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Polycyclic aromatic hydrocarbon metabolizing cytochrome P450s in freshly prepared uncultured rat blood lymphocytes

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ABSTRACT

In an attempt to develop blood lymphocyte cytochrome P450 expression profile as a surrogate to monitor tissue enzyme, the present study aimed to identify the expression and regulation of polycyclic aromatic hydrocarbons (PAHs) responsive CYPs in freshly prepared rat blood lymphocytes. Semi-quantitative and RT-PCR studies demonstrated constitutive and inducible mRNA expression of CYP1A1, 1A2, 1B1 isoenzymes and the associated transcription factors, aryl hydrocarbon receptor (AhR) and AhR translocator (ARNT) in blood lymphocytes. Absolute quantification using RT-PCR revealed several fold lower basal expression of CYP1A1, 1A2 and 1B1 in lymphocytes when compared to the liver. However, significant increase in the mRNA expression of these isoenzymes as well as AhR and ARNT in lymphocytes following pretreatment with 3-methylcholanthrene (MC) have demonstrated that responsiveness is retained in the blood lymphocytes, though the magnitude of increase is several fold lower when compared to liver. This increase in the mRNA expression was found to be associated with an increase in the protein expression of CYP1A1 and 1A2 in blood lymphocytes. Further, CYPs expressed in blood lymphocytes catalysed the O-dealkylation of 7-ethoxy- and 7-methoxyresorufins (ER or MR), though the reactivity was several fold lower in lymphocytes when compared to the liver enzyme. Our data providing quantitative evidence for similarities in the regulation of PAH-regulated CYP in uncultured and non-mitogen stimulated blood lymphocytes with the liver enzyme has led us to suggest that blood lymphocytes could be used as a surrogate to monitor tissue expression of CYPs.

1. Introduction

Majority of the environmental carcinogens including polycyclic aromatic hydrocarbons (PAHs) require metabolic activation by cytochrome P450s (CYPs), the phase I enzymes to produce the carcinogenic and mutagenic reactive intermediates [1,2]. The expression of the CYPs in the target tissues therefore contributes significantly to the process of chemical carcinogenesis [3]. It has been demonstrated that the activity and expression of CYP1A1, 1A2 and 1B1, some of the major carcinogen metabolizing enzymes, are induced by PAHs through activation of aryl hydrocarbon receptor (AhR). On binding of PAHs to AhR, the AhR becomes activated and translocates from cytosol to the nucleus where it heterodimerizes with AhR nuclear translocator (Arnt). This complex then binds to the xenobiotic responsive elements (XRE) in the enhancer regions of various genes, including CYP1A1 and 1B1, the most sensitive targets and up-regulating their expression [4,5]. Although the AhR-dependent pathway is conserved in mammalian species, the expression and inducibility of CYP1A1 and 1B1 isoenzymes is highly variable, attributed primarily to genetic differences, which contribute significantly to the differences in the susceptibility to chemical carcinogenesis [6–9]. Differential CYP1A1 inducibility was also reported in the different strains of mice, which correlated with PAH-induced carcinogenesis in these mice [10].

There has been an interest to develop bioassays using non-invasive procedures that could predict the interindividual variations and responsiveness of PAH-responsive CYPs. CYP1A1 and 1B1 are reported to be expressed in extrahepatic tissues including blood lymphocytes [11–13]. Further, as blood lymphocytes can be easily obtained through minimal invasive procedures and CYP gene expression levels were not influenced by the differences in the cellular composition of blood cells, they could be used as a surrogate for studying the expression of CYP1A1 and 1B1 levels in humans. CYP1A1 inducibility measured in cultured lymphocytes correlated well with that observed in lung tissue explants [6,14]. However, majority of the human studies were carried out with cultured blood lymphocytes which required mitogen stimulation that causes lymphocytes to proliferate resulting in the activation of

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several cell signaling pathways and increases in gene transcription such as expression of CYP1A1, 1B1 and associated AhR levels [15,16]. Uncultured and unstimulated blood lymphocytes were generally considered to be quiescent in vivo in terms of catalytic activity [17]. However, recent studies have shown that several of the CYPs including CYP1A1, 1B1 and their associated transcription factors are expressed in freshly prepared uncultured human blood lymphocytes [13,18]. Freshly isolated blood lymphocytes isolated from rats were also found to express CYP1A1 and that the CYP1A1 enzyme is inducible following pretreatment of rats with prototype as well as non-prototypic inducers of the enzyme [11,12].

Since it is well established that CYP1A1, 1A2 and 1B1 induction occurs at the transcription level, quantification of these CYPs in freshly prepared blood lymphocytes could be a potential tool for measuring basal as well as inducible expression of PAH-responsive CYPs. In an attempt to develop PAH-responsive CYPs in blood lymphocytes as a potential biomarker of exposure to environmental carcinogens, attempts were made to characterize the basal and PAH-inducible expression of CYP1A1, 1A2 and 1B1 isoenzymes together with their associated transcription factors, AhR and Arnt in freshly prepared uncultured blood lymphocytes isolated from control and induced rats. Further, to establish the suitability of PAH-responsive CYPs as a surrogate to monitor tissue enzyme, studies were also carried out to characterize the catalytic activity of CYP1A1 and 1A2 isoenzymes in these uncultured blood lymphocytes.

2. Materials and methods

2.1. Chemicals

7-Ethoxyresorufin, 7-methoxyresorufin, resorufin, histopaque 1077, phenylmethyl sulfonyl fluoride (PMSF), NADPH, dithiothreitol (DTT), protease inhibitor cocktail, 3-methylcholanthrene (MC), β-naphthoflavone (β-NF), furafylline, α-naphthoflavone (α-NF), bromophenol blue, goat anti-rabbit IgG-alkaline phosphatase complex, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), acrylamide, N,N′-methylenebisacrylamide (bis-acrylamide) and other chemicals used in SDS-PAGE were procured from Sigma–Aldrich, St. Louis, MI, USA. Immobilon-P nitrocellulose membrane and rabbit anti-rat Cytochrome P450 enzyme CYP1A1 and 1A2 polyclonal antibody were procured from Millipore Corp. (MA, USA). Glycerol, sodium chloride (NaCl), magnesium chloride ($MgCl_2$) and other routine chemicals were procured from SISCO Research Laboratories Pvt. Ltd., or E. Merck, India. Phenobarbitone sodium salt (PB) was a gift from Biodeal Laboratories, India.

2.2. Animals and treatment

Adult male albino Wistar rats (6–8-week old) were procured from the Indian Institute of Toxicology Research breeding colony and raised on animal pellet diet and water ad libitum. Animal care and experimentation was in accordance with the policy laid down and was approved by the Animal Care Committee of the Centre. For studying PAH-responsive CYPs in blood lymphocytes, the rats were divided into five groups, containing ten animals each. The animals in the first group were treated with 3-methylcholanthrene (MC, 30 mg/kg body wt.), suspended in corn oil, while the second group was treated with phenobarbital (PB, 80 mg/kg body weight) dissolved in normal saline, daily, intraperitoneally for 5 consecutive days. Animals in the third group were treated with β-naphthoflavone (β-NF, 80 mg/kg body weight) once daily for 3 consecutive days. Rats in the fourth and fifth group served as controls and received an equivalent amount of corn oil or normal saline. The animals were sacrificed 24 h after the last dose and blood was drawn from the heart and processed for the isolation of lymphocytes.

2.3. RNA extraction

Total RNA was extracted from whole blood isolated from control and MC pretreated rats by TRIzol LS and from liver by TRIzol reagent (Life Technologies, USA) according to manufacturer's protocol.

2.4. Semi-quantitative PCR analysis

cDNA was synthesized essentially as described in study by Johri et al. [19]. Prior to the amplification of CYPs, normalization was carried out with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the housekeeping gene. Reactions without RNA were also carried out which served as the negative RT control. The sequences of primers for CYP1A1, CYP1A2, CYP1B1, AhR, Arnt and GAPDH were described earlier [20–24]. PCR was carried out as described earlier [19], and products were analyzed by agarose gel electrophoresis using VERSA DOC Imaging System Model 1000 (Bio-Rad, USA). The densitometry was performed using Quantity One Quantitation software of Bio-Rad.

2.5. Quantitative RT-PCR analysis

For quantitative PCR, cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described by Shah et al. [25]. The sequence of primers used for CYP1A1, CYP1A2, CYP1B1, AhR, Arnt and GAPDH have been described in the literature [26–29]. The PCR reaction mixture for CYP1A1, 1A2, 1B1, AhR, Arnt and GAPDH in 20 μl contained 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 10 pM of each gene primers, 4 pM of each gene probe, 2 μl cDNA and nuclease-free H_2O. TaqMan assays for each gene target were performed in triplicate on cDNA samples in 96-well optical plates on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C. For absolute quantification of CYP1A1, 1A2 and 1B1, the number of copies of mRNA was calculated by interpolation from the standard curve generated using known amount of full length cDNA clone of CYP1A1, 1A2 and 1B1.

2.6. Isolation of blood lymphocytes

Blood lymphocytes were isolated by method described in study by Dey et al. [12]. The cell preparations contain >80% of lymphocytes, less than <10% of monocytes, and granulocytes, erythrocytes and platelets (<5%). Therefore, as such these cells should be termed as peripheral blood mononuclear cells (PBMC). However, as majority of the cells are lymphocytes, these preparations for ease of nomenclature and in the literature too, are generally termed as lymphocytes. In brief, 4.0 ml of whole blood was diluted with 4.0 ml of phosphate buffered saline (PBS), pH 7.4, and carefully layered over 2.0 ml of histopaque 1077. After centrifugation at 400 × *g* for 30 min at room temperature, the opaque interface containing mononuclear cells was transferred into a clean centrifuge tube. After repeated washing with PBS and recentrifugation at 250 × *g*, the lymphocyte pellet was resuspended in 0.5 ml of PBS. The number of cells was counted by a haemocytometer and the viability of the cells was assayed by the trypan blue exclusion test.

2.7. Preparation of microsomes and enzymatic analysis

Microsomal preparation from liver and blood lymphocytes were prepared by methods reported earlier [30,31]. The microsomal pellets were resuspended in microsomes dilution buffer containing 0.1 M potassium phosphate buffer, pH7.25, 20% (v/v)

glycerol, 0.25 mM PMSF, 0.01 M EDTA and 0.1 mM DTT and then stored at −70 °C till further analysis.

The activity of 7-ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-deethylase (MROD), marker enzymes of CYP1A1 and CYP1A2 respectively were determined in rat liver and blood lymphocytes by the modified method of Parmar et al. [30]. *In vitro* inhibition studies with polyclonal antibody specific for CYP1A1/1A2 and organic inhibitors of CYP1A1 and CYP1A2 were carried out as described earlier [30]. Protein content of the samples was estimated by the method of Lowry et al. [32] using bovine serum albumin as the reference standard.

2.8. Immunoblot analysis

CYP1A1/1A2 isoenzymes were identified by western blot analysis in liver and lymphocytes solubilized preparations isolated from control and treated animals as described earlier [30]. In brief, the membranes after transfers were incubated with primary antibody (1:500 dilutions) overnight at 32 °C. The membranes after washing were incubated with secondary antibody (alkaline phosphate conjugated goat anti-rabbit) at 1:10,000 dilutions for 30 min at room temperature. After washing, the color was developed by incubating the membrane with 5-bromo 4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The bands in the lanes containing liver samples were clearly visible within 2–3 min while the membranes were left in the color solution for almost 15–20 min to let the bands in the lanes containing lymphocytes samples become distinct. Densitometric analysis of the bands was carried out using Quantity One Quantitation Software version 4.3.1 (Bio-Rad, USA).

2.9. Statistical analysis

Students' *t*-test was employed to calculate the statistical significance between control and treated groups. $p < 0.05$ was considered to be significant when compared with the controls.

3. Results

Approximately (2–3) × 10^6 cells were present in 0.5 ml of rat blood lymphocyte suspension. The viability of these cells was above 95%.

3.1. mRNA expression of PAH-responsive CYPs in blood lymphocytes

The PCR amplification of cDNA prepared from RNA samples isolated from blood lymphocytes or liver of control or MC pretreated rats with the primers of GAPDH, resulted in the formation of PCR products of expected band size of 373 bp (Fig. 1). Densitometric analysis revealed almost equal intensity of the PCR product in the cDNAs samples isolated from control or treated animals (data not shown). Semi-quantitative PCR with primers specific for CYP1A1, 1A2, 1B1, AhR and Arnt resulted in the formation of PCR products of correct sizes of 341 bp (CYP1A1), 793 bp (CYP1A2), 312 bp (CYP1B1), 340 bp (AhR) and 413 bp (Arnt) respectively in cDNA prepared from RNA samples isolated from blood lymphocytes or liver of control and MC pretreated rats (Fig. 1). The mRNA expression of CYP1A1, 1A2, 1B1 and associated transcription factors was found to be several fold lower in freshly prepared control blood lymphocytes when compared to control liver. As observed in the liver, pretreatment of MC was found to increase the expression of these CYP isoenzymes, AhR and Arnt in freshly prepared blood lymphocytes (Fig. 1). Densitometric analysis (data not shown) revealed that the mRNA expression of CYP1A1, 1A2 and 1B1 was increased up to 3.5-, 2.0- and 3.0-fold respectively in blood lymphocytes isolated from MC pretreated rats as against several fold higher magnitude of induction observed in the liver. Likewise, mRNA expression of AhR and Arnt was also increased by approximately 1.5- and 1.0-fold in freshly prepared blood lymphocytes as against several fold higher expression observed in the liver.

Quantitative PCR (RT-PCR) was also carried to quantify the mRNA expression and inducibility of PAH-responsive CYPs in freshly prepared blood lymphocytes. Prior to absolute and relative quantification of CYP and associated transcription factors, each sample was normalized with housekeeping gene (GAPDH), which served as an endogenous control. The expression of GAPDH was found to be uniform in all the samples (control and treated) analyzed, confirming the integrity of RNA used in assays. qRT-PCR data revealed that the levels of CYP1A1, 1A2 and 1B1 were several fold lower in freshly prepared blood lymphocytes isolated from the control rats when compared to the liver enzymes (Table 2). The mRNA expression of CYP1A2 and 1B1 was found to be expressed at relatively higher levels in blood lymphocytes isolated from control rats when compared to CYP1A1. Likewise, as seen in the liver, marked differences were observed in the induction of these CYPs and associated transcription factors in blood lymphocytes isolated from MC pretreated animals (Tables 1 and 2). CYP1A1, 1A2 and 1B1 isoenzymes exhibited 8.0-, 3.0- and 6.0-fold increase in the mRNA in freshly prepared blood lymphocytes isolated from MC pretreated rats when compared to massive increase in the expression of these isoenzymes (CYP1A1-1226-fold, CYP1A2-54-fold, CYP1B1-779-fold) in the liver (Table 1). Likewise, 2.5- and 1.8-fold increase in the mRNA expression of AhR and Arnt was observed in freshly prepared blood lymphocytes as against 23- and 4.37-fold increase observed in the liver isolated from MC pretreated rats (Table 1).

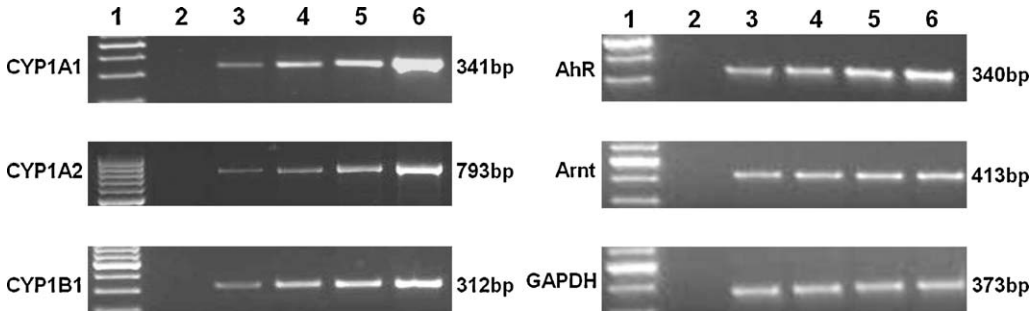

Fig. 1. Ethidium bromide-stained agarose gel in blood lymphocytes and liver of rats pretreated with MC. Lane 1 contains 100 bp ladder. Lane 2 contains RT-PCR product without RNA. Lanes 3 and 4 contain 5 μl of RT-PCR product of RNA isolated from lymphocytes of control and MC pretreated rats respectively. Lanes 5 and 6 contain 5 μl of RT-PCR product of RNA isolated from liver of control and MC pretreated rats respectively.

Table 1
RT-PCR of CYP1A, 1B1 and associated transcription factors in rat.

	Fold change			
	Lymphocytes		Liver	
	Control	MC	Control	MC
CYP1A1	1.0 ± 0.09	7.9 ± 0.59*	1.0 ± 0.11	1226.2 ± 85.84*
CYP1A2	1.0 ± 0.08	3.2 ± 0.37*	1.0 ± 0.09	54.2 ± 7.93*
CYP1B1	1.0 ± 0.11	6.1 ± 0.44*	1.0 ± 0.07	779.1 ± 47.11*
AhR	1.0 ± 0.07	2.5 ± 0.17*	1.0 ± 0.12	23.1 ± 3.75*
Arnt	1.0 ± 0.09	1.8 ± 0.13*	1.0 ± 0.10	4.4 ± 0.51*

Values represent mean ± S.E. of 3 experiments (each reaction was performed in triplicate).
The threshold cycle value (Ct values) of each sample was normalized with Ct values of endogenous control (GAPDH) [ΔCt].
Fold Change is calculated from ΔΔCt# value of each sample.
#ΔΔCt = ΔCt of treated − ΔCt of control.
* $p < 0.05$ when compared with the controls.

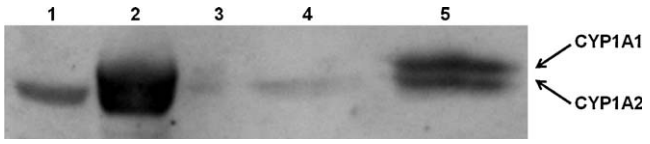

Fig. 2. Western blot analysis of CYP1A1/1A2 in blood lymphocytes and liver of rats pretreated with MC. Lanes 1 and 2 contain 12.5 μg of microsomal protein isolated from liver of control and MC pretreated rats respectively. Lane 3 is blank. Lanes 4 and 5 contain 400 μg of microsomal protein isolated from lymphocytes of control and MC pretreated rats respectively.

3.2. Western blotting studies

Western blotting studies with polyclonal antibody raised against rat liver CYP1A1/1A2 revealed significant immunoreactivity in liver microsomal proteins isolated from control rats. As compared to liver, very faint immunoreactivity was observed with anti-CYP1A1/1A2 in the lane containing freshly prepared blood lymphocytes protein isolated from control rats (Fig. 2). Pretreatment with MC resulted in massive increase in the immunoreactivity comigrating with rat liver CYP1A1 (57 kDa) and 1A2 (53 kDa). As observed with liver protein, pretreatment with MC resulted in several fold increase in immunoreactivity comigrating with rat liver CYP1A1(upper band) and 1A2 (lower band) in the lane containing microsomal proteins isolated from blood lymphocytes (Fig. 2).

3.3. Enzymatic studies

Freshly prepared uncultured and non-stimulated blood lymphocytes were found to catalyze the dealkylation of 7-ethoxyresorufin (ER) and 7-methoxyresorufin (MR). As reported earlier with ER [12], the rate of resorufin formation from ER or MR was linear up to 30 min at protein concentration of 25 μg in the presence of NADPH. No activity of EROD or MROD was observed in the lymphocytes when NADPH was substituted with NADH. Addition of SKF-525A to the complete system significantly inhibited the activity of EROD or MROD. Likewise, when the reaction mixture was saturated with carbon monoxide, significant inhibition was observed in the activity of EROD and MROD in the blood lymphocytes (data not shown).

Pretreatment with 3-methylcholanthrene (MC) or β-naphthoflavone (β-NF) resulted in a significant increase in the activity of EROD and MROD in the blood lymphocytes (2–4-fold), though much higher increase (10–15-fold) was observed in the liver (Table 3). In contrast, treatment with PB did not produce any significant change in the activity of EROD or MROD in freshly prepared blood lymphocytes and liver (Table 3). Lineweaver–Burk plot analysis showed that as observed with EROD in the present study and as reported earlier [12], the O-dealkylation of 7-methoxyresorufin exhibited a monophasic pattern of enzyme kinetics in blood lymphocytes. A significant increase in the affinity (apparent K_m) for the substrate (MR or ER) towards lymphocyte enzyme was observed in blood lymphocytes isolated from MC pretreated rats. This increase in the affinity of the substrates was associated with a significant increase in the apparent V_{max} in freshly prepared blood lymphocytes isolated from MC pretreated rats (Table 4).

3.4. In vitro inhibition studies

The effect of *in vitro* addition of organic inhibitors or polyclonal antibody specific for CYP1A1/1A2 in reaction mixture containing

Table 3
Effect of CYP inducers on the activity of hepatic and lymphocyte EROD and MROD.

	Lymphocytes		Liver	
	EROD	MROD	EROD	MROD
Control	0.9 ± 0.09	1.6 ± 0.09	40.3 ± 4.43	19.6 ± 2.06
MC	2.2 ± 0.11*	4.2 ± 0.20*	606.2 ± 11.71*	285.8 ± 7.11*
β-NF	1.4 ± 0.07*	3.9 ± 0.10*	454.3 ± 8.92*	210.1 ± 6.50*
PB	0.8 ± 0.06	1.4 ± 0.03	37.1 ± 8.07	18.7 ± 1.82

All the values are mean ± S.E. of 3 experiments.
The enzyme activity is expressed as pmoles resorufin/min/mg protein.
* $p < 0.05$ when compared to the controls.

Table 4
Apparent kinetic constants for rat blood lymphocyte EROD and MROD.

	K_m		V_{max}	
	EROD	MROD	EROD	MROD
Control	0.7 ± 0.04	2.3 ± 0.08	1.6 ± 0.09	8.0 ± 0.54
MC	0.5 ± 0.04*	1.6 ± 0.05*	4.4 ± 0.37*	13.8 ± 0.91*

Values represent data ± S.E. of 3 experiments.
K_m is expressed in μM and V_{max} in pmoles resorufin formed/min/mg protein.
* $p < 0.05$ when compared with the controls.

Table 2
Absolute quantification of CYP1A1, 1A2 and 1B1 mRNA by RT-PCR.

	Lymphocytes		Liver	
	Control	MC	Control	MC
	Number of mRNA molecules/μg of total RNA			
CYP1A1	$7.0 \times 10^3 \pm 0.91 \times 10^3$	$5.7 \times 10^4 \pm 4.02 \times 10^{3*}$	$3.3 \times 10^5 \pm 2.54 \times 10^4$	$4.4 \times 10^8 \pm 3.23 \times 10^{7*}$
CYP1A2	$8.2 \times 10^3 \pm 1.14 \times 10^3$	$2.9 \times 10^4 \pm 3.31 \times 10^{3*}$	$1.3 \times 10^7 \pm 9.22 \times 10^5$	$8.1 \times 10^8 \pm 4.90 \times 10^{7*}$
CYP1B1	$1.1 \times 10^4 \pm 1.45 \times 10^3$	$7.3 \times 10^4 \pm 4.70 \times 10^{3*}$	$7.0 \times 10^5 \pm 8.21 \times 10^4$	$6.3 \times 10^8 \pm 4.12 \times 10^{7*}$

Values represent mean ± S.E. of 3 experiments (each reaction was performed in triplicate).
Number of copies of mRNA was calculated by interpolation from the standard curve generated using known amount of full length cDNA clone of CYPs with slope ranges from −3.2 to −3.3 approximately and R^2 ranges ~0.98–0.99.
* $p < 0.05$ when compared with the controls.

Table 5
In vitro effect of organic inhibitors and anti-CYP1A1/1A2 on the activity of blood lymphocyte EROD and MROD.

		EROD		MROD	
		Control	MC	Control	MC
		#pmoles resorufin/min/mg protein			
None		0.9 ± 0.09# (100%)	2.3 ± 0.11# (100%)	1.6 ± 0.06# (100%)	4.2 ± 0.20# (100%)
Acetone		98.9%	93.6%	96.3%	99.3%
α-NF	1×10^{-5} M	87.9%	56.2%*	72.5%	54.0%*
	1×10^{-4} M	82.1%	45.9%*	67.5%	47.4%*
	1×10^{-3} M	75.0%*	29.2%*	59.3%*	26.9%*
SKF-525A	1×10^{-5} M	73.9%*	89.7%	69.4%*	80.4%
	1×10^{-4} M	71.3%*	71.7%*	62.5%*	69.8%*
	1×10^{-3} M	60.0%*	57.9%*	56.9%*	61.1%*
DMSO		99.5%	97.4%	98.1%	99.5%
Furafylline	1×10^{-5} M	85.0%	78.9%	51.3%*	44.6%
	1×10^{-4} M	77.9%	70.8%*	46.9%*	31.6%*
	1×10^{-3} M	72.9%*	66.9%*	30.6%*	18.6%*
Pre-immune IgG	0.3 mg	99.7%	100.0%	91.9%	96.9%
	0.8 mg	99.3%	96.1%	60.0%	48.6%
Anti-CYP1A1/1A2	0.3 mg	87.1%	47.6%*	90.6%	96.0%*
	0.8 mg	72.9%*	29.2%*	45.6%*	14.4%*

Values represent data ± S.E. of 3 experiments.
Values with inhibitor and vehicles represent % activity remaining after assay with inhibitor and respective vehicles.
* $p < 0.05$ when compared with the controls.

blood lymphocytes isolated from control or MC pretreated rats are summarized in Table 5. *In vitro* addition of acetone or DMSO, the solvents used as vehicles to dissolve α-naphthoflavone (α-NF) and furafylline respectively, to the blood lymphocytes isolated from control or MC pretreated rats, did not produce any effect on the activity of EROD or MROD. *In vitro* addition of SKF-525A, an inhibitor of CYP catalyzed reactions, to the reaction mixture containing blood lymphocytes isolated from control rats produced a concentration dependent decrease in the activity of EROD and MROD, particularly at higher concentrations. In contrast, *in vitro* addition of α-NF, a inhibitor of CYP1A, particularly CYP1A1 catalyzed reactions to the blood lymphocytes isolated from control rats produced a small decrease in the activity of EROD or MROD, though significant only at the highest concentration while addition of furafylline, an inhibitor specific for hepatic CYP1A2 catalyzed reactions, produced a small but significant concentration dependent decrease in the activity of MROD at all the concentrations. A decrease in the activity of EROD was also observed on *in vitro* addition of furafylline, particularly at higher concentrations (Table 5).

In contrast to that seen with control blood lymphocytes, *in vitro* addition of α-NF to blood lymphocytes isolated from MC pretreated rats, produced a significant concentration dependent inhibition in the activity of EROD or MROD, though relatively higher magnitude of inhibition was observed in the activity of EROD (Table 5). Likewise, *in vitro* addition of different concentrations of furafylline produced significant concentration dependent inhibition in the activity of EROD or MROD, though a much greater magnitude of inhibition (up to 80%) was observed in the activity of MROD. However the magnitude of inhibition of the activity of EROD or MROD on *in vitro* addition of SKF-525A to the lymphocytes isolated from MC treated rats was almost similar to that seen with control blood lymphocytes (Table 5).

Likewise, *in vitro* addition of polyclonal antibody raised against rat liver CYP1A1/1A2 to the reaction mixture containing blood lymphocytes isolated from MC pretreated rats produced a concentration dependent inhibition of the activity of EROD and MROD. In contrast, a decrease, though much smaller in magnitude, was observed in the activity of EROD and MROD on *in vitro* addition of polyclonal antibody raised against rat liver CYP1A1/1A2 to blood lymphocytes isolated from control rats (Table 5). Pre-immune IgG did not produce any effect on the activity of EROD or MROD, when added *in vitro* to reaction mixtures containing blood lymphocytes obtained either from control or MC pretreated rats (Table 5).

4. Discussion

Our data demonstrated that PAH-metabolizing CYP1A1, 1A2, 1B1 and the associated transcription factors, AhR and Arnt are expressed in freshly prepared uncultured and non-stimulated blood lymphocytes isolated from control rats. CYP1B1, an extrahepatic CYP were found to be expressed at much higher levels in freshly prepared blood lymphocytes when compared to CYP1A1 and CYP1A2. CYP1B1 has been earlier reported to be the major CYP isoenzyme expressed in human blood lymphocytes and monocytes [33,34]. Though most of the earlier studies were performed with cultured blood lymphocytes, CYP1A1 mRNA expression was reported to be almost undetectable in blood lymphocytes. Using sensitive and quantitative reverse transcriptase-PCR, Vanden Heuvel et al. [35] reported reproducible CYP1A1 mRNA expression in uncultured and non-stimulated human blood lymphocytes at levels that were 10–40-fold lower than in cultured and mitogen stimulated lymphocytes.

Significant increase in the mRNA expression of CYP1A1, 1A2, 1B1 and associated transcription factors in freshly prepared blood lymphocytes isolated after 3-MC treatment have demonstrated that responsiveness to PAHs is retained in blood lymphocytes. Similar to that seen in the liver, maximum induction was observed in CYP1A1 mRNA (8-fold) followed by CYP1B1 (6.5-fold) and CYP1A2 (3.5-fold). Previous studies from our laboratory have also shown similarities in the induction profile of CYP1A1, 2E1 and 3A mRNA in freshly prepared blood lymphocytes with the liver enzymes [12,36,37]. Likewise, though significant constitutive and inducible variation in CYP1A1 and 1B1 expression is reported in cultured and mitogen stimulated human blood lymphocytes, enhanced responsiveness of these CYPs towards PAHs was also reported in uncultured lymphocytes isolated from dioxin exposed individuals [34]. It has been suggested that this significant increase

in the CYP1A1 and 1B1 mRNA expression in uncultured blood lymphocytes, which are characterized by low basal expression, may reflect exposure to PAHs as seen in the smokers [34,35,38]. Significant increase in the mRNA expression of CYP1A2 in blood lymphocytes has further shown similarities in the responsiveness of blood lymphocyte CYP1A isoenzymes with the tissue enzymes. Though not much information is available on the expression and inducibility of CYP1A2 in rodent blood lymphocytes, CYP1A2 mRNA has been reported to be expressed in uncultured human blood lymphocytes and its expression was found to be increased in patients suffering from liver cancer and other liver diseases [13,39,40].

The expression of AhR and Arnt in freshly prepared uncultured blood lymphocytes isolated from control rats has provided evidence for similarities in the mechanism regulating PAH-responsive CYP1A1, 1A2 and 1B1 genes in blood lymphocytes with the tissue enzyme. The AhR and Arnt mRNA expression has also been reported earlier in human blood lymphocytes [7,41]. It has been hypothesized that the expression levels of AhR and Arnt contribute to the differences in CYP1A1 and 1B1 inducibility in humans [7,41]. While AhR expression correlated with CYP1A1 inducibility in cultured and mitogen stimulated human lymphocytes and cigarette smoking interfered with the mechanism of AhR-mediated CYP1A1 induction, AhR expression in blood lymphocytes correlated with constitutive CYP1B1 expression but not CYP1B1 inducibility [41]. It has been further suggested that mechanisms, in addition to AhR affinity, that regulate the potency of AhR-dependent gene expression, may exist particularly in the blood lymphocytes [11,42].

Western blotting studies have shown that increased transcription of CYP1A1 and 1A2 mRNA observed in freshly prepared blood lymphocytes following pretreatment of MC leads to increased CYP1A1 and 1A2 protein. Immunoreactivity corresponding to CYP1A1 has been shown earlier with freshly prepared blood lymphocytes isolated from rats pretreated with prototypic inducers (MC, β-NF, cigarette smoke and pyridine) as well as non-prototypic inducer such as dexamethasone while protein expression of CYP1A2 was not observed in either control or induced rats [11,12]. CYP1A1 protein was also found to be expressed in human blood lymphocytes. Smokers were found to have slightly higher levels of CYP1A1 protein when compared to non-smokers while no protein expression of CYP1A2 was observed in the blood lymphocytes of volunteers before or after treatment of omeprazole, a gastric anti-ulcer drug, which induces CYP1A-genes in different human tissues [43]. The differences in the methodology for e.g. solubilization of lymphocyte proteins, the sensitivity of antibody used etc. may account for this disparity in the identification of CYP1A2 protein in freshly prepared blood lymphocytes. The inability to detect CYP1B1 could be due to the very low level expression of CYP1B1 protein in normal tissues suggesting that either CYP1B1 protein is present at very low levels or CYP1B1 mRNA is not translated in normal tissue [44–46]. Tsuchiya et al. [47] have recently shown the CYP1B1 expression is post-transcriptionally regulated by miR-27b. Expression of miR-27b was found to be down-regulated in cancerous tissue when compared with non-cancerous tissue and this down regulation could be one of the causes of higher level of CYP1B1 protein in cancerous tissue.

Further evidence that PAH-responsive CYPs expressed in freshly isolated blood lymphocytes are catalytically active was provided by the present study demonstrating O-dealkylation of 7-ethoxyresorufin (ER) and 7-methoxyresorufin (MR) in freshly prepared blood lymphocytes. Dey et al. [12], have earlier shown that blood lymphocytes catalyze the O-dealkylation of 7-ER, a reaction primarily mediated by CYP1A1 in liver, particularly in MC induced animals, though CYP1A2 and 1B1 are also known to catalyse the activity of EROD [48,49]. The present data indicating the inability of NADH to catalyze the dealkylation of 7-MR and significant inhibition of the enzyme activity by SKF-525A and induction by MC have suggested that like 7-ethoxyresorufin-O-dealkylase (EROD), the activity of 7-methoxyresorufin-O-dealkylase (MROD) is catalyzed by CYPs in blood lymphocytes. As reported earlier with ER [12], a significant increase in the affinity of MR towards the lymphocytes enzyme following pretreatment of rats with MC was associated with an increase in the apparent V_{max} suggesting that like in the liver, exposure of PAHs leads to enrichment of CYP1A1 and 1A2 isoenzymes respectively in blood lymphocytes which catalyze the dealkylation of ER and MR. *In vitro* studies using specific inhibitors for CYP1A1(α-napthoflavone) and 1A2 (furfurayline) catalyzed reactions have further shown that the activity of EROD and MROD is also catalyzed by CYP1A1 and 1A2 isoenzymes in blood lymphocytes.

In conclusion, the present study has provided evidence that PAH-metabolizing CYP1A1, 1A2, 1B1 and their associated transcription factors, AhR and Arnt are expressed in freshly prepared uncultured and non-mitogen stimulated blood lymphocytes. Significant increase in the expression and catalytic activity of these CYPs, though several fold less when compared to liver, following pretreatment with MC has demonstrated that responsiveness of CYPs is retained in freshly prepared blood lymphocytes. Likewise, significant increase in the mRNA expression of AhR and Arnt in blood lymphocytes and similarities in the regulation of blood lymphocyte EROD and MROD with the liver enzymes following pretreatment have further shown that mechanisms similar to that observed in the tissue exist in blood lymphocytes. Our data thus demonstrates that though the expression and responsiveness of PAH-inducible CYPs is several fold lower in freshly prepared blood lymphocytes when compared to liver, similarities in their regulation with the liver enzyme have suggested that the expression profile of these CYPs in uncultured and non-mitogen stimulated blood lymphocytes may be used as a biomarker to predict exposure of environmental carcinogens.

Conflict of interest

The authors declare no conflicts of interest.

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References

[1] Guengerich FP. Metabolic activation of carcinogens. Pharmacol Ther 1992;54:17–61.
[2] Bartsch H, Nair U, Risch A, Rojas M, Wikman H, Alexandrov K. Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. Cancer Epidemiol Biomarkers Prev 2000;9:3–28.
[3] Nebert DW. Role of genetics and drug metabolism in human cancer risk. Mutat Res 1991;247:267–81.
[4] Whitlock Jr JP. Induction of cytochrome P4501A1. Annu Rev Pharmacol Toxicol 1999;39:103–25.
[5] Fujii-Kuriyama Y, Mimura J. Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. Biochem Biophys Res Commun 2005;338:311–7.

[6] Kellermann G, Luyten-Kellermann M, Shaw CR. Genetic variation of aryl hydrocarbon hydroxylase in human lymphocytes. Am J Hum Genet 1973; 25:327–31.
[7] Hayashi S, Watanabe J, Nakachi K, Eguchi H, Gotoh O, Kawajiri K. Interindividual difference in expression of human Ah receptor and related P450 genes. Carcinogenesis 1994;15:801–6.
[8] Schmidt JV, Bradfield CA. Ah receptor signaling pathways. Annu Rev Cell Dev Biol 1996;12:55–89.
[9] Smart J, Daly AK. Variation in induced CYP1A1 levels: relationship to CYP1A1, Ah receptor and GSTM1 polymorphisms. Pharmacogenetics 2000;10:11–24.
[10] Nebert DW. The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. Crit Rev Toxicol 1989;20:153–74.
[11] Fung J, Thomas PE, Iba MM. Cytochrome P450 1A1 in rat peripheral blood lymphocytes: inducibility in vivo and bioactivation of benzo[a]pyrene in the Salmonella typhimurium mutagenicity assay in vitro. Mutat Res 1999;438: 1–12.
[12] Dey A, Parmar D, Dayal M, Dhawan A, Seth PK. Cytochrome P450 1A1 (CYP1A1) in blood lymphocytes evidence for catalytic activity and mRNA expression. Life Sci 2001;69:383–93.
[13] Furukawa M, Nishimura M, Ogino D, Chiba R, Ikai I, Ueda N, et al. Cytochrome p450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. Cancer Sci 2004;95:520–9.
[14] Jacquet M, Lambert V, Todaro A, Kremers P. Mitogen-activated lymphocytes: a good model for characterising lung CYP1A1 inducibility. Eur J Epidemiol 1997;13:177–83.
[15] Whitlock Jr JP, Cooper HL, Gelboin VH. Aryl hydrocarbon (benzopyrene) hydroxylase is stimulated in human lymphocytes by mitogens and benz(a)anthracene. Science 1972;177:618–9.
[16] Baum A, Gatchel RJ, Krantz DS. An Introduction To Health Psychology, 3rd ed., New York: McGraw-Hill; 1997.
[17] Kouri RE, Imblum RL, Sosnowski RG, Slomiany DJ, McKinney CE. Parameters influencing quantitation of 3-methylcholanthrene-induced aryl hydrocarbon hydroxylase activity in cultured human lymphocytes. J Environ Pathol Toxicol 1979;2:1079–98.
[18] Siest G, Jeannesson E, Marteau JB, Samara A, Marie B, Pfister M, et al. Transcription factor and drug-metabolizing enzyme gene expression in lymphocytes from healthy human subjects. Drug Metab Dispos 2008;36:182–9.
[19] Johri A, Dhawan A, Lakhan Singh R, Parmar D. Effect of prenatal exposure of deltamethrin on the ontogeny of xenobiotic metabolizing cytochrome P450 s in the brain and liver of offsprings. Toxicol Appl Pharmacol 2006;214:279–89.
[20] Huang P, Rannug A, Ahlbom E, Håkansson H, Ceccatelli S. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the expression of cytochrome P450 1A1, the aryl hydrocarbon receptor, and the aryl hydrocarbon receptor nuclear translocator in rat brain and pituitary. Toxicol Appl Pharmacol 2000;169:159–67.
[21] Schilter B, Omiecinski CJ. Regional distribution and expression modulation of cytochrome P-450 and epoxide hydrolase mRNAs in the rat brain. Mol Pharmacol 1993;44:990–6.
[22] Piscaglia F, Knittel T, Kobold D, Barnikol-Watanabe S, Di Rocco P, Ramadori G. Cellular localization of hepatic cytochrome 1B1 expression and its regulation by aromatic hydrocarbons and inflammatory cytokines. Biochem Pharmacol 1999;58:157–65.
[23] Timsit YE, Chia FS, Bhathena A, Riddick DS. Aromatic hydrocarbon receptor expression and function in liver of hypophysectomized male rats. Toxicol Appl Pharmacol 2002;185:136–45.
[24] Pustylnyak VO, Gulyaeva LF, Lyakhovich VV. CAR expression and inducibility of CYP2B genes in liver of rats treated with PB-like inducers. Toxicology 2005;216:147–53.
[25] Shah PP, Saurabh K, Pant MC, Mathur N, Parmar D. Evidence for increased cytochrome P450 1A1 expression in blood lymphocytes of lung cancer patients. Mutat Res 2009;670:74–8.
[26] Baldwin SJ, Bramhall JL, Ashby CA, Yue L, Murdock PR, Hood SR, et al. Cytochrome P450 gene induction in rats ex vivo assessed by quantitative real-time reverse transcriptase-polymerase chain reaction (TaqMan). Drug Metab Dispos 2006;34:1063–9.
[27] Vondrácek J, Svihálková-Sindlerová L, Pencíková K, Krcmár P, Andrysík Z, Chramostová K, et al. 7H-Dibenzo[c,g]carbazole and 5,9-dimethyldibenzo[c,g]carbazole exert multiple toxic events contributing to tumor promotion in rat liver epithelial 'stem-like' cells. Mutat Res 2006;596:43–56.
[28] Shirota M, Mukai M, Sakurada Y, Doyama A, Inoue K, Haishima A, et al. Effects of vertically transferred 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) on the reproductive development of female rats. J Reprod Dev 2006;52:751–61.
[29] Qin G, Meng Z. The expressions of protooncogenes and CYP1A in lungs of rats exposed to sulfur dioxide and benzo(a)pyrene. Regul Toxicol Pharmacol 2006;45:36–43.
[30] Parmar D, Dhawan A, Dayal M, Seth PK. Immunochemical and biochemical evidence for expression of Phenobarbital and 3-methylcholanthrene-inducible isoenzymes of cytochrome P450 in rat brain. Int J Toxicol 1998;17:619–30.
[31] Hannon-Fletcher MP, Barnett YA. Lymphocyte cytochrome P450 expression: inducibility studies in male Wistar rats. Br J Biomed Sci 2008;65:1–6.
[32] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[33] Baron JM, Zwadlo-Klarwasser G, Jugert F, Hamann W, Rübben A, Mukhtar H, et al. Cytochrome P450 1B1: a major P450 isoenzyme in human blood monocytes and macrophage subsets. Biochem Pharmacol 1998;56:1105–10.
[34] Spencer DL, Masten SA, Lanier KM, Yang X, Grassman JA, Miller CR, et al. Quantitative analysis of constitutive and 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced cytochrome P450 1B1 expression in human lymphocytes. Cancer Epidemiol Biomarkers Prev 1999;8:139–46.
[35] Vanden Heuvel JP, Clark GC, Thompson CL, McCoy Z, Miller CR, Lucier GW, et al. CYP1A1 mRNA levels as a human exposure biomarker: use of quantitative polymerase chain reaction to measure CYP1A1 expression in human peripheral blood lymphocytes. Carcinogenesis 1993;14:2003–6.
[36] Dey A, Dhawan A, Kishore Seth P, Parmar D. Evidence for cytochrome P450 2E1 catalytic activity and expression in rat blood lymphocytes. Life Sci 2005; 77:1082–93.
[37] Dey A, Yadav S, Dhawan A, Seth PK, Parmar D. Evidence for cytochrome P450 3A expression and catalytic activity in rat blood lymphocytes. Life Sci 2006;79:1729–35.
[38] Cosma GN, Toniolo P, Currie D, Pasternack BS, Garte SJ. Expression of the CYP1A1 gene in peripheral lymphocytes as a marker of exposure to creosote in railroad workers. Cancer Epidemiol Biomarkers Prev 1992;1:137–42.
[39] Finnström N, Thörn M, Lööf L, Rane A. Independent patterns of cytochrome P450 gene expression in liver and blood in patients with suspected liver disease. Eur J Clin Pharmacol 2001;57:403–9.
[40] Haas CE, Brazeau D, Cloen D, Booker BM, Frerichs V, Zaranek C, et al. Cytochrome P450 mRNA expression in peripheral blood lymphocytes as a predictor of enzyme induction. Eur J Clin Pharmacol 2005;61:583–93.
[41] Lin P, Hu SW, Chang TH. Correlation between gene expression of aryl hydrocarbon receptor (AhR), hydrocarbon receptor nuclear translocator (Arnt), cytochromes P4501A1 (CYP1A1) and 1B1 (CYP1B1), and inducibility of CYP1A1 and CYP1B1 in human lymphocytes. Toxicol Sci 2003;71:20–6.
[42] Nohara K, Ao K, Miyamoto Y, Ito T, Suzuki T, Toyoshiba H, et al. Comparison of the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1A1 gene expression profile in lymphocytes from mice, rats, and humans: most potent induction in humans. Toxicology 2006;225:204–13.
[43] Spatzenegger M, Horsmans Y, Verbeeck RK. CYP1A1 but not CYP1A2 proteins are expressed in human lymphocytes. Pharmacol Toxicol 2000;86:242–4.
[44] Murray GI, Taylor MC, McFadyen MC, McKay JA, Greenlee WF, Burke MD, et al. Tumor-specific expression of cytochrome P450 CYP1B1. Cancer Res 1997; 57:3026–31.
[45] McFadyen MC, Breeman S, Payne S, Stirk C, Miller ID, Melvin WT, et al. Immunohistochemical localization of cytochrome P450 CYP1B1 in breast cancer with monoclonal antibodies specific for CYP1B1. J Histochem Cytochem 1999;47:1457–64.
[46] Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP, et al. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. Cancer Res 1996;56:2979–84.
[47] Tsuchiya Y, Nakajima M, Takagi S, Taniya T, Yokoi T. MicroRNA regulates the expression of human cytochrome P450 1B1. Cancer Res 2006;66:9090–8.
[48] Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, et al. Inhibition of cytochromes P4501A by nitric oxide. Proc Natl Acad Sci USA 1994;91:3559–63.
[49] Murray GI, Melvin WT, Greenlee WF, Burke MD. Regulation, function, and tissue-specific expression of cytochrome P450 CYP1B1. Annu Rev Pharmacol Toxicol 2001;41:297–316.