In this review, we cover environmental compounds that are not directly estrogenic, but which are activated by the liver microsomal metabolic system in the mammalian body after ingestion. These include methoxychlor, *trans*-stilbene, diphenyl, diphenylmethane, polycyclic aromatic hydrocarbons, nitropolycyclic aromatic hydrocarbons, α,β-unsatulated ketone, styrene oligomers and others.

ACTIVATION OF *TRANS*-STILBENE AND RELATED COMPOUNDS BY THE CYTOCHROME P450 SYSTEM

It is well known that *trans*-stilbene derivatives, such as diethylstilbestrol (DES), euvestin, 4,4′-diaminostilbene and pinosylvin, have a variety of biological actions, including hormonal, hypocholesterolemic, sympathomimetic, antifungal, antibacterial, antimalarial and anticancer activities.[17–19] DES, a potential estrogen, has been used medically as a substitute for endogenous estrogen and as a hormonal therapy for prostate or breast cancer, and also to prevent threatened abortions.[20, 21] However, it may induce vaginal adenocarcinoma.[22] Its estrogenic activity is similar to that of β-estradiol in *in vitro* estrogen screening tests. However, the estrogenic activity of *trans*-stilbene, which is the parent compound of stilbene derivatives and is used as an industrial material, has not been extensively examined.

trans-Stilbene is not estrogenic in the estrogen screening tests. However, when *trans*-stilbene was incubated with liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited an estrogenic effect in the concentration range of 10^{-5}–10^{-6} M. In contrast, *cis*-stilbene showed little estrogenic activity after incubation with liver microsomes.[9] When *trans*-stilbene was incubated with liver microsomal enzyme system of 3-methylcholanthrene-treated rats, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene were both formed, though *trans*-4-hydroxystilbene was predominant. Human cytochrome P450 1A1 and 1A2 isoforms expressed in human lymphoblastoid cells catalyzed both oxidations.[23] In the case of *cis*-stilbene, such hydroxylated metabolites were not detected (Fig. 1).

trans-4-Hydroxystilbene and *trans*-4,4′-dihydroxystilbene both showed estrogenic activity similar to that of DES in estrogen screening tests. Thus, *trans*-stilbene was converted to the active metabolites, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, by rat liver microsomes, and so the estrogenic activity of *trans*-stilbene might be due mainly to *trans*-4-hydroxystilbene, with some contribution from *trans*-4,4′-dihydroxystilbene. In contrast, *cis*-stilbene was not metabolized to the corresponding hydroxylated metabolites with liver microsomes (Fig. 1). In an *in vivo* estrogenicity test using ovariectomized (OVX) rats, *trans*-stilbene was positive, as well as 4-hydroxylated stilbene.[24] This shows that *trans*-stilbene exhibited estrogenic activity after metabolic activation *in vivo*.

It was demonstrated that resveratrol, which is a derivative of stilbene found in grapes and wine, is an agonist for the estrogen receptor.[25] Among stilbene-related compounds, metabolic activation of *trans*-stilbene oxide, *trans*-α-methylstilbene, tolan, dibenzyl and azobenzene to estrogenic compounds by the cytochrome P450 system was also demonstrated. These compounds did not show

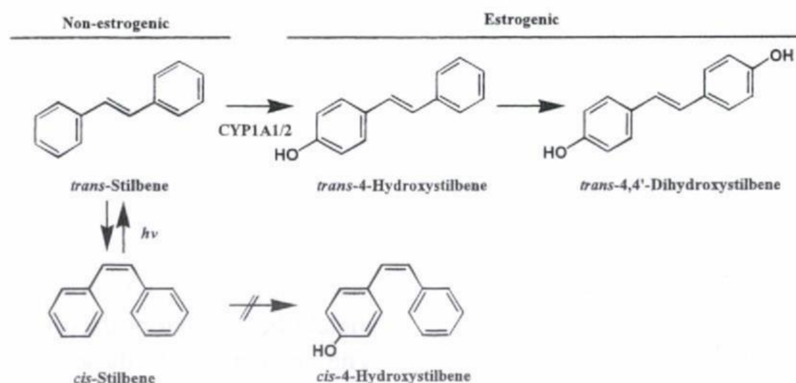

Fig. 1. Metabolic Pathways for the Activation of *trans*-Stilbene to Estrogens by the Cytochrome P450 System

estrogenic activity, but exhibited estrogenic activity after metabolic activation by liver microsomes from 3-methylcholanthrene- or phenobarbital-treated rats (Fig. 2).[26] Furthermore, the estrogenic activities of several stilbene derivatives were compared. The 4-hydroxyl group of the A-ring plays the most important role, but nitro and amino substituents also result in some estrogenic activity. The vinyl linkage is necessary for high activity. The hydrophobic B-ring plays an important role, because the estrogenic activity of hydroxystilbene is higher than that of hydroxystyrene. The structural requirements for the estrogenic activities of stilbene derivatives were proposed to be as shown in Fig. 3. A *p*-hydroxy group in the A-phenyl ring, vinyl linkage, a B-phenyl ring and hydrophobicity of the linkage are necessary for the maximal activity of stilbene derivatives.[26]

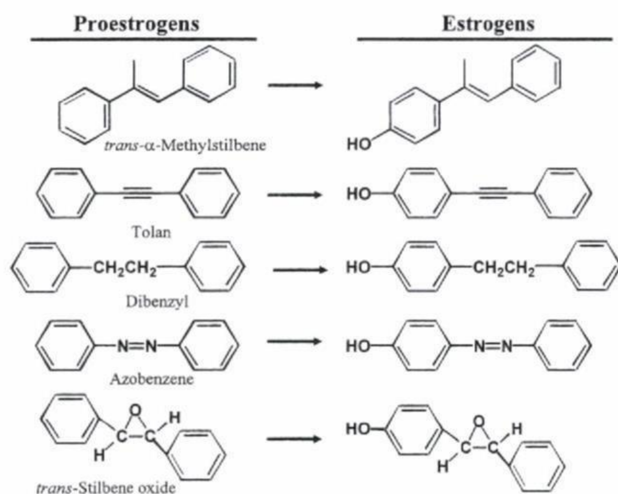

Fig. 2. Metabolic Activation of Stilbene-related Compounds to Estrogens

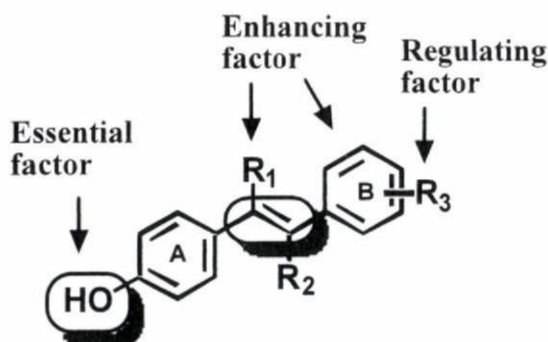

Fig. 3. Structural Requirement of Stilbene Derivatives for Estrogenic Activity

ACTIVATION OF DIPHENYL AND RELATED COMPOUNDS BY THE CYP1A1/2 AND 2B1 SYSTEMS

Diphenyl is used as an antifungal agent for citrus fruits, and also as wrapping paper for impregnated fruit. Under improper conditions, it has been toxic to production workers.[27,28] Diphenylmethane is also used as a dye carrier and synthetic intermediate, similarly to diphenyl. Diphenyl is metabolized to hydroxylated diphenyls. 4-Hydroxydiphenyl, 4,4′-dihydroxydiphenyl and 3,4-dihydroxydiphenyl were identified as urinary metabolites of diphenyl in experimental animals.[29] 2-Hydroxydiphenyl, 2,4′-dihydroxydiphenyl and 3-hydroxydiphenyl were also identified as *in vitro* metabolites with liver microsomes of various animals.[30] These phenylphenols are used as household insecticides, especially for indoor applications,[31] and as intermediates in the manufacture of rubber and resins.

Diphenyl, diphenylmethane and 2,2-diphenylpropane were negative in estrogen screening tests. However, they exhibited estrogenic activity after incubation with liver microsomes of 3-methylcholanthrene-treated rats in the cases of diphenyl and diphenylmethane, or after incubation with liver microsomes of phenobarbital-treated rats in the cases of diphenyl and 2,2-diphenylpropane.[10] When diphenyl was incubated with liver microsomes of phenobarbital- and 3-methylcholanthrene-treated rats in the presence of NADPH for the detection of the estrogenic metabolites, four metabolites (2-hydroxydiphenyl, 3-hydroxydiphenyl, 4-hydroxydiphenyl and 4,4′-dihydroxydiphenyl) were detected. 4,4′-Dihydroxydiphenylmethane and 4-hydroxydiphenylmethane were also detected as metabolites of diphenylmethane with liver microsomes of 3-methylcholanthrene-treated rats. Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] and 2-(4-hydroxyphenyl)-2-phenylpropane were detected as metabolites of 2,2-diphenylpropane with liver microsomes of phenobarbital-treated rats. The estrogenic activity of bisphenol A, which is an active metabolite of 2,2-diphenylpropane in this case, is well-known. However, the amounts of monohydroxyl derivatives of these compounds were much higher than those of the 4,4′-dihydroxyl derivatives (bisphenol A).

These hydroxylated derivatives all showed estrogenic activity. However, 2-hydroxydiphenyl and 3-hydroxydiphenyl showed lower activities than

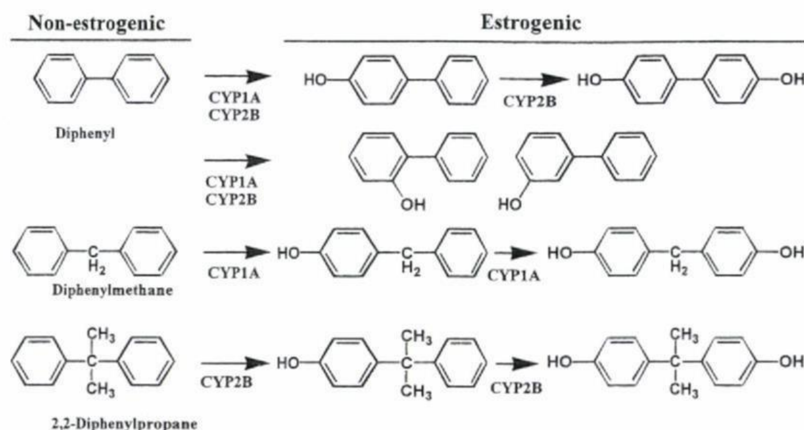

Fig. 4. Metabolic Pathways for the Activation of Diphenyl, Diphenylmethane and 2,2-Diphenylpropane to Estrogens by the Cytochrome P450 System

4-hydroxydiphenyl and 4,4′-dihydroxydiphenyl. Soto *et al.*[32] reported that 2-hydroxydiphenyl, which is used as an antifungal, is a weak estrogen, and the related compounds 4-hydroxydiphenyl and 3-hydroxydiphenyl are also estrogenic. Estrogenic activity of 4,4′-dihydroxydiphenyl has also been reported.[33] A possible metabolic activation pathway of these proestrogenic chemicals with liver microsomes is shown in Fig. 4. The estrogenic activity is likely to be mainly exhibited by the 4-hydroxyl derivatives, because the pathway leading from 4-hydroxyl derivatives to 4,4′-dihydroxyl derivatives does not proceed effectively in this metabolic system.

ACTIVATION OF STYRENE OLIGOMERS BY THE CYTOCHROME P450 SYSTEM

Styrene oligomers, such as *trans*-1,2-diphenylcyclobutane (TCB), *cis*-1,2-diphenylcyclobutane (CCB), 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin are incorporated into polystyrene resin as impurities in the course of manufacture, and may have a variety of biological actions, including hormonal activity.[34,35] Polystyrene has been used to manufacture food containers for take-out, such as coffee cups, meat trays, salad boxes and soup bowls, as well as instant food containers, in which instant foods such as Japanese noodles, buckwheat noodles, Chinese noodles, chow mein, spaghetti and rice are cooked by adding hot water. There are reports indicating that styrene oligomers migrate from these containers into the food contents.[36,37]

These styrene oligomers were negative in the estrogen screening assay. However, TCB exhibited estrogenic activity after incubation with liver microsomes of phenobarbital-treated rats in the presence of NADPH. CCB, 1,3-diphenylpropane and 2,4-diphenyl-1-butene also exhibited estrogenic activity after metabolic activation, but the activities were lower than that of TCB. 2,4,6-Triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin did not show estrogenic activity after such metabolic activation. After incubation of TCB with liver microsomal system of phenobarbital-treated rats, *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane (4-OH-TCB), which exhibited a significant estrogenic activity, was detected. Recombinant human cytochrome P450 2B6 and rat cytochrome P450 2B1 were responsible for the activation. In contrast, cytochrome P450 1A may be mainly responsible for the activation of 2,4-diphenyl-1-butene. Thus, some styrene oligomers, especially TCB, exhibit estrogenic activity after metabolic activation to the 4-hydroxylated metabolite by rat liver microsomes (Fig. 5).[11]

Nobuhara *et al.* reported that styrene oligomers did not induce the proliferation of MCF-7 cells.[38] However, Ohyama *et al.* reported that some styrene dimers and trimers were estrogenic without metabolic activation in a cell proliferation assay with estrogen-responsive MCF-7 cells. They reported that TCB, CCB, 1,3-diphenylpropane and 2,4-diphenyl-1-butene were positive without

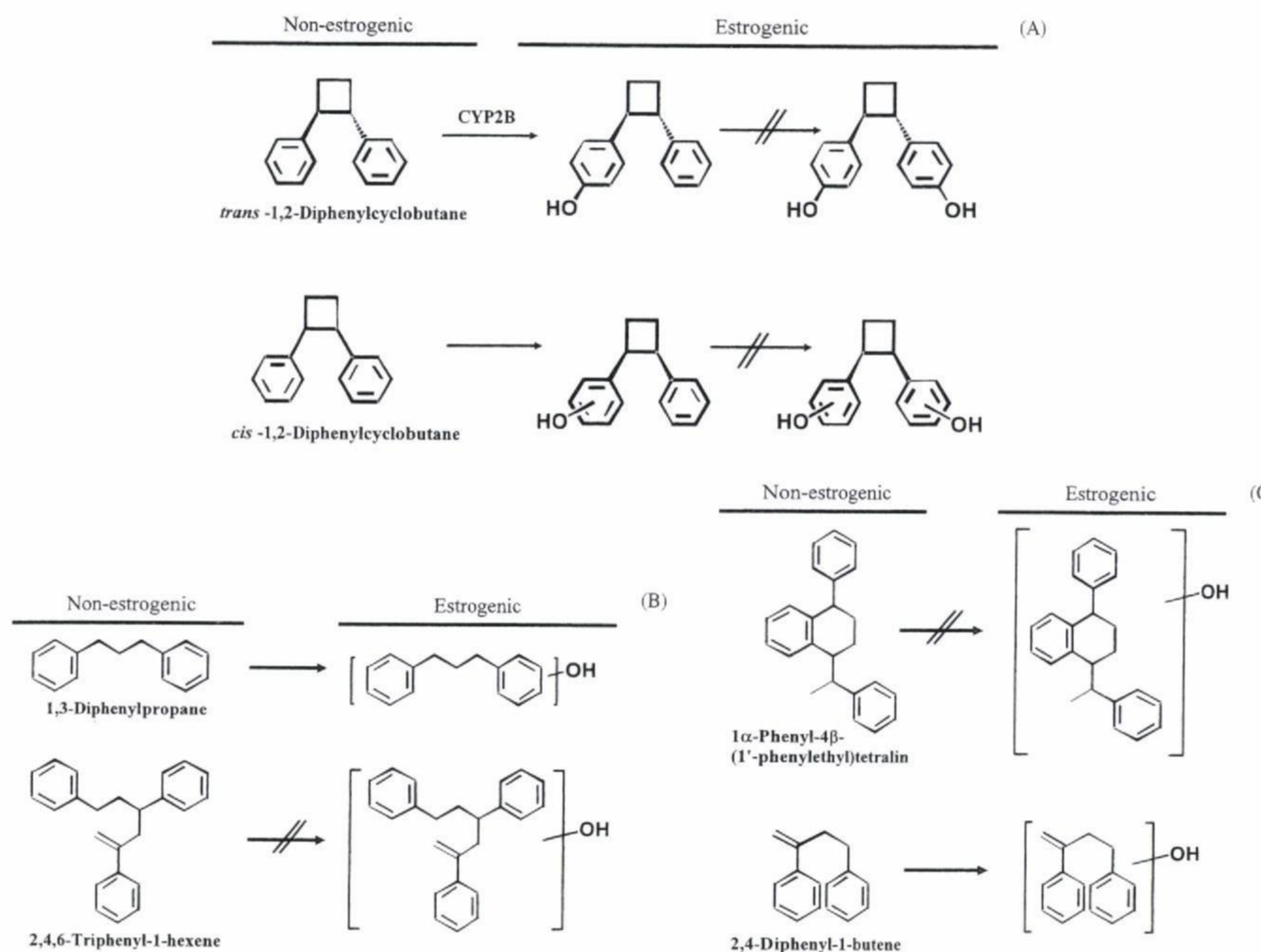

Fig. 5. Metabolic Pathways for the Activation of Styrene Oligomers to Estrogens by the Cytochrome P450 System

metabolic activation.[39] The difference from the above results was not clearly explained. However, one possibility is that, because the substrates were in contact with MCF-7 cells for a long time in their assay, estrogenic metabolites were generated in the cells.

A metabolic activation pathway of proestrogenic styrene oligomers with liver microsomes is shown in Fig. 5. TCB is converted to hydroxylated derivatives by rat liver microsomes. In the microsomal system, the estrogenic activity of TCB is thought to be mainly exhibited by 4-OH-TCB. The further oxidized metabolite, *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane, could not be detected. However, the activity of CCB after metabolic activation was lower than that of the *trans*-isomer. *cis*-1-(4-Hydroxyphenyl)-2-phenylcyclobutane may exhibit lower estrogenic activity than the *trans*-isomer. The activity of CCB after metabolic activation may be due to 4-OH-TCB formed after *cis-trans* isomerization.

ACTIVATION OF POLYCYCLIC AROMATIC HYDROCARBONS AND NITROPOLYCYCLIC AROMATIC HYDROCARBONS BY THE CYTOCHROME P450 SYSTEM

Some reports suggest that hydroxylated polycyclic aromatic hydrocarbons act as xenoestrogens.[40–42] Many polycyclic aromatic hydrocarbons present as environmental pollutants are negative in the estrogen screening test, but the possibility should be considered that these compounds may also be metabolically activated to xenobiotic estrogens via hydroxylation in the body. It was demonstrated that hydroxylated metabolites of benzo[*a*]pyrene exhibit estrogenic activity. In this case, the 1-, 3-, 7- and 9-hydroxylated metabolites had binding affinity for estrogen receptor, but the 7,8- and 9,10-dihydroxy metabolites did not.[40–42] De Wiele *et al.* also reported that polycyclic aro-

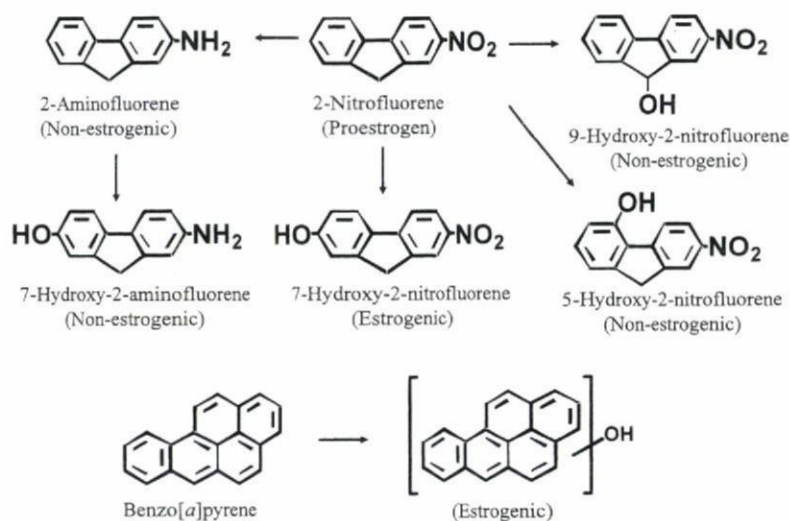

Fig. 6. Metabolic Pathways for the Activation of 2-Nitrofluorene and Benzo[*a*]pyrene to Estrogens by the Cytochrome P450 System

matic hydrocarbons were transformed to estrogenic hydroxylated metabolites by human colon microbiota (Fig. 6).[43]

Nitropolycyclic aromatic hydrocarbons (nitro-PAHs), which are carcinogenic and mutagenic, enter the environment from diesel engine exhaust, urban pollution sources, cigarette smoking and so on.[44–46] Several reports have indicated that nitro-PAHs are mainly metabolized by nitro reduction, ring hydroxylation, acylation and conjugation in mammalian species.[47] Nitro-PAHs should also be examined to see whether nitro reduction or hydroxylation of the aromatic rings activates these compounds to xenobiotic estrogens, as is the case for their carcinogenicity. 2-Nitrofluorene (NF) is a typical carcinogenic nitro-PAH.[48] NF was detected in diesel exhaust particles as a major component, together with nitropyrenes.[49] NF was also detected as a major pollutant in the atmosphere.[50] NF is a potent mutagen and forms DNA adducts in the animal body.[51–53] It was reported that NF is converted to 2-aminofluorene and its acylamino metabolites, and their oxidative metabolites.[54–57]

It was shown that NF exhibits a significant estrogenic activity after activation by rat liver microsomal mixed function oxidase. When the compound was incubated with the liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, 7-hydroxy-2-nitrofluorene (7-OH-NF) was formed as a major metabolite. However, little of the metabolite was formed by liver microsomes of untreated or phenobarbital-treated rats. Rat recombinant cytochrome P450 1A1/2 exhibited a significant oxidase activity toward NF, affording 7-OH-NF. 7-OH-NF exhibited a significant estrogenic activity, while the activity of 5-hydroxy-2-nitrofluorene was much lower.[12]

The estrogenic activity of NF was due to formation of the hydroxylated metabolite at the 7 position by liver microsomes. 2-Aminofluorene and 2-acetylaminofluorene did not exhibit estrogenic activity. These compounds had weak estrogenic activity after metabolic activation by liver microsomes of 3-methylcholanthrene-treated rats. NF is metabolized to hydroxylated derivatives, mainly 7-OH-NF, and is also converted to a reduced metabolite, 2-aminofluorene. In the microsomal system, the estrogenic activity of NF is thought to be mainly due to 7-OH-NF, because reductive metabolism of NF proceeds effectively only under anaerobic conditions. Further, it is possible that 7-OH-NF and 5-OH-NF are reduced to 7- and 5-hydroxy-2-aminofluorene, and acetylated to 7- and 5-hydroxy-2-acetylaminofluorene. However, these reactions are likely to be inactivation routes as regards estrogenic activity (Fig. 6).

ACTIVATION OF METHOXYCHLOR AND RELATED COMPOUNDS BY THE CYTOCHROME P450 SYSTEM

Methoxychlor is a proestrogen which requires demethylation by liver microsomal mixed function oxidase in animals prior to eliciting estrogenic ac-

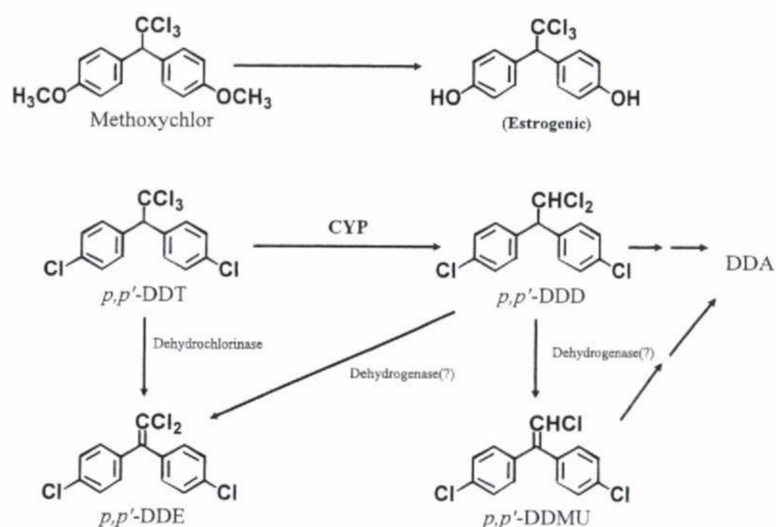

Fig. 7. Metabolic Pathways of Methoxychlor and *p,p'*-DDT in Rat Liver Microsomes

tivity.[13] Methoxychlor requires demethylation by liver microsomal mixed function oxidase, involving CYP 1A2 and 2C19, to elicit estrogenic activity.[58] Elsby *et al.* also reported that methoxychlor was activated through demethylation by human liver microsomes.[14] Schlenk *et al.* reported that methoxychlor was activated to estrogen in fish (Fig. 7).[59]

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (*p,p'*-DDT) is metabolized to 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (*p,p'*-DDD) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p'*-DDE) by reductive dechlorination and dehydrochlorination, respectively, and *p,p'*-DDD is further oxidized to 2,2-bis(4-chlorophenyl)acetic acid (*p,p'*-DDA) in animals and fish (Fig. 7).[60–62] *p,p'*-DDD shows estrogenic activity and *p,p'*-DDE shows anti-androgenic activity.[63] Other DDT isomers exhibited similar endocrine-disrupting activity. The metabolites of *p,p'*-DDT and *o,p'*-DDT described above exhibited estrogenic activity (Fig. 7).[64–66] Nelson reported an *in vivo* uterotropic effect of some of these DDT metabolites.[67] Gray *et al.* reported that *p,p'*-DDT exhibited anti-androgenic activity, like *p,p'*-DDE.[68]

ACTIVATION OF α,β-UNSATULATED KETONES BY THE CYTOCHROME P450 SYSTEM

Naturally occurring phytoestrogens also show estrogenic activity *in vitro* in receptor binding assay, in spite of their beneficial effects, such as anticarcinogenicity.[69] Some flavonoids are phytoestrogens. Chalcones are a source of phytoestrogens, acting as C15 precursors in plant flavonoid biosynthesis.[70] *trans*-4-Phenyl-3-buten-2-one (PBO) also has a flavonoid skeleton. Chalcone (*trans*-1,3-diphenyl-2-propen-1-one) is an α,β-unsaturated ketone that has the skeleton of so-called "chalcones." They are also found in naturally occurring compounds, such as plant allelochemicals, insect hormones and pheromones.[71] PBO (also called *trans*-phenyl styryl ketone or benzalacetone) has a wide range of uses as a industrial material for synthesis of chemicals and drugs, and as a flavoring additive for cosmetics, soaps, detergents, cigarettes and foods.[72] α,β-Unsaturated ketones, in which the double bond is adjacent to the carbonyl group, are reactive compounds due to their electrophilic properties, and undergo nucleophilic attack, *e.g.*, with SH-groups in proteins. They exhibit genotoxicity and mutagenicity, as well as having anti-carcinogenic effects.[73–78]

Chalcone was converted to estrogenically active hydroxylated derivatives by rat liver microsomes. 4-Hydroxychalcone exhibited the highest activity. 4'-Hydroxychalcone and 2-hydroxychalcone were minor metabolites of chalcone, and 2'-hydroxychalcone was not formed. Their estrogenic activities were lower than that of 4-hydroxychalcone. The estrogenic activity of chalcone is thus thought to be mainly due to 4-hydroxychalcone, which is the major metabolite. PBO was also metabolically activated to an estrogen by a

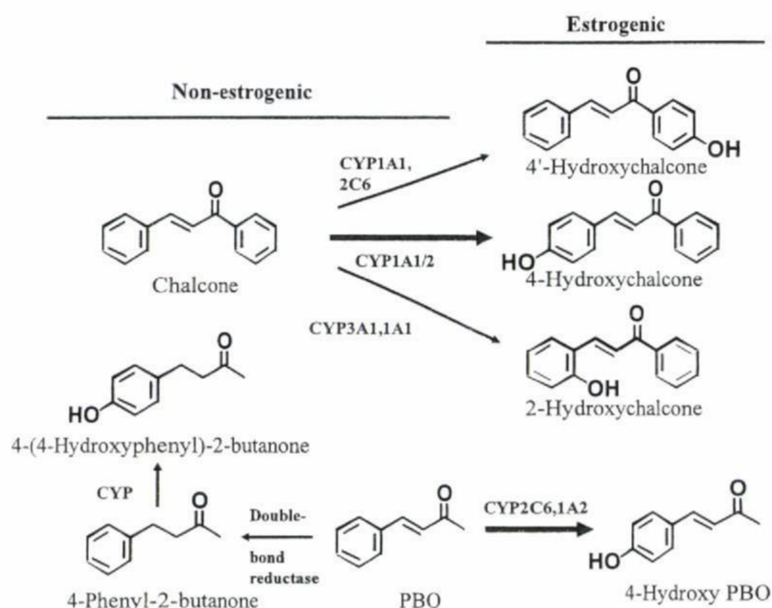

Fig. 8. Metabolic Pathways for the Activation of Chalcone and PBO to Estrogens by the Cytochrome P450 System

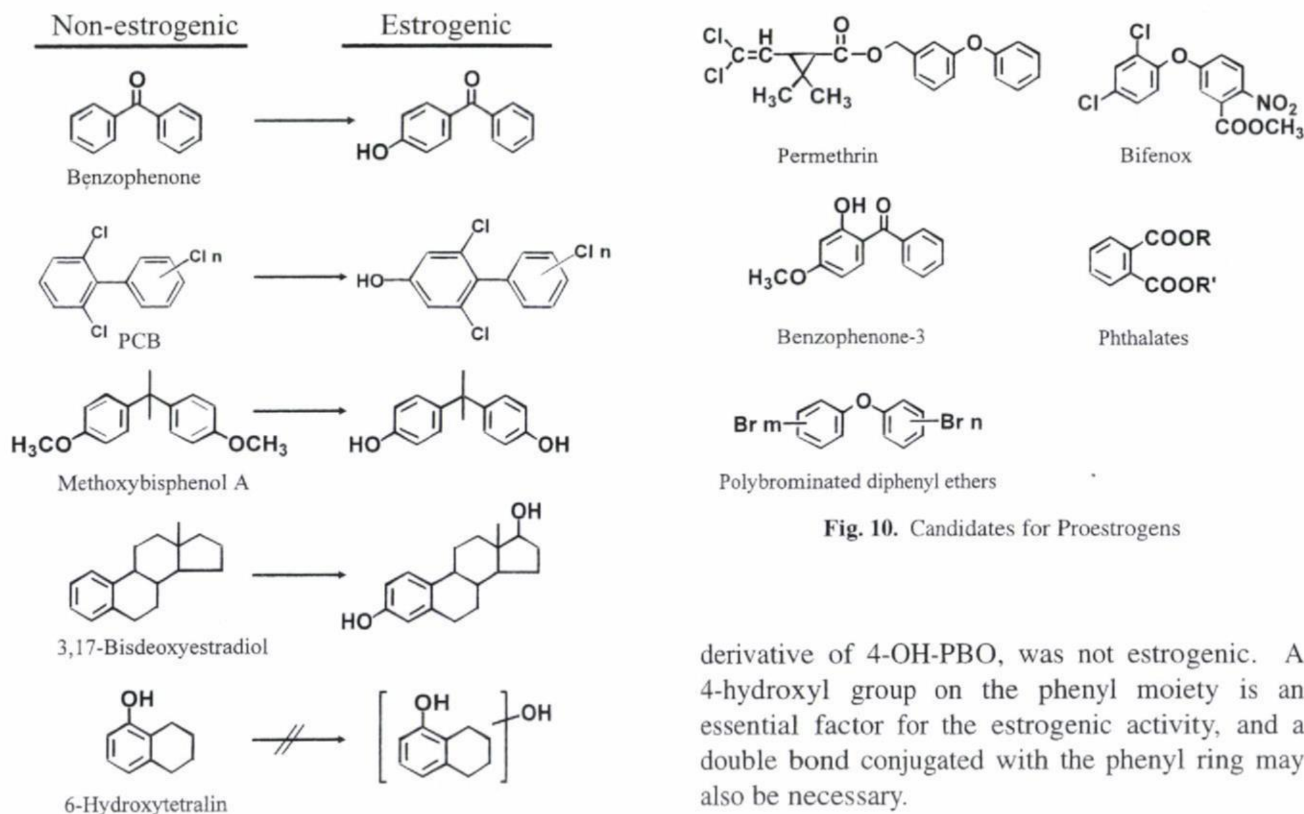

Fig. 9. Metabolic Activation of Other Proestrogens to Estrogens by the Cytochrome P450 System

Fig. 10. Candidates for Proestrogens

microsomal enzyme system, and 4-OH-PBO, which is a major metabolite in the microsomal system, exhibited estrogenic activity (Fig. 8).[16] 4-Hydroxyphenyl-2-butanone, a double-bond-reduced derivative of 4-OH-PBO, was not estrogenic. A 4-hydroxyl group on the phenyl moiety is an essential factor for the estrogenic activity, and a double bond conjugated with the phenyl ring may also be necessary.

ACTIVATION OF OTHER PROESTROGENS IN ENVIRONMENTS BY THE CYTOCHROME P450 SYSTEM

There are other examples of metabolic activation of proestrogens to estrogens, besides

the above examples. Various pesticides might be converted to active estrogens by microsomal oxidase systems, though Sumida *et al.* showed that perimethrine was not metabolically activated.[79)] *p*-Hydroxybenzophenone, which is formed from benzophenone, an antifungal agent, in rat hepatocytes, is also estrogenic.[80,81)] It is also reported that anethole, a flavor agent, is not estrogenic, but 4-hydroxy-1-propenylbenzene, the desmethylated metabolite of anethole, exhibited estrogenic activity.[15)] Some hydroxylated polychlorinated diphenyls (PCBs), which are metabolites of PCB, show estrogenic activity.[82–84)] In the case of PCB, the presence of adjacent chloride substituents decreases the estrogenic activity.[85)] A catechol-type metabolite was also shown to have estrogenic activity.[32)] Elsby *et al.* predicted estrogenicity by a two-stage approach, using a human liver microsome assay and a yeast estrogenicity assay.[14,86)] Methoxychlor, methoxybisphenol A and 3,17-bisdesoxyestradiol were positive, but 6-hydroxytetralin, a degreasing agent, was negative in this screening system (Fig. 9).

Other environmental compounds may be proestrogenic. Candidate proestrogens are illustrated in Fig. 10. They include pyrethroids,[87)] diphenyl ether herbicides (bifenox), polybrominated diphenyl ethers, a flame-retardant, and some benzophenone sunscreens. Some insecticides and medicines are also possible proestrogens. Hydroxylated derivatives of sunscreen,[88)] and phthalate esters[89)] show positive in estrogenicity tests. Thus, the parent compounds may be proestrogens. There may be a variety of other potentially hazardous proestrogens in our environment, too.

CONCLUSION

We have reviewed environmental proestrogens. The estrogenic activity of *trans*-stilbene in rats *in vivo* seems to be a typical example of the metabolic activation of a proestrogen.[24)] It is clearly necessary to consider the activity of metabolites produced from the parent compounds for the assessment of the toxicity of environmental chemicals.

There are also pro-antiandrogen and pro-antithyroid hormonal chemicals.[85,90)] It is therefore necessary, when assessing the potential *in vivo* endocrine-disrupting action of chemicals, to take into account the activities of all the metabolites produced from the parent compounds. For example, bisphenol A, a typical xenoestrogen, is further activated, when it is incubated with rat S-9 mix.[91)] In this case, dimer-type metabolites, which show higher activity than bisphenol A itself, are formed. Such further activation of xenoestrogens must also also considered in the risk assessment of xenoestrogens. Much further work is needed to identify potentially hazardous proestrogens in our environment.

For the activation of proestrogen to estrogen, it is necessary to introduce a hydroxyl group, often at the 4-position of an aromatic ring, in the absence of bulky groups at the adjacent 3,5-positions. When formation of a phenolic hydroxyl group is possible after aromatic ring hydroxylation or dealkylation of chemicals, we should consider the possibility of metabolic activation to estrogens.

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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-

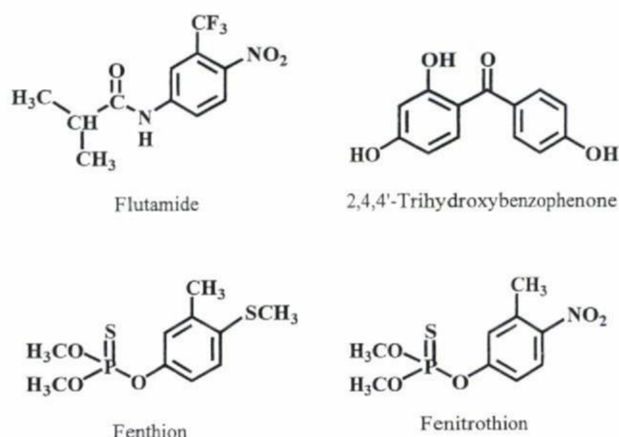

Fig. 1. Structures of Benzophenone, Fenthion and Fenitrothion

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	$0.003 \pm 0.0002^{**}$	$0.0004 \pm 0.0002^{**}$	$0.02 \pm 0.005^{**}$	$1.3 \pm 0.32^{**}$	0.018 ± 0.003
T	7.3 ± 1.1	46 ± 9.0	84 ± 4.1	11 ± 2.0	0.028 ± 0.010
T+Flu	$0.060 \pm 0.016^{**}$	$0.30 \pm 0.010^{**}$	$6.4 \pm 1.7^{**}$	8.7 ± 1.2	0.022 ± 0.002
T+MPP	5.5 ± 0.70	40 ± 4.8	86 ± 10	$19 \pm 2.2^{*}$	0.045 ± 0.011
T+MEP	$4.0 \pm 0.66^{*}$	23 ± 3.1	84 ± 14	15 ± 2.1	0.028 ± 0.003
T+2,4,4′-triOH-BP	7.4 ± 1.2	$93 \pm 10^{*}$	$112 \pm 9.0^{*}$	6.5 ± 1.1	$0.099 \pm 0.020^{*}$

Castrated male F344 rats were treated with T ($0.5\,mg\cdot kg^{-1}\cdot day^{-1}$) and/or MPP ($25\,mg\cdot kg^{-1}\cdot day^{-1}$), MEP ($25\,mg\cdot kg^{-1}\cdot day^{-1}$), BP ($300\,mg\cdot kg^{-1}\cdot day^{-1}$), Flu ($6\,mg\cdot kg^{-1}\cdot day^{-1}$) 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 upregulated 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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In vivo function of the 5′ flanking region of mouse estrogen receptor β gene

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Abstract

The estrogen receptor (ER) subtypes α and β differentially distributed in tissues, and ERβ is present preferentially in epididymis, testis, prostate, ovary and lung. Although transcription promoter activity has been found in the 5′ flanking (5′f) region of the ERβ gene, it is not known whether the proximal 5′f region is responsible for the tissue-specific distribution. In the present study, we examined the *in vivo* promoter activity of this region in transgenic mice with the lacZ reporter. About 2.2 kbp of the proximal 5′f region of ERβ was cloned and inserted into reporter plasmids. This 5′f region of mouse ERβ, which displayed a substantial promoter activity *in vitro*, was very similar to that in rats, but showed limited homology with the human gene. Three independent lines of mice containing ERβ-5′f-lacZ were obtained. Quantitative measurement of mRNA showed that lacZ was expressed only in the testis, in which sertoli cells as well as a part of the spermatogonia were confirmed to be lacZ-positive, in accordance with the known localization of ERβ expression in the testis. The present study suggests that a 2.2 kbp of the 5′f region of the ERβ gene is able to direct testis-specific expression, but is not itself sufficient to determine the expression in other organs.

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1. Introduction

Estrogen-dependent biological function is mediated by two subtypes of estrogen receptor (ER), α and β [1]. ERα mediates classical estrogen responses, such as uterine growth and mammary gland development. In addition, ERα function is essential for sperm fertilization in the testis [2,3]. ERβ, on the other hand, is necessary to maintain a normal ovulation frequency in the ovary and plays anti-proliferative roles in the prostate and mammary glands. While significant amounts of ERα are present in a variety of sites, including bone, liver and skeletal muscle, as well as reproductive tissues, ERβ distribution seems more limited, with significant expression only in thymus, testis, prostate and ovary. Previous studies have indicated that transcription of the ERα gene is determined by multiple untranslated first exons and promoters [4–6]. Six untranslated first exons have been identified in the human case and four in the rat and the mouse [7–9]. The transcripts are generated by differential promoter usage and differ in the 5′ untranslated exon 1 because of alternative splicing events. In addition, intronic promoter activity was recently found to be involved in the transcription of variant ERα [10]. In the case of ERβ, two isoforms of human ERβ mRNA containing different untranslated 5′-ends have been reported, suggesting the existence of two distinct promoter structures for the gene [11,12]. On the other hand, our previous study and others have demonstrated that rat and mouse ERβ mRNA has a single end structure [13,14]. About 0.7 kbp of the 5′ flanking (5′f) region of ERβ was determined in both

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species and found to be highly homologous. *In vitro* reporter gene analysis demonstrated that the region determines basic as well as cell-specific transcriptional activity in both species.

Although *in vitro* studies have suggested that the 5′f region of ERβ gene may be involved in tissue-specific distribution of ERβ, the question of whether it is sufficient for *in vivo* transcriptional regulation has yet to be addressed. In the present study, therefore, promoter activity of the 5′f region of ERβ gene was examined *in vivo*. The 2.2 kbp region from the transcription starting site was cloned and connected into a lacZ reporter gene to create transgenic mice. The reporter expression was determined by quantitative RT-PCR, as well as lacZ staining, in individual tissues.

2. Materials and methods

2.1. Animals

Animal experiments were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals of Hiroshima University and The Animal Welfare Regulations of Yamagata University School of Medicine. BDF1 mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and maintained under constant conditions with free access to basal diet and tap water. Animals were sacrificed under anesthesia and tissues were dissected out, and immediately fixed in RNA Later solution (Ambion, Inc., Austin, TX, USA) for RNA extraction. Portions of the tissues were fixed in 2% formaldehyde/0.2% glutalaldehyde fixation solution, incubated in 20% sucrose solution and embedded in OTC compound (Miles Inc., Elkhart, Indiana, USA) for frozen sectioning.

2.2. Cell culture

The rat prostate cell line, DT3, was maintained in DME medium (Sigma Chemical Co., St. Louis, MO, USA) containing penicillin and streptomycin with 5% FBS (Gibco/Invitrogen Corp., Carlsbad, CA, USA). For hormone treatment, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal treated sera.

2.3. Genomic DNA library screening

To isolate a genomic 5′f region of ERβ exon 1, a 129SVJ library in λFIX II (Stratagene, La Jolla, CA, USA) was screened with a 809-bp cDNA probe containing the previously reported exon 1 and 5′f region of mERβ (Genbank accession AB034985). Two clones were purified and sequenced to the transcriptional starting site with an ABI model 310 sequencer (PerkinElmer Life Sciences, Boston, MA, USA).

2.4. Construction of luciferase reporter plasmids and transient transfection

Truncated fragments of the 5′f region of mouse ERβ were prepared by accurate PCR with LA-Taq (Takara Bio Inc., Ohtsu, Japan) between −2145, −1343, −692, −149, −60 and +193 (relative to the transcriptional starting site) from the cloned fragment. Each was cloned into PCR2.1-TOPO vector (Invitrogen) and then SacI/XhoI fragments were inserted into the same restriction enzyme site of the pGL_3-basic luciferase reporter plasmid (Promega Co., Madison, WI, USA). phRL-CMV (Promega) was employed as an internal control. DT3 cells were plated at 2×10^4 per well in 48-well plates and transiently transfected with 300 ng of a reporter and 5 ng of phRL-CMV with TransFast transfection reagent (Promega). The weight ratio of TransFast reagent to DNA was 1:1. After 24 h incubation, cells were harvested with 30 μl of cell lysis buffer (Promega) and the firefly and renilla luciferase activities were determined with a Dual Luciferase Assay Kit (Promega) by measuring luminescence with a Wallac Micro-Beta Scintillation Counter (PerkinElmer Life Sciences). Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV.

2.5. Construction of lacZ reporter transgene and generation of transgenic mice

A pUC18-based reporter plasmid, placF, containing a nuclear localization signal (nls), lacZ, and mouse protamine polyA was a gift from Richard Palmiter at the University of Washington, Seattle, WA, USA [15]. A 2.3-kb fragment of the 5′f region of mouse ERβ (−2145 to +193, relative to the transcriptional starting site) was cut out from the pCR2.1-TOPO vector and inserted into placF. KpnI/HindIII digestion gave a 6.2 kb fragment of ERβ 5′f connected to nls-lacZ-poly(A). The construct was microinjected into the pronuclei of single-cell fertilized mouse embryos to generate transgenic mice, as previously described [16,17]. To detect the exogenous lacZ gene, genomic DNA was extracted from the tail tissues of pups, and PCR was performed with one primer specific for the ERβ 5′f region and another primer specific for lacZ.

2.6. Quantification of mRNA by real-time RT-PCR

Details were as described previously [18]. Total RNA was isolated from tissue and reverse-transcribed. A QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (Perkin-Elmer Co.) were employed for quantification of cDNA according to the supplied protocol. The following specific forward and reverse PCR primers with a Tm of about 59 °C were designed: 5′-CGATCTTCCTGAGGCCGATAC and 5′-TGTGAGCGAGTAACAACCCGT for lacZ (+231 to +381, 151 bp) and 5′-TCTGGACACCTCTCTCCTTT and 5′-CAACCGCTCCCGCCAAGCTT for mouse ERβ (+112 to +263, 152 bp) and 5′-CTGTCCCTGTATGCCTCTGGTC and 5′-TGAGGTAGTCCGTCAGGTCCC for mouse β-actin

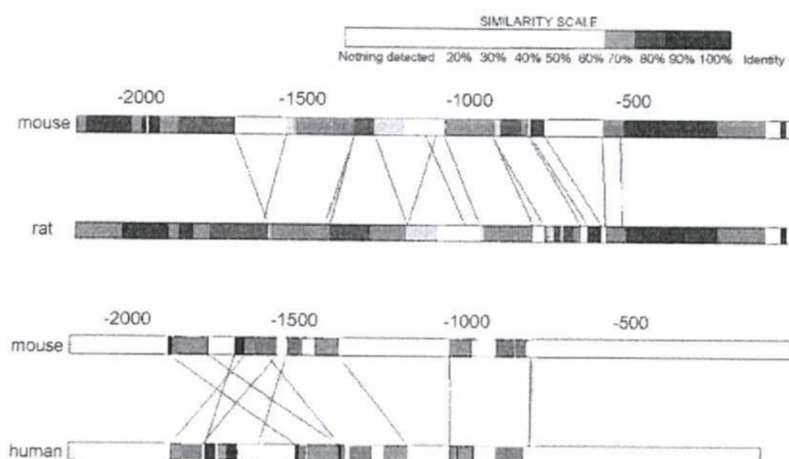

Fig. 1. Sequence comparison of the 5′f regions of mouse, rat and human ERβ. The sequences of 2.2 kbp of the 5′f region of mouse and rat ERβ (DQ273590 and DQ273589) and the corresponding human region (AF191544) were compared by using LFasta (Pôle Bioinformatique Lyonnais).

(+340 to +490, 151 bp). Measured mRNA contents were normalized with reference to β-actin mRNA.

2.7. Quantification of the transgene copy number

The gene copy number of lacZ was also determined with the real-time PCR based method described by Kindich et al. [19]. The genomic DNAs from tails were subject to real-time PCR analysis. The same primer set above was used for the determination of lacZ gene. Androgen receptor gene, a single copy gene in male animals, was employed as a reference. The PCR primers were 5′-CACCATGCAACTTCTTCAGCA (504–524) and 5′-TGAATTGCCCCCTAGGTAACT (+565 to +730 in exon 1, 191 bp).

2.8. Histochemical analysis of lacZ expression in mouse tissues

Sections (40 μm thick) from frozen tissues embedded in OCT compound were mounted onto microscope slides. They were fixed in 0.2% glutalaldehyde in PBS and incubated in PBS containing 1 mg/ml X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$. The reaction was stopped by washing with PBS and the sections were counterstained with 1% eosin, dehydrated in alcohol, treated with xylene, and mounted in Eukitt (O. Kindler GmbH, Freiburg, Germany).

2.9. Statistical analysis

Statistical comparisons were made using Student's *t*-test.

3. Results

3.1. Genomic structure of the 5′ flanking region of mouse ERβ

Two lamda phage DNAs containing a 5′f region of mouse ERβ were obtained from the 129SVJ mouse genomic library. A 2827 bp region from the transcription starting site was sequenced (GenBank accession DQ273590) and compared with the 5′f regions of rat ERβ (DQ273589) and human ERβ (AF191544) by use of the Local Alignment Tool, LFasta, at Pôle Bioinformatique Lyonnais (http://www.pbil.univ-lyon1.fr/pbil.html). High homology is evident between mouse and rat (70–90% similarity throughout the region), but there is only a limited sequence homology of the rodent and human ERβ promoter regions (Fig. 1). Motif analysis (http://www.motif.genome.jp/) showed the 5′f region to contain the consensus promoter motifs AML-1a, SRY, GATA-3 and cdxA (Fig. 2A).

3.2. In vitro promoter activity of mERβ-5′f

The results of examination of the promoter activity of the cloned 5′f region and some of the deletion fragments of mERβ are summarized in Fig. 2B. The luciferase activity of ERβ-5′f (2145/+193)-luc was similar to those of the truncated reporters −1343/+193, −692/+193 and −149/+193, while no activity was seen with −60/+193.

3.3. Generation of mERβ-5′f-lacZ-transgenic mice and lacZ mRNA levels in different tissues at 10 weeks of age

After microinjection and embryo implantation, three lines of transgenic mice (lines 420, 481 and 561) were successfully established. No neonatal or adult deaths were observed in mERβ-5′f-lacZ mice. Quantitative PCR of lacZ in genomic DNA showed that the numbers of transgene copies were 2, 5–6, and 4 per genome in lines 420, 481 and 561, respectively. Table 1 shows the lacZ and ERβ mRNA levels in several tissues of each line of F1 mice at 10 weeks of age. While high ERβ mRNA levels were evident in the epididymis, lung, ventral prostate, testis and ovary, significant lacZ mRNA expression was noted only in testis, although the levels differed among the transgenic mouse lines. In lines 481 and