Effect of Estrogenic Compounds on Superoxide and Nitric Oxide Production by Activated Macrophages Assessed by Sensitive Microplate Assays

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The effect of estrogenic compounds (bisphenol A, genistein, and 17β-estradiol) on the ability of macrophages to produce superoxide and nitric oxide in response to stimulants was investigated. The assays were performed using 96-well microplates to measure multiple samples. Superoxide and nitric oxide were measured by chemiluminescence and fluorescence methods, respectively. Thioglycollate-induced mouse peritoneal macrophages incubated for 24 hr with the estrogenic compounds were stimulated with phorbol 12-myristate 13-acetate and lipopolysaccharide (LPS). The ability of the macrophages to produce superoxide was increased when treated with bisphenol A or genistein, but 17β-estradiol had little effect. Conversely, the ability of the macrophages to produce nitric oxide in response to LPS was strongly suppressed by high concentrations of bispenol A and genistein. 17β-estradiol was ineffective. These results suggest that bisphenol A and genistein similarly affect superoxide and nitric oxide production by a mechanism other than that involving estrogenic activity.

Key words —— endocrine disruptor, bisphenol A, genistein, superoxide, nitric oxide, macrophage

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INTRODUCTION

Endocrine disrupting chemicals (EDCs) or so-called environmental hormones have been suspected to be associated with various disorders of endocrine system, particularly those related to reproduction, increasingly observed in wildlife today.[1] However, there is another concern that EDCs may also disrupt other biological processes involving signal transduction pathways such as the immune system and nervous system.

Cells of the immune system have effector functions to kill invading organisms or tumor cells. These include superoxide anion ($\bullet O_2^-$) production and nitric oxide (NO) production by neutrophils or macrophages. At the site of infection or inflammation, the phagocytes are stimulated by microorganisms or inflammatory cytokines. Upon stimulation, superoxide anion is generated by inner membrane-associated NADPH oxidase complex from extracellular oxygen and cytosolic NADPH,[2] and then converted to hydrogen peroxide (H_2O_2) and more toxic species of hydroxyl radical (•OH). NO is also produced by the stimulated phagocytes. Inducible nitric oxide synthase (iNOS) expression is induced upon cell stimulation, resulting in the production of NO from L-arginine. NO reacts with metals, metalloproteins, and thiols, and exerts its physiological function.[3,4] NO also reacts with radicals such as oxygen (O_2) and superoxide anion. Its reactions with oxygen and superoxide anion, respectively, produce nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) that are highly reactive and thus thought to be deleterious.[5]

Considering the possibility that EDCs affect the effector functions of phagocytes, we have investigated and report here the effect of estrogenic compounds on the ability of macrophages to produce superoxide and NO in response to stimulants. The assays were performed using 96-well microplates to measure multiple samples efficiently. Superoxide and NO were measured by chemiluminescence and fluorescence, respectively, which made sensitive detection of their production possible.

The estrogenic compounds tested in this study were bisphenol A, a raw material for plastic synthesis including polycarbonate and epoxy resins; genistein, a phytoestrogen found in legumes; and 17β-estradiol, a female hormone.

MATERIALS AND METHODS

Reagents —— Bisphenol A was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Genistein and dimethyl sulfoxide (DMSO) was obtained from Wako Pure Chemical Industries (Osaka, Japan). β-Estradiol-watersoluble (17β-estradiol), phorbol 12-myristate 13-acetate (PMA), and superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lipopolysaccharide (LPS) and thioglycollate (TG) medium were the products of DIFCO Laboratories (Detroit, MI, U.S.A.). RPMI-1640 medium without phenol red, fetal bovine serum (FBS), and Hanks' balanced salt solution (HBSS) phenol red free were from GIBCO BRL (Grand Island, NY, U.S.A.), Bio Whittaker (Walkersville, MD, U.S.A.), and Nissui Pharmaceutical Co. (Tokyo, Japan), respectively. 2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), Cell Couting Kit-8, and N^G-monomethyl-L-arginine (L-NMMA) were from Dojindo Laboratories (Kumamoto, Japan). Diaminofluorescein-2 (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). All plasticware including culture dishes, culture plates, and culture flasks used for cell culture were the products of Iwaki Glassware Co. (Tokyo, Japan). According to the manufacturer's information, bisphenol A was not contained in the plasticware. To ensure the reproducibility of the data, the same lots of plasticware and FBS were used for the series of the present experiments.

Preparation of Reagent Solutions —— Bisphenol A and genistein were dissolved in DMSO at 50 mM, and stored at –80°C until use. Before use, aliquots of the stored solutions were diluted with RPMI-1640 medium (without phenol red) supplemented with 20 mM HEPES (pH 7.2), 50 units/ml penicillin, 50 μg/ml streptomycin and 5% FBS (RPMI-HEPES-5% FBS medium [phenol red free]) to adjust the DMSO concentration to 0.04%. All cultures were performed at the final DMSO concentrations of 0.02%. 17β-Estradiol was also used in RPMI-HEPES-5% FBS medium (phenol red free) containing 0.02% DMSO.

Macrophages —— Macrophages were obtained from the peritoneal cavity of 8–12-week old ddY male mice 4 days after intraperitoneal injection of 2–3 ml of 3% TG medium. The peritoneal exudate cells obtained were washed twice with HBSS (phenol red free) by centrifugation ($80 \times g$, 10 min) at 4°C. The cells were then resuspended in RPMI-HEPES-5% FBS medium (phenol red free) at 1×10^6 cells/ml and subjected to the superoxide or NO production assay.

Measurement of Superoxide Production by Stimulated Macrophages —— A solution of estrogenic compound in RPMI-HEPES-5% FBS medium (phenol red free) containing 0.04% DMSO (50 μl) was placed in wells of a 96-well white microplate. To each well, 50 μl of the macrophage suspension (1×10^6 cells/ml) prepared as described above was added, mixed, and the mixture was incubated in a 5% CO_2 incubator at 37°C for 24 hr. At the end of the incubation, the cell supernatant was removed, and the wells with adherent macrophages were rinsed twice with HBSS (phenol red free). To each well, 150 μl of 5.3 μM MCLA in HBSS (phenol red free) was added, and the plate was settled in the chamber of a microplate luminometer (MicroLumat Plus, Berthold, Bad vildbad, Germany) which had been kept at 37°C. Immediately thereafter, luminescence emitted from MCLA[6] was periodically measured according to a predetermined program. Since weak luminescence was emitted and increased for about 15 min in the absence of macrophage stimulants, it was necessary to wait 15 min before the injection of macrophage stimulants. After 15–20 min, 50 μl of a mixture of PMA 0.1 μM and LPS 0.1 μg/ml in HBSS (phenol red free), or HBSS (phenol red free) alone was automatically injected into each well. After injection of the stimulants, the luminescence emitted by the reaction of generated superoxide and MCLA was followed until the emission ceased.

Measurement of NO Production by Stimulated Macrophages —— A solution of estrogenic compound in RPMI-HEPES-5% FBS medium (phenol red free) containing 0.04% DMSO (100 μl) was placed in the wells of a 96-well microplate. To each well, 100 μl of the macrophage suspension (1×10^6 cells/ml) prepared as described above was added, and the cells were cultured in a 5% CO_2 incubator at 37°C for 48 hr. At 48 hr, the culture supernatant was removed, and 200 μl of a solution of LPS 1 μg/ml in RPMI-HEPES-5% FBS medium (phenol red free) or of the medium alone was added to each well, and the wells with adherent macrophages were cultured in a 5% CO_2 incubator at 37°C for another 16 hr. The culture supernatant was then removed from each well, and the wells were rinsed

twice with Krebs-Ringer-phosphate (KRP) buffer. To each well, 200 μl of a solution containing DAF-2 10 μM and L-arginine 1 mM in KRP buffer was added, and the plate was incubated at 37°C for 2 hr. The supernatant of the well was transferred to a well of a 96-well black microplate, and the fluorescence of DAF-2T formed by the reaction of DAF-2[7,8] and NO was measured using a microplate reader (Spectra Fluor Plus Microplate Reader, Tecan, Maennedorf, Switzerland) at excitation of 485 nm and at emission of 535 nm.

The number of viable cells in culture was assessed using a Cell Counting Kit-8 that colorimetrically measured intracellular NADH, according to the manufacturer's instructions.

Statistical Analysis —— The data obtained were expressed as the mean ± S.D. of quadruplicate determinations. Statistical differences between means were determined using an unpaired *t*-test.

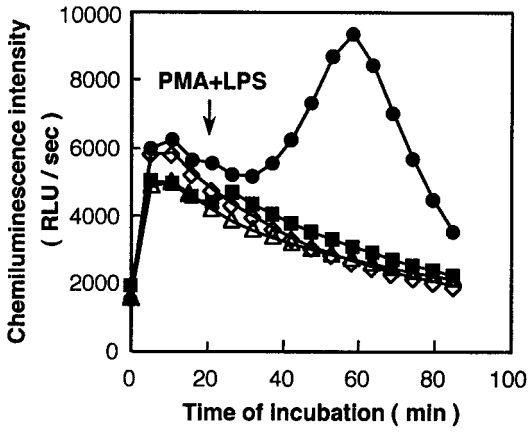

Fig. 1. Measurement of Superoxide Production by Stimulated Macrophages by Chemiluminescence

The experiment was performed as described in Materials and Methods except that macrophages in suspension were used for the measurement. Chemiluminescence intensity was expressed as relative luminescence unit (RLU) per min. The arrow indicates the time of stimulant injection. Each point is the mean of duplicate determinations. ◇, control; ●, PMA+LPS injected; △, SOD (0.1 mg/ml) present; ■, SOD (0.1 mg/ml) present and PMA+LPS injected.

RESULTS AND DISCUSSION

Effect of Bisphenol A, Genistein, and 17β-Estradiol on Superoxide Production by Macrophages

Effect of bisphenol A, genistein, and 17β-estradiol on superoxide production by activated macrophages was investigated using TG-induced mouse peritoneal macrophages. Macrophages were stimulated with a mixture of PMA 0.1 μM and LPS 0.1 μg/ml (PMA+LPS) since the combination of PMA and LPS synergistically enhances macrophage activation.[9,10] Superoxide produced by the activated macrophages was measured by the chemiluminescence emitted by the reaction of superoxide with MCLA, a *Cypridina* luciferin analogue.[10] Figure 1 shows the specificity of this assay system for the measurement of superoxide produced by activated macrophages. The addition of MCLA to a macrophage suspension resulted in an increase in chemiluminescence intensity at 5 min, and the luminescence faded gradually (control). When the cell suspension received PMA+LPS injection at 20 min, the chemiluminescence sharply increased and peaked at about 60 min. However, in the presence of SOD in the assay system, chemiluminescence emission was inhibited, indicating the involvement of superoxide in the increase in chemiluminescence.

Macrophages were incubated for 24 hr in the presence or absence of various concentrations of bisphenol A (0–10^4 nM), genistein (0–10^4 nM), and 17β-estradiol (0–1 nM). Since most cells adhered to the well, the supernatant was removed, and MCLA was added to the cell monolayer. Fifteen minutes after MCLA addition, HBSS or PMA+LPS was injected into the wells. A significant increase in superoxide production that peaked at about 40 min was observed after PMA+LPS injection, while little increase was seen after injection of control HBSS (data not shown). To assess the net value of the stimulant-induced increase in the chemiluminescence intensity, the level of chemiluminescence intensity at the injection time was subtracted from that at 40 min. Figure 2 shows the net increase in chemiluminescence after the stimulation. There were increases in the stimulation-induced chemiluminescence in the bisphenol A-, genistein-, and 17β-estradiol-treated macrophages depending on the concentrations of the compounds, while the unstimulated control (HBSS injection) showed no increase. Bisphenol A was stimulatory at 100 nM and higher concentrations, and genistein tended to be stimulatory at 1 nM and higher concentrations. 17β-Estradiol slightly affected chemiluminescence at 10 pM.

The concentrations of the three estrogenic compounds used here were chosen so that they approximately corresponded with each other, based on recent information on their relative estrogenic activity (*i.e.*, bisphenol A : genistein : 17β-estradiol = 1/10000 : 1/300 : 1) that was assessed using a yeast two-hybrid reporter gene assay.[11] Bisphenol A has been reported to leach from polycarbonate bottles[12]

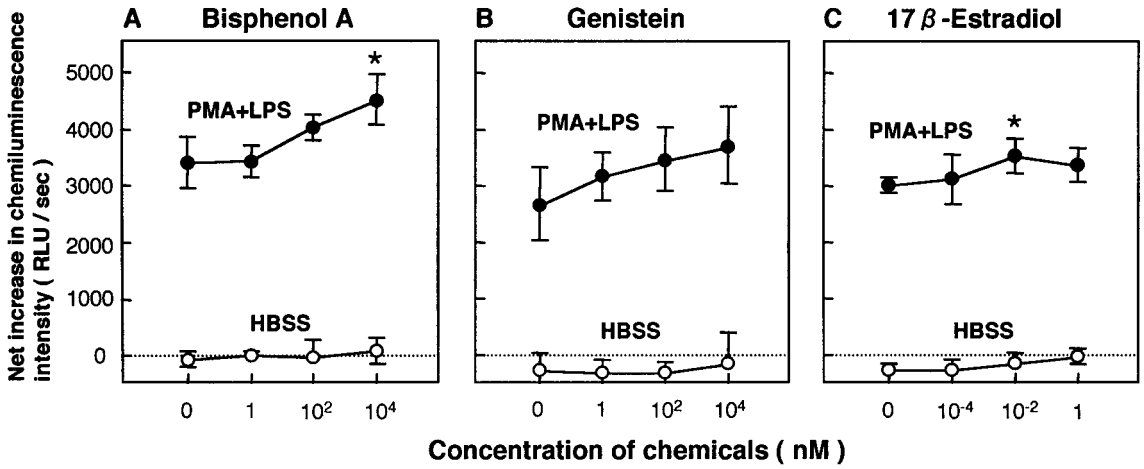

Fig. 2. Effect of Various Concentrations of Bisphenol A, Genistein, and 17β-estradiol on Superoxide Production by Stimulated Macrophages

Experiments were performed as described in Materials and Methods, and the net increase in chemiluminescence was determined as described in the text. Each point is the mean ± S.D. of quadruplicate determinations. ○, HBSS injected; ●, PMA+LPS injected. * Significantly different from control (0 nM). $p < 0.05$.

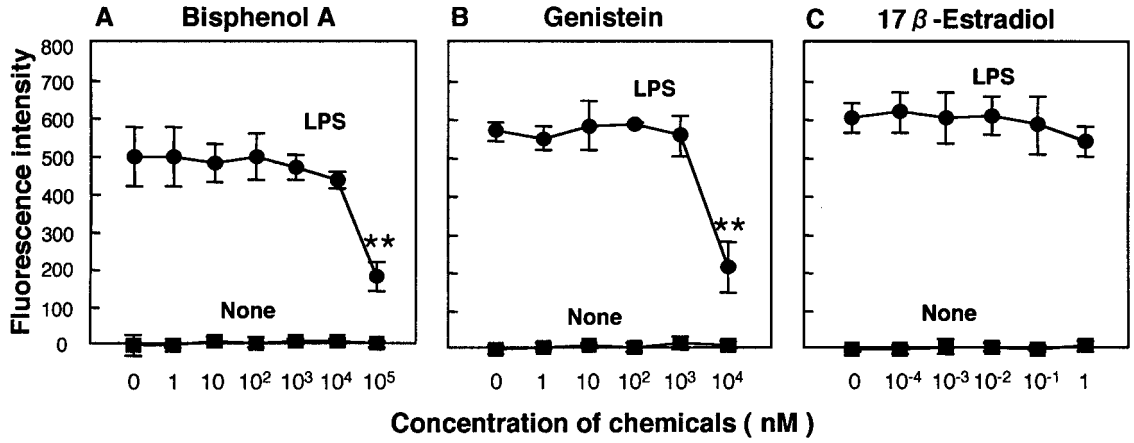

Fig. 3. Effect of Various Concentrations of Bisphenol A, Genistein, and 17β-estradiol on NO Production by Stimulated Macrophages

Experiments were performed as described in Materials and Methods. Each point is the mean ± S.D. of quadruplicate determinations. ■, without LPS; ●, with LPS. ** Significantly different from control (0 nM). $p < 0.01$.

and food can lining.[13] at concentrations of 1–1000 nM. The genistein content in soybean is reported to be about 5–50 mg/100 g,[14] and therefore it would be approximately 20–200 μM if 10 g of soybean were contained in 100 g of food. The concentration of estrogen in mammalian blood is 40–800 pM. Therefore, the present results may suggest that exposure to estrogenic compounds in daily life could cause macrophages to be more sensitive to superoxide-inducing stimuli.

Effect of Bisphenol A, Genistein, and 17β-Estradiol on NO Production by Macrophages

The effect of the three estrogenic compounds on NO production by activated macrophages was investigated by fluorometry using DAF-2.[7,8] Stimulation of TG-induced mouse peritoneal macrophages with LPS resulted in the generation of fluorescence by DAF-2T, and the generation was almost completely inhibited by the iNOS inhibitor L-NMMA (data not shown), indicating that the fluorescence reflects NO production by iNOS. As shown in Fig. 3, LPS effectively stimulated the macrophages to produce NO after 16 hr and the NO production was not significantly affected by 0–10 μM of bisphenol A, 0–1 μM of genistein, and 0–1 nM of 17β-estradiol. The NO production was markedly inhibited by higher concentrations of bisphenol A (100 μM), and

genistein (10 μM). Viability of the cells under these conditions changed little, as determined by the measurement of the intracellular NADH level (data not shown). This concentration of bisphenol A is extraordinarily high, and it is unlikely that humans would encounter this level of exposure. On the other hand, humans could be exposed to 10 μM of genistein. This observed inhibitory effect of genistein must be investigated further.

Mechanisms to Be Clarified

The present methods used to assess the effect of estrogenic compounds on superoxide and NO production by activated macrophages were found to be useful since they were selective, respectively, for superoxide and NO, and reproducible results were obtained for both assays.

Although the concentrations of bisphenol A, genistein, and 17β-estradiol used approximately corresponded with each other with respect to their estrogenic activity, the effect of 17β-estradiol was very weak for both superoxide production and NO production as compared with the effects of bisphenol A and genistein. Therefore the effects of bisphenol A and genistein observed may not be due to their estrogenic acitivity, but to other bioactivity. Because genistein is a potent inhibitor of protein tyrosine kinases,[15] it is conceivable that bisphenol A might have inhibitory activity against protein tyrosine kinases.

For further understanding of the potential toxicity of EDCs other than that to the endocrine system, it is important to clarify whether various EDCs have unknown bioactivity other than estrogenic activity.

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