INDUCTION BY DIFFERENT SUBSTITUTED PCBs AND CHARACTERIZATION OF CYP1A IN CYNOMOLGUS MONKEYS HEPATOCYTES (Macaca Fascicularis)

Aafje S.A.M. van der Burght, Angélique P. Kreikamp, G. Jean Horbach and Martin van den Berg.

Research Institute of Toxicology, Utrecht University, P.O. Box 80.176, NL-3508 TD Utrecht, The Netherlands.

INTRODUCTION

The cytochrome P450 family is involved in the biotransformation of many endogenous and exogenous compounds[1]. Because there are marked species differences in cytochrome P450 activity, it is difficult to extrapolate effects found in animal studies to the human situation. It has been suggested that non-human primates could be a suitable model for humans[2] to study cytochrome P450 activities.

Polychlorinated biphenyls (PCBs) are industrial compounds which are detected as contaminants in a variety of biotic and abiotic environmental samples[3]. Due to their chemical stability, physical properties, inflamability and dielectric properties, PCBs were once widely used as flame retardants, plasticizers, organic diluents and dielectric fluids for capacitors and transformers[4].

Because of their hydrophobic nature and resistence for biotransformation, PCBs accumulate in the food chain and cause a variety of biological and toxicological effects[5].

There are 209 possible congeners, which are generally divided in three groups with different biochemical and toxic responses[5,6].

The *non-ortho* PCBs (planar) with at least two *meta* and both *para* positions substituted, are exhibiting "dioxin-like" properties. The effects are mediated by the *Ah*-receptor, similar to 2,3,7,8-TCDD[5]. Among these effects is the induction of CYP1A(1) enzymes in many vertebrate species. A second group of PCBs has a single *ortho*-chlorine (and some *di-ortho*) substitution(s) besides two *meta*- and *para*- chlorine atoms. This group of congeners has demonstrated not only dioxin-like responses but elicits also toxic and biochemical responses more characteristic for a third group of PCBs, the "phenobarbital-" like congeners.

This last group, with two or more *ortho*- chlorines, is highly abundant in the environment. Congeners belonging to this group present an enzyme induction pattern resembling to some extent phenobarbital, eg. in their ability to induce CYP2B1/2 in the rat[7].

However, no orthologous form of rat CYP2B1/2 is found in humans. Consequently, risk-assessment of PCBs for humans based on the induction of CYP2B1/2 in rodents appears not to be appropriate.

Although *di*- and *tri-ortho* PCBs were not expected to elicit "dioxin-like" responses, like CYP1A1/2 induction, these PCBs demonstrate the capacity of inducing a form of CYP1A in hepatocytes from the cynomolgus monkey. The aim of this study was to determine which CYP1A enzyme was induced in hepatocytes of cynomolgus monkeys (*Macaca fascicularis*) after treatment with different *ortho*- chlorine substituted PCBs.

MATERIAL AND METHODS

Chemicals

PCB#126 (3,3',4,4',5 PeCB), PCB#118 (2,3',4.4',5 PeCB) and PCB#187 (2,2',3,4',5,5',6 HpCB) were synthesized and purified to >99% as determined on GC-MS.

Bovine serum albumine (BSA), gentamicin, insulin, hydrocortison, L-glutamine, Hanks' balanced salt solution (H.B.S.S), ethylene glycol-bis-(ß-amino ethyl ether) N,N,N',N' tetra acetic acid (EGTA), α-naphtoflavone and resorufin were all purchased from Sigma (St.Louis, MO, USA). Fetalclone serum was from Greiner (Alphen a.d. Rijn, The Netherlands). Collagenase and ethoxyresorufin were obtained from Boehringer Mannheim (Mannheim, Germany). Furafylline was purchased from Research Bioch. Int. (Natick, MA USA) and caffeine from Janssen Chimica (Geel, Belgium). Methoxyresorufin was obtained from Brunschwig Chemie BV (Amsterdam, The Netherlands). Human CYP1A1 cDNA[8] was a gift from Dr. P. Kremers (Université de Liège, Belgium), Human CYP1A2 cDNA[9] was a gift from Dr. F.J. Gonzalez (NIH, Bethesda, MA, USA).

Animals, cell isolation and cell culture

Cynomolgus monkeys (*Macaca fascicularis*) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands) and served as donors for kidney cells which are used for the production of the poliomyelitis vaccine.

Hepatocytes were isolated as described by Mennes *et al*[10]. The cells were plated on 6 cm tissue dishes (Greiner, Alphen a/d Rijn, The Netherlands) at a density of $3.2*10^6$ cells/dish in 4 ml Williams'E medium, supplemented with 5% (v/v) fetal clone serum and 1 µM insulin, 10 µM hydrocortisone and 50 mg/L gentamicin.

Cells were incubated in a humified atmosphere of air (95%) and CO_2 (5%) at 37°C. During the first 4 hours, 4 mM $CaCl_2$ and 4mM $MgCl_2$ were added to the medium. After 4 hours, the medium was replaced and the cells were preincubated for 16 hours. Next, the medium was replaced by medium with either inhibitor for one hour, or different inducers (PCBs or DMSO 0.1%, v/v) for 24 hours, in the absence of serum.

Isolation and analysis of RNA

Cells were harvested in ice-cold PBS using a rubber policeman. RNA was isolated using Trizol reagent (GibcoBRL, Breda, The Netherlands), and blotted to a Hybond N^+-membrane. The mRNA expression was determined using a radiolabeled human CYP1A1 or CYP1A2 probe. The membranes were exposed to a x-ray film.

Cytochrome P450 enzyme activity

The demethylation of caffeine[11] was measured in intact hepatocyte monolayers. Cells were incubated with a substrate concentration of 1 mM caffeine in Williams'E medium (without serum) during 24 hours at 37°C. After incubation, 7ß-hydroxypropyltheophilline as internal standard was added and the medium was extracted with a mixture of chloroform: isopropanol (85:15, v/v). The organic fase was evaporated and the residues were dissolved in a mixture of 55 mM NaAc: acetonitril (95:5, v/v), for HPLC analysis.

The methoxyresorufin (MR) and ethoxyresorufin (ER) analysis were performed as described by Burke *et al*[12]. Hepatocyte monolayers were washed with Hanks' balanced salt solution. The incubation was performed by adding 10 µM MR or 5 µM ER and 10 µM dicumarol to the medium. The formation of resorufin was determined fluorimetrically.

Protein content was determined according to Bradford[13] using bovine serum albumin (BSA) as a standard.

RESULTS

Induction of cytochrome P450 1A mRNA

As is shown in figure 1, CYP1A mRNA is induced in hepatocytes after treatment with different PCBs. When blots were hybridized with human CYP1A1 cDNA, mRNA sequences, homologous to human CYP1A1 cDNA, are induced by PCB#187 and PCB#118, which are a *tri* and *mono ortho* substituted congener respectively. RNA hybridized with human CYP1A2 cDNA showed an induction of a mRNA, homologous to human CYP1A2, but less obvious than observed for CYP1A1 cDNA.

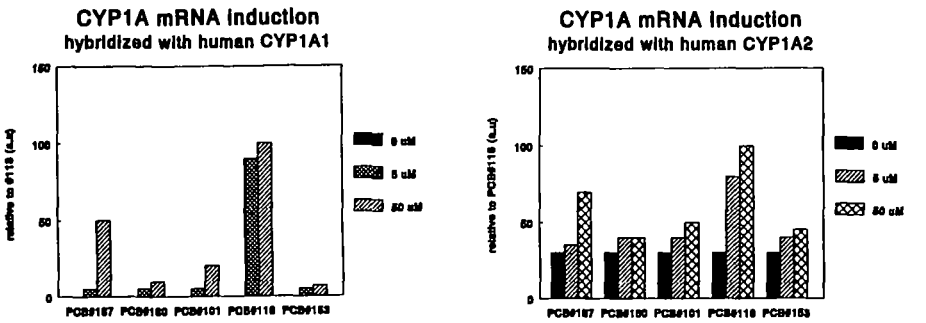

Figure 1. Dot blot mRNA from treated monkey hepatocytes with different PCBs

Induction of MROD and EROD by different PCBs

Methoxyresorufin and ethoxyresorufin are substrates for CYP1A enzymes in different species. The effect of different PCBs on the dealkylation of methoxyresorufin (MROD) or ethoxyresorufin (EROD) in monkey hepatocytes is presented in table 1.

The MROD and EROD activity was increased after treatment with all three PCB congeners.

Table 1: MROD and EROD induction relative to control after treatment with different PCBs

PCB	Concentration (μM)	MROD induction relative to control	EROD induction relative to control
PCB#126 (*non-ortho*)	0.1	74 ± 2	18
PCB#118 (*mono-ortho*)	0.1	1.5 ± 0.1	2
	5	14 ± 1.5	20
PCB#187 (*tri-ortho*)	0.1	1 ± 0.2	1
	50	23 ± 1.1	8

PCB#126, a *non-ortho* (dioxin-like) PCB, induced MROD activity to higher levels than EROD activity. PCB#118, a CYP1A as well as a CYP2B inducer, induced the MROD acitvity less compared to PCB#126, whereas the maximum EROD activity was induced equally but at a 50 times higher concentration. PCB#187 induced both MROD and EROD significantly but at 500 times higher concentrations.

Inhibition of enzyme activity by furafylline and α-naphtoflavone

The effect of different inhibitors on CYP1A activity, measured as either dealkylation of methoxyresorufin or as demethylation of caffeine, in monkey hepatocytes is shown in figure 2. Both furafylline and α-NF have shown to be potent CYP1A inhibitors in different species. The MROD activity was inhibited by both furafylline and α-NF. At concentrations of 7.5 μM furafylline or 7.5 μM α-NF the activity was reduced to 80%, resp. 55%, of the control activity (figure 2a).

Both the formation of paraxanthine (N-3 demethylation) and theophylline (N-7 demethylation), two metabolites of caffeine, was inhibited by furafylline and α-NF. Furafylline decreased the amount of paraxanthine to 37% and α-NF to 23% of the control amount. Theophylline was inhibited by furafylline to 12% and by α-NF to 9% of the control (figure 2c).

In control hepatocytes, the N-7 demethylation of caffeine to theophylline (figure 1b) was 6 times more prevalent than the N-3 demethylation to paraxanthine.

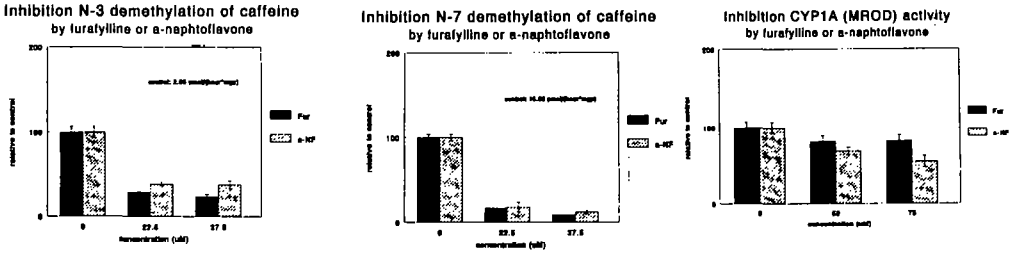

Figure 2. Inhibition of MROD and caffeine demethylating activity by furafylline and α-NF

DISCUSSION AND CONCLUSION

Cytochrome P450 1A mRNA levels were elevated after treatment with different PCBs. Hybridizations with either human CYP1A1 or 1A2 cDNA demonstrated this increase. Based on our dot blot results from Cynomolgus monkey hepatocytes, the presence of only one mRNA, with homology to both human CYP1A1 and CYP1A2 can not be excluded, but the existence of two different mRNAs is more presumable. Northern blot analysis might elucidate the presence of one or two CYP1A mRNAs in Cynomolgus monkey hepatocytes. Up till now only one CYP1A cDNA has been isolated from the liver of a 3-MC treated Cynomolgus monkey[14]. In comparison with the human CYP1A genes there was a 95% similarity on nucleotide sequences (94% on amino acid sequence) with CYP1A1 and 77% (73%) similarity with CYP1A2. Consequently, this enzyme was identified as CYP1A1.

In addition, the CYP1A activity, ie. MROD or EROD activity, in hepatocytes was determined after treatment with PCBs. All three congeners were capable of inducing MROD activity. The maximum induction of MROD-activity (relative to control), after treatment with the *non-ortho* PCB #126 (an *Ah* receptor agonist and CYP1A1 inducer), was higher than that of the of EROD activity. In accordance with our observations, Bullock *et al*[15] found that EROD as well as MROD activity were induced, in microsomes from Cynomolgus monkeys after treatment *in vivo* with ß-NF, another *Ah* receptor agonist. In view of the induction of MROD by the *tri-*

ortho PCB#187. it can be noted that hepatic levels of CYP1A2 protein in C57BL/6J mice increased after treatment with a *di-ortho* substituted PCB#153 (2,2',4,4',5,5' HxCB)[16]. Due to the species specificity of MROD and EROD activities, it is difficult to relate these results to CYP1A enzyme levels. In rat ethoxyresorufin is predominantly catalysed by CYP1A1 and methoxyresorufin by CYP1A2[17,18]. However, human CYP1A2 catalyzes methoxyresorufin and ethoxyresorufin at comparable rates[19]. Thus, until the specific substrate affinity of the monkey CYP1A for methoxyresorufin and ethoxyresorufin is determined, hardly any prediction can be made about the presence of specific enzymes.

The presence of two different CYP1A enzymes in untreated Cynomolgus monkeys is not supported by the results obtained in this study and from other workers. In human hepatocytes, the N-3 demethylation of cafeine is the major path of metabolism and is used as a marker for human CYP1A2 enzyme activity. In our monkey hepatocytes the major pathway of caffeine metabolism in control hepatocytes is N-7 demethylation. This is in agreement with results from experiments with microsomes from untreated Cynomolgus monkeys[20]. Edwards *et al*[21] found in microsomes from untreated Cynomolgus monkeys no CYP1A2 enzyme, which is consistent with the low caffeine N-3 demethylation activity. These results suggests the presence of one enzyme, with CYP1A1 characteristics and only little CYP1A2 properties. However, experiments with two human CYP1A2 inhibitors, furafylline or α-NF, in our control Cynomolgus monkey hepatocytes are not in agreement with the presence of only a CYP1A1 enzyme.

Furafylline is a potent and selective inhibitor of human CYP1A2. N-3 demethylation of caffeine is predominantly inhibited, but furafylline is also partial inhibitor of the N-1 and N-7 demethylation activity in humans[22,23]. In our control Cynomolgus monkey hepatocytes, furafylline inhibited the N-7 demethylation to lower levels than the N-3 demethylation, which is in contrast to experiments in humans. Methoxyresorufin dealkylation was also inhibited by furafylline to 80% of the control activity. Presuming that furafylline is an selective inhibitor in Cynomolgus monkey hepatocytes as well, the presented CYP1A enzyme should have CYP1A2 characteristics. Also the results from a second inhibitor, α-NF, support the existence of CYP1A2 characteristics. α-NF is widely used as CYP1A inhibitor probe, but Tassaneeyakul *et al*[24] demonstrated in human microsomes that α-NF is approximately 10 fold more potent as inhibitor of human CYP1A2 than human CYP1A1.

Summarizing, in hepatocytes of Cynomolgus monkeys a CYP1A enzyme is induced by *non-, mono-* and also *tri-ortho*-substituted PCBs. Dot Blot hybridization with both human CYP1A1 and CYP1A2 cDNA demonstrated an induction of CYP1A mRNA(s), but did not elucidate which mRNA was induced. Results with selective human CYP1A2 inhibitors (furafylline or α-NF) showed the presence of characteristics of some CYP1A2 activities, but did not clarify if one or two CYP1A enzyme(s) are present in Cynomolgus monkeys. In this study, it has not been demonstrated that hepatocytes from Cynomolgus monkeys are a suitable model for the human liver. In control liver of Marmoset monkeys, it has been found that only CYP1A2 enzyme is constitutively expressed. Thus in terms of hepatic levels of CYP1A enzymes, Marmoset monkeys seems to resemble the human liver more than the Cynomolgus monkeys[21].

REFERENCES

1. Guengerich F.P., Ann. Rev. Pharmacol. Toxicol. 29:241 (1989).
2. Jacqz E., Billante C., Moysan F., Mathieu H., Mol. Pharmacol. 34:215 (1988).
3. Rappe C., Buser H.G., Bosshardt H.P., Ann. N.Y. Acad. Sci. 320:1 (1979).
4. *Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products*, 2nd ed. Kimbrough R.D., Jensen A.A., Eds.; Elsevier, Amsterdam pp 3 (1989).

5. Safe S.H., CRC Crit. Rev. Toxicol. 24:87 (1994).
6. McFarland V.A., Clarke J.U., Environ. Health Persp. 81:225 (1989).
7. Parkinson A., Safe S.H., Robertson L.W., Thomas P.E., Ryan D.E., Reik L.M., Levin W., J. Biol. Chem. 258:5967 (1983).
8. Jaiswal A.K., Gonzalez F.J., Nebert D.W., Science 228:80 (1985).
9. Jaiswal A.K., Nebert D.W., Gonzalez F.J., Nucl. Acids Res. 14:6773 (1986).
10. Mennes W.C., van Holsteijn C.W.M., Timmerman A., Noordhoek J., Blaauboer B.J., Biochem. Pharmacol. 41:1203 (1991).
11. Butler M.A., Iwasaki M., Guengerich F.P., Kadlubar F.K., Proc. Nucl. Acad. Sci (USA), 86:7696 (1989).
12. Burke M.D., Mayer R.T., Drug Metab. Dispos. 2:583 (1974).
13. Bradford M.M., Anal. Biochem. 72:248 (1976).
14 Komori M., Kikuchi O., Kitada M., Kamataki T., Biochim. Biophys. Acta, 1131:23 (1992).
15 Bullock P., Pearce R., Draper A., Podval J., Bracken W., Vertman J., Thomas P., Parkinson A., Drug Metab. Disp. 23:736 (1995).
16 De Jongh J., DeVito M., Nieboer R., Birnbaum L.S., Van den Berg M., 25:264 (1995).
17 Nerurkar P., Park S., Thomas P., Nims R., Lubet R., Biochem. Pharmacol. 46:933 (1993).
18 Burke M.D., Thompson S., Weaver R.J., Wolf C.R., Mayer R.T., Biochem. Pharmacol. 48(5):923 (1994).
19 Lee Q., Fantel A., Juchau M., Biochem. Pharmacol. 42:2377 (1991).
20 Berthou F., Guillois B., Riche C., Dreano Y., Jacqz-Aigrain E., Beaune P.H., Xenobiotica 22:671 (1992).
21 Edwards R.J., Bernard P.M., Murray S., Schultz T., Neubert D., Gant T.W., Thorgeirsson S.S., Boobis A.R., Davies D.S., Carcinogenesis 15:829 (1994).
22 Sesardic D., Boobis A.R., Murray B.P., Murray S., Segura J., De La Torre R., Davies D.S., Br. J. Clin. Pharmac. 29:651 (1990).
23 Clarke S.E., Ayrton A.D., Chenery R.J., Xenobiotica. 24:517 (1994).
24 Tassaneeyakul W., Birkett D.J., Veronese M.E., McManus M.E., Tukey R.H., Quattrochi L.C., Gelboin H.V., Miners J.O., J. Pharmacol. Exp. Ther. 265:401 (1993).