

Sulfoconjugation of Bisphenol A in a Human Neuroblastoma Cell Line, NB-1

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Bisphenol A (BPA) is a common environmental endocrine disruptor and BPA sulfate has been demonstrated as one of metabolites of BPA. Since it has been observed to have effects on the central nervous system (CNS), we examined the effects of sulfoconjugation on the toxicity of BPA in a human neuroblastoma cell line, NB-1. NB-1 cells showed limited but significant sulfotransferase activity toward BPA *in vitro* ($K_m = 74 \mu\text{M}$, $V_{\text{max}} = 7.8 \text{ pmol/min/mg}$). Accumulation of BPA sulfate was observed for 12 hr to 4 days after $10 \mu\text{M}$ BPA was added to a culture of NB-1 cells. These results suggest that BPA is partly detoxified by the sulfoconjugation reaction in human neuronal cells.

Key words — brain, bisphenol A, NB-1, neuron, sulfotransferase

INTRODUCTION

Bisphenol A (BPA), one of the most prevalent environmental endocrine disruptors, has been extensively evaluated for toxicity in a variety of rodent models. In particular, the developmental and reproductive toxicity, as well as the carcinogenicity.^{1,2)} Since BPA is released from widely used polycarbonate plastics, we are frequently exposed to the compound in daily life. Due to its lipophilic structure, BPA is absorbed from the gut into the blood and crosses the blood-brain barrier with ease, whereby it can accumulate in the brain. However, little is known about its action on the central nervous system (CNS). Several reports have shown BPA to act on the CNS, especially in the fetus.^{3,4)} As prenatal action of estrogens or aromatizable steroids at the

CNS is responsible for brain sexual differentiation, BPA might alter processes affecting sociosexual behavior through early CNS exposure.

BPA sulfate is a BPA metabolite found in rodent livers and human urine, in addition to BPA glucuronide.^{5,6)} We also have reported that Caco-2 cells, a human colon carcinoma cell line, displayed a significant sulfotransferase (ST) activity toward BPA *in vitro*.⁷⁾ Since it has been established that BPA has effects on the CNS, we investigated whether BPA might be detoxified by sulfoconjugation in a human neuroblastoma cell line, NB-1.

MATERIALS AND METHODS

Materials — 5'-phosphoadenosine 3'-phospho [³⁵S]sulfate ([³⁵S]PAPS) (82.78 Ci/mmol) was purchased from NEN Dupont (Detroit, U.S.A.). Media and supplements for cell culture were purchased from GIBCO BRL (New York, U.S.A.). Snail sulfatase was purchased from Sigma (St. Louis, U.S.A.). Other reagents were obtained from Wako Chemicals (Tokyo, Japan).

Cell Culture — Human neuroblastoma cell line, NB-1, was obtained from Riken Cell Bank, Japan. Cells were grown in 5 ml of the medium consisting of 45% Dulbecco's minimum essential medium (DMEM), 45% RPMI1640, 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin and 10 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of Cytosolic Extracts from NB-1 Cells and Rat Brain — NB-1 cells ($1 - 2 \times 10^7$) were removed from their culture dishes (100 mm²), washed with phosphate buffered saline, and then homogenized in 1 ml buffer A (50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, and 5 μg/ml pepstatin). The debris was removed by centrifugation at $3000 \times g$ for 15 min, after which the supernatant was centrifuged at $105000 \times g$ for 60 min. The clear lysate was used in the following studies. In order to prepare the rat brain and liver extracts, brains and livers were excised from 8-week Sprague-Dawley rats and homogenized in buffer A.

Assay of Phenol Sulfotransferase (P-ST) Activity — Phenol sulfotransferase (P-ST) activity within each sample of lysate was determined using [³⁵S]PAPS as the sulfate donor, and BPA as the sulfate acceptor, according to a slight modification of

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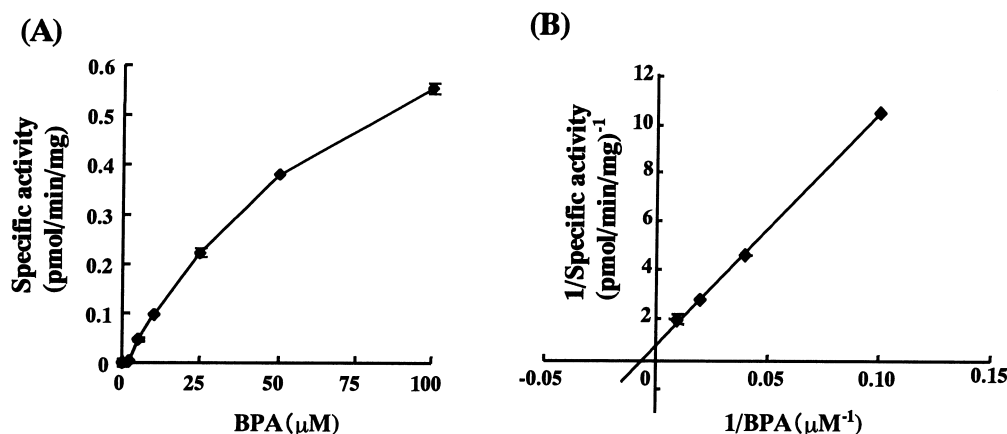


Fig. 1. ST Activity toward BPA in the Extract of NB-1 Cells

(A) ST activities were measured toward BPA ranging 3–100 μM using the NB-1 cell extract (50 μg proteins). Each point represents the mean \pm S.D. ($n = 3$). (B) Lineweaver-Burk plot of the results in (A). From three independent experiments, K_m value and V_{max} value were calculated to be 74 ± 47 mM and 7.8 ± 4.5 pmol/min/mg proteins, respectively.

the procedure of Foldes and Meek.⁸⁾ Briefly, the reaction mixture (250 μl) contained 10 mM phosphate buffer (pH 7.4), 50 μM BPA, 5.0 μM [³⁵S]PAPS (0.4 μCi), and the cytosol extract (50 μg of proteins). The mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 50 μl cold 0.1 M barium acetate. Then, unconverted [³⁵S]PAPS was precipitated by the addition of 50 μl of both 0.1 M Ba(OH)₂ and 0.1 M ZnSO₄. The precipitate was removed by centrifugation at 12000 $\times g$ for 5 min. This precipitation procedure was then repeated. After the second round of precipitation, the remaining supernatant (300 μl) was transferred to a 3 ml liquid scintillator and the amount of radioactivity was counted. Controls were obtained by omitting the acceptor substrate from the reaction mixture.

Identification of BPA Sulfate on HPLC — To identify BPA sulfate on the HPLC, the rat liver cytosolic extract (5 μg proteins) was incubated with 100 μM BPA and 20 μM PAPS for up to 30 min, and then the reaction mixture (5 μl) was subjected to the HPLC analysis as described below. Reaction mixtures lacking either BPA or PAPS were used as controls. The reaction mixture was treated with 8 units of snail sulfatase for 30 min, and then analyzed by HPLC.

Analysis of BPA sulfation in Intact NB-1 Cells — In order to examine the sulfation of BPA in intact NB-1 cells, BPA was added to the medium at a concentration of 1–10 μM , after which the cells were incubated at 37°C. Aliquots (100 μl) were removed at various times, and then 30 μl of the

mixture was filtered and injected into the HPLC. Analysis was performed using an ODS column (Chromolith Performance RP-18e, 100 \times 4.6 mm ϕ , Merck, Darmstadt, Germany). The mobile phase consisted of 10 mM tetrabutylammonium hydrogen sulfate in water and acetonitrile (79.5 : 20.5). A flow rate of 1 ml/min was used with a column temperature of 40°C. Fluorescence intensity was monitored at 313 nm with excitation of 273 nm. The retention times for BPA and BPA sulfate were determined as 6.5 min and 14.5 min, respectively. All signals representative of constituents of the medium were eluted prior to 5 min.

RESULTS AND DISCUSSION

ST Activity toward BPA in the NB-1 Cells

In human tissue, two distinct forms of P-ST activity have been identified, phenol P-ST (P-PST) and monoamine P-ST (M-PST).⁹⁾ These enzymes are functionally distinct, based on differences in substrate and inhibitor specificities, as well as thermal stability. P-PST (a product of the *SULT1A1* gene) catalyzes sulfonation of simple phenols and is thermostable whereas M-PST (a product of *SULT1A3* gene) preferentially catalyzes monoamines and is thermolabile. Previously we have reported NB-1 cells to express both *SULT1* genes.¹⁰⁾ To determine whether NB-1 cells could sulfonate BPA, we measured ST activity toward BPA within the cell extract. As shown in Fig. 1, NB-1 cells showed significant ST activity toward BPA. Kinetic analyses of the ac-

tivity is summarized in Table 1. NB-1 cells displayed a similar K_m value ($74 \mu\text{M}$) to that of the rat brain ($57 \mu\text{M}$). As Suiko *et al.* have demonstrated efficient sulfonation of BPA by recombinant P-PST,⁵⁾ the P-PST enzyme encoded by the *SULT1A1* gene might be responsible for the sulfonation observed in the present experiment.

Sulfoconjugation of BPA in Intact NB-1 Cells

To monitor the sulfoconjugation of BPA in NB-1 cells *in vivo*, we synthesized BPA sulfate from a rat liver extract, followed by analytical HPLC analysis. In addition to the peak presenting BPA (eluted at 6.5 min), a second peak was observed at 14 min, the size of which increased with increasing length of

incubation (Figs. 2A–2D). The appearance of the peak was PAPS-dependent and sensitive to sulfatase treatment, as shown in Figs. 2G and 2E, respectively. Based on these results, we identified the peak eluted at 14 min as BPA sulfate.

After adding 1 to $10 \mu\text{M}$ BPA to the culture medium, the level of BPA sulfate within the culture medium was monitored. As shown in Fig. 3A, BPA sulfate first appeared at 12 hr, and increased up until 4 days in the case of $10 \mu\text{M}$ BPA. Sulfonation of BPA within the cells occurred in a dose-dependent manner (Fig. 3B).

Here we described the ability of a neuroblastoma cell line, NB-1, to sulfonate BPA. Several reports have indicated the neurotoxicity of BPA. Orally administered BPA crosses the placental barrier with relative ease to reach the fetus.¹¹⁾ Exposure to BPA at a point in time when sexual differentiation of the brain normally occurs can influence adult behavior. The sulfoconjugative activity of neuronal cells as described in this report might ameliorate BPA toxicity within the CNS. If the P-PST activity is lower in fetal neuronal cells during the critical period of

Table 1. Kinetic Parameters of ST Activities toward BPA of NB-1 Cells and Rat Brains

cytosol	K_m (μM)	V_{max} (pmon/min/mg)
NB-1	74 ± 47	7.8 ± 4.5
rat brain	57 ± 14	27 ± 12

All values are the mean of three experiments with S.D.

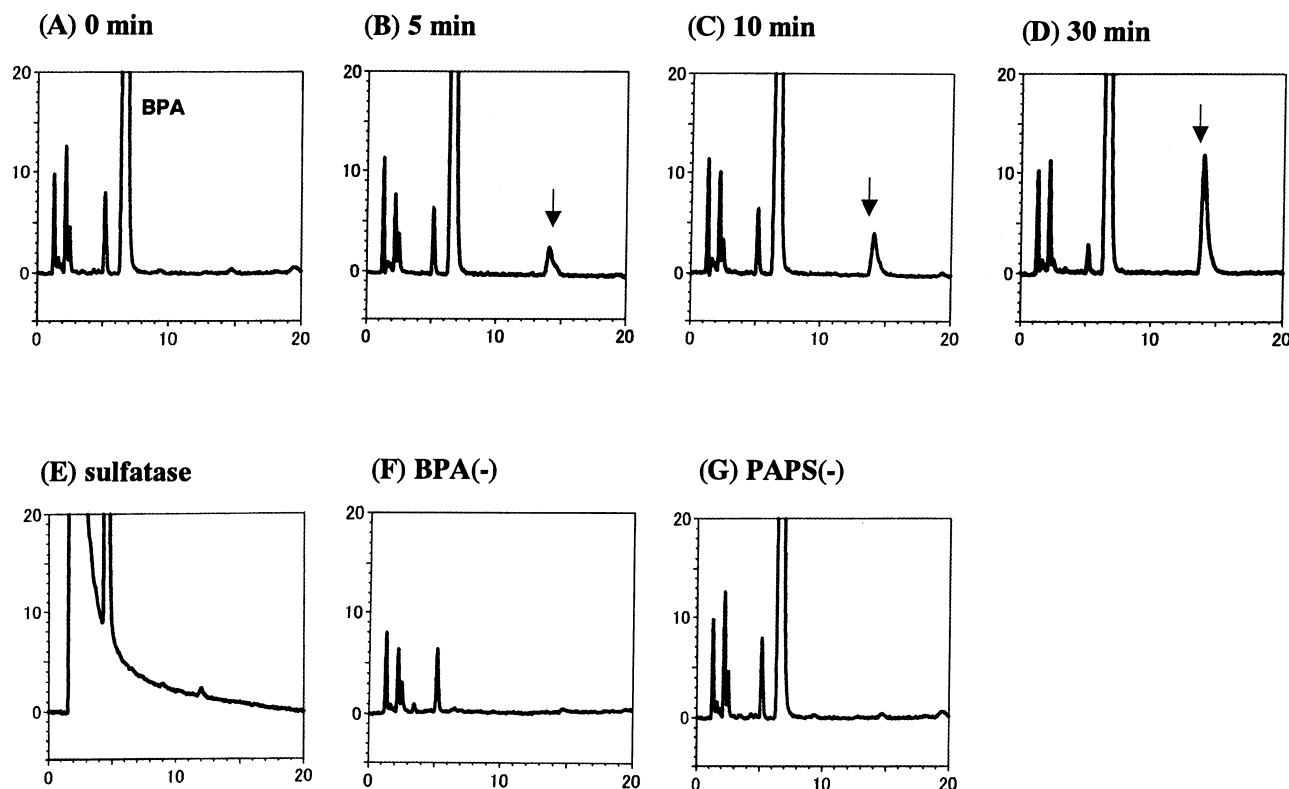


Fig. 2. HPLC Analysis of BPA Sulfate in the Rat Liver Cytosols

HPLC of BPA ($100 \mu\text{M}$) incubated with the rat liver extract ($5 \mu\text{g}$) for 0 min (A), 5 min (B), 10 min (C) and 30 min (D). (E) HPLC of the reaction mixture D treated with 8 units of snail sulfatase for 30 min. (F, G) HPLC of the reaction mixture without either PAPS (F) or BPA (G), respectively. Arrows indicate the peak of BPA sulfate.

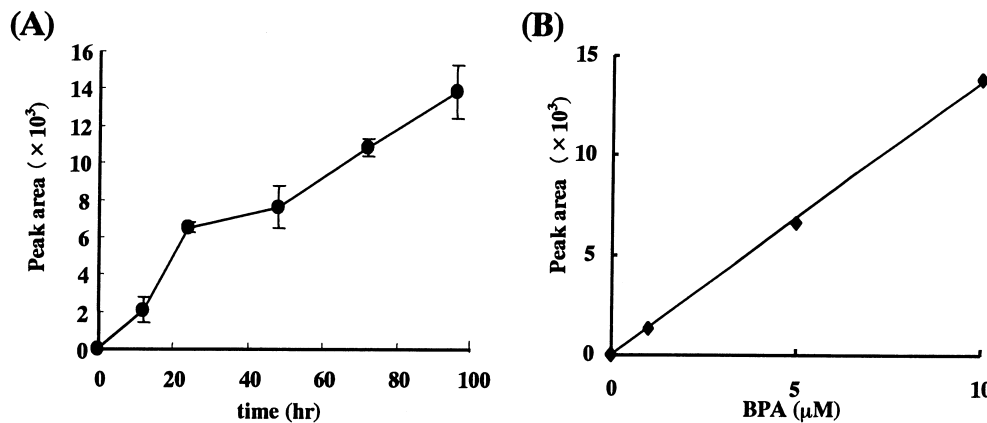


Fig. 3. Production and Secretion of BPA Sulfate in the NB-1 Cells

(A) Time course of BPA sulfation in the NB-1 cells. 10 μM BPA was added to the culture medium and aliquots (100 μl) were removed at the indicated times, and analyzed by HPLC. Each point represents the mean \pm S.D. of the peak area ($n = 3$). (B) Dose response of BPA for the sulfoconjugation reaction. BPA (1–10 μM) was added to the medium and incubated for 5 days. Levels of BPA sulfates in the medium were measured by HPLC.

development, then BPA might affect the normal development of the brain of the fetus. Information regarding the level of P-PST activity within the fetal brain during development might contribute to this theory.

Only a 4% reduction in BPA (unconjugated) was observed in the culture medium containing 10 μM BPA after 5 days incubation (data not shown). This result differs from that of a previous report by Pritchett *et al.*,¹²⁾ in which a rapid loss of BPA from the culture medium of isolated rat hepatocytes was observed. Thus, we theorize that NB-1 cells may have either an active efflux transporter or a weak influx transporter for BPA. If so, this feature of the cells may explain why the NB-1 cells remained largely intact even in the presence of 50 μM BPA for 5 days (data not shown). Since BPA sulfoconjugates account for 1–2% of the initial BPA after 5 days culture (data not shown), which comprises 25–50% of the BPA taken up by the cells, it appears that BPA sulfoconjugation is a major metabolic pathway in neuronal cells. Since glucuronidation has been reported to be a major mean of metabolism of BPA in the rodent liver,¹³⁾ further investigation regarding the extent of glucuronidation of BPA in NB-1 cells is needed.

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