Ebselen Protects Both Gray and White Matter in a Rodent Model of Focal Cerebral Ischemia

H. Imai, MD, PhD; H. Masayasu, PhD; D. Dewar, PhD; D.I. Graham, MB, PhD; I.M. Macrae, PhD

Background and Purpose—The neuroprotective efficacy of an intravenous formulation of the antioxidant ebselen has been comprehensively assessed with specific regard to conventional quantitative histopathology, subcortical axonal damage, neurological deficit, and principal mechanism of action.

Methods—Transient focal ischemia (2 hours of intraluminal thread-induced ischemia with 22 hours of reperfusion) was induced in the rat. Ebselen (1 mg/kg bolus plus 1 mg/kg per hour IV) or vehicle was administered at the start of reperfusion and continued to 24 hours. Neurological deficit was assessed 24 hours after ischemia. Gray matter damage was evaluated by quantitative histopathology. Axonal damage was determined with amyloid precursor protein immunohistochemistry used as a marker of disrupted axonal flow and Tau-1 immunohistochemistry to identify oligodendrocyte pathology. Oxidative damage was determined by 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) immunohistochemistry.

Results—Ebselen significantly reduced the volume of gray matter damage in the cerebral hemisphere (by 53.6% compared with vehicle, \( P < 0.02 \)). Axonal damage was reduced by 46.8% \( (P < 0.002) \) and the volume of oligodendrocyte pathology was reduced by 60.9% \( (P < 0.005) \). The neurological deficit score was reduced by 40.7% \( (P < 0.05) \) and the volume of tissue immunopositive for 8-OHdG and 4-HNE was reduced by 65% \( (P < 0.002) \) and 66% \( (P < 0.001) \), respectively, in ebselen-treated animals.

Conclusions—Delayed (2-hour) treatment with intravenous ebselen significantly reduced gray and white matter damage and neurological deficit associated with transient ischemia. The reduction in tissue displaying evidence of oxidative stress suggests that the major mechanism of action is attenuation of free radical damage. (Stroke. 2001;32:2149-2154.)

Key Words: antioxidants ■ free radicals ■ oxidative stress ■ stroke, experimental ■ white matter ■ rats

Many factors play a role in the development of brain damage after ischemia. In regions with reperfusion, excitatory amino acid release, peri-infarct waves of depolarization, specific gene expression, and oxidative stress may all contribute to the development of ischemic damage. Among these factors, oxidative stress has been shown to play a central role in ischemia/reperfusion-induced damage through increased formation of reactive oxygen species leading to lipid peroxidation and DNA damage.

Ebselen, originally developed as an anti-inflammatory agent, has potent antioxidant effects, acting as a glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase mimic. The oral formulation of ebselen has neuroprotective efficacy, as assessed from gray matter damage in experimental models of cerebral ischemia and, importantly, has been demonstrated to have evidence of benefit in phase 3 clinical stroke trials. Because the optimal administration route is intravenous, providing rapid and controlled delivery of the drug to the brain, a new intravenous formulation of ebselen has been developed. This formulation has already been demonstrated to have significant neuroprotective efficacy in initial studies in which the drug was administered before the onset of rodent focal ischemia.

Over the last decade, many classes of drugs, including antagonists of glutamate release and receptor activation, Ca\(^{2+}\) channel blockers, and antioxidants, have been developed for neuroprotection against ischemic damage, and their efficacy has been demonstrated in experimental animals. While promising results with these drugs have been demonstrated in animal models, the results of clinical trials conducted thus far have been disappointing. The failure to translate preclinical pharmacological insight into therapy is multifactorial. Failure to ameliorate ischemic damage to white matter has been proposed to be one of the major factors, and the importance of white matter damage as well as gray matter in experimental stroke is increasingly recognized.

The first aim of this study was to comprehensively assess the neuroprotective efficacy of delayed treatment with an improved, intravenous formulation of ebselen in an established model of experimental stroke. We used conventional...
histopathology to assess gray matter damage (perikarya), quantification of subcortical white matter damage (axons and oligodendrocytes), and scoring of neurological deficit. The second aim was to explore the mechanism by which ebselen exerts its neuroprotective effect. A reproducible model of transient focal ischemia was used, repurposing the tissue after 2 hours, to promote oxidative stress and provide the best opportunity to assess the antioxidant efficacy of ebselen.

Materials and Methods

General Surgical Preparation

Adult male Sprague-Dawley rats (weight, 300 to 323 g) had free access to laboratory pellet diet and water until the day of operation. All procedures were carried out under license from the Home Office and were subject to the Animals (Scientific Procedures) Act 1986. Anesthesia was induced with 5% halothane and subsequently maintained with 1.0% to 1.9% halothane in nitrous oxide–oxygen (70:30). The rats were intubated transorally and artificially ventilated by a small animal respirator pump. The right femoral artery and vein were cannulated for continuous physiological monitoring and for administration of ebselen or vehicle. Cannulas were exteriorized at the nape of the neck to allow drug infusion and physiological monitoring to be carried out in the conscious animal. Arterial pressure was monitored throughout the surgical procedure and arterial blood samples taken at regular intervals for assessment of respiratory status by means of a direct reading electrode system (Corning). Rats were maintained normotensive (mean arterial blood pressure was monitored throughout the surgical procedure and arterial blood pressure > 80 mm Hg), normocapnic (PaCO₂ < 44 mm Hg), adequately oxygenated (PaO₂ > 100 mm Hg), and normothermic while anesthetized. Rectal temperature was maintained at ∼37°C with a heating lamp during the operation. Laser Doppler flowmetry (Moor Instrument Ltd.) was used to monitor cerebral blood flow, to confirm adequate ischemia and subsequent reperfusion.

Production of Focal Cerebral Ischemia

Focal cerebral ischemia was accomplished by means of a modification of the intraluminal thread model (4-0 nylon monofilament suture), first introduced by Koizumi et al. Briefly, the left common, internal, and external carotid arteries were exposed through a ventral midline neck incision. The external carotid artery was ligated and then cut just proximal to the external carotid bifurcation. The common carotid artery was temporarily occluded with a microvascular clip. A 4-0 nylon monofilament was carefully inserted 20 to 23 mm from the bifurcation of the right common carotid artery, into the internal carotid artery through the external carotid artery, and advanced to block the origin of the right middle cerebral artery (MCA). After 2 hours of ischemia, the monofilament was removed, the wound sutured, and the animal allowed to recover from anesthesia. A subcutaneous injection of saline (2 mL) was administered to prevent postanesthetic dehydration.

Laser Doppler Flowmetry

Local cortical blood flow (LCBF) was monitored in the left hemisphere in the supply territory of the MCA by laser Doppler flowmetry. Each animal was placed supine, and the head was firmly immobilized in a stereotaxic frame (model 900, David Kopf Instruments). Burr holes (1.5-mm diameter) were drilled 5~6 mm lateral and 1~2 mm posterior to bregma, without injury to the dura mater. The laser Doppler flow probe was carefully positioned on the craniectomy site and LCBF was continuously monitored (2-Hz sampling rate) from before the onset of ischemia until 5 minutes after reperfusion. Flow values, averaged over 30-second periods, were collected every 10 minutes, with shorter intervals immediately after induction of ischemia and reperfusion. Decreased levels of LCBF, during intraluminal filament insertion, were expressed as a percentage of baseline flow (ischemic LCBF/preischemic LCBF) × 100. If ischemic LCBF was not sharply reduced with stabilization at <35% of the baseline signal, MCA occlusion was regarded as incomplete, and the animal was excluded from the study. The 35% threshold was based on the results of a pilot study for reproducibility in infarct size (data not shown).

Drug Treatment

Rats were randomly divided into 2 groups (n=9) to receive ebselen or vehicle (control group). Treatment began 2 hours after MCA occlusion, at the onset of reperfusion, as a bolus injection of 1 mg/kg along with a continuous infusion of 1 mg/kg per hour until the end of the experiment, 24 hours after onset of ischemia. This was achieved by means of a jacket-and-tether system attached to a miniature single channel fluid swivel (Harvard Apparatus) to allow animals free movement within the cage.

Pharmacokinetic Study

In a separate group of Sprague-Dawley rats (weight, 275 to 316 g; n=32), blood samples were taken 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours after intravenous administration of 14C-ebselen (1 mg/kg) for pharmacokinetic modeling with the use of Topfit 2.0.

Neurological Assessment

Twenty-four hours after MCA occlusion, the neurological status of each rat was evaluated according to a neurological grading score of 1 to 5: 1, no movements; 2, weakened grip to cage top; 3, circles to the right or left when placed on floor; 4, moves only when stimulated; and 5, dead.

Tissue Processing

After neurological assessment, rats were perfusion-fixed for neuropathological study. Briefly, the rats were deeply anesthetized with 5% halothane, placed in a supine position, and the thorax was opened through a bilateral incision. A catheter was inserted into the left ventricle, the right atrium was incised, and heparinized saline was infused at a pressure equal to the MABP (90 to 110 mm Hg) of the animal until the perfusate from the right atrium was bloodless. The saline was followed by ∼300 mL of PAM (4% paraformaldehyde in PBS). The rat was decapitated immediately after perfusion fixation, and the head was stored in fixative for at least 24 hours before removal of the brain. After detaching the hindbrain, the forebrain was processed, embedded in paraffin wax, and sectioned (6 μm) at multiple levels.

Histology and Immunohistochemistry

Sections at the 8 preselected coronal levels21 were stained with hematoxylin and eosin for assessment of ischemic damage to neuronal perikarya. Adjacent sections were processed for immunohistochemistry with Tau-1 antibody used to label ischemic oligodendrocytes22 in both gray and white matter, anti-amyloid precursor protein (APP) to label ischemically damaged axons (disruption of axoplasmic flow leads to APP accumulation), and 8-OHdG23 and 4-HNE24 antibodies to identify DNA damage and lipid peroxidation, respectively, produced by oxidative stress. Sections were mounted on poly-L-lysine–coated (SIGMA) slides, dried at 37°C overnight, then placed in HistoClear for 20 minutes to remove the wax and dehydrated in absolute alcohol for 20 minutes. For APP and Tau-1 immunohistochemistry, sections were microwaved for 10 minutes in 10 mmol/L citric acid (pH 6.0) and allowed to cool to room temperature for 60 minutes (for 8-OHdG and 4-HNE immunohistochemistry, this step was omitted). Sections were incubated in 3% H₂O₂ in methanol for 30 minutes and for 1 hour in 50 mmol/L PBS (pH 7.2) containing 0.5% bovine serum albumin and 10% normal horse serum. Monoclonal antibodies APP (Clone 22C11; Boehringer, Mannheim, Germany), Tau-1, 8-OHdG, and 4-HNE (both JaICa, Shizuoka, Japan) diluted 1:300, 1:750, 1:100, and 1:750 in PBS, respectively, were applied to sections overnight at 4°C. Sections were then washed in PBS 2×20 minutes. Secondary antibody (biotinylated horse anti-mouse, 1:100, Vector Laboratories) was applied for 1 hour, and the sections were washed again (2×20 minutes). For APP and Tau-1 antibodies, the avidin/biotinylated
horseradish peroxidase complex (ABC kit, Vector Laboratories) was applied for 1 hour. For 8-OHdG and 4-HNE antibodies, the ABC Elite kit (Vector Laboratories) was applied for 1 hour. The washing procedure with PBS was repeated, and the sections were allowed to develop in 3,3′-diaminobenzidine solution (Sigma) for 3 minutes (APP and Tau-1) or 3, 3′-diaminobenzidine + nickel solution (Sigma) for 4 minutes (8-OHdG and 4-HNE). Finally, the sections were dehydrated, cleared, and mounted for light microscopic analysis. Negative controls for each antibody, in which the primary antibody was omitted from the procedure, were included in each protocol, and minimal staining was detected. Subsequent analysis was performed by an investigator blinded to the treatment in each animal.

**Quantification of Gray Matter Damage**

Hematoxylin and eosin–stained sections were viewed by light microscopy by one of us (D.I.G.), without knowledge of the experimental protocol. Areas in which neuronal perikarya and parenchyma displayed the morphological features of ischemic damage26 were delineated on to scale diagrams (>3.36 actual size) of forebrain at 8 predetermined coronal planes from anterior 10.50 mm to anterior 1.02 mm.21 The areas of brain damage were then measured on an image analyzer (MCID, M4, Imaging Research) and integrated, with the known distance between each coronal level, to determine the total volume of ischemic damage in each specimen.

**Quantification of Oxidative Stress and Oligodendrocyte Pathology**

The distribution of tissue immunopositive for 8-OHdG, 4-HNE, or Tau-1 was delineated onto scale diagrams and measured in the same way as for ischemic damage.18

**Quantification of Axonal Damage**

Axonal damage was quantified in the following regions: corpus callosum, external capsule, internal capsule, anterior commissure, median forebrain bundle, fornix, optic tract, caudate putamen, and globus pallidus. Large regions were subdivided according to the Atlas of Paxinos & Watson (1986)27 (e.g., corpus callosum was subdivided into forceps minor, genu of corpus callosum, cingulum, splenium of corpus callosum, and forceps major) or simply into dorsal, ventral, medial, and lateral subregions (e.g., caudate putamen). Each region was assessed across the 8 stereotaxic coronal levels used to measure gray matter damage.21 This provided a total of 65 individual regions of white matter to be assessed in each animal. Axonal damage in each region was identified as intense APP immunoreactivity in swollen or bulbous axons, (APP accumulation as a result of disrupted axonal transport) and assigned a score of 1. If no APP accumulation was present, a score of 0 was assigned. Total APP score for the entire hemisphere (65 regions combined), for a neuroanatomic structure, or a specific stereotaxic coronal level could then be prepared from the sum of appropriate individual components.

**Statistical Analysis**

Physiological variables and the tissue volumes of ischemic damage, oxidative stress, and oligodendrocyte pathology were compared by Student’s unpaired 2-tailed t test. These data are presented as mean±SEM. Axonal damage and neurological deficit were compared between ebselen and vehicle groups by means of the Mann-Whitney test. These data are presented as median (95% CI).

**Results**

**Plasma and Brain Drug Levels**

Pharmacokinetic modeling (3-compartment model) of plasma concentration curves for a 1 mg/kg bolus injection combined with a 1 mg/kg per hour IV infusion revealed an initial spike concentration of 12 μg/mL, which stabilized (within 1 hour) to plateau at a plasma level of between 4 and 6 μg/mL, which was maintained throughout the infusion period (Figure 1). The intravenous formulation of ebselen has good blood-brain penetrability. One hour after a bolus dose of 1 mg/kg IV 14C-labeled ebselen, brain levels of the drug were 21% of the plasma level.

**Physiological Parameters**

Cardiovascular and respiratory parameters in both groups before, during, and after MCA occlusion were within the normal range (Table 1). Ebselen had no significant influence

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**TABLE 1. Physiological Variables Before, During, and After Focal Ischemia**

<table>
<thead>
<tr>
<th></th>
<th>MABP, mm Hg</th>
<th>pH</th>
<th>Pao2, mm Hg</th>
<th>Pao2, mm Hg</th>
<th>Plasma Glucose, mmol/L</th>
<th>Rectal Temperature °C</th>
<th>ICBF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before MCAO</td>
<td>85.6±2.0</td>
<td>7.44±0.03</td>
<td>36.3±2.6</td>
<td>178.7±6.5</td>
<td></td>
<td>36.2±0.1</td>
<td></td>
</tr>
<tr>
<td>MCAO, 2 h</td>
<td>81.8±0.7</td>
<td>7.39±0.02</td>
<td>42.5±3.3</td>
<td>155.3±10.0</td>
<td>9.4±0.3</td>
<td>37.4±0.1</td>
<td>24.2±2.4</td>
</tr>
<tr>
<td>24 h (conscious)</td>
<td>133.0±3.4</td>
<td>7.52±0.01</td>
<td>37.9±1.4</td>
<td>91.2±2.4</td>
<td>7.0±0.4</td>
<td>37.1±0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Ebselen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before MCAO</td>
<td>82.6±1.3</td>
<td>7.47±0.02</td>
<td>30.4±1.2</td>
<td>173.8±5.4</td>
<td></td>
<td>36.2±0.1</td>
<td></td>
</tr>
<tr>
<td>MCAO, 2 h</td>
<td>82.6±0.3</td>
<td>7.42±0.02</td>
<td>40.4±3.0</td>
<td>171.3±7.8</td>
<td>9.3±0.6</td>
<td>37.4±0.1</td>
<td>26.8±1.8</td>
</tr>
<tr>
<td>24 h (conscious)</td>
<td>123.0±3.6</td>
<td>7.49±0.01*</td>
<td>42.0±2.0</td>
<td>94.4±3.8</td>
<td>8.1±0.5</td>
<td>37.2±0.1</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM (n=9 per group).

*P<0.005 compared with vehicle group.
on cardiovascular or respiratory parameters over the 22-hour infusion period. A small decrease in plasma pH at 24 hours was statistically but not physiologically significant.

**Neurological Deficit**
The neurological deficit score at 24 hours after MCA occlusion was significantly higher in the vehicle group than in the ebselen treatment group (3 [2.1 to 3.9] versus 1 [1.0 to 2.5], \(P<0.05\), median [95% CI]), reflecting a 41% reduction in neurological deficit in ebselen-treated animals.

**Gray Matter Damage**
In the ipsilateral cerebral cortex and caudate putamen, neuronal perikarya clearly exhibited the characteristic morphological features of ischemic damage, that is, shrinkage and triangulation of the nucleus and cytoplasm and increased eosinophilia of the cytoplasm (Figure 2A). In both vehicle and ebselen treatment groups, boundaries between ischemic and nonischemic neuronal perikarya were identifiable. Treatment with ebselen significantly reduced the volume of gray matter damage in the cerebral hemisphere (by 53.6% compared with vehicle group, \(P<0.02\)). This reflected a 52.7% reduction in the cerebral cortex (\(P<0.05\)) and a 48.7% reduction in the caudate nucleus (\(P<0.001\)) (Figure 3).

**Axonal Damage**
Axonal damage was recognized as intense APP immunoreactivity in swollen or bulbous axons within ipsilateral subcortical white matter fiber tracts (Figure 2B). The anatomically circumscribed zones of APP immunoreactivity allowed scoring for the presence or absence of APP axonal profiles in each region throughout the rostrocaudal extent of the MCA territory. Axonal damage was less extensive in ebselen-treated animals. Total APP score in the ebselen-treated group was significantly smaller than in the vehicle-treated group (Figure 4A). Drug treatment significantly reduced the axonal damage in the corpus callosum, external capsule, internal capsule, fornix, and caudate putamen (Table 2). APP scores in the anterior commissure, median forebrain bundle, optic tract, and globus pallidus were not significantly different between groups.

**Oligodendrocytes Pathology**
Intensely stained tau-immunopositive cells, with the morphological appearance of oligodendrocytes, were present throughout gray and white matter regions of the ipsilateral MCA territory in vehicle and ebselen treatment groups (Figure 2C). In both groups, boundaries could be identified between areas containing numerous intensely stained Tau-positive oligodendrocytes and areas with few and faintly stained oligodendrocytes located outside the ischemic territory. Treatment with ebselen significantly reduced the volume of tissue containing Tau-1–positive oligodendrocytes by 60.9% in the cerebral hemisphere (\(P<0.005\)), with a 61.4% reduction in cortex (\(P<0.01\)) and a 49.3% reduction in caudate nucleus (\(P<0.01\)) (Figure 4B).

**Oxidative Stress: DNA Damage**
Nuclei, and to a lesser extent cytoplasm, of damaged cells in the ischemic hemisphere were immunoreactive for the DNA damage marker 8-OHdG (Figure 5A). In the vehicle group, the volume of tissue demonstrating 8-OHdG immunoreactivity (Figure 6A) was greater than the volume of infarction determined by hematoxylin and eosin staining (175.0 mm³ versus 146.7 mm³). Ebselen significantly reduced the volume of tissue immunopositive for 8-OHdG in the cerebral hemisphere (by 65%, \(P<0.002\)), cortex (by 66%, \(P<0.005\)), and caudate nucleus (by 49%, \(P<0.005\)). The volume of tissue...
immunopositive for 8-OHdG in this group was similar to the volume of ischemic damage determined by hematoxylin and eosin staining (61.2 mm³ versus 68.2 mm³).

**Oxidative Stress: Lipid Peroxidation**

Immunohistochemistry for 4-HNE revealed positive staining in neuronal perikarya and axons within the region of ischemic damage (Figure 5B). As for 8-OHdG staining, the volume of tissue demonstrating 4-HNE immunoreactivity (Figure 6B) was greater than the volume of infarction determined by hematoxylin and eosin staining in the vehicle group (196.3 mm³ versus 146.7 mm³). Ebselen significantly reduced the volume of tissue immunopositive for 4-HNE in the cerebral hemisphere (by 66%, \( P < 0.002 \)) compared with the vehicle group. Data are volumes of neuronal necrosis derived from area measurements at 8 predetermined coronal planes and are expressed as mean±SEM (n=9 per group). *\( P < 0.05 \), **\( P < 0.02 \), 2-tailed unpaired Student’s \( t \) test.

**TABLE 2. APP Scores of Axonal Damage in Vehicle and Ebselen Groups**

<table>
<thead>
<tr>
<th>Axon-Rich Region</th>
<th>Vehicle</th>
<th>Ebselen</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corpus callosum</td>
<td>10.0</td>
<td>3.0</td>
<td>↑</td>
</tr>
<tr>
<td>External capsule</td>
<td>5.0</td>
<td>1.0</td>
<td>↑</td>
</tr>
<tr>
<td>Internal capsule</td>
<td>6.0</td>
<td>2.0</td>
<td>↑</td>
</tr>
<tr>
<td>Fornix</td>
<td>2.0</td>
<td>2.0</td>
<td>*</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>17.0</td>
<td>8.0</td>
<td>*</td>
</tr>
<tr>
<td>Anterior commissure</td>
<td>1.0</td>
<td>0.0</td>
<td>NS</td>
</tr>
<tr>
<td>Median forebrain bundle</td>
<td>3.0</td>
<td>2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Optic tract</td>
<td>3.0</td>
<td>2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>3.0</td>
<td>3.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as median (95% CI) (n=9 per group) and analyzed by Mann-Whitney test.

*\( P < 0.05 \), †\( P < 0.02 \).

The major finding in this study is that delayed treatment with a new intravenous formulation of ebselen provides significant protection against ischemic damage in both gray and white matter. Quantitative methods have been used to demonstrate reduced ischemic damage to cortical and striatal neuronal perikarya, axons, and oligodendrocytes along with improved neurological recovery after ebselen treatment.

Quantification of gray and white matter damage is equally important for assessing the efficacy of potential therapeutic agents because both affect neurological deficit and functional recovery after stroke. However, to date, gray matter damage has attracted more attention in experimental stroke studies, partly because of the relatively small amount of white matter relative to gray matter in rodents compared with that in the human brain. In addition, the lack of a reliable method to detect and quantify white matter damage has precluded such studies.14 Volumetric assessment of histological damage in gray matter has been established since the 1980s and has become the predominant quantitative measure worldwide for assessing the efficacy of neuroprotective drugs in experimental stroke.21 However, more recently, methods for quantification of white matter damage have been developed.16,17,22,28 With the use of APP accumulation to highlight axonal damage and Tau-1 staining for oligodendrocyte pathology, Valeriani et al18 demonstrated that reperfusion of ischemic tissue salvaged axons and oligodendrocytes, in addition to neuronal perikarya from ischemic damage; Irving et al22 demonstrated that a free radical scavenger but not an NMDA antagonist MK-801 to influence axonal damage while producing a 30% reduction in gray matter damage compared with control. NMDA antagonists are targeted toward salvaging gray matter, the failure to protect against axonal damage most probably reflecting the lack of NMDA receptors in...
white matter.\textsuperscript{29} Therefore, drugs that have proven efficacy in protecting neuronal cell bodies from ischemia but provide no protection to white matter are unlikely to represent an optimal therapy for stroke. AMPA receptors may represent a better target because they mediate excitotoxic damage to oligodendrocytes\textsuperscript{30} and appear more important than NMDA receptors for glutamate-mediated injury to spinal cord white matter.\textsuperscript{31}

In the present study, APP accumulation has been quantified regionally as well as globally to improve the analysis of drug effects on axonal damage. A significant, ebselen-induced reduction of axonal damage in the ischemic hemisphere has been revealed. Interestingly, the drug was not equally effective in all white matter regions examined (Table 2). As occurs in gray matter, the ability of the drug to salvage damaged tissue may depend on the severity of the ischemic insult and the subsequent quality of reperfusion. Ebselen demonstrated significant benefit in the corpus callosum, external and internal capsule, fornix, and fiber tracts within the caudate putamen but had no significant influence on damage in the anterior commissure, median forebrain bundle, optic tract, and globus pallidus. This finding correlates with the severity of cerebral blood flow reduction in these white matter structures during intraluminal filament-induced MCA occlusion.\textsuperscript{31a} The failure of ebselen to protect against axonal damage in these structures may thus reflect levels of ischemia that are associated with irreversible ischemic damage. In addition to its effects on neuronal perikarya and axons, ebselen induced a significant reduction in oligodendrocyte pathology and neurological deficit measured at 24 hours after ischemia.

Ebselen may represent an optimal therapy for stroke in that it has the capacity to attenuate damage to neuronal cell bodies, axons, and oligodendrocytes without obvious cardiovascular or psychotomimetic side effects. Ebselen is an antioxidant, anti-inflammatory, and an antiapoptotic agent,\textsuperscript{32} and in vitro studies reveal a variety of potentially beneficial pharmacological effects. Its main neuroprotective effect is linked to its antioxidant function with multitargeted inhibition of oxidative stress involving attenuation of free radical generation plus detoxification of free radicals and the toxic products of their reactions (eg, 4-HNE). To date, ebselen is reported to mimic glutathione peroxidase\textsuperscript{5,33} and phospholipid hydroperoxide glutathione peroxidase\textsuperscript{6} and to inhibit 5- and 15-lipoxygenases,\textsuperscript{34} inducible nitric oxide synthase,\textsuperscript{35} and NADPH oxidase,\textsuperscript{36} which in pathological conditions such as reperfusion is a major source of superoxide. It also deactivates peroxinitrite, leukotriene B\textsubscript{4}, and 5-hydroxyeicosatetraenoic acid\textsuperscript{37} and inhibits lipopolysaccharide-induced nuclear factor κ-B nuclear translocation and \textit{jun}-N-terminal kinase phosphorylation, which leads to production of tumor necrosis factor-α and nitric oxide.\textsuperscript{38} Therefore, the multiple actions of ebselen can limit damage from all the major oxidants and radicals generated by ischemia, for example, H\textsubscript{2}O\textsubscript{2}, NO*, NOO\textsuperscript{−}, *OH, and *O\textsubscript{2}•.
The data in the present study indicate that ebselen shows a strong antioxidative effect, as has been reported in previous in vitro studies. In the vehicle group, oxidative stress was generated within the ischemic core and the surrounding peri-infarct zone as reflected by increased 8-OHdG and 4-HNE immunoreactivity. Because the infarct continues to increase in size between 24 and 48 hours in this model of focal ischemia, oxidative stress detected in the peri-infarct zone may reveal a potentially evolving target tissue for ebselen to protect. In ebselen-treated animals, the volumes of tissue immunoreactive for 4-HNE and 8-OHdG were significantly reduced in both cortex and caudate compared with the vehicle group and were comparable to the volume of infarct. Inhibition of 4-HNE by ebselen is particularly important because 4-HNE is not only a marker of lipid peroxidation but is also toxic to neurons, axons, and oligodendrocytes in its own right.24,39 4-HNE generated during ischemia and reperfusion is therefore a likely contributory factor to the total amount of damage produced in this model. Both ebselen40 and glutathione, the endogenous substrate for glutathione peroxidase,41 have been reported to inhibit 4-HNE toxicity in cultured neurons. By reducing the accumulation of this aldehyde in damaged tissue, ebselen may also reduce the capacity for 4-HNE to supplement ischemic damage.

In conclusion, delayed intravenous ebselen treatment produced significant attenuation of both gray and white matter damage, improved neurological recovery, and reduced tissue- oxidativedamage. Moreover, there was no evidence of adverse side effects (cardiovascular or behavioral) after ebselen treatment. The intravenous formulation of the drug offers a significant advance both in terms of the time taken to reach therapeutic plasma levels and in maintaining therapeutic levels. The efficacy of ebselen most probably reflects its wide range of antioxidant effects at many points in the ischemic cascade. This and previous studies with the oral formulation of the drug strongly support ebselen as a potent neuroprotective treatment in experimental ischemia, which holds promise as a future therapy in human stroke.

Acknowledgments

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References


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The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/32/9/2149