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

Toshiharu Hirose,[a] Keiko Morito,[a] Ryoichi Kizu,[b] Akira Toriba,[b] Kazuichi Hayakawa,[b] Sumito Ogawa,[c] Satoshi Inoue,[c] Masami Muramatsu,[d] and Yukito Masamune*,[a]

[a]Department of Molecular and Cellular Biology and [b]Department of Hygienic and Analytical Chemistry, Faculty of Pharmaceutical Sciences, Kanazawa University, 13–1 Takara-machi, Kanazawa, Ishikawa 920–0934, Japan, [c]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan, and [d]Department of Biochemistry, Saitama Medical School, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350–0451, Japan

(Received August 6; Accepted August 31)

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 hERβ, 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 hERβ than to hERα. Using the yeast two-hybrid assay system, 1-, 2-, 3-, and 9-OH BaP induced β-galactosidase with hERβ but not with hERα. 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), ERα and ERβ.[1,2] Studies of the distribution of ERs and their expression patterns in the tissues indicate that ERα has a broad expression pattern, whereas ERβ has a more focused pattern, with high levels in the ovary, testis, and thymus.[2] The homology between the ERα ligand binding domain (LBD) and the ERβ LBD is only 53%. Recognition of the structure of the ligand might be different between ERα and ERβ. In fact, ERα and ERβ have almost the same affinity for 17β-estradiol but ERβ has a higher affinity for some phytoestrogens than does ERα.[3]

*To whom correspondence should be addressed: Department of Molecular and Cellular Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, 13–1 Takara-machi, Kanazawa, Ishikawa 920–0934, Japan. Tel. & Fax: +81-76-234-4465; E-mail: masamune@dbs.p.kanazawa-u.ac.jp

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

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 ERα and ERβ, and examined only agonistic activity. Thus, further studies are needed to examine the binding and estrogenic/antiestrogenic activities of BaP and its derivatives to ERα and ERβ separately.

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

MATERIALS AND METHODS

Chemicals —— 17β-Estradiol and BaP were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [2,4,6,7-^{3}H(N)]-17β-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 hERα and hERβ —— hERα and hERβ were prepared as described previously.[11] Sf21 insect cells carrying the gene for hERα or hERβ 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 hERα and hERβ 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 μl of reaction mixture of TKE (20 mM Tris–HCl, pH 7.4, 20 mM KCl, 1 mM EDTA) containing 5 μl hERα or β with 1.25 pmol of [^{3}H]–17β-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 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 μl) were used for scintillation counting.

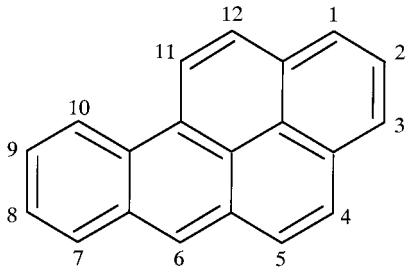

Fig. 1. Structure of BaP
The carbon atoms are numbered.

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 hERα or hERβ 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 $MgSO_4$) 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 Na_2CO_3. 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 17β-estradiol was measured by adding various concentrations of test compound using the yeast system.

RESULTS

Binding of BaP and its Monohydroxy Derivatives to hERα and hERβ

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

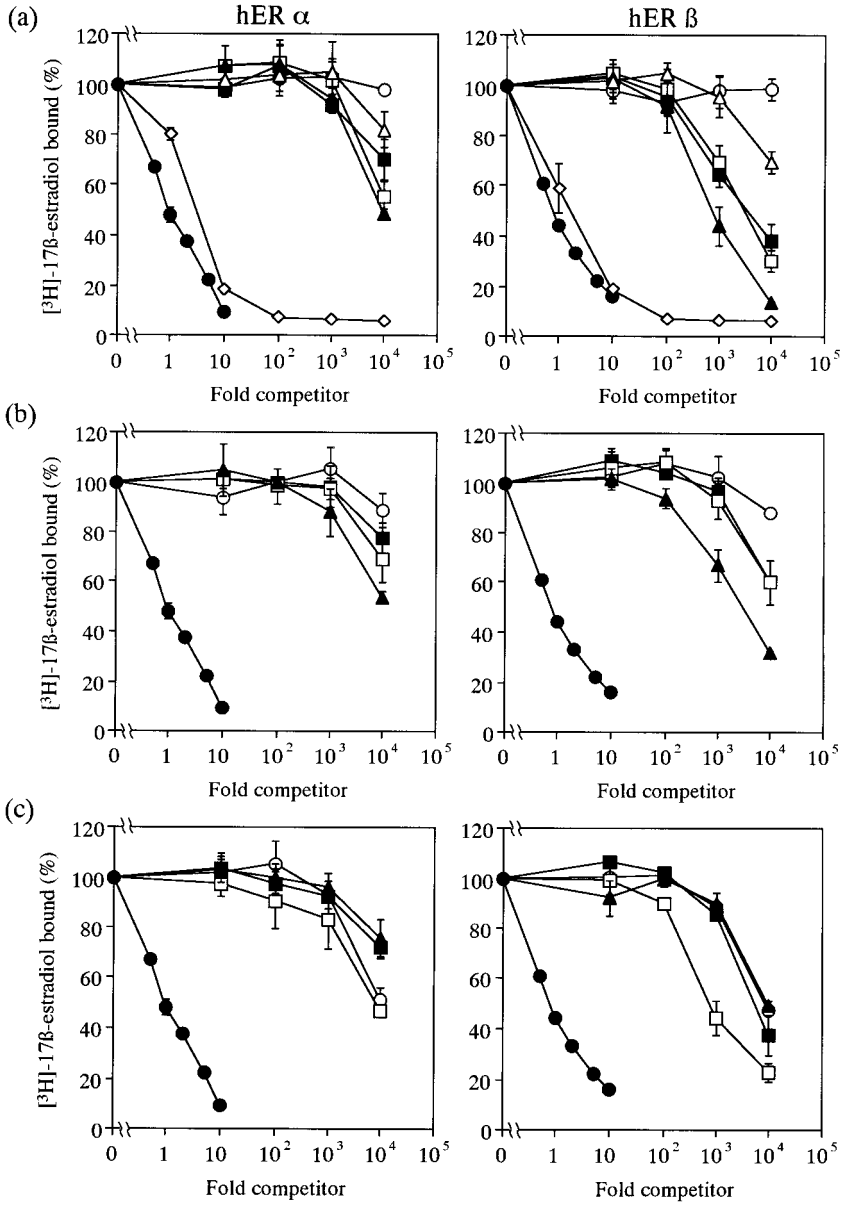

Fig. 2. Assays of Binding of Several Monohydroxy Derivatives of BaP to hERs

The binding to hERs was examined by competition. The concentration of one fold competitor is 1.25 pmol. (a) 17β-estradiol (●), 4-hydroxytamoxifen (◇), 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 (▲). 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]–17β-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]–17β-estradiol for hERα and hERβ. While BaP and 5-OH BaP did not bind to either hER, other monohydroxy derivatives bound to both hERs. hERβ 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. hERα 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 hERβ than to hERα.

Transcriptional Activation by BaP and its Monohydroxy Derivatives

The yeast two-hybrid assay system[12] was used to examine the estrogenic activities of BaP and its

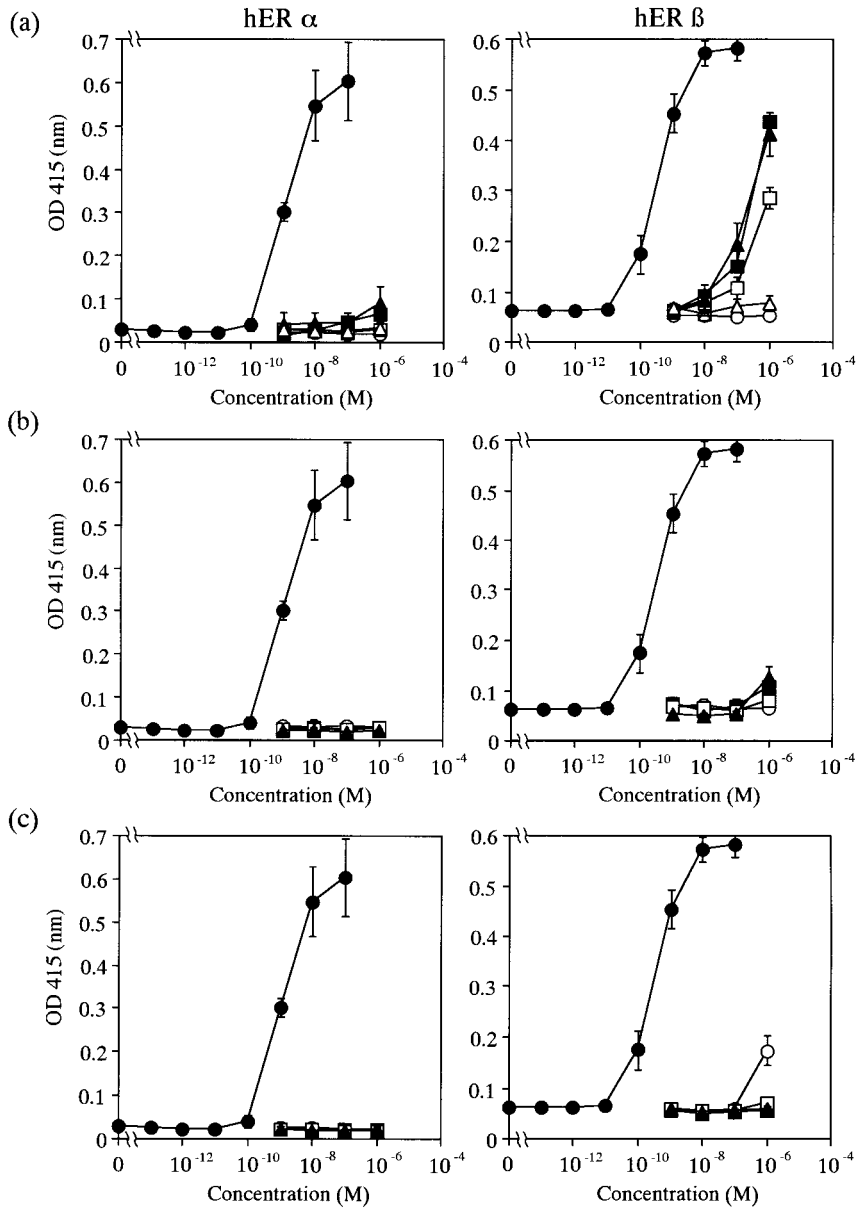

Fig. 3. Assays of ER-Dependent β-Galactosidase Induction

(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 hERβ. Weak induction was observed with 9-OH BaP. However, none of the compounds examined induced any significant β-galactosidase activity in the cells expressing hERα.

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

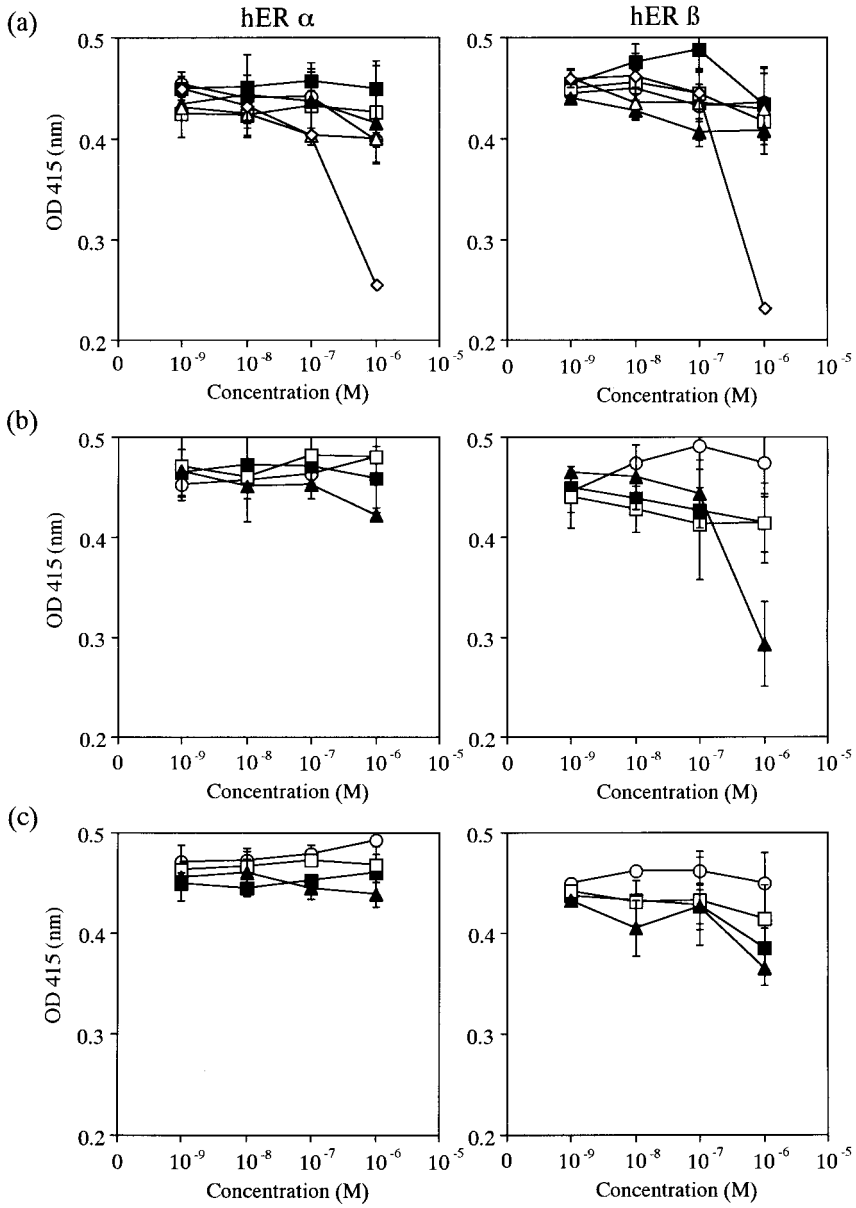

Fig. 4. Assays of Antiestrogenic Activity

(a) OHT (◇), BaP (○), 1-OH BaP (■), 2-OH BaP (□), 3-OH BaP (▲) and 4-OH BaP (△). For assay of antiestrogenic activity, 10^{-9} M of 17β-estradiol was incubated with various concentrations of test chemicals. (b) OHT (◇), 5-OH BaP (○), 6-OH BaP (■), 7-OH BaP (□) and 8-OH BaP (▲). (c) OHT (◇), 9-OH BaP (○), 10-OH BaP (■), 11-OH BaP (□) and 12-OH BaP (▲).

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-estradiol[13] and this was confirmed by our yeast two-hybrid assay system. β-Galactosidase expression in the yeast cells expressing hERβ 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 hERβ, 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 hERα or hERβ. 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 17β-estradiol. Both the induction of transcription by some derivatives and the inhibition of transcription by 8-OH BaP were stronger with hERβ than with hERα.

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

Recently, Brzozowski *et al.*[14] reported the crystal structures of the hER complexed with 17β-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 17β-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 antogonist. 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 17β-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 hERβ. 8-OH BaP has antiestrogenic activity through its binding to hERβ. 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 hERβ than to hERα. In the yeast two-hybrid assay system, the transcriptional responses of monohydroxy derivatives were also higher with hERβ than with hERα. Some monohydroxy derivatives were agonists but only the 8-OH derivative was an antagonist.

Acknowledgements We are grateful to Dr. Shigeaki Kato, The University of Tokyo, for kindly giving us plasmid pBacPAK9/HEG0 which carries hERα and Dr. Tsutomu Nishihara, Osaka University, for kindly giving us *Saccharomyces cerevisiae* Y190 which carries pGBT9-ratER and pGAD424-*hTIF2*. This experiment was partially supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1) Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M. and Chambon, P. (1986) Cloning of the human oestrogen receptor cDNA. *J. Steroid Biochem.*, **24**, 77–83.
2) Mosselman, S., Polman, J. and Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.*, **392**, 49–53.
3) Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B. and Gustafsson, J. Å. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology*, **139**, 4252–4263.
4) Tran, D. Q., Ide, C. F., McLachlan, J. A. and Arnold, S. F. (1996) The anti-estrogenic activity of selected polynuclear aromatic hydrocarbons in yeast expressing human estrogen receptor. *Biochem. Biophys. Res. Commun.*, **229**, 102–108.
5) Clemons, J. H., Allen, L. M., Marvin, C. H., Wu, Z.,

McCarry, B. E., Bryant, D. W. and Zacharewski, T. R. (1998) Evidence of estrogen- and TCDD-like activities in crude and fractionated extracts of PM10 air particulate material using in vitro gene expression assay. *Environ. Sci. Technol.*, **32**, 1853–1860.
6) Sticha, K. R., Staretz, M. E., Wang, M., Liang, H., Kenney, P. M. and Hecht, S. S. (2000) Effects of benzyl isothiocyanate and phenethyl isothiocyanate on benzo[*a*]pyrene metabolism and DNA adduct formation in the A/J mouse. *Carcinogenesis*, **21**, 1711–1719.
7) Yang, N., Castro, A., Lewis, M. and Wong, T. (1961) Polynuclear aromatic hydrocarbons, steroids and carcinogenesis. *Science*, **134**, 386–387.
8) Glusker, J. (1979) Structural aspects of steroid hormones and carcinogenic polycyclic aromatic hydrocarbons. In *Biochemical Actions of Hormones* (Litwack, G., Eds.), New York Academic Press, U.S.A., vol. 6, pp. 122–197.
9) Ebright, R. H., Wong, J. R. and Chen, L. B. (1986) Binding of 2-hydroxybenzo(*a*)pyrene to estrogen receptors in rat cytosol. *Cancer Res.*, **46**, 2349–2351.
10) Charles, G. D., Bartels, M. J., Zacharewski, T. R., Gollapudi, B. B., Freshour, N. L. and Carney, E. W. (2000) Activity of Benzo[*a*]pyrene and its hydroxylated metabolites in an estrogen receptor-α. reporter gene assay. *Toxicol. Sci.*, **55**, 320–326.
11) Morito, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Nohara, T., Ogawa, S., Muramatsu, M. and Masamune, Y. (2001) Interaction of phytoestrogen with estrogen receptor α and β. *Biol. Pharm. Bull.*, **24**, 351–356.
12) Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M. and Nishihara, T. (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.*, **154**, 76–83.
13) Murphy, C. S., Parker, C. J., McCaugue, R. and Jordan, V. C. (1991) Structure-activity relationships of nonisomerizable derivatives of tamoxifen: importance of hydroxyl group and side chain positioning for biological activity. *Mol. Pharmacol.*, **39**, 421–428.
14) Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Öhman, L., Greene, G. L., Gustafsson, J. Å. and Carlquist, M. (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (London), **389**, 753–758.
15) Shimada, T., Yun, C. H., Yamazaki, H., Gautier, J. C., Beaune, P. H. and Guengerich, F. P. (1992) Characterization of human lung microsomal cytochrome P-450 1A1 and its role in the oxidation of chemical carcinogens. *Mol. Pharmacol.*, **41**, 856–864.
16) Bidelman, T. F. (1988) Atmospheric processes. *Environ. Sci. Technol.*, **22**, 361–387.
17) Menzie, C. A., Potocki, B. B. and Santodonato, J. (1992) Ambient concentrations and exposure to carcinogenic PAHs in the environment. *Environ. Sci. Technol.*, **26**, 1278–1283.
18) Santodonato, J. (1997) Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: Relationship to carcinogenicity. *Chemosphere*, **34**, 835–848.
19) Arcaro, K. F., O'Keefe, P. W., Yang, Y., Clayton, W. and Gierthy, J. F. (1999) Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. *Toxicology*, **133**, 115–127.
20) Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*, **132**, 2279–2286.
21) Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G. and vom Saal, F. S. (1999) Exposure to bisphenol A advances puberty. *Nature* (London), **401**, 763–764.
22) Nimrod, A. C. and Benson, W. H. (1996) Environmental estrogenic effects of alkylphenol ethoxylates. *Crit. Rev. Toxicol.*, **26**, 335–364.