Effects of Environmental Antiandrogenic Chemicals on Expression of Androgen-Responsive Genes in Rat Prostate Lobes

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Rat prostate, which is usually used in the Hershberger assay for evaluating the antiandrogenic activity of environmental chemicals *in vivo*, has a complex structure consisting 4 lobes, *i.e.*, the ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to human prostate. However, the Hershberger assay focuses on the VP, not the other lobes. Moreover, there are few other methods for assessment of antiandrogenic activity *in vivo*. We therefore investigated androgen-responsive genes in the DP, as well as VP, following treatment with environmental chemicals reported to be androgen antagonists. Male castrated F344 rats were treated with testosterone (0.5 $mg \cdot kg^{-1} \cdot day^{-1}$) alone or together with flutamide (6 $mg \cdot kg^{-1} \cdot day^{-1}$) as a reference antiandrogen or fenthion (25 $mg \cdot kg^{-1} \cdot day^{-1}$) or fenitrothion (25 $mg \cdot kg^{-1} \cdot day^{-1}$) or 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP) (300 $mg \cdot kg^{-1} \cdot day^{-1}$) for 7 days. Testosterone significantly increased the expression of kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 mRNAs in the VP, and prostate secretory protein of 94 amino acids (PSP94) mRNA, but not stem cell growth factor (SCGF) mRNA, in the DP. Coadministration of flutamide blocked the testosterone-induced increases of all three mRNAs in the VP, but not that of PSP94 mRNA in the DP. Coadministration of fenitrothion significantly reduced the testosterone-induced increase of kallikrein S3 mRNA, while fenthion significantly increased the testosterone-induced increase of PSP94 mRNA. 2,4,4′-TriOH-BP significantly increased the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs. These results indicate that the effects of environmental chemicals on the prostate are very complex. The Hershberger assay alone appears to be inadequate for risk assessment, and it may be useful to employ androgen-responsive genes as additional markers.

Key words —— antiandrogenic activity, androgen-responsive genes, rat prostate lobes, Hershberger assay, quantitative reverse transcriptase polymerase chain reaction

INTRODUCTION

Many environmental xenobiotics exert hormonal effects at the cellular and organism levels. These compounds are able to mimic the biological activity of sex hormones and thyroid hormone, and are called endocrine-disrupting chemicals. Initially, estrogenic chemicals such as alkylphenols and bisphenol A were discovered,[1,2] while more recently, several environmental pollutants were discovered to be androgen antagonists.[3,4]

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The Hershberger assay has been used to detect chemicals with androgen receptor (AR)-mediated activity *in vivo*.[5–7] The advantages of this assay are that is straightforward, quick and relatively specific to androgenic/antiandrogenic compounds. The endpoint of this assay involves weighing the accessory sex organs of castrated male rats treated with an AR agonist and test compounds.[8,9] However, the Hershberger assay is usually focused on the rat ventral prostate (VP), not other lobes. The rat prostate has a complex structure, consisting of a VP, lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP). The rodent VP is considered to have no counterpart in primates, while the LP and DP are histologically similar to the human prostate.[10]

We recently reported the lobe-specific expres-

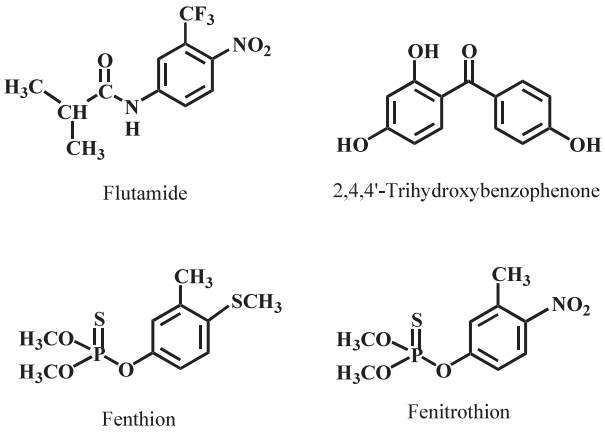

Fig. 1. Structures of Benzophenone, Fenthion and Fenitrothion

sion and lobe-specific response to androgen of several androgen-responsive genes.[11] In the VP, kallikrein S3, cystatin-related protein-1 (CRP-1) and prostatein C3 were highly responsive to androgen treatment. On the other hand, in the LP and DP, prostate secretory protein of 94 amino acids (PSP94), and stem cell growth factor (SCGF) were responsive. In the present study, we used three antiandrogenic chemicals, fenthion, fenitrothion and 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), as well as the reference antiandrogen flutamide (Fig. 1), and quantitatively analyzed the changes of expression of the above genes in the DP and VP after administration of these chemicals to castrated rats using the same schedule as in the Hershberger assay.[4,12,13] Based on the results, we discuss whether androgen-responsive genes might be suitable markers for assessment of the antiandrogenic activity of environmental chemicals.

MATERIALS AND METHODS

Chemicals —— Testosterone propionate, fenthion and fenitrothion were purchased from Wako Junyaku KK, Osaka, Japan, flutamide from Sigma (St. Louis, MO, U.S.A.) and 2,4,4′-triOH-BP from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan.

Animals —— Animal experiments were conducted according to "A Guide for the Care and Use of Laboratory Animals of Hiroshima University." Male F344 rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. All animals were surgically castrated at 5 weeks old. At the age of 7 weeks, they were divided into 6 groups each consisting of 6 animals. The rats were treated once a day for 7 days with subcutaneous doses of 0.3 ml of vehicle (dimethyl sulfoxide), testosterone propionate ($0.5\,mg\cdot kg^{-1}\cdot day^{-1}$), testosterone propionate plus flutamide ($6\,mg\cdot kg^{-1}\cdot day^{-1}$), testosterone propionate plus fenitrothion ($25\,mg\cdot kg^{-1}\cdot day^{-1}$), testosterone propionate plus fenthion ($25\,mg\cdot kg^{-1}\cdot day^{-1}$) or testosterone propionate plus 2,4,4′-triOH-BP ($300\,mg\cdot kg^{-1}\cdot day^{-1}$). Animals were sacrificed under anesthesia and the prostate and seminal vesicles were removed, immediately frozen in liquid nitrogen, and stored at −80 °C.

Quantification of mRNAs by Real-time RT-PCR —— RNA preparation was carried out with a Total RNA Isolation kit (Promega Co., Madison, WI, U.S.A.). Total RNA (2 μg) was reverse-transcribed with 200 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA, U.S.A.) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl of buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37 °C for 60 min. A real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen, Valencia, CA, U.S.A.) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA, U.S.A.) was employed for quantitative measurement, following the supplied protocol.[14] Specific primer sets with a Tm of about 59 °C were designed for each mRNA (Table 1). The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 sec at 94 °C, and 30 sec at 58 °C and 40 sec at 72 °C. Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Extracted fragments were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (PerkinElmer Life Sciences). All mRNA contents were normalized with reference to β-actin mRNA.

Statistical Analysis —— Statistical comparisons were made using ANOVA followed by Scheffe's test.

RESULTS

Effects of Test Chemicals on mRNA Expression of Androgen-responsive Genes

In order to evaluate the effects of several environmental chemicals on the expression of androgen-

Table 1. Primers for Quantitative PCR of Rat Genes

Gene	GenBank Acc#	5′-Primer	3′-Primer
kallikrein S3	M11566	5′-AATTCCCAACCCTGGCAAGT-3′	5′-CGCTGAGCAAAGGGTTCATC-3′
CRP-1	S57980	5′-TGCTCCTACTGGCCATCTTTG-3′	5′-TGTCAGCACTGTGCGTGTTG-3′
prostatein C3	M71245	5′-CAGTGGTTCTGGCTGCAGTATT-3′	5′-CTAGAAAACACTGCTTGAATTGCTTC-3′
PSP94	U65486	5′-GATCACCTGCTGCACCAAAAC-3′	5′-TTCCTGGGTTCGTCCGTTC-3′
SCGF	XM_218611	5′-AGAGGAAACCACCACAACACCT-3′	5′-GTCCAAAACATGCAGACGGAT-3′
β-actin	X03765	5′-CTGTCCCTGTATGCCTCTGGTC-3′	5′-TGAGGTAGTCCGTCAGGTCCC-3′

Table 2. mRNA Levels Expressed by Reportedly Androgen-sensitive Genes in Castrated Rats in the Experimental Treatment Groups

Treatment group	VP			DP	
	Kallikrein S3 mRNA	CRP-1 mRNA	Prostatein C3 mRNA	PSP94 mRNA	SCGF mRNA
Vehicle Control	0.003 ± 0.0002**	0.0004 ± 0.0002**	0.02 ± 0.005**	1.3 ± 0.32**	0.018 ± 0.003
T	7.3 ± 1.1	46 ± 9.0	84 ± 4.1	11 ± 2.0	0.028 ± 0.010
T+Flu	0.060 ± 0.016**	0.30 ± 0.010**	6.4 ± 1.7**	8.7 ± 1.2	0.022 ± 0.002
T+MPP	5.5 ± 0.70	40 ± 4.8	86 ± 10	19 ± 2.2*	0.045 ± 0.011
T+MEP	4.0 ± 0.66*	23 ± 3.1	84 ± 14	15 ± 2.1	0.028 ± 0.003
T+2,4,4′-triOH-BP	7.4 ± 1.2	93 ± 10*	112 ± 9.0*	6.5 ± 1.1	0.099 ± 0.020*

Castrated male F344 rats were treated with T (0.5 mg·kg^{-1}·day^{-1}) and/or MPP (25 mg·kg^{-1}·day^{-1}), MEP (25 mg·kg^{-1}·day^{-1}), BP (300 mg·kg^{-1}·day^{-1}), Flu (6 mg·kg^{-1}·day^{-1}) for a week. Values are mean ± S.E.M. (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* T. Abbreviations: T, testosterone propionate; Flu, flutamide; MPP, fenthion; MEP, fenitrothion; 2,4,4′-triOH-BP, 2,4,4′-trihydroxybenzophenone.

responsive genes, we carried out quantitative analysis of mRNA expression of three genes in the VP and two in the DP. All of these genes have been reported to be androgen-responsive.[11)]

In the VP, expression levels of the kallikrein S3, CRP-1 and prostatein C3 genes in castrated rats were all significantly increased by administration of testosterone (Table 2), while coadministration of flutamide essentially abrogated the effect of testosterone. Coadministration of fenthion had little effect on the action of testosterone, while coadministration of fenitrothion significantly decreased the testosterone-induced increase of kallikrein S3 mRNA. Coadministration of 2,4,4′-triOH-BP significantly enhanced the testosterone-induced increases of CRP-1 and prostatein C3 mRNAs.

In the DP, testosterone increased the expression of PSP94 mRNA, but had no effect on SCGF mRNA, while coadministration of flutamide did not significantly alter the effect of testosterone. Coadministration of fenthion further increased the testosterone-induced expression of PSP94 mRNA, while coadministration of fenitrothion had no effect. Coadministration of 2,4,4′-triOH-BP with testosterone resulted in a significant increase of SCGF mRNA compared with the testosterone-alone group.

DISCUSSION

The Hershberger assay is widely used to study the androgenic and antiandrogenic activity of environmental chemicals. Usually rat prostate is used for this assay. Rat prostate consists of four separate lobes, and although the LP and DP are considered to be homologous to the peripheral zone of human prostate and the AP is similar to the central zone, the VP has no homologous region in human prostate.[15)] However, Hershberger assays generally focus on the VP because of its high sensitivity to androgen ablation and to testosterone supplementation after castration. Moreover, there are few alternatives to the Hershberger assay to assess androgenic/antiandrogenic activity *in vivo*. In this study, we assessed the antiandrogenic activities of some known environmental antiandrogens using androgen-responsive genes expressed in the VP and DP as markers. Fenthion and fenitrothion are organophosphorus insecticides; both have been reported to have antiandrogenic activity *in vivo* in the Hershberger assay.[4, 12)] 2,4,4′-TriOH-BP, a derivative of benzophenone-3 used in sunscreen for humans, is also an antiandrogen.[13)] Kallikrein S3, CRP-1 and prostatein C3 are secreted proteins ex-

pressed abundantly in the VP and regulated by androgen.[16–18] We reported that expression of the mRNAs encoding these proteins was increased 10- to 1000-fold in the VP within 24 hr after testosterone treatment in castrated rats.[11] In this study, all the mRNA levels were confirmed to be greatly increased by testosterone and this increase was blocked by co-treatment with flutamide (Table 2). Although fenthion and fenitrothion have been reported to be antiandrogens *in vivo*, we found that they had no effect on the testosterone-induced increases of gene expression in the VP, except for a modest, but significant, decrease of the testosterone-induced increase of kallikrein S3 mRNA by fenitrothion. The reason for this may be the effect of metabolism *in vivo*. Flutamide is converted to hydroxyflutamide, with an increase of about 50-fold in antagonistic activity, while fenthion is inactivated.[12, 19] On the other hand, coadministration of 2,4,4′-triOH-BP enhanced the testosterone-induced increase in the expression of CRP-1 and prostatein C3 in the VP (Table 2).

PSP94 is one of the secreted proteins abundantly expressed in DP.[20] Expression of PSP94 was reportedly increased about 2-fold in the DP in castrated rats after testosterone treatment for 24 hr.[11] In this study, testosterone treatment increased PSP94 mRNA, but flutamide did not block this increase. Fenthion significantly enhanced the testosterone-induced increase of PSP94 mRNA, but fenitrothion and 2,4,4′-triOH-BP were ineffective. SCGF is one of the growth factors expressed in rat prostate, and is expressed highly in the DP. It is tightly regulated by androgen in the DP, being up-regulated about 5-fold within 1 hr after testosterone treatment.[11] In this study, however testosterone did not significantly increase the mRNA level of SCGF, while coadministration of 2,4,4′-triOH-BP resulted in a significant increase compared with testosterone alone (Table 2). The reason for this may be the estrogenicity of 2,4,4′-triOH-BP, which acts as an estrogen agonist in MCF-7 human breast cancer cells and ovariectomized rats.[13, 21] In the rat, estrogen receptor β (ERβ) is expressed in the prostate, and has a role in prostate growth.[22, 23] Its presence may influence the antiandrogenic activity of environmental chemicals. There are differences in response to fenthion, fenitrothion and flutamide between three genes in the VP and two genes in the DP. The reason for this may be the difference in response to testosterone; expression of three genes in the VP greatly increased, on the other hand two genes in the DP did not show great change.

In conclusion, the effects of environmental chemicals on the prostate are very complex, and the Hershberger assay alone appears to be inadequate to understand them. Androgen-responsive genes especially three genes in the VP may be good markers for assessment of androgenic/antiandrogenic activity of environmental chemicals.

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