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In vitro assay of hydrolysis and chlorohydroxy derivatives of bisphenol A diglycidyl ether for estrogenic activity

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Abstract

Bisphenol A diglycidyl ether (BADGE) is an epoxy resin monomer. Epoxy-based solution coatings are used in many applications as additives for a variety of plastic coatings in food packaging. It is well known that unreacted BADGE can migrate from epoxy-based packing materials into foods. Not only BADGE but also its derivatives can easily migrate into foods and it is likely that we intake BADGE and its derivatives through food or drink. Recently, endocrine disrupting chemicals (EDCs) have attracted attention because they have been shown to affect reproduction in wildlife. The estrogenic activity of BADGE derivatives has not previously been investigated. Therefore, we investigated the estrogenic activity of the BADGE derivatives, dihydrolysed BADGE (BADGE-4OH) and chlorohydroxy BADGE (BADGE-2Cl), using breast cancer cell (T47D) proliferation assay and estrogen receptor (ER) (α) binding assay. These chemicals exhibited T47D cell proliferation at concentrations of 10^{-14}–10^{-4} M. However, these chemicals did not bind to ER (α) in the binding assay.

Keywords: Bisphenol A; Bisphenol A diglycidyl ether; Estrogenic; Cell proliferation assay; Binding assay

1. Introduction

It has been suggested that exposure to certain synthetic chemicals present in the environment can disrupt the endocrine system of humans. These chemicals have been called endocrine disrupting chemicals (EDCs). Many recent assays have indicated various synthetic chemicals such as plastic compounds, plasticizers and additives for plastic materials to be EDCs. Many in vitro (Sumpter and Jobling, 1995; Sonnenschein and Soto, 1998) and in vivo (Steinmetz et al., 1997; Milligan et al., 1998; Ashby et al., 1999; Howdeshell et al., 1999; Welsons et al., 1999; Mehmood et al., 2000; Papaconstantinou et al., 2000) tests have been developed to examine whether these chemicals have estrogenic activity. Some compounds have been reported to exhibit estrogenic activity.

Commercial epoxy resins used as a plastic coating in the food packaging industry are obtained by a condensation reaction between epichlorohydrin and bisphenol A (BPA) (Fig. 1). This reaction leads to the production of bisphenol A diglycidyl ether (BADGE). Many studies have shown that BADGE can migrate from epoxy-based packaging materials into canned fish in oil, meat products, mixed vegetables, milk products and pizzas (Sharman et al., 1995; Biedermann and Grob, 1998; Biles et al., 1999; Theobald et al., 1999). Furthermore, it has been shown that BPA leaches from epoxy can-coatings to canned drinks, vegetables and fruit (Horie et al., 1999; Yoshida et al., 2001) at ppb levels. There is a possibility that individuals might intake epoxy-related compounds from contaminated food. A directive of the Commission of the European Communities established specific migration limits for BPA and BADGE in food or food products: 3 mg/kg for BPA and 0.02 mg/kg for BADGE (European Commission, 1990). In 1996, the Commission also proposed that for BADGE, its hydrolysis products, and

Abbreviations: BADGE, bisphenol A diglycidyl ether; BADGE-2Cl, chlorohydroxy BADGE; BADGE-4OH, dihydrolysed BADGE; BPA, bisphenol A; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; . EDCs, endocrine disrupting chemicals; ER, estrogen receptor; RPE, relative proliferate effect; RPP, relative proliferate potency.

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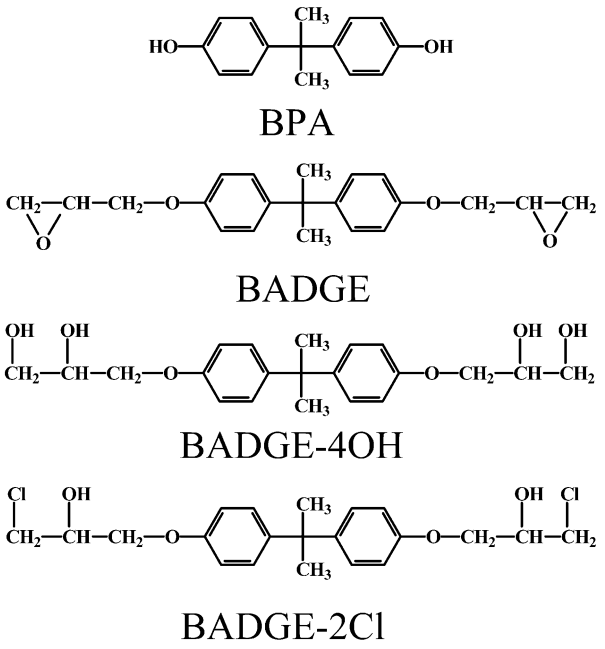

Fig. 1. Structures of epoxy resins chemicals. BPA, bisphenol A; BADGE, bisphenol A diglycidyl ether; BADGE-4OH, bisphenol A bis (2,3-dihydroxypropyl) ether; BADGE-2Cl,bisphenol A bis (3-chloro-2-hydroxypropyl) ether.

chlorohydrin, a specific migration upper limit of 1 mg/kg food should be enforced (SCF, 1996). BADGE has two epoxy rings. It is thought that the epoxy rings in hydrophilic solution are opened to become bisphenol A bis (2,3-dihydroxypropyl) ether (BADGE-4OH), and when the hydrophilic solution contains Cl ions, BADGE will change to bisphenol A bis (3-chloro-2-hydroxypropyl) ether (BADGE-2Cl). Recently, these chemicals were shown to exhibit genotoxicity in the micronucleus test (Suarez et al., 2000). BADGE has also been investigated for estrogenic activity using the human breast cancer cell line MCF-7 and binding assay. Its estrogenic action and other toxicities, in particular carcinogenicity and mutagenicity, were discussed (Olea et al., 1996). BPA is well known to have estrogenic action in in vitro assay (Krishnan et al., 1993). However, the BADGE-related compounds have not previously been investigated for estrogenic activity. Therefore, using cell proliferation assay with T47D human breast cancer cells and estrogen receptor (α) competitor binding assay, we investigated the estrogenic activity of BADGE-4OH and BADGE-2Cl (Fig. 1).

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2), bisphenol A bis (2,3-dihydroxypropyl) ether (BADGE-4OH) and bisphenol A bis (3-chloro-2-hydroxypropyl) ether (BADGE-2Cl) were obtained from Sigma Aldrich (St. Louis, MO, USA). The purity of the BADGE-4OH and BADGE-2Cl standards is up to 97.0%. Bisphenol A (BPA) was obtained from Kanto Chemical Corporation (Tokyo, Japan). ICI 182,780 was obtained from TOCRIS (Ballwin, MO, USA). A Cell Counting Kit was obtained from Dojindo (Kumamoto, Japan). TCH was obtained from CELOX (St. Paul, MN, USA).

2.2. Sample preparation

E2, BPA, BADGE-4OH, BADGE-2Cl and ICI 182,780 were prepared as 10^{-1} M stock solutions in dimethyl sulfoxide (DMSO). Standard solutions of E2, BPA, BADGE-4OH, BADGE-2Cl and ICI 182,780 were prepared in DMSO, and prepared at each concentration as required by the addition of DMSO to each well. Then, stock solutions were dissolved in phenol red free Dulbecco's modified Eagle's medium (DMEM) for cell proliferation assay.

2.3. Cell line and culture conditions

The estrogen receptor positive T47D human breast cancer cell line was donated by Dr S. Kubota, Tokyo University. For routine maintenance, cells were grown in DMEM high glucose supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 25 mM HEPES. Cells were grown at 37 °C in an atmosphere of 5% CO_2/95% air under saturating humidity. Cells were routinely passed at approximately 70% confluence.

2.4. Cell proliferation assay

The E2 was used as a positive control, and the other chemicals were tested in this assay. Cells were seeded in 96-well plates (2500 cells/well) and then incubated for 1 day at 37 °C, in an atmosphere of 5% CO_2/95% air under saturating humidity. The seeding medium contained phenol red. However, because this phenol red exhibits estrogenic activity it was replaced with phenol red free medium supplemented with TCH, a low protein solution, instead of charcoal-dextran-treated fetal bovine serum. Then, test chemicals were added to each well. The plate was incubated for 3 days at 37 °C, in an atmosphere of 5% CO_2/95% air under saturating humidity, and cell proliferation was measured by WST-1 assay using the Cell Counting Kit. Then, absorbance was measured (460 nm, reference 595 nm) using a microplate reader. The estrogenic activity of the chemicals was assessed by the following factors.

1. The relative proliferate potency (RPP): RPP for each assessed chemical was calculated by dividing the maximum response effect of each compound by the maximum response effect of E2.

2. The relative proliferate effect (RPE): RPE for each assessed chemical was calculated by dividing the maximum response concentration of each compound by the maximum response concentration of E2.
3. The O.D./O.D.cont.: The value was calculated by dividing O.D. by O.D. obtained in the control.

2.5. Estrogen receptor antagonist

In order to ensure that the cell proliferation induced by the compounds was mediated by estrogen receptors, we used an estrogen receptor antagonist, ICI 182,780. In the antagonistic test of the cell proliferation assay, ICI 182,780 was added to each well before the test chemicals were added. The final concentration of ICI 182,780 was 10^{-6} M.

2.6. Estrogen receptor (α) binding assay

An estrogen-R (α) competitor screening kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used. This kit consists of human estrogen receptor (α), the ER recombinant coated microplates, and the necessary reagents including fluorescent labeled estrogen as the competitor for assays with a competitive format. An ER reagent (α) binding assay was performed in accordance with protocol.

3. Results

3.1. Cell proliferation assay

All the compounds tested by the cell proliferation assay were found to have estrogenic activity. The control showed minimum cell proliferation. The positive control, E2 exhibited strong estrogenic activity and cell proliferation at concentrations in the range of 10^{-14}–10^{-6} M of T47D cells. The result is shown in Fig. 2. The response curve for E2 showed a dose-dependent response at concentrations of 10^{-11}–10^{-9} M. In this study, the minimum activity was O.D./O.D. cont. less than 1.3. The maximum proliferate effect was obtained at a concentration of 10^{-9} M. The cell proliferation ratio was 2.13-times greater than that in control cultures after 4 days. The proliferation ratio showed a decrease at a concentration of 10^{-4} M, indicating a toxic response. Next, BADGE-4OH and BADGE-2Cl were assayed. The estrogenic activities of these chemicals were tested at concentrations of 10^{-14}–10^{-4} M. These chemicals also exhibited cell proliferation (Fig. 3). The estrogenic activities of BADGE-4OH and BADGE-2Cl (RPE = 88.5 and 75.4, respectively) were stronger than that of bisphenol A (RPE = 72.6) in this assay (Table 1).

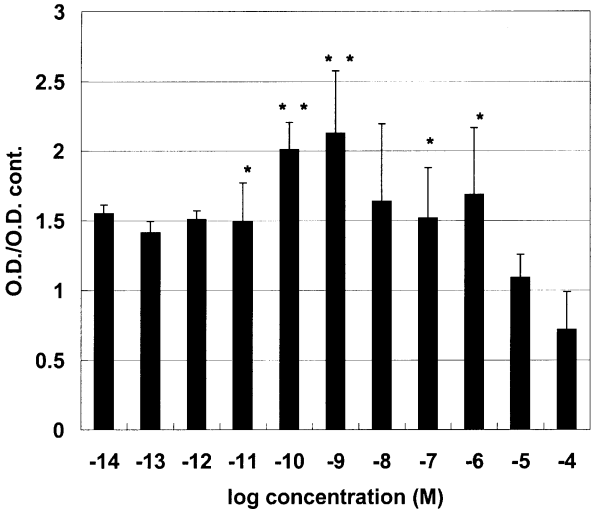

Fig. 2. T47D cell proliferation induced by positive control as 17β-estradiol. Assay concentration levels: 10^{-14}–10^{-4} M. O.D./O.D. cont. value is O.D. divided by O.D. cont. *P <0.05 significantly different from control value; ** P <0.01; significantly different from control value.

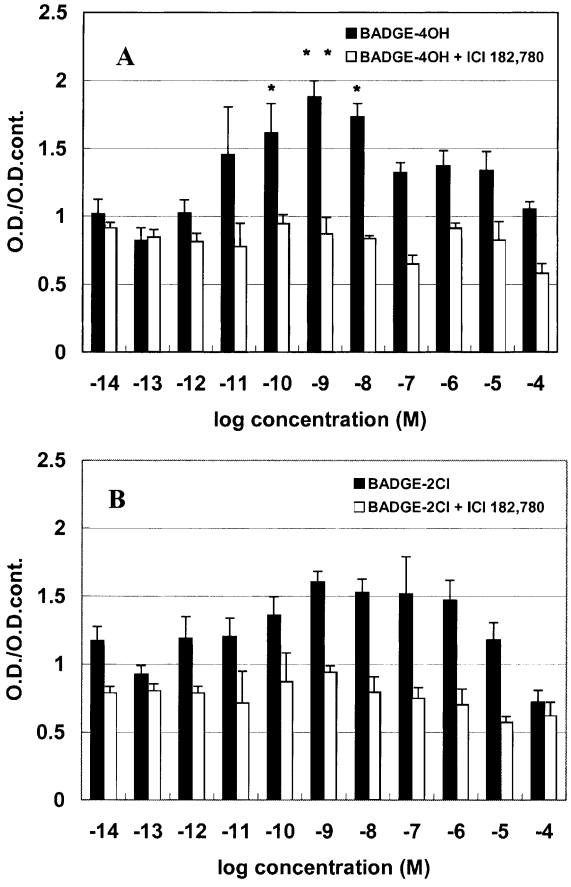

Fig. 3. T47D cell proliferation induced by BADGE-4OH (A) and BADGE-2Cl (B). Assay concentration levels: 10^{-14}–0^{-4} M. O.D./O.D. cont. value is O.D. divided by O.D. cont. ICI 182,780 with a concentration of 10^{-6} M was added to wells. * P <0.05 significantly different from control value; ** P <0.01 significantly different from control value.

The maximum proliferate effects of both the chemicals were obtained at a concentration of 10^{-9} M after 4 days, and the dose–response curve was observed from 10^{-11} M. The cell proliferation ratio in BADGE-4OH was 1.88 and the RPE was 88.5 at the maximum effect. At higher concentrations, cell proliferation started to decrease. BADGE-2Cl exhibited a lower estrogenic activity than BADGE-4OH. The cell proliferation ratio was 1.4 and the RPE was 75.4 at the maximum effect.

In order to confirm that this cell proliferation was mediated by estrogen receptors, an antagonistic test was performed using an estrogen receptor antagonist, ICI 182,780. The proliferation of BADGE-4OH and BADGE-2Cl was inhibited by adding 10^{-6} M ICI 182,780 (Fig. 3).

3.2. Estrogen receptor (α) binding assay

One of the mechanisms of endocrine disruption is the binding of xeno-chemicals to estrogen receptors. The ER binding assay is a simpler and more obvious screening method than any other biological assay of endocrine disruption (Blair et al., 2000). Therefore, we also assayed the estrogenic activity of BADGE-4OH and BADGE-2Cl with the ER binding assay. In Fig. 4, the result of the ER (α) binding assay (E2, BADGE-4OH and BADGE-2Cl) is shown. The assay ranges of these compounds were from 10^{-10} to 10^{-4} M of E2, and 10^{-9} to 10^{-4} M of BADGE-4OH or BADGE-2Cl. Unlabeled E2 competed with fluorescent labeled E2 at a concentration higher than 10^{-10} M. There was a rapid drop in fluorescence intensity between the concentrations of 10^{-10} and 10^{-8} M. At a concentration of 10^{-8} M the composition reached a plateau. In contrast, BADGE-4OH and BADGE-2Cl did not compete with fluorescent labeled E2. From 10^{-9} to 10^{-5} M, the fluorescence intensity remained at the maximum value. In the case of BADGE-2Cl, the fluorescence intensity slightly decreased at 10^{-4} M.

4. Discussion

Perez et al. (1998) investigated whether BPA-related diphenylalkanes, bisphenol F, bisphenol A dimethacrylate, bisphenol A bischloroformate, bisphenol A diglycidylether dimethacrylate and BADGE had estrogenic effects. This was because epoxy resins are also used in dental composites and sealants. In other reports, BADGE was found to be a partial antagonist in the E-SCREEN assay (Brotons et al., 1995; Olea et al., 1996). However, these reports did not provide sufficient evidence to require a safety assessment of food-can-coating epoxy compounds. Hence, it was not investigated whether the major contaminant's compounds such as BADGE-4OH and BADGE-2Cl have estrogenic activity. In addition, Suárez et al. reported that BADGE (tested range 12.5–62.5 μg/ml), BADGE-4OH (25.0–100.0 μg/ml) and BADGE-2Cl (6.25–50.0 μg/ml) induce both cytotoxic and genotoxic effects (Suárez et al., 2000). These tested concentration ranges were comparatively high doses in relation to human exposure levels. Therefore, it was necessary that safety assessments of food-can-coating materials were made studied by using screening assay.

In the studies of contamination of food by can-coating compounds, the total concentration of epoxy compounds (BADGE, hydrolyzed and clorohydrolyzed BADGE) was found at levels up to 12.6 mg/kg in canned food (Hammarling et al., 2000). The half-life of BADGE is very short in acid, therefore the BADGE opening rings were found to be the main contamination in canned foods (Losada et al., 1993). Recently, not only BADGE but also its derivatives were investigated in food contaminations (Biles et al., 1999) and risk assessments. However, their toxicology in these products was limited for these levels of contamination.

When BADGE-4OH and BADGE-2Cl were tested in the cell proliferation assay, proliferations of the T47D breast cancer cell line were induced. In addition, these cell proliferations were inhibited by using ICI 182,780, a

Table 1
Estrogenic effect by T47D cell proliferation

Chemical	Conc. (M)	RPE	RPP
E2	$<10^{-11}$	–	–
BPA	10^{-9}	72.6	100
BADGE-4OH	10^{-9}	88.5	100
BADGE-2Cl	10^{-9}	75.4	100

RPE: Relative proliferative effect, RPP: Relative proliferative potency.

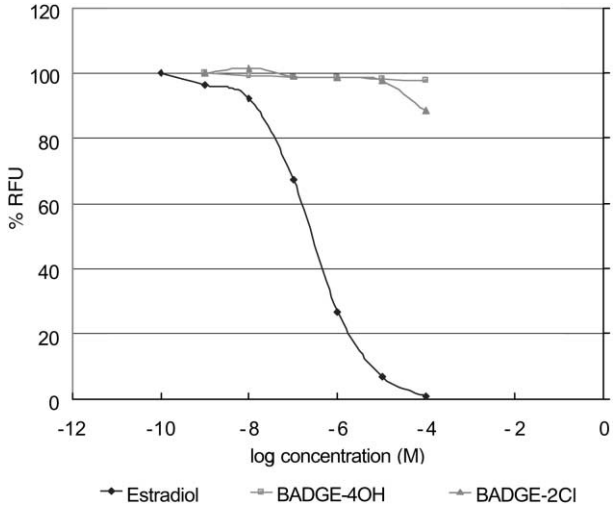

Fig. 4. Representative estrogen receptor binding curve of positive control (17β-estradiol), BADGE-4OH and BADGE-2Cl.

partial agonist and antagonist of estradiol effects (Robertson et al., 2001). However, in the ER (α) binding assay, BADGE-4OH and BADGE-2Cl did not bind to human ER (α). In previous reports (Perez et al., 1998; Blair et al., 2000), there was a correlation between ER binding affinity and proliferation of breast cancer cells. However, the competitive binding assays for ER are limited because they cannot distinguish between estrogenic and anti-estrogenic compounds, and therefore cannot provide insight into a compound's ability to initiate the molecular cascade of events leading to altered gene expression.

Recently, identification of membrane ER from human breast cancer cells was reported (Powell et al., 2001). Thus, it is estimated that there are many instances of unknown receptors acting with antagonist's effects. Therefore, it is reasonable to suppose that the effects of BADGE-4OH and BADGE-2Cl represent unknown estrogenic activity through non-connection with ER. The results of this study raise anxiety about the hormone disrupting activity of BADGE-4OH and BADGE-2Cl from food contamination at human exposure levels. Furthermore, we have studied the reproductive effects of BADGE-4OH on ddY mice during the prenatal and postnatal periods. The food contamination dose of BADGE-4OH exhibits estrogenic activity in this study in vitro. In addition, the reproductive effects of food contamination levels of BADGE-4OH were observed in vivo. These studies will report reproductive effects for the in vivo assay.

It is necessary to examine the effect of BADGE and BADGE derivatives on reproductive organs and function. Exposure to BADGE and its derivatives during the prenatal period may induce reproductive organ malformation. Thus, it is essential to investigate whether these chemicals are harmful to reproductive function.

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