Neuroprotective Effects of Ebselen Following Forebrain Ischemia: Involvement of Glutamate and Nitric Oxide

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Abstract

Ebselen is a mimic of glutathione peroxidase that reacts with peroxynitrite and inhibits nitric oxide (NO) synthase. Ebselen has beneficial effects on the neurological outcome of patients with stroke. In this study, the mechanisms by which ebselen can elicit neuroprotective effects against ischemic brain injury were investigated in male Wistar rats. Experimental forebrain ischemia was induced by bilateral common carotid artery occlusion with hemorrhagic hypotension. Ebselen was administered to animals in the treatment group 2 hours prior to the induction of forebrain ischemia, and placebo was administered in the control group. Cerebral blood flow (CBF) was measured by the hydrogen clearance method. Cortical extracellular levels of excitatory amino acids (EAAs) and NO were evaluated using in vivo microdialysis. Neuronal damage in the CA1 subfield of the hippocampus was assessed in brains harvested after a 24-hour period of survival. CBF did not recover to normal physiological levels after ischemic insults in either the control or treatment groups. The differences in the sequential changes in extracellular EAA and NO levels between groups were not statistically significant. There was a significantly larger mean density of intact, undamaged neurons in the CA1 subfield in the treatment group than in the control group. The neuroprotective effects of ebselen were reflected in the histological findings, without significant inhibition of glutamate release or NO synthesis during the acute phase of experimentally induced cerebral ischemia.

Key words: ebselen, forebrain ischemia, excitatory amino acid, nitric oxide, in vivo microdialysis

Introduction

An increasing amount of experimental evidence has clarified the involvement of oxygen-derived free radicals, which can be generated under conditions of hypoxia or posts ischemic reperfusion, in ischemia-related brain injury.2,15,24) Laboratory studies have established that some antioxidants which scavenge free radicals can provide some protection from the cerebral infarction resulting from experimental cerebral ischemia. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), is a synthetic seleno-organic compound that has been developed as a free radical scavenger.23) This antioxidant has glutathione peroxidase (GSX)-like activity and inhibits lipid peroxidation in vitro. Clinical studies of the effects of ebselen as a free radical scavenger22,32) have shown that the scavenging activity can be explained by this unique GSX-like activity. However, little data is available on the actions of antioxidants in brain tissue during ischemic/hypoxic insults, against which ebselen affords some neuroprotection. Recently, this GSX-like agent has been described as an inhibitor of nitric oxide (NO) synthase (NOS).11) Blockade of NO synthesis causes a dramatic reduction in the volume of the cortical infarct induced by irreversible focal cerebral ischemia in the mouse,21) so application of NOS inhibitors to the cerebral ischemic insults should provide some neuroprotection from any associated neuronal damages. A number of laboratory-based trials testing several types of NOS inhibitors on animal models have now been carried out,5,6,10,18,26,30,33–35) but no conclusion has been reached in the debate about the efficacy of NOS inhibitors as protectors against cerebral ischemic insult. Activation of N-methyl-D-aspartate (NMDA) receptors, overstimulation of which causes massive influx of calcium ions at toxic levels in neurons, has been recently found to be associated with an increase in intracellular NO levels in rat cerebellar slices.11) In addition, NOS inhibitors prevent the neu-
rotoxicity elicited by NMDA and related excitatory amino acids (EAAs), indicating that NO mediates the neurotoxicity of glutamate. Other experimental evidence suggests that endogenous EAAs are released subsequent to the formation of free radicals.  

The present study investigated the possibility that the NOS-inhibitory effects of ebselen might mediate the neurotoxicity elicited by EAAs and NO during cerebral ischemic insult in the rat brain. To determine whether ebselen can reduce the synthesis of NO and/or EAA release from the presynaptic terminal of neurons, the cortical extracellular levels of these factors were measured following experimental forebrain ischemia, using in vivo microdialysis, and the neuroprotective effects investigated in the CA1 subfield of the rat hippocampus.

Materials and Methods

The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine, Japan, and were performed according to the Guidelines for Animal Experimentation of Yamaguchi University School of Medicine and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Male Wistar rats weighing 380–420 g (mean 392 ± 7.3 g) were used. After induction of general anesthesia with halothane, the animals were intubated and connected to a ventilator (Harvard Apparatus, Holliston, Massachusetts, USA). General anesthesia was maintained with 1% halothane and a mixture of 30% oxygen and 69% nitrous oxide. Rats were placed in the supine position, then the bilateral common carotid arteries were exposed, and the femoral artery and vein were cannulated with polyethylene catheters (PE-50; Intramedic A/S, Gentofte, Denmark) for blood gas sampling and withdrawal of venous blood to induce systemic hypovolemic hypotension, respectively. A craniotomy was made in the right parietal skull (8 × 6 mm). A microdialysis probe (CMA/12; Carnegie Medicine, Stockholm, Sweden) and a thermocouple probe (Unique Medical Co., Fukuoka) were implanted into the right parietal cortex of the rat brain using a stereotaxic device. The thermocouple probe for monitoring brain temperature was attached to a platinum electrode, which was used to evaluate cerebral blood flow (CBF) by the hydrogen clearance method. The microdialysis probe was perfused with Ringer’s solution at a flow rate of 0.2 μL/min, and dialysate samples (20 min each) were collected. The concentrations of glutamate and aspartate in the samples were analyzed by high-performance liquid chromatography, and the dialysate content of nitrite and nitrate was analyzed by capillary electrophoresis.

Ebselen was generously provided by Daiichi Pharmaceutical Company (Tokyo). The animals were divided randomly into one of two groups. In the treatment group (n = 12), ebselen (30 mg/kg) dissolved in distilled water was administered through a transoral gastric tube 120 minutes prior to the induction of transient forebrain ischemia, at which point the blood levels of ebselen would peak. In the control group (n = 12), a placebo (lactose and corn starch, 30 mg/kg) was administered transorally 120 minutes prior to the induction of ischemia.

Experimental forebrain ischemia was induced by bilateral common carotid artery occlusion combined with systemic hypovolemic hypotension: 7.5 to 10 ml of blood were withdrawn so that the mean blood pressure was maintained below 50 mmHg. After the 40-minute period of ischemic insult, the clamps on the carotid arteries were released, and the blood withdrawn from the femoral veins was reinjected via the venous catheters. The blood pressure of the animals remained within normal physiological limits.

Animals were fixed by transcardiac perfusion with 4% paraformaldehyde solution after a 24-hour survival period. The brains were then transferred to fixative and stored overnight, then processed routinely for paraffin embedding. Serial 8-μm coronal sections of the brain were cut and stained with hematoxylin and eosin. These sections were studied in detail under a light microscope, and the CA1 subfield of the dorsal hippocampus was enlarged to a magnification of ×20. Neuronal damage in area CA1 of the right hippocampus of the rat brain following the transient ischemic insult was assessed by counting the number of histologically intact CA1 pyramidal neurons. Five adjacent semicontiguous sections from the same area of brain tissue for each animal containing striking neuronal damage to area CA1 of the hippocampus were similarly examined in a blind investigation. The cell counts are expressed as the neuronal density per 1-mm linear length of the CA1 sector. Finally, the mean value of the neuronal density in the CA1 sector was estimated for each animal. The Mann-Whitney U-test was employed to detect statistical significance of differences in the extent of neuronal damages between the control and the treatment groups.

Results

Physiological variables of animals during the experiment are shown in Table 1. The arterial blood pres-
Table 1  Physiological variables

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experimental group</th>
<th>0 Min</th>
<th>30 Min</th>
<th>60 Min</th>
<th>90 Min</th>
<th>150 Min</th>
<th>210 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>Ebselen</td>
<td>105.8 ± 1.9</td>
<td>110.2 ± 1.3</td>
<td>71.8 ± 4.6</td>
<td>121.7 ± 3.1</td>
<td>119.7 ± 2.2</td>
<td>120.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>111.5 ± 2.8</td>
<td>115.8 ± 2.5</td>
<td>69.7 ± 5.1</td>
<td>118.7 ± 3.7</td>
<td>118.5 ± 2.6</td>
<td>122.5 ± 3.0</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>Ebselen</td>
<td>64.8 ± 1.6</td>
<td>71.3 ± 1.2</td>
<td>37.7 ± 3.5</td>
<td>79.3 ± 4.7</td>
<td>70.7 ± 1.7</td>
<td>73.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>70.7 ± 1.6</td>
<td>72.2 ± 3.0</td>
<td>36.8 ± 4.5</td>
<td>76.3 ± 5.0</td>
<td>75.8 ± 4.0</td>
<td>80.2 ± 3.0</td>
</tr>
<tr>
<td>pH</td>
<td>Ebselen</td>
<td>7.42 ± 0.02</td>
<td>7.41 ± 0.02</td>
<td>7.40 ± 0.03</td>
<td>7.40 ± 0.02</td>
<td>7.38 ± 0.01</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>7.44 ± 0.02</td>
<td>7.41 ± 0.01</td>
<td>7.36 ± 0.02</td>
<td>7.38 ± 0.02</td>
<td>7.40 ± 0.02</td>
<td>7.37 ± 0.01</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>Ebselen</td>
<td>35.0 ± 1.3</td>
<td>35.9 ± 1.7</td>
<td>37.2 ± 4.6</td>
<td>35.8 ± 1.8</td>
<td>38.4 ± 1.2</td>
<td>39.3 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>37.6 ± 1.5</td>
<td>40.1 ± 1.6</td>
<td>39.3 ± 3.1</td>
<td>40.3 ± 2.5</td>
<td>37.4 ± 1.4</td>
<td>40.3 ± 1.1</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>Ebselen</td>
<td>144.5 ± 9.1</td>
<td>155.1 ± 6.7</td>
<td>189.3 ± 17.7</td>
<td>172.1 ± 5.6</td>
<td>144.9 ± 8.2</td>
<td>154.0 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>135.3 ± 12.8</td>
<td>153.1 ± 12.5</td>
<td>167.1 ± 24.2</td>
<td>127.4 ± 20.9</td>
<td>121.8 ± 15.7</td>
<td>138.0 ± 12.4</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard error of the mean. DBP: diastolic blood pressure, SBP: systolic blood pressure.

Changes in cerebral blood flow (CBF) was measured prior to termination of the experiment (27.7 ± 7.2 ml/100 g/min in the control group, 57.2 ± 11.4 ml/100 g/min in the treatment group). The differences in the sequential changes in CBF between the two groups were not significant statistically.

Changes in the extracellular levels of amino acids before and after transient forebrain ischemia are shown in Fig. 2. In the control group, the 40-minute period of forebrain ischemia produced an approximately 10-fold increase in the extracellular levels of glutamate to 23.1 ± 11.4 μM/l from the pre-ischemic baseline level of 2.3 ± 0.6 μM/l. In both the control and treatment groups, the range of changes in extracellular levels of aspartate was smaller than that of glutamate. However, in the control group, it increased to a peak level of 4.9 ± 2.7 μM/l during the ischemic insult, and declined gradually to pre-ischemic baseline level of 0.4 ± 0.3 μM/l at 240 minutes after reversal of the insult. In the treatment group, surges in the extracellular levels of glutamate (14.4 ± 8.5 μM/l) and aspartate (3.7 ± 2.5 μM/l) were observed during ischemia. However, levels of these amino acids remained lower in the treatment group than in the control group. Following these ischemia-induced surges, extracellular levels of glutamate and aspartate in the treatment group returned to the baseline by 120 minutes after recanalization of the common carotid arteries. There was no significant difference in these changes between the control and the treatment groups (repeated-measures analysis of variance [ANOVA]).

Sequential changes in the extracellular levels of nitrite and nitrate are shown in Fig. 3. In both the control and treatment groups, nitrite levels in the dialysates gradually increased after a 40-minute period of ischemia, and peaked at 120 minutes after cessation of the transient ischemia. Although these
changes in the treatment group remained slightly lower than in the control group, repeated-measures ANOVA revealed no significant difference in the sequential changes between the two groups. In contrast to the changes in nitrite levels, extracellular levels of nitrate in the treatment group slightly exceeded those in the control group, although the difference was not statistically significant.

The histological study detected CA1 pyramidal neurons in the dorsal hippocampus harvested from the animals that survived for 24 hours after the induction of transient forebrain ischemia. The histological findings of rat brains in the control group of the present study are consistent with those of previous studies.\textsuperscript{9,14} Transient forebrain ischemia produced numerous damaged neurons in the CA1 subfield, which had shrunken cell bodies that were surrounded by empty spaces (Fig. 4 upper). Similarly damaged pyramidal neurons were also observed in the brain of ebselen-treated animals, but significantly fewer were detected (Mann-Whitney U-test), and normal, healthy-looking neurons could be seen in the corresponding CA1 area (Fig. 4 lower). The mean density of intact neurons in the target lesion of hippocampal area CA1 for the control and treatment groups was $25.3 \pm 7.6/\text{mm}$ and $119.4 \pm 36.7/\text{mm}$, respectively ($p < 0.05$).
Discussion

In the present study, the significant neuroprotective effects of ebselen against transient forebrain ischemia were confirmed histologically. The results support those of clinical studies which have suggested that ebselen confers a positive neurological outcome for patients with cerebral infarction. In the present study, treatment with ebselen did not inhibit NO synthesis during or after the ischemic insult, but the surge in the levels of EAAs in the cortex was attenuated, although no significant differences were detected between the groups. Previously, an in vivo microdialysis study measured extracellular lactate, pyruvate, and purine catabolite levels were measured in the brain of rats subjected to transient, four-vessel occlusion after administration of ebselen. In that study, ebselen significantly reduced the maximum levels of lactate and purine catabolites. Ebselen has since been demonstrated to exhibit some beneficial effects on neurons, and provide some protection against ischemic insults. Some evidence has been found showing that ebselen protects neurons against ischemic brain injury, but the mechanisms that account for the effects of this antioxidant remain unclear.

Based on the accumulating evidence obtained in the laboratory setting, whether or not neuroprotection against ischemic brain injury is afforded by the blockade of NO synthesis remains controversial. Since evidence was presented that NOS inhibitors prevent the neurotoxicity elicited by NMDA and EAAs, the involvement of NO in neurotoxicity has been investigated intensively. Some of the resulting data suggest that the administration of NOS inhibitors dramatically reduces the volume of brain edema and/or subsequent cerebral infarction following ischemic insult, which implicates NO in the mediation of neuronal death. On the other hand, conflicting results indicate that NOS inhibitors enhance neuronal death. To date, the exact role of NO in the physiological mechanism of ischemic brain injury appears to be complex, but it is becoming increasingly likely that excessive release of NO is involved in the pathophysiology of neurodegeneration. Furthermore, sustained NO production is accepted to occur in the late phase of cerebral ischemia, and this expansion of NO synthesis is induced mainly by inducible NOS (iNOS). Therefore, blockade of the explosive increase in extracellular levels of NO by inhibition of iNOS at the late phase of cerebral ischemia may have therapeutic potential. Recently, ebselen was confirmed to preferentially inhibit the enzymatic activity of iNOS at a certain dose.

Based on this data, we expected ebselen to cause attenuation in NO synthesis, leading to neuroprotection in ischemic brain injury, but the neuroprotective effects of ebselen reflected in the present histological findings were not based on inhibition of NO synthesis. However, it is important to emphasize that these neuroprotective effects are dependent on reperfusion of the CBF after transient ischemia, which causes reoxygenation of brain tissue and subsequently binds to reperfusion injury, to exhibit the expansion of NO synthesis by iNOS. In contrast to the design of the present study, CBF did not recover sufficiently after the ischemic insult (i.e. to pre-ischemia levels). Prior to this present study, we evaluated experimental ischemic insult with 10-minute and 20-minute periods of bilateral common carotid artery occlusion, but these procedures did not cause significant differences in the histological findings from the control and treatment groups in animals after 24-hour survival. Lastly, systemic hypovolemic hypotension could not be avoided in the 40-minute period of bilateral common carotid artery occlusion. Therefore, in this study, discussion of the relationship between neuroprotective effects of ebselen and reperfusion injury following transient forebrain ischemia is meaningless. However, absence of glutathione peroxidase–1 exacerbates cerebral ischemia-reperfusion injury by reducing post-ischemic microvascular perfusion. Also, in this present study, reduction in the CBF after reversible forebrain ischemia was smaller in the treatment...
group than in the control group. Therefore, the beneficial effects of ebselen on maintaining post-ischemic microvascular perfusion lead to neuroprotection reflected in the histological findings of rat brain harvested 24 hours after the induction of forebrain ischemia. The duration of in vivo microdialysis employed in this study was only 4 hours, which may be one of the reasons why the expansion of NO synthesis was not detected. Ebselen also exerts a neuroprotective effect in a rodent model of permanent middle cerebral artery occlusion. Therefore, the results from the present and this previous study suggest that ebselen provides neuroprotection without reperfusion after ischemic insults.

Ebselen is now thought to react as a free-radical scavenger and an inhibitor of NOS, but in the present study, contrary to expectations, no significant reduction in glutamate release during or after cerebral ischemia was found. The recovery rate of the microdialysis probes employed in this study is 12–46%. Possibly no significant reduction in extracellular levels of glutamate following ischemic insult was found due to the variable recovery rate of these in vivo microdialysis probes. However, ebselen is also known to prevent the excitotoxicity provoked by glutamate in cultured neurons by blocking glutamate-induced lipoperoxidation. Therefore, ebselen may elicit its neuroprotective effects against ischemic brain insult without inhibiting glutamate release.

The present study showed that treatment of rodent animals with ebselen prior to transient ischemic brain injury is beneficial, affording neuroprotection without detecting significant inhibition of either NO synthesis or glutamate release. However, this protection may be attributable to insufficient recovery of CBF after transient cerebral ischemia, as the short time course of the in vivo microdialysis procedure which may not have detected the explosive NO synthesis induced by iNOS in the late phase of ischemia.

Acknowledgment

This study was supported by a Grant from the Japanese Ministry of Health, Labour and Welfare. We are grateful to Mrs. Hitomi Ikemoto for her expert technical assistance.

References


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