Lymphocyte cytochrome P450 expression: inducibility studies in male Wistar rats

M. P. HANNON-FLETCHER* and Y. A. BARNETT†

*Northern Ireland Centre for Diet and Health (NICHE), University of Ulster, Cromore Road, Coleraine, County Londonderry BT52 1SA, Northern Ireland; and †Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK

Accepted: 17 December 2007

ABSTRACT

The cytochrome P450 system plays a key role in the metabolism of endogenous and exogenous compounds. The system is distributed widely in body tissues, with the highest concentration of the enzymes found in liver hepatocytes. Extrahepatic expression of the P450 system has been documented in the lung, pancreas and kidney, and the enzymes are induced by many disease states, including diabetes mellitus and cancer. Little attention has been paid to the expression and inducibility of the system in peripheral blood lymphocytes. In this study, specific P450 inducers are administered *in vivo* to male Wistar rats. The expression and *in vivo* induction of the P450 isoforms CYP2B, CYP2E, CYP3A and CYP4A in liver and lymphocyte samples is determined using Western blot analysis. Following *in vivo* induction, the lymphocyte P450 proteins showed an average three-fold increase in expression (0.003–0.005 μg P450/μg microsomal protein), compared to the control lymphocyte samples. Expression in the induced lymphocyte samples was up to 11-fold lower than that in the induced liver samples, as expected. These results indicate that lymphocytes may provide a relatively simple method by which to monitor the P450 profile in human subjects.

KEY WORDS: Cytochrome P450.
Diabetes.
Lymphocyte P450.
Wistar rats.

Introduction

The cytochrome P450-dependent microsomal mixed-function oxidases (cytochrome P450) are terminal monoxygenases involved in the metabolism of a wide variety of structurally diverse compounds. They play a major role in the metabolism of endogenous substrates[1,2] and exogenous compounds such as drugs, carcinogens and environmental chemicals.[2,3]

Cytochrome P450 exists as a number of families, each comprising one or more proteins with specific but overlapping substrate specificity. This allows the system unprecedented broad substrate specificity.[1,4,5] Cytochrome P450s are regulated by hormones such as growth and sex hormones.[6]

Numerous studies have demonstrated that the P450 proteins, especially those belonging to the CYP1A and CYP2E families, are involved in the bioactivation of many xenobiotics, resulting in the production of metabolites that are highly chemically reactive and may lead to cytotoxic and mutagenic effects.[7-10] *In vivo* animal studies show that those cytochrome P450 isoforms involved primarily in xenobiotic metabolism (i.e., CYP1, CYP2, CYP3)[6] are highly inducible in the liver by chemical exposure and by pathological conditions such as diabetes mellitus in animal models.[11–14]

The cytochrome P450 enzymes are present in the highest concentration in the liver hepatocyte; however, they are expressed in almost every mammalian tissue.[4] Extrahepatic expression of P450 isoforms have been demonstrated in, for example, pancreatic islets of Langerhans,[15,16] kidney,[14,17] brain and intestine[17] and the lung.[18]

Increases in hepatic levels of CYP1A, CYP3A, CYP4A, CYP2B and CYP2E protein have been demonstrated in diabetogen-induced and spontaneous type 1 diabetes in animal models.[19–21] A few studies have demonstrated lymphocyte expression of specific P450 apoproteins (CYP3A expression in rats, using various inducers,[22] and CYP2E1 expression in human subjects with type 1 diabetes[23,24] and in subjects with alcoholic liver disease[25]).

Correspondence to: Dr M. P. Hannon-Fletcher
Email: mp.hannon@ulster.ac.uk

The CYP1A family has been studied extensively in lymphocytes[26–28] and has been used as a biomarker for exposure to environmental pollutants,[29] and as such is not included in the present study.

Modulation of the P450 system can alter the capacity of the individual to metabolise endogenous and exogenous agents (e.g., steroids, prostaglandins, drugs and carcinogens) and may increase the possibility of drug–drug and drug–nutrient interaction.[30] Peripheral lymphocytes are exposed to generally higher concentrations of absorbed chemicals than are tissues, and therefore drug metabolism mediated by lymphocyte P450 proteins may make a significant contribution to xenobiotic metabolism.

In addition, modulation of the P450 proteins by peripheral lymphocytes may result in the bioactivation of various chemicals, which may lead to the production of highly reactive and toxic intermediates. Furthermore, leakage of these reactive intermediates from lymphocytes may result in damage (e.g., lipid peroxidation and DNA damage) to surrounding tissues. If widespread inducibility in

lymphocytes could be demonstrated then the ability to monitor the activity of specific P450 enzymes using a relatively non-invasive procedure (i.e., collection of peripheral blood lymphocytes) would permit the close monitoring of individuals considered to be at higher risk from P450-mediated drug–drug and drug–nutrient interactions. This would be of particularly value to those individuals with specific disease states such as type 1 diabetes.

This study aims to explore the expression of the CYP2B, CYP2E, CYP3A, and CYP4A family of proteins in liver and lymphocytes from male Wistar rats in response to selected inducing agents. In addition, the possible correlation between liver and lymphocyte content of CYP2B, CYP2E, CYP3A, and CYP4A proteins is also examined.

Materials and methods

Animals

Male albino Wistar rats (250–350 g) were obtained from the colony maintained at the Biochemical and Behavioural Research Unit, University of Ulster at Coleraine. All studies were carried out using ethically approved methods and by Northern Ireland Home Office authorised animal handling licence holders. The animals were housed in an air-conditioned room at 22±2°C with a regular lighting schedule (12 h light [07.30–19.30], 12 h dark). Animals were allowed *ad libitum* access to drinking water and a standard pellet diet (Diet 41B, Birmingham, UK).

Animal pretreatment

Four groups (15 animals per group) were administered cytochrome P450-inducing agents (Table 1). Each test group had a respective control group, which received vehicle without inducing agent. All animals were killed by CO_2 inhalation 24 h after the last treatment with inducing agent. Blood (10–15 mL) was aspirated from the heart using a green gauge needle (No. 2 Luer; Terumo, Belgium) and a 10 mL syringe into lithium–heparin blood collection tubes at room temperature. Liver was excised and placed in a sterile 25 mL universal container (Sterilin, UK) on ice. A section of the whole liver and all blood collected from each treatment/control group were then pooled on the same day for further processing.

Preparation of liver microsomal fraction

Microsomes were prepared according to the method described by Ioannides and Parke.[31]

Lymphocyte isolation

Lymphocytes were isolated from whole blood using a modification of the method described by Böyum.[32] Briefly, whole blood was mixed (1:1) with RPMI 1640 (Gibco Life Technologies, UK) then 8 mL diluted blood was layered on 10 mL Histopaque 1077 (Sigma, Poole, UK) in a sterile 25 mL universal container at room temperature.

Following centrifugation at 700 x*g* for 35 min, the mononuclear layer (buffy layer) was aspirated carefully, mixed with 10 mL RPMI 1640 and centrifuged at 400 x*g* for 10 min. After an additional wash in RPMI 1640 the mononuclear cells were incubated for 4 h in RPMI 1640 containing 10% fetal calf serum, 200 µg/mL sodium pyruvate, 100 units/mL penicillin and 100 µg/mL streptomycin (BDH Laboratory Supplies, Poole, UK) at 37°C in a 5% CO_2 in air humidified atmosphere.

Following incubation, the medium, which contained the lymphocyte fraction, was decanted into a labelled centrifuge tube. The lymphocytes were washed briefly (x2) with phosphate-buffered saline (PBS). The resulting cell pellets were then resuspended in 5 mL freeze down medium (70% RPMI 1640, 20% fetal calf serum, 10% glycerol; Sigma, Poole, UK), which was divided equally between two cryotubes and frozen at –70°C until all samples had been collected.

Preparation of lymphocyte microsomal fraction

Once all induction studies had been completed, frozen lymphocytes were thawed on ice and cell pellets collected by centrifugation at 300 x*g* for 10 min. Microsomal fractions were prepared using a modification of the method described by Ioannides and Parke.[31] Briefly, the lymphocyte pellets were resuspended in 2-mL ice-cold 0.1mol/L glycerol phosphate buffer comprising 0.1 mol/L dipotassium hydrogen orthophosphate (pH to 7.4 by adding 0.1 mol/L potassium dihydrogen orthophosphate) containing 20% (v/v) glycerol and a protease inhibitor cocktail (one tablet per 25 mL buffer; Boehringer) and homogenised on ice. Samples were transferred to ultracentrifuge tubes and sonicated on ice for 30 sec (six cycles/sec).

Further processing followed the procedure adopted for liver microsomal samples. Finally, the resultant microsomal pellet was washed (x1) with ice-cold glycerol phosphate buffer and resuspended in 100 µL ice-cold glycerol phosphate buffer before sonicating for 10 sec (six cycles/sec). The protein concentration of the lymphocyte microsomal fraction was determined using the method of Bradford.[33]

Western blotting

Liver and lymphocyte microsomal samples were subjected

Table 1. Protocol for induction of cytochrome P450 enzymes *in vivo* in male Wistar rats.

P450 subfamily	Inducing agent	Daily administration	Duration
CYP2B	Phenobarbitone	0.1% (v/v) in drinking water	7 days
CYP2E	Acetone	1% (v/v) in drinking water	7 days
CYP3A	Clotrimazole	100 mg/kg in saline (i.p.)	3 days
CYP4A	Sodium clofibrate	100 mg/kg in saline (i.p.)	3 days

i.p.: intraperitoneal injection.

Each group had a respective control group that received vehicle without inducing agent.

Table 2. Cytochrome P450 antibody details.

P450 subfamily	Antibody/antiserum	Immunised in
CYP2B	Anti-rat CYP2B1 polyclonal	Goat CYP2B1 purified in rat liver
CYP2E	Anti-rat CYP2E1 polyclonal	Goat CYP2E1 purified in rat liver
CYP3A	Anti-rat CYP3A2 polyclonal	Goat CYP3A2 purified in rat liver
CYP4A	Anti-rat CYP4A1 polyclonal	Goat CYP4A1 purified in rat liver

Microsome standard supplied (Daiichi Pure Chemicals Co., Japan) with each antibody (antiserum).

to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein components were transferred to nitrocellulose and probed with their respective P450 polyclonal antibodies. The method employed was described by Laemmli,[34] with some modification. Briefly, microsomal extracts containing either 40 µg hepatic protein or 100 µg lymphocyte protein were mixed with equal volumes of Laemmli[34] sample buffer (0.01% bromophenol blue [0.1% solution], 15% glycerol, 5% mercaptoethanol, 2.3% SDS [10% solution], 0.625 mol/L Tris) and heated to 98°C for 5 min. Boiled samples were then subjected to SDS-PAGE on a 12% gel. Separated proteins were electrotransferred to Hybond-ECL nitrocellulose membrane for 2 h in buffer containing 15.6 mmol/L Tris, 120 mmol/L glycine and 200 mL methanol. The membranes were incubated with specific P450 polyclonal antibody (1 in 5000 dilution; Daiichi Pure Chemicals Co., Japan; Table 2) for 1 h.

Following washes (5 min x2; 15 min x1) in PBS–Tween, membranes were treated with alkaline phosphatase-linked secondary antibody (1 in 30,000 dilution; Sigma, Poole, UK). Antibody binding was visualised by the addition of two tablets of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma, Poole, UK) in 20 mL distilled H_2O.

Western blot analysis

Western blot images were captured using the UVP-grab system of a real-time recording camera connected to a UV/white light transilluminator. Images were converted to *.tif format. Protein expression on Western blot images were semiquantified by analysis of the *.tif format using the image analysis software program Phoretix 1-D advanced.

All images were analysed in the same manner, which included peak detection, band measurement and quantity calibration. The respective P450 standard on each gel was assigned an arbitrary value of 100%, which permitted comparison of the test samples. As each P450 standard contained 5 µg P450 protein, it was possible to calculate the quantity of P450 protein/µg microsomal protein in each test sample.

Table 3. Quantification of Western blot images and calculated protein content.

Cytochrome P450 family	Sample type	Band analysis Percentage of 100% standard band	Equivalent µg P450/µg microsomal protein in sample	Increase
CYP2B	C-liver	3.70	0.005	
	I-liver	37.51	0.047	9.4-fold
	C-lymphocytes	2.08	0.001	
	I-lymphocytes	7.55	0.004	4.0-fold
CYP2E	C-liver	41.19	0.051	
	I-liver	64.51	0.081	1.6-fold
	C-lymphocytes	4.10	0.002	
	I-lymphocytes	10.41	0.005	2.5-fold
CYP3A	C-liver	33.52	0.042	
	I-liver	49.36	0.062	1.5-fold
	C-lymphocytes	2.92	0.001	
	I-lymphocytes	6.11	0.003	3.0-fold
CYP4A	C-liver	36.34	0.045	
	I-liver	98.63	0.123	2.7-fold
	C-lymphocytes	4.09	0.002	
	I-lymphocytes	5.55	0.003	1.5-fold

C: control, I: induced.

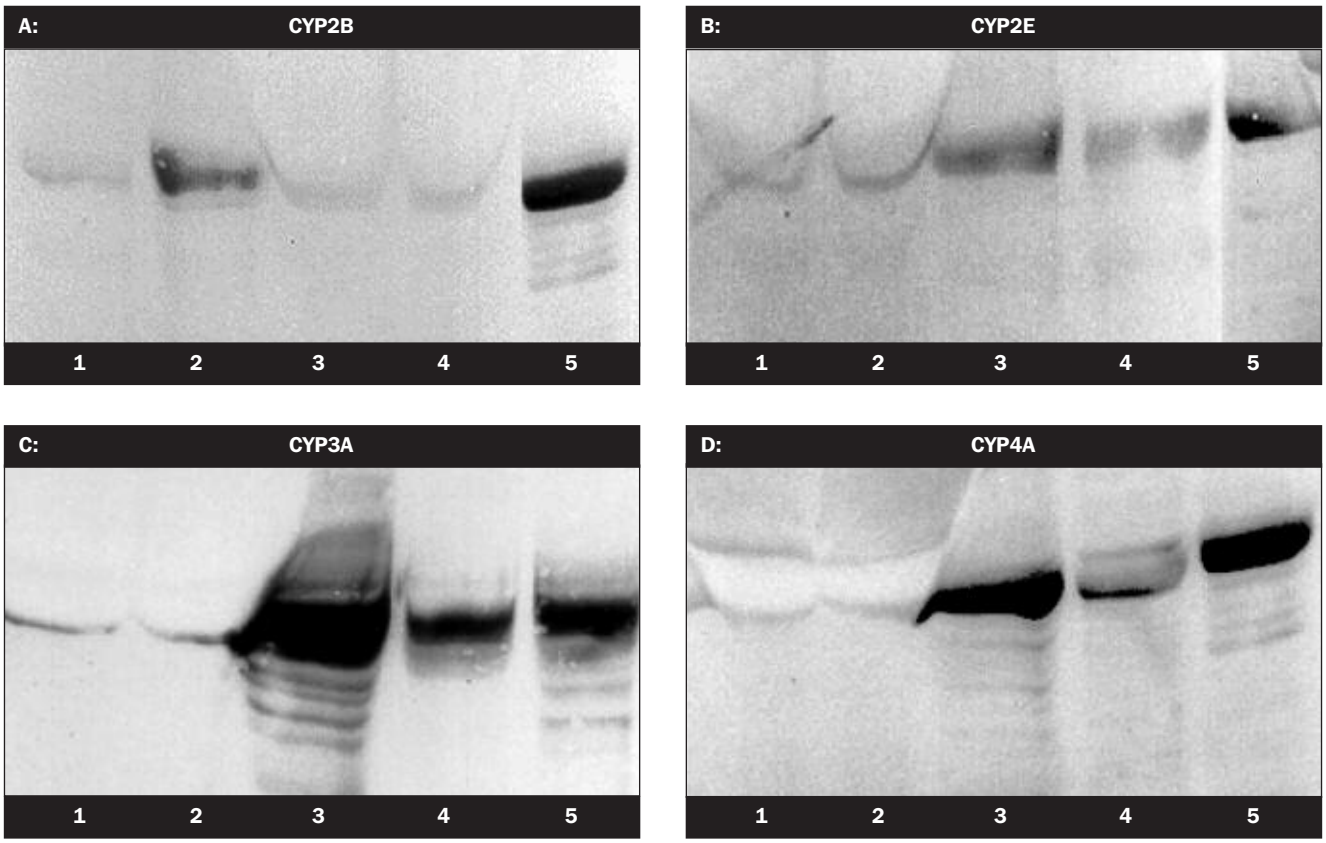

Fig. 1. Western blot analysis of CYP2B, CYP2E, CYP3A, and CYP4A in liver and lymphocyte microsomal proteins from male Wistar rats.
A) 1: control liver, 2: PB-induced liver, 3: control lymphocytes, 4: PB-induced lymphocytes, 5: CYP2B standard.
B) 1: control lymphocytes, 2: acetone-induced lymphocytes, 3: acetone-induced liver, 4: control liver, 5: CYP2E standard.
C) 1: clotrimazole-induced lymphocytes, 2: control lymphocytes, 3: CYP3A standard, 4: control liver, 5: clotrimazole-induced liver.
D) 1: control lymphocytes, 2: Na clofibrate-induced lymphocytes, 3: CYP4A standard, 4: control liver, 5: Na clofibrate-induced liver.

Results

Basal levels (constitutive expression) of liver P450 microsomal proteins determined by Western blotting with polyclonal antibodies against the four isoforms are presented in Figure 1. Analysis of the immunoreactive bands, together with the calculated microsomal protein content of the bands, is presented in Table 3.

In contrast to the control liver (0.005–0.051 µg P450/µg microsomal protein), control lymphocyte microsomes showed up to 18-fold less P450 protein expression (Fig. 1. A: lane 3, B: lane 3, C: lane 2, D: lane 1; Table 3: 0.001–0.002 µg P450/µg microsomal protein).

After *in vivo* treatment with the various inducers (Table 1), the liver P450 isoforms showed up to a 3.4-fold increase (Table 3) in expression (Fig. 1. A: lane 2, B: lane 3, C: lane 4, D: lane 5). Lymphocyte microsomal proteins were also induced following *in vivo* treatment (Fig. 1. A: lane 4, B: lane 2, C: lane 1, D: lane 2), specifically the isoforms CYP2B (four-fold increase), CYP2E (2.5-fold increase), CYP3A (three-fold increase) and CYP4A (1.5-fold increase).

Isoform CYP2B showed the least basal expression of all four isoforms; however, it also showed the greatest degree of induction in the liver and lymphocyte samples (9.4- and four-fold increase, respectively). All four isoforms showed an increase in expression in the liver and lymphocyte samples following *in vivo* induction.

Discussion

This is the first study to investigate basal expression and induction of all four P450 proteins in rat lymphocytes, and shows that there is basal expression, albeit at very low levels (up to 11-fold lower than in liver), of the P450 isoforms CYP2B, CYP2E, CYP3A and CYP4A in control male Wistar rat lymphocytes. The results also demonstrate that CYP2B, CYP2E, CYP3A and CYP4A are inducible *in vivo* in rat lymphocytes. The isoenzymes CYP2B, CYP2E and CYP3A showed slightly stronger expression than CYP4A following *in vivo* induction.

The results agree, in part, with Merk *et al.*,[35] who used polymerase chain reaction (PCR) analysis of P450 messenger RNA (mRNA) expressed in benzanthracene-induced human monocytes, and found expression of CYP2B6, CYP1B1 and CYP2E, but did not detect CYP1A1 activity. Murray *et al.*[36] showed CYP3A isoenzyme expression in control human polymorphonuclear leucocytes, and Raucy *et al.*[25] found that levels of CYP2E were 2.3-fold higher in alcohol abusers than in control subjects.

The present study demonstrates induction of the P450 proteins in the hepatic fractions, and a similar induction profile in the lymphocyte fractions in the same animals. Immunoblotting indicates that lymphocyte expression of lymphocyte P450 is markedly lower than hepatic expression (up to 11-fold), as expected. This agrees, in part, with the

findings of Mahnke *et al*.,[22] who investigated levels of CYP3A in male and female Sprague-Dawley rat lymphocytes and showed expression in lymphocytes to be approximately 1000-fold lower than that found in liver.

The ability of the P450 system to bioactivate many xenobiotics, and the ability of a specific isoform, CYP2E1, to generate reactive oxygen species is well documented.[7–10, 37] In addition to the reactive intermediates produced, modulation of the P450 system may alter the capacity of the individual to metabolise endogenous and exogenous substrates, and increases the possibility of drug–drug and drug–nutrient interaction.[6,30]

Therefore, modulation of the P450 proteins by peripheral lymphocytes could result in the production of highly reactive and toxic intermediates, and leakage of these reactive intermediates from lymphocytes may result in damage to surrounding tissues (lipid peroxidation and DNA damage).

Such damage could have significant implications for patients with atherosclerosis and diabetes, as both diseases are associated with oxidative damage to blood vessels.[38,39] In addition, modulation of the system may have adverse affects on drug–drug interactions, especially in drugs with a narrow therapeutic index.

In conclusion, this study demonstrated that CYP2B, CYP2E, CYP3A and, to a lesser extent, CYP4A are inducible in male Wistar rat lymphocytes following *in vivo* treatment with specific inducers. In addition, a similar induction profile was observed in the liver following *in vivo* induction. Thus, a human lymphocyte P450 profile may be employed as an indicator of the hepatic P450 profile in humans.

This work was supported by a Department of Education Northern Ireland (DENI) Postgraduate Student Distinction Award to Mary P. Hannon-Fletcher. □

References

1 Yang CS, Lu AYH. The diversity of substrates for cytochrome P-450. In: Guengerich. FP ed. *Mammalian cytochrome P-450* Vol 2. Boca Raton: CRC Press, 1987: 1–18.

2 Nelson DR, Koymans L, Stegeman JJ *et al*. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 1996; **6**: 1–42.

3 Gonzalez FJ, Gelboin FJ. Role of human cytochrome P-450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metab Rev* 1994; **26**: 165–83.

4 Gonzalez FJ. The molecular biology of cytochrome P450s. *Pharm Rev* 1989; **40**: 243–88.

5 Nebert DW. Role of genetics and drug metabolism in human cancer risk. *Mutat Res* 1991; **247**: 267–81.

6 Ioannides C, Barnett CR, Flatt PR. The effects of experimental diabetes on the cytochrome P450 system and other metabolic pathways. In: McNeill JH ed. *Experimental models of diabetes*. Boca Raton: CRC Press, 1999: 79–116.

7 Barnett CR, Flatt PR, Ioannides C. Hyperketonaemia markedly modulates the metabolic activation of chemical carcinogens. *Chem Biol Interact* 1990; **74**: 281–9.

8 Lewis DF, Ioannides C, Parke DV. Cytochrome P450 and species differences in xenobiotic metabolism and activation of carcinogen. *Environ Health Perspect* 1998; **106**: 633–41.

9 Ennever FK, Noonan TJ, Rosenkranz HS. The predictivity of animal bioassays and short-term genotoxicity tests for carcinogenicity and non-carcinogenicity to humans. *Mutagenesis* 1987; **2**: 73–8.

10 Lijinsky W. Life-span and cancer: the induction time of tumours in diverse animal species treated with nitrosodiethylamine. *Carcinogenesis* 1993; **14**: 2373–5.

11 Barnett CR, Flatt PR, Ioannides C. Induction of hepatic microsomal P450 I and IIB proteins by hyperketonaemia. *Biochem Pharmacol* 1990; **40**: 393–7.

12 Barnett CR, Gibson GG, Wolf CR, Flatt PR, Ioannides C. Induction of cytochrome P450III and P450IV family proteins in streptozotocin-induced diabetes. *Biochem J* 1990; **286**: 765–9.

13 Ioannides C, Bass SL, Ayrton AD, Trinick J, Walker R, Flatt PR. Streptozotocin-induced diabetes modulates the metabolic activation of chemical carcinogens. *Chem Biol Interact* 1988; **68**: 189–202.

14 Irizar A, Ioannides C. Extrahepatic expression of P450 proteins in insulin-dependent diabetes mellitus. *Xenobiotica* 1995; **25**: 941–9.

15 Clarke J, Flatt PR, Barnett CR. Cytochrome P450 CYP2E expression in the pancreatic islets of Langerhans. *Diabet Med* 1996; **13** (Suppl. 3): 37.

16 Clarke J, Flatt PR, Barnett CR. Cytochrome P450 1A-like proteins expressed in the islets of Langerhans and altered pancreatic β-cell secretory responsiveness. *Br J Pharmacol* 1997; **121**: 389–4.

17 Roberts BJ, Shoaf SE, Jeong KS, Song BJ. Induction of CYP2E in liver, kidney, brain and intestine during chronic ethanol administration and withdrawal: evidence that CYP2E possesses a rapid phase half-life of 6 hours or less. *Biochem Biophys Res Comm* 1994; **205**: 1064–71.

18 Kivisto KT, Fritz P, Linder A, Friedel G, Beaune P, Kroemer HK. Immunohistochemical localization of cytochrome P450 3A in human pulmonary carcinomas and normal bronchial tissue. *Histochemistry* 1995; **103**: 25–9.

19 Barnett CR, Flatt PR, Ioannides C. Role of ketone bodies in the diabetes-induced changes in hepatic mixed-function oxidase activities. *Biochem Biophys Acta* 1988; **967**: 250–4.

20 Barnett CR, Flatt PR, Bone AJ. Hepatic cytochrome P450 profile in BB rats with spontaneous insulin-dependent diabetes mellitus. In: Lechner MC ed. *Biochemistry, biophysics and molecular biology of cytochrome P450*. Paris: John Libbey Eurotext, 1994: 574–50.

21 Ioannides C, Barnett CR, Irizar A, Flatt PR. Drug metabolism and chemical toxicity in diabetes In: Ioannides C, Flatt PR eds. *Drugs, diet and disease* Vol 2. Hemel Hempstead, UK: Ellis Horwood, 1995: 330–53.

22 Mahnke A, Roos PH, Hanstein WG, Chabot GG. *In vivo* induction of cytochrome P450 CYP3A expression in rat leukocytes using various inducers. *Biochem Pharmacol* 1996; **51**: 1579–82.

23 Hannon-Fletcher MP, O'Kane MJ, Moles KW, Barnett CR, Barnett YA. Lymphocyte CYP2E1 expression in human IDDM subjects. *Food Chem Toxicol* 2001; **39**: 25–32.

24 Song BJ, Veech RL, Saenger P. Cytochrome P450IIE1 is elevated in lymphocytes from poorly controlled insulin-dependant diabetics. *J Clin Endocrinol Metab* 1990; **71**: 1036–40.

25 Raucy JL, Schultz ED, Wester MR *et al*. Human lymphocyte cytochrome P450 2E1, a putative marker for alcohol-mediated changes in hepatic chlorzoxazone activity. *Drug Metab Dispos* 1997; **25**: 1429–35.

26 Kouri RE, McKinney CE, Slomiany DJ, Snodgrass DR, Wray NP, McLemore TL. Positive correlation between high aryl hydrocarbon hydroxylase activity and primary lung cancer as

analysed in cryopreserved lymphocytes. *Cancer Res* 1982; **42**: 5030–7.
27 Gurtoo HL, Parker NB, Paigen B, Havens MB, Minowada J, Freeman HJ. Induction, inhibition and some enzymological properties of aryl hydrocarbon hydroxylase in fresh mitogen-activated human lymphocytes. *Cancer Res* 1979; **39**: 4620–9.
28 Kellermann G, Luyten-Kellermann M, Shaw CR. Genetic variation of aryl hydrocarbon hydroxylase in human lymphocytes. *Am J Hum Genet* 1973; **25**: 237–331.
29 Rumsby PC, Yardley-Jones A, Anderson D, Phillimore HE, Davies MJ. Detection of CYP1A1 mRNA levels and CYP1A1 Msp polymorphisms as possible biomarkers of exposure and susceptibility in smokers and non-smokers. *Teratog Carcinog Mutagen* 1996; **16**: 65–74.
30 Lee P-C, Werlin SL. The induction of hepatic cytochrome P450 3A in rats: effects of age. *Proc Soc Exp Biol Med* 1995; **210**: 134–9.
31 Ioannides C, Parke DV. Mechanism of induction of hepatic microsomal drug metabolising enzymes by a series of barbiturates. *J Pharmacol Pharma* 1975; **27**: 739–46.
32 Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Norwegian Defence Research Establishment, Division for Toxicology*. (Paper iv) Kjeller, Norway, 1980;: 77–89.
33 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principal protein-dye binding. *Ann Biochem* 1976; **72**: 248–52.
34 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–5.
35 Merk HF, Baron J, Hertl M, Niederau D, Rübben A. Lymphocyte activation in allergic reactions elicited by small-molecular-weight compounds. *Int Arch Allergy Immunol* 1997; **113**: 173–6.
36 Murray GI, Barnes TS, Sewell HF, Ewen SW, Melvin WT, Burke MD. The immunocytochemical localisation and distribution of cytochrome P450 in normal human hepatic and extrahepatic tissues with a monoclonal antibody to human cytochrome P450. *Br J Clin Pharmacol* 1988; **25**: 465–75.
37 Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. *Biochem Pharmacol* 1994; **48**: 155–9.
38 Oberley LW, Oberley TD. Reactive oxygen species in the aetiology of cancer. In: Ioannides C, Lewis DFV eds. *Drugs, diet and disease*. Vol 1. *Mechanistic approaches to cancer*. Chichester: Horwood, 1995: 47–63.
39 Baynes JW. Reactive oxygen in the aetiology and complications of diabetes. In: Ioannides C, Flatt PR eds. *Drugs, diet and disease* Vol 2. *Mechanistic approaches to diabetes*. Chichester: Horwood, 1995: 201–40.

British Journal of Biomedical Science 2008 65 (1)