DMD Fast Forward. Published on April 29, 2005 as doi:10.1124/dmd.104.002634

Metabolism of the α,β-Unsaturated Ketones, Chalcone and *trans*-4-Phenyl-3-buten-2-one, by Rat Liver Microsomes and Estrogenic Activity of the Metabolites

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a) Running title: Activation of chalcone to estrogen

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c) The number of page: Text 24, Table 0, Figure 7

The total number of references: 40

The number of word: Abstract: 201, Introduction: 812, Discussion: 870

d) Abbreviation: PBO, *trans*-4-phenyl-3-buten-2-one; PBA, 4-phenyl-2-butanone; PBOL, *trans*-4-phenyl-3-buten-2-ol; CYP, cytochrome P450; 4-OH-PBO, *trans*-4-(4-hydroxyphenyl-3-buten-2-one; HPLC, high-performance liquid chromatography.

ABSTRACT:

When chalcone and *trans*-4-phenyl-3-buten-2-one (PBO) were incubated with liver microsomes of untreated rats in the presence of NADPH, 4-hydroxychalcone and *trans*-4-(4-hydroxyphenyl)-3-buten-2-one (4-OH-PBO) were formed as major metabolites, respectively. Two minor metabolites of chalcone, 4'-hydroxychalcone and 2-hydroxychalcone, were also observed. The oxidase activity affording 4-hydroxychalcone was inhibited by SKF 525-A, disulfiram, ketoconazole and α-naphthoflavone. The oxidase activities leading to 4-hydroxychalcone and 4'-hydroxychalcone were enhanced in liver microsomes of 3-methylcholanthrene- and phenobarbital-treated rats, respectively. The activity generating 2-hydroxychalcone was enhanced in liver microsomes of 3-methylcholanthrene- and dexamethasone-treated rats. The oxidation of PBO to 4-OH-PBO was inhibited by SKF 525-A, ketoconazole, disulfiram and sulfaphenazole. This activity was enhanced in liver microsomes of 3-methylcholanthrene-, acetone- and phenobarbital-treated rats. 4-Hydroxylation, 4'-hydroxylation and 2-hydroxylation of chalcone were catalyzed by rat recombinant cytochrome P450 1A1, 1A2 and 2C6; by 1A1 and 2C6; and by 1A1 and 3A1, respectively. PBO was oxidized by cytochrome P450 1A1, 1A2, 2C6 and 2E1. Chalcone and PBO were negative in an estrogen reporter assay using estrogen-responsive human breast cancer cell line MCF-7. However, 4-hydroxychalcone, 2-hydroxychalcone, 4'-hydroxychalcone and 4-OH-PBO exhibited estrogenic activity.

Introduction

α,β-Unsaturated ketones, which are structures in which the double bond is adjacent to the carbonyl group, have been used as starting materials for the synthesis of various chemicals, including plastics, resins, pesticides, dyes and pharmaceuticals (Opdyke, 1973). They are also found in naturally occurring compounds, such as plant allelochemicals, insect hormones and pheromones (Wadleigh and Yu, 1987). In addition, these structures are formed during combustion of carbonaceous materials, and are found in automobile exhausts, tobacco smoke and flue gases (Eder et al., 1991). α,β-Unsaturated ketones are reactive compounds due to their electrophilic properties, and undergo nucleophilic attack, e.g., with SH-groups in proteins. They exhibit genotoxicity and mutagenicity, and show an anti-carcinogenic effect towards cultured tumor cells, such as human colon adenocarcinoma cells, B16 melanoma 4A5 cells and K562 leukemia or melanoma cells (Eder et al., 1993; Prestera et al., 1993; Czerny et al., 1998; Sabzevari et al., 2004). They also showed inhibitory effects on glucose 6-phosphatase, glutathione *S*-transferase, catechol-*O*-methyltransferase, nitric oxide synthase and quinone reductase (Wikberg et al., 1993; Pan et al., 2000; Jørgensen et al., 1992; Chien et al., 1994). Chalcone (*trans*-1,3-diphenyl-2-propen-1-one) is an α,β-unsaturated ketone that has the skeletal of so-called "chalcones". Chalcones are open-chain flavonoids in which two aromatic rings are jointed by a three carbon linker, which are synthesized by chalcone synthetase from 3-malonyl-CoA and a starter CoA ester such as 4-coumaronyl-CoA in plants (Schörder and Schörder, 1990). Chalcone synthetase functions as a key enzyme of flavonoid biosynthesis, utilizing the same substrates as stilbene synthetase (Schörder et al., 1988). Chalcone and chalcones are called "anthochlor pigments". This term was coined to identify a group of yellow pigments which turn red in the presence of alkali. In some plants, chalcones contribute significantly to the corolla pigmentation.

trans-4-Phenyl-3-buten-2-one (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. It has mutagenic and antimutagenic effects (Prival et al., 1982; Motohashi et al., 1997). We have studied the metabolism of PBO in rats and dogs (Kitamura et al., 1999; Okamoto et al., 1999), and identified the double bond-reduced metabolite, 4-phenyl-2-butanone (PBA), and the carbonyl-reduced metabolite, *trans*-4-phenyl-3-buten-2-ol (PBOL), which lacks mutagenic activity, as *in vivo* metabolites of PBO in rats and dogs (Kitamura et al., 1999). Furthermore, it was demonstrated that the reductions of the double bond and carbonyl group were catalyzed in rats by "α,β-ketoalkene double bond reductase" in liver cytosol and by a novel microsomal carbonyl reductase, respectively (Kitamura and Tatsumi, 1990; Okamoto et al., 1999). We also showed that purified α,β-ketoalkene double bond reductase exhibited significant reducing activity towards the double bonds of chalcone and some α,β-unsaturated ketones (Kitamura and Tatsumi, 1990). In contrast, Sauer et al. (1997a; 1997b) found some glutathione conjugates of PBO in the blood and urine of rats and mice dosed with PBO. However, the oxidative metabolism of PBO and chalcone in animals and humans has not been examined.

Various chemicals produced by man mimic the effects of β-estradiol. These chemicals are called environmental estrogens, and include agricultural pesticides, such as kepone, 1,1,1-trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane (*o,p'*-DDT), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (*p,p'*-DDD), dieldrin and methoxychlor, and industrial chemicals such as bisphenol A, nonylphenol and some polychlorinated biphenyl congeners (Andersen et al., 1999). The structural requirements for estrogenic activity include a hindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group (Blair et al., 2002; Hong et al., 2002). Environmental estrogens may be playing a role in the increasing incidence of breast cancer, testicular cancer, and other problems of the reproductive system. Naturally occurring phytoestrogens also show estrogenic activity *in vitro* in receptor binding assay, in spite of their beneficial effects, such as anticarcinogenicity (Branham et al., 2002). Some flavonoids are phytoestrogens. Chalcones are a source of phytoestrogens, acting as C_{15} precursors in plant flavonoid biosynthesis. PBO also has a flavonoid skeleton. Recently, we showed that *trans*-stilbene, the parent compound of diethylstilbestrol, was not itself estrogenic, but exhibited a potent estrogenic activity after metabolic activation by a microsomal cytochrome P450 (CYP) system (Sugihara et al., 2000). We suggested that the estrogenic activity of *trans*-stilbene was due to the hydroxylated metabolites, *trans*-4-hydroxystilbene and *trans*-4,4'-dihydroxystilbene. In spite of the known estrogenic activity of stilbene derivatives such as resveratrol and *trans*-4-hydroxystilbene, the estrogenic action of chalcone and PBO has not been reported (Gehm et al., 1997; Sugihara et al., 2000).

Here, we describe a study of the metabolism of chalcone and PBO by rat liver microsomes. The estrogenic activities of the parent compounds and their metabolites were examined using ERE-luciferase reporter assay in MCF-7 cells. We identified the CYP isoforms functioning in the oxidations of chalcone and PBO, and demonstrated that these hydroxylated α,β-unsaturated ketones exhibit estrogenic activity.

Materials and Methods

Chemicals. Chalcone, 4-hydroxychalcone, 4'-hydroxychalcone, 2-hydroxychalcone, 2'-hydroxychalcone, PBO, PBA and *trans*-4-(4-hydroxyphenyl)-3-buten-2-one (4-OH-PBO) were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). 17β-Estradiol was from Sigma Chemical Co. (St. Louis, MO). Rat recombinant CYP isoforms expressed in a baculovirus system were obtained from Gentest Co. (Woburn, MA). PBOL was synthesized by a reported method (Okamoto et al., 1999).

Animals. Male Sprague-Dawley rats (210-230 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed at 22^{o}C with a 12-hr light/dark cycle, with free access to tap water and a standard pellet diet MM-3 (Funabashi Farm, Funabashi, Japan). In some experiments, rats were given phenobarbital, 3-methylcholanthrene or dexamethasone intraperitoneally once per day for 3 consecutive days at 80, 25 or 100 mg/kg, respectively, or acetone orally once at 3 g/kg at 24 hr before sacrifice.

Preparation of Liver Microsomes. The livers were excised from exsanguinated rats and immediately perfused with 1.15 % KCl. The livers were homogenized in four volumes of the KCl solution using a Potter-Elvehjem homogenizer. The microsomal fraction was obtained from the homogenate by successive centrifugation at 9,000*g* for 20 min and 105,000*g* for 60 min. The fraction was washed by resuspension in the KCl solution and resedimentation. The pellets of microsomes were resuspended in the solution to make 1 ml equivalent to 1 g of liver.

Cell Culture. MCF-7 cell lines were maintained in MEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5 % fetal bovine serum (Life Technologies, Rockville, MD).

Microsomal Incubation Mixtures for Identification of Oxidative Metabolites of Chalcone and PBO. The metabolites of chalcone and PBO were isolated from an incubation mixture, which consisted of 0.2 μmol of chalcone or PBO, 1 μmol of NADPH and 0.1 ml of microsomes in a total volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). After incubation at 37°C for 20 min, the mixture was extracted with 5 ml of diethyl ether. The supernatant fraction was separated by centrifugation for 10 min at 1,000*g*, and evaporated to about 50 µl at 0°C, and 0.1 ml of methanol was added. The solution was injected into an HPLC instrument equipped with a photodiode array UV detector (Beckman Instruments, Inc., Fullerton, CA) or a liquid chromatography-mass spectrometry operated in tandem (LC/MS/MS).

Assay of Liver Microsomal Oxidase Activity. An incubation mixture consisted of 0.1 µmol of chalcone or PBO, 0.5 µmol of NADPH and 0.2 ml of liver microsomes equivalent to 200 mg liver wet weight (0.3 - 0.5 mg protein) or 90 µl of rat recombinant CYP isoforms (CYP 1A1, 1A2, 2B1, 2E1, 2D1, 2C6 and 3A1; about 0.05 nmol CYP equivalent) in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 20 min. After incubation, 0.1 µmol of methyl *p*-aminobenzoate was added as an internal standard and the mixture was extracted with 5 ml of diethyl ether. The extract was evaporated to dryness, the residue was dissolved in 0.1 ml of methanol, and an aliquot (5 µl) was analyzed by HPLC or LC/MS/MS. The 4-hydroxylase activities towards chalcone and PBO were detected by HPLC. The 4'- and 2-hydroxylase activities toward chalcone were detected by LC-MS/MS. Rat recombinant CYP isoforms were expressed using a baculovirus expression system. Baculovirus-infected insect cells were used to prepare microsomes.

Assay of Estrogenic Activity. An estrogen responsive element (ERE)-luciferase reporter assay was performed to assess estrogenic activity (Sugihara et al., 2000). MCF-7 cells were plated at 2×10^4 / well in 48 well plates (Nalgen Nunc International, Rochester, NY). After 24 h, cells in each well were transiently transfected with 0.3 µg of $(ERE)_3$-SV40-luc and 0.01 µg of phRL-CMV (Promega Co., Madison, WI, USA) with 0.3µg TransFast (Promega Co.), a transfection reagent containing a synthetic cationic lipid. After 24 h incubation with chemicals, cells were harvested with 30 µl of cell lysis buffer (Promega Co.). The firefly and renilla luciferase activities were determined with the Dual Luciferase Assay Kit (Promega Co.) by measuring luminescence with a Wallac Micro-Beta scintillation counter (PerkinElmer Life

Sciences, Boston, MA). Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV. For the assay of the activated metabolites of chalcone in the liver microsomal system, chalcone (0.1 μmol) was incubated with 0.1 ml of rat liver microsomes in the presence of 1 μmol of NADPH for 30 min in a final volume of 1 ml of 0.1 M phosphate buffer. After the incubation, the mixture was extracted with 5 ml of ethyl acetate and evaporated to dryness. The residue was dissolved with 1 ml of ethanol and an aliquot was used for the estrogenic activity assay. The total concentration of the substrate and its metabolites was calculated from the original amount of the substrate.

High-Performance Liquid Chromatography (HPLC). HPLC was performed in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 125 x 4 mm Inertsil ODS-3 column (GL-Science, Tokyo, Japan). The mobile phase consisted of acetonitrile-water (2:3, v/v) for 40 min, then a linear gradient to acetonitrile-water (1:1, v/v) over 100 min, followed by 40 min of acetonitrile-water (1:1, v/v) for the separation of chalcone and the metabolites. The chromatograph was operated at a flow rate of 0.5 ml/min with detection at a wavelength of 280 nm. In the case of PBO, the mobile phase was acetonitrile-water (3:7, v/v). The chromatograph was operated at a flow rate of 0.5 ml/min at a wavelength of 254 nm. The elution times of 4-OH-PBO, methyl *p*-aminobenzoate (internal standard for PBO), PBO, methyl *p*-aminobenzoate (internal standard for chalcone), 4-hydroxychalcone, 4'-hydroxychalcone, 2-hydroxychalcone, chalcone and 2'-hydroxychalcone were 13.3, 16.2, 45.2, 8.7, 33.0, 35.4, 46.4, 94.9 and 132.2 min, respectively.

LC/MS/MS Analysis. LC/MS/MS was performed using a API 2000 (Applied Biosystems, Foster, CA) equipped with a triple-stage quadrupole mass spectrometer coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). An electrospray ionization source was used for measurement. A reversed-phase Inertsil ODS-3 column (125 x 4 mm) was used as the separation column, and its temperature was maintained at 30^{o}C. The mobile phase was 40 % acetonitrile/water containing 0.1 % acetic acid for 50 min, then a linear gradient to 80 % acetonitrile/water containing 0.1 % acetic acid over 55 min, followed by a hold for 10 min. The column effluent was monitored in the negative ion mode with multiple reaction monitoring (MRM). The temperature of the Turbo-Ionspray auxiliary gas was 500^{o}C and the ionization voltage was –4500 V. Nitrogen was used as nebulizer, auxiliary, curtain and collision activated dissociation gas, at 40, 80 and 30 psi and a value of 4, respectively (1 psi = 6894.76 Pa). Analytical conditions of the selected ion pair (m/z for Q1/Q3), dwell time, declustering potential, focusing potential, entrance potential, collision energy and collision exit potential (4-hydroxychalcone; 222.7/144.7, 250 msec, -70 V, -260 V, -11 V, -36 V and –20 V, 4’-hydroxychalcone; 222.7/120.7, 250 msec, -81 V, -300 V, -11 V, -40 V and –18 V, 2-hydroxychalcone; 222.7/194.8, 250 msec, -90 V, -330 V, -10.5 V, -24 V and -39 V, n-propylparaben (an internal standard); 178.7/91.7, 250 msec, -46 V, -320 V, -10.5 V, -30 V and -18 V, respectively.) were automatically determined by Analyst® (the application software for quantitative determination with the API2000). MRM chromatograms of the metabolites were compared by those of authentic standards to confirm the identity of metabolites. The elution times of 4-hydroxychalcone, 4’-hydroxychalcone, 2-hydroxychalcone and n-propylparaben (an internal standard) were 32.7 min, 34.9 min, 45.0 min and 21.3 min, respectively. The detection limits (*S/N* value) of 4-hydroxychalcone, 4’-hydroxychalcone and 2-hydroxychalcone were 3, 2 and 2 at the concentration of 100 nM, 500 nM and 10 nM, respectively. Calibration plots were linear up to 10 μM for 4-hydroxychalcone, 4’-hydroxychalcone and 2-hydroxychalcone. Recoveries of 4-hydroxychalcone, 4’-hydroxychalcone and 2-hydroxychalcone were 94, 96 and 97 %, respectively.

Results

Metabolism of Chalcone and PBO by Rat Liver Microsomes. Chalcone was incubated with liver microsomes of untreated rats in the presence of NADPH to obtain metabolites as described in Materials and methods. Four peaks were detected in an HPLC chromatogram of the extract of the incubation mixtures. These peaks were not detected in the control, which was incubated without the substrate. Retention times of the chalcone and its metabolites detected at 33.0, 35.4, 46.4 and 94.9 min corresponded to those of 4-hydroxychalcone, 4'-hydroxychalcone, 2-hydroxychalcone and chalcone, respectively (Fig. 1A). The peak corresponding to 4-hydroxychalcone was much higher than those of the 4'-hydroxyl and 2-hydroxyl derivatives. No peak corresponding to 2'-hydroxychalcone was detected. PBO was also incubated with liver microsomes of untreated rats in the presence of NADPH for the detection of the metabolites. Three peaks, which were not detected in the control, were detected in an HPLC chromatogram of the extract of the incubation mixture. The retention times of 13.3, 33.1 and 45.2 min corresponded to those of 4-OH-PBO, PBOL and PBO, respectively (Fig. 1B). The identities of the hydroxylated metabolites were confirmed by mass and UV spectral comparison with authentic samples (data not shown).

(Fig. 1)

Oxidase Activity of Rat Liver Microsomes. When chalcone or PBO was incubated with liver microsomes of untreated rats, the amounts of hydroxyl derivatives formed increased linearly for 20 min (data not shown). The oxidase activities of rat liver microsomes toward chalcone and PBO were examined using liver microsomes of untreated, and phenobarbital-, 3-methylcholanthrene-, dexamethasone- or acetone-treated rats. When chalcone was incubated with liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, significant oxidase activities affording 4- and 2-hydroxyl derivatives were observed. In contrast, when chalcone was incubated with liver microsomes of phenobarbital-treated rats, a significant oxidase activity affording 4'-hydroxychalcone was observed. The oxidase activity affording 4-hydroxychalcone was much higher than those leading to 4’-hydroxychalcone and 2-hydroxychalcone (Fig. 2A - 2C). When oxidase activity transforming PBO to the 4-hydroxyl derivative was compared in liver microsomes of rats treated with various inducers in the presence of NADPH, the highest activity was observed in liver microsomes of 3-methylcholanthrene-treated rats. The oxidase activity was enhanced to a lesser extent when liver microsomes of acetone- or phenobarbital-treated rats were used instead of those of untreated rats (Fig. 2D). Only marginal oxidase activity toward chalcone and PBO was observed with NADH instead of NADPH (data not shown).

(Fig. 2)

Inhibitory Effect of Chemicals on Microsomal Oxidase Activity. The NADPH-dependent activities of liver microsomes of untreated rats to generate 4-, 4'- and 2-hydroxyl derivatives of chalcone were markedly inhibited by the addition of SKF 525-A, and partly by α-naphthoflavone and disulfiram except 2-hydroxylation, which was stimulated by α-naphthoflavone. Ketoconazole also inhibited these oxidations, especially 2-hydroxylation. Secobarbital and sulfaphenazole did not show inhibitory effects on the oxidation of chalcone by rat liver microsomes (Fig. 3A - 3C). In the case of 3-methylcholanthrene-treated rat liver microsomes, α-naphthoflavone inhibited 4-, 4'- and 2-hydroxylation of chalcone (data not shown). The microsomal oxidase activity with NADPH toward PBO was inhibited by the addition of ketoconazole, disulfiram, sulfophenazole and SKF 525-A (Fig. 3D).

(Fig. 3)

CYP isoforms catalyzing the oxidation of chalcone and PBO. Next, we attempted to identify CYP isoforms involved in the oxidation of chalcone and PBO by using various rat recombinant CYP isoforms. CYP 1A1 and 1A2 mainly catalyzed the oxidation of chalcone to 4-hydroxychalcone, and CYP 2B1 and 2C6 also catalyzed the oxidation. However, CYP 2E1, 2D1 and 3A1 lacked this activity (Fig. 4A). The oxidation to 4'-hydroxychalcone was catalyzed by CYP 1A1 and 2C6 (Fig. 4B). The oxidation to 2-hydroxychalcone was catalyzed by CYP 1A1 and 3A1 (Fig. 4C). CYP 1A2 and 2C6 mainly catalyzed the oxidation of PBO to 4-OH-PBO. CYP 1A1 and 2E1 also catalyzed this reaction (Fig. 4D). The highest oxidase activity was observed in chalcone 4-hydroxylation compared with other oxidations of chalcone and PBO, similarly to the case of liver microsomes.

(Fig. 4)

Estrogenic Activities of Chalcone, PBO and their Hydroxylated Metabolites. The estrogenic activities of chalcone, PBO and their hydroxylated metabolites were examined using ERE-luciferase reporter assay in MCF-7 cells. Chalcone did not show estrogenic activity in this assay. However, 4-hydroxychalcone, 2-hydroxychalcone and 4'-hydroxychalcone showed estrogenic activity. The 2'-hydroxychalcone metabolite was inactive. 4-Hydroxychalcone exhibited the highest activity at 1×10^{-4} M. These compounds were cytotoxic to MCF-7 cells at concentrations above 1×10^{-4} M, except 2-hydroxychalcone, so the apparent estrogenic activities decreased at higher concentrations (Fig. 5A).

The estrogenic activities of PBO and its metabolites were also examined. PBO was negative in the assay with MCF-7 cells. However, the hydroxylated metabolite, 4-OH-PBO, showed estrogenic activity at 1×10^{-5} - 1×10^{-4} M (Fig. 5B). In contrast, PBA, the double bond-reduced metabolite of PBO, and its hydroxylated metabolite, 4-(4-hydroxyphenyl)-2-butanone, did not show estrogenic activity at 1×10^{-4} M (data not shown).

These results suggest that chalcone and PBO were converted to active metabolites by rat liver microsomes.

(Fig. 5)

Estrogenic Activity of Chalcone with a Microsomal Oxidation System. The estrogenic activity of chalcone in the presence of a rat liver microsomal oxidation system was examined. When chalcone was incubated with liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited an estrogenic activity in the range of $1x10^{-6}$ - $1x10^{-5}$ M. In contrast, a smaller effect was obtained when liver microsomes of phenobarbital-treated rats were used (Fig. 6). These results suggest that chalcone is mainly metabolically activated to 4- and 2-hydroxychalcones by liver microsomes from 3-methylcholanthrene-treated rats and to 4'-hydroxychalcone by microsomes from phenobarbital-treated rats.

(Fig. 6)

Discussion

Previously, we demonstrated that the double bond and carbonyl group of α,β-unsaturated ketones were reduced by liver microsomes and cytosol of experimental animals (Kitamura et al., 1990; 1999; Okamoto et al., 1999). Here, we describe the oxidative metabolism of α,β-unsaturated ketones, chalcone and PBO, by rat liver microsomes. These compounds were activated to estrogens by oxidative conversion to their hydroxylated metabolites. α,β-Unsaturated ketones are easily detoxified to reduced metabolites by double bond reductase, a cytosolic enzyme, triple bond reductase, a microsomal enzyme, or microsomal and cytosolic carbonyl reductases. In contrast, when α,β-unsaturated ketones are oxidized at the phenyl ring by CYP, they are converted to active estrogenic compounds. As α,β-unsaturated ketones are taken in through foods, food additives and cigarette smoke, it is necessary to consider the possible endocrine-disrupting activity of these compounds, including their metabolites. In this study, we obtained evidence that chalcone is oxidized to a major metabolite, 4-hydroxychalcone, by CYP 1A1/2 isoforms. PBO was oxidized to 4-OH-PBO by CYP 1A1/2; CYP 2C6 and 2E1 also contributed to this oxidation. Furthermore, CYP 2C6 and 1A1 were major isoforms involved in the 4'-hydroxylation of chalcone. The oxidation of chalcone to 2-hydroxychalcone was catalyzed by CYP 1A1 and 3A1. CYP 1A subfamily commonly catalyze the oxidation of chalcone and PBO.

It is interesting to note that CYP 2C6 contributes to the oxidation of chalcone and PBO. α,β-Unsaturated ketone structure of chalcone and PBO may be important for metabolism by this CYP, as reported in the oxidation of ketones such as tolbutamide, phenytoin and warfarin (Lewis, 1998). Disulfiram is a well known potent inhibitor of CYP 2E1. In this study, the chemical inhibited the 4- and 4'-hydroxylations of chalcone, while CYP 2E1 did not catalyze these oxidations. Martini et al. (1997) reported that disulfiram inhibits not only CYP 2E1-, but also CYP 2C- and 3A-mediated metabolism in rat liver microsomes. Therefore, the decreased 4- and 4'-hydroxylating activities toward chalcone may be due to the inhibitory effect of disulfiram on CYP 2C6 and 3A1. Ketoconazole commonly inhibited the 4-, 4'- and 2-hydroxylations of chalcone, presumably due to its inhibitory effects on rat CYP 1A2, 2C6 and 3A1/2 (Kobayashi et al., 2003). In contrast, 4'-hydroxylation of chalcone was catalyzed by rat CYP 2C6 and 1A1. However, no inhibitory effect of sulfaphenazole, a human CYP 2C9 inhibitor, on 4’-hydroxychalcone formation was observed. This may be due to differential sensitivity to sulfaphenazole in humans and rats, i.e., the involvement of CYP 2C9 and CYP 2C6. Otherwise, the inhibitory effect may be masked by the activity of CYP 1A1. In this study, 2-hydroxylation of chalcone by untreated rat liver microsomes was stimulated by the addition of α-naphthoflavone, an inhibitor of CYP 1A. There are other reports that α-naphthoflavone stimulated various CYP 3A-dependent reactions (Ueng et al., 1995; Harlow and Halpert, 1998). Indeed, the oxidase activity of 3-methylcholanthrene-treated rat liver microsomes for conversion of chalcone to 2-hydroxychalcone was markedly inhibited by α-naphthoflavone.

In this study, we obtained evidence that chalcone is converted to estrogenically active hydroxylated derivatives by rat liver microsomes. 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. In the microsomal system used in this study, the estrogenic activity of chalcone is thought to be mainly due to 4-hydroxychalcone, which is a major metabolite. PBO was also metabolically activated to an estrogen by a microsomal enzyme system, and 4-OH-PBO, which is a major metabolite in the microsomal system, exhibited estrogenic activity. These facts would suggest that chalcone and PBO are metabolically activated to estrogenic hydroxylated derivatives in rats. We reported that PBO was reduced to PBA by liver cytosolic double bond reductase (Kitamura et al., 1990). 4-Hydroxyphenyl-2-butanone, an oxidized metabolite of PBA, was not estrogenic (data not shown). 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 be also necessary. The balance between oxidative and reductive metabolism of chalcone and PBO is an important problem. In our previous paper, the level of microsomal carbonyl reductase activity toward PBO in rat livers is almost equal to that of oxidase activity shown in this study (Okamoto et al., 1999). In our preliminary study, cytosolic double bond reductase activities towards chalcone and PBO were 14 and 5 nmol/min/mg protein, respectively. These specific activities are higher than those of the microsomal oxidase activities observed in this study. Thus, the activation step of chalcone and PBO is not a major reaction. These compounds may pose a relatively low risk to humans and other animals in terms of estrogenic activity, in spite of the in vitro activity shown in this study. Further study in other animals and organs will be necessary to determine the relevance of these reactions in the body. It is worthwhile to investigate further the estrogenic activity of hydroxylated chalcones, because these compounds occur naturally.

(Fig. 7)

Chalcone and PBO were shown to be proestrogens in this study. There are other examples of metabolic activation to estrogens. Methoxychlor requires demethylation by liver microsomal mixed function oxidase involved CYP 1A2 and 2C19, to elicit estrogenic activity (Stresser and Kupfer, 1998). Elsby et al. (2001) also reported that methoxybisphenol A was activated through demethylation by human liver microsomes. *trans*-Stilbene is also a proestrogen, which is metabolically activated to *trans*-4-hydroxystilbene, a potent estrogen, by CYP 1A1/2 (Suguhara et al., 2000; Sanoh et al., 2002). Further, we reported activation of styrene oligomers and 2-nitrofluorene to estrogenic metabolites by CYP 2B1 and 1A1, respectively (Kitamura et al., 2003; Fujimoto et al., 2003). It is known that PCBs are converted to the hydroxylated metabolites in the animal body, and some hydroxylated PCBs shows estrogenic activity (Korach et al., 1988; Connor et al., 1997). Hydroxylated metabolites of benzo[*a*]pyrene also exhibit estrogenic activity (Charles et al., 2000). It is necessary to consider the activity of metabolites produced from the parent compounds for the proper assessment of estrogenic toxicity of many compounds, including α,β-unsaturated ketones.

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Footnotes

a) Unnumbered footnote

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture, and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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Legends to Figures

Figure 1. HPLC chromatograms of the extracts of incubation mixtures of chalcone and PBO with liver microsomes of 3-methylcholanthrene-treated rats.

(A) HPLC chromatogram of the metabolites of chalcone. (B) HPLC chromatogram of the metabolites of PBO. The absorbance (full scale, 0.016 absorbance units) was measured at 254 nm. An incubation mixture consisting of 0.1 μmol of chalcone or PBO, 0.5 μmol of NADPH and 20 μl of liver microsomes was incubated for 20 min. The extract of the mixture with diethyl ether was analyzed by HPLC as described in Materials and methods.

Figure 2. Oxidase activities of rat liver microsomes toward chalcone and PBO.

(A) Chalcone-oxidase activity to 4-OH chalcone. (B) Chalcone-oxidase activity to 4'-OH chalcone. (C) Chalcone-oxidase activity to 2-OH chalcone. (D) PBO-oxidase activity to 4-OH-PBO. Each bar represents the mean ± SD of four rats. A mixture containing 0.1 μmol of chalcone or PBO, 0.5 μmol of NADPH and 20 μl of liver microsomes in 0.1 M phosphate buffer (pH 7.4) was incubated at 37^{o}C for 20 min. The 4-hydroxychalcone, 2-hydroxychalcone, 4'-hydroxychalcone and 4-OH-PBO formed were determined using HPLC as described in Materials and methods. N.D.: not detected. * $p < 0.05$, ** $p < 0.01$ compared with control. Control, 3-MC, Acetone, DEX and PB represent liver microsomes of untreated, 3-methylcholanthrene-treated, acetone-treated, dexamethasone-treated and phenobarbital-treated rats, respectively.

Figure 3. Effects of some chemicals on oxidase activities of rat liver microsomes toward chalcone and PBO.

(A) Chalcone-oxidase activity to 4-OH chalcone. (B) Chalcone-oxidase activity to 4'-OH chalcone. (C) Chalcone-oxidase activity to 2-OH chalcone. (D) PBO-oxidase activity to 4-OH-PBO. Each bar represents the mean ± SD of four rats. A mixture containing 0.1 μmol of chalcone or PBO, 0.5 μmol of NADPH and 20 μl of liver microsomes in 0.1 M phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. Inhibitors were added at the concentration of 10^{-4} M. The 4-hydroxychalcone, 2-hydroxychalcone, 4'-hydroxychalcone and 4-OH-PBO formed were determined using HPLC as described in Materials and methods. N.D.: not detected. * $p < 0.05$, ** $p < 0.01$ compared with control.

Figure 4. Oxidase activities of rat recombinant CYP isoforms toward chalcone and PBO.

(A) Chalcone-oxidase activity to 4-OH chalcone. (B) Chalcone-oxidase activity to 4'-OH chalcone. (C) Chalcone-oxidase activity to 2-OH chalcone. (D) PBO-oxidase activity to 4-OH-PBO. Each bar represents the mean of duplicate experiments. A mixture containing 0.1 μmol of chalcone or PBO, 0.5 μmol of NADPH and 90 μl of rat recombinant CYP isoform (CYP 1A1, 1A2, 2B1, 2E1, 2D1, 2C6 and 3A1; about 0.05 nmol CYP equivalent) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37°C for 20 min. Hydroxylated metabolites formed were determined using HPLC as described in Materials and methods. N.D.: not detected.

Figure 5. Estrogenic activity of chalcone, PBO and their hydroxylated metabolites using ERE-luciferase reporter assay in MCF-7 cells.

(A) Estrogenic activity of chalcone and its hydroxylated metabolites. (B) Estrogenic activity of PBO and its metabolite. Each bar represents the mean ± SD of four experiments. Estrogenic activity was expressed as a relative activity with respect to the control using MCF-7 cells. * $p < 0.05$, ** $p < 0.01$ compared with control.

Figure 6. Estrogenic activity of chalcone with liver microsoms using ERE-luciferase reporter assay in MCF-7 cells.

Each bar represents the mean ± SD of four experiments. Estrogenic activity was expressed as a relative activity with respect to the control using MCF-7 cells. Chalcone was incubated with liver microsomes in the presence of NADPH, and the extract of the incubation mixture was subjected to the screening test. PB: phenobarbital, MC: 3-methylcholanthrene. * $p < 0.05$, ** $p < 0.01$ compared with control.

Figure 7. Metabolic activation pathway of chalcone and PBO to estrogens by rat liver microsomal enzyme system.

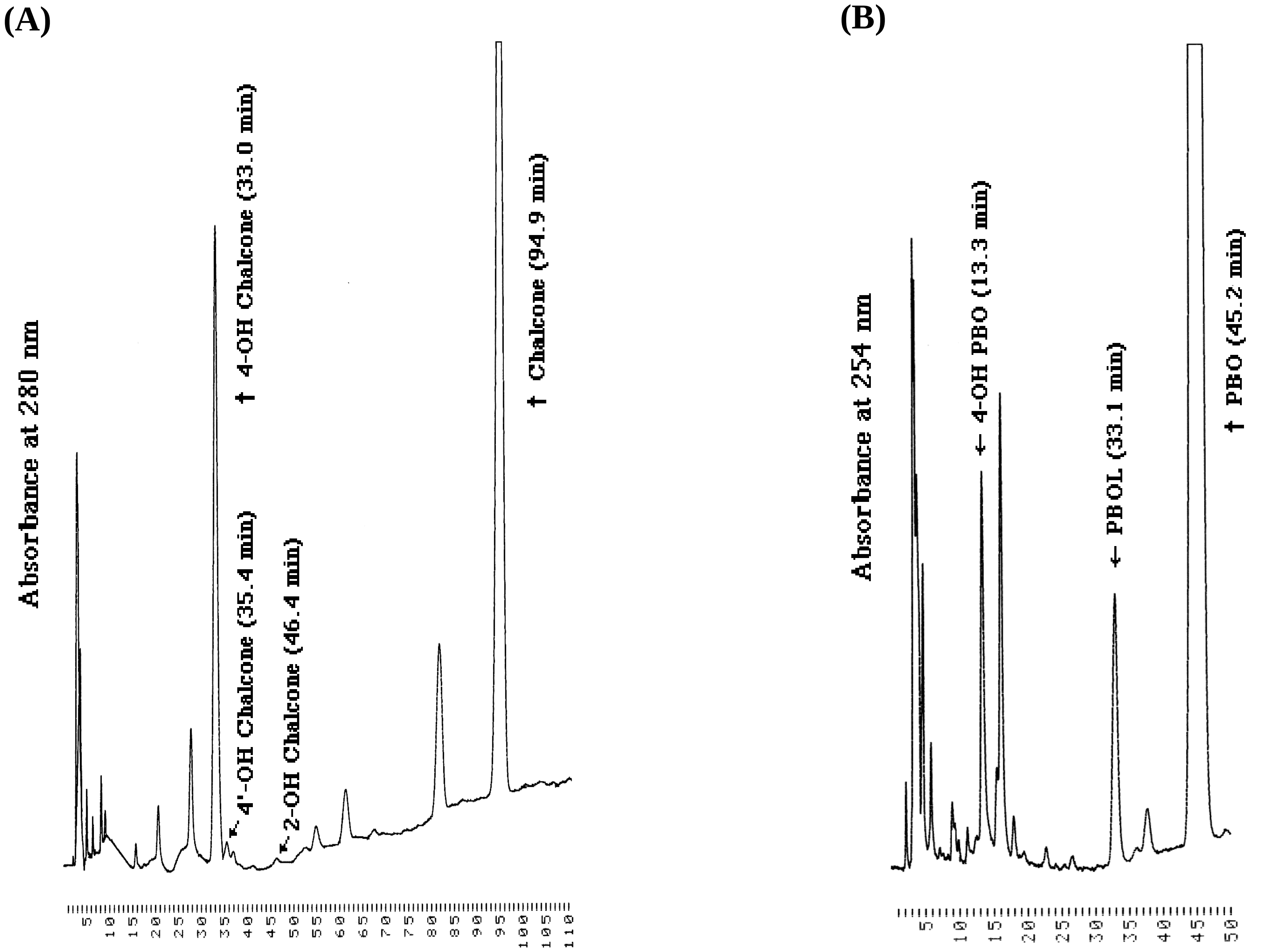

(A)
Absorbance at 280 nm
4-OH Chalcone (33.0 min)
4'-OH Chalcone (35.4 min)
2-OH Chalcone (46.4 min)
Chalcone (94.9 min)
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110
(B)
Absorbance at 254 nm
4-OH PBO (13.3 min)
PBOL (33.1 min)
PBO (45.2 min)
5 10 15 20 25 30 35 40 45 50

Fig. 1.

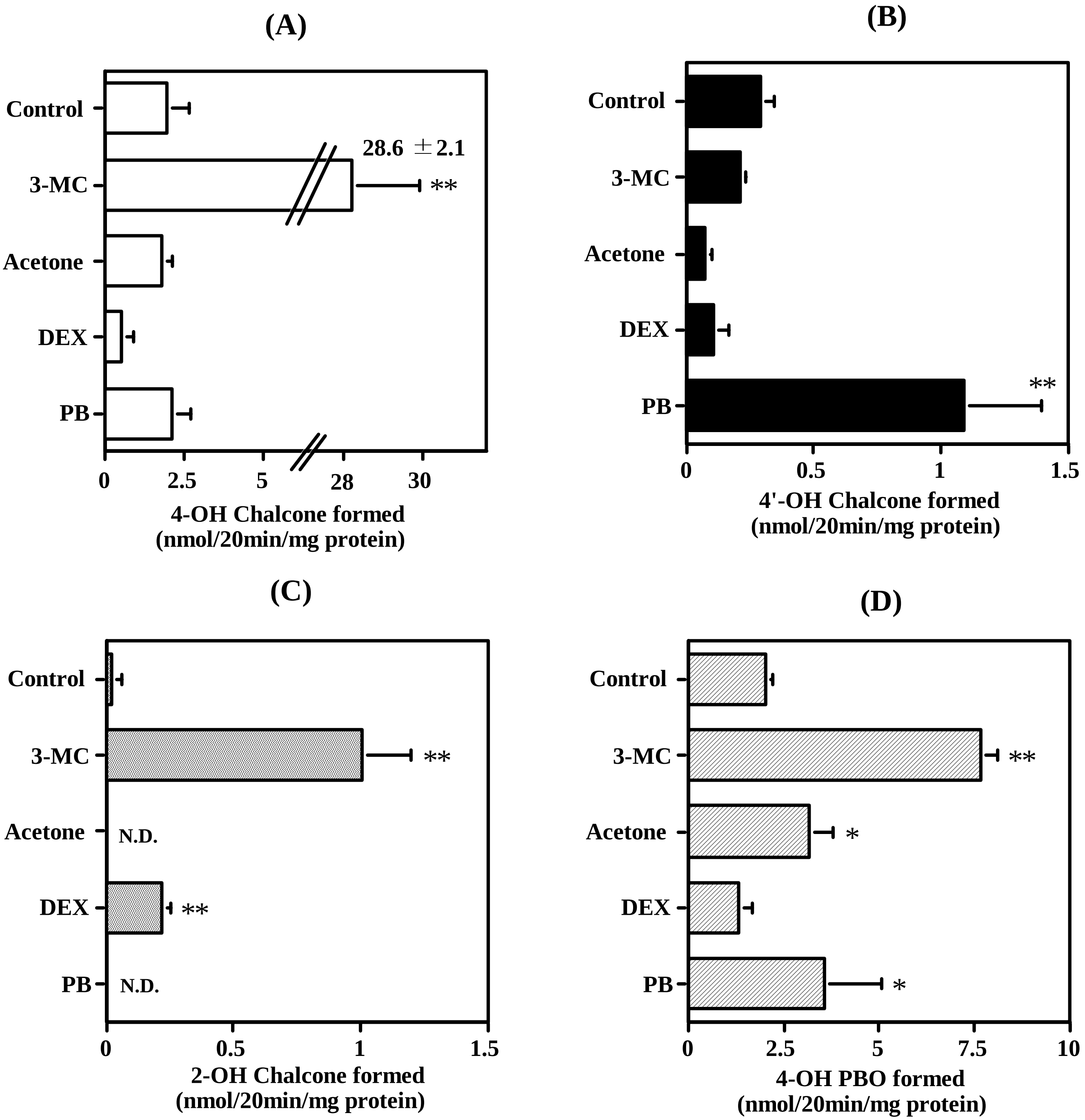

(A)
Control
3-MC
Acetone
DEX
PB
28.6 ±2.1
**
0
2.5
5
28
30
4-OH Chalcone formed
(nmol/20min/mg protein)
(B)
Control
3-MC
Acetone
DEX
PB
**
0
0.5
1
1.5
4'-OH Chalcone formed
(nmol/20min/mg protein)
(C)
Control
3-MC
Acetone
DEX
PB
**
N.D.
**
N.D.
0
0.5
1
1.5
2-OH Chalcone formed
(nmol/20min/mg protein)
(D)
Control
3-MC
Acetone
DEX
PB
**
*
*
0
2.5
5
7.5
10
4-OH PBO formed
(nmol/20min/mg protein)

Fig. 2.

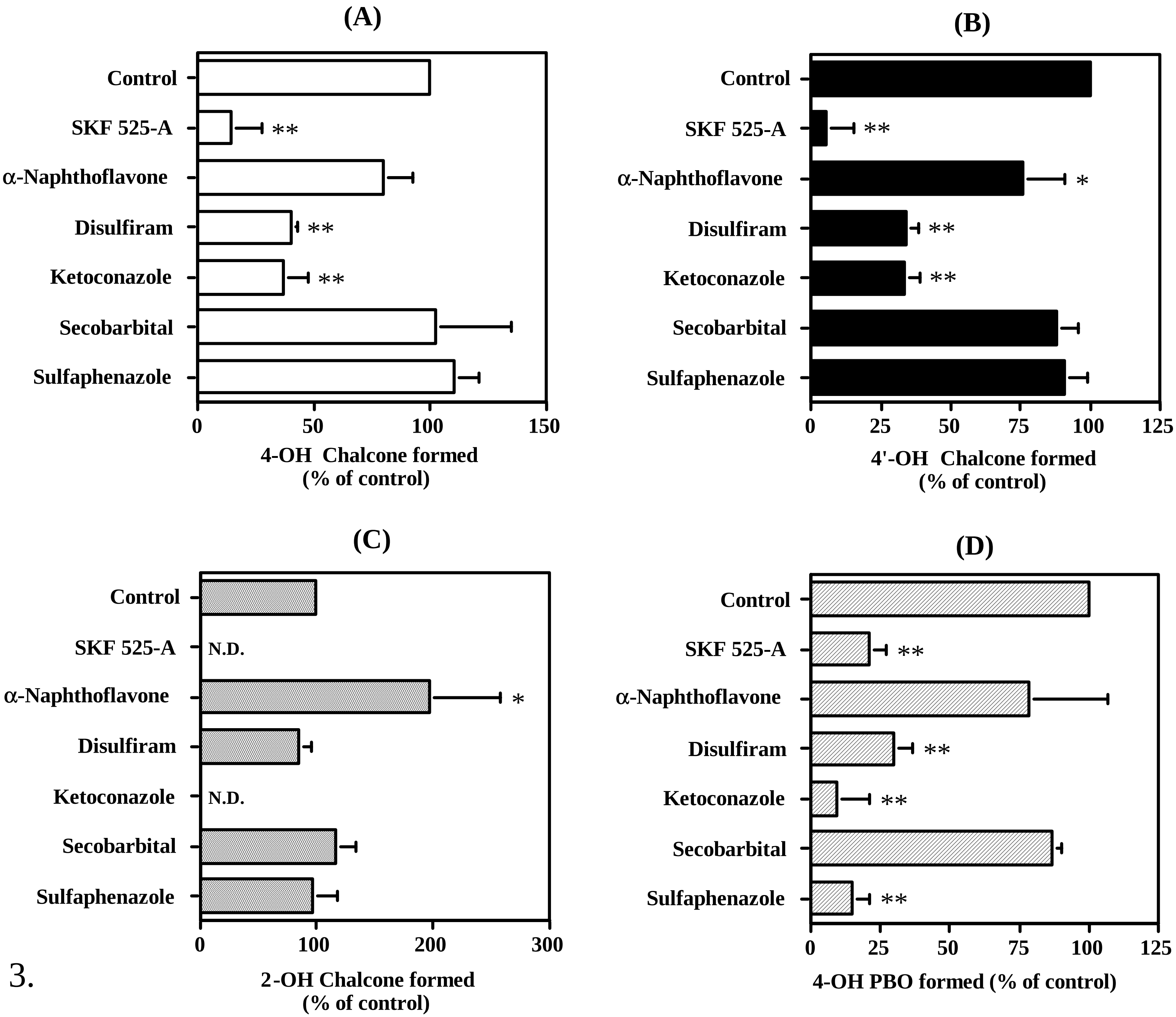

(A)
Control
SKF 525-A
**
α-Naphthoflavone
Disulfiram
**
Ketoconazole
**
Secobarbital
Sulfaphenazole
0
50
100
150
4-OH Chalcone formed
(% of control)
(B)
Control
SKF 525-A
**
α-Naphthoflavone
*
Disulfiram
**
Ketoconazole
**
Secobarbital
Sulfaphenazole
0
25
50
75
100
125
4'-OH Chalcone formed
(% of control)
(C)
Control
SKF 525-A
N.D.
α-Naphthoflavone
*
Disulfiram
Ketoconazole
N.D.
Secobarbital
Sulfaphenazole
0
100
200
300
2-OH Chalcone formed
(% of control)
(D)
Control
SKF 525-A
**
α-Naphthoflavone
Disulfiram
**
Ketoconazole
**
Secobarbital
Sulfaphenazole
**
0
25
50
75
100
125
4-OH PBO formed (% of control)

Fig. 3.

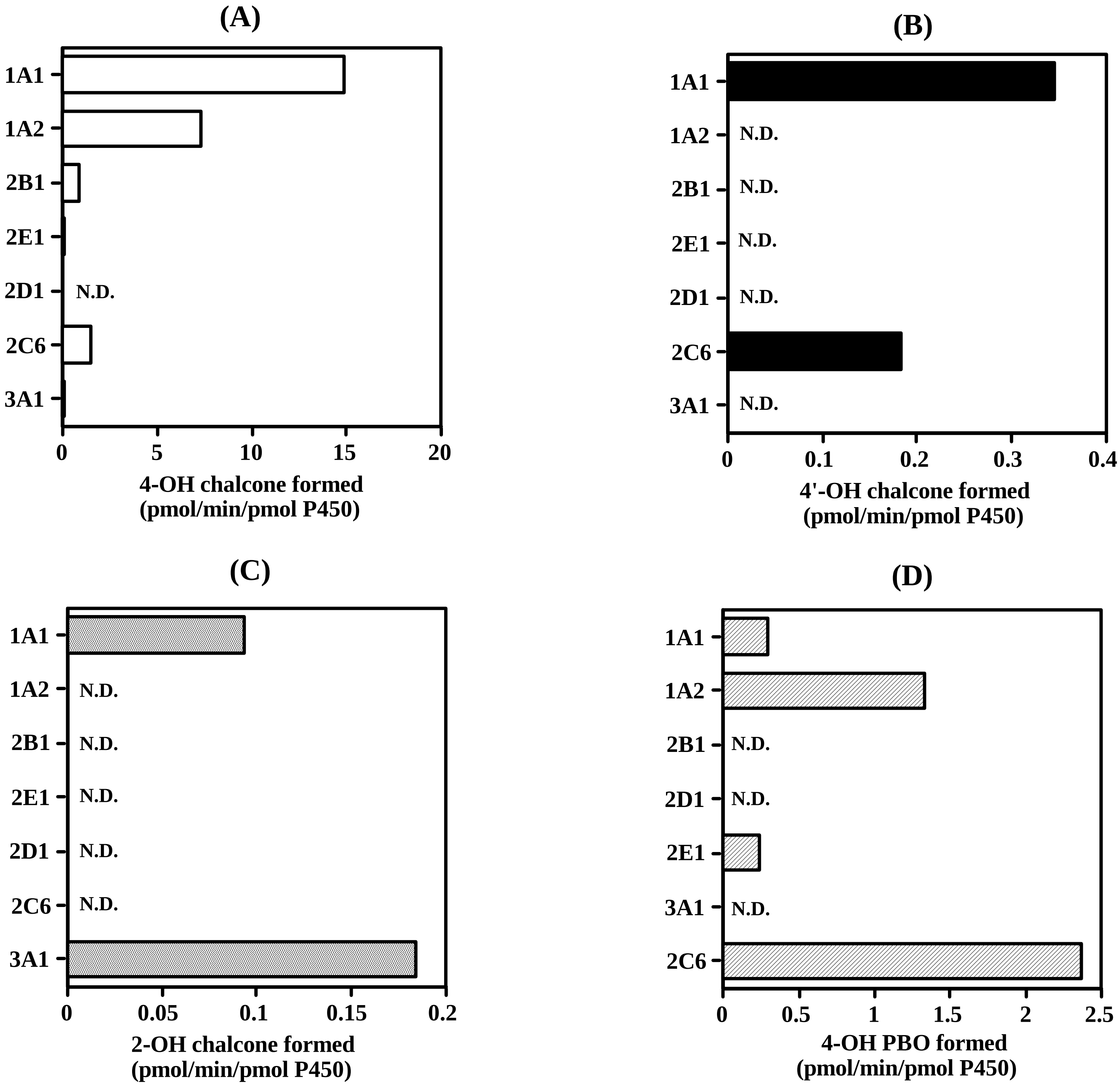

(A)
1A1
1A2
2B1
2E1
2D1
N.D.
2C6
3A1
0
5
10
15
20
4-OH chalcone formed
(pmol/min/pmol P450)
(B)
1A1
1A2
N.D.
2B1
N.D.
2E1
N.D.
2D1
N.D.
2C6
3A1
N.D.
0
0.1
0.2
0.3
0.4
4'-OH chalcone formed
(pmol/min/pmol P450)
(C)
1A1
1A2
N.D.
2B1
N.D.
2E1
N.D.
2D1
N.D.
2C6
N.D.
3A1
0
0.05
0.1
0.15
0.2
2-OH chalcone formed
(pmol/min/pmol P450)
(D)
1A1
1A2
2B1
N.D.
2D1
N.D.
2E1
3A1
N.D.
2C6
0
0.5
1
1.5
2
2.5
4-OH PBO formed
(pmol/min/pmol P450)

Fig. 4.

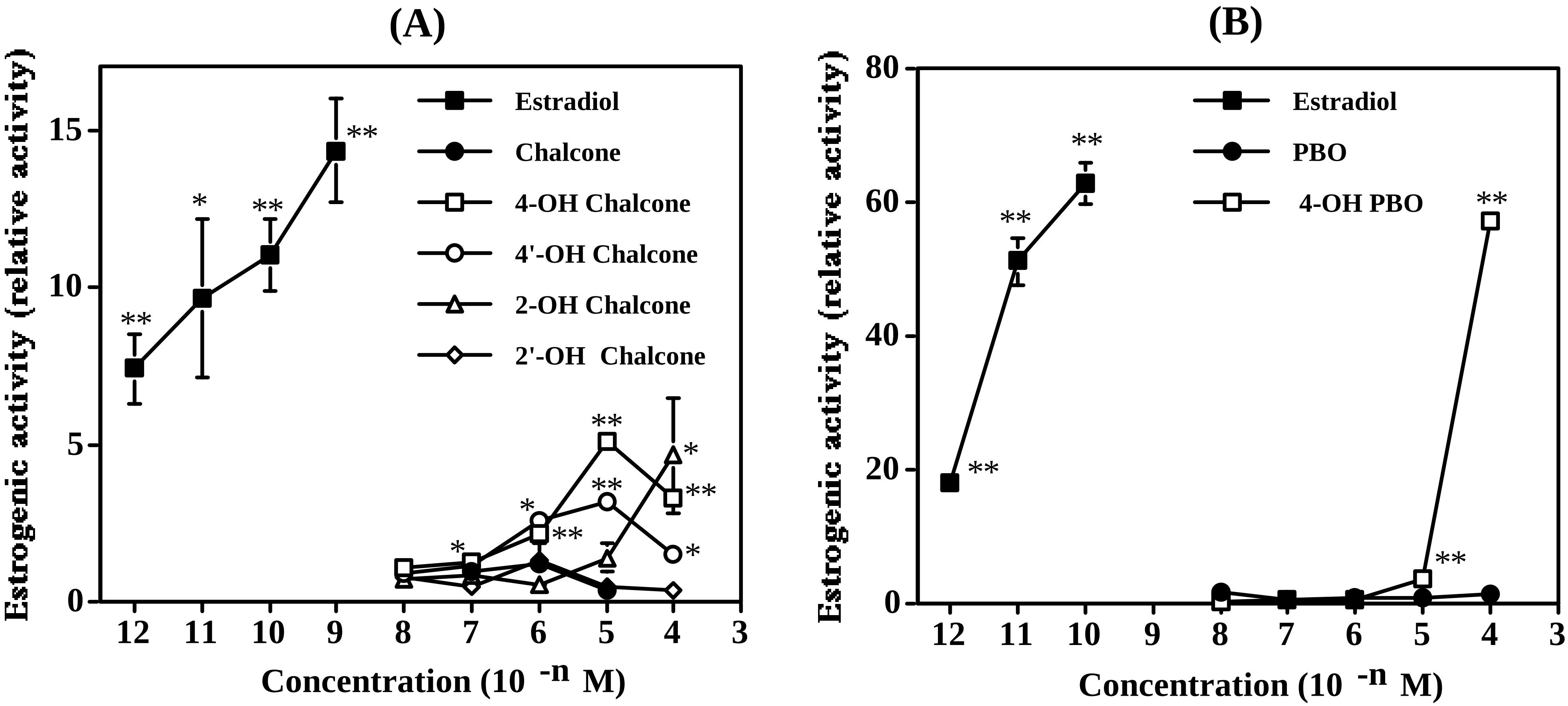

(A)
Estrogenic activity (relative activity)
0
5
10
15
Estradiol
Chalcone
4-OH Chalcone
4'-OH Chalcone
2-OH Chalcone
2'-OH Chalcone
**
*
**
**
*
*
**
**
*
**
*
**
*
12
11
10
9
8
7
6
5
4
3
Concentration (10 -n M)
(B)
Estrogenic activity (relative activity)
0
20
40
60
80
Estradiol
PBO
4-OH PBO
**
**
**
**
**
12
11
10
9
8
7
6
5
4
3
Concentration (10 -n M)

Fig. 5.

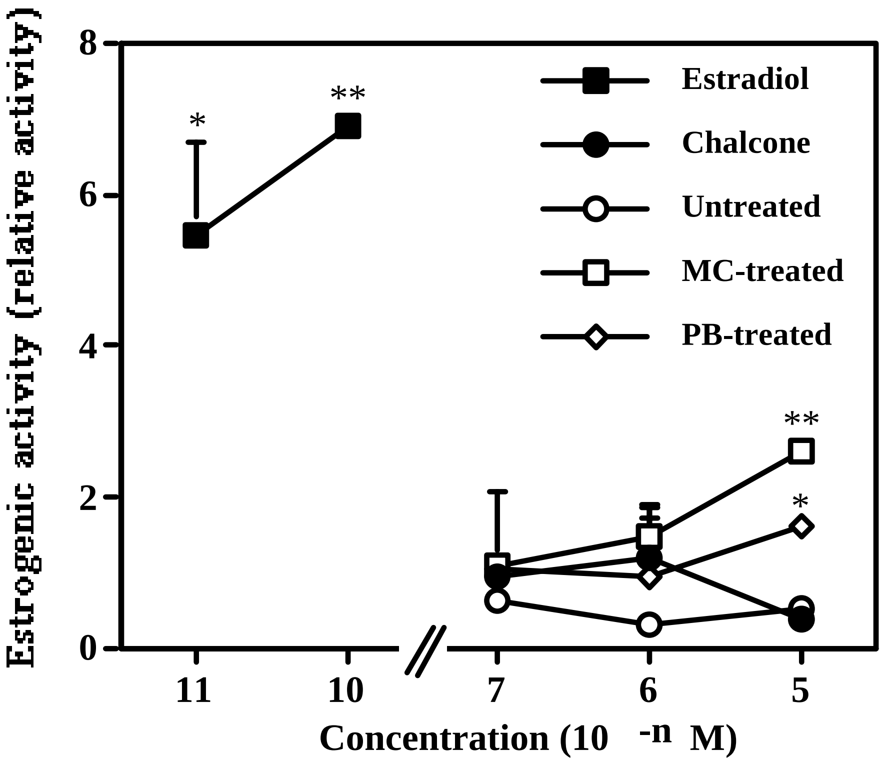
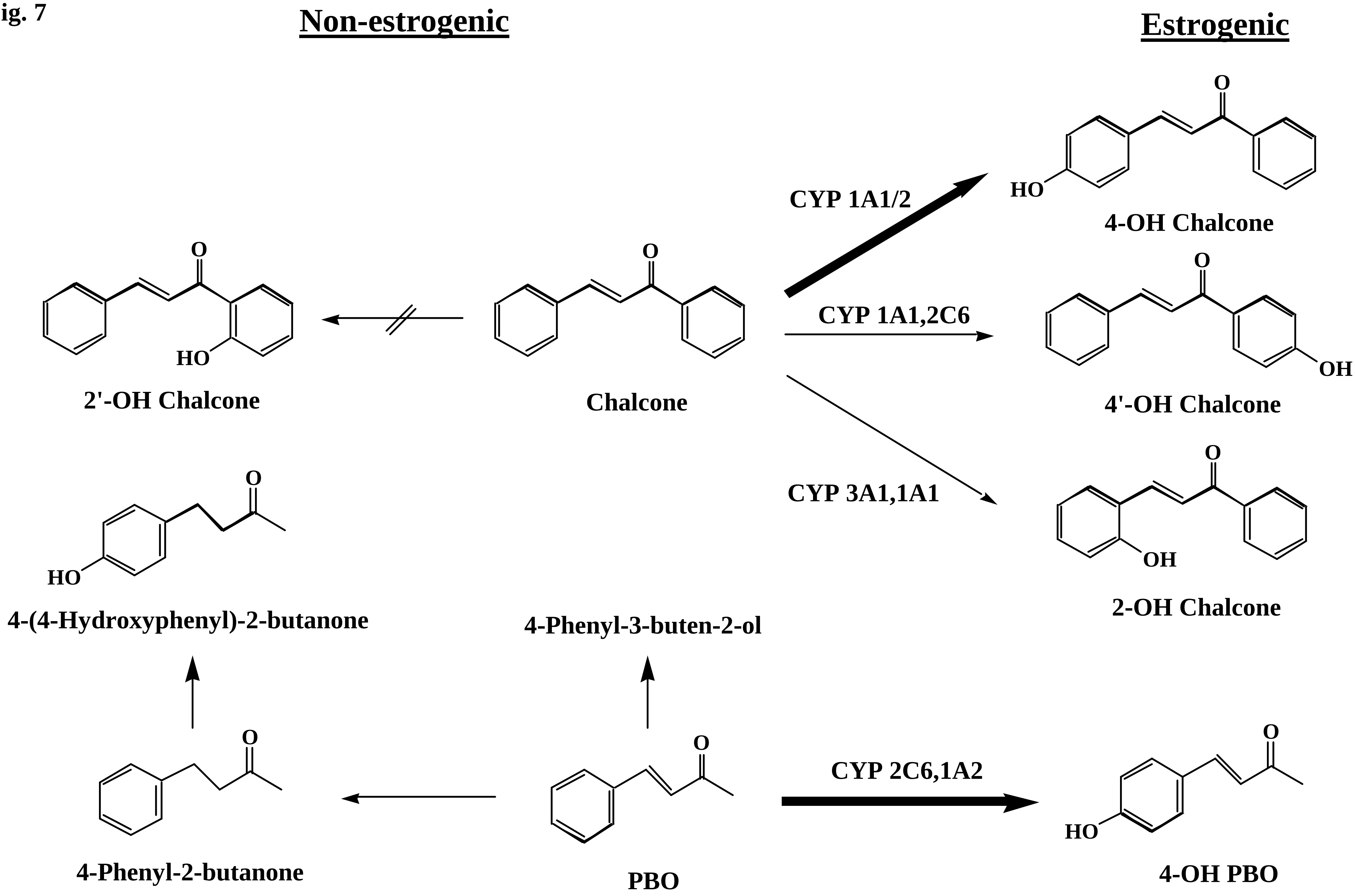

Estrogenic activity (relative activity)
8
6
4
2
0
11
10
7
6
5
Concentration (10 -n M)
Estradiol
Chalcone
Untreated
MC-treated
PB-treated
*
**
**
*

Fig. 6.

Fig. 7


Non-estrogenic
Estrogenic
O
HO
4-OH Chalcone
CYP 1A1/2
O
O
2'-OH Chalcone
HO
Chalcone
O
CYP 1A1,2C6
OH
4'-OH Chalcone
O
HO
4-(4-Hydroxyphenyl)-2-butanone
CYP 3A1,1A1
O
OH
2-OH Chalcone
4-Phenyl-3-buten-2-ol
O
4-Phenyl-2-butanone
O
PBO
CYP 2C6,1A2
O
HO
4-OH PBO