Effect of Ebselen on Bovine and Rat Nitric Oxide Synthase Activity
Is Modified by Thiols

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ABSTRACT—The inhibition of nitric oxide synthase (NOS) by ebselen, 2-phenyl-1,2-benzisoselenazole-3(2H)-one, was reversed by the addition of $10^{-5}$ M dithiothreitol, suggesting that ebselen reacts with a critical thiol group of NOS in the inhibitory mechanism. In the presence of $10^{-4}$ to $10^{-3}$ M dithiothreitol, ebselen dose-dependently enhanced NOS activity, implicating another interaction of ebselen with NOS under these conditions. Thus, the effect of ebselen on the NOS activity is modified by thiols.

Keywords: Nitric oxide synthase, Ebselen, Thiol

The enzyme nitric oxide synthase (EC 1.14.23, NOS) catalyzes the biosynthesis of nitric oxide (NO) from L-arginine. It exists in two principal isoforms: inducible (iNOS) and constitutive (cNOS). We previously reported that ebselen, 2-phenyl-1,2-benzisoselenazole-3(2H)-one (1), preferentially inhibits iNOS at a certain concentration range (2). In the present study, we found that thiols can change the effect of ebselen on the NOS activity from inhibition to enhancement with an increase in their concentrations.

Bacillus Calmette-Guérin was obtained from Kyowa Pharmaceutical Company (Tokyo), lipopolysaccharide (E. coli) from Difco Laboratories (Detroit, MI, USA), Dulbecco’s modified Eagle’s medium from Nissui Pharmaceutical Company (Tokyo), fetal calf serum from Gibco Laboratories (Grand Island, NY, USA), calmodulin from Calbiochem Corporation (La Jolla, CA, USA). All other reagents were of analytical grade.

Activated peritoneal macrophages were obtained from Kbl Wistar rats after intraperitoneal injection of Bacillus Calmette-Guérin. Cells were then incubated for 24 hr on 10-cm culture dishes containing Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 10 ng/ml of lipopolysaccharide to make them cytotoxic (3). The cells were suspended in ice-cold Tris-HCl, pH 7.4 and centrifuged at 10,000 $\times$ g for 10 min at 4°C. The supernatant was used as an enzyme source of iNOS.

Bovine aortic endothelial cells were cultured on 10-cm dishes as previously described (4). Cells were used at confluency in the 5th passage. After the cells were washed 3 times with phosphate-buffered saline, 0.8 ml of ice-cold Tris-HCl, pH 7.4, containing 0.5% Triton X-100 (to dissolve membrane-bound endothelial cNOS) was added to the dish. The cells were scraped, sonicated and centrifuged at 10,000 $\times$ g for 10 min at 4°C. The supernatant was removed and used as an enzyme source of endothelial cNOS (ecNOS).

Total thiol contents in the enzyme source of iNOS and ecNOS were measured according to the method of Ellman (5) employing 5,5'-dithiobis (2-nitrobenzoic acid). Protein concentrations were determined by a dye binding assay (Bio-Rad, Richmond, CA, USA) using bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard.

iNOS was allowed to react for 1 hr at 37°C as previously described (3). The reaction mixture (600 µl) contained the enzyme source, 50 mM Tris-HCl (pH 7.4), 1 mM NADPH, 1 mM L-arginine, 0.1 mM (6R)-5,6,7,8-tetrahydrobiopterin and 1 µM FAD in the absence or presence of various concentrations of ebselen (up to 20 µM) and dithiothreitol (DTT, up to $10^{-3}$ M). Ebselen solutions were freshly prepared in dimethylsulfoxide for each ex-
Experiment. In order to examine the effect of the detergent on the iNOS reaction, the same experiments were performed in the presence of 0.5% Triton X-100. The reaction of eNOS was allowed to proceed essentially as that of iNOS, except that the reaction mixture contained 0.5% Triton X-100, 1 mM Ca^{2+} and 0.1 μM calmodulin. The enzyme reaction was stopped by heating at 100°C for 30 sec. The mixture was then centrifuged at 15,000 x g for 20 min. The resulting supernatant was assayed for nitrite and nitrate, breakdown products of NO (3), to determine the enzyme activity. It was expressed as the ratio (% to the control value (570 pmol/min) obtained in the absence of both ebselen and dithiothreitol.

In the absence of DTT, ebselen showed dual actions on the activities of both iNOS (Fig. 1) and eNOS (Fig. 2). It enhanced the NOS activity at low concentrations and inhibited it at higher concentrations, although the difference was prominent in iNOS as previously described (2). The addition of 10^{-6} M DTT did not change this pattern. At the DTT concentration of 10^{-3} M, the inhibition of iNOS activity by ebselen was abolished (Fig. 1), and the inhibition of eNOS by ebselen at concentrations >5 μM was also attenuated (Fig. 2).

In the presence of 10^{-4} to 10^{-3} M DTT, ebselen dose-dependently raised the iNOS activity, reaching a plateau around 5 μM (Fig. 1). The enzyme activity determined in the absence of ebselen was elevated compared with that obtained under 0 to 10^{-3} M DTT. An increase in the DTT concentration to 10^{-3} M shifted the curve upwards. DTT also induced similar changes in the eNOS activity (Fig. 2). The DTT-induced increase in the eNOS activity at 0 μM ebselen was less prominent.

When the iNOS reaction was performed in the presence of 0.5% Triton X-100, similar results (not shown) were obtained as in Fig. 1. This suggests that the difference between Figs. 1 and 2 was not ascribed to the effect of the detergent. Furthermore, there was no significant difference (unpaired t-test) in the thiol contents between the enzyme source of iNOS and eNOS (46±4 vs 45±5 (mean±S.D., n=5) pmol/mg protein).

The exact mechanism of inhibition of NOS by ebselen has not been clarified. Previously, the mechanism was postulated to be the reaction of ebselen with a thiol group of NOS, which is essential for the catalytic activity of the enzyme, because inhibition of endothelial eNOS by ebselen was prevented by thiols at 10^{-4} M (6). In the present study, the inhibition of both eNOS and iNOS by ebselen was also nearly reversed by the addition of 10^{-3} M DTT. Therefore, the reaction of ebselen with a critical thiol group of NOS is considered to be a possible mechanism of the inhibition.

In the presence of 10^{-4} to 10^{-3} M DTT, however, ebselen dose-dependently enhanced the NOS activity. It is difficult to explain this mechanism. One possibility is that ebselen interacts with different site(s) of NOS that may...
have lower affinity to ebselen than a thiol group of NOS. The blocking of the interaction of ebselen with a thiol group of NOS by DTT enabled such an interaction under these conditions, resulting in the increased enzyme activity.

In the absence of DTT, iNOS was more sensitive to the inhibition by ebselen. Enhancement of enzyme activity by DTT (in the absence of ebselen) was more prominent with iNOS compared with ecNOS. These findings may reflect the difference in the catalytic site between iNOS and ecNOS.

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