Neuroprotective effect of ONO-1078, a leukotriene receptor antagonist, on transient global cerebral ischemia in rats[1]

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ABSTRACT

AIM: To determine whether ONO-1078 {pranlukast, 4-oxo-8-[*p*-(4-phenylbutyloxy)benzoyl-amono]-2-(tetrazol-5-yl)-4*H*-1-benzopyran hemihydrate}, a potent leukotriene receptor antagonist, possesses a neuroprotective effect on global cerebral ischemia in rats, and to explore its possible mechanism of action. **METHODS:** Transient global cerebral ischemia was induced by four-vessel occlusion for 10 min and followed by 72-h reperfusion. ONO-1078 (0.03-0.3 mg/kg) and edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one, a neuroprotective agent) 10 mg/kg were ip injected 30 min before ischemia and 1 h after reperfusion, and once a day afterward. Neurological outcome was evaluated before ischemia and 24, 48, 72 h after reperfusion. Neuron density, the expressions of *N*-methyl-*D*-aspartate (NMDA) receptor subunit proteins (NR1, NR2A, NA2B) and vascular cell adhesion molecule 1 (VCAM-1) in the cerebral cortex and hippocampus were measured at 72 h after reperfusion. **RESULTS:** ONO-1078 (0.1, 0.3 mg/kg) and edaravone (10 mg/kg) improved ischemia-induced neurological deficiency and reduced neuron death. ONO-1078 (0.1, 0.3 mg/kg) significantly inhibited the enhanced expression of NMDA receptor subunit protein NR2A in the cortex and VCAM-1 in the hippocampus of ischemic rats. **CONCLUSION:** ONO-1078 possesses a neuroprotective effect on global cerebral ischemia in rats, and its mechanism may be partly related to the inhibition of the upregulation of NR2A and VCAM-1 in different regions of the brain.

INTRODUCTION

ONO-1078 {pranlukast, 4-oxo-8-[*p*-(4-phenyl-butyloxy)benzoyl-amino]-2-(tetrazol- 5-yl)-4*H*-1-benzo-pyran hemihydrate}, a potent antagonist of cysteinyl leukotrienes (LTC$_4$, D$_4$, and E$_4$), possesses anti-inflammatory and anti-asthma effects, and is used as a therapeutic drug of bronchial asthma[1-3]. ONO-1078 inhib-

ited subarachnoid hemorrhage-induced delayed cerebral vasospasm[4]. We recently found that this compound protected mice and rats from focal cerebral ischemic injury[5,6]. However, whether ONO-1078 possesses a protective effect against other ischemic injuries, such as global cerebral ischemia, has not yet been studied.

Global cerebral ischemia resulting from cardiac arrest, stroke and hypoxia is a problem of increasing clinical significance. A four-vessel occlusion (4-VO) model of rats has been developed in an attempt to determine the mechanisms underlying global ischemia-related neural injury, and to develop the treatments for cerebral ischemic injury. The pattern of neural injury

in this model is similar to that reported in global ischemia in humans, including substantial neuron death in the cerebral cortex and hippocampal CA1 region[7,8].

N-methyl-*D*-aspartate (NMDA) receptors play an important role in the pathological processes of ischemic neuron injury[9-14]. Cerebral ischemia induces the abnormal expression and activation of NMDA receptors, and an excessive calcium influx, which further results in progressive irreversible neuronal damage and delayed cell death. On the other hand, inflammatory cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), are also involved in cerebral ischemic injury[9,14-16]. Cerebral ischemia upregulates inflammatory cell adhesion molecules, which may induce the subsequent inflammatory reactions and neural injury. But whether ONO-1078 influences these molecules in cerebral ischemia is unknown.

Therefore, the purpose of this study was to determine whether ONO-1078 protects rats against global cerebral ischemia in a 4-VO model, and to explore the effects of ONO-1078 on NMDA receptor subunit and VCAM-1 expressions for the possible underlying mechanism(s). Edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one), a novel neuroprotective agent for ischemic stroke[17,18], was used as a positive control in this study.

MATERIALS AND METHODS

Chemicals ONO-1078 was kindly gifted by Dr TSUBOSHIMA Masami (Ono Pharmaceutical Co, Osaka, Japan). This compound was dissolved in 100 % ethanol (10 g/L), and diluted with saline. Edaravone injection was from Hangzhou Conba Pharmaceutical Co. Goat polyclonal antibody against VCAM-1 was purchased from Santa Cruz Biotechnology Inc, USA; specific antibodies for NR1, NR2A, or NA2B were from Dr LUO JH (School of Medicine, Zhejiang University, China); rabbit anti-goat, goat anti-rabbit and goat anti-mouse IgG-HRP were from Zhongshan Biotech Co, Beijing. Enhanced chemiluminescence (ECL) reagent was from Renaissance, New England Nuclear-Dupont. Other reagents were commercial products with analytic purity.

Global cerebral ischemia Male Sprague-Dawley rats weighting 250-300 g were from the Experimental Animal Center of Zhejiang Academy of Medical Sciences (Grade II, Certificate No 2001001). Rats were housed in groups of four per cage at a constant temperature (22-25 ℃) and allowed free access to laboratory chow and water. Global cerebral ischemia was carried out in a two-stage procedure. Rats were anesthetized with chloral hydrate (350 mg/kg, ip), and then fixed on a stereotaxic apparatus. An incision was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, the right and left alar foramina of the first cervical vertebrae were exposed. A 0.5-mm electrocautery needle was inserted through each alar foramen, and both vertebral arteries were electro-cauterized and permanently occluded. Meanwhile, through a ventral midline neck incision, an atraumatic arterial clasp was loosely placed around both common carotid arteries without interrupting carotid blood flow. After these procedures, the incisions were closed, and the animals were fasted overnight.

Twenty-four hours later, the rats were randomly divided into groups and subjected to either 10 min of global cerebral ischemia or sham operation (the same manipulation but the carotid arteries were not occluded). Global cerebral ischemia in the conscious rats was induced by tightening the carotid artery clasps to block brain blood flow. At the end of 10 min, the carotid artery clasps were released to allow recirculation of the brain. Criteria for global cerebral ischemia were determined as bilateral loss of righting reflex, paw extension ability and mydriasis. Before and during 4-VO and 10 min after reperfusion, the arterial blood pH, p_{A,O_2} and p_{A,CO_2} (Blood gas analyzer ABL 50, Denmark), blood pressure (MPS-2000M computer-assisted system, Shanghai Honglian Medical Apparatus Development Co, LTD) and blood glucose (One Touch™ Basic Complete Blood Glucose Monitoring System, Lifescan Inc) were monitored, and rectal temperature was maintained at 37 ℃ by means of heating mat and lamp. Animals that regained their righting reflex before release of the carotid artery clasps, and convulsed during the recirculation period were excluded from the study. ONO-1078 (0.03, 0.1, or 0.3 mg/kg), edaravone (10 mg/kg) or the same volume of saline were ip injected 30 min before 4-VO and 1 h after reperfusion, and once a day afterward.

Evaluation of neurological outcome Neurological outcome was evaluated before ischemia, and 24, 48, and 72 h after reperfusion using a modified method described previously[19,20]. In rotating screen test, rats were placed on a horizontal wire mesh screen. The screen was then slowly rotated through vertical posi-

tion [270º] to inverted position [180º]. Score 0, grasp screen to 180º for >5 s; 1, grasp screen to 180º for <5 s; 2, grasp screen past 270º for 5 s but not to 180º; 3, falls from vertical screen. Beam balance task consisted of placing the rats on suspended, narrow wooden beam (1.5-cm width) and measuring the duration they remained on the beam for up to 60 s. Training consisted of 3 trials, approximately 1 h prior to cerebral ischemia, which also served as pre-ischemic baseline measures.

Histopathological assessment Seventy-two hours after reperfusion**,** rats were reanesthetized with chloral hydrate, then decapitated. The brains were rapidly removed and frozen. Serial coronal sections (10 μm) were cut by cryoultramicrotomy (Leica CM-1900, Germany), and stained with hematoxylin and eosin. The numbers of survival neurons in the cortex and hippocampal CA1 region (3.3 to 3.5 mm posterior to the bregma) were counted using an image analyzer (AnalyPower 1.0, Zhejiang University, Hangzhou, China).

Membrane protein preparation The cortex and hippocampus were dissected out, and homogenized twice in 100 volumes (100×wet weight) of ice-cold Tris HCl buffer 10 mmol/L, pH 7.4, containing sucrose 320 mmol/L, for 10 s at a 20-s interval between bursts. The homogenate was centrifuged at $700 \times g$ for 10 min, 4 °C. The supernatant was transferred to another tube, and centrifuged at $37\,000 \times g$, 4 °C for 40 min. The pellet was resuspended in Tris-HCl buffer 10 mmol/L, pH 7.4. Protein concentration was determined by Lowry method.

Gel eletrophoresis and immunoblotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer of proteins to nitrocellulose membranes were performed according to the method with minor modifications[21]. The protein samples (5-7.5 μg) were separated on 7.5 % polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad) in transfer buffer (Tris 25 mmol/L, glycine 192 mmol/L, 20 % methanol, 0.05 % SDS, pH 8.3). The membranes were incubated with blocking buffer [5 % nonfat dry milk in TBST (Tris-HCl 20 mmol/L, NaCl 140 mmol/L, 0.1 % Tween-20, pH 7.4)] for 30 min at room temperature. The membranes were then incubated in blocking buffer with the specific affinity-purified antibodies directed against NR1, NR2A, NA2B or VCAM-1 overnight at 4 °C, respectively. After washing with TBTS, the membranes were respectively incubated with goat anti-mouse, goat anti-rabbit or rabbit

anti-goat IgG-HRP at a dilution of 1:2000 in blocking buffer for 2 h at room temperature followed by repeated washing for 60 min with TBST. At the end, the membranes were incubated in an ECL solution for 1 min and exposed on an X-ray film. The protein bands on an X-ray film were quantitatively analyzed with a laser densitometer (Ultro Scan XL, Pharmacia LKB Co, Sweden). A protein sample from normal rat brains was used as a standard, and the relative expression of NMDA receptor subunits NR1, NR2A, NA2B, and VCAM-1 was calculated as the ratio of tested/standard sample densities.

Statistical analysis All values were presented as mean±SD. Independent-sample *t*-test or one-way ANOVA was used for calculating a significant difference (SPSS 11.0 for Windows, SPSS inc, USA). $P<0.05$ was considered statistically significant.

RESULTS

Changes in physiological variables Procedure of 4-VO resulted in hyperventilation. In the rats treated with saline ($n=4$) and ONO-1078 0.3 mg/kg ($n=5$), arterial blood p_{A,O_2} was siginificantly higher during 4-VO (125±27 and 119±14, respectively) than that of pre-ischemia (93±13 and 93±15, $P<0.05$), while arterial blood p_{A,CO_2} was markedly lower during 4-VO than pre-ischemia (28±3 and 24±7 *vs* 32.4±2.8 and 35±4, respectively, $P<0.05$). Both p_{A,O_2} and p_{A,CO_2} recovered to baseline levels after ischemia. However, there were no siginificant differences in body temperature, blood pressure, arterial blood pH and blood glucose before, during 4-VO and 10 min after reperfusion among the groups.

Effects of ONO-1078 and edaravone on neurological outcome in ischemic rats The rats with 4-VO exhibited neurological deficiency ($P<0.05$ or 0.01). ONO-1078 (0.3 mg/kg) and edaravone (10 mg/kg) significantly improved ischemia-induced neurological deficiency. The neurological scores in rotating screen test were reduced in both ONO-1078- and edaravone-treated rats after ischemia. The durations of beam balance task were longer in the rats treated with ONO-1078 or edaravone than those in saline controls ($P<0.05$, Tab 1).

Effects of ONO-1078 and edaravone on neuron densities in the cerebral cortex and hippocampal CA1 region of ischemic rats Neuropathological changes in the brains of ischemic rats were character-

**Tab 1. Effects of ONO-1078 and edaravone on neurological outcome after global cerebral ischemia in rats. Mean±SD.
[b]$P<0.05$, [c]$P<0.01$ *vs* sham operation. [e]$P<0.05$ *vs* saline control.**

Drug /mg·kg⁻¹	n	Vertical screen test/score				Beam balance task/se			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Sham operation	8	0.4±0.5	0.5±0.5	0.4±0.5	0.1±0.4	54.1±2.1	54±6	57±4	57.8±2.9
Saline control	10	0.5±0.5	2.5±0.8[c]	2.2±0.8[c]	2.1±0.6[c]	55±3	18±9[c]	24±10[c]	32±11[c]
ONO-1078 0.03	8	0.6±0.5	2.9±0.4[c]	2.5±0.8[c]	2.1±0.8[c]	56.5±2.9	13±7[c]	19±12[c]	28±12[c]
0.1	8	0.5±0.5	2.6±0.5[c]	2.6±0.5[c]	2.3±0.7[c]	57±4	18±9[c]	23±8[c]	29±9[c]
0.3	8	0.4±0.5	1.9±0.8[c]	1.5±0.5[ce]	1.5±0.5[ce]	53±4	29±9[ce]	35±8[ce]	44±6[ce]
Edaravone 10.0	8	0.5±0.5	1.6±0.5[ce]	1.3±0.9[be]	1.3±0.7[ce]	56±5	29±10[ce]	39±11[ce]	44±8[ce]

ized by ischemic cell changes with eosinophilic cytoplasm, pyknotic nucleus (Fig 1), and the survival neuron counts decreased after global cerebral ischemia ($P<0.05$ or 0.01, Tab 2). In the cortex of the rats treated with ONO-1078 (0.03, 0.1, 0.3 mg/kg) and edaravone (10 mg/kg), neuron counts decreased slightly, but had no significant differences compared with sham operated rats ($P>0.05$). Hippocampus CA1 region was vulnerable to ischemia, the survival neuron counts in this region were markedly decreased in saline controls ($P<0.01$). ONO-1078 dose-dependently reduced hippocampal neuron death, the effective doses were 0.1 and 0.3 mg/kg, and edaravone also exhibited this effect ($P<0.05$, Tab 2).

Tab 2. Effects of ONO-1078 and edaravone on neuron densities (1×10^3 cells per mm²) in the cerebral cortex and hippocampal CA1 region after global cerebral ischemia in rats. Mean±SD. [a]$P>0.05$, [b]$P< 0.05$, [c]$P<0.01$ *vs* sham operation. [e]$P<0.05$ *vs* saline control.

Drug/mg·kg⁻¹	n	Cortex	Hippocampus
Sham operation	8	0.62±0.05	0.69±0.06
Saline control	10	0.48±0.14[b]	0.22±0.08[c]
ONO-1078 0.03	8	0.53±0.12[a]	0.23±0.13[c]
0.1	8	0.57±0.10[a]	0.42±0.23[be]
0.3	8	0.55±0.10[a]	0.46±0.27[be]
Edaravone 10.0	8	0.54±0.12[a]	0.41±0.22[be]

Effects of ONO-1078 and edaravone on the expressions of NMDA receptor subunit proteins and VCAM-1 in the cerebral cortex and hippocampus of ischemic rats In the cortex, NR2A expression in-creased in ischemic rats, and ONO-1078, not edaravone, inhibited this increase dose-dependently ($P<0.05$ or 0.01), but NR1 and NR2B expressions had no significant changes. The expression of VCAM-1 in the cortex had a tendency to increase in ischemic rats, and ONO-1078 had an inhibitory tendency, but not significantly. In the hippocampus, NR1 and NR2A expressions had a tendency to decrease in ischemic rats, but no significant differences were found among ischemic rats. NR2B expression showed no remarkable changes. However, VCAM-1 expression in the hippocampus was increased, ONO-1078 dose-dependently inhibited this increase ($P<0.05$), but edaravone did not show a significant effect (Fig 2 and Tab 3).

DISCUSSION

In the present study, we found that ONO-1078 significantly attenuated neurological deficiency, and increased the survival neurons in the global cerebral ischemic rats induced by 4-VO. The effective dose was 0.1 mg/kg, and the most effective dose was 0.3 mg/kg. These effects are similar to that of edaravone, a clinically available radical scavenger and antioxidant for the treatment of stroke[17,18]. These results clearly confirm that ONO-1078 possesses the neuroprotective effect on global cerebral ischemia in rats, similar to those on rat and mouse focal cerebral ischemia[5,6]. Increasing evidence has demonstrated the involvement of cysteinyl leukotrienes in cerebral ischemia. The production of cysteinyl leukotrienes increases in gerbil global cerebral ischemia[22] and rat focal cerebral ischemia[23], and 5-lipoxygenase inhibitors inhibit both cysteinyl leukotriene production and neurological injury[22-24]. These findings support the therapeutic potentials of ONO-1078 in
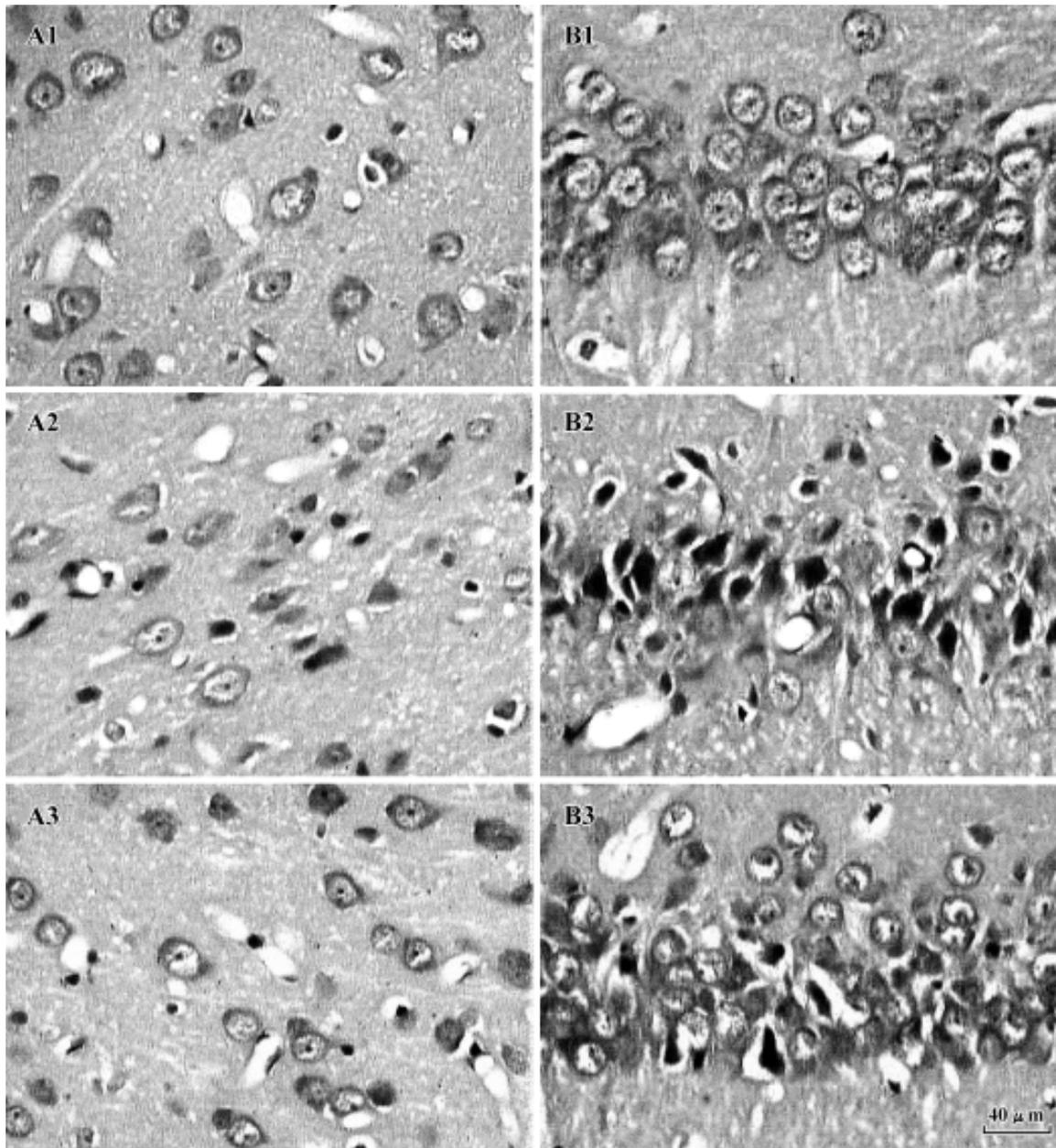
Fig 1. Histopathologic changes of the cerebral cortex (A) and hippocampal CA1 region (B) 72 h after global cerebral ischemia in rats. 1: sham operation: neurons are intact; 2: saline control: severe ischemic damage is present in this histological field; 3: treated with ONO-1078: neurons are preserved. (HE stain, ×250).
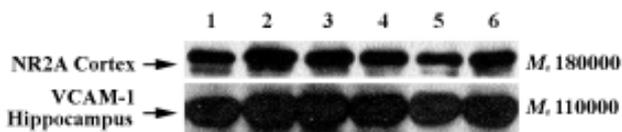

Fig 2. Expressions of NMDA receptor subunit NR2A in the cerebral cortex and VCAM-1 in the hippocampus 72 h after global cerebral ischemia in rats. 1: sham operation; 2: saline control; 3: treated with ONO-1078 0.03 mg/kg; 4: ONO-1078 0.1 mg/kg; 5: ONO-1078 0.3 mg/kg; 6: edaravone 10.0 mg/kg.

treatment of cerebral ischemia.

Furthermore, our results showed that ONO-1078 significantly inhibited the enhanced expression of NR2A in the cortex and VCAM-1 in the hippocampus 72 h after global cerebral ischemia and reperfusion. Global cerebral ischemia increased NMDA receptor subunit (NR1, NR2A, NR2B) and VCAM-1 levels in the cerebral cortex and hippocampus within 1-14 d after reperfusion[9]. This study further found that ONO-1078 dose-dependently inhibited the enhanced expressions.

Tab 3. Effects of ONO-1078 and edaravone on the expressions of NMDA receptor subunit proteins and VCAM-1 in the cerebral cortex and hippocampus after global cerebral ischemia in rats.   Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* sham operation. $^eP<0.05$, $^fP<0.01$ *vs* saline control.

Drug /mg·kg⁻¹	*n*	Cortex				Hippocampus			
		NR1	NR2A	NR2B	VCAM-1	NR1	NR2A	NR2B	VCAM-1
Sham operation	8	1.05±0.29	1.05±0.14	0.97±0.21	1.01±0.10	1.13±0.19	1.16±0.14	1.03±0.21	1.06±0.18
Saline control	10	1.13±0.20	1.30±0.20^c	1.07±0.21	1.20±0.27	0.99±0.22	1.05±0.18	1.11±0.17	1.34±0.21^b
ONO-1078 0.03	8	1.02±0.18	1.15±0.22	1.2±0.4	1.21±0.19^b	0.94±0.08^b	1.11±0.20	1.00±0.29	1.22±0.16
0.1	8	1.25±0.38	1.11±0.15^e	1.21±0.24	1.03±0.22	0.96±0.10	1.03±0.27	0.96±0.17	1.17±0.22
0.3	8	1.18±0.15	1.02±0.13^f	1.05±0.23	1.10±0.10	0.93±0.11^b	0.98±0.09^b	1.08±0.18	1.13±0.16^e
Edaravone 10.0	8	1.04±0.24	1.21±0.21	1.1±0.3	0.97±0.25	1.05±0.10	0.94±0.17^b	1.16±0.27	1.2±0.3

In contrast, edaravone showed no remarkable effect on the expressions of both NMDA receptor subunits and VCAM-1 in ischemic rats, although it showed the same protective effect as ONO-1078.

The events in the ischemic brain include excitotoxicity in the early phase and inflammation in the late phase[25], and NMDA receptors and VCAM-1 play important roles in the excitotoxicity and inflammation. In rat focal cerebral ischemia, NMDA receptor antagonist MK 801 inhibiting cysteinyl leukotriene production, suggests that excitatory amino acids may regulate 5-lipoxygenase activity and its responses to cysteinyl leukotrienes through NMDA receptor activation[23]. In this study, the modulation of the expression of NMDA receptor subunit NR2A by ONO-1078 may be another aspect of the interaction of cysteinyl leukotrienes and NMDA receptors. On the other hand, ONO-1078 inhibited the enhanced VCAM-1 expression that was associated with inhibition of eosinophil infiltration in allergic inflammation in rat airways[21]. This study showed the same effect in the hippocampus of ischemic rats. As indirect evidence, Satio *et al*[26] reported that leukotrienes augmented VCAM-1 expression slightly in human umbilical vein endothelial cells. Since serum VCAM-1 levels were increased in ischemic stroke patients[27], VCAM-1 may be involved in the ischemic cerebral injury. Therefore, our results suggest that inhibition of VCAM-1 expression in certain brain regions may be one of the possible mechanisms of neuroprotection by ONO-1078.

However, whether the effect of ONO-1078 on these molecular expressions is via antagonism of leukotriene receptors or other mechanism(s) remains to be clarified. Also, the actions of cysteinyl leukotrienes and their antagonists on the inflammation initiation and severity in the ischemic injury should be investigated.

In conclusion, ONO-1078 possesses neuroprotective effect on transient global cerebral ischemia in rats, and its mechanism may be partly related to inhibiting the expression of NMDA receptor subunit protein NR2A and VCAM-1 in special regions of the brain.

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