

Full-length article

ONO-1078 reduces NMDA-induced brain injury and vascular cell adhesion molecule-1 expression in rats¹

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Key words

leukotriene antagonists; pranlukast; *N*-methylaspartate; vascular cell adhesion molecule-1; neurotoxicity; brain injuries

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Abstract

Aim: To determine whether ONO-1078 (pranlukast), a potent cysteinyl leukotriene receptor 1 (CysLT₁) antagonist, has an effect on *N*-methyl-*D*-aspartate (NMDA)-induced brain injury and vascular cell adhesion molecule-1 (VCAM-1) expression in rats. **Methods:** Brain injury was induced by direct microinjection of NMDA (0.3 μmol in 1 μL of sterile 0.1 mol/L PBS, pH 7.4) into the cerebral cortex. The lesion volume (area), brain edema and neuron density were assessed by an image analyzer and the expression of VCAM-1 in the cortex was detected by Western blot 24 h after NMDA injection. ONO-1078 (0.03, 0.1, or 0.3 mg/kg) and edaravone (MCI-186, 10 mg/kg), a neuroprotective agent, were ip injected 30 min before and after NMDA injection. **Results:** NMDA microinjection produced well-defined focal lesions (Figure 1) dose- and time-dependently. ONO-1078 (0.1, 0.3 mg/kg) and edaravone (10 mg/kg) decreased the total lesion volume, lesion area and brain edema induced by NMDA. Furthermore, ONO-1078 (0.1, 0.3 mg/kg) significantly inhibited the enhanced expression of VCAM-1 in the injured cortices, but edaravone did not have this effect. **Conclusion:** CysLT₁ receptor antagonist ONO-1078 attenuates NMDA-induced brain damage in rats, and this might relate to the attenuation of NMDA receptor-dependent neurotoxicity and the inhibition of the upregulation of VCAM-1 expression.

Introduction

It is well known that *N*-methyl-*D*-aspartate (NMDA) receptors play a critical role in the initiation of cerebral ischemia [1]. The excitotoxicity mediated by NMDA receptors contributes to the damage that occurs in the early stages (min to h), and inflammation and programmed cell death in the late stages (h to weeks) of cerebral ischemia [1,2]. Among many factors involved in inflammation, adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), can modulate ischemic injury in the brain [3,4]. However, cysteinyl leukotrienes (CysLTs, including LTC₄, LTD₄, and LTE₄), 5-lipoxygenase (5-LOX) metabolites of arachidonic acid, are potent inflammatory mediators [5] that are also involved in pathogenic processes of cerebral ischemia. CysLT levels were elevated after global and focal cerebral ischemia and 5-lipoxygenase inhibitor attenuated

CysLT production and ischemic brain injury [6–8]. Furthermore, we have previously found that ONO-1078 {pranlukast, 4-oxo-8-[*p*-(4-phenylbutyloxy)benzoyl-amono]-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate}, a CysLT₁ receptor antagonist, protects rats and mice against focal cerebral ischemic injury [9,10].

Recently, we reported that global cerebral ischemia induces brain injury and time-dependently increases the expressions of NMDA receptor subunit proteins and VCAM-1 in different regions of the brain in rats [11]. We also found that ONO-1078 attenuates ischemic injury and inhibits the increased expressions of NMDA receptor subunit NR2A and VCAM-1 [12]. However, whether ONO-1078 protects rats from chemically induced injury, and if NMDA receptor activation directly links to the increased VCAM-1 expression, remains unknown. Thus, in the present study, we induced cortical injury by NMDA microinjection to evaluate the

protective effect of ONO-1078. Also, we observed VCAM-1 expression after NMDA receptor activation and the influence of ONO-1078. Edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one), a novel neuroprotective agent for ischemic stroke^[13,14], was used as a control drug.

Materials and methods

Drugs and reagents NMDA was purchased from Sigma (St Louis, MO, USA); ONO-1078 was provided by Dr Masami Tsuboshima (Ono Pharmaceutical Co, Osaka, Japan). This compound was dissolved in 100% ethanol (10 g/L), and freshly diluted with saline before use. Edaravone injection was obtained from Hangzhou Conba Pharmaceutical Co; polyclonal goat antibody against VCAM-1 from Santa Cruz, CA, USA; rabbit anti-goat IgG-HRP was obtained from Zhong-Shan Biotech Co, Beijing; enhanced chemiluminescence (ECL) reagent was obtained from Renaissance, New England Nuclear-Dupont. Other reagents were commercial products with analytic purity.

NMDA microinjection in neocortex 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 4 per cage at a constant temperature (25 °C) and allowed free access to laboratory chow and water. In chloral hydrate-anesthetized (350 mg/kg, ip) rats, the dura overlying the parietal cortex was exposed, and a microinjector was inserted into the parietal cortex at a site 3.0 mm caudal to bregma, 5.0 mm from the midline, and 1.5 mm below the dural surface. NMDA (0.1, 0.2, and 0.3 μmol in 1 μL of sterile 0.1 mol/L PBS, pH 7.4) or 0.1 mol/L PBS alone (control) was injected according to the method described by Iadecola *et al*^[15]. The microinjector was left in place for 10 min, to minimize the back-flux of NMDA, and then removed.

After these procedures, the incisions were closed and rats were returned to their cages. ONO-1078 (0.03, 0.1, and 0.3 mg/kg), edaravone (10 mg/kg) or the same volumes of saline were ip injected 30 min before and after NMDA injection.

Evaluation of tissue injury Twenty-four hours after NMDA microinjection, the rats were anesthetized with chloral hydrate again and decapitated. Brains were removed immediately and sectioned coronally into 6 slices (2 mm-thick). Brain slices were stained in 0.5% 2,3,5-triphenyl tetrzolium chloride at 37 °C for 30 min, followed by fixation with 10% formalin, overnight. All the brain slices were photographed using a CCD camera (MV-CP-230, Panasonic, Japan) on an anatomy microscope (XTL 2600, Shanghai, China). The volume and area of NMDA-injured cortex were measured by an image analyzer (AnalyPower1.0, Zhejiang University,

Hangzhou, China).

Histopathological assessment Under chloral hydrate anesthesia, the rats were infused with 100 mL of heparinized saline followed by 300 mL of 10% formalin, before being decapitated. Brains were removed and fixed in 10% formalin for 7 d; paraffin sections (5 μm) were then cut and stained with hematoxylin and eosin. The densities of survival neurons in the cortex were counted using the image analyzer (AnalyPower1.0, Zhejiang University, Hangzhou, China).

Western blot analysis Western blot analysis was performed as described by Kang *et al*^[16]. The injured and contralateral cortices were dissected and homogenized in buffer A (10 mmol/L Tris-HCl, pH 7.4, containing 320 mmol/L sucrose). The homogenate was centrifugated at 700×g for 10 min at 4 °C, and the supernatant was further centrifuged at 37 000×g for 40 min at 4 °C. The resultant pellet was resuspended in buffer B (10 mmol/L Tris-HCl, pH 7.4), and protein concentration was determined by the Lowry method.

The protein samples of 7.5 μg were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane in transfer buffer (Tris 25 mmol/L, glycine 192 mmol/L, 20% methanol, 0.05% SDS, pH 8.3). The nonspecific binding was blocked in 5% fat-free drymilk 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 membrane was incubated with polyclonal goat antibody against VCAM-1 (1:500) overnight at 4 °C. After washing with TBTS, the membrane was then incubated with HRP-conjugated rabbit anti-goat IgG (1:2000) for 2 h at room temperature followed by repeated washing with TBST. At the end, the membrane was incubated in enhanced chemiluminescence (ECL) solution for 1 min and then exposed to X-ray film. The protein bands on an X-ray film were scanned by a GS-800 Laser Densitometer (Bio-Rad, USA) and analyzed by Met Imaging Series 5.0 (Bio-Rad, USA). As a standard, a protein sample from normal rat brains was used and the relative expression of VCAM-1 was calculated as the ratio of tested/standard sample densities.

Statistical analysis Data were reported as mean±SD. Statistical evaluation was carried out by the independent-sample *t*-test or one-way ANOVA (SPSS 11.0 for Windows, SPSS Inc, USA), according to the experimental design. *P*<0.05 was considered statistically significant.

Results

NMDA-induced injury *in vivo* NMDA microinjection produced well-defined focal lesions (Figure 1) dose- and time-dependently in TTC-stained brain slices. The lesion volumes induced by NMDA 0.3 μmol (32.4±10.7, *n*=5) were

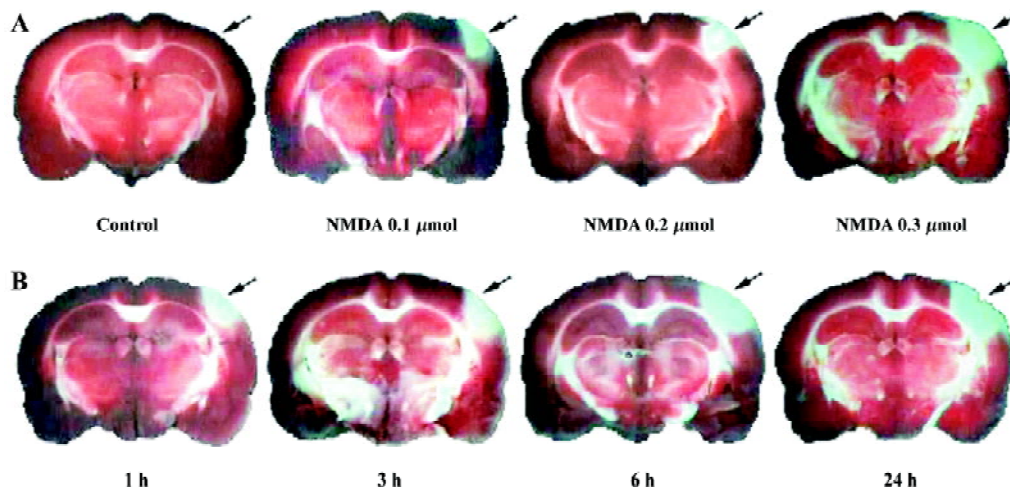


Figure 1. TTC-stained brain slices after direct microinjection of NMDA into the cerebral cortex in rats. A: brain slices 24 h after NMDA microinjection with various dose; B: brain slices 1–24 h after NMDA microinjection (0.3 μmol). Arrowheads show the injection sites.

significantly larger than those by 0.1 and 0.2 μmol (11.1 ± 5.5 and 15.6 ± 7.6 , $n=5$, $P<0.01$, respectively) 24 h after NMDA injection. The lesion volumes at 24 h after NMDA (0.3 μmol) microinjection (35.4 ± 7.0 , $n=4$) were larger than those at 1, 3, or 6 h after injection (21.8 ± 8.0 , 25.1 ± 8.8 , or 30.7 ± 9.2 , respectively, $n=4$, $P<0.05$).

Histopathological changes in the lesion regions 24 h after NMDA (0.3 μmol) injection were characterized by neu-

ronal damage with eosinophilic cytoplasm and serious pyknotic nucleus, and survival neuron decreased markedly in the injured cortices; however, no cell damage was observed in the contralateral cortices or in PBS-injected control cortices (Figure 2).

Effects of ONO-1078 and edaravone on NMDA-induced injury ONO-1078 at 0.1 and 0.3 mg/kg significantly reduced the lesion volume ($P<0.05$ or $P<0.01$, Table 1) and the lesion

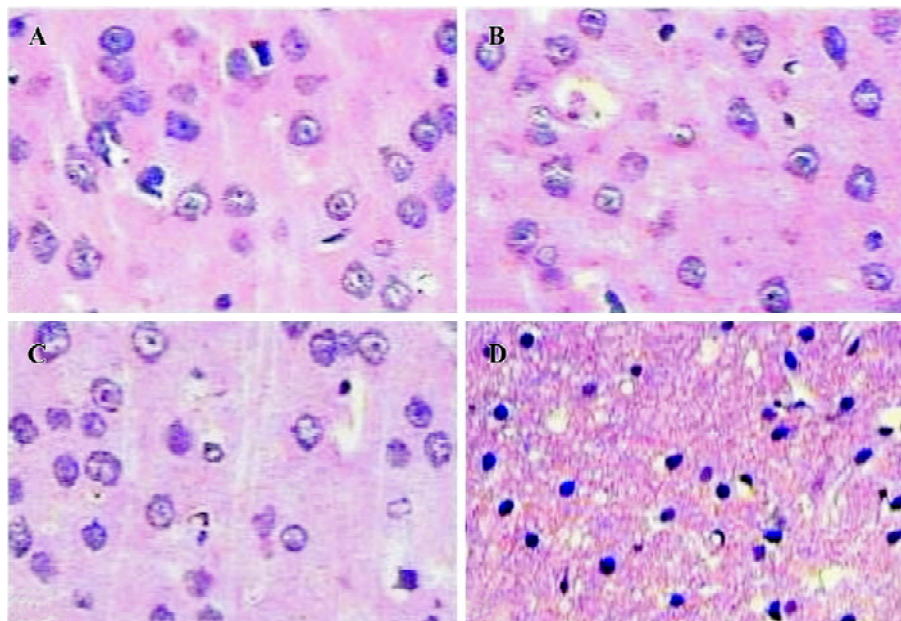


Figure 2. Histopathologic changes 24 h after direct microinjection of PBS (control) or NMDA into the cerebral cortex in rats. A: contralateral cortex of control rat; B: PBS-injected cortex of control rat; C: contralateral cortex of NMDA-injected rat; D: NMDA (0.3 μmol)-injected cortex. (HE staining, $\times 250$).

areas in individual 2 mm-thick consecutive slices ($P<0.05$ or $P<0.01$, Table 2), and the enlargement of injected cortices (indicated as I/C ratio) at 0.1 mg/kg ($P<0.05$, Table 1). The survival neuron densities in NMDA-injured regions showed a tendency to increase in the rats treated with ONO-1078, but no significant difference was found among these groups ($P>0.05$, Table 1). Edaravone (10 mg/kg) exhibited a similar effect to ONO-1078 (Tables 1, 2).

Effects of ONO-1078 and edaravone on VCAM-1 expression in NMDA-injected cortex To assess the relation between the protection of ONO-1078 and the expression of inflammatory adhesion molecule VCAM-1, we detected the local expression of VCAM-1 in contralateral and injured cortices 24 h after NMDA injection. VCAM-1 level did not remarkable change among the groups in the contralateral cortices without damage. In the injured cortices, however, VCAM-1 expression was up-regulated. ONO-1078 (0.1 and 0.3 mg/kg) significantly inhibited the increased VCAM-1 expression ($P<0.05$ or $P<0.01$), however, edaravone did not show a significant effect ($P>0.05$, Figure 3).

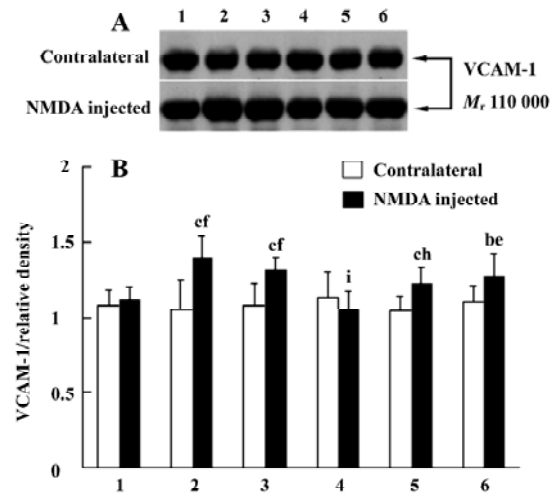


Figure 3. Effects of ONO-1078 and edaravone on VCAM-1 expression in rat cerebral cortex 24 h after NMDA microinjection. A: Western blot results; B: statistical analysis of VCAM-1 expression. 1: control rats with PBS-injected; 2: saline-treated rats with NMDA-injected; 3: ONO-1078 0.03 mg/kg; 4: ONO-1078 0.1 mg/kg; 5: ONO-1078 0.3 mg/kg; 6: edaravone 10.0 mg/kg. $n=6-8$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs contralateral. ^e $P<0.05$, ^f $P<0.01$ vs control. ^h $P<0.05$, ⁱ $P<0.01$ vs saline.

Table 1. Effects of ONO-1078 and edaravone on the lesion volume, the ratio of I/C cortex area and viable neuron counts (10^3 cells per mm^2) in cerebral cortex 24 h after NMDA microinjection in rats. Mean \pm SD. ^e $P<0.01$ vs control. ^f $P<0.05$, ⁱ $P<0.01$ vs saline. ^j $P<0.01$ vs contralateral cortices. I: NMDA injected, C: contralateral.

Drug/mg \cdot kg ⁻¹	<i>n</i>	Lesion volume (mm ³)	Ratio of I/C cortex area	Neuron densities in cortex	
				Contralateral	NMDA injected
Control	6	0 \pm 0	0.99 \pm 0.04	0.68 \pm 0.07	0.65 \pm 0.07
Saline	8	35.75 \pm 10.36 ^e	1.15 \pm 0.08 ^c	0.63 \pm 0.11	0.14 \pm 0.04 ^{si}
ONO-1078 0.03	8	29.92 \pm 12.26	1.12 \pm 0.09	0.64 \pm 0.12	0.15 \pm 0.04 ⁱ
0.1	8	21.12 \pm 7.10 ^f	1.05 \pm 0.06 ^e	0.66 \pm 0.07	0.19 \pm 0.07 ⁱ
0.3	8	23.74 \pm 10.76 ^e	1.08 \pm 0.06	0.65 \pm 0.10	0.18 \pm 0.06 ⁱ
Edaravone 10.0	8	21.46 \pm 7.57 ^f	1.06 \pm 0.07 ^e	0.64 \pm 0.10	0.17 \pm 0.05 ⁱ

Table 2. Effects of ONO-1078 and edaravone on lesion area in 2 mm-thick consecutive brain slices 24 h after NMDA microinjection in rats. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs saline.

Drug/mg \cdot kg ⁻¹	<i>n</i>	Lesion area (mm ²)/slice number			
		3	4	5	6
Control	6	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
Saline	8	1.93 \pm 3.50	11.61 \pm 2.48	4.16 \pm 4.90	0.19 \pm 0.52
ONO-1078 0.03	8	3.23 \pm 2.75	9.87 \pm 1.67	1.87 \pm 3.15	0 \pm 0
0.1	8	0 \pm 0	7.86 \pm 2.04 ^c	2.70 \pm 1.98	0 \pm 0
0.3	8	0.83 \pm 2.35	8.53 \pm 1.82 ^b	2.52 \pm 2.25	0 \pm 0
Edaravone 10.0	8	1.64 \pm 3.19	7.80 \pm 1.94 ^c	1.30 \pm 2.02	0 \pm 0

Discussion

The findings in the present study show that NMDA microinjection produces serious neocortical damage and a CysLT₁ receptor antagonist ONO-1078 possessed protective effect on NMDA-induced brain injury. This effect of ONO-1078 on chemically-induced brain injury is consistent with its protective effect on brain ischemic injuries discussed in our previous studies^[12]. Furthermore, the results of the present study indicates that NMDA microinjection up-regulates the local expression of VCAM-1, and ONO-1078 inhibits the enhanced VCAM-1 expression in the injured cortices.

The excessive release of excitory amino acids and activation of NMDA receptor are important events in cerebral ischemia and other brain injuries. NMDA receptor activation has been reported to be related to CysLT production in focal cerebral ischemia because NMDA receptor antagonist MK 801 reduces both CysLT production and ischemic injury^[17]. Also, LTC₄ synthesis inhibitor azelastine protects cultured hippocampal slices from hypoxic or NMDA-induced injury *in vitro*^[18]. Therefore, the protective effect of ONO-1078 in this study might be via antagonizing the actions of resultant CysLTs after NMDA receptor activation.

However, the increased VCAM-1 expression by NMDA injection suggests a possibility that excitotoxicity might initiate inflammation in the brain. It has been reported that NMDA receptor activation can up-regulate the expression of inflammatory cytokines, such as TNF- α and MCP-1^[19,20], and induce neuronal signal transduction-associated adhesion molecules, such as PSA-NCAM and NCAM-180^[21,22]. Our results further confirmed that NMDA increases the expression of inflammatory adhesion molecule VCAM-1. Evidence has shown the involvement of VCAM-1 in cerebral ischemic injury and inflammation. Serum level of soluble VCAM-1 increased in patients with ischemic stroke, at its peak 5 d after ischemia^[4]. VCAM-1 expression also increased in astrocytes and endothelial cells from the infarcted brain areas in patients who died of acute ischaemic stroke^[23], and in cultured human cerebrovascular endothelial cells after being subjected to ischemia-like insults or inflammatory cytokinins (IL-1 and TNF- α)^[24]. VCAM-1 promotes intracerebral inflammation by affecting leukocyte rolling, adhesion^[25] or earlier steps of interaction between inflammatory cells (such as T lymphocytes) and microvascular endothelial cells^[26]. Furthermore, we found that ONO-1078 inhibits NMDA-increased VCAM-1 expression. In other reported studies, CysLTs only slightly augmented VCAM-1 expression in human umbilical vein endothelial cells^[27]; CysLT₁ receptor antagonist ONO-1078^[16, 28] and montelukast^[29] sup-

pressed VCAM-1 expression in the lung after allergen challenge. Based on these findings, we hypothesized that NMDA receptor activation induces CysLT production, CysLTs activate their receptors followed by VCAM-1 expression. This might partially explain our previous results that ONO-1078 reduces VCAM-1 expression in rat global ischemia^[12].

As a control drug used in this study, edaravone protected NMDA-induced injury but did not reduce VCAM-1 expression compared with ONO-1078. Edaravone is a scavenger reacting with hydroxyl radical (OH)^[30] and might act on the signal pathways other than those of ONO-1078. In cultured human dermal microvascular endothelial cells, antioxidants show different actions on TNF- α -induced expressions of VCAM-1, ICAM-1 and E-selectin: pyrrolidine dithiocarbamate had inhibiting effects, but N-acetylcysteine did not^[31]. In our previous study of rat global ischemia, edaravone also did not significantly inhibit VCAM-1 expression^[12]. Its neuroprotective effect might be mediated by other actions, such as inhibition of mitochondrial permeability transition pore, upregulation of Bcl-2^[32], normalization of irradiation-reduced endothelial nitric oxide synthase expression^[33], and protection against hypoxia/ischemia-induced endoplasmic reticulum dysfunction^[34].

In the present study, we found that ONO-1078 reduced NMDA-induced lesion size, but did not increase survival neurons in the lesion, suggesting incomplete protection. We have not yet explained the details of NMDA receptor activation in CysLTs production, VCAM-1 expression and brain injury, however, the results of the present study indicate that CysLT₁ receptor antagonist ONO-1078 inhibits NMDA-induced brain injury and pro-inflammatory VCAM-1 expression, supplying further evidence for its neuroprotective effect on ischemic brain injury.

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