Antioxidative Activity of Indenestrol A, a Diethylstilbestrol Metabolite

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To elucidate the various activities of synthetic estrogens, the antioxidative activities of diethylstilbestrol (DES) and related metabolic analogs were examined. The antioxidative activities were assessed in terms of the inhibitory effect on Fe^{2+} - and ascorbic acid-induced peroxidation of egg phosphatidylcholine (egg PC), and also superoxide scavenging ability using cyclic voltammetry. Moreover, after *in vivo* administration of the test compounds to mice, the animals were subjected to hyperoxia, and catalase, glutathione peroxidase and superoxide dismutase activities in the brain, lungs and liver were measured. The results indicated that indenestrol A, one of the metabolites of DES, had the strongest antioxidative activity among the test compounds under both *in vivo* and *in vitro* conditions.

Key word antioxidative activity; indenestrol A; thiobarbituric acid reactive substance; catalase; superoxide dismutase; glutathione peroxidase

Natural and synthetic estrogens and their derivatives have been demonstrated to exert physiological and pharmacological actions.¹⁾ For several decades, many scientists have focused on actions other than the estrogenic actions, and our group has also reported non-estrogenic activities of natural and synthetic estrogens. These compounds, including metabolites and analogs of 17β -estradiol (E_2) and diethylstilbestrol (DES), have been shown to have potent inhibitory effects on the polymerization of microtubule proteins *in vitro*, and to exert cytotoxic and aneuploidy-inducing effects on Chinese hamster V79 cells.²⁾ Competitive cytosol estrogen receptor-binding assays using synthetic estrogen and its analogs have demonstrated no correlation between the affinity of estrogen receptor binding and cytotoxicity.³⁾

It is generally recognized that vitamin E (VE), especially α -tocopherol, is an important component of biological membranes, and plays a role as a free radical chain-breaking antioxidant, preventing peroxidation of polyunsaturated lipids in membranes. Such biological activities are presumed to arise from the chemical reactivity of the chromanol ring of α -tocopherol in a redox system. Many *in vivo* observations have clarified the role of VE as a prominent antioxidant.⁴⁾ Because of its delayed efficacy in *in vitro*, however, many efforts have been made to find a more efficient antioxidant than VE from both natural and synthetic compounds. Since it was recently shown that E_2 has antioxidative activity,⁵⁾ we investigated the activity of DES and its analogs. Our results indicate that indenestrol A (IA), one of the metabolites of DES, has stronger antioxidative activity than VE.

EXPERIMENTAL

Materials DES, *meso*-hexestrol, and *E,E*-dienestrol were purchased from Sigma Chemical Co. (+)- and (-)-DES oxide monomethyl ether, (+)- and (-)-IA and indenestrol B (IB), and their monomethyl ether derivatives were prepared as described previously⁶⁾ (Fig. 1). Thiobarbituric acid (TBA), L-ascorbic acid, and FeSO_4 were from Tokyo Kasei Kogyo Co., Daichi Kagaku Yakuhin Co., and Kanto Chemical Co. Inc., Tokyo, respectively. *dl*-Tocopherol was obtained from Sigma Chemical Co. A surfactant, EaO160-Z106 for injection of compounds was obtained from Eisai Co., Tokyo.

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Fe^{2+} and Ascorbic Acid-Induced Peroxidation of Egg Phosphatidylcholine (Egg PC) and Assessment of Antioxidant Activity Lipid peroxidation was assessed in terms of formation of thiobarbituric acid reactive substance (TBARS), as described previously.⁷⁾ A mixture of a solution of egg PC in chloroform (200 μl ; 10 mg/ml) and each sample (50 nmol/mg lipid) was evaporated to dryness under nitrogen gas. A control and a reference sample including α -tocopherol (50 nmol/mg lipid) were also tested for comparison. Each mixture was then mixed with 3 ml of 1% phosphoric acid and 1 ml of a 0.7% TBA solution, and heated at 98 °C for 45 min. The mixture was extracted with 4 ml of *n*-butanol, and the absorbance at 535 nm was measured using a Hitachi 220A spectrophotometer (Tokyo, Japan).

Superoxide Scavenging Effect of the Compounds The effect of the compounds on superoxide scavenging ability was determined by cyclic voltammetry. The reduction of O_2 to O_2^- and reoxidation of O_2^- to O_2 were measured in the presence and absence of the test compounds. The peak current for the reoxidation of O_2^- (i_{pc}) decreased in the presence of the compounds. It is known that the change in the peak current provides a measure of the reaction, and that comparison of the cathodic and anodic peak currents (i_{pc} and i_{pa} , respectively) provides a measure of the rate constant.⁸⁾ In the present study, cyclic voltammetry was performed with a BAS 100B electrochemical analyzer (Bioanalytical Systems Inc., Lafayette, IN) employing a glassy carbon-inlay working electrode, an Ag/AgCl cracked bead reference electrode filled with aqueous tetramethylammonium chloride solution and adjusted to 0.000 V vs. saturated calomel electrode (SCE) and a Pt auxiliary electrode. All solutions (acetonitrile) for electrochemistry were 1 mM in substrate and 0.1 M in tetraethylammonium perchlorate. Scan rate was 0.1 V s^{-1} .

Assessment of Antioxidant Enzyme Activities The effect of the compounds on antioxidant enzyme activities in 6 weeks old C57 BL male mice was investigated. Standard food and water were provided *ad libitum*. Each compound (100 nmol) was dissolved in a surfactant, EaO160-Z106, and three animals were given an intraperitoneal injection of each compound in each experiment. After 24 h, the same concen-

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Fig. 1. Structures of Synthetic Estrogens

tration of the compound was administered, and the animals were subjected to hyperoxia with 100% oxygen for 48 h. The activities of antioxidant enzymes catalase (CAT), glutathione (GSH) peroxidase (GSHPx), and superoxide dismutase (SOD) from the brain, liver and lungs were then evaluated. Each organ (brain, liver, lungs) from the mice was homogenized in 0.1 M phosphate buffer (pH 7.0) to obtain a final 10% (w/v) homogenate, which was centrifuged at 3000 rpm for 10 min. The supernatant was used for measurement of the activity. Experiments carried out without administration of the compounds and without exposure to 100% oxygen were used for control assessments.

Measurement of TBARS To 400 μ l of the homogenate obtained above or 0.1 M phosphate solution (blank solution), 0.5 ml of 5 mM EDTA, 3 ml of 1% phosphoric acid and 1 ml of aqueous 0.7% TBA solution were added, and then heated at 98 °C for 45 min. The mixture was extracted with 3 ml of *n*-butanol, and centrifuged at 3000 rpm for 10 min, and the absorbance of the supernatant measured at 535 nm with a Hitachi 220A spectrophotometer. Using the molar absorption coefficient of malondialdehyde (156000 mol^{-1}), the concentration of TBARS was expressed as nanomole equivalents of malondialdehyde per milligram protein in the samples.⁷⁾

Effects of Compounds on CAT Activity The supernatant (100 μ l) was added to 1.9 ml of 5 mM potassium phosphate buffer and 30 mM H_2O_2 , and the decrease in absorbance at 240 nm for 1 min was measured. The slope of the decrease was corrected by the absorption coefficient per milligram protein.⁹⁾

Effects of Compounds on GSHPx Activity The supernatant (100 μ l) was mixed with 500 μ l of 0.1 M potassium phosphate buffer (pH, 7.0), 100 μ l of GSH reductase (2.4 U/ml) and 100 μ l of 10 mM GSH solution, and the mixture was incubated at 37 °C for 10 min. Thereafter, to 100 μ l of the incubation mixture, 0.1% NaHCO_3 solution (containing 1.5 mM NADPH) was added, and the decrease in absorbance at 340 nm for 1 min was measured. The difference in absorbance between the starting time and after 1 min was measured as GSHPx activity, as reported previously.¹⁰⁾ For the control experiments, 100 μ l of 0.1 M potassium phosphate buffer was used as the standard.

Effects of Compounds on SOD Activity The super-

natant (100 μ l) was mixed with 0.2 ml of 65 mM phosphate buffer (pH 8.2), 0.2 ml of 0.5 mM hypoxanthine, 0.1 ml of 10 mM hydroxylamine·HCl–1 mg/ml hydroxylamine-*O*-sulfonic acid and 0.2 ml of distilled water, and incubated for 5 min at 37 °C as described previously.¹¹⁾ For the control, the same reaction was performed except that distilled water was used in place of the above solution. Then, 0.2 ml of 5×10^{-3} U/ml xanthine oxidase was added to the reaction mixture, and incubated at 37 °C for 5 min. Two milliliters of 30 μ M *N*-naphthylethylenediamine·HCl, 3 mM sulfanilic acid, and 25% acetic acid were added to the reaction mixture, and stirred for 30 min at room temperature. The mixture was analyzed by the absorbance at 550 nm and the SOD activity was calculated as the amount that produced 50% inhibition.

Protein Concentration Protein concentration was determined by the Bradford method using bovine serum albumin as a standard in each sample.¹²⁾

RESULTS AND DISCUSSION

Effect of Indenestrols on Antioxidant Activity in an *in Vitro* System The effect of DES metabolites and their analogs on antioxidant activity was examined in terms of the inhibitory effect on Fe^{2+} and ascorbic acid-induced peroxidation of egg PC. As shown in Fig. 2, (+)-, (–)- and (±)-IA and IB and (+)- and (–)-4'-*O*-methyl ethers of IA showed strong antioxidant activities. Moreover, we tested whether there were activity differences between (+)- and (–)-IA and IB, but no such differences were evident. Therefore, we used the (±)-IA and IB forms for further study of the antioxidative activities. The superoxide scavenging activity of the compounds was measured by voltametric analysis. The results, shown in Fig. 3, indicated that (±)-IA and IB had higher antioxidative activities.

Effects of Indenestrols on Antioxidant Activity in an *in Vivo* System First, we examined the effects of (±)-IA and IB on antioxidant activity using mouse liver homogenate. The results showed that the antioxidative activity of the two compounds was almost 1.5 times higher than that of *dl*-tocopherol (data not shown).

Next, we examined the inhibitory effect of the compounds *in vivo* under hyperoxia by exposure to 100% oxygen for 48 h, and determined the rate of lipid peroxidation in each

Fig. 2. Inhibitory Effect of Compounds Derived from DES on Lipid Peroxidation

Fig. 3. Reactivity of Compounds Derived from DES toward Superoxide Ion

Fig. 4. Effects of IA, IB and VE on Amount of Lipid Peroxides (TBARS) and Activities of CAT, SOD and GSHPx in Tissues Isolated from 100% O₂ Stressed Mouse

organ tested. The results are shown in Fig. 4a. The strongest antioxidative compound among those tested was IA, and its activity, as measured by the inhibitory effect on TBARS production, was higher than that of α -tocopherol.

Effects of Indenestrols on Antioxidative Enzyme Activity in Organs from Mice Subjected to Hyperoxia In order to clarify the action of IA on the living body, the effects of the test compounds on CAT, GSHPx, and SOD activities were examined under oxidative stress (100% O₂) or normal (20% O₂) conditions. Under oxidative stress in control mice, SOD activity decreased at rates of 83% in the liver, 43% in the lungs and 54% in the brain, and CAT activity also decreased at the rates of 62% in liver and 35% in lungs. However, other studies have demonstrated that oxidative stress increases CAT and GSHPx activity in the rat brain.¹³⁾ This discrepancy is probably due to differences in the animals and experimental conditions employed. The results obtained here

in the presence of IA, IB or tocopherol are shown in Figs. 4b—d. Although changes in the level of enzyme activity differed considerably among IA, IB and tocopherol, it was also shown that the effects varied among the organs studied.

In conclusion, the above results indicate that IA has the most potent antioxidative activity among the compounds tested in *in vitro* and *in vivo* systems, and moreover that its activity is stronger than that of α -tocopherol, a natural antioxidant. On the basis of the results of cyclic voltametric analysis, it is considered that IA and IB may scavenge superoxide directly rather than break the free radical chain reaction, as with α -tocopherol. Since no mutagenic activity for IA has been detected,¹⁴⁾ the present results demonstrate that estrogens and their analogs may be effective compounds for providing protection against oxidation in the living body.

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