ELSEVIER

Biochemical Pharmacology 62 (2001) 713–721

Biochemical Pharmacology

Relationships between non-occupational cadmium exposure and expression of nine cytochrome P450 forms in human liver and kidney cortex samples

Jason R. Baker[a], Soisungwan Satarug[a]*, Paul E.B. Reilly[b], Robert J. Edwards[c], Noritaka Ariyoshi[d], Tetsuya Kamataki[d], Michael R. Moore[a,e], David J. Williams[e]

[a]*National Research Centre for Environmental Toxicology, Brisbane, Australia*
[b]*Department of Biochemistry, University of Queensland, Brisbane, Australia*
[c]*Section of Clinical Pharmacology, Imperial College School of Medicine, London, UK*
[d]*Laboratory of Drug Metabolism, Hokkaido University, Sapporo, Japan*
[e]*Queensland Health Scientific Services, JohnTonge Centre for Forensic Pathology, Queensland Department of Health, Brisbane, Australia*

Received 5 February 2001; accepted 7 May 2001

Abstract

This study was undertaken to assess associations between age, gender, cigarette smoke and non-workplace cadmium exposure, and liver pathology and inter-individual variation in cytochrome P450 (CYP) expression in human tissues. Autopsy specimens of twenty-eight Queensland residents whose ages ranged from 3 to 89 years were analyzed for the presence of nine CYP protein isoforms by immunoblotting. All subjects were Caucasians and their liver cadmium contents ranged from 0.11 to 3.95 μg/g wet weight, while their kidney cadmium contents were in the range of 2 to 63 μg/g wet weight. CYP1A2, CYP2A6, CYP2D6, CYP3A4, and CYP3A5 were detected in liver but not in kidney, and CYP1A1 and CYP1B1 were not found in liver or kidney. Lowered liver CYP2C8/19 protein contents were found to be associated with liver pathology. Importantly, we show elevated levels of CYP2C9 protein to be associated with cadmium accumulation in liver. No mechanism that explains this association is apparent, but there are two possibilities that require further study. One is that variation in CYP2C9 protein levels may be, in part, attributed to an individual's non-workplace exposure to cadmium, or an individual's CYP2C9 genotype may be a risk factor for cadmium accumulation. A positive correlation was found between liver CYP3A4 protein and subject age. Levels of liver CYP1A2 protein, but not other CYP forms, were increased in people more exposed to cigarette smoke, but there was no association between CYP1A2 protein and cadmium. CYP2A6 protein was found in all liver samples and *CYP2A6* gene typing indicated the absence of *CYP2A6* null allele (*CYP2A6(D)*) in this sample group, confirming very low prevalence of homozygous *CYP2A6(D)* in Caucasians. CYP2A6 gene types *W*/*W*, *W*/*C*, and *C*/*C* were not associated with variations in liver microsomal CYP2A6 protein. CYP2D6 protein was absent in all twenty-five kidney samples tested but was detectable in liver samples of all but two subjects, indicating the prevalence of the *CYP2D6* null allele (*CYP2D6(D)*) in this sample group to be about 7%, typical of Caucasian populations.

Keywords: Human autopsy; Environmental cadmium; Cytochrome P450; Individual differences; Tissue differences; Cigarette smoke; CYP1A2 induction

1. Introduction

Remarkable inter-individual variations in abundance and activity of some human CYP forms have been reported [1–7]. For example, in one study [3] using a human liver bank of twenty-one samples (fourteen males, six females, and one unknown), the fold variations (number in parentheses) in catalytic activity found for each CYP were reported as follows: 1A2 (3), 2A6 (21), 2C9 (8), 2C19 (175), 2D6 (18), 2E1 (5), 3A4 (18). It is considered that such variation may be due to exposure to particular classes of drugs and compounds of endogenous and exogenous origin in some individuals [1,3,8,9] and to genetically determined variation in CYP expression, since a number of genetic polymorphisms of human genes have been identified to contribute to variability in the abundance of the corresponding CYP proteins [10–14]. A recent study on genetic polymorphism

* Corresponding author. Tel.: +61-7-3274-9016; fax: +61-7-3274-9003.
E-mail address: s.satarug@mailbox.uq.edu.au (S. Satarug).
Abbreviation: CYP, cytochrome P450.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00716-X

reported striking differences in *CYP1A1*, *1B1*, and *2D6* gene polymorphism in Caucasians and Japanese [13,14].

Among the environmental factors that are important sources of CYP variability among individuals, cigarette smoke exposure has been subject to the most intense research. Results have shown that among the many chemicals in tobacco smoke, polycyclic aromatic hydrocarbons may be inducers of CYP1A2 in liver and CYP1A1 in lung and placenta in tobacco smokers [1,8,9]. Cadmium is another common environmental toxin found in low levels in cigarette smoke and in most foods [15–21], but the possible influence of environmental cadmium exposure on CYP expression and inter-individual variation has not been clarified.

In the non-occupationally exposed population, food and tobacco smoke are known to be major sources of cadmium [15,16,19,22]. Cadmium intake in the range of 9–15 μg/day was estimated for an average Australian consumer, using data from the 1996 Market Basket Survey [21]. Potatoes, wheat, cocoa, and meat constitute 46%, 16%, 12%, and 7%, respectively of total cadmium intake. Crustaceans, liver, peanuts, and vegetables each constitute only 2–3% of cadmium in the diet, providing a further 11% to the total intake. Cadmium of dietary and tobacco smoke origin is known to accumulate in the tissues where CYP enzymes are found, including the placenta, although its accumulation in the proximal tubular cells of the kidney cortex appears to be most extensive [20,22–28], which explains why kidney is a target for cadmium toxicity in the non-occupationally exposed population. Cadmium in the kidneys accounts for one-third of the total body cadmium burden, while cadmium in the placenta has been found to increase with age of mothers [27]. Cadmium in lung was found to derive mainly from cigarette smoke and from polluted air [22,29].

The purpose of this present study was to reveal variations in the expression of selected CYP proteins in liver and kidney that may be attributable to human exposure to environmental cadmium, as reflected by levels of cadmium accumulated in liver and kidney cortex samples. Formulation of this hypothesis developed out of work by ourselves and others [30–34] showing changes in tissue CYP content associated with cadmium administration to rats and rabbits. Other variables were also taken into consideration in this analysis. These variables were donor age, gender, *CYP2A6* genotype, levels of exposure to cigarette smoke, and liver histopathology, some of these being established to be a cause of inter-individual variation in CYP expression [35–44]. The present paper focuses on the following CYP forms: CYP1A1, 1A2, 1B1, 2A6, 2C9, 2C8/19, 2D6, 3A4, and 3A5. Levels of these CYP forms in liver and kidney samples were determined by Western immunoblotting with a panel of polyclonal antipeptide antibodies possessing high specificity and which have previously been validated for use in studying CYP expression in human liver, lung and placenta tissues [1,6,8,9].

2. Materials and methods

2.1. Chemicals

Bovine liver standard (SRM 1577a) was purchased from the US National Institute of Standards (NST) and ICP multi-element standards were from EM Science. To achieve the highest purity possible, analytical grade HNO_3 (69%, w/v) was distilled before use. All containers used in specimen collection and preparation were tested prior to their use to ensure they contained undetectable amounts of cadmium or were metal-free. The Qiagen DNeasy Tissue Extraction Kit was obtained from Qiagen Pty Ltd., Australia and pre-stained protein molecular weight markers were obtained from Amrad Pharmacia Bitotech. Antipeptide antibodies raised in rabbits and specific for the following human CYP forms: 1A1, 1A2, 1B1, 2A6, 2A6/2B6, 2C8/9/19, 2D6, 3A4, and 3A5 were used [1,6]. Polyacrylamide/bis solution, goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase, and alkaline phosphatase colour development reagents (BCIP/NBT) were obtained from Bio-Rad Laboratories Pty Ltd. All other chemicals were of reagent grade.

2.2. The sample group

The sample group of this study consisted of 28 individuals, 22 males and 6 females, aged from 3 to 89 years. It was a subset of samples drawn from a wider investigation, designed to quantify human exposure to environmental cadmium [22]. The study was approved by the Queensland Health Scientific Services Ethical Committee and was carried out in accordance with the standard code of practice prescribed by the Australian National Health and Medical Research Council and no donor's consent was required at the time this study was undertaken. The sample group included those who had died from accidental causes in 1997/1998 and were subject to post-mortem examination at the John Tonge Centre for Forensic Sciences, Queensland Health Scientific Services, Brisbane, Australia. About 95% of subjects in this sample population were residents of Queensland. None were Aboriginal Australians and none had been exposed to cadmium in the workplace. None of the cases showed gross pathology in any tissues and histological examination of liver was used to assess any pathological changes in these samples. The time lapse between death and collection of samples was usually within 24 hr. Causes of death were as follows; motor vehicle accident (58%), drowning (14%), workplace accident (10%), fall (8%), electrocution and all other accidents (10%). Age, gender, body weight, organ weight, smoking habits, alcohol use, and histology of the lung, liver and kidney of the cases were obtained from autopsy reports.

2.3. Specimen preparation

Liver and kidney specimens were kept on ice during collection and preparation in the laboratory of the National

Research Centre for Environmental Toxicology. The tissue samples were rinsed in ice-cold normal saline and blotted dry. The medulla and cortex of the kidney samples were dissected apart. The liver samples were cut into small pieces. Immediately after dissection, 1–2 g samples of kidney cortex and liver were frozen in liquid nitrogen and stored at −80° for later analysis. For metal analysis, samples of the kidney cortex and liver about 1–2 g each were frozen in liquid nitrogen and lyophilized at −40° for 24 hr. Lyophilized samples were digested in distilled HNO_3 equilibrated on a boiling water bath for 2 hr. Samples of the acid digest were analysed for Cd by inductively coupled plasma mass spectrometry (ICP/MS). The accuracy and precision of our analysis for tissue metals were assessed by a simultaneous analysis of a standard reference bovine liver sample (SRM 1577a).

2.4. DNA preparation and CYP2A6 gene typing

Samples of liver (about 25 mg each) were cut into small pieces and digested in buffer containing proteinase K for 2–3 hr. Protein-free solutions were treated with buffer and ethanol before loading onto the Qiagen Dneasy spin column. The bound DNA was eluted with Tris–EDTA buffer to a final volume of 100 μL after washing. The DNA samples were analyzed for *CYP2A6* gene types by the restriction fragment length polymorphism technique as described previously [45].

2.5. Microsomal preparation

Microsomal fractions were prepared from the liver and kidney cortex samples as described previously [46]. The tissue samples were homogenised using a motor-driven glass pestle and mortar in 0.1 M Tris–acetate buffer containing 0.1 M KCl, 1 mM EDTA, and 20 μM butylated hydroxytoluene (BHT) (pH 7.4). The pellet resulting from centrifugation of the homogenate (20 min, 4°, 10,000 *g*) was discarded and the supernatant filtered through Miracloth™ (Calbiochem) and centrifuged at 100,000 *g* for 1 hr. The pellet was collected and washed twice in 100 mM potassium pyrophosphate buffer containing 1 mM EDTA and 20 μM BHT by resuspension and centrifugation at 100,000 *g* for 1 hr at 4°. The microsomal pellet was collected and resuspended in 10 mM Tris–acetate buffer containing 1 mM EDTA and 20% glycerol (pH 7.4). Protein concentrations of the microsomal samples, using bovine serum albumin as a standard, were in the range of 22–138 μg/μL and 24–75 μg/μL for the liver and kidney cortex samples, respectively [47]. Microsomal suspensions were kept at −80° for later analysis.

2.6. Immunoblotting

Selected CYP forms in the liver and kidney microsomal suspensions were analysed by immunoblotting using liver and kidney cortex microsomal fractions containing fixed amounts of protein. For the liver microsomal samples, 10 μg of protein was used for detection of CYP apoproteins. For the kidney cortex microsomal samples, 40 μg of protein was used. The liver and kidney cortex microsomal protein samples were separated by SDS–PAGE (4.5% acrylamide stacking gel, 9% acrylamide resolving gel) in a Tris–glycine buffer system [48]. The prestained protein molecular weight markers (See-Blue) were included in every SDS–PAGE which was run along with 10 liver or kidney cortex microsomal samples. The proteins on each gel were blotted onto a PVDF membrane for 90 min at 25V [49]. Membranes were incubated overnight in 3% non-fat, dry milk-TBS to block membrane non-specific binding sites, and individual CYP forms were detected with antibodies diluted 1:4000 in 1% non-fat, dry milk-Tween-TBS. After incubation with primary antibody, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin. Incubations with primary antibody preparation were for 1.5 to 2 hr and with secondary antibody for 45 min. Immunoreactive proteins were visualized by incubation with the alkaline phosphatase colour development reagents (BCIP/NBT) used according to the manufacturer's instructions. The specificities of the antipeptide antibody preparations had been demonstrated previously, using recombinant CYPs expressed in the microsomal fraction of lymphoblastoid cells or insect cells which showed extremely high specificity of the antibody preparations used in this present study and hence their suitability for purposes of measuring CYP apoprotein abundance [1,6].

2.7. Scoring of immunoblots

Qualitative assessment of the CYP apoprotein abundance in tissues was achieved by using a scoring system based on visual inspection of individual immunoreactive bands. This analysis was conducted blind, i.e., without knowledge of any information on the sample donors or the CYP isoform being analysed. Each band on all blots was scored as trace, +1, +2, and +3 levels, where a trace level indicates a definite band at the expected molecular weight region but having the lowest intensity relative to the one with the highest intensity of the same blot. The +1, +2, and +3 levels indicate increasing immunoreactive protein band intensity from low, medium, and high, respectively.

2.8. Statistical analysis

Data were analysed by the Statistical Package for Social Sciences (SPSS) for Windows (version 6.1). Kruskal–Wallis one-way ANOVA was used to determine statistical significance levels for differences in the levels of particular CYP forms in more than two groups of subjects. The Mann–Whitney U-Wilcoxon Rank Sum W test was used to determine significance levels for differences in CYP protein abundance in pairs of subject groups. Spearman's rank correlation test was used to reveal correlations between age and individuals' CYP protein levels.

Table 1
Age, gender, cigarette smoke exposure levels, liver and kidney cadmium contents, and liver histopathology state of the subjects

Subject	Age (yr)	Gender	Cigarette[a] smoke	Liver[b] cadmium	Kidney[b] cadmium	Liver histopathology
1	3	Male	Low	0.23	2.12	Normal
2	5	Male	Low	0.35	1.99	Normal
3	15	Male	Low	0.30	4.71	Normal
4	15	Male	Medium	1.22	9.00	Normal
5	18	Male	Medium	0.58	4.56	Normal
6	18	Male	Low	0.29	4.19	Fatty change
7	18	Female	Low	0.40	5.53	Normal
8	19	Male	Medium	0.20	2.22	Normal
9	21	Male	High	1.50	14.27	Normal
10	22	Male	Medium	0.29	3.82	Normal
11	32	Female	Medium	2.56	27.35	Normal
12	32	Male	Medium	0.87	8.37	Normal
13	38	Male	Low	0.11	6.85	Fatty change
14	44	Male	High	1.88	27.77	Normal
15	46	Male	Low	0.43	6.89	Fatty change
16	47	Male	High	3.23	43.03	Normal
17	49	Male	Medium	0.31	22.28	Chronic hepatitis
18	54	Female	Medium	2.58	61.05	Fatty change
19	56	Male	Low	0.27	9.23	Chronic hepatitis
20	60	Male	Medium	1.45	36.87	Fatty change
21	60	Male	Medium	0.17	11.14	Chronic hepatitis
22	61	Male	High	1.93	37.57	Fatty change
23	64	Male	Low	0.16	6.43	Fatty change
24	72	Female	Medium	3.95	63.72	Normal
25	74	Female	Medium	2.29	19.72	Normal
26	79	Female	Medium	1.31	18.53	Chronic hepatitis
27	81	Male	Medium	1.55	14.01	ND
28	89	Male	Medium	0.44	4.38	Normal

Kidney samples from all subjects show normal histology except for subject number 24, whose kidney histology showed mild tubular atrophy and interstitial nephritis. ND: not determined.

[a] Cigarette smoke exposure level of each individual was assessed by smoking history, lung cadmium level, and lung histology.

[b] Liver and kidney cadmium contents are expressed in μg/g wet tissue weight.

3. Results

The sample group was composed of twenty-two males and six females with ages ranging from 3 to 89 years. Each individual's age, gender, level of exposure to cigarette smoke, liver and kidney cadmium contents, and liver histopathology results are shown in Table 1. The overall mean age for the sample group was 42.5 years. The mean ages for males and females were not significantly different. The level of exposure to cigarette smoke of each subject was assessed as being high, medium, or low based on the individual autopsy report, the lung cadmium content relative to liver and kidney levels, and lung histology showing evidence of exposure to cigarette smoke such as carbon residues in lung macrophages. Four individuals were found to have been extensively exposed to cigarette smoke and were reported to be heavy smokers. Twelve individuals whose cigarette smoke exposure was found to be at the medium level were considered to be light smokers or passive smokers. Nine individuals were minimally exposed to cigarette smoke as judged by the above criteria and were reported to be nonsmokers or ex-smokers. Four cases had chronic hepatitis, seven cases showed fatty change, which may be indicative of relatively high alcohol intake, and sixteen individuals had histologically normal livers. Liver histology was not determined for one individual. Kidney samples from all subjects showed normal histology with the exception of a 79-year-old female, who had the highest kidney cortex cadmium level (63.7 μg/g wet tissue weight), and whose kidney histology showed mild tubular atrophy and interstitial nephritis. None of the subjects were on regular medication.

Fig. 1 shows examples of immunoblots obtained with liver microsomal samples where the scoring system for qualitative analysis is depicted on the CYP1A2 panel. The antipeptide antibody preparations used in the blotting of these CYP proteins each gave one immunoreactive band corresponding to each individual CYP form except for CYP2C8/9/19, where two immunoreactive bands were identified. The upper band was assessed as CYP2C9 while the lower band corresponded to CYP2C8 plus CYP2C19 that were not separable because of very close electrophoretic migration [50,51]. This enabled the relative abundance of nine CYP isoforms to be assessed.

Table 2 shows a summary of immunoblotting of liver and kidney microsome samples using the eight antibody preparations. CYP1A1 and CYP1B1 were not detectable in those liver or kidney samples subjected to these analyses. CYP1A2 was expressed in all the liver samples but was not

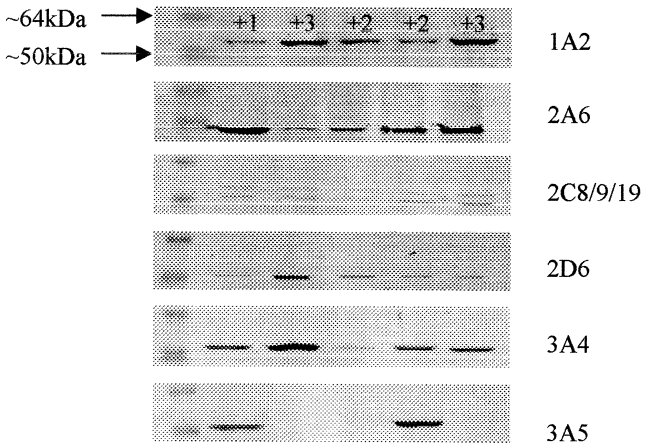

Fig. 1. Samples of Western immunoblots for CYP proteins in livers of five individuals. The CYP forms are indicated next to each blot. The protein molecular weight markers are at the left corner of each blot and are indicated by arrows at the left corner of the CYP1A2 blot. An example of the scoring system which was applied is also shown on the liver CYP1A2 blot.

identified in the nine kidney samples tested. CYP2A6 apoprotein was identified in all liver samples and in no kidney samples. CYP2C9 was detected in 27/28 liver samples and CYP2C8/19 was detected in all liver samples. Analysis of these CYP forms in kidney was inconclusive due to very low intensity of the immunoreactive bands. CYP2D6 was found in 26/28 liver samples but was not identified in the nine kidney samples tested. CYP3A4 apoprotein was identified in all liver samples but not in the nine kidney samples tested. CYP3A5 was detected in 16/28 liver samples but not in any of the kidney samples.

Table 3 gives details of immunoblotting scores for CYP forms in families 1, 2, and 3 in liver and kidney samples of the twenty-eight subjects. *CYP2A6* gene types determined for 23/28 subjects are also shown. None (0/23) carried the *CYP2A6* null allele and the *W*/*W*, *W*/*C*, *C*/*C* gene types were found in 9/23 (39%), 8/23 (35%), and 6/23 (26%) samples, respectively.

Table 2
A summary of results of Western immunoblotting for various CYP proteins in liver and kidney cortex microsomal samples

CYP forms	Liver microsomes	Kidney cortex microsomes
	Positive samples	Positive samples
1A1	0/10	0/25
1A2	28/28	0/9
1B1	0/10	0/9
2A6	28/28	0/25
2C9	27/28	Inconclusive
2C8/19	28/28	Inconclusive
2D6	26/28	0/9
3A4	28/28	0/9
3A5	16/28	0/25

Inconclusive results were indicated where reliable assessment was not possible due to very low intensity of immunoreactive bands.

The immunoblot scores of the CYP forms shown in Table 3 were subject to non-parametric statistical analysis to reveal potential associations with individuals' age, gender, exposure to cigarette smoke, liver cadmium content, and liver histopathology. Results of these tests are summarized in Table 4, where the statistical tests used for association analysis are indicated in the table legend. None of the CYP forms expressed in liver showed a significant association with gender. CYP3A4, but no other forms, showed a positive correlation with age (Spearman's rank correlation coefficient = 0.35, $P = 0.03$). CYP3A5 was found in 16/28 samples. However, no correlation was found between level of CYP3A5 in the 16 positive cases with age ($r = -0.05$, $P = 0.43$). Levels of CYP1A2, but not other forms, showed significant association with levels of exposure to cigarette smoke, while the levels of CYP2C8/19 showed statistically significant association only with liver histopathology state. CYP2C9 apoprotein levels were positively correlated with tissue cadmium content. These associations are detailed in Table 5.

A test for association between variations in liver CYP2A6 protein levels and *CYP2A6* gene types revealed that variation in the abundance of liver microsomal CYP2A6 protein was not attributable to differences in *CYP2A6* gene types (Kruskal–Wallis one-way ANOVA, $\chi^2 = 0.35$, df = 2, 21, $P = 0.84$).

4. Discussion

In our attempt to document environmental sources of variation in human CYP expression, we elected to analyse CYP expression in the liver and kidney samples from people who died of accidental causes. This sample group was likely to include "apparently healthy" individuals and is the closest to a random sample of the general population since an accidental cause of death may be viewed as a random incident. Since disease and other host factors are also known to contribute to CYP expression, analysis of samples from apparently healthy individuals should minimize confounding effects that may be encountered when samples from hospital patients are used. Analysis of CYP expression in different tissues of the same individuals was also possible. An additional advantage of using autopsy specimens is the availability of tissue histology in confirming the health status of the subjects, while cigarette smoke exposure status could be verified using lung histology in conjunction with lung cadmium content.

We note, however, that there was a sensitivity limit in our approach to assessment of CYP protein levels. Under these conditions, only strong effects (associations) could be identified. Possible associations between environmental cadmium exposure, gender, and age affecting variation of CYP forms in human liver not revealed in the present study may be revealed with larger sample size and quantitative assessment of CYP proteins aided by use of recombinant CYP protein standards.

In the present study, we showed that there was a strong

Table 3
CYP2A6 gene types, relative levels of CYP1, CYP2, and CYP3 family proteins in liver microsomal samples for each of the 28 individuals

Subject	CYP1A1	CYP1A2	CYP1B1	*CYP2A6* gene type	CYP2A6	CYP2D6	CYP2C		CYP3A4	CYP3A5
							2C9	2C8/19		
1	—	+3	—	*W*/*C*	+3	+2	+1	+1	+1	+3
2	ND	+3	ND	*W*/*C*	+2	+2	+1	+1	Trace	+3
3	—	+1	—	*C*/*C*	+1	+2	Trace	Trace	Trace	—
4	ND	+3	ND	*C*/*C*	+3	+1	+2	+1	Trace	Trace
5	—	+3	—	*W*/*W*	+2	+1	+2	+2	+2	+3
6	—	+3	—	*W*/*W*	+3	—	+1	+1	Trace	—
7	ND	+2	ND	*W*/*W*	+3	+3	+1	+1	+1	Trace
8	ND	+3	ND	ND	+2	+1	+2	+2	+2	+1
9	—	+3	—	*C*/*C*	+3	+2	+2	+1	+1	+3
10	—	+3	—	*C*/*C*	+3	+2	Trace	+1	+2	Trace
11	ND	+3	ND	*W*/*W*	+3	Trace	+1	+1	+1	—
12	ND	+3	ND	ND	+3	+1	+2	+2	+1	Trace
13	—	+2	—	*W*/*C*	+3	+1	Trace	Trace	Trace	—
14	ND	+3	ND	*W*/*C*	+3	+1	+1	+1	Trace	—
15	—	+3	—	*C*/*C*	+3	Trace	+3	+2	+1	—
16	ND	+3	ND	*W*/*W*	+3	—	+1	+2	+1	Trace
17	ND	+3	ND	*W*/*C*	+3	+2	+1	+1	Trace	—
18	ND	+2	ND	ND	+1	+2	+2	+1	+2	Trace
19	ND	+2	ND	*W*/*W*	+2	+1	+1	+1	+2	—
20	ND	+2	ND	ND	+1	+2	+2	+1	+1	—
21	—	+3	—	*W*/*C*	+2	+2	Trace	Trace	Trace	—
22	ND	+3	ND	*W*/*W*	+2	Trace	Trace	Trace	+1	Trace
23	—	+2	—	*W*/*C*	+2	+2	—	Trace	Trace	—
24	ND	+3	ND	*W*/*C*	+3	+3	+3	+2	+3	+1
25	ND	+3	ND	*C*/*C*	+3	+3	+2	+2	+1	—
26	ND	+3	ND	*W*/*W*	+3	+1	+1	Trace	+1	+1
27	ND	+3	ND	ND	+3	+3	+3	+2	+3	+3
28	ND	+3	ND	*W*/*W*	+3	+3	+2	+2	+3	+3

For *CYP2A6* gene types, *W* is wild type allele and *C* is conversion-type mutant allele. ND: not determined; —: not detectable.

positive association between cadmium content in the livers of donors and CYP2C9 apoprotein content (Table 5). This is the first evidence for a potential effect of environmental cadmium on the expression of CYP2C9 in human livers. No mechanism to explain this association is evident from our study and no published data appear to offer insights into this finding. Further work is required to clarify this relationship because allelic variations in CYP2C9 are known in human populations [52]. They are important because they cause altered catalytic activity towards a wide range of commonly used drugs and are associated with altered clinical effectiveness of some drugs such as phenytoin and warfarin [53,54]. In light of the positive association we have shown between liver cadmium content and CYP2C9 apoprotein abundance in liver, it is possible that an individual's CYP2C9 genotype may be a risk factor for tissue cadmium accumulation.

Table 4
Results of tests for associations between variations in levels of certain CYP forms in the liver microsomal samples with age, gender, cigarette smoke exposure and liver histopathology

CYP forms	Host factors		Environmental factors		Liver histopathology[c]
	Age[a]	Gender[b]	Cadmium[b]	Cigarette smoke[c]	
1A2	0.47	0.65	0.26	0.03 ↑	0.15
2A6	0.45	0.41	0.34	0.84	0.18
2C9	0.12	0.37	0.05 ↑	0.06	0.34
2C8/19	0.25	0.83	0.41	0.08	0.04 ↓
2D6	0.12	0.28	0.72	0.44	0.35
3A4	0.03 ↑	0.77	0.55	0.12	0.82

Numbers shown are *P* values and only the *P* values which are equal to or less than 0.05 were considered to identify statistically significant associations. ↑, indicates increased CYP levels. ↓, indicates decreased CYP levels.

[a] Analysis was done by Spearman's rank correlation test.

[b] Analysis was done by Mann–Whitney U-Wilcoxon Rank Sum W test.

[c] Analysis was done by Kruskal–Wallis one-way ANOVA.

Table 5
Associations between liver CYP2C9 and Cd exposure (panel 5a), CYP1A2 and cigarette smoke exposure (panel 5b), and CYP2C8/19 and liver histopathology (panel 5c) by Mann–Whitney U-Wilcoxon Rank Sum W test and Kruskal–Wallis one-way ANOVA

Panel 5a. Cd exposure[a] levels	Number of cases N = 28	CYP2C9 protein levels			Mean rank* values
		Trace	+1	+2	
Low	15	5	6	4	11.8
High	13	1	4	8	17.6

[a] The lower exposure group had liver Cd concentrations ranging from 0.11 to 0.58 μg/g wet tissue weight whereas in the higher exposure group liver Cd concentration ranged from 0.87 to 3.95 μg/g wet tissue weight.
(*$P = 0.04$).

Panel 5b. Cigarette smoke exposure levels	Number of cases N = 28	CYP1A2 protein levels			Mean rank* values
		+1	+2	+3	
Low	9	1	4	4	10.1
Medium	15	0	2	13	16.2
High	4	0	0	4	18.0

(* $P = 0.03$).

Panel 5c. Liver histopathology	Number of cases[a] N = 27	CYP2C8/19 protein levels				Mean rank* values
		Trace	+1	+2	+3	
Normal	16	1	8	5	2	17.0
Fatty change	7	3	3	1	0	10.8
Chronic hepatitis	4	2	2	0	0	8.2

[a] In this analysis, the case number 27 was excluded due to lack of data on liver histology.
* $P = 0.04$.

Liver pathology, manifested as fatty change and/or chronic hepatitis, was found to be associated with reduced levels of liver CYP2C8/19 protein, compared to those of histologically normal livers. In an earlier study, however, no changes in CYP2C protein were detected in cirrhotic liver samples [44]. This may be related to the severity of the liver pathology affecting the samples in that study. It should be noted here that the fatty change observed in our subjects was of a mild, possibly reversible form, and that the reduction in CYP2C8/19 apoprotein identified here was only possible due to the high selectivity of the antibody preparation used here. It remains unclear what factors underlie the reduction in CYP2C8/19 protein in the livers showing fatty change and/or chronic hepatitis. Impaired nutrition, cachexia, and elevated serum bilirubin levels were attributed to a reduction in CYP2C8/10 protein levels in another study conducted on 71 patients, 21 with histologically normal livers and 50 with chronic liver disease [40].

Age, gender, and liver histopathology were not associated with variation in liver CYP1A2 protein, but increased levels of liver CYP1A2 were found in people more exposed to cigarette smoke. This confirms results of several other studies [35–38]. In addition, no association was found between liver cadmium contents and liver CYP1A2 protein levels (Table 4). This suggests that involvement of cadmium in liver CYP1A2 induction in smokers was unlikely, although smokers have higher liver cadmium levels compared to non-smokers (Table 1). We also note that the increase in liver CYP1A2 protein levels by cigarette smoke was selective, since no other forms were affected (Table 4). This isoform-specific effect would be expected if induction of liver *CYP1A2* gene is mediated by the aryl hydrocarbon receptor pathway [55].

The lack of CYP1A1 protein expression in kidney was perhaps unexpected, since expression and induction of the CYP1A1 in extrahepatic tissues including the lung and placenta is well established in people [8,9] and its expression in kidney of rats treated with TCDD has recently been reported [56]. The lack of detectable CYP1A1 protein in liver and kidney samples studied here may indicate endogenous tissue-specific constraints or lack of appropriate xenobiotic exposure. As with CYP1A1, CYP1B1 was not expressed in detectable quantities in liver or kidney samples (Table 3). CYP1B1 has been found to be expressed in human skin cells and its expression is induced by UV light [57]. CYP1B1 has been detected in many types of human cancer cells but was not found in the corresponding normal human non-tumor tissue [58]. This is in agreement with the presently reported lack of detection of this isoform in the liver and kidney samples used here.

All twenty-eight liver samples contained detectable CYP2A6 protein and in a majority of samples this protein was in high abundance. Variation in CYP2A6 protein was not associated with age, gender, or *CYP2A6* gene type (Table 4). A

lack of association between *CYP2A6* gene type and CYP2A6 apoprotein, however, does not rule out variations in catalytic activity in individuals with different *CYP2A6* gene types, since immunoquantitation does not measure enzyme activity. None of the subjects was found to carry the *CYP2A6* null allele, confirming a low frequency of the allele in Caucasians [10–12]. In contrast, homozygous *CYP2A6(D)* is found in 4–5% of Japanese and Chinese [11,45,59]. This is the same prevalence found in our previous study [39] and in a study conducted recently with 200 Thai volunteers, phenotyped for CYP2A6 by coumarin hydroxylation test and genotyped by the method used here (unpublished observations).

CYP3A4 was found in all liver samples whereas CYP3A5 protein was found only in 16/28 liver samples. CYP3A5, but not CYP3A4, is known to be expressed in lung [9,60] and in small intestine in 70% of individuals [61]. The variation in CYP3A5 in liver samples recorded in this present study is consistent with polymorphic expression also revealed in other studies [2,4,7,62]. Interestingly, a positive correlation between hepatic CYP3A4 apoprotein content and age ($r = 0.35$, $P = 0.03$) was revealed in our present study. This is in opposition to findings on CYP2E1, whose tissue levels have been shown to decrease with age [41,42].

In conclusion, a number of sources of variation in CYP expression have been analysed using Western immunoblotting with a panel of highly specific antipeptide antibodies using liver and kidney cortex samples of Queensland residents whose non-workplace cadmium exposure and health status were also characterised. Our study reveals for the first time a possible association between environmental cadmium exposure and variation of CYP2C9 apoprotein abundance in human liver; however, further research is required to fully characterise this relationship.

Acknowledgments

We thank all staff of the John Tonge Centre for Forensic Sciences for their assistance in sample collection. We are grateful to Dr. Alan Greig in the ICP/MS laboratory in the Department of Earth Sciences for his analysis of tissue metal contents. We thank Professor Don Davies of Clinical Pharmacology section, University of London for his support. The National Research Centre for Environmental Toxicology was funded by the Australian National Health and Medical Research Council, Queensland Department of Health and the University of Queensland.

References

[1] Boobis AR, Edwards RJ, Adams DA, Davies DS. Dissecting the function of cytochrome P450. Br J Clin Pharmacol 1996;42:81–9.
[2] Mugford CA, Kedderis GL. Sex-dependent metabolism of xenobiotics. Drug Metab Rev 1998;30:441–98.
[3] Wrighton SA, VandenBranden M, Ring BJ. The human drug metabolizing cytochromes P450. J Pharmacokinet Biopharm 1996;24:461–72.
[4] Iyer KR, Sinz MW. Characterization of phase I and phase II hepatic drug metabolism activities in a panel of liver preparations. Chem Biol Interact 1999;118:151–69.
[5] Pearce RE, McIntyre C, Madan A, Sanzgiri U, Draper AJ, Bullock PL, Cook DC, Burton L, Latham J, Nevins C, Parkinson A. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. Arch Biochem Biophys 1996;331:145–69.
[6] Edwards RJ, Adams DA, Watts PS, Davies DS, Boobis AR. Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. Biochem Pharmacol 1998;56:377–87.
[7] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994;270:414–23.
[8] Hakkola J, Pasanen M, Hukkanen J, Pelkonen O, Maenpaa J, Edwards RJ, Boobis AR, Raunio H. Expression of xenobiotic-metabolizing cytochrome P450 forms in human full-term placenta. Biochem Pharmacol 1996;51:403–11.
[9] Raunio H, Hakkola J, Hukkanen J, Lassila A, Pelkonen O, Anttila S, Piipari R, Boobis AR, Edwards RJ. Expression of xenobiotic-metabolising CYPs in human pulmonary tissue. Exp Toxicol Pathol 1999;51:412–7.
[10] Fernandez-Salguero P, Hoffman SM, Cholerton S, Mohrenweiser H, Raunio H, Raunio A, Pelkonen O, Huang JD, Evans WE, Idle JR, Gonzalez FJ. A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles. Am J Hum Genet 1995;57:651–60.
[11] Oscarson M, McLellan RA, Gullsten H, Yue Q, Lang MA, Bernal ML, Sinues B, Hirvonen A, Raunio H, Pelkonen O, Ingelman-Sundberg M. Characterisation and PCR-based detection of a *CYP2A6* gene deletion found at a high frequency in a Chinese population. FEBS Lett 1999;448:105–10.
[12] Chen G-F, Tang Y-M, Green B, Lin D-X, Guengerich FP, Daly AK, Caporaso NE, Kadlubar FF. Low frequency of *CYP2A6* gene polymorphism as revealed by a one-step polymerase chain reaction method. Pharmacogenetics 1999;9:327–32.
[13] Inoue K, Asao T, Shimada T. Ethnic-related differences in the frequency distribution of genetic polymorphisms in *CYP1A1* and *CYP1B1* genes in Japanese and Caucasian population. Xenobiotica 2000;30:285–95.
[14] Shimada T, Tsumura F, Yamazaki H, Guengerich FP, Inoue K. Characterization of (±)-bufuralol hydroxylation activities in liver microsomes of Japanese and Caucasian subjects genotyped for *CYP2D6*. Pharmacogenetics 2000;10:1–14.
[15] Galal-Gorchev H. Dietary intake, levels in food and estimated intake of lead, cadmium and mercury. Food Addit Contam 1993;10:115–28.
[16] Satarug S, Haswell-Elkins MR, Moore MR. Safe levels of cadmium intake to prevent renal toxicity in human subjects. Br J Nutr 2000; 84:791–802.
[17] Elinder CG, Kjellstorm T, Lind B, Linnman L, Piscator M, Sundstedt K. Cadmium exposure from smoking cigarettes: variations with time and country where purchased. Environ Res 1983;32:220–7.
[18] Foulkes EC. The concept of critical levels of toxic heavy metals in target tissues. Crit Rev Toxicol 1993;20:327–39.
[19] Muller M, Anke M, Illing-Gunther, Thiel C. Oral cadmium exposure of adults in Germany 2: market basket calculations. Food Addit Contam 1998;15:135–41.
[20] Elinder CG, Lind B, Kjellstorm T, Linnman L, Friberg L. Cadmium in kidney cortex, liver and pancreas from Swedish autopsies: estimation of biological half time in kidney cortex, considering calorie intake and smoking habits. Arch Environ Health 1976;31:292–301.
[21] Australia New Zealand Food Authority. The Australian Market Basket Survey 1996, Melbourne, Australia.
[22] Satarug S, Baker JR, Reilly PEB, Moore MR, Williams DJ. Cadmium levels in the lung, liver, kidney cortex and urine samples from

Australians without occupational exposure to metals. Arch Environ Health, (in press).
[23] Lyon TDB, Aughey E, Scott R, Fell GS. Cadmium concentration in human kidney in the UK: 1978–1993. J Environ Monit 1999;1:227–31.
[24] Chung J, Nartey NO, Cherian MG. Metallothionine levels in liver and kidney of Canadians—A potential indicator of environmental exposure to cadmium. Arch Environ Health 1986;41:319–23.
[25] Tiran E, Karpf E, Tiran A. Age dependency of selenium and cadmium content in human liver, kidney and thyroid. Arch Environ Health 1995;50:242–6.
[26] Yoshida M, Ohta H, Yamauchi Y, Seki Y, Sagi M, Yamazaki K, Sumi Y. Age-dependent changes in metallothionein levels in liver and kidney of the Japanese. Biol Trace Element Res 1998;63:167–75.
[27] Fiala J, Hruba D, Rezl P. Cadmium and zinc concentrations in human placentas. Cent Eur J Public Health 1998;6:241–8.
[28] Nilsson U, Schutz A, Skerfving S, Mattsson S. Cadmium in kidneys in Swedes measured *in vivo* using x-ray fluorescence analysis. Int Arch Occup Environ Health 1995;67:405–11.
[29] Fortoul TI, Osorio LS, Tova AT, Salazar D, Castilla ME, Olaiz-Fernandez G. Metals in lung tissues from autopsy cases in Mexico City residents: comparison of cases from the 1950s and 1980s. Environ Health Perspect 1996;6:630–2.
[30] Iszard MB, Liu J, Klaassen CD. Effect of several metallothionein inducers on oxidative stress defense mechanisms in rats. Toxicology 1995;104:25–33.
[31] Anjum F, Raman A, Shakoori AR, Gorrod JW. An assessment of cadmium toxicity on cytochrome P450 and flavin monooxygenase-mediated metabolic pathways of dimethylaniline in male rabbits. J Environ Path Toxicol Oncol 1992;11:191–5.
[32] Satarug S, Ujjin P, Reilly PE. Changes in CYP1A specific monooxygenase in rat liver caused by cadmium and LPS administration. In the 13th International Symposium on Microsomes and Drug Oxidations, Abstract number 114, p. 138, 10–14 July 2000.
[33] Kadiiska M, Stoytchev T, Serbinova E. On the mechanism of the enzyme-inducing action of some heavy metal salts. Arch Toxicol 1985;56:167–9.
[34] Kadiiska M, Stoytchev T, Serbinova E. Effect of some heavy metal salts on hepatic monooxygenases after subchronic exposure. Arch Toxicol Suppl 1985;8:313–5.
[35] McLemore T, Adelberg S, Liu MC, McMahon NA, Yu SJ, Hubbard WC, Czerwinski M, Coudert BP, Mosco JA, Stinson S, Storeng RA, Eggleston JC, Boyd MR, Hines RN. Cytochrome P450 1A1 gene expression in living cancer patients: Evidence for cigarette smoke-induced expression in normal lung and altered gene regulation in primary pulmonary carcinomas. J Natl Cancer Inst 1990;82:1333–9.
[36] Nordmark A, Lundgren S, Cnattingius S, Rane A. Dietary caffeine as a probe agent for assessment of cytochrome P450 1A2 activity in random urine samples. Br J Clin Pharmacol 1999;47:397–402.
[37] Chung W-G, Kang J-H, Park C-S, Cho M-H, Cha Y-N. Effect of age and smoking on *in vivo* CYP1A2, flavin-containing monooxygenase, and xanthine oxidase activities in Koreans: determination by caffeine metabolism. Clin Pharmacol Ther 2000;67:258–66.
[38] Welfare MR, Murray A, Bassendine ME, Daly AK. Detailed modelling of caffeine metabolism of the *CYP1A2* gene: lack of a polymorphism in CYP1A2 in Caucasians. Pharmacogenetics 1999;9:367–75.
[39] Satarug S, Lang MA, Yongvanit P, Sithithaworn P, Mairiang E, Mairiang P, Pelkonen P, Barstch H, Haswell-Elkins MR. Induction of cytochrome P450 2A6 expression in humans by the carcinogenic parasite infection, opisthorchiasis viverrini. Cancer Epidemiol Biomarkers Prev 1996;5:795–800.
[40] George J, Byth K, Farrell GC. Influence of clinicopathological variables on CYP protein expression in human liver. J Gastroenterol Hepatol 1996;11:33–9.
[41] Tanaka E. Clinical importance of non-genetic and genetic cytochrome P450 function tests in liver disease. J Clin Pharm Ther 1998;23:161–70.
[42] Rodighiero V. Effects of liver disease on pharmacokinetics: an update. Clin Pharmacokinet 1999;37:399–431.
[43] Pasanen M, Rannala Z, Tooming A, Sotaniemi EA, Pelkonen O, Rautio A. Hepatitis A impairs the function of human hepatic CYP2A6 *in vivo*. Toxicology 1997;123:177–84.
[44] Guengerich FP, Turvy CG. Comparison of levels of several human microsomal cytcochrome P-450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. J Pharmacol Exp Ther 1991;256:1189–94.
[45] Yokoi T, Kamataki T. Genetic polymorphism of drug metabolizing enzymes: New mutations in *CYP2D6* and *CYP2A6* genes in Japanese. Pharm Res 1998;15:517–24.
[46] Guengerich FP. Analysis and characterization of enzymes. In: Wallace Hayes A, editor. Principles and methods of toxicology. New York: Raven Press, 1994. p. 1267–8.
[47] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[48] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[49] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets; procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.
[50] Richardson TH, Griffin KJ, Jung F, Raucy JL, Johnson EF. Targeted antipeptide antibodies to cytochrome P450 2C18 based on epitope mapping of an inhibitory monoclonal antibody to P450 2C5. Arch Biochem Biophys 1997;338:157–64.
[51] Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM, Ghanayem BI. Evidence that CYP2C19 is the major (*S*)-mephenytoin-4′-hydroxylase in humans. Biochemistry 1994;33:1743–52.
[52] Goldstein JA, de Morais SM. Biochemistry and molecular biology of the human CYP2C subfamily. Pharmacogenetics 1994;4:285–99.
[53] Aynacioglu AS, Brockmoller J, Bauer S, Sachse C, Guzelbey P, Ongen Z, Nacak M, Roots I. Frequency of cytochrome P450 CYP2C9 variants in a Turkish population and functional relevance for phenytoin. Br J Clin Pharmacol 1999;48:409–15.
[54] Freeman BD, Zehnbauer BA, McGrath S, Borecki I, Buchman TG. Cytochrome P450 polymorphisms are associated with reduced warfarin dose. Surgery 2000;128:281–5.
[55] Shimizu Y, Nakatsuru Y, Ichinose M, Takahashi Y, Kume H, Mimura J, Fuji-Kuriyama Y, Ishikawa T. Benzo[*a*]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. Proc Natl Acad Sci USA 2000;97:779–82.
[56] Badawi AF, Cavalieri EL, Rogan EG. Effect of chlorinated hydrocarbons on expression of cytochrome P450 1A1, 1A2 and 1B1 and 2- and 4-hydroxylation of 17 beta-estradiol in female Sprague–Dawley rats. Carcinogenesis 2000;21:1593–9.
[57] Katiyar SK, Matsui MS, Muktar H. Ultraviolet-B exposure of human skin induces cytochromes P450 1A1 and 1B1. J Invest Dermatol 2000;114:328–33.
[58] Murray GI, Taylor MC, McFadyen MCE, McKay JA, Greenlee WF, Burk MD, Melvin WT. Tumor-specific expression of cytochrome P450 CYP1B1. Cancer Res 1997;57:3026–31.
[59] Miyamoto M, Umetsu Y, Dosaka-Akita H, Sawamura Y, Yokota J, Kunitoh H, Nemoto N, Sato K, Ariyoshi N, Kamataki T. *CYP2A6* gene deletion reduces susceptibility to lung cancer. Biochem Biophys Res Commun 1999;261:658–60.
[60] Anttila S, Hukkanen J, Hakkola J, Stjernvall T, Beaune P, Edwards RJ, Boobis AR, Pelkonen O, Raunio H. Expression and localization of CYP3A4 and CYP3A5 in human lung. Am J Resp Cell Mol Biol 1997;16:242–9.
[61] Lown K, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA, Watkins PB. Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Drug Metab Dispos 1992;22:947–55.
[62] Jounaidi Y, Hyrailles V, Gervot L, Maurel P. Detection of CYP3A5 allelic variant: a candidate for the polymorphic expression of the protein? Biochem Biophys Res Commun 1996;221:466–70.