Evaluation of Endocrine Disrupting Activities of Monohydroxylated Derivatives of 1-nitropyrene by Yeast Two-hybrid Assay

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Endocrine disrupting activities of three isomers of monohydroxylated 1-nitropyrene (1-NP) [3-, 6-, and 8-hydroxy-1-nitropyrenes (OHNPs)] were evaluated for the first time by yeast two-hybrid assay. OHNPs, which are not only metabolites of 1-NP but are also found in airborne particles, did not exhibit androgenic activity but exhibited estrogenic, antiestrogenic, and antiandrogenic activities. 6-OHNP showed the strongest estrogenic activity among the three OHNP isomers examined in this study. Concentrations of the OHNP isomers that gave 10% of activity of 1.0×10^{-6} M 17β-estradiol (E_2) were as follows: 3-OHNP, 6.0×10^{-7} M; 6-OHNP, 6.0×10^{-8} M; 8-OHNP, 9.0×10^{-7} M. On the contrary, 8-OHNP exhibited the strongest antiestrogenic and antiandrogenic activities of the three isomers. 1.0×10^{-6} M of 8-OHNP inhibited 32 and 90% of β-galactosidase activity induced by 1.0×10^{-9} M of E_2 and 1.0×10^{-8} M of 5α-dihydrotestosterone (DHT), respectively. These findings point out the necessity for detailed investigation of environmental sources and distributions of OHNPs as well as the parent 1-NP.

Key words —— endocrine disruptor, hydroxynitropyrene, polycyclic aromatic hydrocarbon, nitrated polycyclic aromatic hydrocarbon, nitropyrenol

INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (NPAHs) are a class of mutagens/carcinogens found in the atmosphere, and some of them exhibit stronger mutagenicity/carcinogenicity than their parent PAHs. 1-Nitropyrene (1-NP) is a representative NPAH formed through combustion processes of fossil fuel such as diesel fuel combustion[1] and one of the most abundant NPAHs in the atmosphere.[2] 1-NP taken into human or animals is transformed to various metabolites such as hydroxy-1-nitropyrenes (OHNPs; Fig. 1) in the presence of cytochrome P450 enzymes.[3,4] Additionally, OHNPs were observed in airborne particles[5,6] and diesel exhaust particles.[7,8] The mutagenicity of OHNPs has been investigated by several groups, and they concluded that most of OHNP isomers showed lower activity than the parent 1-NP.[8–10] However, other biological effects of OHNPs are still uncertain.

Recently several kinds of PAHs and their derivatives have been found to act as endocrine disruptors which may cause dysfunction of human and wildlife endocrine systems, abnormalities associated with the developing reproductive systems, and deficiencies of immune systems.[11] In addition to showing estrogenic activity in transfected MCF-7 cells,[12] it was reported that PAHs showed antiestrogenic responses by binding to the aryl hydrocarbon (Ah) receptor in MCF-7 cells[13] or by blocking activation of the estrogen receptor (ER) in a yeast assay system.[14] Significant estrogenic/antiestrogenic activities for several monohydroxylated derivatives of PAHs (OHPAHs) were also observed by using a

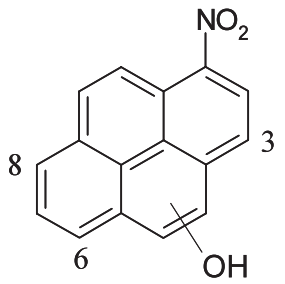

Fig. 1. Structure of Hydroxy-1-nitropyrenes

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reporter gene assay[15] or a yeast two-hybrid assay system based on the ligand-dependent interaction of ER and its co-activator.[11] Furthermore, it has been reported that mono- and dihydroxy metabolites of PAHs might act as antiandrogenic chemicals in a reporter gene assay based on CHO cells transiently cotransfected with a human androgen receptor (hAR) vector and an MMTV-LUC vector.[16] These results imply that OHNPs having similar structure to OHPAHs also exhibit endocrine disrupting activities. To test this hypothesis, we examined estrogenic, antiestrogenic, androgenic and antiandrogenic activities of three isomers of OHNP (3-, 6-, and 8-OHNPs) using a yeast two-hybrid assay.

MATERIALS AND METHODS

Synthesis of OHNPs —— 3-, 6-, and 8-OHNPs were synthesized according to the previously reported procedure.[17] Briefly, acetoxypyrene which was prepared from pyrene by the treatment with lead tetraacetate in benzene/acetic acid (9/1, v/v) was nitrated using concentrated HNO_3 in acetic acid. The obtained mixture of three isomers of acetoxynitropyrenes was treated with CH_3ONa in methanol/tetrahydrofuran (1/1, v/v) to obtain a mixture of OHNPs. Each isomer of OHNPs was purified by preparative normal phase HPLC (silica-gel column, 25 cm × 21.2 mm *i.d.*, eluted with CH_2Cl_2 containing 0.5 mM CH_3COOH at 10 ml/min). To identify the synthesized compounds, the GC-MS and proton NMR spectra of them were compared with the data in the literature.[3,4,18]

Chemicals —— 17β-Estradiol (E_2) and 5α-dihydrotestosterone (DHT) were purchased from Wako Pure Chemicals (Osaka, Japan). 4-Hydroxytamoxifen (4-OHT) and hydroxyflutamide (OHFl) were obtained from Sigma (St. Louis, MO, U.S.A.) and Toronto Research Chemical Inc. (North York, Canada), respectively. Test compounds were dissolved in ethanol and stored at −20°C until use. All other chemicals were of the highest quality available from commercial sources.

Yeast Two-hybrid Assay —— Estrogenic, antiestrogenic, androgenic, and antiandrogenic activities of OHNPs were evaluated with the yeast two-hybrid assay following Nishikawa's method with some modifications.[11,19] Briefly, yeast cells (*Saccharomyces cerevisiae* Y190) expressing hERα and hAR or two-hybrid system control yeast cells (*Saccharomyces cerevisiae* Y190 transfected with the pGBK7-53 and pGADT7-T) were grown overnight at 30°C with shaking in synthetic defined medium free from tryptophan and leucine, and treated with each test compound at 30°C for 4 hr. After the incubation, the treated cells were collected and enzymatically digested with 1 mg/ml Zymolyase 20T at 37°C for 30 min. 2-Nitrophenyl-β-D-galactoside was added to the lysate to a final concentration of 4 mg/ml. After incubation at 30°C for 45 min, the reaction was terminated 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. Estrogenic activity was evaluated by the 10% relative effective concentration (REC_{10}), which is defined as the concentration of the test compounds showing 10% of the highest β-galactosidase activity of E_2. Antiestrogenic and antiandrogenic activities were evaluated by IC_{20}, which is the concentration of the test compounds that inhibit 20% of β-galactosidase activity induced by 1.0×10^{-9} M E_2 and 1.0×10^{-8} M DHT, respectively.

RESULTS AND DISCUSSION

Figure 2 shows the results of the estrogenic activities for the three isomers of OHNP and E_2. β-Galactosidase activity increased with increasing E_2 concentration, reaching a plateau at 1.0×10^{-6} M. Each of the three OHNPs also showed dose-dependent activities at concentrations between

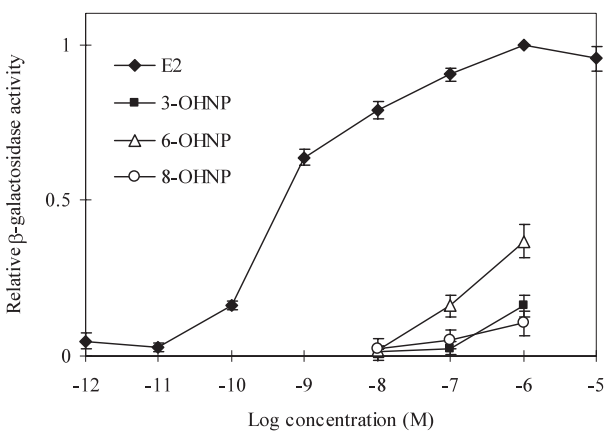

Fig. 2. Dose Response Curves of Estrogenic Activity of 17β-estradiol (E_2) and Hydroxy-1-nitropyrenes (OHNPs) in Yeast Two-hybrid Assay System
Each data point is the mean ± S.D. (n = 3).

Table 1. REC_{10} and IC_{20} Values for Hydroxy-1-nitropyrenes and Reference Chemicals in Yeast Two-hybrid Assay

Compound	REC_{10} (M) [c]	IC_{20} (M) [d]	
	Estrogenic activity	Antiestrogenic activity	Antiandrogenic activity
3-OHNP [a]	6.0×10^{-7}	1.1×10^{-6}	2.3×10^{-7}
6-OHNP [a]	6.0×10^{-8}	1.0×10^{-6}	3.1×10^{-7}
8-OHNP [a]	9.0×10^{-7}	7.0×10^{-7}	5.1×10^{-8}
17β-Estradiol [a]	6.0×10^{-11}		
Bisphenol A [b]	3×10^{-6}		
4-Hydroxytamoxifen [a]		5.3×10^{-6}	
Hydroxyflutamide [a]			5.3×10^{-6}

OHNP: hydroxy-1-nitropyrene. *a*) This study. *b*) Taken from reference 11. *c*) Concentration of the test compounds showing 10% of the highest β-galactosidase activity of 17β-estradiol. *d*) Concentration of the test compounds that inhibit 20% of β-galactosidase activity induced by 10^{-9} M 17β-estradiol or 10^{-8} M 5α-dihydrotestosterone.

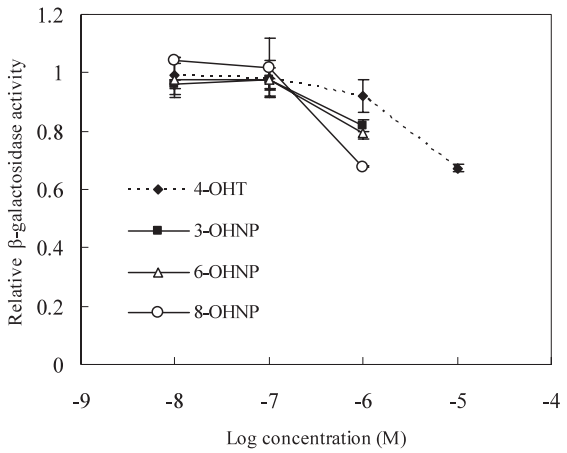

Fig. 3. Antiestrogenic Activity of 4-hydroxytamoxifen (4-OHT) and Hydroxy-1-nitropyrenes (OHNPs) against the Estrogenic Activity of 17β-estradiol (E_2) in Yeast Two-hybrid Assay System

Antiestrogenic activities of 4-OHT and OHNPs were expressed as relative β-galactosidase activity to the level induced by 1.0×10^{-9} M E_2. Each data point is the mean ± S.D. ($n = 3$).

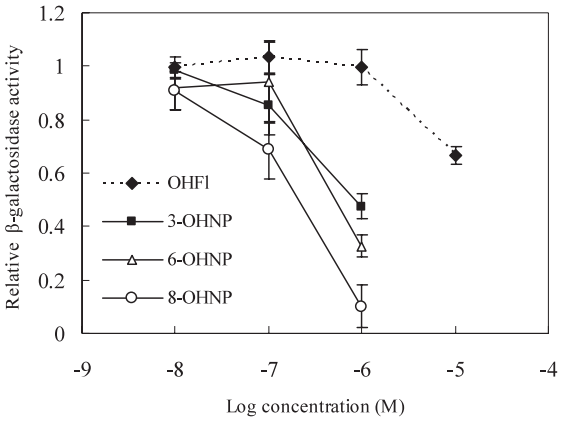

Fig. 4. Antiandrogenic Activity of Hydroxyflutamide (OHFl) and Hydroxy-1-nitropyrenes (OHNPs) against the Androgenic Activity of 5α-dihydrotestosterone (DHT) in Yeast Two-hybrid Assay System

Antiandrogenic activities of OHFl and OHNPs were expressed as relative β-galactosidase activity to the level induced by 1.0×10^{-8} M DHT. Each data point is the mean ± S.D. ($n = 3$).

1.0×10^{-8} and 1.0×10^{-6} M. A significant induction of β-galactosidase activity was observed for 6-OHNP ($REC_{10} = 6.0 \times 10^{-8}$ M), and 3- and 8-OHNP also exhibited strong estrogenic activity ($REC_{10} = 6.0 \times 10^{-7}$ and 9.0×10^{-7} M, respectively). The REC_{10} and IC_{20} values for OHNP isomers obtained in this study and reference chemicals are summarized in Table 1. As shown in Table 1, the estrogenic activities of OHNPs were higher than that of bisphenol A ($REC_{10} = 3 \times 10^{-6}$ M),[11] a known estrogenic compound. Figure 3 shows the results of the antiestrogenic activities for the OHNPs in the concentration range from 1.0×10^{-8} to 1.0×10^{-6} M. We used 1.0×10^{-9} M of E_2 to assess the antiestrogenic activity, which induced near 50% of the highest β-galactosidase activity of E_2. Each of the three OHNP isomers, especially 8-OHNP, decreased the induction of β-galactosidase activity by E_2 at a concentration of 1.0×10^{-6} M. The OHNPs showed 5–8 times higher antiestrogenic activity than 4-OHT, a typical ER antagonist (Table 1). Figure 4 shows the results of the antiandrogenic activities for the OHNPs. In the presence of OHNPs at concentrations between 1.0×10^{-8} and 1.0×10^{-6} M, the activity of 1.0×10^{-8} M DHT, which induced near 50% of the highest β-galactosidase activity of DHT, was inhibited concentration-dependently. The highest inhibitory effect among the three isomers was observed with 8-OHNP as is the case with antiestrogenic activity. The antiandrogenic activities of OHNPs were 20–100 times higher than that of OHFl (Table 1). At concentrations less than 1.0×10^{-6} M, none of the OHNP isomers were cytotoxic to the control yeast cells, which supports that the decreases of β-

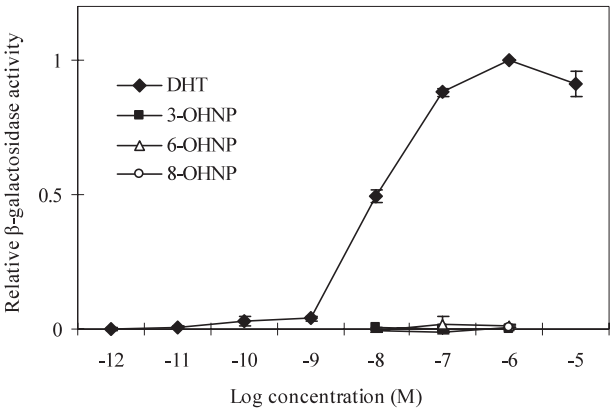

Fig. 5. Dose Response Curves of Androgenic Activity of 5α-dihydrotestosterone (DHT) and Hydroxy-1-nitropyrenes (OHNPs) in Yeast Two-hybrid Assay System

Each data point is the mean ± S.D. (n = 3).

galactosidase induction observed in this study were due to antiestrogenic/antiandrogenic effects rather than cytotoxic effects. None of the OHNP isomers showed androgenic activity at concentrations between 1.0×10^{-8} and 1.0×10^{-6} M (Fig. 5).

OHPAHs having four aromatic rings, such as 4-hydroxybenz[a]anthracene and 3-hydroxybenzo[c]phenanthrene, were shown to have strong endocrine disrupting activities.[20] Furthermore, it was found that the four rings and a phenol group needed to be in a rectangular plane in order for OHPAHs to bind to the site of the receptor.[20] The OHNPs examined in this study have the same planar structure, which could explain their endocrine disrupting activities.

Our results suggest that investigations of environmental sources, sinks, and distributions of OHNPs are essential for assessing their risks.

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