Formation of Active Oxygen Species from Diethylstilbestrol, a Synthetic Estrogen, and Its Metabolite in the Presence of RAW 264.7 Cells

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Previously, we reported that (±)-IA but not DES produces O_2^- spontaneously in PBS. We are interested in the possibility that these compounds might produce active oxygen species under mild cell culture conditions. On incubation of RAW 264.7 cells with (±)-IA, the signal of 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO)-OH adducts increased but no more than the additive effect. However, stimulation of RAW cells with LPS and INF-γ enhanced the formation of DMPO-OH adducts slightly more than the additive effect, especially when the concentration of (±)-IA increased. In the case of DES, the spectra of DMPO-OH adducts did not increase concentration-dependently in the absence of RAW 264.7 cells, however in their presence, they increased concentration-dependently, especially when these cells were stimulated with LPS and IFN-γ. The results were interpreted to mean that DES would have a higher oxidation potential than (±)-IA, not be oxidized to semiquinoes spontaneously, and therefore not produce DMPO-OH adducts in the absence of RAW cells. In their presence, DES might be easily oxidized to semiquinones by the reaction with O_2^- produced from RAW 264.7 cells.

Key words synthetic estrogens; indenestrol A; active oxygen species; RAW 264.7 cells

In the late sixties, McCord and Fridovich showed that superoxide free radical anion (O_2^-) could be produced enzymatically in mammalian tissues, and demonstrated that superoxide dismutase (SOD) catalyzed the dismutation of O_2^-.[1] The role of O_2^- has been recognized for some time in nonspecific host defence, and more recently in signal transduction for physiological communication as well as in the pathophysiological mechanisms of various processes.[2] Often, the contribution of reactive oxygen species (ROS) to these processes is demonstrated indirectly through the use of antioxidant molecules. Indeed, the half-life of ROS as O_2^- is very short and the assessment of its production is not easy, particularly in nonphagocytic cells such as endothelial cells.

It has been reported that synthetic and natural estrogens such as diethylstilbestrol (DES) and its metabolite, 17β-estradiol, produce free radicals and active oxygen species *via* redox reactions.[3] These active oxygen species are implicated in the carcinogenesis by these compounds.

Previously, we reported that (±)-indenestrol A (IA) but not DES produces O_2^- spontaneously in phosphate buffered saline (PBS).[4] Although these compounds produce free radicals or O_2^- in the presence of peroxidase or under alkaline conditions, we are interested in the possibility that they might produce active oxygen species under mild cell culture conditions. In the presence of the murine macrophage cell line RAW 264.7 cells, which produce O_2^- and NO following stimulation by LPS and/or IFN-γ,[5] we examined the production of active oxygen species from these compounds. For the detection of active oxygen species, we used electron spin resonance spectroscopy with a spin trapping agent, 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO).

MATERIALS AND METHODS

Materials DES was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (±)-IA was prepared as described previously.[6] The structures of the compounds were defined by ^{1}H-NMR analysis. All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were of the highest grade available commercially. The structures of the test compounds are shown in Fig. 1.

Cell Culture The murine macrophage-like cell line, RAW 264.7 cells, were cultured in COS-001 (Cosmo Bio Co., Ltd.) without serum, in a humidified 5% CO_2 incubator at 37 °C.

Incubation of DES and (±)-IA with RAW Cells RAW 274.7 (1×10^7) cells were collected and cultured in 90-mm Petri dishes with 10 ml of medium. When necessary, cells were stimulated with LPS (1 μg/ml) and IFN-γ (100 U/ml) for 8 h. They were collected, suspended in 900 μl of PBS in a 1.5-ml tube, mixed with 100 μl of diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC) (10 mM pH 7.5) and DES (1, 10, 30 mM) or (±)-IA (1, 10, 30 mM) dissolved in 1 μl of DMSO, and incubated for 10 min at 37 °C. After centrifugation at 5000 rpm for 2 min, 500 μl of the supernatant was discarded. The cell pellet was suspended in 500 μl of supernatant. Then, 250 μl of the suspension was transferred to a new tube, with 16 μl of DMPO, and incubated for 30 min at 37 °C. After centrifugation at 15000 rpm for 2 min, the supernatant was used for ESR spectroscopy.

ESR Spectroscopy ESR measurements were recorded on a JEOL JES-RE1X spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation operating at 9.4240 GHz and at room temperature. The following instrumental parameters were employed: modulation amplitude, 0.100 mT; microwave power, 8.0 mW; scan time, 10.0 min.

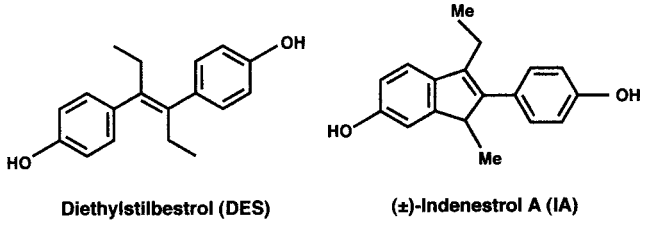

Fig. 1. Structures of Diethylstilbestrol (DES) and (±)-Indenestrol A (IA)

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RESULTS AND DISCUSSION

Spontaneous Production of DMPO Adducts from DES and (±)-IA in PBS Previously we detected production of O_2^- from (±)-IA but not from DES by a reduction of cytochrome c.[4] However, the assay depends on the rate of O_2^- production and is not suitable for the comparison of relative amounts of O_2^- produced in various systems. In this report, we detected active oxygen species by electron spin resonance spectroscopy using a spin trapping agent, DMPO. As shown in Fig. 2A, we could detect spectra of DMPO adducts concentration-dependently in the presence of (±)-IA. The main signals observed (aN=aH=1.5 mT, Fig. 2A) were assigned to DMPO-OH produced at 1 μM (±)-IA in PBS, but were superimposed on small signals of DMPO-OOH in 30 μM (±)-IA in PBS based on the following results. First, these main signals have the same hyperfine splitting constants as those obtained by a Fenton reaction *in vitro*; second the superimposed signals (estimated as, aN=1.43, aHb=1.17, aHg=0.125) have the same hyperfine splitting constants as those obtained by a reaction between xanthine and xanthine oxidase (data not shown). Probably, primary DMPO-OOH adducts were decomposed to DMPO-OH, whose signals we detected as main signals. This interpretation was supported by the fact that in the presence of SOD, we could not detect any signals from DMPO adducts in 30 μM (±)-IA in PBS. The possibility that these DMPO-OH adducts are produced from a Fenton reaction by H_2O_2 dismutated from O_2^- was excluded by the fact that catalase had no effect on the spectra from (±)-IA in PBS. As shown by the relative intensity of the signals in Fig. 3, calculated partly from Fig. 2A, signals of DMPO-OH adducts increased concentration-dependently in (±)-IA in PBS. In the case of DES, however, the signals did not increase concentration-dependently. These results are consistent with our previous results.[4]

Production of DMPO Adducts from (±)-IA and DES during Culture with RAW 264.7 Cells Before the measurement of O_2^- from DES and (±)-IA in the presence of ac-

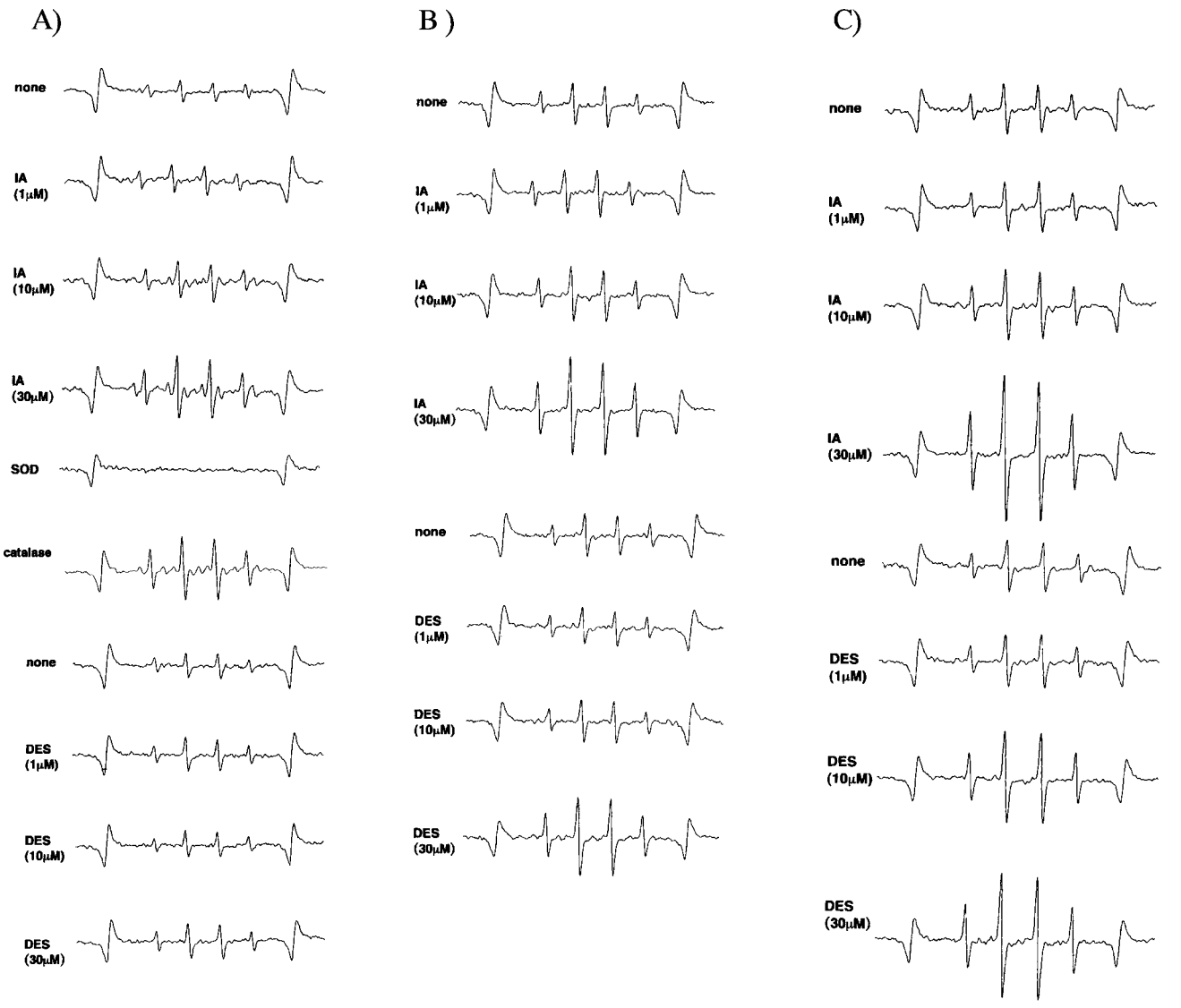

Fig. 2. ESR Spectra of DMPO Adducts Generated in the Presence of (±)-IA or DES under Various Conditions

A) ESR spectra of DMPO adducts generated in the presence of various concentrations of (±)-IA or DES in PBS. B) ESR spectra of DMPO adducts generated in the presence of various concentrations of (±)-IA or DES in RAW 264.7 cells. C) ESR spectra of DMPO adducts generated in the presence of various concentrations of (±)-IA or DES in LPS/IFN-γ-stimulated RAW 264.7 cells. The experimental conditions for A, B and C were described in the experimental section.

tivated RAW cells, the production of O_2^- by RAW cells was examined. As shown in Fig. 2B, the spectrum of DMPO adducts from RAW cells was similar to that obtained in (±)-IA solution. The main signals observed (aN=aH=1.5 mT, Fig. 2B) were assigned to DMPO-OH based on the fact that they have the same hyperfine splitting constants as those obtained by a Fenton reaction *in vitro* as shown previously (Fig. 2A). Since the signal for DMPO-OH adducts from RAW cells was missing in the presence of SOD but not in the presence of catalase, the DMPO-OH adducts were ascribed to decomposed products of DMPO-OOH, as in the case of (±)-IA in PBS. On incubation of RAW 264.7 cells with (±)-IA, the signal of DMPO-OH adducts increased but no more than the additive effect. However, stimulation of RAW cells with LPS and INF-γ enhanced the formation of DMPO-OH adducts slightly more than the additive effect, especially when the concentration of (±)-IA increased (Fig. 2C). We interpret this to mean that (±)-IA has low oxidation potential and easily suffers from autoxidation. In the presence of excess O_2^-, the efficiency of oxidation to semiquinones and resultant production of O_2^- from semiquinones would not be much increased. In the case of DES, the spectra of DMPO-OH adducts were not increased concentration-dependently in the absence of RAW cells, however in their presence, they increased concentration-dependently, especially when these cells were stimulated with LPS and IFN-γ. These results were interpreted to mean that DES would have a higher oxidation potential than (±)-IA as shown in Fig. 2C, not be oxidized to semiquinoes spontaneously, and therefore not produce DMPO-OH adducts in the absence of RAW cells. However, in their presence, DES might be easily oxidized to semiquinones by the reaction with O_2^- produced from RAW 264.7 cells. These semiquinones would be converted to hydroquinones, producing O_2^- which would be trapped by DMPO. Producing O_2, DES would be converted to quinones, which might be converted again to semiquinones due to a themodynamic equilibration of quinones and hydroquinones.[7] Since semiquinones would disappear, the equilibration would proceed toward producing semiquinones (Fig 4).

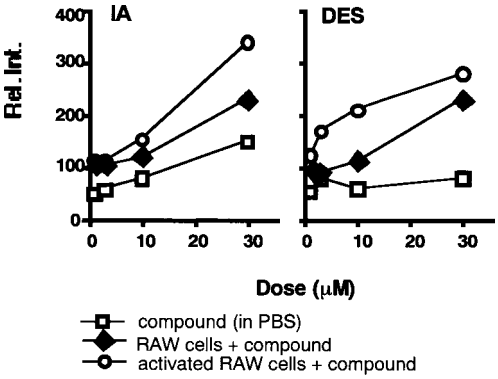

Fig. 3. The Relative Intensity of ESR Spectra of DMPO Adducts Generated in the Presence of (±)-IA or DES under Various Conditions

All values were corrected using Mn signal as a standard. The data are means of three independent experiments.

Recently, it was elucidated that DES is one of the few substances for which a clear association with carcinogenicity has been established in humans.[8] However, DES and (±)-IA (a DES metabolite), in contrast to most other carcinogens, did not induce mutations in the *Salmonella*/*microsome* test[9,10] or the malignant transformation of eukaryotic cells in culture.[11] The results support that active oxygen species from DES and (±)-IA are implicated in the carcinogenesis by these compounds.

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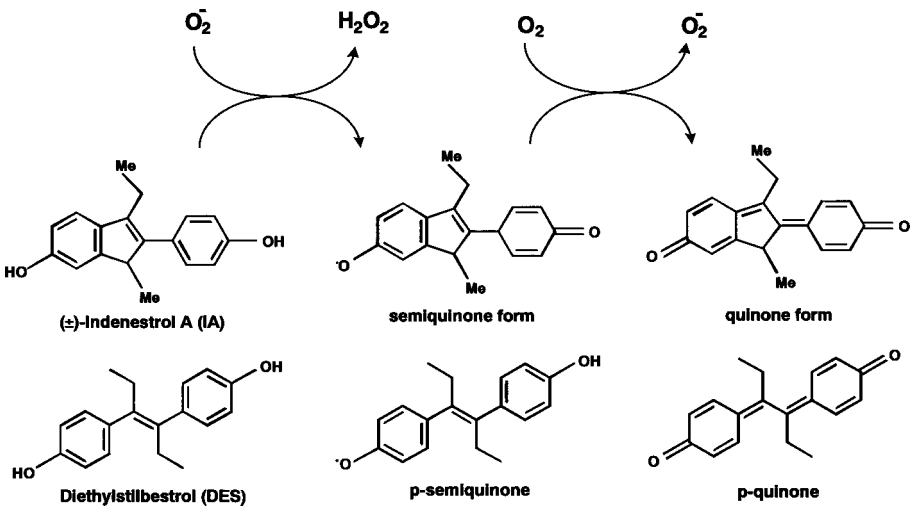

Fig. 4. Super Oxide Production from (±)-IA or DES

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