Effects of Stilbene and Related Compounds on Reproductive Organs in B6C3F1/Crj Mouse

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trans-Stilbene exhibits estrogenic activity in an estrogen reporter assay after metabolic activation. In this study, uterotrophic assay was conducted using ovariectomized B6C3F1/Crj female mice treated with *trans*-stilbene, *trans*-4-hydroxystilbene, *trans*-4,4′-dihydroxystilbene and diethylstilbestrol. Administration of *trans*-4-hydroxystilbene, *trans*-4,4′-dihydroxystilbene and diethylstilbestrol elicited increases in absolute and relative uterus weight. Furthermore, the uterine response caused by the hydroxylated stilbenes was accompanied with an increase in the thickness of epithelial cell layers. *trans*-Stilbene itself also showed estrogenic activity, affecting uterine weight and causing histological changes of the uterus. This suggests that *trans*-stilbene is activated to hydroxylated metabolites to exhibit its estrogenic activity. Indeed, *trans*-hydroxystilbenes were detected in the urine and feces of mice dosed with *trans*-stilbene. The effect of stilbene derivatives on testis in newborn B6C3F1/Crj mouse was examined. Administration of diethylstilbestrol slightly decreased the testis weight and atrophy of seminiferous tubules. However, *trans*-stilbene and *trans*-4-hydroxystilbene affected neither testis weight nor the histological appearance of the testis.

Key words —— *trans*-stilbene, hydroxystilbene, diethylstilbestrol, uterotrophic assay, proestrogen, testis

INTRODUCTION

Stilbene derivatives, such as diethylstilbestrol (DES), resveratrol, euvestin, 4,4′-diaminostilbene and pinosylvin, have a variety of biological actions, including hormonal, hypocholesterolemic, sympathomimetic, antifungal, antiallergic, antibacterial, antimalarial and anticancer activities.[1–4] DES is the most well-known stilbene derivative, and has been used medically as a substitute for endogenous estrogen and as hormonal therapy for prostate or breast cancer, and also to prevent threatened abortions.[5,6] However, it may induce vaginal adenocarcinoma.[7] Stilbene derivatives such as resveratrol (*trans*-4,3′,5′-trihydroxystilbene) are synthesized by stilbene synthase, which utilizes 3-malonyl-CoA and a starter CoA ester such as *p*-coumaroyl-CoA, in plants.[8,9] Some hydroxystilbenes and their oligomeric derivatives play an important role as stilbenoid phytoalexins, and have been implicated in the process of induced resistance in some plants.[8] Resveratrol, which is found in grapes, peanuts and pines, and also grape products such as wine, exerts potent antioxidant and anti-inflammatory activities.[10,11] This hydroxylated stilbene inhibited tumor formation in mammary glands and skin in mice exposed to dimethylbenz[*a*]anthracene. It is also an antagonist at the aryl hydrocarbon receptor, and has estrogenic and antiestrogenic activities.[12–14]

Recently, we examined the estrogenic activity of *trans*-stilbene using ERE-luciferase reporter assay in MCF-7 cells and estrogen-responsive growth assay in MtT/E-2 cells. In that study, we found that *trans*-stilbene was not estrogenic, but exhibited potent estrogenic activity after metabolic activation by the liver microsomal oxidation system, and we suggested that the estrogenic activity was due to hydroxylated metabolites formed by cytochrome P450 1A1/2.[15,16] Estrogens have been shown to have multiple sites of activity and to exert various biological actions. Many of the environmental estrogens are known to produce a wide variety of toxic effects in animals. They may play a role in the increasing incidence of breast cancer, testicular cancer, and other

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problems of the reproductive system in humans. In this study, we investigated potential estrogenic activities of *trans*-stilbene and its derivatives, including its metabolites, using *in vivo* uterotrophic assay in ovariectomized (OVX) female B6C3F1/Crj mouse. We also examined the influence of these compounds on testis in newborn male mouse.

MATERIALS AND METHODS

Chemicals —— 17β-Estradiol was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.), *trans*-stilbene (99%) and DES (99%) from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and *trans*-4-hydroxystilbene (98%) from Lancaster Synthesis Ltd. (Lancashire, England). *trans*-4,4′-Dihydroxystilbene (mp. 275–278°C, 99% pure) was synthesized by the previously reported method.[15]

Animals —— Female B6C3F1/Crj, male C3H/HeNCrj and female C57BL/6NCrj mice (20–22 g) were obtained from Charles River Japan (Hino, Japan). The animals were housed at 22°C with a 12-hr light/dark cycle, with free access to tap water and a standard pellet diet MF (Oriental Yeast, Tokyo, Japan).

Uterotrophic Assay —— The procedure was based on the OECD protocol (OECD 1999). All animals were acclimatized to laboratory conditions for at least 1 week before ovariectomy, which was performed on 6-week-old animals. The ovary was pulled out and cut at the junction of the oviduct and the uterus body. After confirming that no massive bleeding had occurred, the abdominal wall was closed by a needle with strings and then skin by autoclips. All animals were allowed to acclimatize for at least 4 weeks after OVX. Test compounds (DES, *trans*-stilbene, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene) were dissolved in Panacete 810 (a mixture of medium-chain triglycerides, Nippon Oils and Fats Co., Ltd., Tokyo, Japan) as a vehicle. All animals were dosed once per day for 3 days by intraperitoneal injection and were killed 24 hr after the last dose. Dosing solutions were prepared in Panacate at a concentration of 0.1, 1 or 3 mg/ml and used at a dosing volume of 0.2 ml/20 g body weight. The dose level of DES, used as a positive control, was 0.1 mg/kg body weight. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterus horns, and weighed. The uterus was placed in 10% buffered formalin solution, and paraffin sections were prepared. They were stained with an anti-PCNA antibody (Dako Co., Kyoto, Japan) used with the avidin-biotin complex method, and stained with hematoxylin and eosin for histological examination.

Effect of Stilbenes on Testis —— Male C3H/HeNCrj mice were mated with female C57BL/6NCrj mice for 1 week for the purpose of copulation. The newborn pups on postnatal day 1 (PND1) were dosed once by subcutaneous injection. Dosing solutions were prepared in Panacete at a concentration of 100 or 300 mg/ml and used at a dosing volume of 0.02 ml/2 g body weight. The dose levels of DES, used as a positive control, were 3 and 30 mg/kg body weight. After 8 weeks, all animals were killed, and testes were taken. In addition, one testis from each mouse was fixed in Bouin solution containing saturated picric acid solution, 5% formalin and 1% acetic acid for 24 hr. Paraffin sections were prepared and stained with hematoxylin and eosin for histological examination.

Determination of *in Vivo* Metabolites of *trans*-Stilbene in Mice —— *trans*-Stilbene was given intraperitoneally to male mice at a single dose of 50 mg/kg. Collected urine (1 ml) was supplemented with 0.1 ml of 2 N HCl and 0.1 μmol of phenothiazine (an internal standard). The urine was extracted twice with two volumes of ethyl acetate and the combined extracts were evaporated to dryness. The extract was analyzed by high-performance liquid chromatography (HPLC). The urine (1 ml) was also incubated with β-glucuronidase 3000 units/arylsulfatase 24000 units in 1 ml of 0.1 M citrate-phosphate buffer (pH 6.0) at 37°C for 16 hr. After the incubation, the mixture was extracted as described above. The feces (1 g) were dried, pulverized in a mortar and extracted twice with 10 volumes of methanol by sonication and shaking for 20 min each. The combined extracts were passed through a Florisil (Wako Pure Chemical Industries Ltd., Osaka, Japan, 5 × 30 mm) column, and analyzed by HPLC, using a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 250 × 4.6 mm Shiseido Capcell Pak C18 type UG 120A 5 μm column (Shiseido Co., Ltd. Tokyo, Japan). The mobile phase was acetonitrile-water (1 : 1, v/v). The chromatograph was operated at a flow rate of 1.0 ml/min at a wavelength of 254 nm. The elution times of *trans*-4,4′-dihydroxystilbene, *trans*-4-hydroxystilbene, phenothiazine (an internal standard) and *trans*-stilbene were 5.5, 15.3, 22.2 and 56.5 min, respectively.

Statistics —— Results are expressed as the mean ± S.D. Multiple comparison was made by analysis of

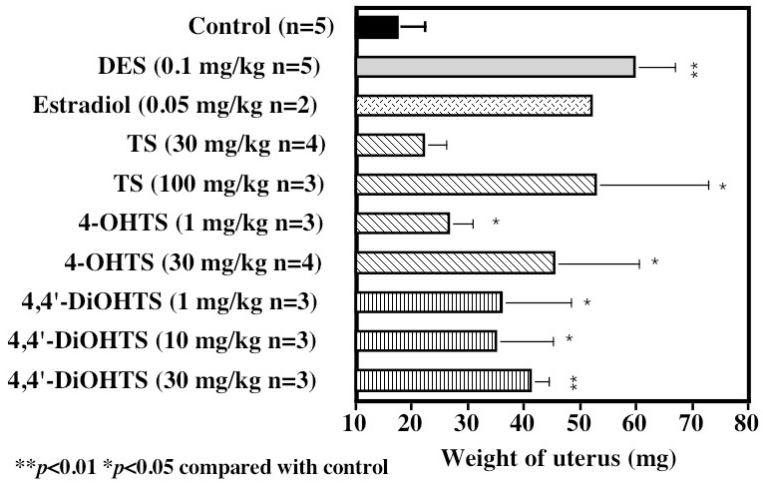

Fig. 1. Effect of *trans*-Stilbene (TS), its Metabolites, and DES on Uterus Wet Weight in OVX Mouse

Each bar represents the mean ± S.D. Animals received a single daily dose for 3 days and were killed 24 hr after the last dose. Control (Panacete), DES (0.1 mg/kg), TS (30 and 100 mg/kg), *trans*-4-hydroxystilbene (4-OHTS; 1 and 30 mg/kg) and *trans*-4,4′-dihydroxystilbene (4,4′-DiOHTS; 1, 10 and 30 mg/kg) groups were used. Significant differences between the control group and treated groups are indicated (*$p < 0.05$, **$p < 0.01$).

variance (ANOVA) followed by Scheffe's test.

RESULTS

Uterotrophic Test of Stilbene and Related Compounds

Previously, we reported that *trans*-stilbene was oxidized to 4-hydroxyl and 4,4′-dihydroxyl derivatives by cytochrome P450 1A1/2 in rat liver microsomes, and was thereby activated to exhibit estrogenic activity.[15,16] Fifty percent effective concentration values (EC50) of 17β–estradiol, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene in estrogen responsive element (ERE) luciferase reporter assay using MCF-7 were 1.3×10^{-12} M, 1.0×10^{-7} M and 8.0×10^{-8} M, respectively.[17] To confirm the estrogenic potential of *trans*-stilbene and its metabolites in female reproductive tract, we evaluated the uterotrophic activities of *trans*-stilbene, *trans*-4-hydroxystilbene, *trans*-4,4′-dihydroxystilbene, and DES as a positive control using the test protocol recommended by the OECD (1999). These compounds were intraperitoneally injected once per day for 3 days into OVX mouse. As shown in Fig. 1, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene increased the uterus weight at 1.0 mg/kg/day or higher concentrations. DES (0.1 mg/kg/day) caused an increase in uterus weight of approximately threefold compared to control (Panacete-treated) mice. On the other hand, administration of *trans*-stilbene (100 mg/kg) significantly increased uterus weight.

Histological Changes of Uterus of Mice Dosed with Stilbenes

Histological changes in uterus of OVX mouse induced by 3-day treatment with DES and other stilbene derivatives were examined. Administration of DES, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxy-stilbene increased the luminal epithelial height and the thickness of the stromal layers in the uterus. Treatment with *trans*-stilbene (100 mg/kg/day) also altered the histological phenotype in the epithelial cells in OVX mouse (Figs. 2 and 3). As illustrated in Fig. 4, there was a high correlation between uterus weight and the thickness of epithelium in OVX mouse treated with stilbene derivatives.

Effect of Stilbenes on Newborn Pups

To evaluate the effect of stilbene derivatives on testis, these compounds were administered to newborn pups on PND1 by subcutaneous injection, and testis weights were measured after 8 weeks (Fig. 5). Administration of DES (3 and 30 mg/kg) caused a slight decrease in testis weight. However, *trans*-stilbene and *trans*-4-hydroxystilbene did not affect testis weight. The group treated with *trans*-4,4′-dihydroxystilbene died within 1 week after treatment. Fig. 6 shows the histological changes in testis of mouse induced by treatment with DES, *trans*-stilbene and *trans*-4-hydroxystilbene. DES (3 and 30 mg/kg) induced atrophy of seminiferous tubes, and

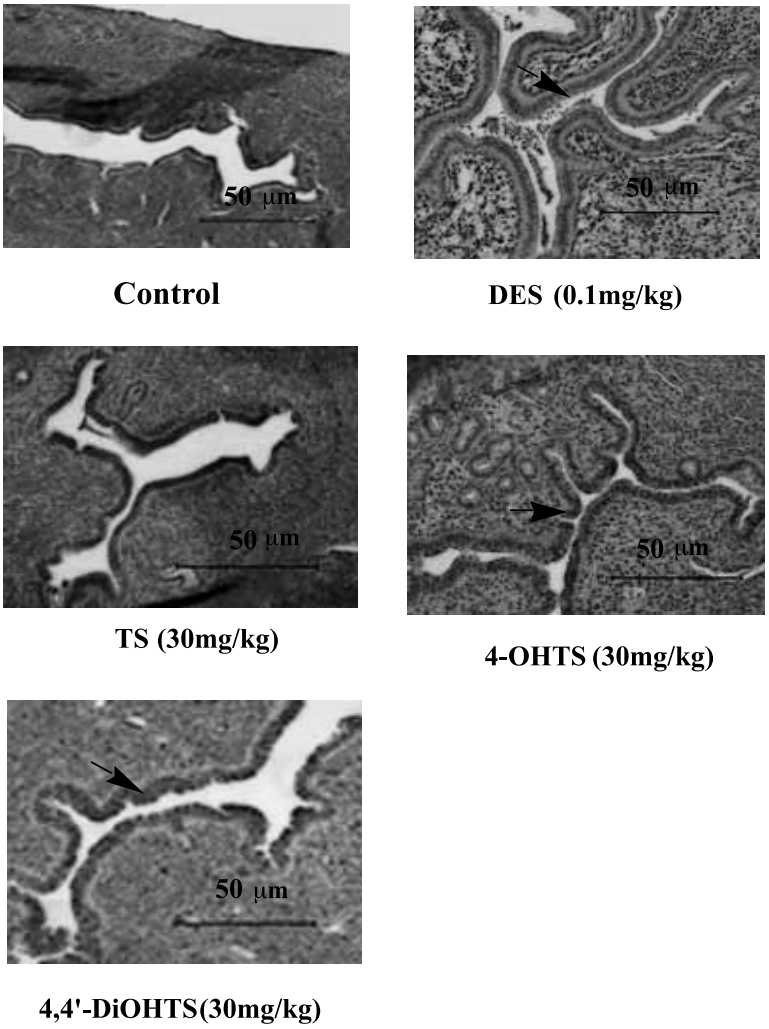

Fig. 2. Pathological Changes in Uterus Epithelium of OVX Mouse

Control (Panacete), diethylstilbestrol (DES; 0.1 mg/kg), *trans*-stilbene (TS; 30 mg/kg), *trans*-4-hydroxystilbene (4-OHTS; 30 mg/kg), and *trans*-4,4′-dihydroxystilbene (4,4′-DiOHTS; 30 mg/kg) groups were used. Luminal epithelium in the uterus is indicated by a closed arrow.

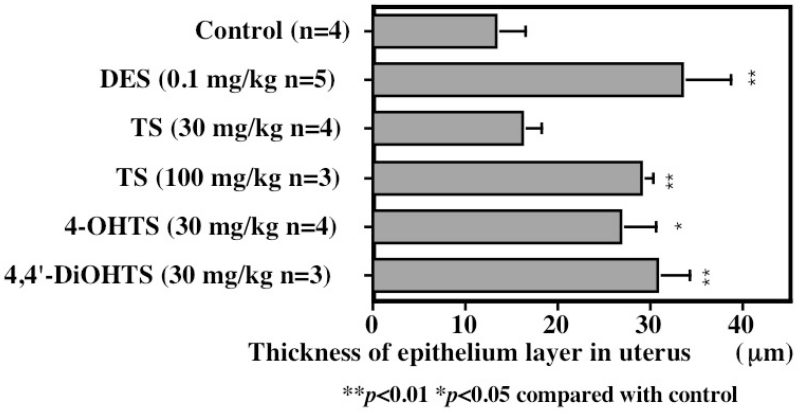

Fig. 3. Effect of TS, its Metabolites, and DES on Uterus Epithelium in OVX Mouse

Each bar represents the mean ± S.D. The luminal epithelial height and the thickness of stromal layers of uterus was measured in animals treated with DES, TS, *trans*-4-hydroxystilbene (4-OHTS) and *trans*-4,4′-dihydroxystilbene (4,4′-DiOHTS).

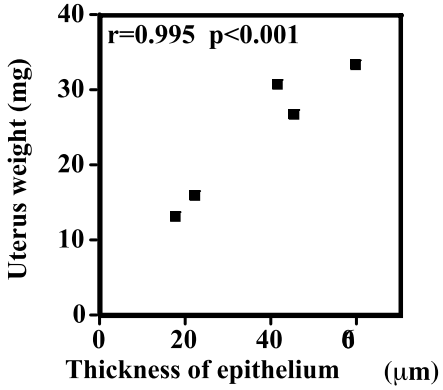

Fig. 4. Correlation between Uterus Weight and Thickness of Epithelium in OVX Mouse

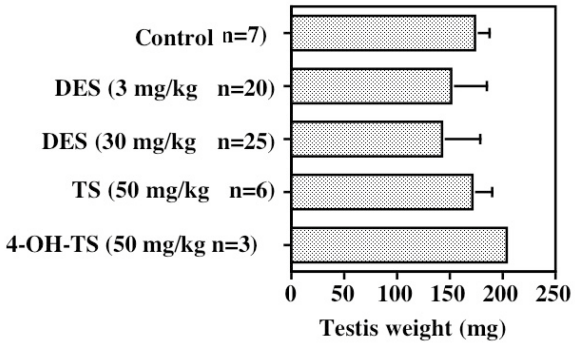

Fig. 5. Effect of TS, its Metabolites, and DES on Testis Weight in Newborn Mouse

Each bar represents the mean ± S.D. Animals received a single daily dose at PND 0 and were killed 8 weeks after the last dose. Control (Panacete), DES (3 and 30 mg/kg), TS (50 mg/kg) and *trans*-4-hydroxystilbene (4-OHTS; 50 mg/kg) groups were used.

tubular atrophy in seminiferous tubules (Fig. 7). *trans*-Stilbene and *trans*-4-hydroxystilbene did not alter the histological phenotype in testis at this dosage. When these compounds were subcutaneously injected once into mature mouse, none of them caused any change in the weight or histology of the testis (data not shown).

in Vivo Metabolism of *trans*-Stilbene

When *trans*-stilbene was intraperitoneally administered to male mice, *trans*-stilbene and its metabolites were detected in the HPLC chromatogram of the urine and feces extracts. Retention times of the peaks corresponded to those of authentic *trans*-stilbene, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene. The identity of these hydroxylated metabolites isolated by HPLC was confirmed by comparison of the mass and UV spectra with those of authentic samples (data not shown). The amounts of the metabolites excreted in the urine and feces are shown in Table 1. Conjugated hydroxylated metabolites were determined after treatment of the urine with β-glucuronidase/arylsulfatase. The results are consistent with view that *trans*-stilbene exhibits estrogenic activity after activation to its hydroxylated metabolites *in vivo*.

DISCUSSION

trans-Stilbene is used as an industrial raw material for stilbene dyes and fluorescent brightening agents, and its derivatives, such resveratrol, are biosynthesized in plants in response to fungal attack or other stress conditions.[9] Previously, we presented evidence that *trans*-stilbene is converted to an active estrogen by liver microsomal enzymes. The estrogenic activity of *trans*-stilbene in rats *in vivo* seems to be a typical example of the metabolic activation of a proestrogen. It will be necessary to consider the activity of metabolites produced from the parent compounds for the assessment of the toxicity of stilbene derivatives. In rats and rabbits *in vivo*, *trans*-4-hydroxystilbene, *trans*-4,4′-dihydroxystilbene and *trans*-3,4,4′-trihydroxystilbene have been found as urinary metabolites of *trans*-stilbene.[18] 4,4′-Dihydroxydibenzyl was also identified as a metabolite.[19] We have also found that in mouse, *trans*-stilbene is converted to *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, and to their glucuronide- or sulfate-conjugated metabolites as shown in the metabolism of resveratrol.[20] Interconversion between *trans*-stilbene and *trans*-stilbene oxide may also occur.[21] *trans*-Stilbene seems to be transformed by cytochrome P450 1A1/2 to hydroxylated metabolites, which exhibit estrogenic activity *in vivo* (Fig. 8).

The uterotrophic action of estrogenic compounds in OVX mouse increases the uterus wet weight, which is an end point utilized in the standard *in vivo* assay for estrogenicity. Uterus wet weight and cytological changes have been used extensively as indicators of uterus response to estrogen-like effects.[22] In fact, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, as well as DES, caused increases in uterus weight and thickness of epithelium. Since these were a high correlation between the changes of uterus weight and thickness of epithelium, we suggest that the increase in uterus weight is largely attributable to the increase in thickness of epithelum

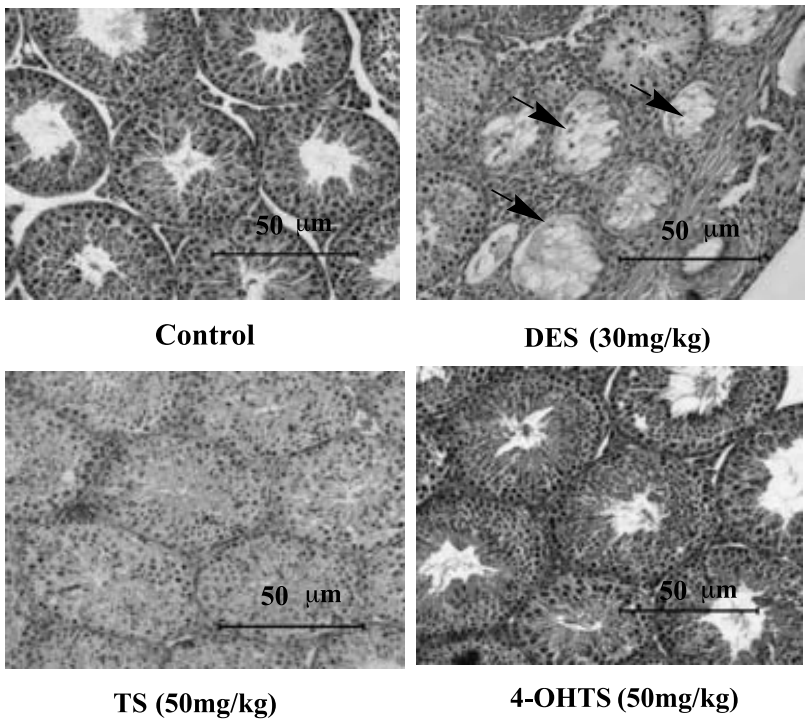

Fig. 6. Pathological Changes in Testis of Newborn Mouse

Control (Panasate), DES (30 mg/kg), TS (50 mg/kg) and 4-OHTS (50 mg/kg) groups were used. Atrophy of seminiferous tubules is indicated by closed arrows.

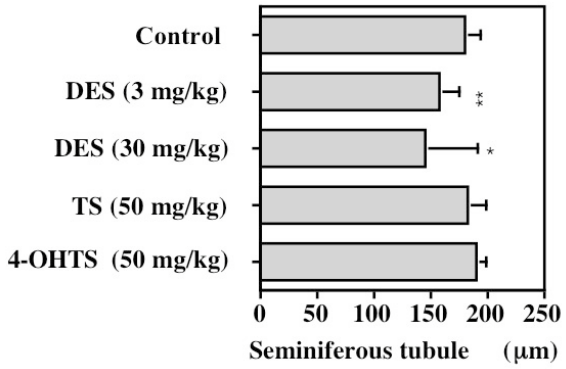

Fig. 7. Effect of TS, its Metabolites, and DES on Tubular Atrophy in Newborn Mouse

Each bar represents the mean ± S.D. Animals received a single daily dose at PND 1 and were killed 8 weeks after the last dose. The diameter of seminiferous tubules was measured. Control (Panasate), DES (3 and 30 mg/kg), TS (50 mg/kg) and 4-OHTS (50 mg/kg) groups were used. Significant differences between the control group and treated groups are indicated (*$p < 0.05$, **$p < 0.01$).

owing to absorption. There is a brief report that *trans*-stilbene showed a positive estrus response on vaginal smears in OVX rats after six doses of 25 mg/kg over three days.[23] In MCF-7 luciferase reporter assay, ICI 182780, a pure estrogen receptor antagonist, markedly inhibited the estrogenic activity of *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene.[15] ICI 182780 also inhibited estrone-stimulated uterine weight increase in OVX mouse.[24] These findings suggest that the uterotrophic effects of *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene are mediated through the estrogen receptor. Xeno-estrogenicity has been reported to be related to the presence of a phenolic ring, since phenolic compounds interact with the estrogen receptor in a similar manner to endogenous estrogens.[25–27] Treatment with *trans*-stilbene at the high dose of 100 mg/kg increased uterus weight and thickness of the epithelium, suggesting that *trans*-stilbene may be a proestrogen that is activated to *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene via aromatic hydroxylation by cytochrome P450. However, these metabolites would be rapidly conjugated *in vivo* to inactive forms, as reported in bisphenol A and *p*-tert-octylphenol.[28–30] This may be the reason why *trans*-stilbene exhibited estrogenic activity *in vivo* only at a high dose. Other potentially proestrogenic aromatic compounds that may be activated to estrogens by hydroxylation *in vivo* include methoxychlor, benzophenone, benzo[*a*]pyrene, diphenyl, 2-nitrofluorene and styrene oligomers.[31–37] These compounds, which are

Table 1. Amounts of Oxidative Metabolites Excreted in Urine and Feces from C3H Mice Given *trans*-Stilbene

Sample	% of Administered dose				Total
	24 hr	48 hr	72 hr	96 hr	
Urine					
4,4′-DiOHTS					
free	N.D.	N.D.	0.107	N.D.	0.107
conjugated	0.039	0.038	0.155	0.526	0.758
4-OHTS					
free	0.014	0.007	0.011	N.D.	0.032
conjugated	0.370	0.047	0.0001	N.D.	0.417
TS	0.050	0.044	0.038	0.056	0.188
Total	0.473	0.136	0.311	0.582	1.502
Feces					
4,4′-DiOHTS free	0.184	0.123	N.D.	N.D.	0.307
4-OHTS free	0.606	0.752	0.171	0.012	1.541
TS	0.154	0.370	0.053	0.021	0.598
Total	0.944	1.245	0.224	0.033	2.446
Total	1.417	1.381	0.535	0.615	3.948

Each value represents the mean of three animals. TS was given intraperitoneally to male mice at a single dose of 50 mg/kg. The extract of urine and feces was analyzed by HPLC. Conjugated metabolites were determined after treatment of the urine with β-glucuronide/arylsulfatase. 4,4′-DiOHTS: *trans*-4,4′-dihydroxystilbene, 4-OHTS: *trans*-4-hydroxystilbene. N.D.; not detected.

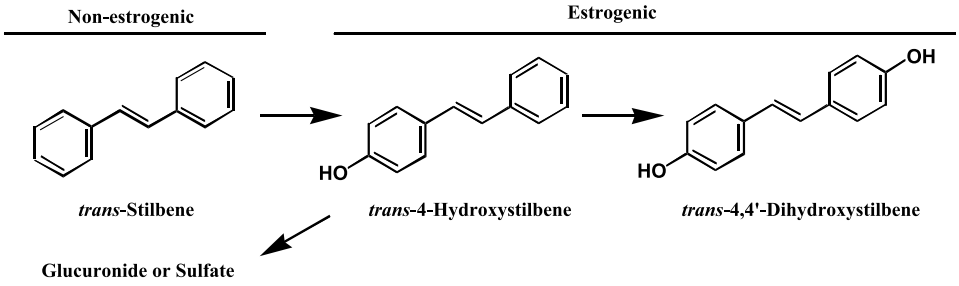

Fig. 8. Possible Metabolic Pathway for the Activation of *trans*-Stilbene in Mice *in Vivo*

present as enviromental pollutants are negative in estrogen screening tests, but the possibility should be considered that they may also be metabolically activated to xeno-estrogens via hydroxylation in the body. Much further work is needed to identify potentially hazardous proestrogens in our environment.

We also investigated the effect of stilbene derivatives on testis in male newborn mouse. When DES was administered at a dose of 3 or 30 mg/kg, decreased testis weight and vacuolation of spermatids in the testis were seen at 8 weeks after the treatment. Furthermore, we observed tubular atrophy. Marked atrophy of the seminiferous tubules has been observed in the testis of rats exposed to dibromoacetic acid and 1,3-dinitrobenzene, which are thought to be non-estrogenic.[38,39] Estradiol decreased testicular spermatid numbers and epididymal sperm numbers, and caused atrophy of epididymal tubules in male rats given ppm-level dietary exposure.[40] It is also known that neonatal exposure to DES (0.37 mg/kg/day or less for 18 days) can alter the structure of the testicular excurrent ducts, while change in testis weight, distension of the rete testis and efferent ducts, changes of epithelial cell height in the efferent ducts and immunoexpression of the water channel aquaporin-1 have been observed in adult rats.[41] However, we did not observe the above end points. Frederick *et al.*[42] reported that DES induced prostate enlargement in mice subjected to low-dose fetal exposure, but inhibited prostate

development at high doses. It is difficult to establish whether these effects were direct or indirect. Our current study at least indicates that exposure to DES at high dose causes testicular damage in newborn mouse. On the other hand, administration of *trans*-stilbene and *trans*-4-hydroxystilbene had no detectable effect on any parameter examined. It is difficult to assess the action of these chemicals on testis unlike uterotrophic assay, because of the effect of the blood-testis barrier and the influence of negative feedback by the hypothalamus-pituitary system. Administration of stilbene derivatives to adult mouse did not cause histological changes. We suggest that hydroxylated stilbenes such as *trans*-4-hydroxystilbene and DES are transformed to non-estrogenic conjugates *in vivo*, because adults have a greater capacity for conjugation as compared with newborn mouse. However, there may be differences in metabolism between DES and hydroxylated stilbenes.

In conclusion, administration of *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, metabolites of *trans*-stilbene, increased uterus weight in OVX mouse. Non-estrogenic *trans*-stilbene may be converted to estrogenic hydroxystilbene *in vivo*.

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