

Estrogenic/Antiestrogenic Activities of Benzo[*a*]pyrene Monohydroxy Derivatives

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Benzo[*a*]pyrene (BaP), a major environmental pollutant, is metabolized *in vivo* and produces many hydroxy derivatives. The estrogenic/antiestrogenic activities of twelve monohydroxy derivatives of BaP (1- through 12-OH species) were investigated using competition binding to human estrogen receptor (hER) α and hER β , and the gene expression assay of the yeast two-hybrid system. BaP and 5-OH BaP did not bind to either hER. The other monohydroxy derivatives bound to both hERs. These compounds bound more strongly to hER β than to hER α . Using the yeast two-hybrid assay system, 1-, 2-, 3-, and 9-OH BaP induced β -galactosidase with hER β but not with hER α . This suggested that these compounds were estrogenic. In the presence of 10^{-9} M 17β -estradiol, 8-OH BaP inhibited the induction of β -galactosidase. Because 8-OH BaP did not affect cell growth, it appeared to be an antiestrogen. The present study shows that most of the monohydroxy derivatives of BaP bind to estrogen receptors (ERs), and several of them have estrogenic or antiestrogenic activity.

Key words — benzo[*a*]pyrene, benzo[*a*]pyrene monohydroxy derivative, estrogen receptor α , β , estrogenic activity

INTRODUCTION

Estrogens are critical to the functioning and maintenance of a diverse array of tissues and physiological systems in mammals. The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors (ERs), ER α and ER β .^{1,2} Studies of the distribution of ERs and their expression patterns in the tissues indicate that ER α has a broad expression pattern, whereas ER β has a more focused pattern, with high levels in the ovary, testis, and thymus.² The homology between the ER α ligand binding domain (LBD) and the ER β LBD is only 53%. Recognition of the structure of the ligand might be different between ER α and ER β . In fact, ER α and ER β have almost the same affinity for 17β -estradiol but ER β has a higher affinity for some phytoestrogens than does ER α .³

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic organic chemicals numbering in the hundreds that are ubiquitous in the environment and in foodstuffs. They are produced and released into the environment by incomplete combustion of fossil fuel, oil spills, and industrial processes. Benzo[*a*]pyrene (BaP) is one of the main components of PAHs and has served as a prototypical chemical carcinogen. BaP is abundantly distributed in the environment.

Tran *et al.*⁴ reported PAHs acted as antiestrogens in a yeast assay system. Clemons *et al.*⁵ found, on the contrary, that these compounds acted as estrogens in MCF-7 cells.

BaP is metabolized by cytochrome P450 enzymes (CYPs) to dihydrodiols, phenols, and quinone derivatives.⁶ Among these metabolites, some hydroxylated species seem to have a structural similarity to 17β -estradiol,^{7,8} and some of the hydroxylated metabolites will interact with ERs. Several studies have been conducted to evaluate the estrogenic and antiestrogenic activities of BaP metabolites. Ebright *et al.*⁹ found that 1- and 2-OH BaP bound strongly to ER in rat cytosol and the other

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derivatives (4-, 5-, 6- and 12-OH BaP) bound poorly. Charles *et al.*¹⁰ found that *trans* 9,10-diOH BaP, 7,8-diOH BaP, 3-OH BaP and 9-OH BaP were produced in MCF-7 cells and that 3- and 9-OH BaP induced transcription of luciferase. Their experiments used mixtures of ER α and ER β , and examined only agonistic activity. Thus, further studies are needed to examine the binding and estrogenic/antiestrogenic activities of BaP and its derivatives to ER α and ER β separately.

In this study, we examined the estrogenic/antiestrogenic activities of BaP and its monohydroxy derivatives by (1) their binding to full-length hER α and hER β , and (2) their effect on estrogen receptor-dependent transcription of β -galactosidase.¹¹

MATERIALS AND METHODS

Chemicals — 17 β -Estradiol and BaP were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [2,4,6,7-³H(N)]-17 β -Estradiol (72 Ci/mmol) was purchased from Dai-Ichi Pure Chemicals Co., Ltd. (Tokyo, Japan). 4-Hydroxytamoxifen (OHT) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BaP monohydroxy derivatives were purchased from NCI Chemical Carcinogen Repositories (Kansas City, MO, U.S.A.). BaP and its monohydroxy derivatives were dissolved in ethanol and stored at -20°C. All other chemicals were of reagent grade. Figure 1 shows the structures and abbreviations of BaP and its monohydroxy derivatives used in this experiment.

Preparation of the Extract of hER α and hER β — hER α and hER β were prepared as described previously.¹¹ Sf21 insect cells carrying the gene for hER α or hER β were grown and lysed. The lysate was diluted with TKEG buffer (40 mM Tris-HCl, pH 7.4, 200 mM KCl, 0.5 mM EDTA, 10% glycerol). The concentrations of hER α and hER β were 0.6% and 0.3% of the total protein, respectively. These receptors were stable at -80°C for several months.

Competition Binding Assay of BaP and its Monohydroxy Derivatives — The competition binding assay was carried out as described previously.¹¹ Two hundred and fifty μ l of reaction mixture of TKE (20 mM Tris-HCl, pH 7.4, 20 mM KCl, 1 mM EDTA) containing 5 μ l hER α or β with 1.25 pmol of [³H]-17 β -estradiol were incubated at 0°C (for 16 hr) in the presence of various concentrations of test compound. Free and bound ligands were separated by

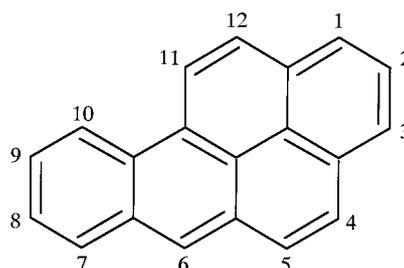


Fig. 1. Structure of BaP
The carbon atoms are numbered.

addition of an equal volume of dextran-coated charcoal in TKE. Samples were treated for 5 min on ice with periodic mixing and centrifuged at 15000 rpm for 1 min. Aliquots of the supernatant (300 μ l) were used for scintillation counting.

ER-Dependent Transcriptional Expression Induced by BaP and its Monohydroxy Derivatives

— The yeast two-hybrid assay was carried out as described previously.¹¹ Briefly, yeast cells expressing hER α or hER β were grown overnight at 30°C with shaking in S.D. medium lacking tryptophan and leucine. Yeast cells were treated with a test compound for 4 hr at 30°C. After the incubation, β -galactosidase activities were determined as follows. The treated cells were collected and lysed by incubation with Z-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO₄) containing 1 mg/ml Zymolyase at 37°C for 30 min. 2-Nitrophenyl- β -D-galactoside (ONPG) was added to the lysate to a final concentration of 4 mg/ml. After incubation at 30°C for 45 min, the reaction was stopped by the addition of 1 M Na₂CO₃. The yeast debris was removed by centrifugation and the absorbance of supernatant was measured at 415 nm.

Anti-Estrogen Assay — To examine the antagonistic activity of BaP and its monohydroxy derivatives, the inhibition of β -galactosidase activity induced by 1 nM 17 β -estradiol was measured by adding various concentrations of test compound using the yeast system.

RESULTS

Binding of BaP and its Monohydroxy Derivatives to hER α and hER β

In this study, a crude extract prepared from Sf21 insect cells infected with hER α or hER β recombinant virus was used for an estrogen binding assay as reported previously.¹¹ Since there was no non-spe-

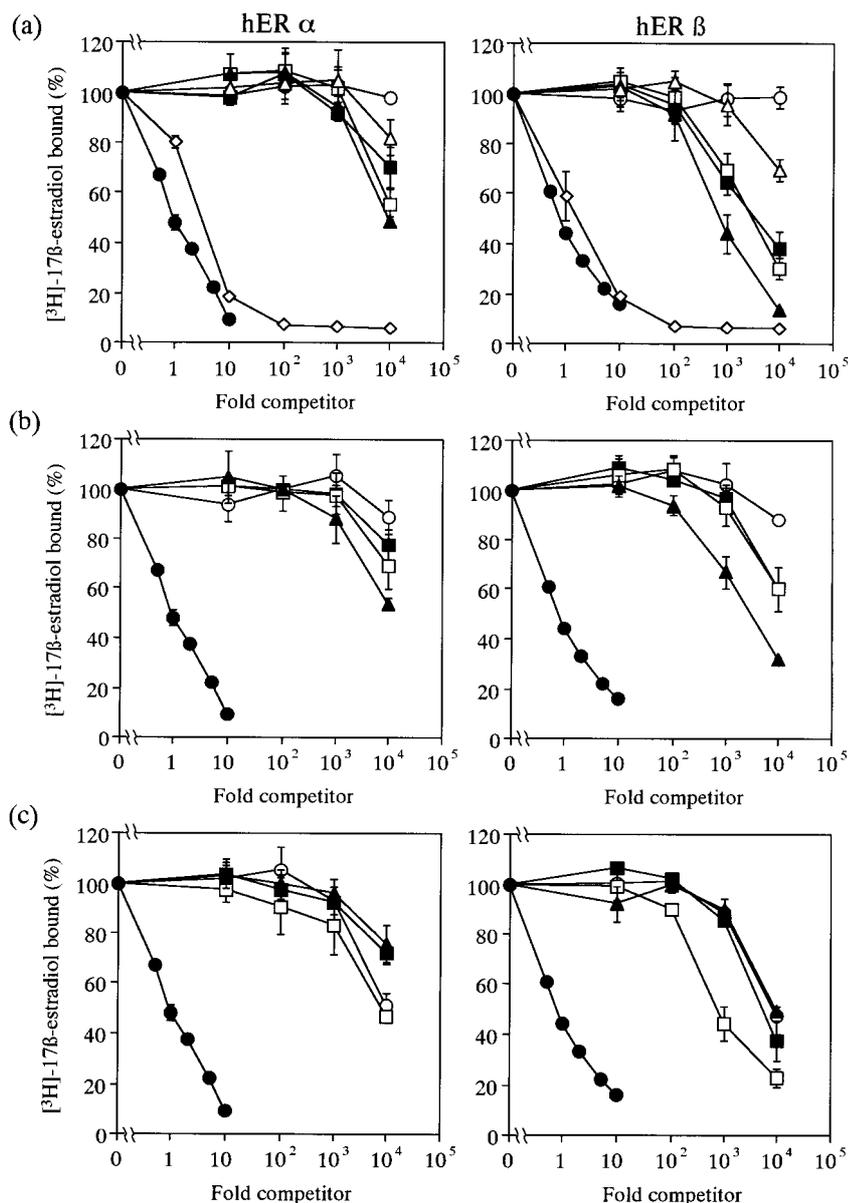


Fig. 2. Assays of Binding of Several Monohydroxy Derivatives of BaP to hERs

The binding to hERs was examined by competition. The concentration of one fold competitor is 1.25 pmol. (a) 17 β -estradiol (●), 4-hydroxytamoxifen (◇), BaP (○), 1-OH BaP (■), 2-OH BaP (□), 3-OH BaP (▲) and 4-OH BaP (△). (b) 17 β -estradiol (●), 5-OH BaP (○), 6-OH BaP (■), 7-OH BaP (□) and 8-OH BaP (▲). (c) 17 β -estradiol (●), 9-OH BaP (○), 10-OH BaP (■), 11-OH BaP (□) and 12-OH BaP (▲). The experiments shown in Figs. 2–4 were carried out more than twice and the bar in each point represents the standard deviation. When the two experimental results were almost the same, we did not try further.

cific estrogen binding to an extract of uninfected cells, any decrease of bound [3 H]-17 β -estradiol that was observed would have been due to competitive binding of the test compound to hER. Figure 2 shows the competition of BaP and its monohydroxy derivatives with [3 H]-17 β -estradiol for hER α and hER β . While BaP and 5-OH BaP did not bind to either hER, other monohydroxy derivatives bound to both hERs. hER β had high affinity for 3- and 11-OH BaP, moderate affinity for 1-, 2-, 8-, 9-, 10-, and 12-OH BaP,

and weak affinity for 4-, 6- and 7-OH BaP. hER α had moderate affinity for 2-, 3-, 8-, 9- and 11-OH and weak affinity for the remaining derivatives. Monohydroxy derivatives of BaP generally bound more strongly to hER β than to hER α .

Transcriptional Activation by BaP and its Monohydroxy Derivatives

The yeast two-hybrid assay system¹²⁾ was used to examine the estrogenic activities of BaP and its

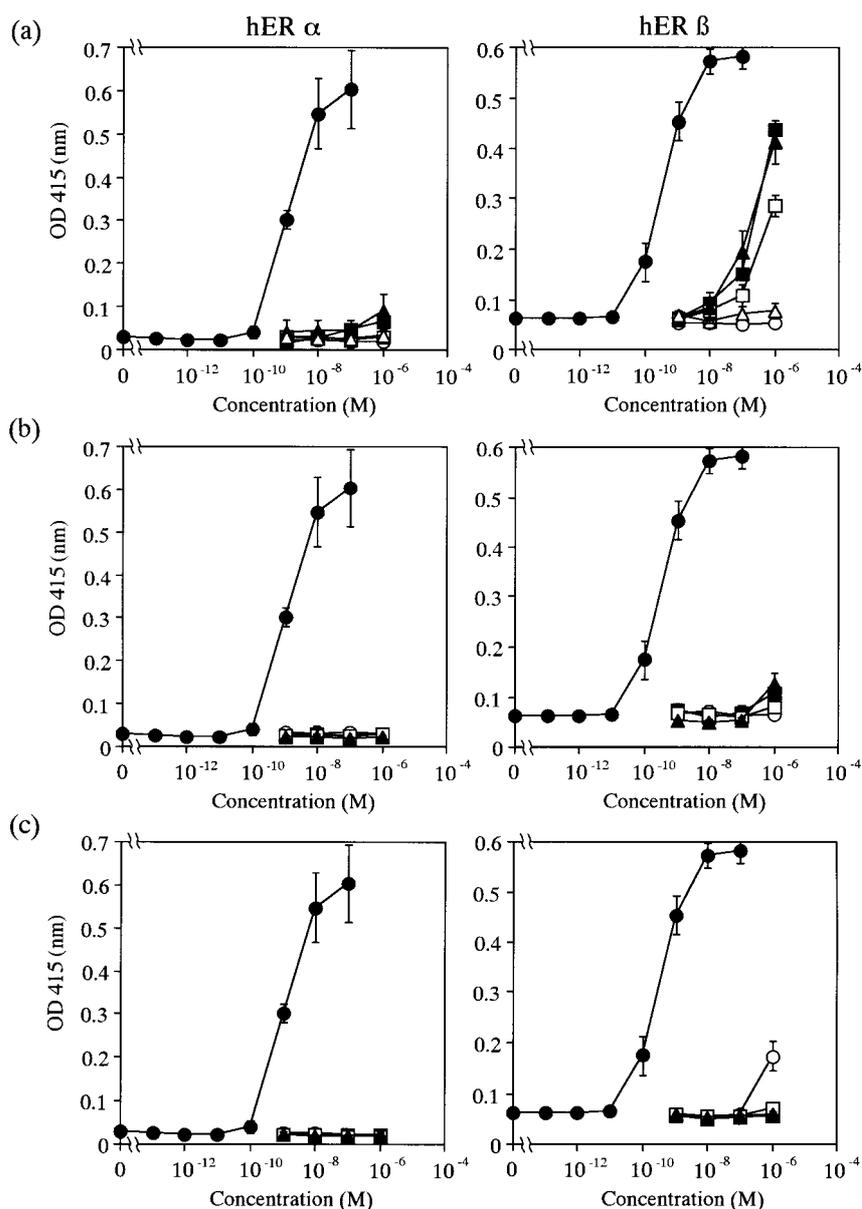


Fig. 3. Assays of ER-Dependent β -Galactosidase Induction

(a) 17β -estradiol (●), BaP (○), 1-OH BaP (■), 2-OH BaP (□), 3-OH BaP (▲) and 4-OH BaP (△). (b) 17β -estradiol (●), 5-OH BaP (○), 6-OH BaP (■), 7-OH BaP (□) and 8-OH BaP (▲). (c) 17β -estradiol (●), 9-OH BaP (○), 10-OH BaP (■), 11-OH BaP (□) and 12-OH BaP (▲).

monohydroxy derivatives. Estrogenic activity was measured as β -galactosidase activity. Since the toxic effect of these compounds on the cells will interfere with the assay of these effects, it was examined by the inhibition of cell growth. No toxic effect was detected at concentrations less than 10^{-6} M. Therefore, the assay was performed at concentrations up to 10^{-6} M. Figure 3 shows the β -galactosidase activities of the cells treated with BaP and its monohydroxy derivatives. A significant induction of β -galactosidase was observed with 1-, 2- and 3-OH BaP in cells expressing hER β . Weak induction was

observed with 9-OH BaP. However, none of the compounds examined induced any significant β -galactosidase activity in the cells expressing hER α .

Antiestrogenic Activities of BaP and its Monohydroxy Derivatives

Yeast cells for the two-hybrid assay system were treated with each monohydroxy derivative in the presence of 10^{-9} M of 17β -estradiol to examine the antiestrogenic activity. As shown in Fig. 3, β -galactosidase activity was increased with an increase of 17β -estradiol concentration and reached a plateau

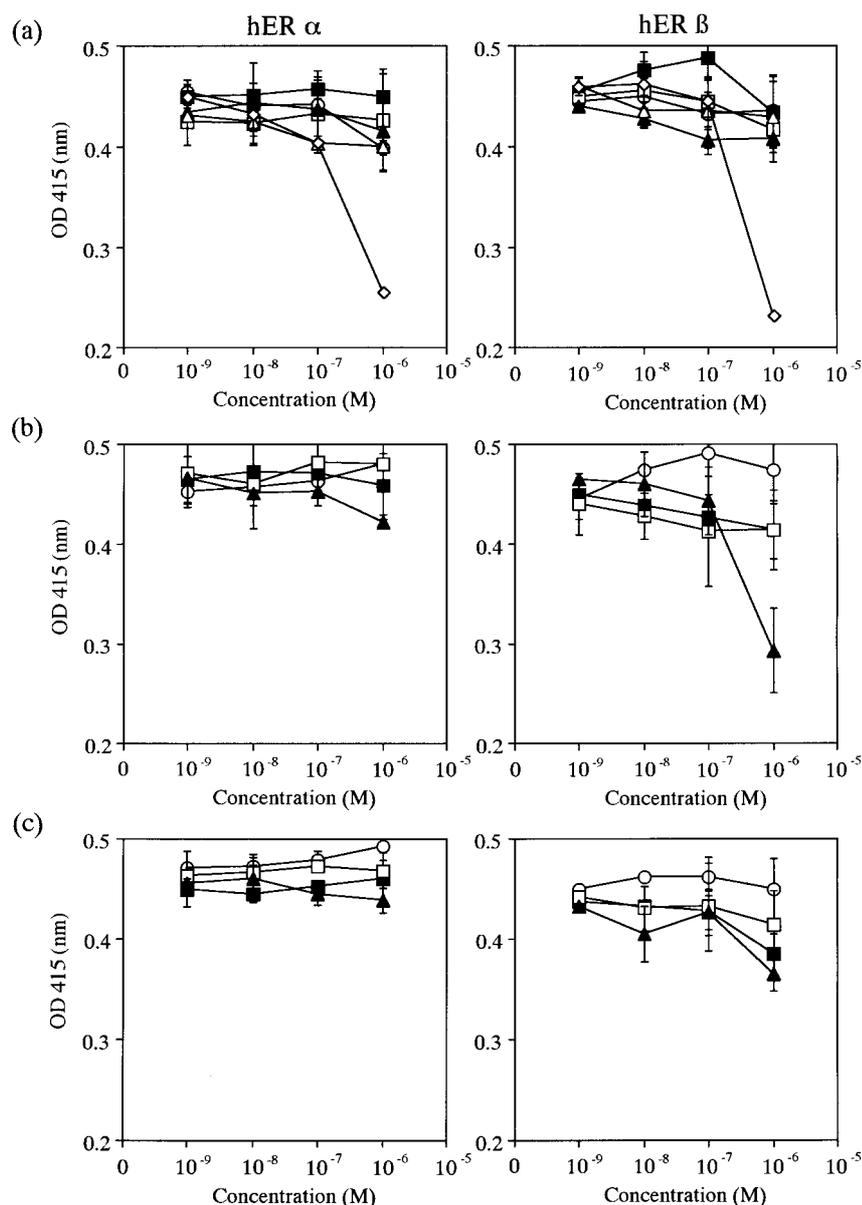


Fig. 4. Assays of Antiestrogenic Activity

(a) OHT (\diamond), BaP (\circ), 1-OH BaP (\blacksquare), 2-OH BaP (\square), 3-OH BaP (\blacktriangle) and 4-OH BaP (\triangle). For assay of antiestrogenic activity, 10^{-9} M of 17β -estradiol was incubated with various concentrations of test chemicals. (b) OHT (\diamond), 5-OH BaP (\circ), 6-OH BaP (\blacksquare), 7-OH BaP (\square) and 8-OH BaP (\blacktriangle). (c) OHT (\diamond), 9-OH BaP (\circ), 10-OH BaP (\blacksquare), 11-OH BaP (\square) and 12-OH BaP (\blacktriangle).

at 10^{-8} M. We used 10^{-9} M of 17β -estradiol to assess the antiestrogenic activity. As shown in Fig. 4, OHT strongly inhibited the induction of β -galactosidase by 17β -estradiol. OHT is a well-known antagonist of 17β -estradiol¹³) and this was confirmed by our yeast two-hybrid assay system. β -Galactosidase expression in the yeast cells expressing hER β was strongly inhibited by 8-OH BaP, but only weakly inhibited by the other compounds. 8-OH BaP is an antagonist of 17β -estradiol with hER β , but other compounds are not antagonists of either receptor.

DISCUSSION

The aim of this study was to clarify the actions of BaP and its monohydroxy derivatives on hER α or hER β . In this study, we examined the estrogenic/antiestrogenic activities of these compounds. The results showed that BaP did not bind to either hER or induce β -galactosidase activity by interacting with either hER. Several monohydroxy derivatives bound to both hERs. In the yeast two-hybrid assay system, some monohydroxy derivatives induced transcrip-

tions and did not affect the transcription of β -galactosidase induced by 17 β -estradiol. Both the induction of transcription by some derivatives and the inhibition of transcription by 8-OH BaP were stronger with hER β than with hER α .

The phytoestrogens that we examined also bound more strongly to hER β than to hER α and induced β -galactosidase more strongly with hER β than with hER α .¹¹⁾ Thus, the structural requirements for a compound to bind to hER β seems to be less strict than those for binding to hER α .

Recently, Brzozowski *et al.*¹⁴⁾ reported the crystal structures of the hER complexed with 17 β -estradiol and hER complexed with the antiestrogen raloxifene. Their result suggests that the position of LBD helix 12 with raloxifene interfered with the binding co-activator. However, the position of the helix 12 with 17 β -estradiol or genistein (one of the strongest phytoestrogens) opened the co-activator binding site on the receptor. The positioning of LBD helix 12 will change significantly when an agonist or antagonist binds to the receptor.

Our result shows that BaP and 5-OH BaP did not bind and they were neither agonists nor antagonists. 1-, 2-, 3- and 9-OH BaP bind and act as agonists. 8-OH BaP binds and acts as an antagonist. 4-, 6-, 7-, 10-, 11- and 12-OH BaP bind but they are neither agonists nor antagonists. Yeast cells may be impermeable to these five compounds. Monohydroxy derivatives of BaP have structures that are similar to the structure of 17 β -estradiol. The slight difference of OH positions may affect the interaction with LBD significantly and make some compounds agonists and others antagonists.

In *in vitro* experiments, CYP1A1, the most abundant CYP in lung,¹⁵⁾ produced 3-, 7- and 9-OH BaP from BaP. MCF-7 cells produced 3- and 9-OH BaP by metabolizing BaP.¹⁰⁾ Since these compounds were produced by treatment with CYP1A1 or by incubation with MCF-7 cells and because they acted as agonists as confirmed in this study, it seems that they would be endocrine disrupters in mammals.

Diesel exhaust is a complex mixture of particulate and vapor phase components. The fraction of particulate matter in diesel exhaust that is soluble in organic solvents contains hundreds of organic compounds. These compounds include a variety of PAHs and dioxins.^{16,17)} and have been recognized as a potential public health hazard. Although most studies have focused on the mutagenic and carcinogenic properties of PAHs and their derivatives, recently, there has been a concern that the PAHs and their

derivatives that are found in motor vehicle exhaust may contain endocrine-disrupting chemicals.^{18,19)}

Our results suggest that BaP has no estrogenic activity, but that 1-, 2-, 3- and 9-OH BaP have estrogenic activity through their binding to hER β . 8-OH BaP has antiestrogenic activity through its binding to hER β . Since the estrogenic activities of these compounds are comparable to or stronger than those of bisphenol A and nonylphenol,^{11,20-22)} these monohydroxy derivatives are expected to act as endocrine disruptors.

In conclusion, several monohydroxy derivatives of BaP bound to both hERs, and they bound much more strongly to hER β than to hER α . In the yeast two-hybrid assay system, the transcriptional responses of monohydroxy derivatives were also higher with hER β than with hER α . Some monohydroxy derivatives were agonists but only the 8-OH derivative was an antagonist.

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