NBTI attenuates neuroinflammation and apoptosis partly by ENT1/NLRP3/Bcl2 pathway after subarachnoid hemorrhage in rats
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**Objectives** Neuroinflammation and apoptosis are two key factors contributing to early brain injury (EBI) after subarachnoid hemorrhage (SAH) and are strongly associated with a poor prognosis. Recently, equilibrative nucleoside transporter 1 (ENT1) was emerged to accelerate the severity of inflammation and cell apoptosis in several nervous system diseases, including cerebral ischemia, neurodegeneration and epilepsy. However, no study has yet elaborated the expression levels and effects of ENT1 in EBI after SAH.

**Methods** Sprague–Dawley rats were subjected to SAH by endovascular perforation. Nitrobenzylthioinosine (NBTI) was intranasally administered at 0.5 h after SAH. The protein expression levels of ENT1, NLRP3, Bcl2, Bax, ACS, Caspase-1, IL-1 were detected by western blot. The modified Garcia score and beam balance score were employed to evaluate the neurologic function of rats following SAH. In addition, hematoxylin-eosin, fluoro-jade C and TdT-mediated dUTP nick-end labeling staining were then used to evaluate brain tissue damage and neuronal apoptosis.

**Results** Analysis indicated that endogenous levels of ENT1 were significantly upregulated at 24-hour post-SAH, accompanied by NLRP3 inflammasome activation and Bcl2 decline. The administration of NBTI, an inhibitor of ENT1, at a dose of 15 mg/kg, ameliorated neurologic deficits and morphologic lesions at both 24 and 72h after SAH. Moreover, ENT1 inhibition efficiently mitigated neuronal degeneration and cell apoptosis. In addition, NBTI at 15 mg/kg observably increased Bcl2 content and decreased Bax level. Furthermore, suppression of ENT1 notably reduced the expression levels of NLRP3, apoptosis associated speck like protein containing CARD, caspase-1 and IL-1β.

**Conclusions** NBTI relieved SAH-induced EBI partly through ENT1/NLRP3/Bcl2 pathway. NeuroReport 2021; 32:1341–1348 Copyright © 2021 The Author(s). Published by Wolters Kluwer Health, Inc.

Keywords: apoptosis, equilibrative nucleoside transporter 1, neuroinflammation, subarachnoid hemorrhage

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**Introduction** Subarachnoid hemorrhage (SAH) is a devastating subtype of stroke characterized with a high mortality rate (45–50%) and a poor neurologic prognosis, including cognitive and functional deficits [1]. Abundant evidence manifested that early brain injury (EBI), a term that describes immediate injury within 72h after SAH, rather than vasospasm, is the key factor that determines the prognosis of patients with SAH [2,3]. Additionally, an increasing body of evidence suggests that neuroinflammation and apoptosis play a decisive role in the pathologic mechanism of EBI post-SAH [4].

There are four members of the equilibrative nucleoside transporter (ENT) protein family: ENT1, ENT2, ENT3 and ENT4. ENT1 exhibits 47, 33 and 24% sequence consistency with ENT2, ENT3 and ENT4, respectively [5] and is the most widely expressed nucleoside transporter in mammals. ENT1 has been identified in various cell-types including neurons. Recent studies have shown that the inhibition of ENT1 exerts a protective role in cerebral ischemia, lipopolysaccharide-induced neuroinflammation, acute lung injury and ischemic liver injury [6–9], and can also suppress the inflammatory response and apoptosis. However, the specific effects of ENT1 have yet to be investigated in EBI after SAH in rats.

Activation of the NLRP3 inflammasome is involved in EBI after SAH and acts by increasing the expression of caspase-1 and IL-1β. Also, it has been reported that the inhibition of the NLRP3 inflammasome can confer neuroprotective roles by alleviating inflammation and apoptosis in EBI post-SAH [10]. Intriguingly, recent studies have shown that the reduction of ENT1 can restrain NLRP3 inflammasome activation in acute lung injury [8]. Although the link between ENT1 and NLRP3 has been mentioned, the specific relationship between them after SAH has yet to be elucidated.
In this study, we explored the underlying role of ENT1 following SAH and the therapeutic effect of Nitrobenzylthioinosine (NBTI), an ENT1 inhibitor, on NLRP3 inflammasome activation and Bcl-2 expression after SAH. First, we examined the time course of ENT1, NLRP3 and Bcl-2 in the cerebral cortex following SAH in rats. Then, we utilized NBTI, an antagonist of ENT1, to assess the effects of ENT1 inhibition on neurologic impairment, morphologic lesions, neuronal degeneration and apoptosis. Finally, we investigated the activation of the NLRP3 inflammasome, and the levels of Bcl2 and Bax, to determine the underlying effects of NBTI on neuroinflammation and apoptosis induced by SAH.

Methods

Animals
All experiment procedures were approved by the experimental animal ethics committee of Jinmen NO.1 hospital, Hubei. Adult male Sprague–Dawley rats, weighing 280–320g, were purchased and raised in a 12-h light-dark cycle environment with access to water and food ad libitum.

Subarachnoid hemorrhage model
The SAH rat model was established by endovascular perforation as described before [2]. First, rats were anesthetized by intraperitoneal injection of 4% sodium pentobarbital. Following skin preparation, fixation and disinfection, the skin was cut open and the subcutaneous tissue was bluntly separated to expose the left carotid artery. Then, a sharpened nylon suture was inserted from the external carotid artery and passed through the common carotid bifurcation and the internal carotid artery in a sequential manner until the nylon suture punctured the blood vessels at the bifurcation of the anterior and the middle cerebral arteries. The same procedure was applied to the sham group but without intravascular puncture.

Drug treatment
NBTI, an ENT1 inhibitor (Sigma, USA), was dissolved in 10% dimethyl sulfoxide (DMSO) solution. Rats were then administered via intraperitoneal injection with either NBTI (5, 15 or 45 mg/kg) or the same volume of DMSO at 1 and 12 h after SAH ictus.

Experimental design

Experiment 1
The expression change trend of endogenous ENT1, NLRP3 and Bcl-2 in the left cerebral cortex within 72 hours after SAH-induced was detected by western blot analysis. Rats were evenly assigned to five groups: sham, 3 h after SAH, 12 h after SAH, 24 h after SAH and 72 h after SAH (n = 4).

Experiment 2
To assess the effects of NBTI on neurologic behavior at 24 and 72 h after SAH, we randomly divided the rats into five groups (n = 4 per group): sham, SAH+vehicle, SAH+NBTI(5 mg/kg), SAH+NBTI(15 mg/kg) and SAH+NBTI(45 mg/kg). Based on the above neurobehavioral scores, we chose 15 mg/kg NBTI for subsequent experiments.

Experiment 3
To explore the impact of NBTI on apoptosis and neuroinflammation, we assigned rats equally to three groups (n = 4 rats per group): sham, SAH+vehicle and SAH+NBTI. Then, we applied hematoxylin-eosin staining on brain tissues collected at 24 and 72 hours after SAH. At 24 h after SAH, we also carried out TdT-mediated dUTP nick-end labeling (TUNEL) staining, fluoro-jade C (FJC) staining and western blotting.

Western blotting
Western blot analysis was applied to detect protein levels. Briefly, proteins were extracted from the injured cerebral cortex with a protein extraction kit purchased from Beyotime Bio Inc. Then, 30 µg protein of each sample was separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes by wet transfer. The PVDF membranes were blocked by 5% BSA or 5% milk and then incubated at 4 °C overnight with primary antibodies against ENT1 (1:1000, ab223851, Abcam), NLRP3 (1:1000, ab5207, Abcam), Bcl2 (1:1000, AF6139, affinity), β-actin (1:5000, affinity), Bax (1:1000, AF0120, affinity), apoptosis associated speck like protein containing CARD (ASC) (1:1000, DF6304, affinity), caspase-1 (1:1000, ab1871, Millipore) and IL-1β (1:1000, ab9722, Abcam). After washing with tris buffered saline tween, the PVDF membranes were incubated with secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence Plus Chemiluminescence Reagent Kit was used to detect positive protein bands, results were quantified by Image J software.

Neurofunction assessment
The modified Garcia scale and beam balance test were carried out at 24 and 72 h after SAH in a blinded manner to evaluate neurologic function. The modified Garcia scale consists of six parts, spontaneous activity, symmetry of limb movement, forepaw outstretching, vibrissa touch, trunk touch and climbing, the total score ranges from 3 to 18. The beam balance test was calculated the walking distance of rats on beam for 1 min, the total score ranged from 0 to 4. The scores obtained related directly to neurobehavioral function.

Hematoxylin-Eosin staining
The brain tissue was fixed with 4% polyformaldehyde and then dehydrated, paraffin-embedded and cut into 4 µm slices. Paraffin sections were then dewaxed, rehydrated, stained for 60 s with hematoxylin, stained for 3 s with 1% ethanol hydrochloride, and finally, stained for 1 min with eosin. Neutral gum was used to seal the sections onto glass slides. For all rats, we quantified the degree of histomorphologic damage by calculating the...
number of cells showing evidence of edema, vacuolization and nuclear shift.

**TUNEL staining and FJC staining**
Cell apoptosis was detected with a TUNEL kit (Roche, USA). In brief, sections were first dewaxed and then incubated with the TUNEL reagent for 1 h at room temperature, then sections were stained with (4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). Neuronal degeneration was determined by FJC staining as previously. Simply, sections were successively stained with potassium permanganate, FJC and DAPI. sections were then obtained under a light microscope system (Leica, DM2500, Germany). The number of stained cells was quantified by Image J software.

**Statistical analysis**
Data are exhibited as mean ± SD and all statistical analysis were carried out with SPSS 22.0 version. First, the data were tested for normality and homogeneity of variance, if there conditions were met, we performed one-way analysis of variance (ANOVA), otherwise, the nonparametric test was used. Two-way-repeated-measures ANOVA was applied to the neurobehavioral. P < 0.05 was considered statistically significant.

**Results**

**Mortality**
As shown in Table 1, 91 rats were used in this experiment, including 20 in sham group and 71 in model group. None of the rats in the sham group died; however, 14 rats died in the SAH group (a mortality rate of 15.21%).

**Temporal expression profiles of ENT1, NLRP3 and Bcl2 after subarachnoid hemorrhage**
Figure 1a shows a representative image of the SAH model at 24 h. Western blotting was used to measure the expression levels of ENT1, NLRP3 and Bcl2 in the injured cerebral cortex at 3, 12, 24 and 72 h after SAH. Compared to the sham group, the expression of endogenous ENT1 was significantly increased after SAH and reached maximal levels at 24 h after the induction of SAH (Fig. 1b,c). Accordingly, the expression of NLRP3 was also significantly increased and remained at high levels until 72 h when compared with the sham group (Fig. 1b and d). In contrast, the expression levels of Bcl2 gradually decreased until 24 h after SAH (Fig. 1b and e).

**NBTI treatment improved neurological function at 24 and 72 h after subarachnoid hemorrhage**
Modified Garcia and beam balance scores were used to evaluate the neuroprotective effect of NBTI at 24 and 72 h after SAH. As shown in Fig. 2a–d, rats in the SAH+vehicle group showed obvious neurologic impairments when compared with the sham group at 24 and 72 h after SAH. NBTI treatment at doses of 15 mg/kg and 45 mg/kg significantly enhanced the modified Garcia score and beam balance score at 24 and 72 h after SAH. However, the administration of NBTI at a dose of 5 mg/kg showed no obvious neurobehavioral improvement. Based on these short-term results, we used NBTI at a dose of 15 mg/kg in all subsequent experiments.

**NBTI ameliorated morphological lesions at 24 and 72 h after subarachnoid hemorrhage**
Hematoxylin-eosin staining was used to investigate the effect of NBTI on morphologic lesions. The observation area for all rats was the left basal temporal cortex (marked in black box on the image shown in Fig. 2e). As shown in Fig. 2f,g, the morphology of cortical cells in the sham group was regular and round, without edema and vacuoles; the nucleus was round and located in the middle of the cells. Compared with the sham group, the SAH+vehicle group showed distinctly morphologic lesions: a large number of cells showed edema and vacuolization (as shown by the black arrow); the nucleus appear to be irregularly shaped and extruded to one side of the cell (as shown by the blue arrow). After treatment with NBTI (15 mg/kg), the degree of cortex damage in the same area was reduced: a small amount of cell edema, cell vacuolization and irregular nuclei can be seen.

**NBTI alleviated neuronal degeneration at 24 h after subarachnoid hemorrhage**
FJC staining was applied to research whether NBTI could alleviate neuronal degeneration at 24 h after SAH. As shown in Fig. 3, the number of FJC-positive neurons in the ipsilateral cortex in the SAH+vehicle group increased obviously at 24 h after SAH when compared with the sham group, the administration of NBTI dramatically reduced the number of FJC-positive cells.

**NBTI mitigate cell apoptosis at 24 h after subarachnoid hemorrhage**
TUNEL staining was employed to see whether NBTI could mitigate cell apoptosis at 24 h after SAH. As shown

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**Table 1. Animal usage and mortality data of the experimental groups**

| Groups          | Mortality |      |
|-----------------|-----------|------|
| Experiment 1    |           |      |
| Sham            | 0% (0/4)  |      |
| SAH(3h, 12h, 24h, 72h) | 20.00% (4/20) |      |
| Experiment 2    |           |      |
| Sham            | 0% (0/4)  |      |
| SAH+vehicle     | 33.33% (2/6) |      |
| SAH+NBTI (5mg/kg) | 33.33% (2/6) |      |
| SAH+NBTI (15mg/kg) | 0% (0/4)  |      |
| SAH+NBTI (45mg/kg) | 20.00% (1/5) |      |
| Experiment 3    |           |      |
| Sham            | 0% (0/12) |      |
| SAH+NBTI (15mg/kg) | 25.00% (4/16) |      |
| Total           |           | 14.29% (2/14) |
| Sham            | 0% (0/20) |      |
| SAH             | 15.21% (14/71) |      |

NBTI, nitrobenzylthioinosine; SAH, subarachnoid hemorrhage.
in Fig. 4a,b, the number of TUNEL-positive cells in the ipsilateral cortex in SAH+vehicle group increased obviously at 24 h after SAH when compared with the sham group. Administration of NBTI dramatically reduced the number of TUNEL-positive cells. Additionally, western blotting further confirmed that the Bcl2 family of proteins were regulated by NBTI, accompanied by the decreased level of Bax and the increased expression of Bcl2 when compared with SAH+vehicle group (Fig. 4c–f).

**NBTI deterred NLRP3 inflammasome activation**

To address whether NBTI protects against SAH-induced EBI via suppression of NLRP3 inflammasome, we evaluated the effects of NBTI blockade on NLRP3 inflammasome activation. As shown in Fig. 5, SAH-induced NLRP3 inflammasome activation, including the elevated expression of NLRP3, ASC, IL-1β and caspase-1, was remarkably attenuated by NBTI.

**Discussion**

In this study, we investigated the neuroprotective effects of NBTI in SAH rat models and explored the potential molecular mechanisms. The findings of this research are as follows: (1) the expression levels of ENT1 and NLRP3 increased, whereas the levels of Bcl2 decreased in the cerebral cortex during the early stage after SAH. (2) Administration of NBTI (15 mg/kg), an ENT1 inhibitor, ameliorated neurologic impairment and morphologic lesions at 24 and 72 h after SAH. (3) Treatment with NBTI alleviated neuronal degeneration and apoptosis at 24 h after SAH. (4) NBTI also suppressed NLRP3 inflammasome activation and Bax expression, while upregulating the levels of Bcl2. Collectively, our results suggested that NBTI relieved SAH-induced EBI via the ENT1/NLRP3/Bcl2 pathway, at least in part.

Recent studies have demonstrated that ENT1 inhibition can protect against a range of diseases in the central nervous system. For instance, ENT1 was reported to be upregulated in the cerebral cortex of a rat model of cerebral ischemia-reperfusion; furthermore, an intraperitoneal injection of an ENT1 inhibitor reduced the focal area of cerebral infarction by activating the CREB/Bcl2 pathway [7]. Similarly, elevation of ENT1 was observed in the cortex and hippocampus of rats with epilepsy, decreasing the expression of ENT1 could alleviate the pathologic injury of neurons and prolong the seizure cycle [11]. In a mouse model of progressive spinal disease, the expression of ENT1 was shown to be related to the impairment of paraspinal muscle contraction [12]. In line with previous research, we also demonstrated that the levels of
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ENT1 in the cerebral cortex increased within 72 h after SAH, and inhibition of ENT1 with NBTI improved the neurobehavioral score and morphologic lesions at both 24 and 72 h after SAH. These results jointly hinted that there is a clear relationship between ENT1 and EBI after SAH.

Fig. 2.

NBTI treatment attenuated neurologic dysfunction and improved morphologic lesions at 24 and 72 h after subarachnoid hemorrhage (SAH). (a,b) Modified Garcia scores and beam balance scores at 24 h after SAH. (c,d) Modified Garcia scores and beam balance scores at 72 h after SAH. (e) Observation area in the cerebral cortex. The yellow circle in the bottom view of the brain represents the part of the slices; the black box in the coronal section represents the area observed under microscope. (f,g) Representative images acquired from hematoxylin-eosin staining of damaged cerebral cortex at 24 and 72 h after SAH. n=4 per group. The bars represent the mean±SD. *P<0.05 versus sham, # P<0.05 versus SAH+vehicle. NBTI, nitrobenzylthioinosine.

Fig. 3.

NBTI alleviated neuronal degeneration at 24 h after subarachnoid hemorrhage (SAH). (a) representative microphotographs of FJC staining in the damaged cerebral cortex at 24 h after SAH. (b) Quantitative analysis of FJC-positive cell. n=4 per group. The bars represent the mean±SD. *P<0.05 versus sham, # P<0.05 versus SAH+vehicle. FJC, fluoro-jade C; NBTI, nitrobenzylthioinosine.
It is widely recognized that EBI is characterized by inflammation, oxidative stress, apoptosis, pyroptosis, ferroptosis and breakdown of the blood–brain barrier [13–15], among which inflammation and apoptosis are generally considered as crucial contributors for brain injury after SAH [16]. In recent years, numerous studies have shown that the NLRP3 inflammasome activation can exert a powerful pro-inflammatory property in varying central nervous system diseases, including SAH [10], intracerebral hemorrhage [17], traumatic brain injury [18], brain ischemia/reperfusion injury [19] and neurodegenerative disease [20]. Our present results also manifested that NLRP3 activation was directly related to EBI after SAH. Therefore, it is of great significance to probe into how to effectively inhibit the activation of NLRP3 inflammasome, thereby ameliorating the brain damage in SAH.

NBTI mitigated cell apoptosis at 24 h after subarachnoid hemorrhage (SAH). (a) Representative microphotographs of TUNEL staining in the injured cerebral cortex at 24 h after SAH. (b) Quantitative analysis of TUNEL-positive cell. (c) Representative western blotting analysis of equilibrative nucleoside transporter 1 (ENT1), Bcl2 and Bax levels in sham, SAH+vehicle and SAH+NBTI. (d) Quantitative analyses of ENT1. (e) Quantitative analyses of Bcl2. (f) Quantitative analyses of Bax. *P<0.05 versus sham. # P<0.05 versus SAH+vehicle. NBTI, nitrobenzylthioinosine; TUNEL, TdT-mediated dUTP nick-end labeling.
The NLRP3 inflammasome is an intracellular multiprotein complex composed of NOD-like receptor family member NLRP3, adaptor protein ASC and precursor caspase-1, activation of which leads to the release of pro-inflammatory cytokines (IL-1β and IL-18) via the cleavage of caspase-1 [21]. Numerous researchers have investigated the role of NLRP3 in SAH, and shown that obstructing the activation of the NLRP3-ASC inflammasome can reduce neuroinflammation and attenuate short-term and long-term neurobehavioral function after SAH [22]. In present study, we found that NBTI alleviated NLRP3 inflammasome expression (NLRP3, ASC, caspase-1), reduced the level of pro-inflammatory cytokine IL-1β, attenuated neuronal degeneration and apoptosis, as well as improved short-term neurologic function after SAH. The effects of NBTI on NLRP3 inflammasome expression and subsequent neurologic behavior were consistent with previous research in that blockade of ENT1 with NBTI can guard against acute lung injury by suppressing the NLRP3 inflammasome.

The NLRP3 inflammasome is an important regulator of neuronal degeneration and apoptosis. A tremendous amount of basic studies have demonstrated that the pharmacologic inhibition of NLRP3 can stimulate the expression of the antiapoptotic protein Bcl2 and block the upregulation of the pro-apoptosis protein Bax, thereby improving neurologic function after SAH in rat models.
Similarly, our present data showed that the inhibition of NLRP3 inflammasome was accompanied by the upregulation of Becl2, downregulation of Bax and reduction of neuronal degeneration and apoptosis. Therefore, it is reasonable to conclude that NBTI exerts a neuroprotective role via the ENT1/NLRP3/Becl2 signaling pathway in EBI after SAH in rats.

However, our current research has some limitations that need to be considered. First, the present study focused predominantly on the short-term behavioral effects of NBTI after SAH in rats; further research now needs to investigate the long-term behavioral effects of NBTI after SAH. Second, in addition to the regulation of inflammation and apoptosis, previous studies have shown that ENT1 also exerts multiple biologic properties, such as regulation of nucleotide metabolism [25]. Hence, future research are needed to investigate the other functions of ENT1 after SAH and the potential mechanisms involved.

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Conflicts of interest

There are no conflicts of interest.

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