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Metabolic activation of *o*-phenylphenol to a major cytotoxic metabolite, phenylhydroquinone: role of human CYP1A2 and rat CYP2C11/CYP2E1

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1. The *in vitro* metabolic activation of *o*-phenylphenol has been evaluated as yielding a toxic metabolite, 2,5-dihydroxybiphenyl (phenylhydroquinone), by *p*-hydroxylation in liver microsomes of rat and human. The involvement of rat CYP2C11, CYP2E1 and human CYP1A2 in the *p*-hydroxylation of *o*-phenylphenol is suggested.

2. 2,3- and phenylhydroquinone, which induced DNA single-strand scission in the presence of 1 μM $CuCl_2$, were the most cytotoxic chemicals examined to cultured mammalian cell lines among *o*-phenylphenol, *m*-phenylphenol, *p*-phenylphenol, 2,2′-, 4,4′-, 2,3- and phenylhydroquinone.

3. Rat and human liver microsomes catalysed the formation of phenylhydroquinone, but not 2,3-dihydroxybiphenyl, using *o*-phenylphenol as a substrate. A higher rate of metabolic activation of *o*-phenylphenol was observed with livers of the male than the female rats by 5·6- and 2·6-fold respectively.

4. Inhibitory antibodies against the male-specific CYP2C11 inhibited hepatic *o*-phenylphenol *p*-hydroxylation in the male F344 and Sprague–Dawley rat by >70%. Liver microsomes from the isoniazid-treated rats produced 1·8- and 3-fold induction of *o*-phenylphenol *p*-hydroxylation and chlorzoxazone 6-hydroxylation (a CYP2E1-dependent activity) respectively.

5. Human CYP1A2, expressed by baculovirus-mediated cDNA expression systems, exhibited a remarkably higher capacity for *o*-phenylphenol *p*-hydroxylation at concentrations of 5 (>5-fold), 50 (>2-fold) and 500 μM (>2-fold) than CYP2A, CYP2B, CYP2Cs, CYP2D6, CYP2E1 and CYP3A4 on the basis of pmol P450.

6. Among various CYP inhibitors tested here, 7,8-benzoflavone and furafylline, typical human CYP1A2 inhibitors, inhibited the microsomal *p*-hydroxylation of *o*-phenylphenol in human livers most potently by 70 and 50% respectively.

7. The results thus indicate the involvement of rat CYP2C11/CYP2E1 and human CYP1A2 in the hepatic *p*-hydroxylation of *o*-phenylphenol.

Introduction

o-Phenylphenol (OPP) is used as a fungicide for harvested citrus fruits and its toxicity has been extensively studied, including acute toxicity, mutagenicity and teratogenic effects (Luster *et al*. 1981). Long-term carcinogenicity testing (Hodge *et al*. 1952) did not give clear evidence on any carcinogenic effects by OPP. From these early results, no significant hazardous effect was anticipated with the exposure of

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man to low doses of OPP. However, in 1981 (Hiraga and Fujii 1981), OPP was first shown to induce urinary bladder tumours by administration of an OPP-containing diet to the male F344 rat. Subsequently, a clear sex-related difference was observed in the susceptibility to bladder carcinogenicity by OPP (Hasegawa *et al*. 1991), with males being more sensitive than females.

OPP was shown to undergo oxidative metabolism in livers of mouse and rat to produce a toxic metabolite, 2,5-dihydroxybiphenyl or phenylhydroquinone (PHQ) (Ushiyama *et al*. 1992). PHQ is thought to be readily auto-oxidized to phenylbenzoquinone (PBQ) via a semiquinone radical (Tayama *et al*. 1994). A significant correlation was reported between the incidence of bladder cancer and the level of reactive intermediates including PBQ in urine after administration of OPP to the male F344 rat (Morimoto *et al*. 1987, 1989). Several lines of evidence have accumulated to support the concept that the OPP metabolite, PHQ, causes oxidative damage to exposed cells by the induction of DNA damage, to produce 8-hydroxy deoxyguanosine, adduct formation and DNA strand breakage (Nagai *et al*. 1990, 1995, Sasaki *et al*. 1997, Murata *et al*. 1999). Among the several forms of xenobiotic-metabolizing enzymes in rat and mouse, neither phenobarbital- nor 3-methylcholanthrene-induced cytochrome P450 (CYPs) were likely to be the major forms catalysing the oxidative metabolism of OPP to yield PHQ (Halpaap-Wood *et al*. 1981). Furthermore, in spite of use of OPP as a food additive, little is known about human CYP forms involved in the metabolic activation of OPP. An attempted, therefore, is made to elucidate which CYPs play major roles in the oxidative metabolism of OPP to produce the reactive metabolite, PHQ.

In the present study, microsomal oxidation of OPP was quantitated by HPLC using hepatic microsomes from the male and female rat and human. Inhibition of PHQ formation catalysed by male rat liver microsomes was examined by the aid of inhibitory antibodies against various rat CYPs. Also compared was the oxidation of OPP to produce PHQ catalysed by human CYPs expressed by baculovirus-mediated systems. Typical inhibitors of human CYPs were used to confirm the results obtained from the cDNA-mediated human CYP expression systems. Thus, rat CYP2C11 and possibly CYP2E1, and human CYP1A2 were shown to be the major CYPs involved in the metabolic activation of OPP to PHQ in the present *in vitro* systems.

Materials and methods

Reagents and hepatic microsomes

All reagents used were of the highest grade available. OPP, *p*-phenylphenol (PPP), 2,2′-dihydroxybiphenyl, 2,3-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl, 7,8-benzoflavone, coumarin and diethyldithiocarbamate were from Wako Pure Chemical Industries Ltd (Tokyo, Japan). *m*-Phenylphenol (MPP) and PHQ were from Tokyo Kasei Organic Chemicals (Tokyo, Japan). The purity of OPP, PPP, 2,2′-dihydroxybiphenyl, 2,3-dihydroxybiphenyl, 4,4′-dihydroxybiphenyl, MPP and PHQ was $>99\%$, since no other hydroxybiphenyl was detected on HPLC with any of the hydroxylated biphenyls used in the present study. The chemical structures of the hydroxybiphenyls studied and PBQ are shown in figure 1. Diclofenac, troleandomycin, cyclophosphamide and β-naphthol were from Sigma Chemical Co. Ltd (St. Louis, MO, USA). Furafylline was from Ultrafine Chemical Co. (Manchester, UK) through Daiichi Chemicals (Tokyo, Japan), omeprazole was from Astra Japan Ltd (Osaka, Japan), and quinidine was from Kanto Chemicals (Tokyo, Japan). SKF-525A was from Smith Kline & French Labs. $NADP^+$ and NADPH were from Oriental Yeast (Tokyo, Japan) and glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Boehringer Mannheim. Mixed liver microsomes of human individuals (six males, six females) and inhibitory antibodies against rat CYP forms were from GENTEST Corp. (Woborn, MA, USA) through Daiichi Chemicals (Tokyo, Japan). Male and female rat liver microsomes were prepared from 10 male and 10 female F344 rats (8 weeks old; Japan SLC, Inc., Hamamatsu, Japan) respectively, and those from 20 male and 42 female Sprague–Dawley rats were from

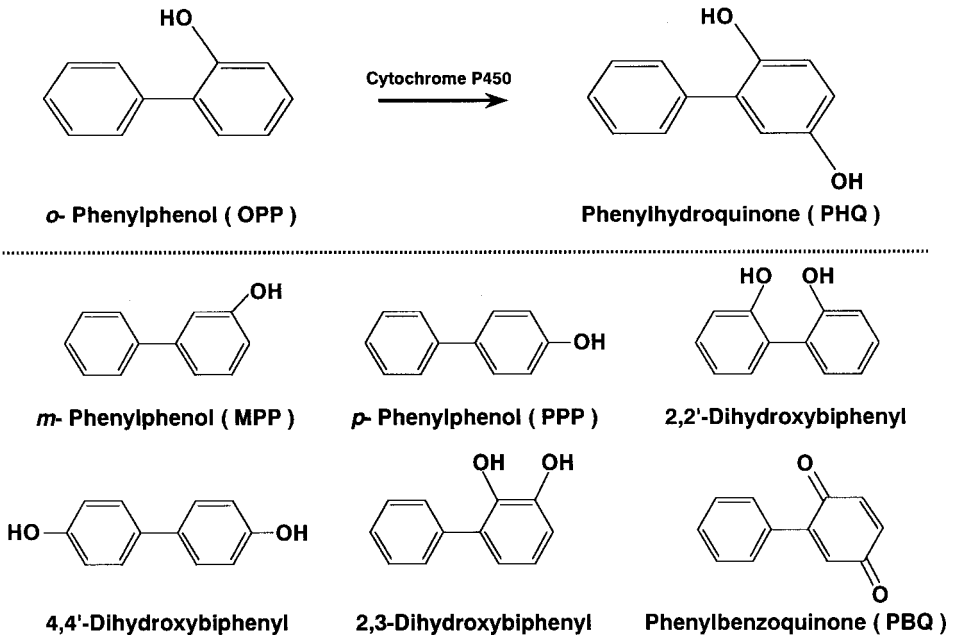

Figure 1. Enzymatic reaction of *o*-phenylphenol *p*-hydroxylation and the chemical structures of other related hydroxybiphenyls and phenylbenzoquinone. Metabolic activation of *o*-phenylphenol catalysed by cytochrome P450 is depicted as well as the chemical structures of other related hydroxybiphenyls and phenylbenzoquinone.

Xenotech LLC (Kansas City, KS, USA) to examine sex-related differences in hepatic PHQ formation. Male rat liver microsomes from corn oil- or saline-administered Sprague–Dawley rats (20 each) and various CYP inducer-treated 15 (phenobarbital-treated), 16 (5,6-benzoflavone-treated) or 18 (isoniazid-treated) male Sprague–Dawley rats (8 weeks old) were from Xenotech LLC (Yokohama, Japan). 5,6-Benzoflavone (a CYP1A inducer; Sigma) dissolved in corn-oil was administered to 16 male Sprague–Dawley rats at 100 mg.kg^{-1} body weight once per day on days 1–4, and liver microsomes were prepared on day 5. Hepatic 7-ethoxyresorufin *O*-dealkylation (the CYP1A-dependent activity) was 3940 pmol.mg^{-1} protein.min^{-1} in mixed microsomes of the inducer-treated 16 animals and 280 pmol.mg^{-1} protein.min^{-1} in corn oil-administered 20 animals. Phenobarbital (a CYP2B inducer; Spectrum Chemical Mfg. Co.) dissolved in saline was administered to 15 male Sprague–Dawley rats at 80 mg.kg^{-1} body weight once per day on days 1–4, and liver microsomes were prepared on day 5. Hepatic 7-penthoxyresorufin *O*-dealkylation (the CYP2B-dependent activity) was 1490 pmol.mg^{-1} protein.min^{-1} in mixed microsomes of the inducer-treated 15 animals and 18 pmol.mg^{-1} protein.min^{-1} in saline-administered 20 animals. Isoniazid (a CYP2E1 inducer; Sigma) dissolved in saline was administered to 18 male Sprague–Dawley rats at 200 mg.kg^{-1} body weight once per day on days 1–4, and liver microsomes were prepared on day 5. Hepatic chlorzoxazone 6-hydroxylation (the CYP2E1-dependent activity) was 13.3 nmol.mg^{-1} protein.min^{-1} in mixed microsomes of the inducer-treated 18 animals and 4.4 nmol.mg^{-1} protein.min^{-1} in saline-administered 20 animals.

Cell kill and growth inhibitory assays in cultured mammalian cells

HepG2 cells were cultured in Dulbecco's MEM supplemented with 10% foetal calf serum (FCS). Culture media for MIAPaCa-2, V79 and rabbit cornea derived SIRC cells were Eagle's MEM supplemented with 10% FCS and that for Morris5123DTC was RPMI1640 with 10% FCS. Cell kill assays were done according to the inhibition of colony formation induced by OPP and related compounds using human cancer cell lines, HepG2 (liver) and MIAPaCa-2 (pancreas), rat cancer cell line, Morris5123DTC (liver) and Chinese hamster fibroblast cell line, V79 (lung). Concentrations of *o*-, *m*-, *p*-phenylphenol and 2,2′-dihydroxybiphenyl used were 1000, 500, 250, 125, 62.5, 31.3 and 15.6 μM; those of 4,4′-dihydroxybiphenyl were 1000, 500, 250, 125, 62.5, 31.3 and 15.6 μM; and those of 2,3-dihydroxybiphenyl and PHQ were 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.9 and 1.0 μM. Hydroxybiphenyls were dissolved in H_2O. Cells (100–4000) were inoculated in 60-mm (diameter) dishes and cultured overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Either OPP or related compounds were added at various concentrations and further cultured for 48 h. Culture media were changed, and cultured for 1–2 weeks depending on the growth rates of each cell line. Colonies were counted after 0.4% crystal

violet staining. A growth inhibition assay was performed according to Itagaki *et al*. (1995) and Ohno *et al*. (1998). Briefly, SIRC cells were inoculated at a cell density of 20 000 cells per well of 96-well plates. The cells were further cultured with various concentrations of hydroxybiphenyls for 72 h at 37 °C. Inhibition of cell growth was determined after the cells were stained with 0·4% crystal violet.

In vitro DNA strand scission induced by OPP and related compounds

Covalently closed circular (ccc) DNA (pTrcHisB, Invitrogen, Carlsbad, CA, USA) was treated with various concentration of OPP and related compounds. The reaction mixture (10 μl) consisted of 50 mM Tris-HCl (pH 8·0), 1 μM $CuCl_2$, 0·4 μg cccDNA and various concentrations of OPP and related compounds. DNA strand breakage was analysed on 1% agarose gel electrophoresis according to mobility differences between cccDNA, open circular DNA and linearized DNA, after incubation performed at room temperature for 30 min.

Assays of microsomal OPP-hydroxylating activities by rat and human hepatic microsomes

The microsomal oxidation of OPP to PHQ was examined using rat and human liver microsomes. A typical incubation mixture (250 μl) contained 100 mM potassium phosphate buffer (pH 7·4), 0·5 mM OPP, 0·33 mM NADPH and 50 μg hepatic microsomes from rat or 100 μg mixed human microsomes. The reaction was initiated by the addition of NADPH, incubated for 20–40 min and terminated by the addition of 250 μl ethyl ether. The reaction was linear with time up to 40 min. OPP and hydroxylated metabolites were extracted twice with ethyl ether. Amounts of OPP metabolites formed were analysed together with β-naphthol as an internal standard by reverse-phase HPLC system (Shimadzu LC-VP series) equipped with a L-column ODS (4·6 × 250 mm). The initial mobile phase was acetonitrile : 0·5% acetic acid (3 : 7), and was kept for 2 min with a flow rate of 0·8 $ml.min^{-1}$. The acetonitrile concentration was then raised as a linear gradient up to acetonitrile : 0·5% acetic acid (8 : 2) over 22 min. Metabolites were detected at 312 nm by a UV-VIS detector (SPD-10Avp). The elution times of PHQ, β-naphthol and OPP were 14·7, 20·3 and 28·6 min respectively.

Inhibitory antisera against rat CYP1A1/2, CYP2B1, CYP2C11, CYP2E1 and CYP3A1 (0, 10, 20 or 50 μl) were incubated with rat hepatic microsomes at room temperature for 30 min as suggested by the manufacturer. Normal goat antisera were added so that total volume of antisera was 50 μl. The mixture was added to 100 mM potassium phosphate buffer (pH 7·4), 0·5 mM OPP, 0·33 mM NADPH, in a total volume of 250 μl. The reaction was started by the addition of 0·33 mM NADPH, incubated for 20 min, and was terminated by the addition of 250 μl ethyl ether. The aqueous layer was extracted twice with ethyl ether and the PHQ extracted into the organic layer was quantitated by HPLC.

Also measured was OPP hydroxylation in microsomes from rat treated with CYP inducers.

Hydroxylation of OPP by human CYP forms, which had been expressed together with cytochrome P450 reductase in baculovirus-mediated cDNA-expression systems (SUPERSOMES™, GENTEST Corp., Woburn, MA, USA), was examined. The CYP forms used were: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Insect cell microsomes expressing cytochrome b_5 and cytochrome P450 reductase were also used. The reaction mixture for OPP hydroxylation (250 μl) consisted of 100 mM potassium phosphate buffer (pH 7·4), either 500 or 5 μM OPP, 0·33 mM $NADP^+$, 3·3 mM glucose 6-phosphate, 0·4 U/ml glucose 6-phosphate dehydrogenase, 3·3 mM $MgCl_2$ and insect cell microsomes expressing each human CYP form. Reactions were performed for 20 min and metabolites analysed by HPLC.

Effects of typical CYP inhibitors or substrates on OPP hydroxylation catalysed by human liver microsomes were examined (5 μM OPP). The following substances were used for inhibition of CYP-dependent enzymatic activities: 7,8-benzoflavone and furafylline for CYP1A2, coumarin for CYP2A6, cyclophosphamide for CYP2B6, diclofenac for CYP2C9, omeprazole for CYP2C19, quinidine for CYP2D6, diethyldithiocarbamate for CYP2E1, troleandomycin for CYP3A4 (Ono *et al*. 1996, Teramura *et al*. 1997). Human microsomes (100 μg) were pre-incubated with various concentrations of CYP inhibitors or substrates at 37 °C for 15 min. OPP was then added at a concentration of 5 μM. The reaction was performed at 37 °C for 20 min and terminated by the addition of 250 μl ethyl ether. The aqueous layer was extracted twice with ethyl ether, and then metabolites formed were quantified by HPLC.

Results

Cell kill and growth inhibitory effects of OPP and biphenyl-related compounds with hydroxyl moieties

The potency of cytotoxicity and growth inhibition of OPP and other hydroxylated biphenyls were compared using established cell lines derived from various animals. Based on the IC_{50}, 2,3-dihydroxybiphenyl and PHQ were the most cytotoxic of the hydroxy biphenyls studied (table 1). 2,3-Dihydroxybiphenyl, PHQ

Table 1. Inhibition of colony formation or cell growth of various mammalian cells treated with OPP and related compounds.

	IC_{50} (μM)				
	Human		Rat	Chinese hamster	Rabbit
	HepG2	MIAPaCa-2	Morris5123DTC	V79	SIRC
o-Phenylphenol	270	100	420	130	290
m-Phenylphenol	210	70	250	160	250
p-Phenylphenol	230	110	170	200	170
2,2′Dihydroxybiphenyl	400	310	560	340	420
4,4′Dihydroxybiphenyl	42	90	16	90	40
2,3′Dihydroxybiphenyl	50	5	19	9	70
2,5′Dihydroxybiphenyl	30	3	21	9	40

Inhibition of colony formation of HepG2, MIAPaCa-2, Morris5123DTC and V79 cells was examined; and growth inhibition of SIRC cells was determined as described by Ohno *et al*. (1998) respectively. IC_{50} shown are the average of three determinations.

and 4,4′-dihydroxy biphenyl showed a tendency to be the most potent inhibitors of colony formation (cell lines listed in table 1 except for SIRC cells) or cell growth (SIRC cells) among the hydroxylated biphenyl derivatives studied.

Induction of DNA strand breakage by OPP and hydroxylated derivatives of biphenyl

Induction of DNA strand breakage by OPP and other mono- and di-hydroxylated biphenyl derivatives was examined in the presence of 1 μM $CuCl_2$. Both PHQ and 2,3-dihydroxybiphenyl (at 10–20 μM) caused nicks on the plasmid DNA molecules used, and DNA strand breakage was much weaker with OPP, MPP, PPP, 2,2′-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl at even 500 μM (figure 2).

Microsomal PHQ formation catalysed by rat liver microsomes

Microsomal PHQ formation was compared between livers of the male and female F344 and Sprague–Dawley rat and a clear male dominance was observed (table 2), namely 5·64-fold higher in the male F344 rat than in the female, and 2·60-fold in the male Sprague–Dawley rat than in the female.

Effects of inhibitory antibodies of rat CYPs on PHQ formation

Inhibitory antibodies against rat CYP forms were used to elucidate the specific rat CYPs involved in PHQ formation by the male F344 and Sprague–Dawley rat liver microsomes. In both the male F344 and Sprague–Dawley rat, antisera against the male-specific CYP2C11 showed the most effective and concentration-dependent inhibition of microsomal PHQ formation from OPP by 88 and 70% respectively (figure 3A and B).

Hydroxylation of OPP in liver microsomes from rat treated with various CYP-inducers

It was examined whether hepatic microsomes from rat treated with CYP inducers exhibit induction of OPP hydroxylation. 5,6-Benzoflavone (a CYP1A inducer) failed to induce microsomal OPP *p*-hydroxylation in the Sprague–Dawley

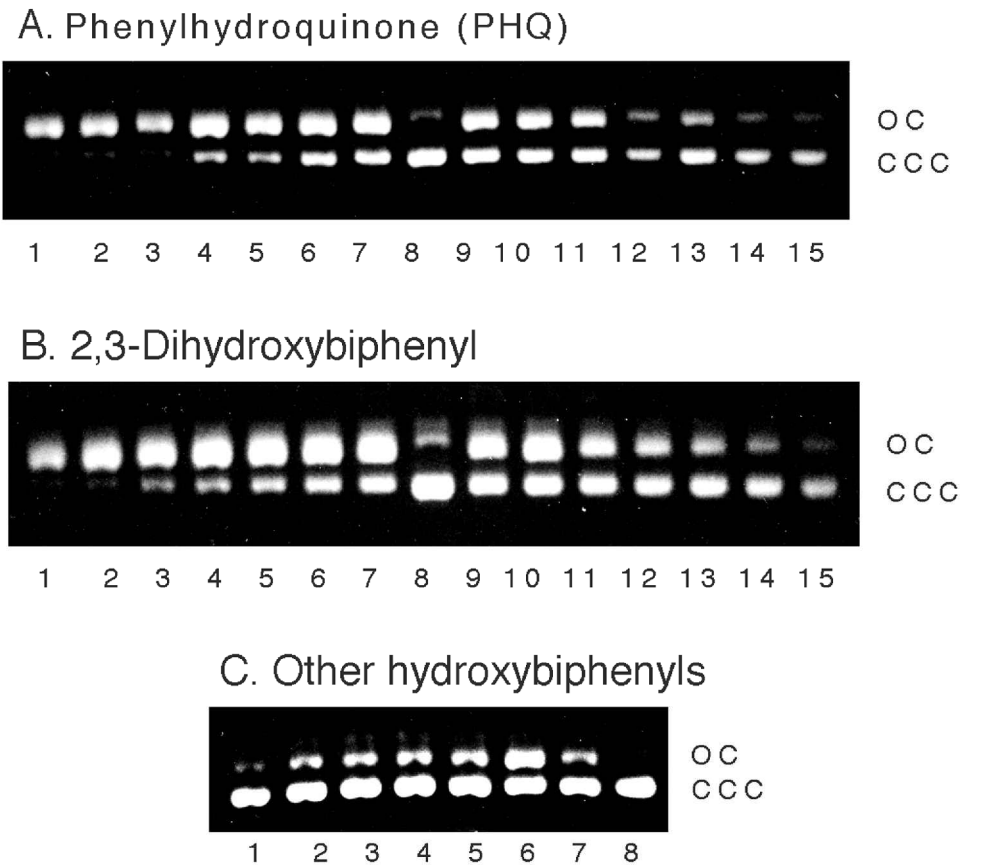

Figure 2. DNA single-strand breaks induced by OPP and other hydroxylated biphenyl derivatives. Covalently closed circular DNA (cccDNA) was treated with various concentration of OPP and related compounds in the presence of 1 μM $CuCl_2$. DNA strand breakage was analysed according to the reduction in the amount of cccDNA and the increase in the amount of nicked open circular (OC) DNA. (A) Concentrations (μM) of PHQ used were: 12·5 (lane 1); 10 (2); 8 (3); 6·4 (4); 5·1 (5); 4·1 (6); 3·3 (7); 0 (control, 8); 2·6 (9); 2·1 (10); 1·7 (11); 1·3 (12); 1·1 (13); 0·9 (14); 0·7 (15). (B) Concentrations (μM) of 2,3-dihydroxy-biphenyl used were: 25 (lane 1); 20 (2); 16 (3); 12·8 (4); 10·2 (5); 8·2 (6); 6·5 (7); 0 (control, 8); 5·2 (9); 4·2 (10); 3·4 (11); 2·6 (12); 2·1 (13); 1·7 (14); 1·3 (15). (C) Biphenyl and other hydroxylated biphenyl (500 μM): biphenyl (lane 1); 2-hydroxy (*o*-phenylphenol; 2); 3-hydroxy (*m*-phenylphenol; 3); 4-hydroxy (*p*-phenylphenol; 4); 2,2′-dihydroxybiphenyl (5); 4,4′-dihydroxybiphenyl (6); 3-hydroxy (*m*-phenylphenol; 7); none (control; 8).

Table 2. Male-dominant *p*-hydroxylation of OPP in liver microsomes of the F344 and Sprague–Dawley rat.

Strain	Male	Female	p[a]
F344	3.67 ± 0.40	0.65 ± 0.10	< 0.001
Sprague–Dawley	2.06 ± 0.05	0.79 ± 0.02	< 0.001

Hepatic *p*-hydroxylation of OPP (500 μM, nmol·min^{-1}·mg microsomal protein) was determined using mixed liver microsomes from 10 male and 10 female F344 rats and those from 20 male and 42 female Sprague–Dawley rats as described in the Materials and methods.

Data are the mean ± SD of four determinations.

[a] Degree of statistical significance.

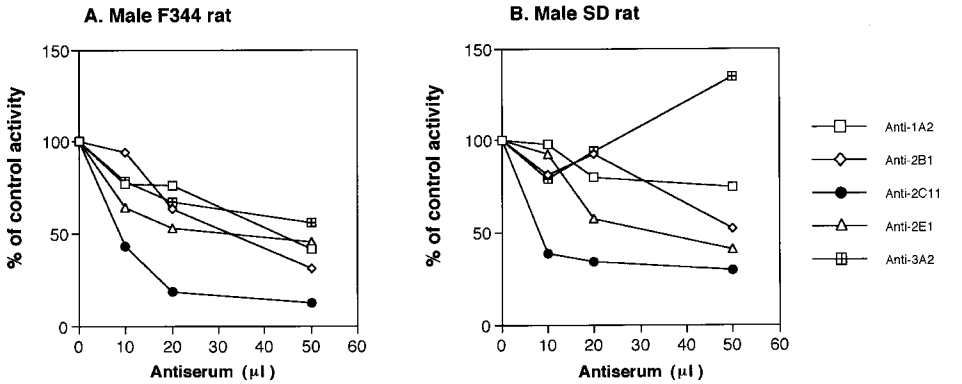

Figure 3. Effects of antisera against rat CYP forms on OPP *p*-hydroxylation in rat liver microsomes. Effects of inhibitory antibodies on microsomal OPP *p*-hydroxylation in rat livers were examined as described in the Materials and methods. The concentration of OPP was 500 μM and OPP was incubated for 40 min with rat liver microsomes (50 μg protein). Control activities (mean ± SD, nmol.min^{-1}.mg^{-1} microsomal protein) in the male F344 rat and male Sprague–Dawley (SD) rat were 1·17 ± 0·10 and 1·61 ± 0·05 respectively. Data points are the average of four determinations.

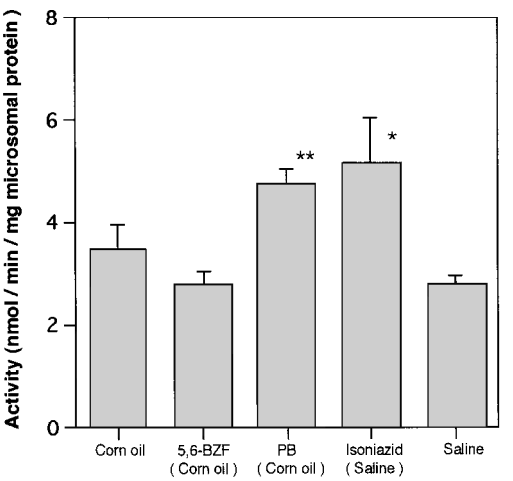

Figure 4. OPP *p*-hydroxylation in microsomes from the male Sprague–Dawley rat pretreated with CYP inducers. The concentration of OPP was 500 μM and OPP was incubated for 40 min with microsomes (50 μg protein). Data from 5,6-benzoflavone-treated (5,6-BZF) and phenobarbital-treated (PB) rats were compared with those from the corn oil-treated rat as controls. Data from the isoniazid-treated rat were compared with those from the saline-treated rat as controls. Each column represents the mean ± SD of triplicate determinations. Control activities (nmol.min^{-1}.mg^{-1} microsomal protein) in the presence of corn oil and saline were 3·48 ± 0·46 in quadruplicate determinations and 2·81 ± 0·15 in triplicate determinations respectively. The degree of statistical significance is expressed as $*p < 0{\cdot}02$ or $**p < 0{\cdot}01$.

rat liver, although 7-ethoxyresorufin *O*-deethylation, a typical CYP1A-dependent activity, was induced by 14-fold. Microsomal OPP *p*-hydroxylation was minimally induced (1·36-fold) by the administration of phenobarbital (a CYP2B inducer), whereas 7-pentoxyresorufin *O*-depentylation, a typical CYP2B-dependent activity, was induced by 85-fold. These results indicated that both CYP1A and CYP2B were not the major isoforms involved in the microsomal of *p*-hydroxylation OPP (figure 4). On the other hand, the largest induction (1·8-fold) of OPP *p*-hydroxylation was observed by isoniazid-administration.

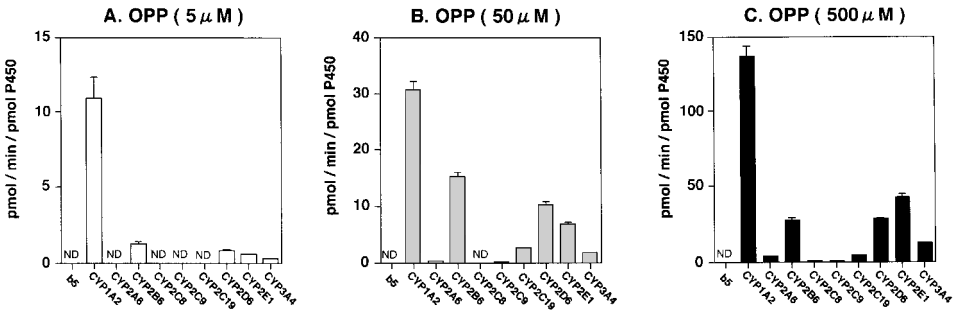

Figure 5. OPP *p*-hydroxylation in microsomes from baculovirus-infected insect cells expressing individual human CYPs. OPP was incubated for 20 min with CYP-expressing microsomes (10 pmol CYP) and the concentration of OPP was 5 μM (A), 50 μM (B) and 500 μM (C). Each column represents the mean ± SD of three separate experiments. ND (not detectable) indicates that metabolic activity was < 0·18 pmol.min^{-1}/pmol CYP.

Human CYPs responsible for PHQ formation

Mixed liver microsomes derived from human individuals (n = 12) also catalysed the *p*-hydroxylation of OPP (500 μM) at 2·54 ± 0·48 nmol.min^{-1}.mg^{-1} protein in triplicate determinations, which was as high as the rate of formation in male rat liver microsomes. Forms of human CYP expressed in baculovirus-mediated cDNA expression systems were used to examine the specific CYPs catalysing OPP hydroxylation. In the reaction mixtures using 10 pmol of each expressed CYP, CYP1A2 catalysed PHQ formation the most efficiently of the CYP forms tested in the present study (figure 5A-C). The PHQ formation was tested at the OPP concentration of 5 μM (figure 5A), 50 μM (B) and 500 μM (C). At 5 μM substrate concentration, CYP1A2 predominantly exhibited OPP *p*-hydroxylation at 11·94 pmol.min^{-1}/pmol CYP. The next highest was observed with CYP2B6 (1·26 pmol.min^{-1}/pmol CYP). CYP2D6, CYP2E1 and CYP3A4 showed lower activities at rates of 0·76, 0·58 and 0·23 pmol.min^{-1}/pmol CYP respectively. At 50 μM substrate concentration, catalytic activities by CYP1A2, CYP2B6, CYP2D6, CYP2E1 and CYP3A4 were 30·6, 15·2, 10·1, 6·7 and 1·7 pmol.min^{-1}/pmol CYP respectively. At 500 μM, CYP1A2, CYP2E1, CYP2D6, CYP2B6 and CYP3A4 converted OPP to PHQ at 136·8, 42·4, 27·9, 27·2 and 12·5 pmol.min^{-1}/pmol CYP respectively. In addition, CYP2C19, CYP2C9, CYP2C8 and CYP2A6, which did not exhibited catalytic activity at 5 μM, showed lower activities at 50 and 500 μM.

Hence, the overall conclusion is that CYP1A2 was the most efficient isoform for OPP *p*-hydroxylation.

Inhibition of OPP hydroxylation catalysed by human liver microsomes

Effects of typical CYP substrates or inhibitors on human hepatic OPP hydroxylation were examined to support results obtained using the microsomes of insect cells expressing different forms of human CYP. 7,8-Benzoflavone and furafylline, typical CYP1A inhibitors, exhibited the most potent inhibition of OPP *p*-hydroxylation (figure 6). By contrast, inhibitors of other forms of CYP showed lesser extent of inhibition of OPP *p*-hydroxylation. These results supported the results obtained by the recombinant CYP experiments in that CYP1A2 most efficiently catalysed OPP *p*-hydroxylation.

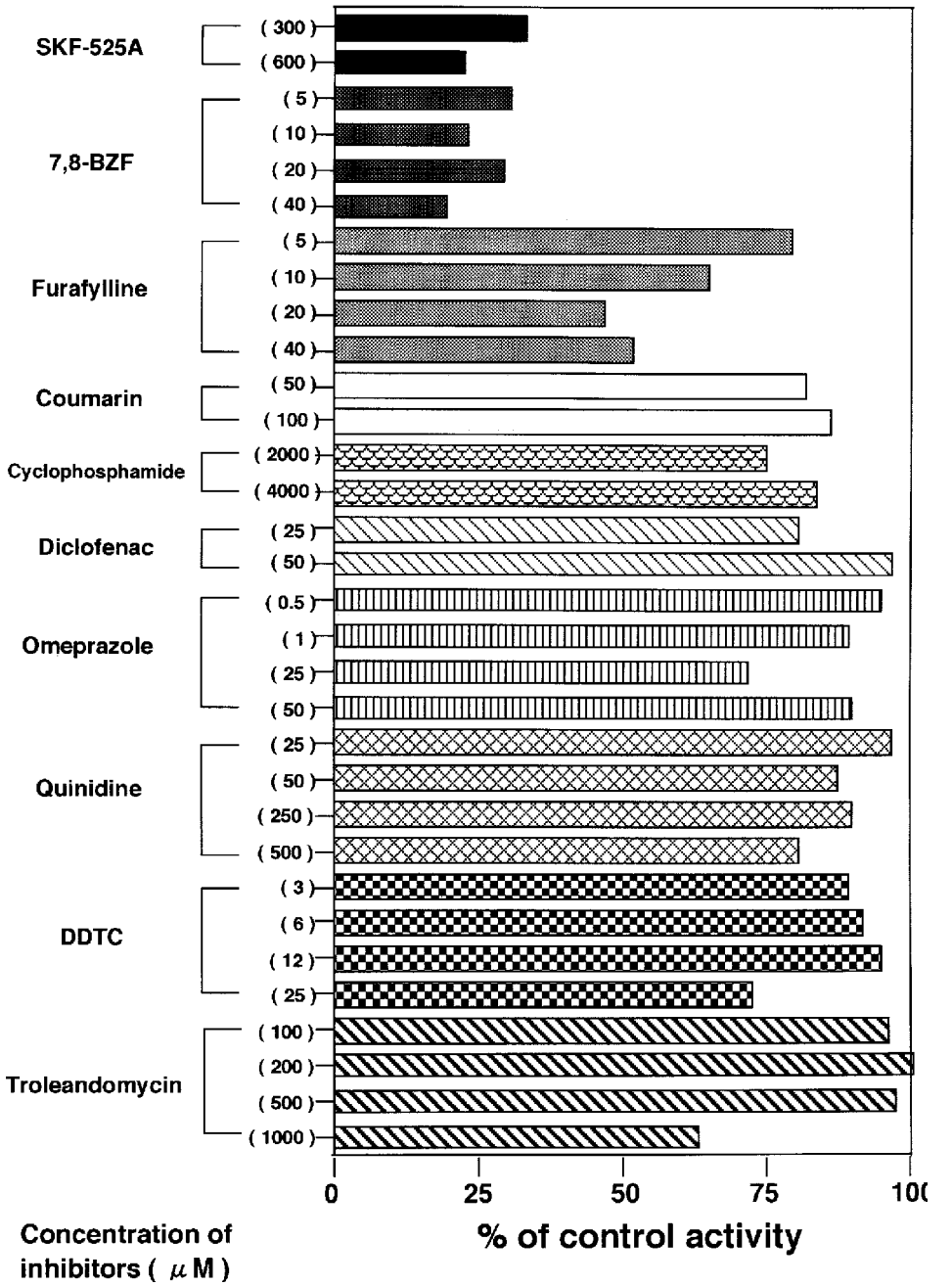

Figure 6. Effect of CYP inhibitors on OPP *p*-hydroxylation in human liver microsomes. The concentration of OPP was 5 μM and each column represents the average of duplicate experiments. OPP was incubated for 20 min with pooled human microsomes (100 μg protein). SKF-525A, furafylline, coumarin, cyclophosphamide, diclofenac, omeprazole, quinidine, diethyldithiocarbamate (DDTC) and troleandomycin were dissolved in distilled water. 7,8-Benzoflavone (7,8-BZF) was dissolved in methanol. Control activities (mean ± SD, nmol.min^{-1}.mg^{-1} protein) in the presence of distilled water and methanol were 0·32 ± 0·06 in six determinations and 0·31 ± 0·04 in triplicate determinations respectively.

Discussion

The involvement of rat CYP2C11 (a male-specific form), rat CYP2E1 and human CYP1A2 has been demonstrated *in vitro* for the metabolic activation of OPP to the toxic metabolite, PHQ by hepatic microsomes of the male rat and human.

2,3-Dihydroxybiphenyl and PHQ were cytotoxic in cell lines derived from several animal species and also for DNA strand break at the lowest concentration of the hydroxylated biphenyl derivatives used in this study. 4,4′-Dihydroxybiphenyl failed to induce efficient DNA strand break but was relatively cytotoxic, suggesting different mechanisms of cytotoxicity from those of 2,3-dihydroxybiphenyl and PHQ (table 1 and figure 2).

2,3-Dihydroxybiphenyl was not detected in the microsomal systems from either rat or human. Male dominance in the liver microsome-mediated conversion of OPP into PHQ was observed (table 2), consistent with the fact that a higher incidence in OPP-induced bladder cancer was also observed in the male F344 rat as compared with the female (Hasegawa *et al*. 1991). It was also observed that hepatic OPP *p*-hydroxylation catalysed by the male F344 and Sprague–Dawley rat was best inhibited by inhibitory antibodies against the male-specific rat CYP2C11 (figure 3). Antibodies against CYP3A seemed to enhance hepatic OPP *p*-hydroxylation. These results might suggest a possible interaction of OPP with CYP3A, although the exact mechanisms are unknown at present (figure 3). Recently, OPP was reported to induce cell injury in hepatocytes, associated with a substantial decrease in intracellular glutathione (Sugihara *et al*. 1997). Potencies of damage in cultured cells and induction of DNA strand breaks seemed to correlate among the hydroxybiphenyls, including PHQ used in the present study. Renal expression of the male-specific CYP2C11 has been documented (Ryan *et al*. 1993). Thus, male-specific rat CYP2C11, which has been proposed to be involved in the metabolic activation of OPP in this study, might play an important role in mediating cell damage in OPP-exposed rat. Whether the observed male-dominance in hepatic OPP *p*-hydroxylation could also be attributed to the bladder cancer incidence in the male rat clearly depends on the stability of the resultant toxic metabolite (PHQ) in the circulating blood and uro-epithelial tracts.

PHQ is further oxidized to yield PBQ (figure 1; Lambert and Eastmond 1994). CYP-catalysed one-electron reductive bioactivation has been reported as an important metabolic activation of compounds with a quinone moiety (Goeptar *et al*. 1995). The resultant PBQ is likely to undergo reductive bioactivation by CYP form(s). Elucidation of forms of CYP involved in the PBQ formation and the reductive bioactivation is required for clarification of the complete metabolic activation pathway of OPP.

Neither phenobarbital- nor 5,6-benzoflavone-administered rat liver microsomes exhibited substantially higher OPP *p*-hydroxylation than those from the corn oil-administered rat (figure 4). Failure of 5,6-benzoflavone to induce OPP *p*-hydroxylation is consistent with the results reported by Halpaap-Wood *et al*. (1981).

Among various human CYPs expressed in the baculovirus-mediated systems, CYP1A2 catalysed the OPP *p*-hydroxylation most efficiently among the CYP forms which are known to be expressed in human livers, over the OPP concentration examined (i.e. 5, 50 and 500 μM; figure 5). At higher OPP concentrations, CYP2D6 catalysed the OPP *p*-hydroxylation. Apparently, the K_m of CYP2D6 for the reaction was higher than that of CYP1A2 (figure 5). CYP2D6 is known to catalyse

C-hydroxylation of debrisoquin. It has been shown that ring-hydroxylation of debrisoquin was catalysed by CYP2D6 (Lightfoot *et al*. 2000). These results may support our results on OPP being a substrate of CYP2D6 to form PHQ, though further investigation is required.

It was decided to examine the major hepatic CYP forms catalysing the reaction at an OPP concentration of 5 μM, since this lowest concentration adopted in the present study might be of the highest toxicological significance. The results on the potent inhibition by 7,8-benzoflavone and furafylline, typical CYP1A inhibitors, on OPP hydroxylation catalysed by mixed human liver microsomes were quite consistent with the efficient catalytic activities by cDNA-expressed CYP1A2. K_m for microsomal OPP hydroxylation was $\sim$ 110 μM in human liver systems (data not shown). The approximate hepatic levels (pmol.mg^{-1} protein) of CYP forms in human livers are (Shimada *et al*. 1996): 55 $\pm$ 28 for CYP1A; 83 $\pm$ 29 for CYP2C; 10 $\pm$ 5 for CYP2D6; 33 $\pm$ 17 for CYP2E1; and 147 $\pm$ 57 for CYP3A. Thus, taking the substantially high rate of OPP *p*-hydroxylation by the recombinant CYP1A2 and the hepatic CYP levels into consideration, CYP1A2 likely plays a major role in PHQ formation in human livers. It was reported that 7,8-benzoflavone stimulated human CYP3A4-mediated phenacetin *O*-deethylation (Nakajima *et al*. 1999). 7,8-Benzoflavone inhibited OPP *p*-hydroxylation by human liver microsomes at concentrations of 5–40 μM (figure 6), which also enhanced CYP3A4-mediated phenacetin *O*-deethylation (data not shown). These results suggested the important role of CYP1A2 in hepatic OPP-hydroxylation, although CYP3A4 was also shown to catalyse the reaction (figure 5A).

CYP1A2-dependent activity is known to show remarkable individual difference and to be polymorphically distributed in the human population (Minchin *et al*. 1985, Butler *et al*. 1992, Nakajima *et al*. 1994). Thus, the present results on the role of CYP1A2 in the metabolic activation of OPP raise the possibility of potential toxic effects by this food additive in human individuals with high CYP1A2 expression.

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