Ebselen Reduces Cytochrome c Release From Mitochondria and Subsequent DNA Fragmentation After Transient Focal Cerebral Ischemia in Mice

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Background and Purpose—The seleno-organic compound ebselen has both antioxidant and anti-inflammatory properties. Although ebselen has been shown to protect the brain against stroke, it is unclear how ebselen provides neuroprotection. In the present study the authors examined whether ebselen inhibits neuronal apoptosis resulting from transient focal cerebral ischemia in mice. The cytochrome c release and DNA fragmentation, both of which are biochemical markers of apoptosis, were compared between vehicle- and ebselen-treated mice.

Methods—Cerebral ischemia was induced by transient middle cerebral artery occlusion for 30 minutes in ICR mice under halothane anesthesia. Ebselen (10 mg/kg) was given orally twice, 30 minutes before ischemia and 12 hours after reperfusion. By Western blot analysis, we examined release of mitochondrial cytochrome c. To evaluate brain damage, the brain sections were treated for terminal deoxynucleotidyl transferase–mediated DNA nick-end labeling (TUNEL) and Nissl staining. Prolonged neuroprotective efficacy of ebselen was determined by counting neuronal nuclei (NeuN) immunopositive cells at 21 days after ischemia.

Results—Cytochrome c release was detected in the ischemic hemisphere at 3 to 24 hours after ischemia. Ebselen treatment diminished the cytochrome c release at 12 and 24 hours. In addition, ebselen decreased both DNA fragmentation determined by TUNEL and brain damage volume at 3 days after ischemia. Furthermore, ebselen increased the number of NeuN immunopositive cells at 21 days after ischemia.

Conclusions—These results indicate that ebselen attenuates ischemic neuronal apoptosis by inhibiting cytochrome c release. Ebselen may be a potential compound in stroke therapy. (Stroke. 2001;32:1906-1911.)

Key Words: apoptosis ■ cerebral ischemia, focal ■ neuroprotection ■ oxidative stress ■ mice

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Reactive oxygen species (ROS) have been implicated in a number of neurological disorders, including stroke, Parkinson’s disease, and Alzheimer’s disease.1–3 ROS cause lipid peroxidation, protein oxidation, and DNA damage, all of which are deleterious to cells. Oxidative stress resulting from the ROS production is also implicated in apoptosis.4,5 Regarding brain injury resulting from focal ischemia, apoptosis has been shown to play an important role in neuronal death.6–8 The antioxidant enzymes block ROS, thereby playing a protective role against cerebral ischemia. For example, gene overexpression of copper zinc superoxide dismutase (SOD1) decreases brain damage due to focal ischemia in mice.9 Heterozygous gene deletion of mitochondrial manganese superoxide dismutase (SOD2) exacerbates brain damage following permanent focal ischemia.10 Overexpression of human glutathione (GSH) peroxidase protects brain against transient focal ischemia.11 Fujimura et al12,13 presented a hypothesis that SOD1 and SOD2 can reduce apoptosis resulting from focal cerebral ischemia by blocking cytosolic release of cytochrome c, which interacts with apoptotic protease activating factor-1 (Apaf-1) and caspase-9, thereby playing a crucial role in apoptosis mediated by mitochondria.14,15 Thus, it is suggested that antioxidant compounds are potential therapeutic agents for stroke.

The seleno-organic compound ebselen [2-phenyl-1,2-benzisoselenazol-3 (2H)-one] has antioxidant properties as well as anti-inflammatory activities. Its unique character is the mimicry of GSH peroxidase and phospholipid hydroperoxide GSH peroxidase.16 Furthermore, a variety of actions by ebselen has been shown: the inhibition of lipoxigenases,19 NADPH oxidase, and protein kinase C.20 Recently, a new property of ebselen to inhibit apoptosis has been demonstrated in several paradigms of apoptosis.21–23 Although ebselen has been shown to protect brain from stroke in humans24–26 as well as in experimental animals,27,28 there...
have been limited published works studying the mechanisms for the neuroprotective efficacy of ebselen. In this work we examined the effects of ebselen on cytochrome c release from mitochondria, caspase-3–like activity, and subsequent DNA fragmentation, all of which are biochemical markers of apoptosis, in a transient focal cerebral ischemia model using ICR mice.

Materials and Methods

Cerebral Ischemia Model

Male ICR mice (n = 98; weight, 25 to 30 g; SLC, Hamamatsu, Japan) were anesthetized with 1.0% halothane in 70% N\textsubscript{2}O and 30% O\textsubscript{2} with a vaporizer (Muraco Medical). In each mouse, regional cerebral blood flow was monitored by laser-Doppler flowmetry (FLO-C1, Omegawave) with the use of a flexible probe affixed to the skull (2 mm posterior and 6 mm lateral to the bregma). Rectal and temporal muscle temperatures were maintained at approximately 37°C with a thermostat-controlled heating pad (Neuroscience and BAT-12) during surgery and monitored for 3 hours after reperfusion. Middle cerebral artery occlusion (MCAO) was induced with an 8.0-m diameter nylon monofilament coated with silicone resin and hardener mixture (Heraeus). The filament was introduced into the left internal carotid artery through the external carotid artery and left for 30 minutes. For reperfusion, the filament was withdrawn, and the external carotid artery was coagulated with bipolar forceps (Muraco Medical). Animal protocols followed the guidelines of the National Cardiovascular Center animal experiments and were approved by the Committee on Research of the National Cardiovascular Center.

Ebselen Treatment

Mice were randomly assigned into 2 groups to receive ebselen (10 mg/kg) or vehicle. Ebselen or vehicle was given orally twice, 30 minutes before ischemia and 12 hours after reperfusion. We chose this dose and treatment protocol on the basis of previously published work. Vehicle control animals were given 0.5% carboxymethyl cellulose (5 mL/kg). For therapeutic window studies, the first ebselen administration was at 3, 6, 9, or 12 hours after reperfusion, followed by a second administration 12 hours thereafter. For long-term studies, ebselen was given 3 times (30 minutes before ischemia and 12 and 24 hours after reperfusion).

Western Blot Analysis

Brain tissue was homogenized in ice-cold HEPES buffer containing 250 mmol/L sucrose, 0.1 mmol/L PMSF, 2 μg/mL aprotinin, 10 μg/mL leupeptin, 5 μg/mL pepstatin, and 12.5 μg/mL N-aceetyl-leuc-leu-norleucinal. The homogenates were centrifuged at 750g and then at 8000g for 20 minutes at 4°C. The pellets were used to obtain the mitochondrial fraction. The supernatant was centrifuged at 100 000g for 60 minutes at 4°C, and the obtained supernatant was used as the cytosolic fraction. Protein concentrations were determined by the Bradford method. Western blot analysis was performed by using antibody for cytochrome c (1:1200; Santa Cruz Biotechnology), or cytochrome oxidase subunit IV (COX-IV) (1 μg/mL; Molecular Probes). The membrane was then incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG for cytochrome c and COX-IV, respectively (Dako). Immunoblots were visualized with ECL immunodetection system kit (Amersham). Protein amounts loaded were analyzed by Western blot analysis of β-actin (1:5000; Sigma).

Caspase-3–like Activity Assay

Caspase-3–like activity assay was performed with a commercially available kit (Promega). According to the manufacturer’s protocol, samples obtained as the cytosolic fraction were incubated with 50 μmol/L acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) at 37°C. Fluorescence (excitation, 360 nm/emission, 460 nm) was measured with a fluorescence plate reader (Cytofluor 4000, PerSeptive Biosystems), and caspase-3–like DEVDase activity was calculated according to Fink et al.

Histological Examinations

Three days after MCAO, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused by transcardial injection of 10% formalin in 0.1 mol/L PBS (pH 7.4). The brains were removed quickly and kept in the same fresh buffer containing 20% sucrose. The brains were cut into 50-μm-thick coronal sections on a freezing microtome (HM400R, Microm). Every 10 sections from the frontal pole, the brain sections were mounted onto glass slides, and the sections were processed for Nissl staining (0.1% cresyl violet) and terminal deoxynucleotidyl transferase (TdT)–mediated DNA nick-end labeling (TUNEL).

Ischemic Lesion Size Measurement

The boundaries of the area containing injured cells that showed abnormal morphology, such as cell shrinkage and chromatin condensation, under a light microscope were marked with the use of the Olympus Image analysis system (Olympus). The entire volume of lesion was calculated by summing the lesion area.

TUNEL

TUNEL was performed as described previously. The sections were incubated with buffer containing TdT (Takara Shuzo) and biotin-16-dUTP (Boehringer Mannheim) for 70 minutes at 37°C. After we stopped the reaction by transferring the sections to termination buffer, biotin-16-dUTP was visualized by the avidin-biotin-peroxidase method using 3,3′-diaminobenzidine as chromogen. On the section through the anterior commissure, the striatum was assigned to 3 subregions (500×500 μm square): medial, center, and lateral; the number of TUNEL(+) cells within each subregion was counted under a light microscope by using a ×20 objective lens (BX50, Olympus).

Neuronal Nuclei Immunostaining

Immunostaining for neuronal nuclei (NeuN) was performed by a free-floating method. The sections were incubated with mouse anti-NeuN monoclonal antibody (1:1000; Chemicon International) overnight at 4°C. After wash with 0.1 mol/L PBS (pH 7.4), the sections were incubated with biotin-conjugated anti-mouse IgG1 antibody (Southern Biotechnology Associates). The staining was visualized by standard avidin-biotin-peroxidase technique with the use of 3,3′-diaminobenzidine. NeuN immunopositive cells were counted on the section through the anterior commissure, as described in the TUNEL method.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were made by Mann-Whitney U test for immunoblot densitometric analysis and caspase-3–like activity assay in vehicle- and ebselen-treated mice. ANOVA followed by Bonferroni was used for time-dependent caspase-3–like activity changes and lesion size. Cell number was compared by Student’s t-test. The software StatView (version 5.0) was used. P<0.05 was considered statistically significant.

Results

Ebselen Reduces Mitochondrial Cytochrome c Release

We first studied intracellular cytochrome c distribution in the brain tissues after 30 minutes of MCAO. We detected cytochrome c immunoreactivity as a single band at 15 kDa in the cytosolic fraction from the ischemic hemisphere, as well as the mitochondrial fractions from both ischemic and contralateral hemispheres at 24 hours after ischemia (Figure 1A). We also detected a band at 15 kDa in the cytosolic fraction from the contralateral hemisphere; however, it was very weak. COX-IV immunoreactivity was found in the mitochon-
Ebselen Attenuates Brain Damage

We examined neuroprotective efficacy of ebselen. Three days after reperfusion, damaged cells were predominantly found in the striatum and to minor extent in the cortex, which was consistent with the previously published work using 129/Sv mice. Pretreatment with ebselen decreased brain damage volume by 39% and 34% ($P<0.05$) compared with ischemic control and vehicle-treatment, respectively (Figure 3). We next determined the therapeutic time window of ebselen. Ebselen initially given at 3 and 6 hours after reperfusion significantly decreased damage volume by 42% and 37% ($P<0.05$), respectively, when compared with ischemic control (Figure 3). Consistent with previous reports, ebselen did not affect body temperature and regional cerebral blood flow (Table). Therefore, the neuroprotection by ebselen was unlikely to be due to alterations in physiological parameters.

**Ebselen Decreased DNA Fragmentation**

To ask whether ebselen inhibits apoptosis in ischemic brain, we assessed DNA fragmentation by TUNEL, a marker of apoptosis. TUNEL(+) cells were seen throughout the striatum at 3 days after ischemia (Figure 4A). Only densely labeled cells that showed cell shrinkage, chromatin condensation, and fragmented nuclei indicating apoptotic body were considered positive (Figure 4B). Cells with light diffuse

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**Figure 1.** Western blot analysis using the cytosolic and mitochondrial fractions. A, Western blot analysis of cytochrome c (cyto-c) and COX-IV in the cytosolic (cytosol.) and mitochondrial (mitochon.) fractions of contralateral (C) or ischemic (I) hemisphere at 24 hours after 30 minutes of MCAO. β-Actin was used as an internal control (bottom). The cytosolic fraction (B) and mitochondrial fraction (C) were collected from the ischemic (I) and contralateral (C) hemispheres in the sham-control (S) or ischemic mice after the indicated reperfusion periods after 30 minutes of MCAO and were analyzed by immunoblotting with cytochrome c antibody. The amount of protein loaded in the cytosolic fraction was examined by reblotting the membrane with β-actin antibody. Increased cytochrome c immunoreactive bands were observed in the cytosolic fraction from the ischemic hemispheres at 3 to 24 hours after reperfusion (B), while there was no remarkable change in the mitochondrial fraction during these reperfusion periods (C). Data were reproduced by 4 independent experiments. The results from a representative experiment are shown.

We then studied time-dependent changes in the intracellular cytochrome c distribution after ischemia (n=4 in each group). We detected cytochrome c in the cytosolic fraction from the ischemic hemisphere as early as 3 hours after reperfusion. The cytochrome c immunoreactivity was increased until 24 hours (Figure 1B). However, the cytochrome c immunoreactivity in the mitochondrial fraction did not change during reperfusion periods (Figure 1C).

We next examined the effect of ebselen (10 mg/kg) on mitochondrial cytochrome c release after 30 minutes of MCAO. Pretreatment with ebselen significantly reduced the level of cytochrome c immunoreactivity in the cytosolic fraction at 12 and 24 hours after reperfusion ($P<0.05$) (Figure 2).

**Ebselen Reduces Caspase-3-like Activity**

DEVDase activity was initially increased to $15.0\pm6.2$ pmol/mg per minute at 12 hours after reperfusion (n=4) and returned to $9.6\pm3.6$ pmol/mg per minute at 24 hours (n=4), consistent with a previous work. Pretreatment with ebselen inhibited DEVDase activity by 61% when examined at 12 hours after reperfusion ($P<0.05$; n=7 and n=4 in vehicle- and ebselen-treated mice, respectively).

**Figure 2.** Ebselen decreases the mitochondrial cytochrome c (cyto-c) release into the cytosol. Western blot analysis of cytochrome c in the cytosolic fractions in the ischemic hemisphere from vehicle- and ebselen-treated mice at 12 (A) and 24 hours (B) after ischemia. β-Actin was used as an internal control. Reduced immunoreactive bands were observed in ebselen-treated mice at both time points. Data from 4 mice in each group were presented. C. Densitometric analysis of the cytochrome c immunoreactive bands in the cytosolic fraction demonstrating a significant reduction in ebselen-treated mice. Bars show SEM; n=4 in each group. $^*P<0.05$.

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**Figure 3.** Brain damage volume determined at 3 days after 30 minutes of MCAO. Ebselen or vehicle was given at the indicated time after reperfusion and followed by a second administration 12 hours thereafter. Ischemic control group was subjected only to ischemia without administration. The damage volume was expressed as a percentage of the contralateral hemisphere volume. Bars show SEM; n=6 to 9 in each group. $^*P<0.05$ compared with ischemic control; $^\#P<0.05$ compared with vehicle.

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**Figure 4.** Ebselen decreased DNA fragmentation. A, TUNEL staining of ischemic brain. Only densely labeled cells that showed cell shrinkage, chromatin condensation, and fragmented nuclei indicating apoptotic body were considered positive. Cells with light diffuse
Core Temperature, Regional Cerebral Blood Flow, and Body Weight Loss

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle (n=9)</th>
<th>Ebselen (10 mg/kg) (n=8)</th>
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<td>Core temperature, °C</td>
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<td>During</td>
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<tr>
<td>3 hours</td>
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<tr>
<td>3 days</td>
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<td>36.9±0.4</td>
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<tr>
<td>Regional cerebral blood flow, %</td>
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<tr>
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<td>100</td>
</tr>
<tr>
<td>During</td>
<td>21.2±1.9</td>
<td>20.7±2.0</td>
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<tr>
<td>After</td>
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<td>17.6±1.9</td>
<td>20.1±4.2</td>
</tr>
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Values are mean±SEM. Body weight loss at 3 days after ischemia was calculated by the following formula: 100×(body weight (before)−body weight (3 days)/body weight (before). Ebselen or vehicle (0.5% carboxymethyl cellulose, 5 mL/kg) was administered twice, 30 minutes before ischemia and 12 hours after reperfusion.

These data indicate that ebselen inhibits neuronal apoptosis induced by focal cerebral ischemia.

Neuroprotection by Ebselen Is Sustained for 21 Days

Finally, to ask whether ebselen delays or inhibits neuronal death, we evaluated neuronal survivability in mice that survived for 21 days after ischemia. We counted the number of NeuN immunopositive cells in the striatum that were considered viable. In the contralateral hemisphere, many NeuN immunopositive cells were found throughout the striatum in both vehicle- and ebselen-treated mice (Figure 5A). In contrast, NeuN immunopositive cells were occasionally found in the ischemic striatum (Figure 5B). Ebselen significantly increased the number of NeuN immunopositive cells in the medial and lateral parts of the striatum compared with vehicle (Figure 5C).

Discussion

The authors presented 2 main findings. First, ebselen reduced mitochondrial cytochrome c release resulting from transient focal cerebral ischemia (30 minutes). Second, ebselen decreased DNA fragmentation in the brain after ischemia. The cytochrome c release from the mitochondria into the cytosol is an early molecular event in apoptosis.14,15 The cytochrome c induces caspase activation by making a complex with Apaf-1, caspase-9, and dATP.14,31 Activated caspase-9 in turn activates caspase-3, which cleaves various intracellular substrates, playing a critical role for the execution step in apoptosis. Caspase-3 also activates DNase by cleaving inhibitor of caspase-activated deoxyribonuclease (ICAD), thereby contributing to DNA fragmentation, which is a hallmark of apoptosis.32 Moreover, we observed that ebselen reduced caspase-3–like activity in ischemic brain. Taken together, these results suggest that ebselen inhibits apoptosis by blocking the mitochondrial cytochrome c release in ischemic brain. This is the first demonstration that ebselen inhibits apoptosis resulting from cerebral ischemia.

Although we observed a low level of cytochrome c release as early as 3 hours after reperfusion, increased caspase-3–like activity was first detected at 12 hours. The difference in onset...
of these 2 molecular events may indicate that minor levels of cytochrome c release are not sufficient to induce caspase-3 activation in ischemic brain. Another explanation for the delayed caspase-3 activation is that other required factors for the activation of caspase-3, such as Apaf-1, caspase-9, and dATP, may not be sufficient during these reperfusion periods. A previous work provided evidence that cytochrome c release is a reversible phenomenon. 33

Postischemic treatment with ebselen was effective when given at 6 hours after reperfusion, at which time the caspase-3–like activity had not yet appeared. The level of cytosolic cytochrome c was dramatically increased from 6 to 24 hours after reperfusion. It is highly likely that ebselen given at 6 hours provided protection by inhibiting this delayed but major cytochrome c release. This needs to be examined to establish the causal link between the reduced mitochondrial cytochrome c release and neuroprotection by ebselen.

We have not clarified the exact mechanisms by which ebselen blocks the mitochondrial cytochrome c release into the cytosol. The antioxidant activity of ebselen may contribute to this effect by diminishing mitochondrial oxidative stress. Previous studies demonstrated that antioxidant molecules such as SOD 232 and U-74389G, 34 a free radical scavenger, inhibit both ROS production and mitochondrial cytochrome c release into the cytosol. Another possibility is that ebselen modifies the protein expression or function of cell death–related molecules such as Bcl-2 family members that are important in maintaining of the mitochondrial membrane integrity. Although we did not find upregulated protein expression of Bclx-L in ebselen-treated ischemic brains (data not shown), other Bcl-2 family members remain to be studied. In addition, ebselen may block cytochrome c release by altering caspase-8 activity. Caspase-8, which plays a crucial role in Fas and tumor necrosis factor–α–mediated apoptosis, has been demonstrated to induce the mitochondria-mediated caspase activation. Caspase-8 cleaves the cytosolic molecule Bid, and the cleaved Bid translocates to the mitochondria from the cytosol, resulting in cytochrome c release. 35,36 In fact, ebselen was shown to inhibit tumor necrosis factor–α–mediated liver apoptosis. 23

In conclusion, we demonstrated that ebselen reduces cytochrome c release and subsequent DNA fragmentation after 30 minutes of MCAO in ICR mice. In addition, we observed that the neuronal protection by ebselen was sustained for 21 days after ischemia. These data indicate that ebselen not only postpones but indeed inhibits neuronal apoptosis resulting from cerebral ischemia. Thus, the present study provides further evidence that ebselen may be a useful intervention for the treatment of ischemic stroke.

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