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RESEARCH

## Research Report

# Neutrophil elastase inhibitor attenuates hippocampal neuronal damage after transient forebrain ischemia in rats

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## ABSTRACT

Inflammatory responses have been known to contribute to the development of neuronal damage after brain ischemia in experimental animals. Also, neutrophil elastase activity in the plasma has been elevated in the patients with acute cerebral infarction. In order to clarify whether neutrophil elastase distributes into the brain parenchyma and exacerbates neuronal damage following ischemia, we examined the effects of specific neutrophil elastase inhibitor, ONO-5046, on hippocampal CA1 neuronal death in relation to neutrophil elastase activity in the plasma and its distribution in the brain and to caspase-3/7 activity. ONO-5046 (5 and 10 mg/kg) or saline (control group) was administrated after 8 min of forebrain ischemia in rats. Ratio of surviving neurons (median, [range]) in hippocampal CA1 seven days after ischemia was significantly higher in the ONO-5046 5 mg/kg (31% [12–57%]), and 10 mg/kg groups (69% [39–76%]) than in the control group (3.2% [0–10%]). Plasma neutrophil elastase activity in the ONO-5046 10 mg/kg group was significantly lower than in the control group (14 [11–25] vs. 41 [35–68] nmol/ml). Neutrophil elastase distributed in the extracellular space in the hippocampal CA1 neuronal layer in the control group, while, in the ONO-5046 10 mg/kg group, trace of neutrophil elastase was detected only in the endothelium. Caspase-3/7 activity was elevated after ischemia over 8 h in the control group, while, in the ONO-5046 10 mg/kg group, no elevation was observed. The results suggest that neutrophil elastase may contribute to neuronal death in hippocampal CA1 following forebrain ischemia and that neutrophil elastase inhibitor attenuates neuronal death.

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## 1. Introduction

Transient global cerebral ischemia causes delayed neuronal death in vulnerable regions such as hippocampal CA1. A

complex series of events has been suggested as the mechanisms, including excessive release of glutamate, accumulation of intracellular calcium, formation of free radicals and degradation of membrane lipids (Siesjö, 1988; Siesjö and

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Abbreviations: MABP, mean arterial blood pressure; ANOVA, analysis of variance; IL, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase

**Table 1 – Physiological variables for the histopathological outcome study**

	pH	PaCO <sub>2</sub> (mmHg)	PaO <sub>2</sub> (mmHg)	Hb (g/dl)	Glucose (mg/dl)	Temperature (°C)
Pre-ischemia						
Control	7.36±0.02	41±2	108±4	12.4±1	119±17	37.0±0.2
ONO-5046 5 mg/kg	7.36±0.03	41±2	108±5	13.4±1	125±14	37.0±0.1
ONO-5046 10 mg/kg	7.35±0.04	41±2	109±7	12.9±1	116±10	37.0±0.1
Post-ischemia						
Control	7.35±0.04	42±2	110±6	11.9±1	127±14	37.1±0.2
ONO-5046 5 mg/kg	7.36±0.04	41±3	115±9	12.7±1	135±15	37.1±0.2
ONO-5046 10 mg/kg	7.37±0.02	40±3	112±6	11.5±1	126±10	37.0±0.1

Values presented mean±SD. Post-ischemic values were obtained 30 min after forebrain ischemia. There were no significant differences in pre- and post-ischemic values in each group and no differences among three groups.

Bengtsson, 1989). In addition, the contribution of proinflammatory cytokines (e.g., IL-1 $\beta$ , IL-6, TNF $\alpha$ ) to delayed neuronal death has been suggested (Saito et al., 1996). Neutrophil elastase derived from activated neutrophils following inflammation or various stresses degrades a broad spectrum of extracellular matrix and cell surface proteins (Janoff, 1985; Mainardi et al., 1980; McDonald and Kelley, 1980). Intracerebral injection of neutrophil elastase has been shown to disrupt blood–brain barrier and to produce brain damage with intraparenchymal hemorrhage (Armao et al., 1997). It is of great interest that plasma neutrophil elastase levels were found to be increased in the patients with acute cerebral infarction (Iwatsuki et al., 1998). Furthermore, recent animal studies reported that specific neutrophil elastase inhibitor, ONO-5046, (Sodium N-[2-[4-(2, 2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl] aminoacetate tetrahydrate), has a protective effect against central nervous system damage induced by ischemia (Shimakura et al., 2000; Yamauchi et al., 2006) and trauma (Tonai et al., 2001), suggesting that neutrophil elastase plays an important role in the development of central nervous system damage. Thus, elucidation of the role of neutrophil elastase in developing ischemic neuronal damage and its modification by elastase inhibitor may provide an important therapeutic strategy in cerebral ischemia. We hypothesized that neutrophil elastase activated in plasma following brain ischemia exacerbates neuronal damage by distributing in the brain parenchyma. In the present study, we sought to investigate the involvement of inflammatory responses in aggravating selective neuronal death in the hippocampal CA1 following forebrain ischemia. We focused, in particular, on whether activation of neutrophil elastase and its distribution in the brain parenchyma is produced and whether pharmacological inhibition of activated neutrophil elastase attenuates neuronal death. Because the nature of the selective neuronal death in hippocampal CA1 is characterized, though not exclusively, by apoptotic cell death, we measured the chronological changes of caspase-3 activity which has been known to be involved in apoptotic signaling (Cao et al., 2002).

## 2. Results

### 2.1. Physiological variables for pre- and post-ischemia

In all series of experiments, pericranial temperature was maintained close to 37 °C throughout the experiments. Mean

arterial blood pressure (MABP) as well as PaCO<sub>2</sub> and PaO<sub>2</sub> was maintained in the normal ranges (except for MABP during ischemia). Tables 1 and 2 show physiological variables (pH, PaCO<sub>2</sub>, PaO<sub>2</sub>, hemoglobin, glucose, or pericranial temperature) and MABP in each group for the histopathological outcome study. There were no significant differences in physiological variables and MABP among the three groups. The physiological variables in another series of studies were comparable to those of the histopathological outcome study (data not shown).

### 2.2. Histopathological outcome 7 days after forebrain ischemia

Fig. 1 shows the representative microphotographs of the hippocampus in each group (7 days after ischemia) and percentage of surviving neurons in each group is illustrated in Fig. 2. Hippocampal CA1 neurons were selectively and extensively damaged in the control group, exhibiting only limited number of normal neurons (3.2% [0–10%] (median [range]) of those in the age matched normal rats). Pyramidal neuronal shrinkage and chromatin condensation of nuclei as well as reactive gliosis were observed. In the ONO-5046 5 mg/kg and 10 mg/kg groups, neuronal damage was significantly less compared with that in the control group, exhibiting normal neurons 31% [12–57%], and 69% [39–76%] ( $p < 0.001$ ), respectively. Administration of 10 mg/kg ONO-5046 most strongly attenuated hippocampal CA1 neuronal damage induced by transient forebrain ischemia.

### 2.3. Time course of neutrophil elastase activity in the plasma during reperfusion

Time course of neutrophil elastase activity in the plasma after ischemia in the control and the ONO-5046 10 mg/kg groups is shown in Fig. 3. In the control group, neutrophil elastase activity was time-dependently elevated over 60 min. After peaking at 60 min (41 [35–68] nmol/ml (median [range]) vs. baseline), the activity was declined to baseline at 4 and 8 h (0 [0–1] and 0 [0–4] nmol/ml, respectively). In the ONO-5046 10 mg/kg group, neutrophil elastase activity showed only slight insignificant increase at 60 min (14 [11–25] nmol/ml) and decreased to the baseline values at 4 and 8 h (0 [0–7] and 0 [0–15] nmol/ml, respectively) after ischemia. Neutrophil elastase activity at 60 min after ischemia in the ONO-5046 10 mg/kg group was

**Table 2 – Mean arterial blood pressure (MABP, mmHg) for the histopathological outcome study**

	Pre-ischemia	Ischemia		Post-ischemia
		4 min	8 min	
Control	97±9	47±2	47±2	99±8
ONO-5046 5 mg/kg	99±7	46±2	47±3	98±7
ONO-5046 10 mg/kg	99±7	46±2	47±2	99±9

There were no significant differences in MABP among three groups.

significantly lower than in the control group (14 [11–25] vs. 41 [35–68] nmol/ml,  $p=0.009$ ).

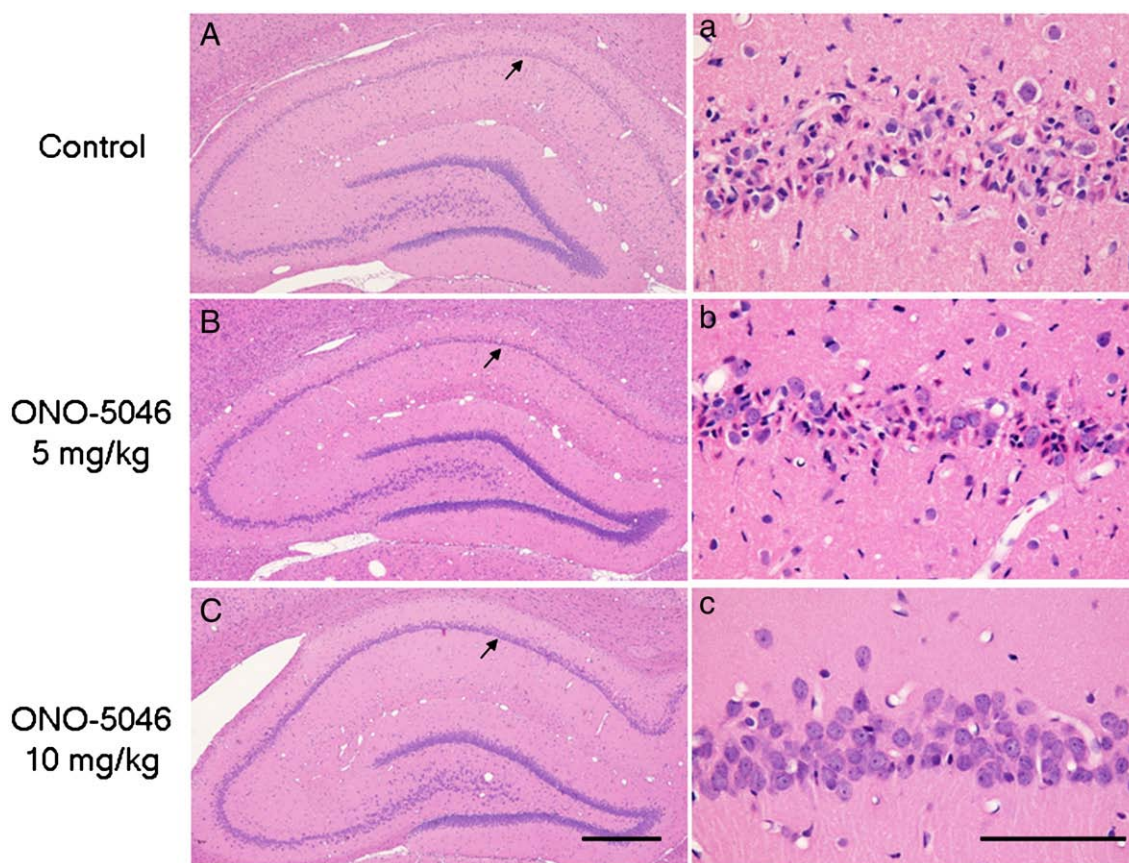
#### 2.4. Distribution of neutrophil elastase in the brain

Fig. 4 shows the distribution of immunohistochemical staining for neutrophil elastase in the area of hippocampus. In the control group (ischemia), neutrophil elastase immu-

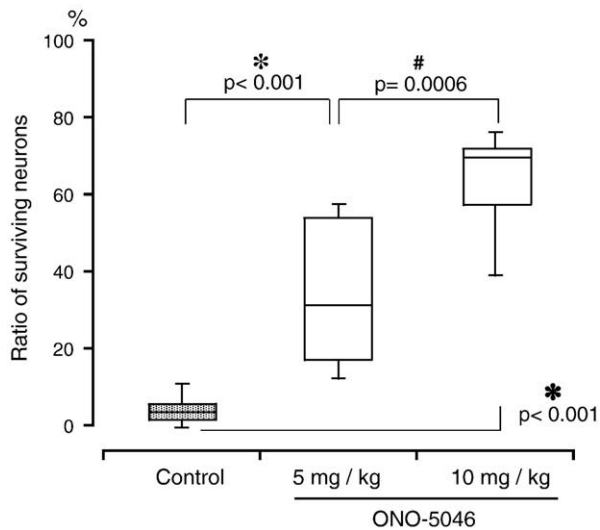
nostaining (intense brown-staining compared to the sham group (Figs. 4A, a)) was detected in endothelial cells and in peri-vascular space (Fig. 4c), but not in the hippocampal neuronal layer (Fig. 4C) at 4 h after ischemia. At 8 h, diffuse positive staining, as compared with negative control (Figs. 4B, b), was observed in the extracellular space adjacent to hippocampal CA1 neurons (Figs. 4D, d). In the ONO-5046 10 mg/kg group (Ischemia+ONO), elastase staining was detected only in endothelial cells, not in the peri-vascular space at 4 h (Fig. 4e) and not in the extracellular space adjacent to hippocampal CA1 neurons at 8 h (Fig. 4f).

#### 2.5. Time-course changes in caspase-3/7 activity in the hippocampus

Fig. 5 shows the changes in caspase-3/7 activity in the hippocampal CA1 region after ischemia. In the control group, caspase-3/7 activity was significantly elevated at 8 h (145% [114–190%] (median [range]) of baseline,  $p=0.003$ ) after ischemia. In the ONO-5046 10 mg/kg group, no elevation of caspase-3/7 activity was observed and the activity at 8 h (75%



**Fig. 1 – Histopathological outcome in hippocampal CA1 (at –3.8 mm from bregma) after forebrain ischemia (8 min)–reperfusion (7 days). Light microphotographs of the hippocampus with hematoxylin-eosin stain in the control group (A and a), ONO-5046 5 mg/kg group (B and b) and ONO-5046 10 mg/kg group (C and c). Panels (A and a), control group: selectively and extensively damaged neurons in CA1 are observed. Pyramidal neuronal shrinkage and chromatin condensation of nuclei as well as reactive gliosis is observed (a). Panels (B and b), ONO-5046 5 mg/kg group: Neurons with shrinkage and chromatin condensation with normal surviving neurons in pyramidal cell layer are observed. Panels (C and c), ONO-5046 10 mg/kg group: neurons in pyramidal cell layer of hippocampal CA1 are markedly preserved. Arrows indicate the area examined with a high power micrograph. (Scale bar: A–C, 500  $\mu$ m; a–c, 100  $\mu$ m).**



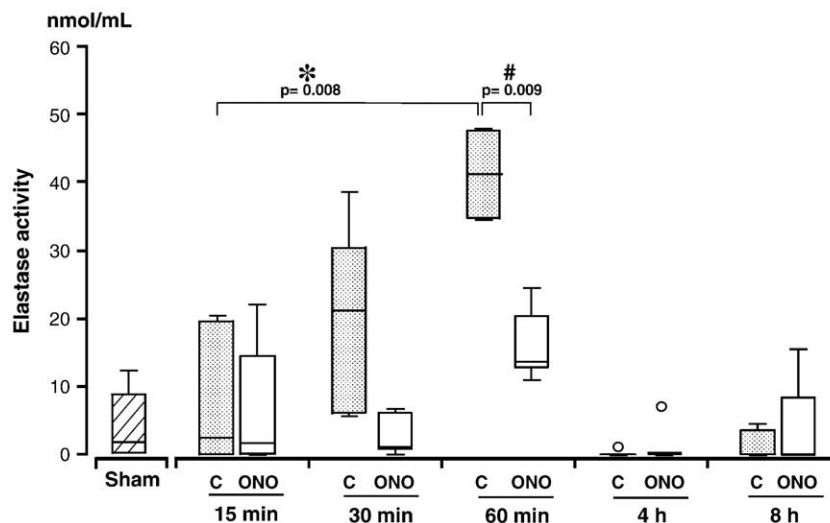
**Fig. 2 – Histopathological outcome in hippocampal CA1 after forebrain ischemia (8 min)–reperfusion (7 days).** Y axis represents ratio of surviving neurons (percentage of normal neurons in hippocampal CA1 in each group as referenced to those of the age matched normal rats). Box plots show the median (middle line of box), 25th–75th percentiles (box) and minimum/maximum values (lower/upper whiskers). In the ONO-5046 5 mg/kg and 10 mg/kg groups, neuronal damage is significantly less compared with the control group. ONO-5046 10 mg/kg administration leads to a greater attenuation of ischemic neuronal damage compared to that with 5 mg/kg administration. (\* $p < 0.01$  vs. control group, # $p < 0.01$  vs. ONO-5046 5 mg/kg group, respectively).

[66–116%],  $p = 0.006$ ) was significantly lower than that of the control group.

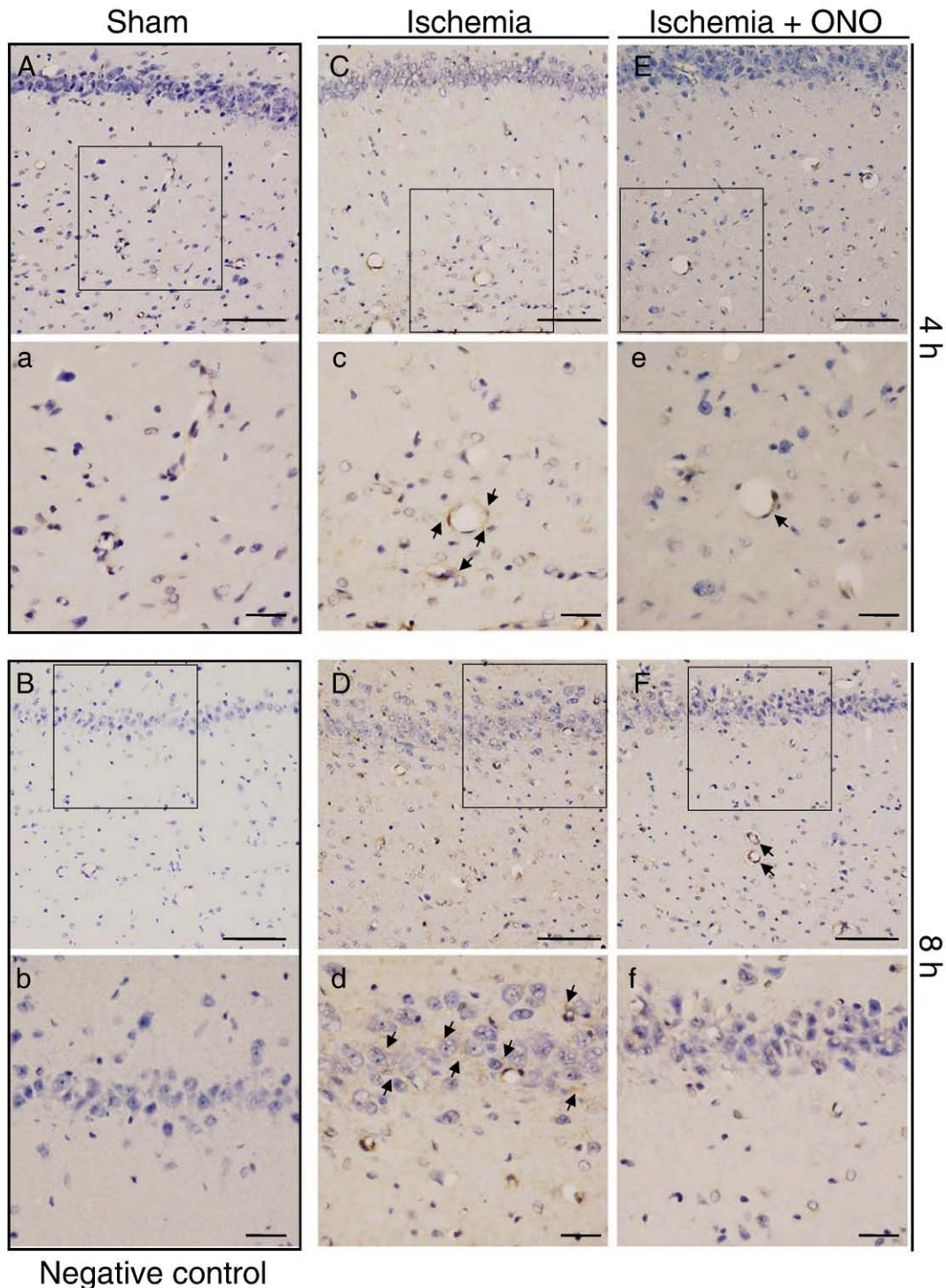
### 3. Discussion

In the present study, we demonstrated that a specific neutrophil elastase inhibitor, ONO-5046, ameliorated ischemic neuronal damage in hippocampal CA1 7 days after 8-min forebrain ischemia in rats. Ameliorating effect was associated with an inhibition of activated neutrophil elastase in the plasma and distribution in the brain parenchyma as well as an inhibition of caspase-3/7 activity. These results suggest that elastase contributes to the development of ischemic neuronal damage in hippocampal CA1 induced by transient forebrain ischemia.

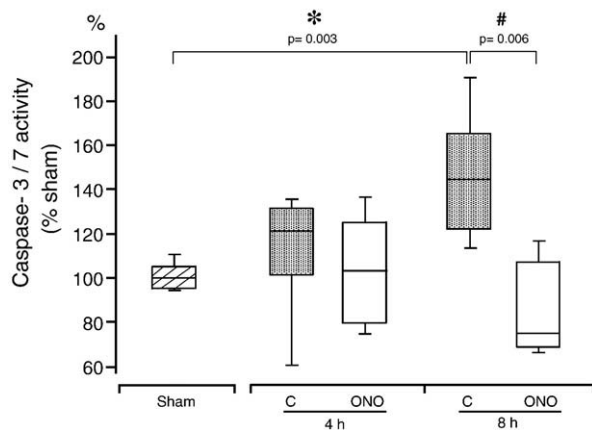
There have been a few reports regarding the protective effects of neutrophil elastase inhibitor, ONO-5046, in various models of central nervous system damage (Shimakura et al., 2000; Tonai et al., 2001; Yamauchi et al., 2006). Shimakura et al. (2000) first demonstrated in focal cerebral ischemia, induced by occlusion of middle cerebral artery (MCA), that ONO-5046, given just after occlusion of MCA or from 3 h after ischemia, reduced brain damage (as evaluated by the size of damaged area and water content) 24 h following ischemia, while not affecting the accumulation of neutrophils. Attenuating effects of ONO-5046 have also been reported in spinal cord damage induced either by ischemia (Yamauchi et al., 2006) or compression (Tonai et al., 2001). Our finding that ONO-5046 attenuated neuronal damage is in accordance with these reported results, despite the different experimental models. Shimakura et al. (2000) suggested that the protective



**Fig. 3 – Time-course changes of neutrophil elastase activity in plasma during reperfusion.** Y axis represents neutrophil elastase activity, quantified by spectrophotometrical analysis. Box plots show the median (middle line of box), 25th–75th percentiles (box) and minimum/maximum values (lower/upper whiskers). In the control group (C), neutrophil elastase activity was significantly elevated at 60 min. In the ONO-5046 10 mg/kg group (ONO), elastase activity showed only slight insignificant increase at 60 min and decreased to the baseline values at 4 and 8 h. At 60 min after ischemia, neutrophil elastase activity in the ONO-5046 10 mg/kg group (ONO) was significantly lower than in the control group. (\* $p < 0.01$  vs. baseline, # $p < 0.01$  vs. control group, ○ = outliers (outside threefold of the whole box length)).



**Fig. 4** – Distribution of neutrophil elastase in the brain. Panels (A and a), sham group. Panels (B and b), negative control (reperfusion 8 h after ischemia), shows lack of neutrophil elastase staining in peri-vascular area (B) and in the hippocampal neuronal layer (b). Panels (C and c), 4 h after ischemia in the control group: immunohistochemical staining is observed in the endothelial cell and peri-vascular area (c, arrow), not in the hippocampal neuronal layer (C). Panels (D and d), 8 h after ischemia in the control group: diffuse staining is observed in the extracellular space adjacent to hippocampal CA1 neurons (d, arrow). Panels (E and e), 4 h after ischemia in ONO-5046 10 mg/kg group: elastase staining is detected in endothelial cells, not in the peri-vascular space (e, arrow). Panels (F and f), 8 h after ischemia in ONO-5046 10 mg/kg group: elastase staining localized within endothelial cells (F, arrow), not in the extracellular space adjacent to hippocampal CA1 neurons (f). Panels (a-f) respectively show high power magnification of the boxed area in corresponding low power picture. All photographs were taken under same setting (shutter speed; 1/40 under low-magnification and 1/30 s under high-magnification view). (Scale bar: A-F, 100  $\mu$ m; a-f, 25  $\mu$ m).



**Fig. 5 – Caspase-3/7 activity (% sham) in the hippocampal CA1 region after ischemia. The values are expressed as percent of those values obtained in the normal (sham) rats (% sham). Box plots show the median (middle line of box), 25th–75th percentiles (box) and minimum/maximum values (lower/upper whiskers). In the control group (C), caspase activity was significantly elevated at 8-hour reperfusion. In the ONO-5046 10 mg/kg group (ONO), caspase activity was significantly inhibited at 8-hour reperfusion. (\* $p < 0.01$  vs. sham group, # $p < 0.01$  vs. control group, respectively).**

mechanism of ONO-5046 against ischemic damage may be related to an inhibition of various profiles induced by infiltrating neutrophils, such as an adherence of neutrophils to the endothelium, production of toxic metabolites, proinflammatory cytokines and elastase, or activation of phospholipase. In the spinal cord compression model (Tonai et al., 2001), protective effect was suggested to be the result of blocking cytokine-induced neutrophil chemo-attractant-1 mRNA and protein, preventing neutrophil activation-infiltration and vascular endothelial injury. It has been known that, in addition to proinflammatory cytokines (Owen et al., 1997), reactive oxygen species (Demopoulos et al., 1980) and proteolytic enzymes (Taoka et al., 1997) were produced with infiltrated leukocytes, which all can exacerbate ischemic neuronal damage. However, in the transient global forebrain ischemia model used in the present study, characteristic neuronal damage is selective and delayed neuronal death in hippocampal CA1 without recruitment of hematogenous inflammatory cells (Stoll et al., 1998). In the present study, significant increase in activated neutrophil elastase in the plasma and its distribution in the brain parenchyma was observed in the control group. A specific neutrophil elastase inhibitor, ONO-5046 (Kawabata et al., 1991) inhibited plasma elastase activity and elastase presence in the extracellular matrix after ischemia. It is therefore suggested that neutrophil elastase contributes to the development of ischemic neuronal damage by elastase distribution in the matrix component. Armao et al. (1997) demonstrated that intracerebral injection of neutrophil elastase disrupted blood-brain barrier and provoked brain tissue damage with intraparenchymal hemorrhage. Our immunohistochemical examination showed neutrophil elastase in the endothelium of hippocampal vessels,

peri-vascular regions and even in parenchyma in the hippocampal CA1 region. Although the nature of brain damage differs between the studies by Armao et al. (1997) and ours, it is possible that neutrophil elastase, when transferred into parenchyma, can exacerbate ischemia-induced brain damage. The present study is the first to demonstrate in forebrain ischemia that neutrophil elastase inhibitor, ONO-5046, significantly ameliorates neuronal damage in hippocampal CA1, when given immediately after reperfusion.

The mechanism for delayed neuronal death has been argued from the intracellular signaling pathways such as glutamate acting via *N*-methyl-*D*-aspartate receptors, disintegration of cellular ion metabolism with subsequent intracellular calcium accumulation or caspase-mediated apoptosis (MacManus et al., 1993; Siesjö, 1988; Siesjö and Bengtsson, 1989). Interestingly, in a recent study in spinal cord ischemia, Yamauchi et al. (2006) reported that attenuation of delayed motor neuron death with ONO-5046 was accompanied by an inhibition of caspase-3 expression. Our result that attenuation of neuronal death in hippocampal CA1 with ONO-5046 was accompanied by an inhibition of caspase-3/7 activity is in accordance with the result reported by Yamauchi et al. (2006) in spinal cord ischemia. The present study demonstrated that the elevation of caspase-3/7 activity in hippocampal CA1 in untreated (control) rats was accompanied by expression of neutrophil elastase not only in the peri-vascular space but also into the extracellular space in the neuronal layer of hippocampal CA1. In contrast, in the rats treated with ONO-5046, no staining was detected for neutrophil elastase in the extracellular space and caspase-3/7 activity was significantly inhibited at 8 h after ischemia. The attenuation of neuronal death in hippocampal CA1 with a specific neutrophil elastase inhibitor ONO-5046 was accompanied by an inhibition of caspase-3/7 activity and by inhibition of neutrophil elastase activity in the plasma and of its distribution in the brain parenchyma. Although it is undetermined whether the decreased activities of caspase-3/7 is the cause of or a result of attenuated damage, the results of the present study indicate that activation of neutrophil elastase is involved in the development of ischemic neuronal damage in hippocampal CA1 induced by transient forebrain ischemia. Neutrophil elastase is likely to operate both directly and indirectly in modulating fibrotic extracellular matrix at different levels (Chua and Laurent, 2006). Matrix metalloproteinase-9 (MMP-9) was suggested to be representative measurements of extracellular proteolysis. Our preliminary investigation showed that MMP-9 activity at 1 day-reperfusion ( $n=4$ , in each group) in the ONO-5046 group tended to be decreased compared to the control group (see Supplementary Fig. 1).

There are some limitations of the present study. First, because the neutrophil elastase inhibitor was given immediately after reperfusion, clinical relevance is limited. Second, the assessment of neuropathological outcome 7 days after ischemia may not be long enough and the possibility that the drug may only delay neuronal injury cannot completely be excluded. Third, current experimental design is not sufficient to claim the source of the elastase present in the ischemic brain because the possibility that microglia can secrete elastase-like protease (Nakajima et al., 1992). Although these limitations need to be addressed in the future work, the

current study suggests that activation of neutrophil elastase following ischemia is involved in exacerbating selective neuronal damage in hippocampal CA1.

In summary the results suggest that neutrophil elastase possibly, but may not exclusively, derived from activated leukocytes contributes to the development of selective neuronal death in hippocampal CA1 after 8 min-forebrain ischemia in rats and that a specific neutrophil elastase inhibitor, ONO-5046, ameliorates neuronal damage via inhibition of neutrophil elastase distribution in the extracellular matrix after ischemia. Further study may be needed to determine the therapeutic time window for ONO-5046 and the effects of higher or repeated doses as well as the detailed mechanisms for interaction between neutrophil elastase and activation of caspase.

## 4. Experimental procedures

### 4.1. Drugs

Sodium N-[2-[4-(2, 2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl] aminoacetate tetrahydrate (ONO-5046) was kindly provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). The elimination half life [ $t_{1/2}$  (2–60 min), and  $t_{1/2}$  (240–480 min)] of ONO-5046 (evaluated as radioactivity) has been reported to be  $15.6 \pm 1.8$  and  $164 \pm 51$  min, respectively after a single intravenous administration of 10 mg/kg  $^{14}\text{C}$ -ONO-5046 in male rats.

### 4.2. Animals and experimental design

The study protocol was reviewed and approved by the Committee of the Ethics on Animal Experiment and carried out under the control of Guidelines for Animal Experiment in Yamaguchi University Graduate School of Medicine. All experiments were carried out in male Wistar rats aged 8 to 9 weeks (280–330 g, Kyudo, Kumamoto, Japan) subjected to 8 min period of forebrain ischemia produced by bilateral carotid artery occlusion and hemorrhagic hypotension. We sought to evaluate neuroprotective effect of the post-ischemic treatment with neutrophil elastase inhibitor, ONO-5046, in hippocampal CA1 and the mechanism for neuroprotection, if any, in relation to neutrophil elastase activity in the plasma and its distribution in the brain as well as to the temporal profile of caspase activity.

### 4.3. Forebrain ischemia

Fasted rats for 12 h were anesthetized with 3% isoflurane and their trachea intubated, and mechanically ventilated with a gas mixture of 2% isoflurane in  $\text{O}_2$  and nitrous oxide (30:70). A polyethylene catheter was placed into the tail artery for continuously monitoring blood pressure and for obtaining arterial blood samples. Pericranial temperature (temporalis muscle temperature) was monitored and maintained at close to 37 °C throughout the experiments. Bilateral carotid arteries were carefully isolated for later temporal occlusion. A vascular catheter was placed via right external jugular vein with its tip at the right atrium for drug administration and for inducing

hemorrhagic hypotension. Before starting ischemia the following criteria were assured:  $\text{PaO}_2$  90–140 mmHg;  $\text{PaCO}_2$  35–45 mmHg; pH 7.35–7.45; and blood glucose level 90–140 mg/dl. Forebrain ischemia of 8 min duration was induced by the method of Smith et al. (1984) with a slight modification (Hirata et al., 2007). First, hypotension was induced by 0.01 mg of phentolamine and then bilateral carotid arteries were occluded with vascular clips and MABP was maintained at 45–50 mmHg by withdrawing or infusing blood using heparinized syringe (see Results, Table 2). After 8 min ischemia, vascular clips were removed, and withdrawn blood was infused. The vascular catheters were removed and wounds were sutured. Thereafter anesthesia was discontinued and animals were extubated after recovery of spontaneous respiration and placed in a temperature controlled room. The transient forebrain ischemia model used in the present study is the one that produces selective and delayed neuronal death in hippocampal CA1 with no mortality.

### 4.4. Histopathological outcome study

Thirty six rats were randomly assigned to one of the following three groups: control group, ONO-5046 5 mg/kg group, and ONO-5046 10 mg/kg, respectively ( $n=12$  in each). Rats in the treated groups received a single intravenous injection of ONO-5046 at doses of 5 or 10 mg/kg, dissolved in 1 ml of saline, immediately after reperfusion. In the control group, 1 ml of saline was administered in the same manner. We have attempted to evaluate the effect of three doses, 5 mg/kg, 10 mg/kg and 30 mg/kg. However, 30 mg/kg of ONO-5046 was not adequately dissolved in 1 ml of saline. Seven days after ischemia, the rats were reanesthetized with 4% isoflurane and their brains were fixed by trans-cardiac perfusion with saline containing heparin (4 U/ml) followed by 4% paraformaldehyde. Coronal sections (6  $\mu\text{m}$  thickness) of the brain embedded in paraffin including the dorsal hippocampus (at the level of  $-3.8$  mm from bregma) were stained with hematoxylin and eosin (HE). The number of intact neurons was counted in a blind fashion along the pyramidal layer of the hippocampal CA1 region for 1 mm using light microscopy. The neuronal densities (cell/mm) obtained in both right and left hemispheres were averaged and presented as the percentage of the normal neurons referenced to those of the age-matched eight normal rats. Normal neurons were defined as the neurons with no shrinkage and no chromatin condensation of nuclei.

### 4.5. Time-course changes in neutrophil elastase activity in the plasma

In fifty rats, we investigated the time-course changes in neutrophil elastase activity after ischemia (15, 30, 60 min, 4, 8-h of reperfusion,  $n=5$  at each time-point) in the control and ONO-5046 10 mg/kg groups, respectively. Preparation and ischemia protocol are the same as the first series of experiment. ONO-5046 10 mg/kg was selected because this dose showed greater neuroprotective effect than with 5 mg/kg (see Results, Fig. 2).

Blood samples (1 ml/rat) were obtained through the venous catheter placed in the right atrium. Because the blood volume

required for assay is rather large, only one time point measurement for each rat was done. Plasma samples were isolated from blood anticoagulated with EDTA by centrifugation (3000  $\times$ g for 3 min) and were stored frozen. Assay of neutrophil elastase activity was performed at the central laboratory (Mitsubishi Chemical Medience Co. Ltd., Tokyo, Japan). Briefly, neutrophil elastase activity was determined with the synthetic substrate *N*-methoxysuccinyl-Ara-Ara-Pro-Val *p*-nitroanilide, which is highly specific for neutrophil elastase (Yoshimura et al., 1994). Samples were incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate for 24 h at 37 °C. After incubation, *p*-nitroaniline was measured spectrophotometrically at 405 nm, and absorbance, corrected for baseline activity, was used as an index of neutrophil elastase activity.

#### 4.6. Immunohistochemistry of neutrophil elastase in the brain

In two groups of rats (control and ONO-5046 10 mg/kg), distribution of activated neutrophil elastase in the brain was assessed at two time points, 4 and 8 h after ischemia ( $n=6$  at each time-point). Sham group of rats ( $n=6$ ) were prepared without subjecting to ischemia. The brains were perfused transcardially with ice-cold Tris-buffered saline (TBS), pH 7.4, followed with ice-cold 4% paraformaldehyde in TBS. The brains were removed, immersed with the same fixative overnight. The brains were cryoprotected in 30% sucrose in PBS, and frozen with hexane cooled in liquid nitrogen. Frozen coronal sections (10  $\mu$ m thick) were prepared using a cryostat. After blocking endogenous peroxidase with 0.3% hydrogen peroxide in methanol and blocking nonspecific background with TBS containing 3% normal rabbit serum, sections were incubated overnight at 4 °C with neutrophil elastase antibody (diluted in 1:100; Santa Cruz Biotechnology, Santa Cruz, CA). Detection of the primary antibody was performed by the supersensitive Multilink-HRP/DAB detection kit (BioGenex, San Ramon, CA) using the biotin-streptavidin-peroxidase technique, followed by counterstaining with hematoxylin. The staining procedures were carried out using an automated immunostainer, Optimax plus (BioGenex).

#### 4.7. Time-course changes in caspase activity in the brain

To investigate how an inhibition of neutrophil elastase activity and its penetration into the brain parenchyma influence on neuronal death, we attempted to see biochemical events by measuring temporal profile of caspase (caspase-3/7) in hippocampal CA1 in the control group and ONO-5046 10 mg/kg group (4 and 8-hour reperfusion,  $n=6$  at each time point). The method for obtaining the tissue sample containing hippocampal CA1 region was previously described (Hirata et al., 2007). Samples in each group were stored  $-80$  °C until later analysis. Measurement of caspase-3/7 activity was performed by using a homogeneous luminescent assay which measures caspase-3 and -7 (Caspase Glo™ 3/7, Promega, Madison, WI) according to the manufacturer's instruction (Liu et al., 2004). Briefly, the frozen tissues were lysed using a tungsten carbide bead in hypotonic extraction buffer (25 mM HEPES, pH 7.5,

5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 1  $\mu$ g/ml each pepstatin, leupeptin, and aprotinin, and 1 mM phenylmethanesulfonyl fluoride) with a Mixer Mill MM300 (Rescht, Haan, Germany) at 30 Hz for 30 s. The lysates were centrifuged (12 000  $\times$ g, 15 min, 4 °C), and the supernatants were used for protein determination. The protein concentrations of supernatant were adjusted to 1 mg/ml with extraction buffer. The extracted protein (10  $\mu$ g/ml) was added to equal volume of Caspase-Glo reagents in a white-walled 96-well plate and incubated at room temperature for 1 hour. The luminescence of each samples was measured in a plate-reading luminometer (MicroLumat LB 96P, EG and G Berthold, Bad Wildbad, Germany). The values are expressed as percent of those values obtained in the normal rats (sham group,  $n=6$ ).

#### 4.8. Statistical analysis

Physiological parameters analyzed for one-way ANOVA followed by post hoc Bonferroni–Dunn test were used to detect significant difference and were expressed as mean  $\pm$  SD. Statistical analysis for ratio of surviving neurons, elastase activity, and caspase-3/7 activity was performed by nonparametric test (Mann–Whitney *U*-test for two-independent samples and Kruskal–Wallis for multiple comparison) to determine significance. Results were expressed as medians [range] and box plots were used to graphically display data distributions. Differences were considered significant with *p* value less than 0.01.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.brainres.2008.12.070](https://doi.org/10.1016/j.brainres.2008.12.070).

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