Journal of Neurochemistry, 2001, **78**, 1307–1314

The neuroprotective agent ebselen modifies NMDA receptor function via the redox modulatory site

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Abstract

Ebselen is a seleno-organic compound currently in clinical trials for the treatment of ischemic stroke and subarachnoid hemorrhage. Its putative mode of action as a neuroprotectant is via cyclical reduction and oxidation reactions, in a manner akin to glutathione peroxidase. For this reason, we have investigated the effects of ebselen on the redox-sensitive NMDA receptor. We have found that ebselen readily reversed dithiothreitol (DTT) potentiation of NMDA-mediated currents in cultured neurons and in Chinese hamster ovary (CHO) cells expressing wild-type NMDA NR1/NR2B receptors. In contrast, ebselen was unable to modulate NMDA-induced currents in neurons previously exposed to the thiol oxidant 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), or in CHO cells expressing a mutant receptor lacking the NR1 redox modulatory site, suggesting that ebselen oxidizes the NMDA receptor via this site. In addition, ebselen was substantially less effective in modifying NMDA responses in neurons exposed to alkylating agent *N*-ethylmaleimide (NEM) following DTT treatment. Ebselen also reversed DTT block of carbachol-mediated currents in Cos-7 cells expressing the $\alpha_2\beta\delta\varepsilon$ subunits of the acetylcholine receptor, an additional redox-sensitive ion channel. Ebselen was observed to significantly increase cell viability following a 30-min NMDA exposure in cultured neurons. In contrast, other more typical antioxidant compounds did not afford neuroprotection in a similar paradigm. We conclude that ebselen may be neuroprotective in part due to its actions as a modulator of the NMDA receptor redox modulatory site.

Keywords: cerebral cortex, ebselen, excitotoxicity, NMDA receptor, redox modulation, tissue culture.

J. Neurochem. (2001) **78**, 1307–1314.

Ebselen (2-phenyl-1,2-benzisoselenazol-3[2*H*]-one) is a seleno-organic compound that has been shown to be cytoprotective in various ischemia-reperfusion models in both heart (Maulik *et al*. 1998) and brain (Takasago *et al*. 1997). Ebselen is currently in clinical trials for the treatment of ischemic stroke (Yamaguchi *et al*. 1998) and aneurysmal subarachnoid hemorrhage (Saito *et al*. 1998). Although generally considered to be an antioxidant, ebselen is not a free radical scavenger *per se*, but mimics the enzyme glutathione peroxidase (Mueller *et al*. 1984; Wendel *et al*. 1984; Sies 1993) and a closely related enzyme, phospholipid hydroperoxide glutathione peroxidase (Maiorino *et al*. 1988). Ebselen, like these enzymes, acts with glutathione through a selenium core to eliminate hydroperoxides and lipoperoxides. Although the mechanism of ebselen is similar to these macromolecules, ebselen does not have a substrate-binding site, and therefore, has a much wider range of specificity (Sies 1993; Schewe 1995). Importantly, ebselen can interact with cysteine residues contained in proteins such as metallothionein (Jacob *et al*. 1998), glutathione-*S*-transferase (Nikawa *et al*. 1994), and the IP3 receptor (Dimmeler *et al*. 1991), leading to thiol oxidation and formation of disulfides and an ebselen diselenide product (Schewe 1995).

Several ligand-gated ion channels contain labile cysteine residues that are modified by redox-active compounds. These include the nicotinic acetylcholine receptor (Karlin and Bartels 1966), the $GABA_A$ receptor (Pan *et al*. 1995), and the NMDA subtype of glutamate receptor (Aizenman *et al*. 1989; Tang and Aizenman 1993a). Of these, a

Received March 12, 2001; first revision received May 10, 2001; second revision received June 25, 2001; accepted June 28, 2001.

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Abbreviations used: AChR, acetylcholine receptor; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), DTT, dithiothreitol; EBSS, Earle's balanced salt solution; LDH, lactate dehydrogenase; MEM, minimum essential medium; NEM, *N*-ethylmaleimide; PQQ, pyrroloquinoline quinone.

physiological role for redox modulation has been evaluated extensively on the NMDA receptor (Aizenman 1994; Aizenman *et al*. 1998), due to the importance of this protein in such processes as neuronal development, learning and memory, and excitotoxicity (Dingledine *et al*. 1999). Physiological responses in neurons mediated by NMDA receptors are potentiated by disulfide reducing agents such as dithiothreitol (DTT; Aizenman *et al*. 1989; Tang and Aizenman 1993a). Conversely, sulfhydryl oxidants such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Aizenman *et al*. 1989), lipoic acid (Tang and Aizenman 1993b), and pyrroloquinoline quinone (PQQ; Aizenman *et al*. 1992) are able to reverse DTT potentiation. Oxidants can also depress NMDA receptor function from baseline levels, depending on the native redox state of the receptor (Aizenman *et al*. 1989; Colton *et al*. 1989; Gozlan *et al*. 1994; Sinor *et al*. 1997). Indeed, it was recently demonstrated that PQQ, previously shown to be neuroprotective *in vitro* and *in vivo* (Aizenman *et al*. 1992; Jensen *et al*. 1994), was able to reverse the chemical reduction of the NMDA receptor redox site that occurred as a result of seizure activity (Sanchez *et al*. 2000). This condition appeared to induce the release of yet unidentified endogenous reducing agents (Sanchez *et al*. 2000). As ebselen can alter the function of various proteins via thiol oxidation, we investigated whether this drug could also interact with the redox-sensitive NMDA receptor to modulate its function. Such an interaction would be suggestive of a novel mechanism of ebselen in altering glutamatergic synaptic transmission and associated pathophysiologies.

Materials and methods

Tissue culture

Cortical neurons were dissociated from E16 Sprague–Dawley rats as described previously (Hartnett *et al*. 1997). Cortices were dissociated in Earle's balanced salt solution (EBSS) with 0.03% trypsin at 37°C. Plating suspension was adjusted to a density of 335,000 cells/mL growth media. Growth media contained 80% (v/v) Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD, USA) with 10% heat-inactivated, iron-supplemented bovine calf serum (Hyclone, Logan, UT, USA), 10% Ham's F-12 media (Sigma, St Louis, MO, USA), 25 mM HEPES, 24 U/mL penicillin, 24 U/mL streptomycin and 2 mM L-glutamine. Dissociated cells were plated in six-well plates containing five 12 mm glass coverslips that had been previously treated with poly-L-lysine. Cells were refed on a Monday/Wednesday/Friday basis and maintained in 37°C, 5% CO_2. Two weeks after plating, non-neuronal cell growth was arrested with a 72-h treatment with 2 μM cytosine arabinoside, after which the growth media contained only 2% serum and no F-12. Cells were used for electrophysiology experiments in the fourth week after dissociation.

For toxicity experiments, forebrain neuronal-enriched cultures were prepared as previously described (Aizenman *et al.* 2000). Dissociated cells from E17 rat fetuses were plated on poly-L-ornithine-treated tissue culture plates in a growth medium containing 80% DMEM (high glucose with L-glutamine and without sodium pyruvate; Gibco BRL), 10% Ham's F-12 nutrients, and 10% heat-inactivated bovine calf serum, and 1X antimycotic/antibiotic mixture with amphotericin B and streptomycin sulfate (Gibco BRL). Cultures were maintained at 37°C in 5% CO_2. Glial cell proliferation was inhibited after 48 h in culture with 1–2 μM cytosine arabinoside. Serum-containing medium was replaced after three days *in vitro* with a serum-free medium containing Neurobasal medium (without L-glutamine; Gibco BRL), B27 supplement (Gibco BRL), and antimycotic–antibiotic mixture as above.

Heterologous expression systems

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 media containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were passaged (less than 30 times) at a 1 : 10 dilution when 80% confluent, usually every two days. The cDNAs for NMDA receptor subunits had been previously ligated into mammalian expression vectors containing the cytomegalovirus promoter (Boeckman and Aizenman 1996; Brimecombe *et al.* 1999). CHO cells were seeded at 2.8×10^5 cells/well into six-well plates 24 h previous to transfection. Cells were transfected in serum-free medium with 6 μL LipofectAMINE reagent (Gibco BRL) and a total of 1.4 μg of DNA/well. A ratio of 0.3 : 1 : 3 green fluorescent protein: NR1: NR2 subunit ratio was employed. Four hours after transfection, cells were refed with media containing 10% serum and, 24 h or less after transfection, 300 μM ketamine was added to prevent the excitotoxic cell death that occurs following functional receptor expression (Boeckman and Aizenman 1996).

Cos-7 cells were maintained in DMEM containing 10% FBS and 2 mM L-glutamine and were passaged at a 1 : 5 dilution at 70–80% confluency, approximately every 2 days. Acetylcholine receptor subunit (α, β, δ and ε) cDNA in pSM with an SV40 promoter was the kind gift of Dr Zuo-Zhong Wang (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Cos-7 cells were transiently transfected with the following subunit ratios: 1.32 α : 0.66 β : 0.32 δ : 1.00 ε. The transfection protocol was similar to that described for CHO cells, without the ketamine addition. Cells were used for recording 40–60 h after transfection (Gu *et al*. 1990).

Electrophysiology

Electrophysiological recordings were performed at room temperature (25°C) using the whole-cell configuration of the patch-clamp technique. Cells were bathed in external solution containing (in mM): 150 NaCl, 1.0 $CaCl_2$, 2.8 KCl, 10 HEPES, 10 glycine, 25 tetrodotoxin (Calbiochem, La Jolla, CA, USA) and pH was adjusted to 7.2 with NaOH. Electrodes were pulled on a Sutter P-87 electrode puller (Sutter Instruments, Novato, CA, USA) to a resistance of 1.5–3 MΩ when filled with internal solution containing (in mM): 140 CsF, 10 EGTA/CsOH, 1 $CaCl_2$ and 10 HEPES (pH adjusted to 7.2 with CsOH). Signals were amplified using an Axopatch 200B integrating patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), filtered using an 80 dB/decade filter at 1 kHz, and digitized at 2 kHz with a DigiData 1200b (Axon Instruments) computer interface. The reference electrode was an Ag/AgCl wire bridged with 2 M KCl/1% agarose in PE-90 tubing.

Drugs were applied via a perfusion system with a stepper motor for fast solution changes (Warner Instruments Corp., Hamden, CT, USA). NMDA, DTT, DTNB, NEM and ebselen (Sigma) were all dissolved in external solution for recording. Ebselen was diluted from a 50-mM stock solution in DMSO. Data were collected and analyzed using commercially available software (pCLAMP 8, Axon Instruments).

Toxicity assays

Coverslips containing forebrain neurons were transferred from six-well plates to 24-well plates and treated in triplicate. Coverslips were maintained in serum-containing media at 37°C until ready for treatment. Cells were gently washed twice with 2 mL of a solution containing minimum essential media (MEM; no phenol red), 0.01% bovine serum albumin (BSA; Sigma) and 25 mM HEPES (MEM/BSA). Drugs were diluted in 1 mL MEM/BSA, and wells were incubated in treatment solutions for the indicated period of time. After treatment, cells were washed as before. Cells were incubated for 20 h in MEM/BSA, at which point neuronal viability was measured with a lactate dehydrogenase (LDH)-based *in vitro* toxicology assay kit (Sigma; Hartnett *et al*. 1997). Media samples (40 μL) were analyzed spectrophotometrically (490–630 nm) according to the manufacturer's protocol, to obtain a measure of cytoplasmic LDH release from dead and dying neurons. Relative toxicity is expressed in optical density (OD) units.

Results

Ebselen modifies NMDA receptor function

Whole-cell responses to 30 μM NMDA were recorded at −60 mV under control conditions, after a 3-min application of 4 mM DTT, and following a 30-s incubation in varying concentrations of ebselen. Ebselen was able to readily reverse DTT-mediated NMDA current potentiation in a concentration-dependent manner (Fig. 1a). At higher concentrations of ebselen (10–30 μM), post-ebselen currents were sometimes smaller than control responses (i.e. before DTT treatment). Cells were treated further with repeated iterations of this protocol; DTT and ebselen were able to exert opposing effects on each cell for as long as the recording configuration was held. Furthermore, the effects of ebselen in depressing NMDA-induced currents could only be reversed by an additional DTT treatment. This effect

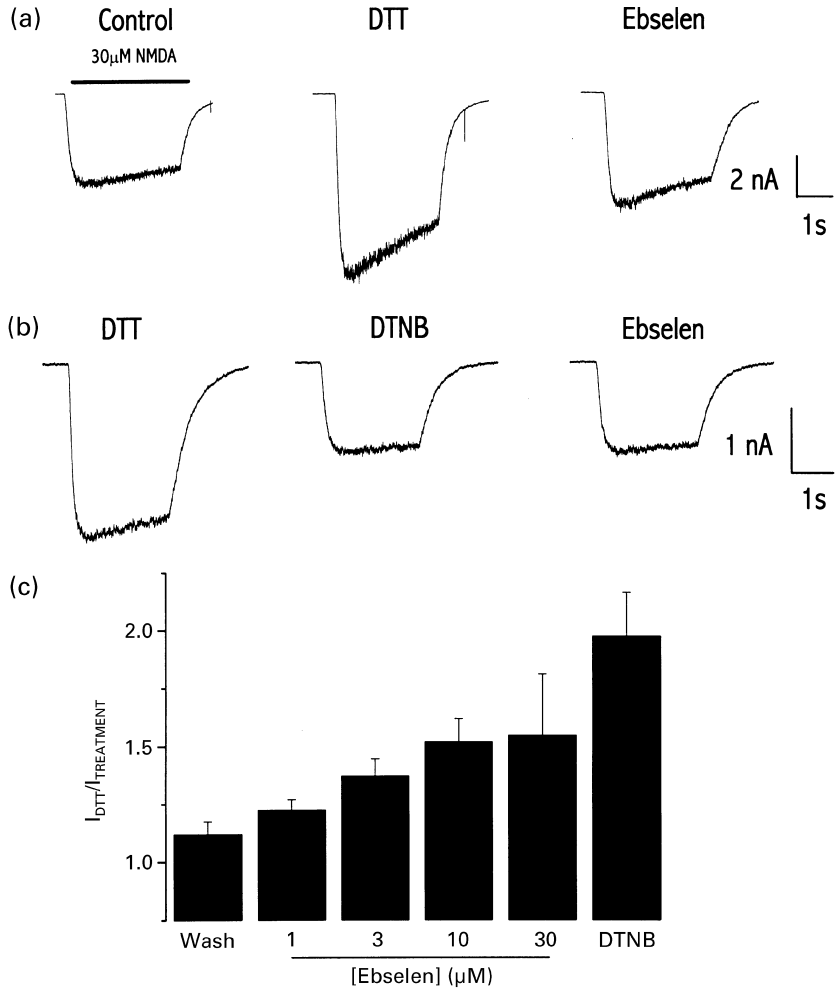

Fig. 1 Ebselen reverses NMDA-induced whole-cell current potentiation by the reducing agent DTT. (a) Representative whole-cell recordings from a cortical neuron during activation by 30 μM NMDA at −60 mV. Cells were bathed in 4 mM DTT for 3 min, which potentiated NMDA currents by an average of two-fold. Immediately following DTT, cells were treated with 10 μM ebselen for 30 s, reversing the actions of DTT. Bar depicting NMDA application is only shown in the first trace in this and subsequent figures for clarity. (b) Whole-cell responses to 30 μM NMDA from a cell previously exposed to 4 mM DTT, and following a 30-s exposure to 500 μM DTNB, and following a wash period, to an additional 30 s treatment with 30 μM ebselen. Note that ebselen does not alter the amplitude of the currents after prior oxidation with DTNB. Similar observations were made in a total of six cells. (c) Reversal of DTT potentiation is concentration dependent. Each bar of the histogram represents the mean ratio (± SEM $n = 4–8$) between the peak potentiated current (3 min DTT) and the peak current after 30 s treatment with wash, ebselen, or 500 μM of the oxidizing agent DTNB. A Kruskal–Wallis test revealed a significant treatment effect by ebselen ($p < 0.05$). Controls utilizing the maximal concentration of DMSO used (0.06%) had no effect on the currents.

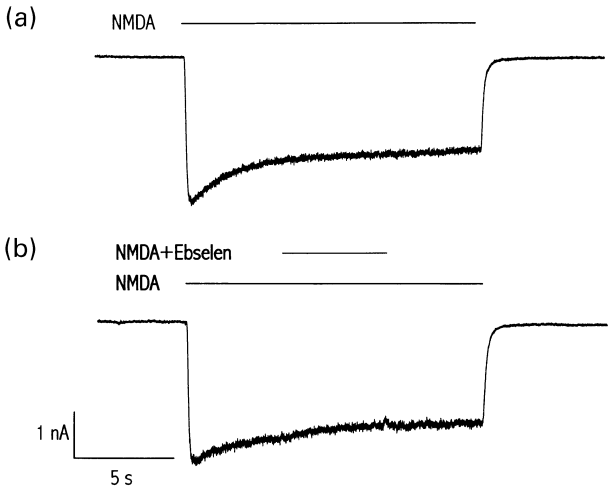

Fig. 2 Ebselen does not directly antagonize NMDA receptors. (a) NMDA (30 μM)-induced current elicited from a 4-week-old cortical neuron. (b) When ebselen was applied for 4 s during application of NMDA, ebselen did not alter whole cell responses to agonist. Similar results were obtained in a total of five cells.

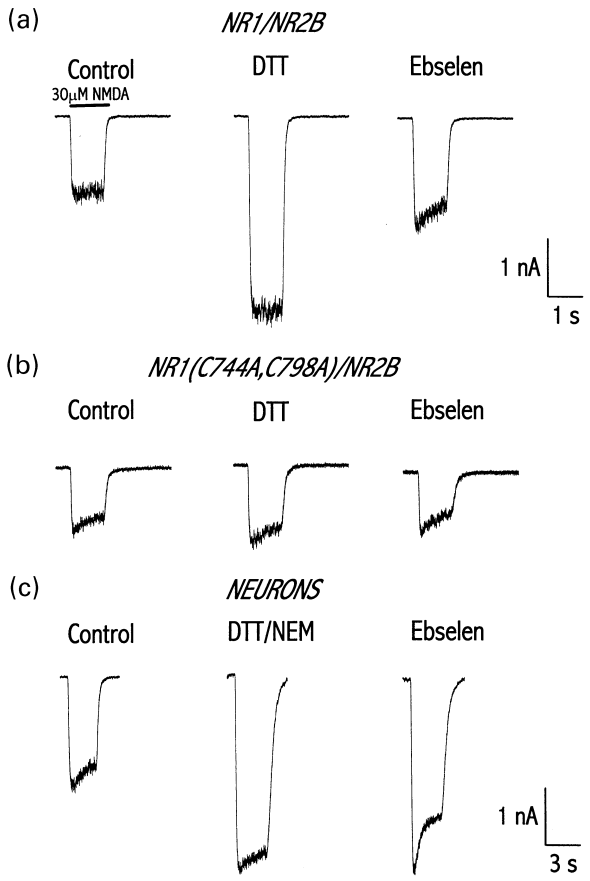

Fig. 3 Modulation of NMDA currents by ebselen requires a functional redox site. (a) Representative whole cell currents recorded from a CHO cell transiently expressing recombinant NR1/NR2B receptors during application of 30 μM NMDA. Currents were measured under control conditions, following a 3-min application of 4 mM DTT, and a subsequent 30 s application of 10 μM ebselen. DTT typically potentiated currents 1.5–2 –fold, reversible by ebselen. (b) Ebselen has no effect on responses from a CHO cell transfected with NR1(C744A,C798A)/NR2B receptors, which lack a functional NR1 redox site. Note that DTT does not significantly enhance responses to NMDA, nor does ebselen depress responses from baseline. Similar observations were noted in a total of 9 cells expressing wild-type receptors and in 12 cells expressing the mutated NR1 subunit. (c) Treatment with the irreversible alkylating agent *N*-ethylmaleimide (NEM) causes a permanent potentiation of NMDA-mediated whole-cell currents in cortical neurons. Cells were treated with the reducing agent DTT (6 mM; 6 min), and immediately bathed in 300 μM NEM (1 min), which substantially prevented further peak current modification by ebselen. Changes in the desensitization profile of the response were not reproducible.

of ebselen closely resembles the actions of the oxidizing agent DTNB (Aizenman *et al*. 1989; Tang and Aizenman 1993a), implying that ebselen is able to oxidize the redox site of the NMDA receptor. We noted that incubations with ebselen at concentrations in the 100–300 μM range quickly destabilized the recordings. Therefore, we were unable to determine whether this compound could oxidize the NMDA receptor to the same extent as DTNB (500 μM; Fig. 1c). Nonetheless, pretreatment with DTNB (500 μM) occluded any potentially additional actions of ebselen (30 μM), further strengthening the notion that the seleno-organic compound acts via the NMDA receptor redox site (Fig. 1b).

An additional set of experiments confirmed that ebselen did not alter membrane conductance by itself or directly antagonize NMDA responses. No currents were observed during a 2-min application of 10 μM ebselen (not shown). In addition, 10 μM ebselen co-applied during application of 30 μM NMDA did not appreciably alter whole cell responses (Fig. 2).

To confirm that ebselen reversed the actions of DTT in neuronal receptors by oxidizing the known redox-sensitive site on the NR1 subunit (Sullivan *et al*. 1994), we transiently expressed cDNAs for both wild-type (NR1/NR2B) and a redox-insensitive double cysteine mutant [NR1(C744A, C798A)/NR2B; Sullivan *et al*. 1994; Brimecombe *et al*. 1999] NMDA receptor in CHO cells. Currents recorded from cells expressing NR1/NR2B NMDA receptors showed properties similar to those obtained from cultured neurons. DTT potentiation was approximately 1.5–2 -fold, and a 30-s application of ebselen (10–30 μM) reversed this potentiation (Fig. 3a). However, in NR1(C744A,C798A)/NR2B expressing cells, little or no DTT potentiation was observed, and ebselen did not appreciably alter the magnitude of currents (Fig. 3b). The lack of effect of ebselen in this redox-insensitive NMDA receptor configuration strongly implicates cysteines 744 and 798 in the modulation of NMDA receptor function by this compound. In an additional set of studies, we incubated neurons with the alkylating agent

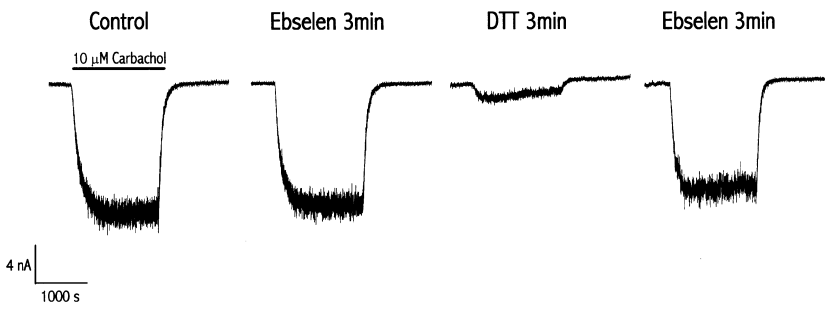

Fig. 4 Ebselen reverses the effects of DTT on carbachol-induced currents in nicotinic acetylcholine receptors. Responses to 10 μM carbachol obtained from $\alpha_2\beta\varepsilon\delta$ recombinant nicotinic acetylcholine receptors transiently expressed in Cos-7 cells were unchanged by a three minute incubation in ebselen prior to treatment with DTT. A subsequent 3 min treatment with 4 mM DTT substantially diminished carbachol-induced currents. Application of 10 μM ebselen (3 min) returned currents to control levels. Similar results were observed in a total of 6 cells.

NEM (300 μM) immediately following a 6-mM DTT treatment. Under these conditions, we have previously shown that the NMDA receptor can be alkylated and rendered relatively insensitive to further reduction or oxidation (Tang and Aizenman 1993a). Following alkylation, the actions of ebselen in modulating NMDA receptor function were dramatically diminished (Fig. 3c).

Ebselen can oxidize cysteine residues in the nicotinic receptor

We evaluated whether ebselen could modify another redox-sensitive, ligand-gated ion channel. These experiments were performed to confirm that ebselen has no substrate specificity and that it can readily oxidize cysteine residues in unrelated members of the ligand-gated channel superfamily. Nicotinic acetylcholine receptors (nAchRs) contain a disulfide bond that lies very close to the agonist-binding site of the receptor complex, and reduction of this bond abolishes receptor function (Karlin and Bartels 1966; Kao and Karlin 1986). Oxidation can readily restore activity in these channels. Carbachol (10 μM)-induced currents were recorded from Cos-7 cells expressing $\alpha_2\beta\delta\varepsilon$ subunits of the nAChR under control conditions and following incubation with 4 mM DTT (3 min) or 10 μM ebselen. As expected, carbachol-induced currents were unaffected by ebselen (10 μM) but diminished substantially following DTT treatment. Treatment with 10 μM ebselen after DTT returned currents to near control levels (Fig. 4). DTNB (500 μM; 7 min) mimicked the actions of ebselen in reversing the effects of DTT (not shown), suggesting that ebselen also acts as an oxidant at the nicotinic receptor.

Ebselen is neuroprotective against NMDA toxicity *in vitro*

We sought to determine whether the interaction of ebselen with the NMDA receptor could protect cells from NMDA in a neurotoxicity paradigm. When applied to neurons overnight, 30 μM ebselen showed no intrinsic toxicity (data not shown). Exposure of cultured rat cortical neurons to 100 μM NMDA for 30 min caused significant neurotoxicity (~80%; Sinor *et al*. 2000), reflected as an increase in LDH release from control (Fig. 5). Neuronal toxicity was nearly completely blocked by a co-application with the NMDA receptor blocker MK-801 (10 μM), confirming the NMDA-receptor dependency of the neuronal death observed. Ebselen (10 μM) significantly blocked cell death by approximately 40%, a level of protection similar to what had previously been described for PQQ (Aizenman *et al*. 1992). In these experiments, ebselen was present 30 min prior to NMDA exposure to ensure that all the receptors were in the oxidized state before activation. Ebselen was also present during and following agonist exposure. The reason for including ebselen following agonist exposure was to eliminate the possibility that secondary glutamate release might obfuscate the neuroprotective actions of ebselen, as this drug does not antagonize the agonist actions of NMDA *per se*. In order to ensure that ebselen induced neuroprotection by oxidizing the NMDA receptor prior to NMDA exposure, we performed additional experiments where 10 μM MK-801 substituted for ebselen in the post-exposure period. Under these conditions, we observed 43% neuroprotection in the ebselen/post-MK-801 treatment group, compared to 19% protection in the post-MK-801 only treatment group (not shown). Hence, the complete neuroprotective effects of ebselen in our paradigm are likely mediated by maintenance of the receptors in the oxidized state during the secondary release of glutamate.

Ebselen has been shown in several systems to abrogate oxidative injury (Mueller *et al*. 1984; Wendel *et al*. 1984; Sies 1993). In fact, a recent study has suggested that ebselen is protective against glutamate toxicity in cultured rat cerebellar granule cells by acting as an antioxidant (Porciuncula *et al*. 2001). Therefore, we evaluated whether other, more traditional, antioxidant compounds could also inhibit NMDA toxicity under our exposure conditions. We utilized three different antioxidants: trolox (100 μM), glutathione methyl ester (1 mM), and *N*-acetylcysteine (1 mM). None of these agents provided any measurable protection against NMDA-induced neurotoxicity.

Discussion

We have found that ebselen reversed DTT potentiation of NMDA-induced currents in cortical neurons in a concentration-dependent manner. This effect was also observed in recombinant NR1/NR2B receptors expressed in CHO cells, but not in cells expressing an NR1(C744A/C798A)/NR2B mutant receptor, which lacks a functional redox modulatory site. Furthermore, alkylation of native receptors also substantially diminished the actions of ebselen. In addition, recombinant nicotinic acetylcholine receptors are modulated by ebselen in a manner analogous to other thiol oxidizing agents such as DTNB. This leads us to conclude that ebselen acts as an oxidant at the NMDA receptor redox modulatory site, a previously undescribed mechanism of action for this drug. We believe that the final oxidation products of the interaction of ebselen with the NMDA receptor are an intramolecular disulfide within the cysteines of the redox site itself and an ebselen diselenide product (Schewe 1995). This is due to the fact that an ebselen-cysteinyl adduct would be structurally similar to the alkylated form of the receptor, a conformation that results in a permanent potentiation of NMDA-induced currents (Fig. 3c; Tang and Aizenman 1993a). Whole-cell recordings revealed that ebselen seldom decreased baseline NMDA-induced currents; that is, the actions of ebselen were mostly apparent after the NMDA receptor had been chemically reduced by DTT. This suggests that under our recording configuration, most of the NMDA receptors in the culture system exist primarily in the oxidized state. Although this situation is normally encountered in cultured cells that are being rapidly perfused in the recording set-up (e.g. Aizenman *et al*. 1992), it may not be reflective of the redox state of NMDA receptors present in neurons in more intact preparations, especially during pathophysiological conditions (e.g. NMDA exposure or seizure activity; Aizenman *et al*. 1992; Sanchez *et al*. 2000).

There is a substantial body of evidence linking excessive NMDA receptor-mediated calcium influx to the widespread neuronal death that occurs in primary cultures upon exposure to glutamate (Lee *et al*. 1999). Due to the high calcium permeability of the receptor, modulation of the receptor by reducing and oxidizing agents accordingly modulates calcium influx (Reynolds *et al*. 1990; Sucher *et al*.

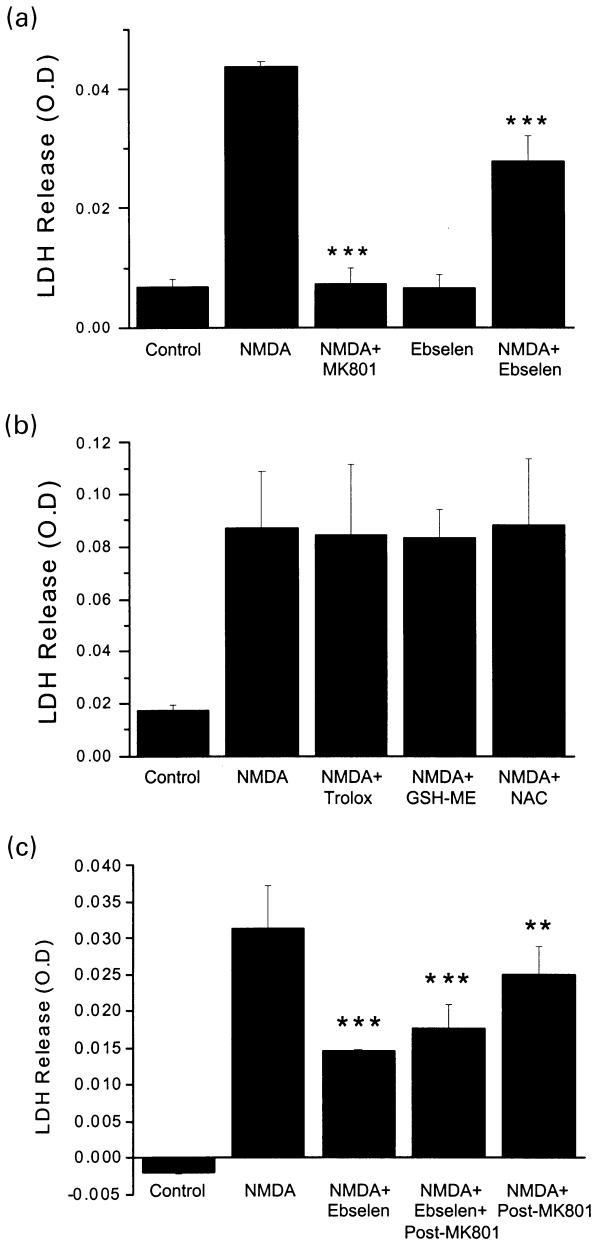

Fig. 5 Ebselen is neuroprotective in an excitotoxicity paradigm. (a) Neuronal cultures were exposed for 30 min to vehicle (control), 100 μM NMDA alone, or in the presence of either 10 μM ebselen or 10 μM MK-801, or ebselen alone. Ebselen was present 30 min prior to exposure, during, and following NMDA exposure. MK-801 was present only during NMDA exposure. The decrease in LDH values from NMDA represent increased neuronal viability, and when normalized against total NMDA toxicity, a significant increase in neuronal survival was observed in the NMDA + ebselen and NMDA + MK801 treatment groups (***$p < 0.001$, ANOVA followed by Bonferroni's *post hoc* tests). Results represent the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained in a total of six experiments. (b) An additional set of cultures were exposed for 30 min to vehicle (control), 100 μM NMDA alone, or in the presence of 100 μM Trolox, 1 mM glutathione methyl ester (GSH-ME), or 1 mM *N*-acetylcysteine (NAC). Antioxidants were present 30 min prior, during, and following NMDA exposure. No observable neuroprotection was afforded by these compounds, nor were they toxic on their own (not shown). Results represent the mean ± SD of a representative experiment performed in triplicate. Similar results were obtained in a total of three experiments.

1990). As such, DTT and other reducing agents exacerbate excitotoxic neuronal death in culture (Aizenman *et al*. 1990; Aizenman and Hartnett 1992), and oxidizing agents that act at NMDA receptors are neuroprotective *in vitro* (Aizenman *et al*. 1992) and *in vivo* (Jensen *et al*. 1994). We observed here that ebselen is neuroprotective against NMDA-mediated neurotoxicity in rat cortical cultures. We propose that the observed neuroprotection induced by ebselen *in vitro* and *in vivo* (Takasago *et al*. 1997; Saito *et al*. 1998; Yamaguchi *et al*. 1998) may be due, at least in part, to modulation of the NMDA receptor redox site.

We recognize that NMDA receptor oxidation may not fully account for the degree of neuroprotection afforded by ebselen *in vivo*. For instance, ebselen is cardioprotective against ischemia (Maulik *et al*. 1998). As NMDA receptors are irrelevant to ischemia/reperfusion-induced cardiopathology, this drug probably exerts its protective effects through some other function. However, hypoxic neuronal injury in cortical culture is mediated primarily by activation of NMDA receptors (Goldberg and Choi 1993; Sattler *et al*. 2000; Sinor *et al*. 2000). Hence, exposure of cortical neurons to NMDA in culture is a good model of ischemic injury in this system. In addition, a recent study suggests that oxidative stress may not be a primary component in triggering NMDA-mediated excitotoxicity in culture, at least under certain conditions (Rudolph *et al*. 2000). Therefore, it is likely that neuroprotection by ebselen in our model is due primarily to a direct interaction of this drug with the NMDA receptor redox modulatory site. This is supported by our observation that more typical antioxidants, such as trolox, glutathione methyl ester, and *N*-acetylcysteine are not neuroprotective against NMDA toxicity under identical conditions.

NMDA receptors have been implicated in the pathogenesis of neurological disorders including stroke, Parkinson's disease, chronic pain, and epilepsy (Dingledine *et al*. 1999). Though under heavy investigation as therapeutic agents, NMDA receptor blockers have met with limited success in clinical trials due to untoward side-effects associated with blockade of physiological glutamatergic transmission (Lee *et al*. 1999). However, redox modulatory agents do not cause total receptor function loss, and thus are less likely to interfere with normal synaptic transmission. In fact, PQQ, an NMDA receptor oxidant, has been shown to minimize epileptic seizures *in vitro* and *in vivo* while not significantly affecting LTP in hippocampal slices (Sanchez *et al*. 2000). However, PQQ may be toxic at high doses (Jensen *et al*. 1994). In contrast, ebselen shows little toxicity in humans at therapeutic doses (Saito *et al*. 1998; Yamaguchi *et al*. 1998). The findings herein may extend the clinical applicability of this compound beyond stroke to other disorders where the pathophysiological consequences of abnormal glutamatergic transmission have been implicated.

Acknowledgements

We would like to thank Dr BethAnn McLaughlin and Sumon Pal for helpful advice. NMDA receptor subunit cDNAs were the gift of S. Nakanishi, D. Lynch, and S. Traynelis. AChR subunit cDNAs were kindly provided by Z. Z. Wang. This work was supported by a Grant-In-Aid from the American Heart Association, The Charitable Leadership Foundation, and by NIH grant NS29365.

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