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Measurement of immunoreactive cytochrome P450 2E1 in human leucocytes

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We describe initial results on a Western blotting method, using a ploy clonal antibody and chemiluminescence detection, for the measurement of cytochrome P450 2E1 in human lymphocytes. The method has been used to study the levels of 2E1 in lymphocytes isolated from 5 ml blood samples collected from a small group of well-controlled type 1 diabetics and healthy individuals. The described method offers increased sensitivity compared with a previously published method and does not need *in vitro* culturing of the lymphocytes prior to 2E1 measurement. The apparent molecular weight of the lymphocyte P450 2E1 was 55 kDa. There was approximately a six-fold difference in expression levels of 2E1 detected by this immunochemical technique across the study population.

Keywords: cytochrome P450, CYP2E1, leucocytes, metabolism.

Introduction

Microsomal cytochromes P450 are terminal monooxygenases that are involved in the biotransformation of a variety of unrelated compounds. Their substrates include exogenous compounds such as drugs, carcinogens and pesticides as well as endogenous molecules such as steroids and prostaglandins. Typical characterstics of P450s are that they exist in multiple forms with overlapping substrate specificity, and they are often induced by their own substrates. The results of P450-dependent reactions may potentially lead to more toxic metabolites, which exert their effects by interaction with biological macromolecules including proteins, DNA and RNA. Alternatively, the metabolites are less toxic, easily excretable compounds after conjugation with glucuronide or sulphate. With the exception of liver, characterization of P450s in human tissues has been historically hindered by their low concentration, catalytic instability and limited access to extra-hepatic human tissue. A recent study (Raunio *et al.* 1998) used both reverse transcriptase-polymerase chain reaction (RT-PCR) and immunochemical methods to characterize P450 expresssion in the human lung, alveolar macrophages and blood lymphocytes. Lung tissue expressed a wide range of P450s, whilst in blood lymphocytes only 2E1 and 1B1 were consistently expressed.

The cytochrome P450 2E1 (CYP P450 2E1) subfamily is toxicologically a very important metabolic system. CYP P450 2E1 is inducible by its substrates and catalyses the metabolism of numerous low molecular weight agents encountered occupationally and environmentally, including acetaldehyde, ethanol, various ketones, aromatic hydrocarbons and halogenated chemicals. 2E1 can catalyse chemicals to metabolites that are more chemically reactive than the parent compound.

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There is evidence of interindividual variation in hepatic expression of this enzyme in human liver samples (Shimada *et al.* 1994b), an observation confirmed by Nedelcheva *et al.* (1995) who reported a four-fold variability in the 2E1 content of liver microsomes prepared from 13 individuals. Chlorzoxazone, a centrally acting muscle relaxant, has been reported to be specifically hydroxylated *in vitro* by human 2E1 (Peter *et al.* 1990), and thus 6-hydroxylation of this compound is often used as a probe of the inter-individual differences *in vivo* metabolic potential of human 2E1. However, there are concerns about both the specificity of chlorzoxazone 6-hydroxylation in reflecting only CYP 2E1 activity as compared with other P450 activities, such as the CYP 3A subgroup family (Gorski *et al.* 1997) and CYP 1A1 (Carriere *et al.* 1996). Also the possible risk associated with using such invasive probe drugs has been raised (FDA 1996).

The extra-hepatic expression of 2E1 has been reported in human lymphocytes (Song *et al.* 1990, Raunio *et al.* 1998) and in various hamster tissues (Ueng *et al.* 1993). Ueng *et al.* (1993) showed that a protein which is cross-reactive with a rabbit antibody raised against human 2E1 is ethanol-inducible in hamster liver, kidney and lung but not intestine. If the extra-hepatic induction of 2E1 also occurs in human lymphocytes and parallels liver 2E1 levels, then monitoring lymphocyte 2E1 expression in a blood sample could be a useful, readily available biomarker. It could be used as an effect-biomarker of occupational exposure to the many xenobiotics metabolized by 2E1. A similar approach has been used in the study of urinary 6-β hydroxy cortisol excretion in solvent-exposed workers as an index of P450 3A-induction, the predominant liver P450 (Mason *et al.* 1994). Lymphocyte 2E1 levels may also be used as an explanatory variable in the study of inter-individual variation found in monitoring exposure by measurement of the parent chemical or its metabolites in body fluids. Recently a correlation has been reported for alcohol-abusers between metabolism of an *in vivo* dose of chlorzoxazone and lymphocyte 2E1 (molecular weight 50.5 kDa) measured by immunoblot (Raucy *et al.* 1997). However, this immunoblot method needed large volumes of blood. Using a Western blotting approach, Song *et al.* (1990) measured 2E1 in lymphocytes prepared from poorly controlled type I diabetics and showed that there was a positive correlation between induced CYP2E1 with HbA1, a well established metabolic marker of diabetic control. This assay did not have the required sensitivity to measure CYP2E1 in lymphocytes prepared from normal individuals and needed *in vitro* culture of lymphocytes prior to immunochemical detection. The molecular weight was reported to be 48 kDa (Song *et al.* 1990). If the sensitivity of such assays could be increased to allow the measurement of 2E1 in the lymphocytes of normal individuals, this approach may be applicable to measuring 2E1 induction following occupational exposure to various xenobiotics which are metabolized by this P450.

This report describes a method for the measurement of 2E1 in the lymphocytes of normal individuals as well as well-controlled type 1 diabetics.

Methods

The study population comprised five well controlled type I diabetics with Hb A1c values in the range 6.0–8.9 %; mean = 7.6 % (normal range < 7.8 %) and six volunteers from the laboratory. The diabetics (mean age 28, range = 24–30 years) were younger than the laboratory volunteers (mean 34, range = 28–45 years); 3/5 and 3/6 of the diabetic and laboratory staff were female. Blood samples (5 ml) were collected into bottles containing potassium EDTA. Blood samples were centrifuged at 100 *g* for

10 min, the platelet-rich plasma was then removed, taking care not remove any of the buffy white cell layer, and replaced with an equal volume of isotonic saline. Lymphocytes were prepared from this plasma-depleted blood sample using Lymphoprep preparation media, density 1.077 g ml^{-1} (Nycomed), and Accuspin separation tubes (Sigma Diagnostics). After centifugation of the Accuspin tube for 15 min at 1000 *g* , the lymphocyte cell layer was removed, washed by centrifugation for 10 min at 800 *g* and resuspended in 1 ml of isotonic saline. The lymphocyte concentration was counted using a COBAS MINOS clinical haematology analyser.

A known concentration of the lymphocytes was centrifuged, the supernatent removed and the pellet resuspended in double strength Laemelli sample buffer (Laemelli 1970) to give a concentration of approximately 500 000 cells (equivalent to approximately 100–200 µl of whole blood) per 30 µl of buffer. Samples were vortexed and could then be stored at –700 ºC before analysis. Solubilization of the cells prior to electrophoresis was effected by heating in a boiling water bath for 5 min. Purified human liver microsomes were used as a comparative standard in this study and were a generous gift from the Department of Medicine and Pharmacology, University of Sheffield. They were prepared as described by Otton *et al.* (1988) and prior to analysis were diluted in double strength Laemelli sample buffer (Laemelli 1970) and heated in a boiling water bath for 5 min. Each gel used at least one track which contained 6.5 µg of liver microsomal protein as standard.

Electrophoresis was performed essentially as described by Laemelli on 10 % T (2.6 %C) slab gels incorporating a 5.3 % T (2.6 %C) stacking gel. For each individual, the protein extract prepared from 500 000 lymphocytes was analysed (approximately 30 µl of sample). Biotinylated molecular weight markers (Sigma) were run on each gel to facilitate molecular weight estimation. The remainder of the experimental procedure was as described by Scobbie *et al.* (1994). Essentially, gels were run at a constant current of 35mA per gel until the pyronin Y tracking dye reached the bottom of the gel. Cooling to approximately 15 ºC was incorporated throughout. The separated protein patterns were transferred electrophoretically to nitrocellulose and probed using a polyclonal antibody and chemiluminescence detection.

The antibody used in these studies was a polyclonal anti-human 2E1 raised in rabbit, and was generous gift from Professor F.P. Geungerich, Vanderbuilt University, Nashville. Blocking of the unreacted groups on the nitrocellulose filter and all antibody incubations were performed using PBS pH 7.2/3 % dried fat milk/0.1 % Tween 20. Washing steps were performed using PBS pH 7.2/0.1 % Tween 20. The anti-2E1 antibody was used at a dilution of 1/1250 and the second antibody (peroxidase conjugated swine anti rabbit (Dako)) was used at a dilution of 1/10 000. Control incubations were performed by replacing the anti-CYP2E1 antibody with normal rabbit serum (Sigma). Avidin-peroxidase was used to visualize biotinylated molecular weight markers, but using a buffer system without dried fat milk. Filters were processed and visualized using enhanced chemiluminescence (ECL) (Amersham) as described by our laboratory (Scobbie *et al.* 1994). Exposure times were of the order of 6–10 min. Densitometry, by integration of optical density associated with a band, and molecular weight analysis of the exposed ECL films was performed using a Millipore Bioimage System.

Results and discussion

Using this method CYP2E1 was detected in all of the lymphocyte preparations analysed ($n = 11$). Figure 1 shows a typical example of an ECL film obtained from the measurement of CYP2E1 in lymphocyte extracts. The apparent molecular weight of the CYP2E1 band in the liver microsomes (lane 1) was 53 500 daltons, which agrees well with a previously published values of 51 500 daltons (Shimada *et al.* 1994b), and 54 000 daltons (Song *et al.* 1990, Raucy *et al.* 1997). The apparent molecular weight of the CYP2E1 band in the lymphocyte extracts was 55 000 compared with a previously published value of 48 000 (Song *et al.* 1990). Raucy *et al.* (1997) reported a molecular weight for lymphoctye 2E1 of 3500 daltons less than that found in human liver microsomes. This contrasts with our findings of an apparently slightly higher molecular weight in the lymphocyte enzyme (figure 2). Figure 1 also shows that the amount of CYP2E1 measured in half a million lymphocytes prepared from each member of the study population was different, with an approximate 6.5-fold difference in expression across the study population. The levels of lymphocyte 2E1 in well-controlled diabetics were not significantly different from the laboratory volunteers (figure 3). The difference in expression of CYP2E1 by the various members of the study population is not unexpected.

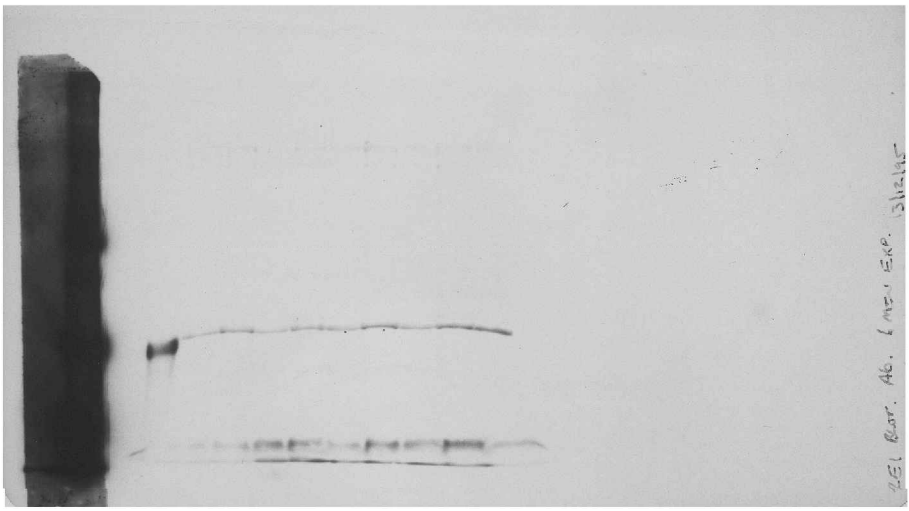

Figure 1. Gel lane1; 6.5 μg microsomal human liver extract, lanes 2–10; lymphocyte preparations from 500 000 cells per blood sample. Lanes are numbered from left to right.

CYP2E1 is regulated at many different cellular levels and inducers affect gene transcription, mRNA stabilization, translation and protein degradation. Ethanol in particular is involved in stabilization of the CYP2E1 gene product (Rannug *et al.* 1995), hence increased ethanol intake may be associated with increased CYP2E1 expression *in vivo* (Raucy *et al.* 1997). The ethanol intake of the study population was not established, hence it was not possible correlate CYP2E1 expression with ethanol consumption. A four-fold inter-individual variability of CYP2E1 expression in human liver microsomes has previously reported by Nedelcheva *et al.* (1995), a value which agrees well with the inter-individual variability reported in present study.

Figure 2 shows a gel with the molecular weight markers where the time of ECL development has been used to explore minor bands in the gel. The antisera staining in the lymphocytes corresponded to a minor band found in human liver microsomal liver preparation just above the major band which comprised 74 % of the signal at 53 500 daltons; two well defined bands at 48 400 daltons and 47 300 daltons, not showing in lymphocytes, comprised 25 % of the signal in the liver microsomal extract. The specificity of the assay largely depends on the specificity of the antisera used. Blank gels run using normal rabbit sera instead of primary anti-2E1 antibody did not give any response in the molecular weight area of interest. The antisera was noted to have some small cross-reactivity to P450 2A6 (personal communication, F.P. Guengerich). However, it has been reported that, unlike human liver, expression of P450 2A6 is not found in human lymphocytes and P450 expression in these peripheral cells is restricted to 2E1 and 1B1 (Raunio *et al.* 1998). Therefore we are confident that the assay is measuring 2E1 in lymphocytes and that the minor bands in liver microsomes at approximately 48 kDa, which are well resolved from the major signal at the higher molecular weight, are likely to be from the 2A family. The molecular weights of 2E1 and 2A6 in human liver microsomes have been reported to be 51.2 and 49.5 kDa respectively (Shimada *et al.* 1994a).

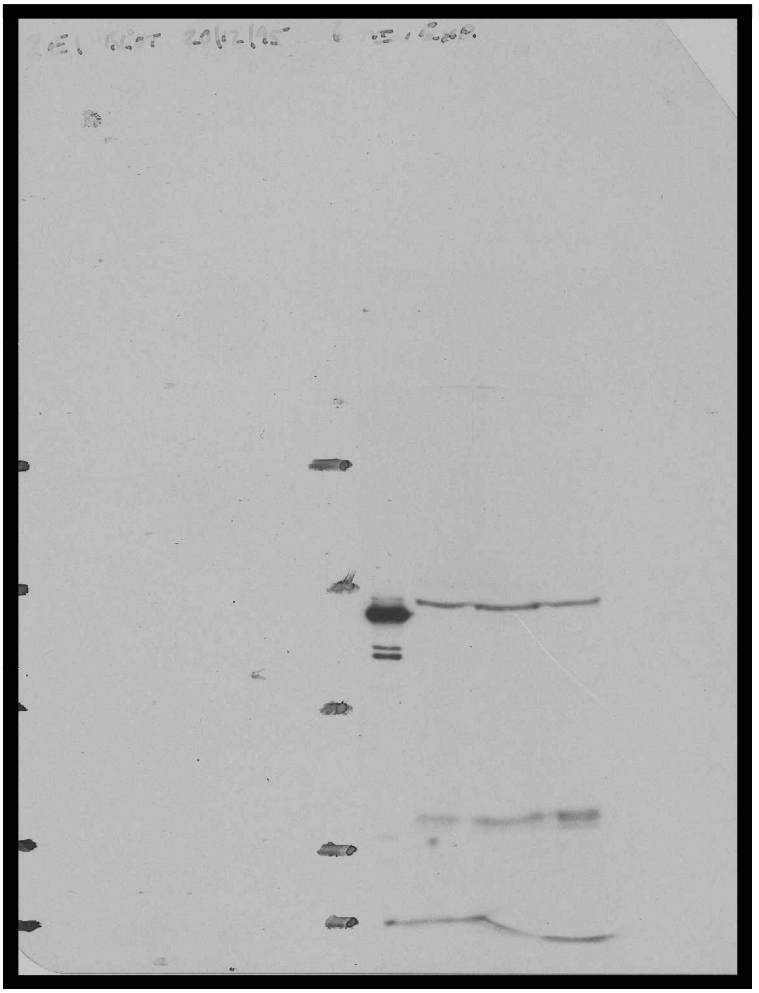

Figure 2. Gel with molecular weight markers bands on left hand side, transcribed from blot not using dried fat milk containing buffers. From top to bottom molecular weights are 97.4 kDa (phosphorylase b), 58.1 kDa (catalase), 39.8 kDa (alcohol dehydrogenase), 29 kDa (carbonic anhydrase) and 20.1 kDa (trypsin inhibitor) daltons. From the left the sample lanes are liver microsomal extract and then three lymphocyte preparations.

When compared with the previously published methods for the measurement of CYP2E1 in human lymphocytes (Song *et al.* 1990, Raucy *et al.* 1997) the present method has several improvements. Using the original Song method, measurement of CYP2E1 in lymphocytes was only possible following the *in vitro* culture of the isolated lymphocytes for 4 days prior to immunochemical assay for CYP2E1. In this form their assay could only measure CYP2E1 in the lymphocytes of poorly controlled diabetics (mean HbA1c value = 12.1 %) and not in normal individuals. Raucy (1997) proved a significant relationship between chlorzoxazone hydroxylation and lymphocyte 2E1 levels but used 320 ml of blood for their 2E1 measurement in lymphocytes. The assay described in this report does not require the *in vitro* culture of lymphocytes prior to assay and can measure CYP2E1 in

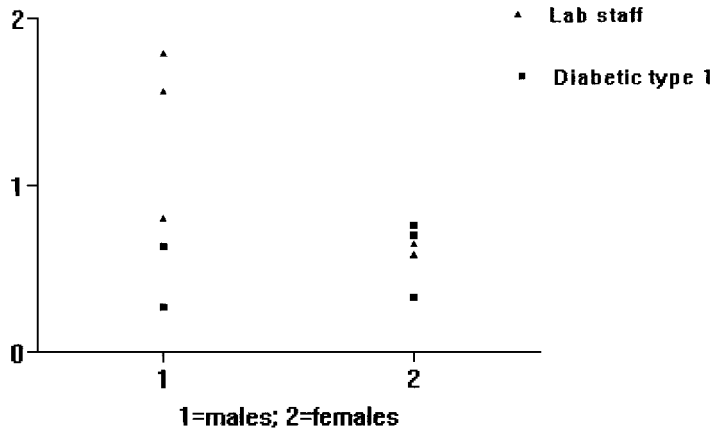

Figure 3. Scatter plot of arbitary integrated optical density units of 2E1 bands per 0.5 million white blood cells (*y* axis) from diabetics and laboratory staff divided by sex (*x* axis).

lymphocytes isolated from normal individuals in the equivalent of about 200 ml of whole blood.

In our experience the use of chemiluminescence rather than chromogenic detection in Western blotting techniques can afford potentially increased sensitivities of 2–3 decades. However, key factors, such as the specific antibody, the buffers and blocking reagents used, are involved in increasing the sensitivity of Western blotting techniques. This may be particularly true using chemiluminescence detection techniques, where increased incubation times with chemiluminescent substrate lead to increased sensitive detection of the specific antibody–antigen interaction without increased non-specific background signal. We believe that our optimization of these parameters has led to a method capable of measuring CYP 2E1 in practical amounts of white blood cells.

In its present form the assay described in this paper may be suitable for use in a number of studies involving chemicals of occupational and environmental interest which involve CYP2E1 metabolism. It may be useful in exploring the relationship between extra-hepatic expression of CYP2E1 as an indicator of enzyme induction caused by occupational exposure to xenobiotics metabolized by CYP2E1. Further evidence of a good correlation between lymphocyte 2E1 levels and *in vivo* chlorzoxazone metabolism may indicate the former's value as a simple means of defining the activity level of this important metabolizing enzyme that is applicable in large scale field studies as well as in controlled exposure studies. The sensitivity of the assay also make it potentially applicable to CYP2E1 measurement in small, non-hepatic tissue samples or *in vitro* cell culture studies where 2E1 expression may be relatively low.

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