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An *in Vitro* Reporter Gene Assay Method Incorporating Metabolic Activation with Human and Rat S9 or Liver Microsomes

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A metabolic activation system with an S9 fraction or liver microsomes was applied to a reporter gene assay *in vitro* for the screening of estrogenicity of chemicals. The endpoint (luciferase) was luciferase induction in cells transfected with a reporter plasmid containing an estrogen-responsive element linked to the luciferase gene. Compounds were applied to the reporter gene assay system after pretreatment or simultaneous treatment with an S9 fraction or liver microsomes. Both *trans*-stilbene and methoxychlor themselves showed no or little estrogenicity, but when they were treated with an S9 fraction or liver microsomes, they demonstrated strong effects, indicating their metabolites to be estrogenic. When four pyrethroid insecticides were subjected to this assay system, however, they showed no estrogenicity even with liver microsome or S9 mix treatment. © 2001 Academic Press

Key Words: **metabolic activation; metabolites; reporter gene assay; estrogen receptor; pyrethroid insecticides.**

Recently, it is a social concern that both man-made and naturally occurring compounds in the environment may cause adverse effects on the endocrine system of mammals, including human and wildlife. The main cause is considered to be agonistic or antagonistic effects on hormone receptors. Development of efficient and rapid assay systems is strongly to be desired, because there are a vast number of chemicals which must be examined for hormonal effects. So far the following assay systems have been reported for *in vitro* application: (i) receptor binding assays (1–3); (ii) an MCF-7 cell proliferation assay (E-screen) (4); (iii) reporter gene assays utilizing animal cell lines (5, 6); (iv) a reporter gene assay utilizing yeast cells (YES) (7); and (v) a yeast two-hybrid assay (8). However, the absence of a satisfactory metabolising system for use in *in vitro* established cell line systems limits the utility of such receptor mediated assays. It has been noted that PCBs, which may require metabolic activation to exert estrogenicity, are not active in *in vitro* assay systems (9) as is also the case with methoxychlor (10, 11). It is thus necessary to include a biotransformation step of compounds in the assay system to mimic the *in vivo* situation. To this end, we selected a reporter gene (luciferase) assay system with the estrogen receptor α using mammalian cells (MCF-7 and HeLa). Our reporter gene assay with metabolic activation was then employed to ascertain whether metabolism of four pyrethroid insecticides leads to estrogenicity, because they are among the most common pesticides in current use worldwide. In line with the earlier report of Saito *et al.*, their significant estrogenic or anti-estrogenic activity was not found (12).

In this study we incorporated metabolic activation with human and rat S9 or liver microsomes into a reporter gene assay to evaluate the estrogenicity of metabolites. We report the optimized metabolizing conditions and the species difference in the reporter gene responses with rat and human liver microsomes. We also made a comparison between the normal and Aroclor-1254 induced rat liver microsomes.

MATERIALS AND METHODS

Chemicals. Chemicals tested in the present study were: *trans*-stilbene (purity >98.0%), methoxychlor (purity >97.8%) and 17β-estradiol (purity >97.0%), purchased from Wako Pure Chemical Industries (Osaka, Japan), dimethyl sulfoxide (DMSO) from Kanto Chemical Co. (Tokyo, Japan), and DES from Nacalai Tesque (Kyoto, Japan). Four pyrethroid insecticides (Fenvalerate, *d*-Phenothrin, Permethrin, and Cypermethrin) were synthesized in Sumitomo Chemical Co. (Osaka, Japan) and their purity (>93%) confirmed by HPLC analyses. Human S9 and liver microsomes were purchased from XENO TECH (Kansas City, Kansas), and rat S9 and liver microsomes from Charles River Japan Inc. (Kanagawa, Japan). SKF525A and 1-aminobenztriazole were obtained from ULTRA-

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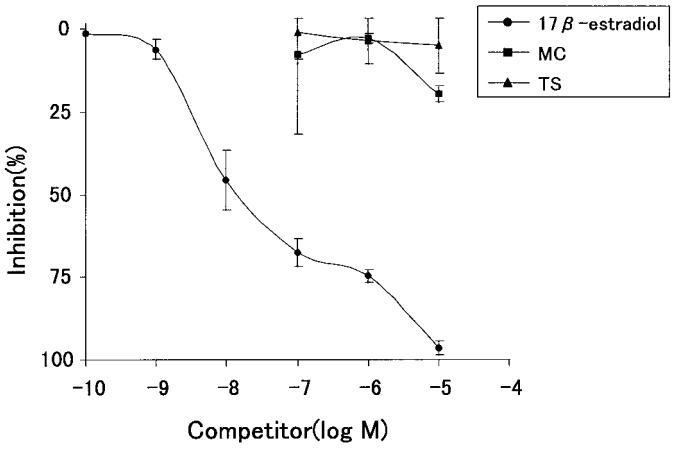

FIG. 1. Displacement of [^{3}H]estradiol from hERwith the fluorescence polarization method. Data presented are means ± SD (n = 3). TS, *trans*-stilbene; MC, methoxychlor.

FINE chemicals (Manchester, England), and Penicillin/Streptomycin from ICN Biomedicals Inc. (Aurora, Ohio).

Cell lines and cell culture conditions. MCF-7 cells purchased from ATCC (Rockville, MD) were maintained in Eagle MEM (Nissui Pharmaceutical Co., Tokyo, Japan) without phenol red and supplemented with 10% natural FCS (GIBCO-BRL, Rockville, MD) plus 6 ml of 3% L-glutamine (Wako Pure Chemical Industries), 9 ml of 10% sodium bicarbonate (Wako Pure Chemical Industries), 5.8 ml of 100 mM sodium pyruvate (ICN Biomedicals Inc.) and 5.8 ml of 10 mM nonessential amino acids (NEAA, ICN Biomedicals Inc.) at 37°C in an atmosphere of 5% CO_2/95% air at saturated humidity.

HeLa cells purchased from ATCC were maintained in Eagle MEM without phenol red and supplemented with 10% twice-dextran/charcoal-treated FCS plus 6 ml of 3% L-glutamine, 9 ml of 10% sodium bicarbonate at 37°C in an atmosphere of 5% CO_2/95% air at saturated humidity.

Vector construction. Human liver cDNAs were prepared from human liver mRNAs (Clontech Laboratories, Palo Alto, CA) with polydT primers (Pharmacia Biotech, Uppsala, Sweden) and Superscript II reverse transcriptase (GIBCO-BRL). The estrogen α receptor sequence (fragment with blunt ends) prepared from human liver cDNA by PCR was inserted into the pRc/RSV vector (Invitrogen, San Diego, CA). The resultant plasmid was named pRc/RSV-hER α. To construct the reporter plasmid, the herpes simplex virus thymidine kinase promoter sequence inserted into the pGV-P or pGL3 vectors (Toyo Ink, Tokyo, Japan) to give pGV-tk and pGL3-tk. The five tandem repeat synthetic oligonucleotide for the perfectly palindromic estrogen-responsive element (ERE) from the *Xenopus* vitellogenin gene was inserted into the pGV-tk and pGL3-tk vector, to give reporter plasmids pGV-tk-vEREx5 and pGL3-tk-vEREx5, respectively.

Incubation Procedure with S9 or Liver Microsomes

Pretreatment. 17β-estradiol and DES were dissolved in DMSO at the concentration of 100 nM and the other compounds at 10 mM. Then a 10 μl aliquot of each compound solution was mixed with 100 μl of 30 mM β-NADPH (Wako Pure Chemical Industries), 20 μl of S9 fraction or liver microsomes, 20 μl of Penicillin/Streptomycin (5000 I. U./ml and 5000 μg/ml) and 850 μl of 10 mM Hepes buffer (pH 7.2) (Wako Pure Chemical Industries), containing 12.5 mM $MgCl_2$ (Wako Pure Chemical Industries) and 82.5 mM KCl (Wako Pure Chemical

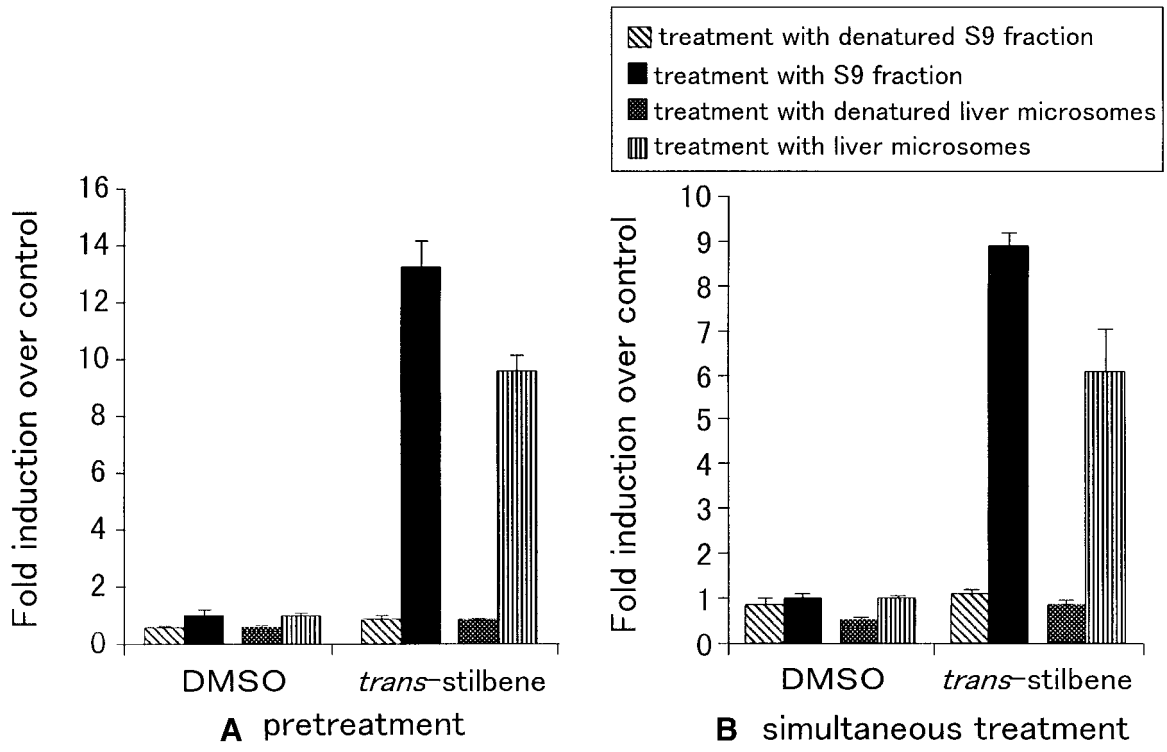

FIG. 2. Analysis of estrogenic activity of *trans*-stilbene and its metabolites by the luciferase reporter gene assay. HeLa cells were transiently transfected with the reporter plasmid pGV-tk-vEREx5 together with the expression vector pRc/RSV-hER. The chemical was treated with intact or denatured S9 or liver microsomes before addition to HeLa cells. Data presented are means ± SD (n = 4).

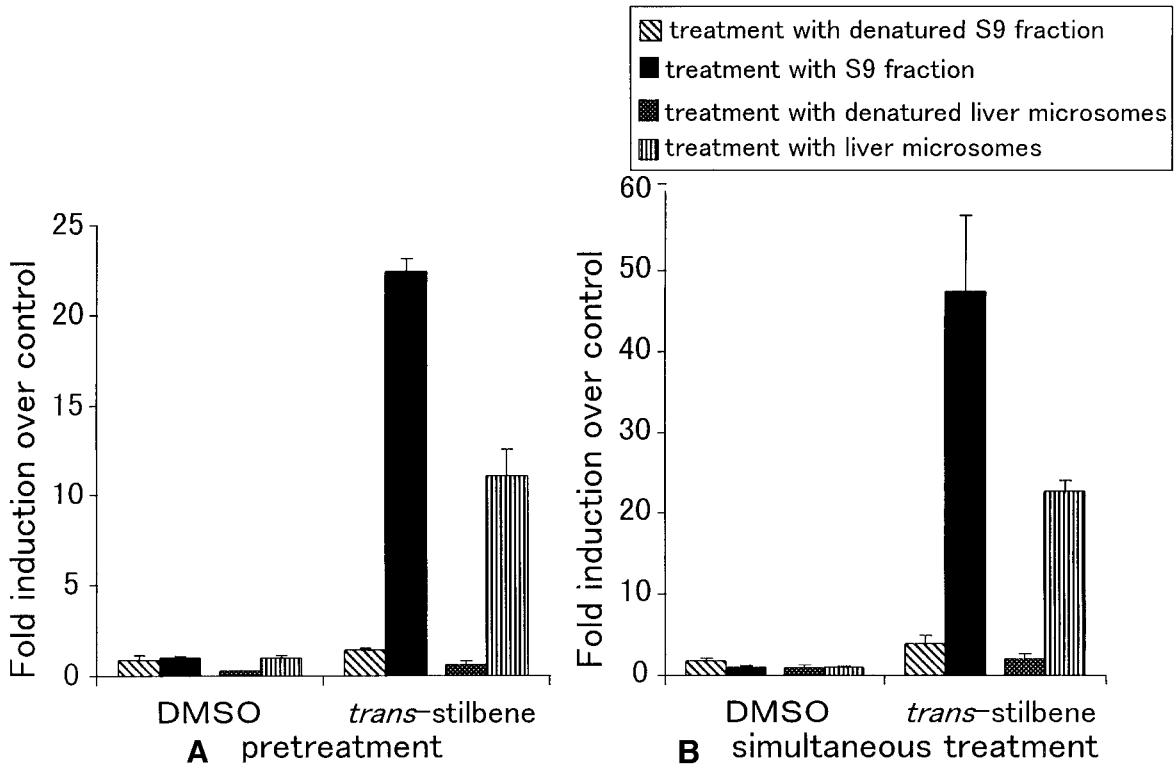

FIG. 3. Analysis of estrogenic activity of *trans*-stilbene and its metabolites by the luciferase reporter gene assay. MCF-7 cells were transiently transfected with the reporter plasmid pGV-tk-vEREx5 together with the expression vector pRc/RSV-hER. The chemical was treated with intact or denatured S9 or liver microsomes before addition to MCF-7 cells. Data presented are means ± SD (n = 4).

Industries). The total volume of the reaction mixture was 1 ml in a test tube, which was incubated and shaken in 37°C water bath overnight. As a control, S9 fraction or liver microsomes denatured at 95°C for 5 min was added to the reaction mixture. A 600 μl aliquot of reaction mixture was mixed with 2400 μl of fresh medium and added to the cells, with the final concentration of compound 1/1000 of that of the stock solution. To inhibit P450 activity, SKF525A was added to the reaction mixture at concentrations of 10 μM or 1-aminobenztriazole (ABT) at 100 μM.

Simultaneous treatment. The reaction mixture described above was prepared and mixed with fresh medium and immediately added to the cells. Briefly, 1 μl of the compound was mixed with 10 μl of 30 mM-NADPH, 2 μl of S9 fraction or liver microsomes, 2 μl of Penicillin/Streptomycin (5000 I. U./ml and 5000 μg/ml) and 85 μl of 10 mM Hepes buffer containing 12.5 mM $MgCl_2$ and 82.5 mM KCl. The total volume was 100 μl. As a control sample, S9 fraction or liver microsomes denatured at 95°C for 5 min were used. The reaction mixture was immediately mixed with 400 μl of fresh medium and added to the cells.

Reporter gene assay. Cells were plated at a density of 4×10^4 cells/well in 24-well multi-well plates with culture medium and incubated overnight. The medium for MCF-7 contained 10% twice-dextran/charcoal-treated FCS. The cells were transfected using lipofectin (GIBCO-BRL) or lipofectamine (GIBCO-BRL) in serum free medium following the manufacturer's protocol. HeLa cells were transfected with pRc/RSV-hERand the reporter plasmid. In the MCF-7 case, cells were transfected with the reporter plasmid only because this cell line intrinsically expresses estrogen receptors. Five hours after transfection, cells were incubated in 0.5 ml of fresh medium overnight. Compounds were added to the cells as the total volume was adjusted to 1 ml per well. The preparation of compounds is referred to under Incubation Procedure with S9 or Liver Microsomes. The cells were cultured for an additional 24–28-h period and thereafter washed twice with PBS(−) (Nissui Pharmaceutical Co.) after aspiration of the medium. Cell lysates were prepared by treatment with PicaGene cell lysis buffer (Toyo Ink). After 30 min at room temperature, luciferase activity was measured using a PicaGene kit (Toyo Ink) following the manufacturer's protocol with a Lumat LB 9501 luminometer from Berthold (Wildbad, Germany). Assays were carried out using not only 24-well but also 96-well multi-well plates (n = 4–8).

Analysis of metabolites. The reaction mixture consisted of 100 μl of 10 mM *trans*-stilbene or methoxychlor, 1000 μl of 30 mM-NADPH, 200 μl of S9 fraction or liver microsome and 8700 μl of 0.1 M potassium phosphate buffer (pH 7.4). Total volume was 10 ml. This reaction mixture was extracted with about 30 ml acetonitrile and centrifuged at 3000 rpm (1500g) for 10 min using a Himac CR 20B3 Superspeed Refrigerated Centrifuge and a RR14A-159 Rotor (Hitachi, Tokyo, Japan). The reaction mixture was further extracted twice with acetonitrile, then, supernatants were collected and extracts concentrated and fractionated by TLC (Art.5744, E. Merck, Damstadt, Germany) using hexane/ethyl acetate, 2/1 (v/v). Metabolites were analyzed by ^{1}H-NMR (GSX-270, JEOL, Tokyo, Japan) and EI-MS (AX-505W, JEOL, Tokyo, Japan).

Binding assay. The binding ability of *trans*-stilbene or methoxychlor to estrogen receptor α was measured with a Beacon 2000 (Takara, Kyoto, Japan) and an FP Screen-for-Competitor (Takara) Kit ER α following the manufacturer's protocol.

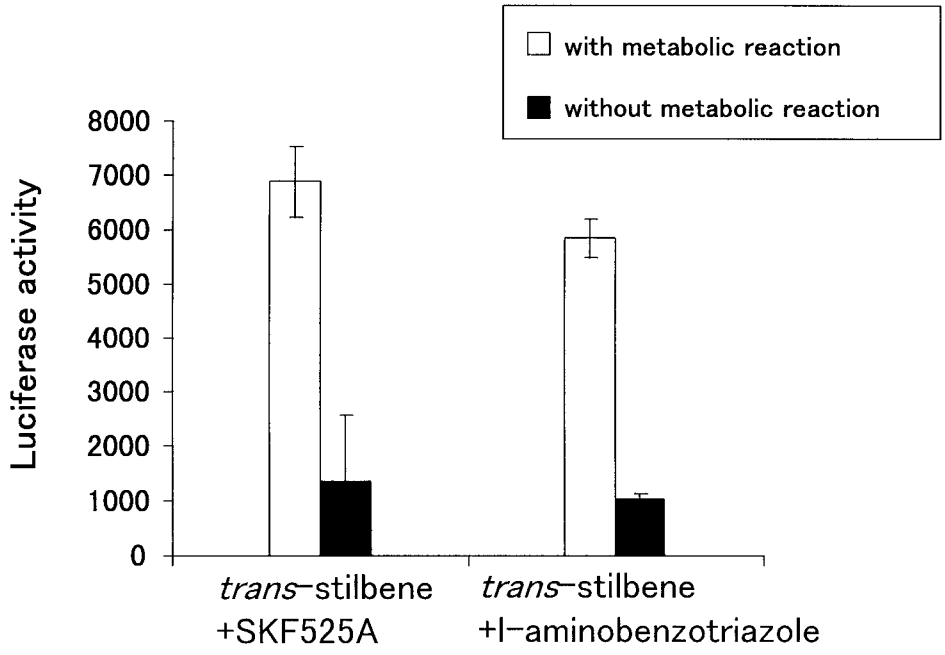

FIG. 4. Analysis of estrogenic activity of *trans*-stilbene and its metabolites by luciferase reporter gene assay. HeLa cells were transiently transfected with the reporter plasmid pGV-tk-vEREx5 together with the expression vector pRc/RSV-hER. The chemical was treated with intact S9, with or without additional SKF525A or 1-aminobenzotriazol before addition to HeLa cells. Data presented are means ± SD (n = 4).

RESULTS AND DISCUSSION

Estrogenicity of methoxychlor and trans-stilbene. It was found from analysis of metabolites in this study (data not shown) that both S9 fraction and liver microsomes demethylated methoxychlor, yielding mono-OH-methoxychlor and bis-OH-methoxychlor. These two

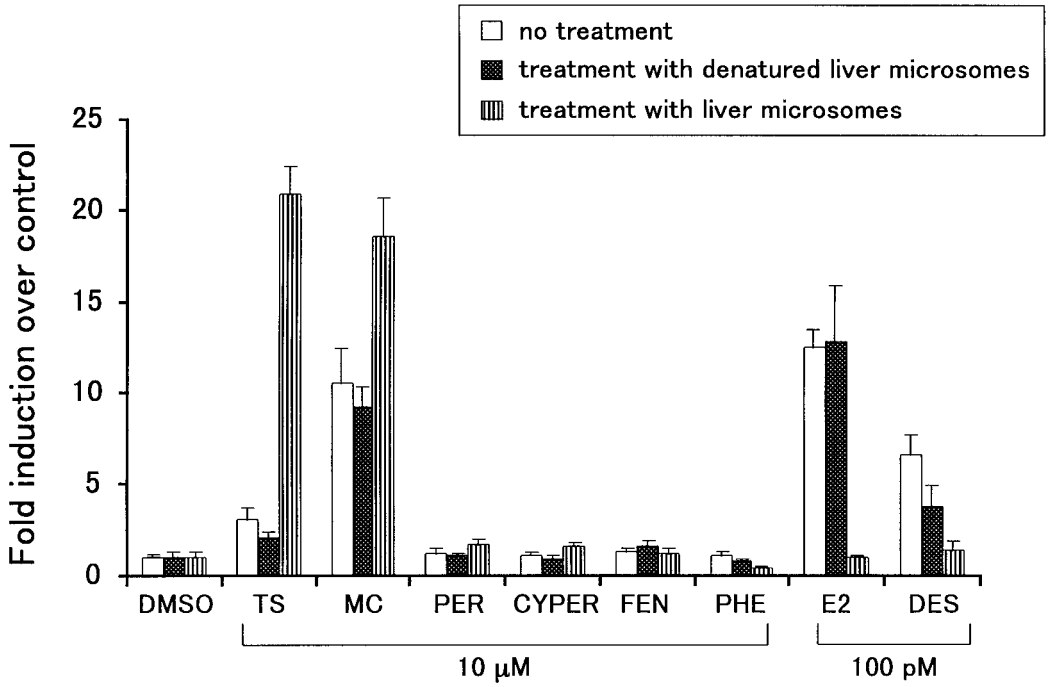

FIG. 5. Analysis of estrogenic activity of pyrethroid insecticides and control compounds by the luciferase reporter gene assay. HeLa cells were transiently transfected with the reporter plasmid pGL3-tk-vEREx5 together with the expression vector pRc/RSV-hER. Chemicals were treated with intact or denatured aroclor-1254 induced rat liver microsomes before addition to HeLa cells. Data presented are means ± SD (n = 8). TS, trans-stilbene; MC, methoxychlor; PER, permethrin; CYPER, cypermethrin; FEN, fenvalerate; PHE, phenothrin; E2, 17β-estradiol; DES, diethylstilbesterol.

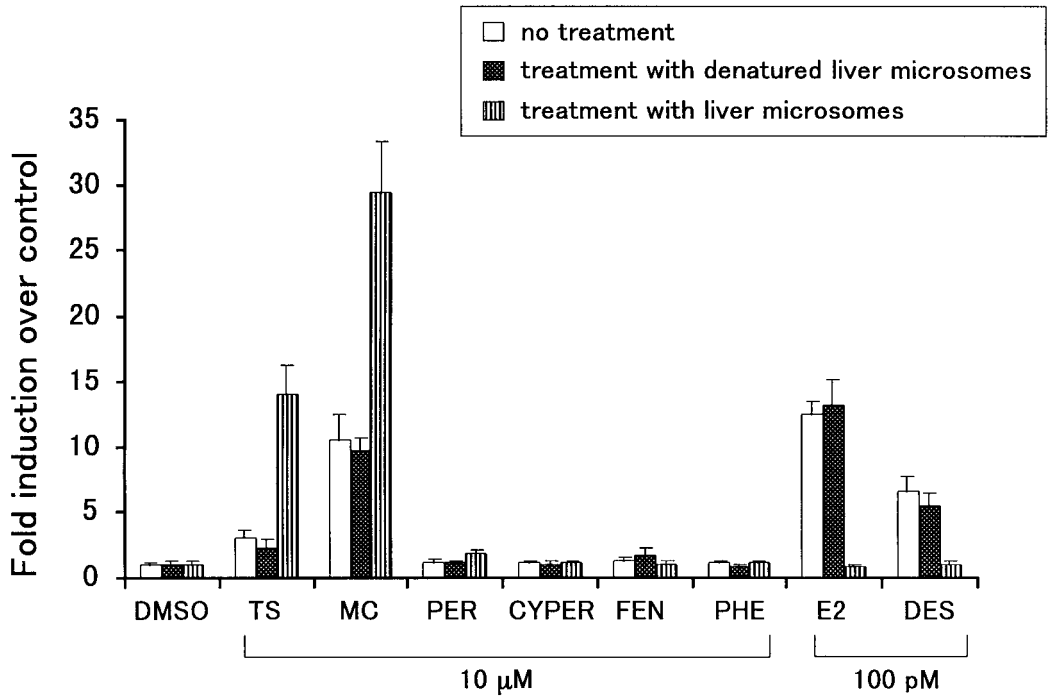

FIG. 6. Analysis of estrogenic activity of pyrethroid insecticides and control compounds by the luciferase reporter gene assay. HeLa cells were transiently transfected with the reporter plasmid pGL3-tk-vEREx5 together with the expression vector pRc/RSV-hER. Chemicals were treated with intact or denatured rat liver microsomes before addition to HeLa cells. Data presented are means ± SD (n = 8). TS, *trans*-stilbene; MC, methoxychlor; PER, permethrin; CYPER, cypermethrin; FEN, fenvalerate; PHE, phenothrin; E2, 17β-estradiol; DES, diethylstilbesterol.

have also been identified as *in vivo* metabolites of methoxychlor (13–16). Kupfer *et al.* reported methoxychlor to be a proestrogen, since the presence of microsomal enzymic activity was required for estrogenic activity *in vitro* (14). Metabolites of methoxychlor were reported to be estrogenic compounds *in vivo* (13, 17–19).

Biotransformation of *trans*-stilbene to 4-hydroxy-*trans*-stilbene was observed with both S9 fraction and liver microsomes. Hydroxylated metabolites were also identified *in vivo* using rats and rabbits (20, 21). It has not been reported whether *trans*-stilbene and its metabolites are estrogenic compounds. However, we here used *trans*-stilbene as a proestrogen because its metabolites have the structural similarity to DES.

The result of the binding assay by a fluorescence polarization method are shown in Fig. 1. Binding of *trans*-stilbene to the estrogen receptor was not noted at concentrations from 1×10^{-7} to 1×10^{-5} M. As for methoxychlor, slight inhibition was observed at the concentration 1×10^{-5} M, this being considered attributable to small amounts of impurities, methoxychlor itself not having estrogenic activity as described above (13).

Optimization of conditions for metabolic activation. A series of preliminary experiments was carried out in order to find suitable conditions for metabolic activation in HeLa and MCF-7 cells (data not shown). Investigations with two buffers, 10 mM Hepes buffer containing 12.5 mM $MgCl_2$ and 82.5 mM KCl, used in the genotoxicity assay (22) and 0.1 M potassium phosphate buffer (pH 7.4) employed for measurement of cytochrome P450 activity (23), revealed the former to be the more suitable for this assay. Another preliminary experiment was conducted to assess β-NADPHsupply with/without a NADPH regeneration system using D-glucose 6-phosphate monosodium salt. Suitable amounts of S9 fraction or liver microsomes were determined, with reference to toxicity. Metabolites of *trans*-stilbene were yielded without toxic effects at about 2 μl/well. From the results of these preliminary experiments, the conditions described under Materials and Methods were selected.

Metabolic activation of trans-stilbene. The response to metabolites of *trans*-stilbene, in terms of induction of luciferase activity, was investigated in MCF-7 and HeLa cells (Figs. 2 and 3). The 1229-fold induction relative to DMSO controls was observed with S9 fraction and liver microsomes in both cases. As expected, *trans*-stilbene thus proved to be a proestrogen *in vitro.* Pretreatment was compared with simultaneous treatment in each cell line with S9 fraction and liver microsomes. Both treatment methods luciferase activities with metabolites of *trans*-stilbene increased in comparison with *trans*-

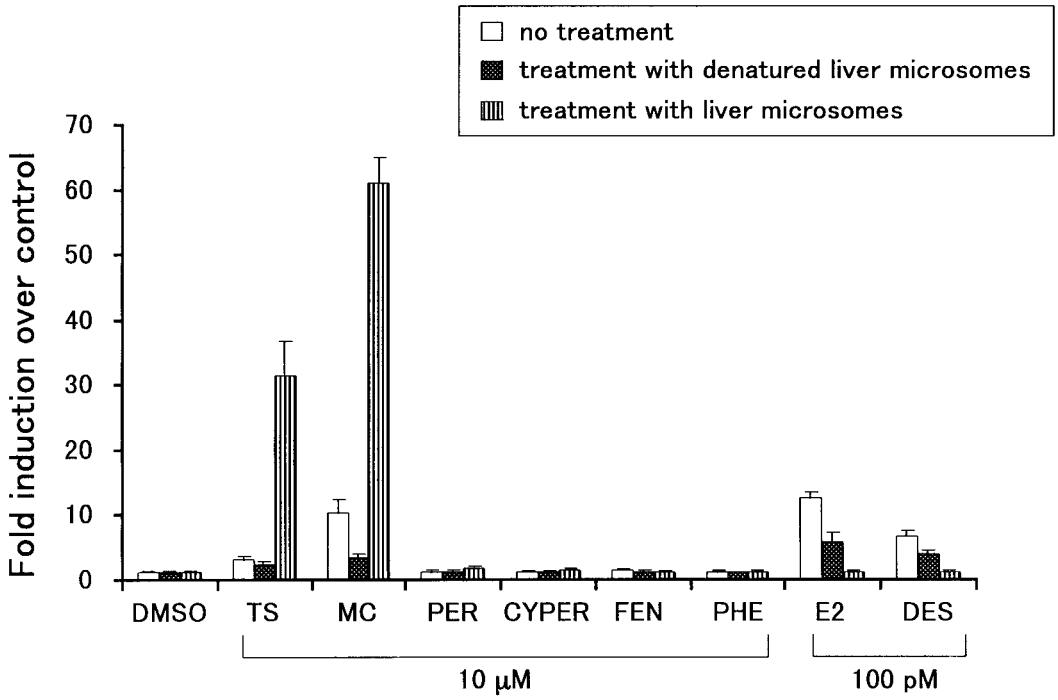

FIG. 7. Analysis of estrogenic activity of pyrethroid insecticides and control compounds by the luciferase reporter gene assay. HeLa cells were transiently transfected with the reporter plasmid pGL3-tk-vEREx5 together with the expression vector pRc/RSV-hER. Chemicals were treated with intact or denatured human liver microsomes before addition to HeLa cells. Data presented are means ± SD (n = 8). TS, *trans*-stilbene; MC, methoxychlor; PER, permethrin; CYPER, cypermethrin; FEN, fenvalerate; PHE, phenothrin; E2, 17β-estradiol; DES, diethylstilbesterol.

stilbene itself. In both cells, the induction was higher with S9 fraction and the overall response was higher in MCF-7 cells. The exact reason is unknown, but this might be due to the intrinsic estrogen receptor α expression in MCF-7 cells. From the results, the increase in luciferase activity was considered attributable to the metabolic activation of *trans*-stilbene. To confirm this, metabolic activation was investigated with the addition of SKF525A or 1-aminobenztriazole, which are well known inhibitors of cytochrome P450, to the reaction mixture (Fig. 4). Luciferase activities with metabolites of *trans*-stilbene were decreased by treatment with SKF525A or 1-aminobenztriazole in Hela cells, providing direct evidence in support of our conclusion.

Effects of enzyme induction on metabolic activation. We examined the effects of enzyme induction using S9 fraction or liver microsomes from Aroclor-1254 induced and normal rats in HeLa cells. (Figs. 5 and 6). In this test assay, the positive control, *trans*-stilbene, was found to induce the 21-fold activity relative to the DMSO control with liver microsomes and only 2-fold on treatment with denatured liver microsomes. With methoxychlor the induction was 18-fold with liver microsomes and 9-fold with denatured liver microsomes. Michael *et al.* carried out a transcriptional activation assay in ER-transfected HeLa cells and reported bis-OH-methoxychlor to be a metabolite of methoxychlor (10). This was consistent with our result. It is remarkable that the value for fold induction over control without metabolism was very high, perhaps pointing to the existence of impurities. On the other hand, 17β-estradiol or DES showed similar induction to the DMSO control on treatment with liver microsomes, and induction of 13-fold or 4-fold by treatment with denatured liver microsomes, indicating rapid loss of estrogenic activity on metabolism. Four pyrethroid insecticides subjected to this assay showed similar induction to the DMSO controls by treatment with liver microsomes and denatured liver microsomes, in line with earlier findings of no estrogenic activity (19). Results with normal rat and human liver microsomes are shown in Figs. 6 and 7, respectively. The response pattern was similar in each case *trans*-stilbene and methoxychlor going high induction relative to the DMSO control by treatment with liver microsomes, but not when they were denatured. The four pyrethroid insecticides again exhibited no estrogenic activity with or without the metabolic reaction. In HeLa cells, the patterns of metabolic activation with S9 fraction were also the same (data not shown). Thus, in conclusion, the four pyrethroid insecticides show no estrogenic activity in the present *in vitro* system incorporating metabolic activation.

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