Free Rad. Res. Comms., Vol. 2, No. 3, pp. 179–185
8755-0199/86/0203–0179 $25.00/0

Printed in Great Britain

INHIBITORY EFFECTS OF EBSELEN ON LIPID PEROXIDATION IN RAT LIVER MICROSOMES

M. HAYASHI† and T.F. SLATER

Department of Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K.

(Received June 6th 1986)

The effects of ebselen(2-phenyl-1,2-benzoisoselenazol-3(2H)-one), a synthetic seleno-organic compound with glutathione peroxidase-like activity were investigated on lipid peroxidation in rat liver microsomes. Ebselen inhibited malondialdehyde production coupled to the lipid peroxidation stimulated by either ADP-iron-ascorbate or CCl_4. The inhibitory activity of ebselen on each system was strongly increased by a 5-min preincubation with liver microsomes; the IC_{50} values against ADP-Fe-ascorbate-stimulated and CCl_4-stimulated lipid peroxidation were 1.6 μM and 70 μM respectively. Ebselen also inhibited the endogenous lipid peroxidation with a NADPH-generating system, but it slightly stimulated the endogenous activity of ADP-Fe-ascorbate-stimulated lipid peroxidation (without a NADPH-generating system). Furthermore, ebselen inhibited oxygen uptake coupled to the lipid peroxidation by ADP-Fe-ascorbate and NADPH-ADP-iron; the IC_{50} values were 2.5 μM and 20.3 μM respectively. Ebselen also prolonged the lag-time of onset of ADP-Fe-ascorbate-stimulated lipid peroxidation significantly, but not that observed with NADPH-ADP-Fe-stimulated lipid peroxidation.

These findings suggest that ebselen penetrates into the membrane lipid and acts as an effective antioxidant, and that there may be some differences between the modes of inhibitory action on the several types of lipid peroxidation.

KEY WORDS: Ebselen, lipid peroxidation, MDA production, oxygen uptake.

INTRODUCTION

Lipid peroxidation is generally believed to be important in several types of tissue injury and disease[1,2]. Protective mechanisms against free radical-mediated lipid peroxidation can therefore be seen as important in relation to tissue damage and some diseases. One mechanism of protection involves the metabolism or chemical degradation of lipid hydroperoxides that are intermediates in the overall complex process of lipid peroxidation. An enzymatic mechanism for removing lipid hydroperoxides involves glutathione peroxidase(GSH-Px)[3,4]. However, GSH-Px does not penetrate easily into lipid membrane where lipid peroxidation may be initiated[5–7].

Ebselen(PZ 51;2-phenyl-1,2-benzoisoselenazol-3(2H)-one) is a new seleno-organic compound that exhibits GSH-Px-like activity[8–10]. In this study, we have used ebselen to investigate its actions on lipid peroxidation in liver microsomes.

†Permanent address: Research Department, Santen Pharmaceutical Co., Ltd., 3-9-19, Shimoshinjo, Higashiyodogawa-ku, Osaka 533, JAPAN.

MATERIALS AND METHODS

Animals and Chemicals

Male Wistar rats were fed on normal laboratory food and water *ad libitum*, and were starved for one night prior to experimental use.

Chemicals were obtained as follows: ebselen was kindly provided by A. Nattermann & Co. GmbH(Cologne, F.R.G.) and was dissolved in dimethylsulfoxide-(DMSO) in all experiments. Glucose-6-phosphate(G-6-P), NADP disodium salt, glucose-6-phosphate dehydrogenase(G-6-P-D; from yeast Grade II), and adenosine 5′-diphosphate(ADP) were purchased from Boehringer Mannheim (Germany). Thiobarbituric acid, DMSO, and NADPH were purchased from SIGMA Chemical Company (U.S.A.). Carbon tetrachloride(CCl_4; microanalytical grade) was purchased from Hopkin and Williams Ltd. (U.K.); and ferrous sulfate and L-ascorbate were purchased from BDH chemicals LTD. (U.K.).

Preparation of Microsomal Suspension

Liver microsomes were prepared according to the procedure described by Slater and Sawyer[11].

Lipid Peroxidation Stimulated by CCl_4

A stock solution contained 74 mM-KCl, 33 mM-tris HCl buffer; pH 7.4, and 93 mM-acetamide. Stock solution (2.55 ml) was mixed with 0.05 ml of ebselen solution and 0.3 ml of microsomal suspension (approx. 20 mg protein/ml), and preincubated at 37°C for a fixed period (0 or 5 min). After the preincubation, lipid peroxidation was started by the addition of 0.1 ml of G-6-P/NADP/G-6-P-D mixture (containing 112 mM G-6-P, 5.08 mM NADP, and 5.6 U/ml G-6-P-D) and 0.010 ml of 20% (v/v) CCl_4/DMSO solution. Simultaneously, a similar number of tubes, to which were added 0.010 ml of DMSO instead of 20% CCl_4/DMSO solution, were prepared. The tubes were firmly sealed and incubated for 15 min at 37°C in the dark with gentle shaking (approx. 60 shakes/min).

The Estimation of Malondialdehyde(MDA)

The reaction was stopped by the addition of 6 ml of 10% (w/v) trichloroacetic acid and the tubes were placed in ice for 10 min. The precipitates were separated by centrifugation and 2 ml of supernatant was mixed with 2 ml of 0.67% thiobarbituric acid and heated in a boiling-water bath for 10 min. The solution was cooled and the absorption at 535 nm was determined. The thiobarbituric acid determination on each sample was carried out in duplicate.

The results are expressed in terms of MDA production, using the molar extinction coefficient, 1.49×10^5 l/mol/cm[11], and the IC_{50} values were calculated. The protein concentration of the microsomal suspension was estimated by the procedure of Lowry *et al.*[12]

Lipid Peroxidation Stimulated by ADP/Fe/ascorbate

A stock solution contained 90 mM-KCl and 40 mM-tris HCl buffer (pH 7.4). Stock solution (2.45 ml) was mixed with 0.3 ml of microsomal suspension and 0.05 ml of

ebselen solution, and was preincubated at 37°C for a fixed period (0 or 5 min). The lipid peroxidation was started by the addition of 0.1 ml of ADP/Fe mixture (containing 60 mM-ADP and 0.48 mM-$FeSO_4$) and 0.1 ml of 15 mM-ascorbate solution (which had been neutralized with 1 N NaOH). Simultaneously, a similar number of tubes was prepared to which were added 0.2 ml of water instead of ADP/Fe mixture and ascorbate solution. The tubes were firmly sealed and incubated at 37°C for 15 min with gentle shaking.

The estimation of MDA production was carried out by the same procedure as described above and the IC_{50} values were calculated.

The Estimation of Oxygen Uptake

The uptake of oxygen coupled to the process of lipid peroxidation induced by NADPH/ADP/Fe or ADP/Fe/ascorbate was measured by the following modification of the method described by Slater[13].

Stock solution (1.625 ml; containing 0.1 M KCl and 0.033 M tris HCl buffer; pH 7.4), 0.1 ml of microsomal suspension, and 0.03 ml of ebselen solution were incubated at 37°C for 5 min in the electrode chamber. After this preincubation, the lipid peroxidation was started by the addition of 0.03 ml of ADP/Fe mixture (containing 120 mM ADP, and 0.96 mM-$FeSO_4$) and 0.015 ml of ascorbate (60 mM), or by the addition of 0.015 ml of NADPH (14.4 mM) and ADP/Fe mixture (containing 48 mM ADP and 0.55 mM $FeSO_4$).

The results are given as nmol of oxygen uptake/min/mg of microsomal protein.

RESULTS

Effects of Ebselen of Lipid Peroxidation Stimulated by CCl_4 or ADP/Fe/ascorbate

As shown in Table I, column c, ebselen inhibited the stimulation of MDA production by CCl_4. The IC_{50} value of ebselen with no preincubation was approximately 130 μM. However, this inhibitory activity was potentiated by 5 min-preincubation with microsomes, when the IC_{50} value was 70 μM. In addition, ebselen inhibited the endogenous lipid peroxidation (without CCl_4) in a dose-dependent manner (Table 1, column 6).

As shown in Table II, ebselen also inhibited the stimulation of MDA production by ADP/Fe/ascorbate. The potentiation of its inhibitory activity by a period of preincubation was more striking than that observed with CCl_4-stimulated lipid peroxidation; The IC_{50} value was over 200 μM with no preincubation, but decreased to 1.6 μM after a period of 5 min-preincubation. This latter inhibitory activity was similar to that reported by Müller *et al.*[8]. It was also noticeable that, in contrast with CCl_4-stimulated lipid peroxidation, ebselen significantly stimulated the endogenous lipid peroxidation (without ADP/Fe/ascorbate; 5 min pre-incubation, Table II, column b).

Effects of Ebselen on Oxygen Uptake Stimulated by NADPH/ADP/Fe or ADP/Fe/ascorbate

As shown in Figs 1 and 2, ebselen inhibited the oxygen uptake coupled to the lipid peroxidation stimulated by NADPH/ADP/Fe or by ADP/Fe/ascorbate. The IC_{50}

TABLE I
Effects of ebselen on the stimulation of MDA production by carbon tetrachloride in rat liver microsomes

Conc. of ebselen (μM)	MDA production (nmoles/min/mg protein)		
	$-CCl_4$	$+CCl_4$	stimulation
Exp. I; 0 min-preincubation			
0	0.242 ± 0.015	0.343 ± 0.034	0.101 ± 0.020
20	0.236 ± 0.026	0.319 ± 0.031	0.083 ± 0.016
50	0.165 ± 0.017**	0.287 ± 0.020	0.121 ± 0.006
100	0.097 ± 0.008**	0.224 ± 0.009*	0.127 ± 0.006
200	0.047 ± 0.002**	0.088 ± 0.012**	0.041 ± 0.012
IC_{50} value	78 μM	131 μM	185 μM
Exp. II; 5 min-preincubation			
0	0.054 ± 0.001	0.172 ± 0.004	0.118 ± 0.005
10	0.004 ± 0.004	0.160 ± 0.005	0.116 ± 0.004
20	0.040 ± 0.002**	0.156 ± 0.006	0.116 ± 0.005
50	0.029 ± 0.001**	0.136 ± 0.004**	0.107 ± 0.003
100	0.018 ± 0.001**	0.032 ± 0.004**	0.015 ± 0.004**
200	0.011 ± 0.001**	0.012 ± 0.0003**	0.002 ± 0.001**
IC_{50} value	57 μM	70 μM	72 μM

Each value represents the mean ± S.E. of 4 or 5 experiments.
*, **; Significant differences between control MDA production (0 μM of ebselen) and that in the presence of ebelen at $p < 0.05$ and $p < 0.01$, respectively.

TABLE II
Effects of ebselen on the stimulation of MDA production by ADP/Fe/ascorbate in rat liver microsomes

Conc. of ebselen (μM)	MDA production (nmoles/min/mg protein)		
	− ADP/Fe/asc	+ ADP/Fe/asc	simulation
Exp. I; 0 min-preincubation			
0	0.054 ± 0.002	0.464 ± 0.005	0.410 ± 0.007
10	0.068 ± 0.001**	0.438 ± 0.006*	0.370 ± 0.006*
20	0.078 ± 0.003**	0.450 ± 0.014	0.372 ± 0.012*
50	0.101 ± 0.004**	0.427 ± 0.010*	0.326 ± 0.010**
100	0.110 ± 0.007**	0.414 ± 0.010*	0.304 ± 0.014**
200	0.032 ± 0.001**	0.360 ± 0.026*	0.328 ± 0.026*
IC_{50} value		> 200 μM	> 200 μM
Exp. II; 5 min-preincubation			
0	0.086 ± 0.001	0.381 ± 0.013	0.295 ± 0.013
0.5	0.087 ± 0.002	0.352 ± 0.008	0.265 ± 0.010
1.0	0.097 ± 0.004*	0.332 ± 0.027	0.235 ± 0.026
2.0	0.104 ± 0.006*	0.109 ± 0.051**	0.005 ± 0.047**
5.0	0.106 ± 0.005*	0.024 ± 0.004**	−0.082 ± 0.005**
10.0	0.118 ± 0.008**	0.022 ± 0.002**	−0.097 ± 0.006**
IC_{50} value		1.6 μM	1.3 μM

Each value represents the mean ± S.E. of 3 or 4 experiments.
*, **; Significant differences between control MDA production (0 μM of ebselen) and that in the presence of ebselen at $p < 0.05$ and $p < 0.01$, respectively. Abbreviation; asc, ascorbate.

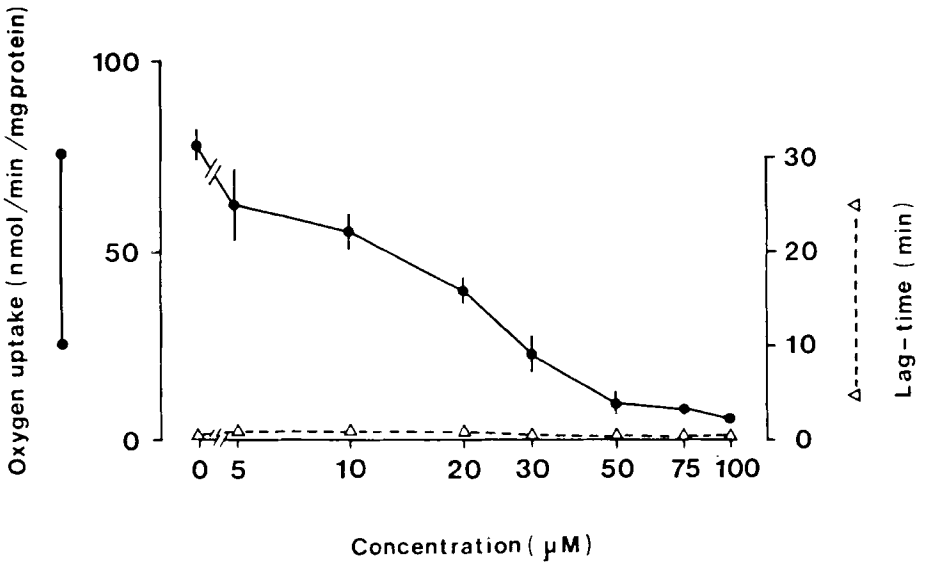

FIGURE 1 Effects of ebselen on oxygen uptake stimulated by NADPH/ADP/Fe. After microsomes were preincubated with each concentration of ebselen at 37°C for 5 min, the reaction was started by the addition of NADPH, ADP, and Fe. Each value represents the mean ± S.E. of 3 or 4 experiments.

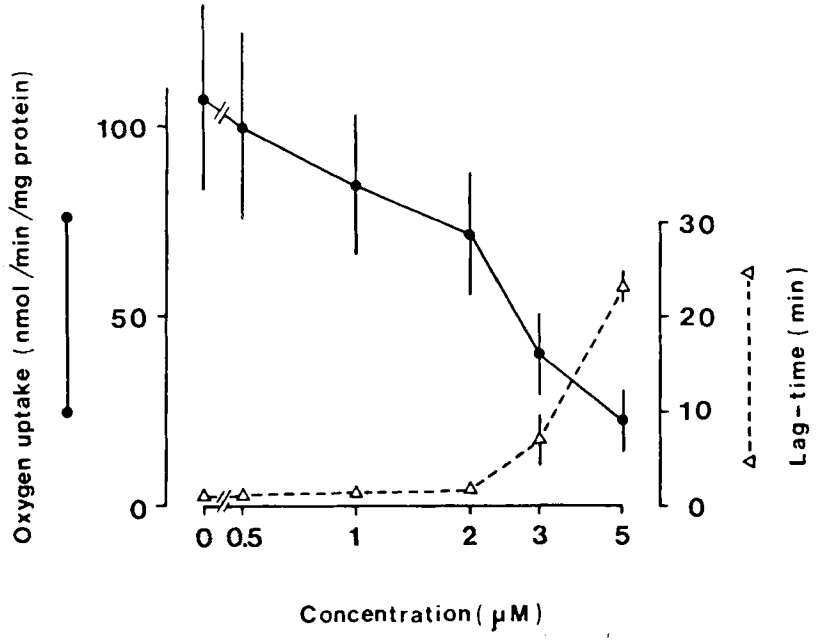

FIGURE 2 Effects of ebselen on oxygen uptake stimulated by ADP/Fe/ascorbate. After microsomes were preincubated with each concentration of ebselen at 37°C for 5 min, the reaction was started by the addition of ADP, Fe, and ascorbate. Each value represents the mean ± S.E. of 4 experiments.

value for each reaction was 20.3 μM and 2.5 μM, respectively. Ebselen also significantly prolonged the lag-time of ADP/Fe/ascorbate-stimulated lipid peroxidation, but no effect was observed on the lag-time of NADPH/ADP/Fe-stimulated lipid peroxidation.

DISCUSSION

Ebselen has been reported to exhibit GSH-Px-like activity and inhibit the ADP/Fe/ascorbate-stimulated lipid peroxidation[8–10]. In this study, we confirmed that ebselen inhibited MDA production by ADP/Fe/ascorbate and, additionally, we have found that it inhibits the oxygen uptake stimulated by ADP/Fe/ascorbate. Moreover, ebselen inhibited MDA production stimulated by CCl_4 and the Oxygen uptake coupled to NADPH/ADP/Fe-stimulated lipid peroxidation. The inhibitory activity of ebselen was significantly increased by the preincubation of ebselen with liver microsomes for 5 min at 37°C. Considering the hydrophobic nature of ebselen, these findings suggest that this compound enters into the membrane lipid rather easily and acts as an antioxidant. In addition, ebselen inhibited the endogenous lipid peroxidation due to NADPH-stimulated lipid peroxidation (Table I, column b), but stimulated the endogenous production of MDA in the absence of NADPH (Table II, column b).

The inhibitory activity of ebselen varied with the method used to stimulate lipid peroxidation (the range of IC_{50} values was 1.6–70 μM). In particular, its inhibitory activity against CCl_4-stimulated lipid peroxidation was very low. Poli *et al.*[14] reported that peroxidations of liver microsomes stimulated by ADP/Fe or CCl_4 had different stoichiometries and suggested that this may be due to the two pro-oxidant stimuli acting on the polyunsaturated fatty acids in different ways, or through a different path way of degradation of lipid hydroperoxides. The differences in the inhibitory activities of ebselen observed in this study may be due to differences of intermediates or pathways of lipid peroxidation. Moreover, ebselen could not retard the lag-time of NADPH/ADP/Fe-stimulated lipid peroxidation, even though it significantly inhibited the oxygen uptake. NADPH/ADP/Fe-stimulated lipid peroxidation is considered to be an enzymatic reaction involving NADPH-cytochrome P_{450} reductase that is a component of the cytochrome P_{450}-drug metabolism system. Wendel *et al.*[9] have reported that NADPH-dependent aminopyrine dealkylation was totally inhibited in the presence of 50 μM of ebselen, and this concentration range is similar to that inhibiting of oxygen uptake by NADPH/ADP/Fe-stimulated lipid peroxidation in this study. In addition, some compounds which inhibit the enzymes of drug metabolism have been reported to inhibit MDA production stimulated by NADPH/ADP/Fe[16]. Therefore, the fact that ebselen inhibited the oxygen uptake without prolonging the lag-time of NADPH/ADP/Fe-stimulated lipid peroxidation suggest that ebselen may have an inhibitory action on the enzymes of the cytochrome P_{450} pathway. In fact, ebselen has been found to inhibit microsomal NADPH-cytochrome P_{450} reductase with an IC_{50} value of 0.13 μM (Prof. A. Wendel, personal communication).

GSH-Px constitutes a powerful cellular defence system against oxidative stress. Recently, it has been reported with Se-deficient mice that impaired lymphocyte responses to mitogens are associated with decreased macrophage GSH-Px activity and increased macrophage H_2O_2 release during phagocytes[17]. In addition, anti-inflammatory effects of selenite have been reported[18]. These reports suggest that GSH-Px

participates deeply in control of inflammation; however, enzyme proteins, such as GSH-Px do not easily reach intracellular target sites[5-7]. Ebselen has been reported to exhibit GSH-Px-like activity, and in this study it has been found that ebselen penetrates into membrane lipid easily and inhibits several types of lipid peroxidation. Ebselen may therefore be considered to be a very useful compound for the study of lipid peroxidation, and having some potential for the therapy of inflammatory diseases and other diseases in which lipid peroxidation participates.

References

1. Slater, T.F. in Free Radical Mechanisms in Tissue Injury (Pion: London, 1972).
2. Slater, T.F. *Biochem. J.*, **222**, 1 (1984).
3. Mills, G.C. *J. Biol. Chem.*, **234**, 502 (1959).
4. Rotruch, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G. and Hoekstra, W.G. *Science*, **179**, 588 (1973).
5. McCay, P.B., Gibson, D.D., Fong, K. and Roger, K. *Biochem. Biophys. Acta*, **431**, 459 (1976).
6. Michelson, A.M., Dangon, O., Puget, K., Durosay, P., Perdereau, B. and Carbaroux, C. *Molec. Physiol.*, **3**, 27 (1983).
7. Grossman A. and Wendel, A. *Eur. J. Biochem* **135**, 549 (1983).
8. Müller, A., Cadenas, E., Graf, P. and Sies, H. *Biochem. Pharmac.*, **33**, 3235 (1984).
9. Wendel, A., Fausel, M., Safayhi, H., Tiegs, G. and Otter, R. *Biochem. Pharmac.*, **33**, 3241 (1984).
10. Parnham, M.J. and Kindt, S. *Biochem. Pharmac.*, **33**, 3247 (1984).
11. Slater, T.F. and Sawyer, B.C. *Biochem. J.*, **123**, 805 (1971).
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. *J. Biol. Chem.*, **193**, 265 (1951).
13. Slater, T.F. *Biochem. J.*, **106**, 155 (1968).
14. Poli, G., Dianzani, M.U., Cheeseman, K.H, Slater, T.F., Lang, J. and Esterbaur, H. *Biochem. J.*, **227**, 629 (1985).
15. Mead, J.F., in Free Radicals in Biology ed by W.A. Pryor, Academic Press: London (1976).
16. Wills, E.D., *Biochem. J.*, **133**, 333 (1969).
17. Parnham, M.J., Winkelemann, J. and Leyck, S. *Int. J. Immunopharmac.*, **5**, 455 (1983).
18. Spallholz, J.E. *Adv. exp. Med. Biol.*, **135**, 43 (1981).

Accepted by Dr. J.V. Bannister