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Changes in the mutagenic and estrogenic activities of bisphenol A upon treatment with nitrite

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Abstract

Bisphenol A (4,4′isopropylidenediphenol: BPA), an endocrine-disrupting chemical, is contained in food-packaging and can-coating agents as well as in dental sealants. Nitrite is present in vegetables, fish and tap water as an ingredient or contaminant, and also in human saliva. Here, we explored the possible generation of genotoxicity from the reactions of BPA and nitrite under acidic conditions, a situation simulating the stomach. We determined the changes in the mutagenic and estrogenic activities of BPA before and after nitrite treatment. Untreated BPA did not exhibit any mutagenicity. However, the mixture of BPA and sodium nitrite after incubation at pH 3.0 showed strong mutagenic activity toward *Salmonella typhimurium* strains TA 100 and TA 98 either with or without a metabolic activation system (S9 mix). The clastogenic properties of nitrite-treated and untreated BPA were analyzed by a micronucleus test with male ICR mice. A single gastric intubation of nitrite-treated BPA induced a significantly higher frequency of micronucleated reticulocytes (MNRETs) in mice. The results of analysis of electron spin resonance (ESR) suggest that the expression of the mutagenic activity of nitrite-treated BPA is related to the generation of radicals in the reaction mixture. By applying ^{1}H and ^{13}C NMR, AB-MS and APCI/LC/MS, we identified two compounds 3-nitrobisphenol A and 3,3′-dinitro-bisphenol A. These compounds were synthesized by the reaction of BPA with nitric acid. 3,3′-Dinitro-bisphenol induced a significantly greater frequency of MNRETs in male ICR mice. By applying a green fluorescent protein (GFP)-reporter expression system and an estrogen R(α) competitor screening kit, we found that nitrite-treated BPA and 3,3′-dinitro-bisphenol A showed weak estrogenic activity compared to that of untreated BPA.

Keywords: Bisphenol A; Nitrite; Mutagenic activity; Estrogenic activity

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1. Introduction

We are currently being exposed to many kinds of chemicals, such as food additives, pesticides and environmental contaminants. Many of these compounds have adverse effects on aquatic animals and mammals, including humans [1]. Among these compounds, polychlorodibenzo-*p*-dioxins and polychlorinated biphenyls, nonylphenol, octylphenol and certain pesticides are known to be endocrine-disrupting chemicals [2]. When endocrine-disrupting chemicals bind to the estrogenic receptor in the cell, they cause abnormal hormone secretion and subsequent related diseases. Bisphenol A (BPA) (2,2-bis(4-hydroxyphenyl)propane) is used as an ingredient in the manufacture of epoxy carbonate, polycarbonate and polyester-styrene. These compounds are often used in food packaging and can-coating agents as well as dental sealants, and are readily ingested by humans orally [3–5].

BPA is not generally recognized as a mutagen through the use of several in vitro and in vivo mutagenicity assays. Haworth et al. reported that BPA showed negative results in bacterial reverse mutation tests using *Salmonella typhimurium* (*S. typhimurium*) TA 98, TA 100, TA 1535 and TA 1537 [6]. Ivett et al. demonstrated that BPA did not exhibit mutagenic activity in in vitro genotoxicity assays with Chinese hamster ovary cells [7]. Gudi et al. reported that BPA was not associated with an increase in the frequency of micronucleated reticulocytes (MNRETs) in bone marrow of mice [8]. Recently, however, there have been several positive reports regarding the mutagenic activity of BPA. Hilliard et al. verified that BPA showed positive results in an in vitro chromosome aberration test using CHO cells without S9 mix [9]. Tsutsui et al. demonstrated that quinone compounds derived from BPA produced DNA adducts in an in vitro ^{32}P-postlabeling assay [10]. As described above, the mutagenic activity of BPA varies with the test system. However, Haighton et al. recently reported that BPA presents little mutagenic/carcinogenic risk to human beings [11].

The estrogenic activity of BPA has been widely studied in in vitro and in vivo systems [12–15]. Several researchers have investigated the estrogenic activity of structurally modified BPA. Hu et al. observed that the estrogenic activity of chlorinated BPA, which is formed during chlorination in water-purification plants or paper factories, was greater than that of non-chlorinated BPA [16]. Kitamura et al. observed an increase in the estrogenic activity of BPA after bromine treatment [17]. Yoshihara et al. demonstrated that the estrogenic activity of BPA was increased upon metabolic activation with rat S9 mix [18]. Matthews et al. reported that BPA glucuronide, which is a metabolic conjugate of BPA, did not exhibit estrogenic activity in vitro [19].

Humans regularly consume nitrite and nitrate in vegetables, tap water and in their daily diet as food additives [20]. Nitrate is readily reduced to nitrite by oral bacteria. Mutagenic/carcinogenic nitrosamines can be formed by the reaction of nitrite and secondary amines [21]. Several phenolic compounds also show mutagenic activity after nitrite treatment under acidic conditions. Wakabayashi et al. demonstrated that some phenol and indole derivatives present in the environment are changed to mutagenic compounds by nitrosation [22]. Kikugawa and Kato reported that diazoquinone compounds, which show mutagenicity, were formed by the interaction between phenol and nitrite [23,24]. Consequently, BPA might induce mutagenicity after it reacts with nitrite under acidic conditions. Schrader et al. demonstrated that nitrite-treated BPA exhibited mutagenic activity using *S. typhimurium* strains TA 100 and TA 98 [25]. They found that nitrite-treated BPA produced base-pair mutations at a variety of sites including T:A > A:T, G:C > A:T, C:G > A:T and C:G > G:C, as well as G:C frameshift mutations [24]. However, they did not address how changes in the structure of chemically modified BPA affected estrogenic activity.

In the present study, we examined the reaction of BPA with nitrite and the change in mutagenic activity using *S. typhimurium* strains TA 100 and TA 98 with and without S9 mix. The clastogenic activities of nitrite-treated BPA and other products were examined by the micronucleus test using male ICR mice. The estrogenic activities of nitrite-treated BPA and products were determined by a green fluorescent protein (GFP)-reporter expression system and estrogen R(α) competitor screening kit. The production of radicals in the reaction solution of nitrite-treated BPA was examined by an electron spin resonance method. The chemical structure of nitrite-treated BPA was determined by using instrumental analysis.

2. Materials and methods

2.1. Chemicals

BPA was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Sodium nitrite, sodium citrate, citric acid monohydrate, sodium acetate, acetic acid, ammonium sulfamate, 17β-estradiol and dimethylsulfoxide (DMSO), L-cysteine, nitric acid, magnesium sulfate and silica gel were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl acetate, hexane and dichloromethane were bought from Kanto Kagaku Co., Ltd. (Tokyo, Japan). 5,5-Dimethylpyrroline-1-oxide (DMPO) was purchased from Sigma–Aldrich Japan K.K. (Tokyo, Japan).

2.2. Nitrite treatment of BPA

BPA was dissolved in DMSO at 100 mM, and 1 ml of this solution was added to 100 ml of 200 mM citrate buffer (pH 3.0) or 200 mM acetate buffer (pH 4.0 or 5.0), containing 100 mM sodium nitrite. The mixture was incubated at 37 °C for 1 h. Adding an equal molar amount of ammonium sulfamate dissolved in the same buffer then stopped the reaction. The reaction mixture was treated with three 50 ml washes of ethyl acetate. All ethyl acetate extracts were collected and the solvent was evaporated to dryness. The residue was dissolved in DMSO for the mutagenicity tests. We also used the reaction mixtures or ethyl acetate extracts as test samples in other assays.

2.3. Ames test

Each test solution was subjected to the Ames test [26]. The test was carried out using *S. typhimurium* test strains TA 98 and TA 100 obtained from Dr. Bruce N. Ames (University of California, Berkeley, USA). The mutagenic activity of each sample was examined with and without S9 mix (Kikkoman, Chiba, Japan). One hundred microliters of each sample was added to a sterilized tube. Five hundred microliters of S9 mix or phosphate buffer (pH 7.5) and 100 μl of suspensions of *S. typhimurium* TA 98 or TA 100 were added to the tube and mixed. The mixture was incubated at 37 °C for 20 min. Two milliliters of soft agar was added to the tube and mixed. The mixture was put in a petri dish and kept at 37 °C for 48 h. The number of revertant colonies was counted. The number of colonies presented here is the average of three plates.

2.4. Micronucleus test with mouse peripheral blood reticulocytes

This assay was performed according to the acridine orange-coated slide method developed by Hayashi et al. [27]. Eleven-week-old male ICR mice (Japan SLC, Hamamatsu, Japan) were used. They were housed in an air-conditioned room and given CE-2 commercial food pellets (Crea Japan, Tokyo, Japan) ad libitum. Five mice were assigned to each experimental group. Samples dissolved in DMSO were administered by gastric intubation at a dose of 1 mmol/kg body weight. Five microliters of peripheral blood was collected from a tail blood vessel at 24, 48 and 72 h after sample administration. Blood samples were stained on acridine orange-coated glass slides. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Shizuoka. At least 1000 RNA-containing erythrocytes were observed by fluorescence microscopy (magnification ×400) and the numbers of micronucleated cells were recorded.

2.5. Analysis of nitrite-treated BPA by HPLC coupled with mass spectrometry

APCI/LC/MS with an ultraviolet (UV) detector was used to characterize nitrite-treated BPA. Liquid chromatography was carried out on an HPLC apparatus equipped with a model L-7100 intelligent pump (Hitachi Ltd., Tokyo, Japan), a model L-7300 column oven (Hitachi, Tokyo Japan), and a model SPD-6AV UV detector (Shimadzu Co. Ltd., Kyoto, Japan). A UV wavelength of 225 nm was used. Each sample was chromatographed on a YMC-Pack R-ODS-5-A (4.6 mm i.d., 250 mm in length, YMC Co. Ltd., Kyoto, Japan) at 40 °C. The flow rate was 0.7 mL/min. The mobile phase was a mixture of methanol and water (70/30, v/v) containing 0.1% acetic acid, which was eluted isocratically. Mass spectrometry was carried out in a negative-ion mode on a model M-1200H LC/MS (Hitachi Ltd., Tokyo, Japan) quadrupole mass spectrometer equipped with an APCI interface. The temperatures of the nebulizer and desolvator were set at 185 and 400 °C, respectively. The drift voltage was controlled at 80 V.

2.6. Determination of electron spin resonance spectra

Electron spin resonance (ESR) spectra were determined on a JES RE3X spectrometer (JEOL, Tokyo, Japan) with a Mn^{2+} marker at room temperature using a capillary tube. The instrumental conditions were a field setting at 335.1 mT, scan range of $\pm 7.5 \times 1$ mM, modulation frequency of 9.4 GHz microwave power of 10 mW, modulation amplitude of 0.079 mT, gain of 5×100, time constant of 0.1 s, and scanning time of 4 min. Ethyl acetate extract, evaporated to dryness, was dissolved in DMSO and put into a tube, and then DMPO was added. The final volume of the mixture was adjusted to 500 μl so that the concentrations of DMPO and samples were always 100 and 10 mM, respectively. The mixture was stirred for several seconds after the addition of DMPO. Untreated BPA was used as a blank sample. Radical intensity was measured within 1 min after mixing the sample and DMPO.

2.7. Synthesis of 3-nitrobisphenol A and 3,3′-dinitro-bisphenol A

BPA (500 mg) was dissolved in acetic acid (4.0 ml). A 10% nitric acid/acetic acid solution (0.1 ml) was added to the mixture dropwise, and then mixed for 30 min at 37 °C. The reaction mixture was poured in ice water and extracted with dichloromethane. The dichloromethane extract was washed with saturated saline and dried over magnesium sulfate. It was then concentrated under reduced pressure and subjected to column chromatography on silica gel (eluent: 20% ethyl acetate in hexane), after which we obtained two compounds.

2.8. Spectroscopic measurement

Spectroscopic measurement was taken with the following instruments: NMR, Jeol Delta-500 spectrometers (tetramethylsilane as an internal reference at 0 ppm for ^{1}H and ^{13}C NMR); FAB-MS, Jeol JMS-700.

2-(4-Hydroxy-3-nitrophenyl)-2-(4-hydroxyphenyl) propane (3-nitrobisphenol A). HRFAB-MS $[M+H]^+$, m/z 274. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.65(6H, s, $2 \times CH_3$), 4.97(1H, s, 4′-OH), 6.75(2H, d $J = 8.6$ Hz, 3′- & 5′-H), 7.02(1H, d, $J = 9.2$ Hz, 5-H), 7.06(2H, d, $J = 8.6$ Hz, 2′- & 6′-H), 7.34(1H, dd, $J = 9.2$, 2.3 Hz, 6-H), 8.04(1H, d, $J = 2.3$ Hz, 2-H), 10.53(1H, s, 4-OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 30.6($2 \times CH_3$), 41.8(quaternary-C), 115.1(3′- & 5′-C), 119.6(5-C), 121.5(2-C), 127.8(2′- & 6′-C), 132.8(3-C), 137.4(6-C), 141.3(1′-C), 143.6(1-C), 153.3(4-C), 153.7(4′-C). HMBC correlation: CH_3-H to CH_3-C, quaternary-C, 1-C and 1′-C, 4′-OH-H to 3′-C and 5′-C, 3′-H to 5′-C, 1′-C and 4′-C, 5-H to 3-C, 1-C and 4-C, 6′-H to 3′-C, 2′-C and 4′-C, 6-H to 2-C and 4-C, 2-H to 3-C, 6-C and 4-C, 4-OH-H to 5-C, 3-C and 4-C.

2,2′-Bis (4-hydroxy-3-nitrophenyl) propane (3,3′-dinitro-bisphenol A). HRFAB-MS $[M+H]^+$, m/z 319. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.71(6H, s, $2 \times CH_3$), 7.07(2H, d, $J = 9.2$ Hz, 5′- & 5′-H), 7.34(2H, dd, $J = 9.2$, 2.3 Hz, 6- & 6′-H), 8.04(2H, d, $J = 2.3$, 2- & 2′-H), 10.54(2H, s, $2 \times$ OH). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 30.3($2 \times CH_3$), 42 (quaternary-C), 120.2(5- & 5′-C), 121.8(2- & 2′-C), 133(3- & 3′-C), 136.8(6- & 6′-C), 141.6(1- & 1′-C), 153.6(4- & 4′-C). HMBC correlation: CH_3-H to CH_3-C, quaternary-C and 1-C, 5-H to 3-C, 1-C and 4-C, 5′-H to 3′-C, 1′-C and 4′-C, 6-H to 2-C and 4-C, 6′-H to 2′-C and 4′-C, 2-H to 3-C, 6-C, 1-C and 4-C, 4-OH-H to 5-C, 3-C and 4-C, 4′-OH-H to 5′-C, 3′-C and 4′-C.

2.9. Cell proliferation assay and image analysis of estrogenicity (GFP expression system)

These analyses were performed according to the following method described by Kuruto-Niwa et al. [28]. Human breast carcinoma MCF-7 cells, to which had been transferred a green fluorescent protein (GFP) reporter vector regulated by an estrogen response element (ERE), were used. ERE-GFP-MCF-7 cells (1 or 2×10^4) were plated in each well of a 96-well plate (Costar, Corning, NY) in assay medium (phenol red-free DMEM (Cosmo Bio, Tokyo, Japan). Cell proliferation was measured using a Cell Counting Kit-8 (Dojin, Kumamoto, Japan), in which a water-soluble tetrazolium salt WST-8, i.e., 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, is used as a substrate. The counting solution containing WST-8 and 1-methoxyphenazinium salt (final respective concentrations of 0.5 mM and 20 M) was added to each well, and the cells were incubated at 37 °C for 2 h in a CO_2 incubator. The absorbance of the well was measured at 450 nm with a reference

wavelength at 630 nm. The number of cells was determined by use of a standard curve. After the counting assay, the medium was removed, and the intensity of green fluorescence in the cells was scanned by a FluorImager SI (Amersham Pharmacia Biotech). Fluorescence intensity was estimated with the Image Quant program (Amersham). Each experiment was performed in triplicate, and mean values are shown.

2.10. Screening of binding activity to estrogen receptor

Estrogen R(α) competitor screening kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to examine the binding activity of samples to estrogen receptor. Six microliters of sample was dissolved in DMSO with 114 μl of reaction solution in a test tube or a well microplate that had been prepared separately. One hundred microliters of the prepared mixture was pipetted into each well to be used in the supplied ERα-coated microplate. Ninety-five microliters of reaction solution and 5 μl of DMSO were added to the well as a blank. The mixtures were incubated at room temperature for 2 h. The plate was washed with 200 μl of wash solution and residual solutions were drained from the wells by inverting the plate. One hundred microliters of assay solution was then added to each well. The fluorescence intensity in each well was determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm by a fluorescence microplate recorder (Perkin-Elmer Japan, Yokohama, Japan).

2.11. Statistics

A *t*-test was used to evaluate the significance of the differences in the frequency of micronucleated cells in the micronucleus test between ICR male mice treated with nitrite-treated BPA and with untreated BPA group; *p*-values lower than 0.01 or 0.05 were considered to be statistically significant. To determine the EC_{50}, we fitted the dose response curves (sigmoid fit) using GraphPad Prism 4.0 (GraphPad Software, Inc., CA, USA). The EC_{50} was calculated by determining the concentration at which 50% of the maximum fluorescence intensity was reached on the sigmoidal fit equation.

3. Results and discussion

The mutagenic activity of nitrite-treated BPA was examined by the Ames test using *S. typhimurium* strains TA 100 and TA 98 in the presence or absence of S9 mix. The data are shown in Fig. 1. Before nitrite treatment, BPA did not show any mutagenic activity toward either strain. Upon treatment with sodium nitrite under acidic conditions, mutagenic activity emerged and increased with an increase in the sodium nitrite concentration in either strain. In *S. typhimurium* strain TA 100, treatment with 50 mM nitrite induced 183 (+S9 mix) and 276 (−S9 mix) revertants/100 μl reaction solution. Treatment with 100 mM nitrite induced 331 (+S9 mix) and 383 (−S9 mix) revertants. BPA that had been treated with 100 mM sodium nitrite exhibited 175 (+S9 mix) and 200 (−S9 mix) revertants toward *S. typhimurium*

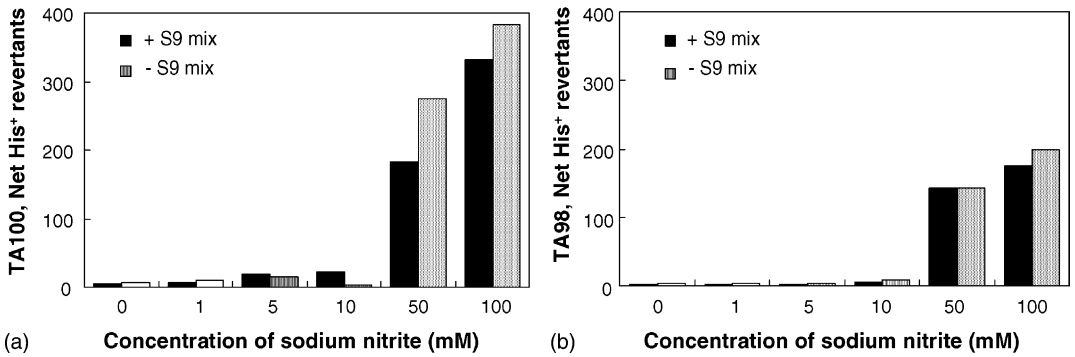


Fig. 1. Mutagenic activity of nitrite-treated BPA toward *S. typhimurium* (a) TA 100 and (b) TA 98 in the presence and absence S9 mix. The concentration of BPA was 1 mM in each reaction mixture at pH 3.0. One hundred microliter samples of the reaction mixtures were tested.

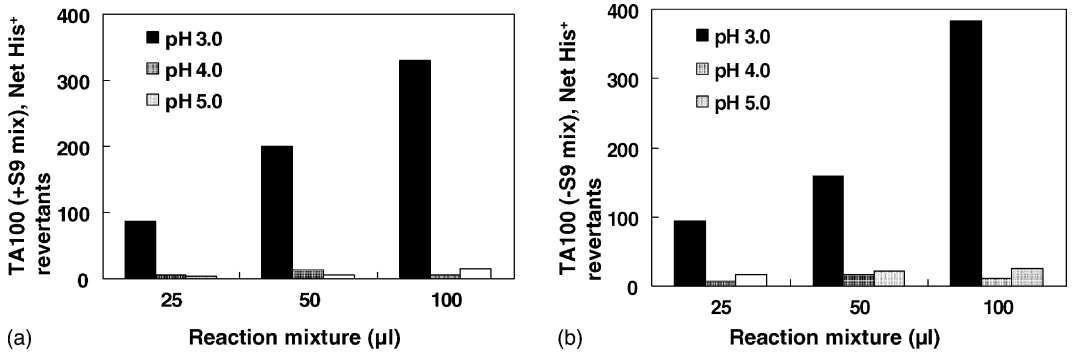


Fig. 2. Effect of pH on the mutagenic activity of the reaction mixtures toward *S. typhimurium* TA 100 in the (a) presence or (b) absence of S9 mix. The concentration of BPA was 1.0 mM in each reaction mixture. One hundred microliter samples of the reaction mixtures were used.

strain TA 98. However, BPA treated with less than 10 mM nitrite did not show any mutagenic activity in either strain. Since nitrite-treated BPA showed positive results in both the presence and absence of S9 mix, the products might be direct-acting mutagens that induce frameshift and base-pair-change mutations. Therefore, we examined the effect of pH during nitrite treatment on the mutagenic activity of the products (Fig. 2). The pH was adjusted to 3.0, 4.0 or 5.0 with 200 mM citrate buffer or 200 mM acetate buffer. The mutagenic activity was found in the reaction mixture at pH 3.0, but not at pH 4.0 or 5.0. These results show that BPA might exhibit mutagenicity by reacting with nitrite under acidic conditions, as in the stomach. We examined free radical formation in the reaction mixture of nitrite with BPA by electron spin resonance (ESR). Fig. 3 shows the free radical intensity in the reaction mixture without BPA, with untreated BPA and with nitrite-treated BPA. The intensity of the peaks observed for nitrite-treated BPA increased with time after the addition of DMPO: at 10 min the intensity was about twice that at 1 min. The mixture of DMSO, citrate and nitrite after reaction without BPA and untreated BPA showed several small peaks, but these peaks were weak. This suggested that the mutagenicity of nitrite-treated BPA may be induced by free radical produced in the reaction mixture of BPA and nitrite. Therefore, we examined if mutagenicity of nitrite-treated BPA was suppressed by the addition of L-cysteine, which is a radical scavenger, using *S. typhimurium* TA 100 in the absence of S9 mix (Fig. 4). The mutagenic activity of nitrite-treated BPA showed 465 revertants. With the addition

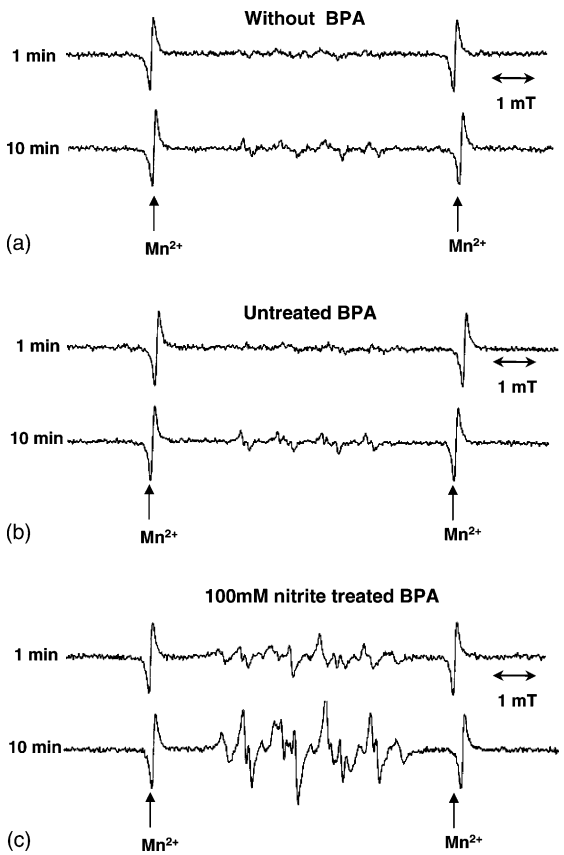


Fig. 3. ESR spectra of spin adducts of reaction mixtures (a) without BPA, (b) with untreated-BPA and (c) with nitrite-treated BPA. Each concentration of samples was 0.01 M in DMSO. DMPO was added to the samples to a final concentration of 0.1 M.

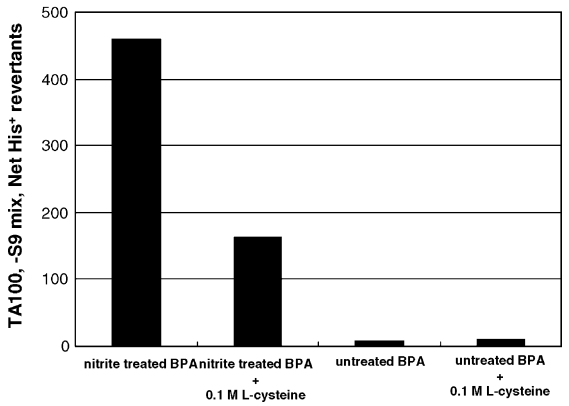


Fig. 4. Inhibitory effect of L-cysteine on the mutagenic activity of nitrite-treated BPA toward *S. typhimurium* TA 100 without S9 mix. The concentrations of BPA and L-cysteine in the reaction mixture were 1 mM and 0.1 M, respectively. One hundred microliter samples of the reaction mixtures were tested.

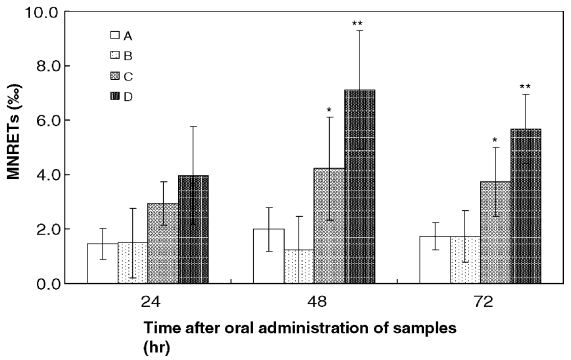


Fig. 5. The frequency of micronucleated reticulocytes (MNRETs) in male ICR mice with the administration of nitrite-treated BPA. Group A: DMSO, B: untreated BPA (1 mmol/kg body weight), C: nitrite-treated BPA (0.5 mmol/kg body weight), D: nitrite-treated BPA (1 mmol/kg body weight). Blood was collected at 24, 48 and 72 h after the oral administration of samples. RNA-containing erythrocytes were counted by fluorescence microscopy (magnification ×400) and the numbers of micronucleated cells were recorded. $^{**}p < 0.01$ (vs. group B), $^{*}p < 0.05$ (vs. group B).

of L-cysteine, the mutagenicity was decreased to 67% compared to that without L-cysteine. Untreated BPA and L-cysteine-treated BPA showed no mutagenic activity. These results demonstrate that the mutagenic activity of nitrite-treated BPA could be attributed to the production of free radical in the reaction mixture.

We examined the clastogenic activity of nitrite-treated BPA using a micronucleus test with male ICR mice. Before nitrite treatment, BPA did not show any MNRETs at any of the sampling times, as shown in Fig. 5. However, nitrite-treated BPA significantly increased the frequency of MNRETs compared to untreated BPA at 48 and 72 h after oral administration in a nitrite dose-dependent manner. The clastogenic activity was the highest at 48 h in the treated group. The treated group showed higher activity than that with untreated BPA at 24 h after administration.

In the next experiment, we sought to identify the products formed by the reaction of BPA and nitrite. The concentration of BPA in the reaction mixture was determined using the APCI/LC/MS (negative ion mode) method. The concentration of BPA was decreased to 35.9 and 2.78% in 10 and 50 mM nitrite-treated reaction mixtures, respectively. BPA was not detected in the reaction mixture with 100 mM sodium nitrite. Several new peaks were seen upon the treatment of BPA with nitrite. We confirmed that the $[M-H]$ m/z values for compounds A and B were 272 and 317, respectively, and considered that these products was formed by the addition of one or two nitro groups to BPA, as shown in Fig. 6. Both peaks were collected with an HPLC apparatus and the chemical structures of compound A and B were investigated by ^{1}H and ^{13}C NMR, HMBC data and FAB-MS. Compounds A and B were identified as 2-(4-hydroxy-3-nitrophenyl)-2-(4-hydroxyphenyl)propane (3-nitrobisphenol A, CAS number: 5329-21-5) and compound B as 2,2′-bis (4-hydroxy-3-nitrophenyl)propane (3,3′-dinitrobisphenol A CAS number: 127244-29-5), respectively (Fig. 7). We synthesized 3-nitrobisphenol A and 3,3′-dinitro-bisphenol A formed by the reaction of BPA and nitric acid. The ^{1}H and ^{13}C NMR data of these compounds matched those of compounds A and B formed by the reaction of BPA and nitrite. 3-Nitrobisphenol A and 3,3′-dinitro-bisphenol A were obtained in amounts of 180 and 400 mg from 500 mg bisphenol A, respectively.

We examined the mutagenic activity of these compounds toward *S. typhimurium* TA 100 using the Ames test. Neither compound showed any mutagenic activity toward *S. typhimurium* strain TA 100 (−S9 mix) (data not shown). However, 3,3′-dinitro-bisphenol A significantly increased the frequency of MNRETs compared to untreated BPA at 48 and 72 h after oral administration (Fig. 8). Therefore, the clastogenic activity of

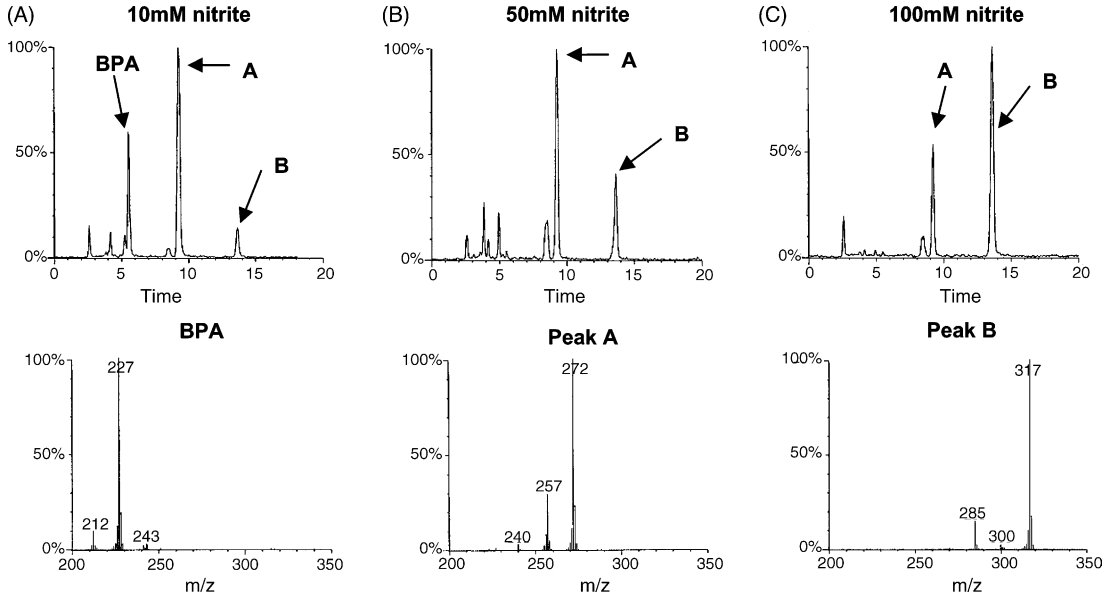
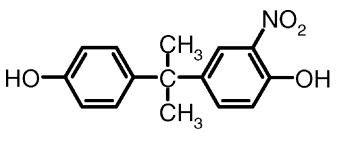


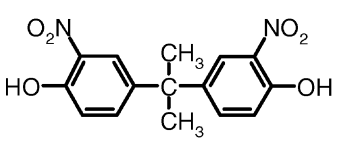
Fig. 6. Chromatograms and mass spectra of nitrite-treated BPA determined by an analysis of APCI/LC/MS. Sodium nitrite was used at concentrations of (a) 10 mM, (b) 50 mM and (c) 100 mM. Mass spectrometry was determined in negative-ion mode with an APCI interface.

nitrite-treated BPA may be attributed to 3,3′-dinitrobisphenol A. We supposed that several kinds of mutagens might be formed by the reaction of nitrite and BPA.

Several investigators have reported that BPA did not exhibit any mutagenic or genotoxic activity in in vitro assays including the Ames test with and without S9 mix [6], chromosomal aberrations or sister chromatid exchange [7], unscheduled DNA synthesis in rat primary hepatocytes [29], mutation at the thymidine kinase and HGPRT gene foci and cellular transformation [30]. Furthermore, BPA has not shown positive results in in vivo tests such as sex-linked recessive lethality in *Drosophila melanogaster* [31] and the frequency of micronuclei in male ICR mice [8]. In recent reports, BPA has been found to exhibit positive results in mutagenicity tests [9,10,32]. Although these assays are not standard methods, Haighton et al. reported that BPA



Compound A (3-nitrobisphenolA)



Compound B (3,3'-dinitrobisphenol A)

Fig. 7. Chemical structures of products of the treatment of BPA with nitrite.

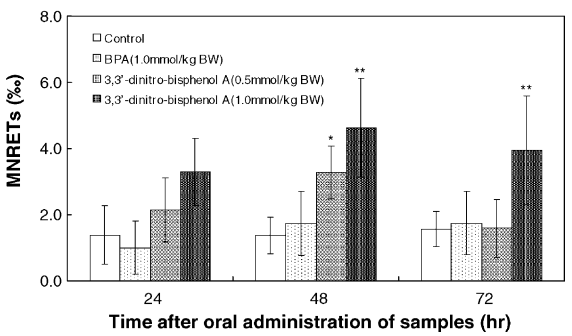


Fig. 8. The frequency of micronucleated reticulocytes (MNRETs) in male ICR mice administered compound A (3,3′-dinitro-bisphenol A). Blood was collected at 24, 48 and 72 h after oral administration of the sample. RNA-containing erythrocytes were counted by a fluorescence microscopy (magnification ×400) and the numbers of micronucleated cells were recorded.

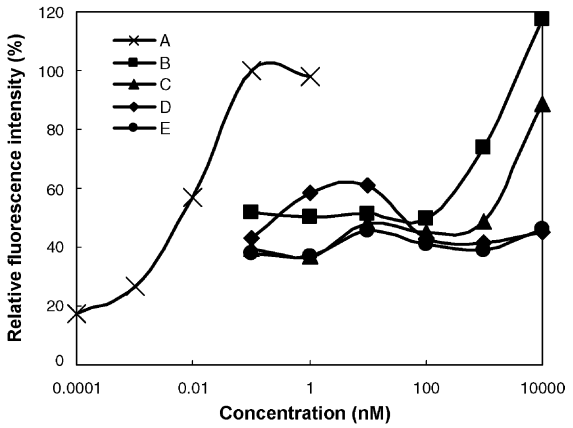


Fig. 9. Estrogenic activity of nitrite-treated BPA using a GFP expression system. (A) Estradiol, (B) BPA, (C) 10 mM nitrite-treated BPA, (D) 50 mM nitrite-treated BPA, (E) 100 mM nitrite-treated BPA.

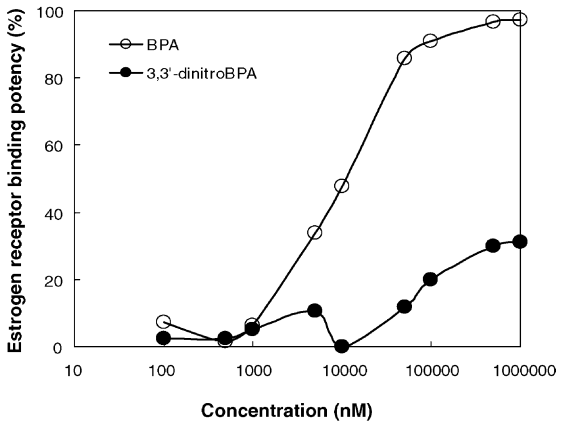


Fig. 10. Binding potency of BPA and compound A (3,3′-dinitrobisphenol A) to estrogenic receptors using an estrogen R(α) competitor screening kit.

does not present a mutagenic/carcinogenic risk to human beings [11]. However, we demonstrated that BPA induced mutagenic activity after reacting with nitrite under acidic conditions.

The estrogenic activity of nitrite-treated BPA was examined using a GFP expression system (Fig. 9). The EC_{50} value of the estrogenic activity of treated BPA at each nitrite concentration was calculated and compared with that of 17β-estradiol. While the EC_{50} value of untreated BPA was 1.4×10^{-7} M, that for 10 mM nitrite-treated BPA was 1.3×10^{-6} M. The EC_{50} values for 50 mM and 100 mM nitrite-treated BPA were not calculated because the activity completely disappeared. Next, the estrogenic activity of 3,3′-dinitro-bisphenol A formed in the reaction of BPA and nitrite was determined using an estrogen R(α) competitor screening kit (Fig. 10). The activity of 3,3′-dinitro-bisphenol A was weak compared to that of untreated BPA.

In the present study, we demonstrated that BPA could be changed to a mutagen by reacting with nitrite under acidic conditions. BPA is eluted from polycarbonate or epoxy-linked containers. Nitrite is contained in foodstuffs, food additives and tap water. There is a possibility of ingesting of BPA in combination with a nitrite-rich diet, which could lead to the production of nitrite-treated BPA. However, nitrite-treated BPA has not been detected in humans or animals. At present, the health risks associated with BPA in humans and other organisms have not yet been evaluated by considering chemical and metabolic modifications. Therefore, it is important to evaluate the risk of BPA after it is consumed. Our next goal is to elucidate the generation of nitrite-treated BPA in vivo. We also need to monitor mutagens formed by nitrite treatment in our environment for a thorough risk assessment. This work may represent a standard method for examining new toxic compounds in our environment, such as endocrine disrupters.

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