical analysis - The whole cell protein extraction and nuclear fractions were prepared as previously described (15). For western analysis, 35 μg of total protein were loaded in each lane of SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 2 h to overnight at 4°C. The primary antibodies were against cleaved-caspase-3 (cat# 9661), p-ERK1/2 (cat# 4370P), p-JNK (cat# 4668P), p-c-Jun (cat# D47G9), p-P38 (cat# 4511), histone 3 (H3, cat# 9715), or β-actin (cat# 4967) (1:1000, from Cell Signaling Technology, Danvers, MA), interleukin (IL)-1β (cat# ab9722), occludin (cat# ab31721), p-TAK1(Ser439, cat# ab109404) and phospho-TAK1 (Thr187, cat# ab192443) (1:1000, Abcam, Cambridge, MA), TAK1 (cat# sc-7162), p65(cat# sc-372), IκBα (cat# sc-371), zonula occludens-1 (ZO-1, cat# sc-10804), and tumour necrosis factor (TNF)-α (cat# sc-52746), (1:200; from Santa Cruz Biotechnology, Santa Cruz, CA). Signal was detected with horse radish peroxidase-conjugated immunoglobulin G (IgG) using enhanced chemiluminescence detection reagents (Amersham International, Buckinghamshire, UK). Blot bands were quantified by densitometry with Image J software (NIH).
Histological examination - Rats were anesthetized and transcardially perfused with saline and 4% paraformaldehyde. The brains were removed and postfixed in paraformaldehyde for 24 h. For immunofluorescence microscopy, the brains were frozen in O.C.T. media, sectioned (10 μm), and mounted onto slides. Immunofluorescence staining was performed as described previously (15). The specificity of immunofluorescence reaction was evaluated by replacement of the primary antibody with rabbit IgG.

Terminal dUTP Nick-end Labeling (TUNEL) assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (In situ Cell Death Detection Kit- Roche Applied Science, Indianapolis, IN). In brief, each section was incubated with 50 μl TUNEL reaction mixture (contains 5 μl Enzyme solution and 45 μl Label Solution) for 1 h at 37°C in the dark and then washed with PBS. Slides were then counter-stained with 4,6-diamidino-2-phenylindole (DAPI), washed, cover slipped with a water-based mounting medium, and sealed with nail polish. For the negative control, the sections were only incubated with Label solution. The positive cells were identified, counted and analyzed under the light microscope by an investigator blinded to the grouping. The extent of brain damage was evaluated by the apoptotic index, defined as the average percentage of TUNEL-positive cells in each section counted in 10 cortical microscopic fields (at × 400 magnification). A total of four sections from each animal were used for quantification. The final average percentage of the four sections was regarded as the data for each sample.

Formalin-fixed brains (72 h post-SAH) were dehydrated, embedded in paraffin and sliced into 4 μm thick sections which were stained with Cresyl Violet. The sampled region for each subfield was demarcated in the inferior basal temporal lobe and cresyl-violet neuronal cell bodies were counted. To quantify the amount of Nissl staining, ten random high-power fields (400×) in each coronary section were chosen, and the mean number of intact neurons in the ten views was regarded as the data of each section. A total of four sections from each animal were used for quantification. The final average number of the four sections was regarded as the data for each sample.

Brain water content (Brain edema) - Brains were removed 24 h after surgery in experiment 2 and weighed immediately (wet weight) and reweighed after drying in 100°C for 72 h (dry weight). The percentage of water content was calculated as [(wet weight- dry weight)/wet weight] × 100%.

Neurologic scoring - Three behavioral activity examinations to record appetite, activity, and neurological deficits (Table 1) were performed by an investigator blinded to the study groups at 24 and 72 h after SAH using the scoring system reported previously (22,23).

Statistical analyses - Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey test was used to analyze differences between groups except for the neurobehavioral scores, which were analyzed with nonparametric tests (Kruskal-Wallis, followed by Dunn's post-hoc test). SPSS 16.0 was used for the statistical analysis (SPSS, Inc., Chicago, IL).
Differences were determined to be significant with p<0.05.

RESULTS

The mortality and general observation were recorded - No significant changes in body temperature or injected arterial blood gas data were detected in any of the experimental groups. Intracerebroventricular injection of vehicle or OZ did not significantly alter arterial blood gas and heart rate in rats. The mortality rate was 0% (0/41) in the control group and 18.6% (36/194) in the SAH group. The mortality among SAH, vehicle and OZ treatment groups was not significantly different in experimental design 2 and 3 (data not shown).

TAK1 was activated in rats post SAH - To determine whether TAK1 is activated after SAH, tissue extracts from basal temporal lobe were examined for the levels of TAK1, p-TAK1 (Thr187) and p-TAK1 (Ser439) by western blotting. One-way ANOVA analysis showed a significant difference in p-TAK1 (Thr187) expression among the seven groups (F=5.88, p=0.003). Unexpectedly, the level of total TAK1 and p-TAK1 (Ser439) kept constant after SAH (Fig. 1A and 1B, both p>0.05). However, the level of p-TAK1 (Thr187) showed substantial increase at 24 h (Fig. 1B, p<0.01) and 48 h (Fig. 1B , p<0.05) after SAH. Double immunofluorescence also demonstrated that TAK1 in the basal temporal lobe mainly co-localized with NeuN, a neuronal marker, without difference between control and SAH groups (Fig. 1C).

OZ reduced the levels of TAK1 and p-TAK1 in SAH rats - To evaluate the role of TAK1 in the early brain injury following SAH, TAK1 specific inhibitor OZ was used to treat SAH and control rats. OZ treatment did not affect levels of p-TAK1 and TAK1 in control rats as compared to normal rats (data not shown), which is in line with data from a previous study (17). Statistical significance was revealed in TAK1 (F=7.928, p=0.001) and p-TAK1 (F=17.5, p=0.001) expression among the four groups. OZ treatment significantly reduced the levels of TAK1 (Fig. 2A, p<0.01) and p-TAK1 (Thr187) (Fig. 2B, p<0.001) compared to the vehicle-treated rats. Double immunofluorescence staining further confirmed dramatic reduction of TAK1 by OZ administration in neuronal cells (Fig. 2C).

Neuronal apoptosis was attenuated by TAK1 inhibition - TAK1 inhibition was shown to play a neuroprotective effect in animal model of some diseases (15,17). Neuronal apoptosis is the main pathological process in EBI (1,24). Thus, TUNEL staining was performed and cleaved-caspase-3, an indicator in the early phase of apoptosis (25), was detected. The results revealed significant difference in apoptosis index (F = 43.5, p<0.001) and cleaved-caspase-3 expression (F = 6.849, p=0.002) among the groups. SAH increased the number of TUNEL positive cells in the basal temporal lobe at 24 h after SAH (Fig. 3A and 3B, p<0.001). Blockage of TAK1 decreased the amount of TUNEL positive cells in SAH rats compared to the untreated animals (Fig. 3A and 3B, p<0.01). Likewise, we found a significant increase of cleaved-caspase-3 in SAH and SAH+vehicle groups compared to the control group (Fig. 3C, p<0.05). Notably, OZ treatment significantly decreased the level of cleaved-caspase-3 when compared to the SAH+vehicle-treated samples (Fig. 3C, p<0.05).

TAK1 inhibition downregulated SAH-induced activation of MAPK signaling pathway - To investigate the cellular mechanisms by which treatment with OZ may attenuate the apoptosis, further terminate the development of early brain injury, we evaluated the activation of MAPK family such as p-JNK, p-ERK1/2, and p-p38 by
western blot. One-way ANOVA analysis revealed significant differences in p-p38 (F=15.34, p<0.001), p-ERK1/2 (F=4.745, p=0.012), p-JNK (F=4.369, p=0.016) and p-c-Jun (F=10.12, p<0.001) expression among the four groups. A significant increase in p-ERK1/2 (Fig. 4B, p<0.05) and p-p38 (Fig. 4C, p<0.001) expression was observed in SAH rats. Although the expression of p-JNK was found to increase in SAH group as compared to the control group, this difference was not statistically significant (Fig. 4A, p>0.05). Single-dose intracerebroventricular administration of OZ prevented the SAH-induced phosphorylation of JNK (Fig. 4A, p<0.05) and p38 (Fig. 4C, p<0.05), but not ERK (Fig. 4B, p>0.05). C-Jun, a downstream target for phosphorylation by JNK and an important factor in neuronal apoptosis induced by SAH, was evaluated. The result showed that OZ treatment reduced the level of the activated c-Jun (Fig. 4D, p<0.05), confirming the neuroprotective effect of OZ after SAH.

Effect of TAK1 inhibition on SAH-induced nuclear translocation of NF-κB p65 and degradation of IκBα protein – In addition to MAPK signaling pathways, NF-κB also plays a key role in the pathogenesis of early brain injury after SAH (6,26). To determine the effect of TAK1 inhibition on NF-κB signaling in EBI in this study, we investigated the effect of OZ on IκB, an inhibitor of NF-κB. Our results revealed significant differences in IκBα (F=13.4, p<0.001) and p65 (F=10.21, p<0.001) expression among the four groups. A basal level of IκBα was high in the basal temporal lobe from the rats of the control group, whereas it was substantially reduced in SAH rats and reversed in OZ treatment group (Fig. 5A), suggesting that OZ administration prevented the SAH-induced IκBα-degradation. Then, nuclear fractions were isolated from basal temporal lobe and probed for NF-κB subunit p65 via western blot. The results demonstrated that the level of p65 significantly increased at 24 h after SAH compared to those in the control group and returned to the basal level after OZ treatment (Fig. 5B). To further analyze the downstream of NF-κB and MAPKs signaling, the levels of TNF-α and IL-1β were also measured. Likewise, statistically significant difference was revealed in TNF-α (F=4.97, p=0.009) and IL-1β (F=4.852, p=0.011) expression among the four groups. The levels of both IL-1β (Fig. 6A, p<0.05) and TNF-α (Fig. 6B, p<0.05) increased following SAH as has been reported previously (18). However, TAK1 inhibition had no effect on TNF-α and IL-1β levels (Fig. 6A and B, both p>0.05).

The cerebral edema after SAH were not improved by OZ administration - Brain edema, along with the development of neuronal apoptosis, is the main pathological process in EBI (1). To determine whether TAK1 inhibition reduce cerebral edema, brain water content was evaluated at 24 h post SAH. One-way ANOVA analysis revealed significant differences in water content (F=4.444, p=0.015), ZO-1 (F=4.478, p=0.015) and occludin expression (F=20.03, p<0.001) among the four groups. Although a significant increase in water content of the whole brain at 24 h after SAH was revealed compared to that in control group (Fig. 7A, p<0.05), the mean value was not decreased by TAK1 inhibition (Fig. 7A, p>0.05). The results suggested that TAK1 inhibition could not attenuate brain edema in this rat-SAH model. To confirm this at molecular levels, we assessed the effect of TAK1 inhibition on the expression levels of the tight junction proteins occludin and ZO-1, which play important roles in maintaining the functional blood brain barrier (BBB) integrity. Western blot analysis was performed using tissue homogenates from cortex harvested 24 h after SAH. The levels of occludin and ZO-1 were significantly lower in SAH and vehicle groups compared to the control group (Fig. 7B and C, both p<0.05). TAK1 inhibition failed to
attenuate SAH-induced decreases of the protein levels of ZO-1 and occludin (Fig. 7B and C, p<0.05).

**TAK1 inhibition ameliorated clinical neurological function and brain tissue damage after SAH** — To further justify the protective effect of OZ on behavioral improvement, mean neurological scores were evaluated with a separate cohort of rats. Nonparametric tests revealed significant differences in neurological scores among different groups (Fig. 8A, both 24 h and 72 h post-SAH: p<0.001). SAH induced prominent impairment of the clinical behavioral function at 24 h and 72 h (Fig. 8A, p<0.001 vs. control group). There were no significant differences between SAH and SAH+vehicle- treated rats at both time points (Fig. 8A, p<0.05). However, OZ-treated rats exhibited significant improvement in clinical behavioral function at both 24 and 72 h after injury when compared to the vehicle-treated rats (Fig. 8A, p<0.05). Furthermore, OZ also ameliorated clinical behavioral function even when administered up to 4 h post SAH (Fig. 8B). Finally, we stained the brain sections with cresyl violet (Nissl stain) to count the number of intact versus non-intact neurons 72 h post SAH with and without OZ treatment. One-way ANOVA analysis showed a significant difference in the number of intact neurons among the four groups (F=10.65, p<0.001). A large proportion of neurons in the vehicle treated group exhibited pyknotic or fragmented nuclei, whereas OZ treatment significantly increase the number of intact neurons compared to the vehicle group even when OZ administration was delayed by 4 h after SAH (Fig. 9).

**DISCUSSION**

The main findings of this study are summarized as follows. First, TAK1 was activated by SAH although the levels of total TAK1 had no difference between the control and SAH groups. Secondly, direct intracerebroventricular delivery of OZ, a TAK1 inhibitor, reduced the severity of experimental SAH. Thirdly, delayed treatment with clinical relevant time window improved the neurologic deficits. Fourthly, selectively reduced levels of p-p38, p-JNK, and its downstream target p-c-Jun and prevented nuclear translocation of NF-κBp65 may be involved in the mechanism of neuroprotection of TAK1 inhibition.

TAK1 can be activated by many upstream cytokines, such as TGF-β, IL-1β, TNF-α, and toll-like receptor ligands as illustrated in Fig. 10. Stimulation of IL-1R and TLRs activates myeloid differentiation primary response gene 88 (MyD88)-dependent pathways and MyD88 recruits TNF receptor-associated factor 6 (TRAF6), IL-1 receptor-associated kinase (IRAK1), and IRAK4. TRAF6 acts with ubiquitinconjugating enzyme 13/ubiquitin E2 variant 1a to catalyze lysine (K)63 polyubiquitination of the TAK1 protein kinase complex. For full activation, TAK1 forms a heterotrimeric complex with TAB1 and TAB2 or TAB3, which strongly interact with K63-linked polyubiquitin chains to drive TAK1 activation by ubiquitination and autophosphorylation. The activated TAK1 complex triggers phosphorylation and activation of MAPKs and NF-κB pathways (27). The downstream effects of TAK1 are mediated via phosphorylation of multiple residues in its activation loop such as Ser192, Thr178, Thr184/187 and Ser412 (28-30). Despite the expression of TAK1 showed no prominent alternation after SAH in the basal cortex where acute brain injury was the most severe. Our western blot analysis revealed that the phosphorylation of p-TAK1 (Thr187) was significantly increased after SAH, whereas the phosphorylation of p-TAK1 (Ser439) showed no significant alteration. Taking into account that the phosphorylation of the Thr-187 residue in TAK1...
could simultaneously induced the activation of NF-κB and MAPK signaling pathways (28), we speculated that Thr187 phosphorylation might play an important role rather than Ser439 in this animal model of SAH. It has been well demonstrated that OZ acts as a potent and selective inhibitor of TAK1 (Fig. 10) which displays > 33-fold and > 62-fold selectivity over MEKK1 and MEKK4 respectively (31). Mechanistically, OZ forms a covalent complex with TAK1 and inhibits both the kinase and the ATPase activity of TAK1 following a bi-phase kinetics (32). Importantly, OZ is a relatively safe drug found in vivo and has been previously reported to cross the blood-brain barrier (17,33,34). The target dose used in the current study was chosen based on our published study (15) and treatment of SAH rats with 25 μg OZ did not induce significantly difference in body temperature, injected arterial blood gas data and mortality rate compared to SAH vehicle-treated groups. Our data also showed that OZ did not only suppress the expression of p-TAK1 but also TAK1, which was consistent with our previous and other studies (15,35,36). However, the exact mechanism that OZ decreased TAK1 expression is needed to be elucidated in future.

Several studies have reported that TAK1 is a critical regulator of apoptosis, which is mainly mediated by the downstream pathways p38 MAPKK, JNK, ERK-1/2 and NF-κB p65 (15,16,37). Evidences show that the activation of the MAPK and NF-κB pathways contributes to EBI after SAH, and inhibition of these pathways offer neuro-protective effects against SAH (5,24,26). To identify the molecular mechanisms by which TAK1 inhibition provides neuroprotection after SAH, we first focused on the activation of the MAPK pathway. Inhibition of TAK1 was found to be selectively prevented the SAH-induced phosphorylation of p38 and JNK, but not ERK1/2. Then we found that inhibition of TAK1 resulted in less NF-κB activation and less neuronal apoptosis in vivo (15). NF-κB has emerged as one of the most promising molecular targets in the prevention of EBI (6,26). NF-κB resides in the inactive state in the cytoplasm as a heterotrimer consisting of p50, p65 and IκBα subunits. An IκBα kinase, IKK, phosphorylates serine residues in IκBα at position. Upon phosphorylation and subsequent degradation of IκBα, NF-κB activates and translocates to the nucleus, where it binds to DNA and activates the transcription of various genes (38). In this study, we report that SAH was associated with significant IκB-degradation as well as increased nuclear localization of p65 in the basal temporal lobe at 24 h after SAH. TAK1 inhibition significantly reduced IκB-degradation as well as NF-κB p65 nuclear translocation. Together, the above results demonstrate a selective involvement of TAK1 in the various signaling pathways mobilized in the pathological process of EBI following SAH.

TAK1 activation is also known to induce pro-inflammatory gene expression leading to the production of pro-inflammatory cytokines and chemokines and activation of immune cells (14). Importantly, conditional depletion of TAK1 in microglia only significantly reduced CNS inflammation and diminished axonal and myelin damage by cell-autonomous inhibition of the NF-κB, JNK, and ERK1/2 pathways (39). SAH has long been known to induce an inflammatory response in blood vessels and neuronal tissues (40-42). Elevation of TNF-α and IL-1β were reported in the early stage after SAH and leads to an exacerbation of EBI (10,43). Consistent with the previous work, our data demonstrated that SAH induced a significant increase of TNF-α and IL-1β levels. However, there were no differences in TNF-α and IL-1β levels between vehicle and drug-treated SAH animals, possible explanation for the phenomenon is that TAK1 was expressed...
mainly in neurons but TNF-α and IL-1β are generally thought to be released from glia (44). Thus, we speculate that the anti-inflammatory properties of OZ may play a less significant role in neuroprotection.

Increasing evidences support that SAH significantly decreases the levels of tight junction proteins, occludin and ZO-1 (45). Altered expression of these tight junction proteins could cause BBB breakdown following SAH leading to brain edema (45). Global edema is an independent risk factor for mortality and poor outcome after SAH (46). In the present study, our data demonstrated that SAH degraded occludin and ZO-1, whereas TAK1 inhibition could not restore these proteins. As a result, there was no difference between vehicle and OZ treatment group with single treatment in brain water content at 24 h after SAH. These data suggest that inhibition of TAK1 by OZ could not alleviated brain edema and blood-brain barrier disruption after SAH.

In conclusion, the present study identified TAK1 as a novel upstream mediator of the apoptosis in EBI following SAH (Fig. 10). Selective inhibition of TAK1 by OZ reduced the activation of JNK, p38, and NF-κB, effectively prevented neuronal apoptosis and attenuated neurological deficits in SAH. These data shed new light on the treatment of SAH, and suggest that OZ may be an effective drug therapy for EBI after SAH. However, we used only one single-dose intracerebroventricular treatment to evaluate the neuroprotective role of OZ in the current study. Whether systemic injection of OZ provided similar beneficial effects remains to be further investigated.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

DZ performed the studies and wrote the manuscript. HY performed the IF and analyses. SH and YY contributed to the western blotting and Nissl staining. ZZ, ML and QS designed and performed the animal studies. HL and MZ analysed the samples and data. CH and KL contributed to the design and analysis of the study and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.
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FOOTNOTES

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The abbreviations used are: SAH, Subarachnoid hemorrhage; EBI, early brain injury; TAK1, transforming growth factor beta-activated kinase 1; OZ, 5Z-7-oxozeaenol; BBB, blood brain barrier; ZO-1, zonula occludens-1; ICV, intracerebroventricular.

FIGURE LEGENDS

FIGURE 1. Expression and cellular distribution of TAK1 in the basal cortex after SAH. Western blot analysis showed that the level of total TAK1 (A) kept constant after SAH and p-TAK1 (Thr187) (B) increased significantly at 24 and 48 h after SAH. C. Overlapped images with TAK1 and NeuN demonstrated that TAK1-positive cells in the cortex colocalized with NeuN, a neuronal marker, in control and SAH rats (24 h post SAH). D. Representative photographs of rat brains after surgery. In the sham-operated rats, there is no blood clotting throughout the brain. In the SAH group, the inferior basal temporal lobe was always stained by blood. Hence, the brain tissue adjacent to the clotted blood was taken to the analysis. The site of the obtained sample was indicated with ovals. ##p<0.01, #p<0.05 vs. control; n=6 in each group.

FIGURE 2. Effect of OZ treatment on the expression of TAK1 and p-TAK1. A and B. OZ treatment reduced levels of TAK1 and p-TAK1 at 24 h post-SAH compared with vehicle treated group. C. Representative photomicrographs showed immunofluorescent staining for TAK1 and NeuN in vehicle and OZ treatment groups after SAH. TAK1-positive cells in the inferior basal temporal lobe colocalized with NeuN in vehicle treated group; however, TAK1-positive neurons with very low expression of TAK1 were seen in rats treated with OZ. ##p<0.01 vs. control; **p<0.01, ***p<0.001 vs. vehicle; n=6 in each group.

FIGURE 3. OZ treatment inhibited cell apoptosis in the anterior basal temporal lobe at 24 h post-SAH. A. Representative photomicrographs of TUNEL staining in the inferior basal temporal lobe (×400). B. Statistical data revealed that OZ treatment significantly reduced the TUNEL-positive cells compared with vehicle treated group. C. TAK1 inhibition reduced the levels of cleaved-caspase-3 in the anterior basal temporal lobe at 24 h post SAH. n=6 each group, #p<0.05, ###p<0.001 vs. control; *p<0.05, **p<0.01 vs. vehicle.

FIGURE 4. Effect of TAK1 inhibition on the expression of activated JNK, ERK, p38, and c-Jun at 24 h post SAH. Levels of activated ERK1/2 (p-ERK1/2), p-p38, and p-c-Jun were significantly increased after SAH. TAK1 inhibition reduced the levels of p-JNK, p-p38, and p-c-Jun, but not that of p-ERK1/2 at 24 h post-SAH. n=6 in each group. #p<0.05, ##p<0.01, ###p<0.001 vs. control; * p<0.05 vs. vehicle.

FIGURE 5. Effects of TAK1 inhibition on IκB and p65 expression 24 h post SAH. Western blot analysis of IκB (A) and p65 (B) expression from control, SAH, vehicle, and OZ-treated rats. β-actin and H3 are the loading controls for figure (A) and (B), respectively. Bottom panels are the quantitative
data for IκB and p65. n=6 in each group. ##p<0.01, ###p<0.001 vs. control; *p<0.05, **p<0.01 vs. vehicle.

**FIGURE 6.** Effects of TAK1 inhibition on IL-1β and TNF-α levels 24 h post-SAH. A substantial increase in IL-1β (A) and TNF-α (B) was found in anterior basal temporal lobes from SAH rats 24 h after SAH. TAK1 inhibition failed to reduce SAH-induced increase of IL-1β and TNF-α expression. n=6 in each group. #p<0.05 vs. control; NS=not significant, p>0.05 vs. vehicle.

**FIGURE 7.** TAK1 inhibition failed to attenuate SAH-induced cerebral edema. OZ treatment did not reverse brain water content (A) and the levels of ZO-1 (B) and occludin (C) 24 h post-SAH. #p<0.05, ###p<0.001; NS=no significance, p>0.05 vs. vehicle. n=6 in each group.

**FIGURE 8.** Effect of OZ treatment on clinical recovery after SAH. A. Neurological assessment of SAH animals treated with OZ or vehicle (ICV 10 min post-SAH) in experiment design 2. In comparison with the control group, SAH significantly increased the neurological scores both at 24 h and 72 h post-SAH. The numbers of rats in each group were: control, n=14; SAH, 24 h, n=11; 72 h, n=11. Vehicle, 24 h, n=12; 72 h, n=9. OZ, 24 h, n=11; 72 h, n=10. B. Neurological assessment of SAH animals treated with OZ or vehicle (ICV 4 h post-SAH) in experiment design 3. Neurologic deficits were significantly alleviated in rats receiving OZ compared to vehicle-treated rats at 24 h and 72 h post-SAH. The numbers of animals used in each group were: vehicle, 24 h, n=11; 72 h, n=9. OZ, 24 h, n=13; 72 h, n=12. ###p<0.001 vs. control; *p<0.05 vs. vehicle.

**FIGURE 9.** Effect of OZ treatment on histological alteration of the inferior basal temporal lobe at 72 h post SAH. Representative nissl-stained brain sections showed that there was a significant increase in the number of condensed nuclei in the vehicle group (B) compared with control group (A). At 72 h after injury, both timely (10 min post-SAH; C) and delayed (4 h post-SAH; D) treatment with OZ significantly increased the number of intact neurons compared to the vehicle groups. E. Diagram of a coronal section of rat brain showing the regions taken for assay (red line circled). F. Statistical data from A-D to present the significant improvement by OZ administration. n=6 in each group. ###p<0.001 vs. Control; *p<0.05 vs. vehicle.

**FIGURE 10.** Schematic illustrating the proposed mechanism of neuroprotective role of TAK1 inhibition by OZ. TAK1 plays a central role in several signaling pathways involved in early brain injury after SAH. As illustrated, several toxic stimuli (e.g., TNF-α, IL-1β) induced by SAH cause an increase in TAK1 activity in neurons, leading to NF-κB, JNK and p38 activation, which further activate the apoptotic pathway. Inhibition of TAK1 by OZ will block the signal transduction to protect the neurons from apoptosis, eventually prevent the EBI.

**Table 1 Behavior and activity scores**
TAK1 inhibition attenuates early brain injury after SAH

<table>
<thead>
<tr>
<th>Category</th>
<th>Behavior</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appetite</td>
<td>Finished meal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left meal unfinished</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Scarcely ate</td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td>Walk and reach at least three corners of the cage</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Walk with some stimulations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Almost always lying down</td>
<td>2</td>
</tr>
<tr>
<td>Deficits</td>
<td>No deficits</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unstable walk</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Impossible to walk</td>
<td>2</td>
</tr>
</tbody>
</table>

FIGURE 1
TAK1 inhibition attenuates early brain injury after SAH

FIGURE 2

A

B

C

vehicle

OZ

p-TAK1(Thr187) / β-actin

0.5

1.0

1.5

2.0

control SAH SAH+ vehicle OZ

p-TAK1(Thr187) / β-actin

0.5

1.0

1.5

2.0

control SAH SAH+ vehicle OZ

30 μm
TAK1 inhibition attenuates early brain injury after SAH

FIGURE 3

A

B

C

cleaved-caspase3

β-actin

Apoptosis index (%)

control SAH SAH+ vehicle SAH+ OZ

cleaved-caspase3/β-actin

control SAH SAH+ vehicle SAH+ OZ
TAK1 inhibition attenuates early brain injury after SAH

FIGURE 4

A

control SAH SAH+ vehicle OZ

p-JNK

β-actin

B

control SAH SAH+ vehicle OZ

p-ERK

β-actin

C

control SAH SAH+ vehicle OZ

p-p38

β-actin

D

control SAH SAH+ vehicle OZ

p-c-Jun

β-actin
TAK1 inhibition attenuates early brain injury after SAH

FIGURE 5

FIGURE 6
TAK1 inhibition attenuates early brain injury after SAH

FIGURE 7

FIGURE 8
FIGURE 9
TGFβ-activated kinase 1 (TAK1) inhibition by 5Z-7-oxozeaenol attenuates early brain injury after experimental subarachnoid hemorrhage

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