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KINETIC PARAMETERS OF LYMPHOCYTE MICROSOMAL EPOXIDE HYDROLASE IN CARBAMAZEPINE HYPERSENSITIVE PATIENTS

ASSESSMENT BY RADIOMETRIC HPLC

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Abstract—Idiosyncratic hypersensitivity reactions with carbamazepine have been postulated to be due to a deficiency of microsomal epoxide hydrolase (HYL1), although this is based on indirect evidence. Using ^{3}H-*cis* stilbene oxide (0.5 Ci/mmol) as a substrate, we have developed a radiometric HPLC assay sensitive enough to measure the kinetic parameters of HYL1 in lymphocytes. The intra-assay coefficient of variation was 8%. Enzyme activity has been measured in lymphocytes from six carbamazepine hypersensitive patients, six patients on carbamazepine without any adverse effects, and twelve drug-naive healthy volunteers. No significant difference was observed in three kinetic parameters of the enzyme among these three groups. The values for K_m, V_{max}, and intrinsic clearance ranged from 6.1–89.9 μM, 3.0–23.2 pmoles diol formed/min/mg protein, and 0.147–0.493 μl/min/mg protein. There was no difference in enzyme activity between patients currently on carbamazepine and healthy volunteers, indicating a lack of induction of lymphocyte HYL1 by carbamazepine. Co-incubation of lymphocytes with 1,1,1-trichloropropene oxide, an inhibitor of hepatic HYL1, resulted in an 82% inhibition of activity, similar to that observed with the hepatic enzyme. The healthy volunteers were genotyped as being either GSTM1 positive ($n = 6$) or GSTM1 negative ($n = 6$). This did not affect the kinetic parameters of lymphocyte microsomal epoxide hydrolase. Our results suggest that there is normal HYL1 activity in lymphocytes of hypersensitive patients using *cis*-stilbene oxide as a substrate.

Key words: microsomal epoxide hydrolase; carbamazepine; *cis*-stilbene oxide

Idiosyncratic hypersensitivity reactions caused by carbamazepine (CBZ), although rare, can be severe, and affect many organ systems [1, 2]. Factors determining individual predisposition are not known, but a deficiency of microsomal epoxide hydrolase (termed HYL1, as per the nomenclature proposed by Beetham *et al.* [3]) has been postulated [4, 5]. Supportive evidence for this is largely indirect, as follows: (a) lymphocytes from hypersensitive patients were more sensitive to drug metabolites (generated *in situ* by a microsomal drug metabolising system) *in vitro* than control cells [4, 5]; (b) the sensitivity of control was increased to a level observed using cells from hypersensitive patients by trichloropropene TCPO,‡ an HYL1 inhibitor [5]; (c) the metabolism-dependent cytotoxicity and covalent binding of CBZ was reduced by adding purified liver HYL1 [6]; and (d) various metabolites including the 2,3- and 1,4-dihydrodiols, and aromatic hydroxylated compounds, have been identified in human urine, suggesting that epoxidation also occurs on the aromatic ring moiety of CBZ [7].

There is no direct evidence for a functional deficiency of HYL1 in CBZ-hypersensitive patients. This may reflect the lack of an assay of sufficient sensitivity to measure HYL1 in lymphocytes where activity is 1000-fold lower than that observed in the liver [8]. Assays have been developed using specific substrates such as styrene oxide, benzo[a]pyrene-4,5-oxide, and *cis*-stilbene oxide (CSO) [9]. However, the application of these assays to lymphocyte HYL1 is limited by factors such as their insensitivity, high background, and reliance on differential extraction with no definitive chemical identification of the products formed. A sensitive HPLC-based fluorometric assay using benzo[a]pyrene-4,5-oxide has been reported, although with lymphocytes, concomitant inhibition of glutathione-S-transferase activity was necessary [10].

In this study, a radiometric HPLC assay using ^{3}H-*cis*-stilbene oxide, which is sensitive enough to measure the kinetic parameters of HYL1 in lymphocytes, has been developed. Enzyme activity has been measured in lymphocytes taken from CBZ-hypersensitive patients, patients on CBZ without adverse effects, and drug-free, normal, healthy volunteers. In the latter group, the effect of glutathione S-transferase μ (GSTM1) on the hydrolysis of CSO to the diol has also been investigated using individuals with and without a deficiency of the enzyme. This is important because GSTM1, like HYL1, can also metabolise epoxides [11] but, unlike HYL1, is known to be polymorphically expressed, being absent in 50% of individuals [12, 13].

METHODS

Patients and controls

Six CBZ-hypersensitive patients took part in the study (Table 1). Three of the patients (patients 1, 2, and 3)

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‡ Abbreviations: CSO, *cis*-stilbene oxide; TCPO, 1,1,1-trichloropropene oxide.

have been described previously [5, 14, 15]; their lymphocytes have been shown to be more sensitive to CBZ metabolites than those from controls using the *in vitro* cytotoxicity assay. In patient 6, the episode described was a rechallenge which, after a long illness, ultimately resulted in the patient's death. The previous episode had resulted in a rash and fever after about 4 weeks' exposure.

Two control groups were used:

1. 6 patients (age range 17–60 years) who had been on CBZ (mean daily dose 850 mg, range 600–1200 mg/day) for at least a year without adverse effects; and
2. 12 healthy volunteers (age range 20–48 years) who had never been exposed to anticonvulsants. Six of the patients had been genotyped as being GSTM1-positive, whereas the other six were GSTM1-negative (GSTM1*O).

Materials

Sodium borohydride, CSO and its *trans*-dihydrodiol (±)-hydrobenzoin, and TCPO were purchased from Sigma Chemical Co. (Poole, U.K.). All solvents were of analytical grade and were products of Fisons plc (Loughborough, U.K.). The PCR reagents and *Taq* polymerase were purchased from Cetus (Beaconsfield, U.K.).

Radiolabelled ^{3}H-CSO of high specific activity (0.5 Ci/mmol) and high purity (99.9%) was synthesised from desylchloride (Aldrich Chemicals, U.K.) by reduction with 250 mCi of sodium borohydride (^{3}H; 5.8 Ci/mmol; Amersham, U.K.) using the method of Gill *et al.* [16]. Direct exposure to light was avoided.

Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes (viability upon isolation >95%) were isolated from venous blood (30–40 mL) by centrifugation over a density gradient (Lymphoprep®, Nycomed, Birmingham, U.K.) as described previously [17].

Determination of the glutathione-S-transferase M1 (GSTM1) status

Genomic DNA was isolated from lymphocytes using standard methodology. The GSTM1 genotype was determined using the PCR method of Brockmoller *et al.* [18]. Of the individuals genotyped, six who were GSTM1-positive and six who were GSTM1*O were used as controls in the study.

Measurement of microsomal epoxide hydrolase activity in lymphocytes

In our previous study [19], HYL1 activity towards CSO was measured under linear conditions using 8×10^6 lymphocytes in a total volume of 100 μL. To assess the kinetics of the lymphocyte enzyme, it was necessary to reduce the final incubation volume and cell count by fivefold, whilst maintaining a constant protein concentration. Lymphocytes were suspended in sodium phosphate buffer (0.2 M; pH 7.4), and lysed by freeze-thawing twice in a methanol/dry ice bath. The lysate from 1.6×10^6 cells (in a volume of 19 μL) was incubated with several concentrations (1.3 μM–139 μM) of ^{3}H-CSO (1 μL; 0.01 μCi–1.4 μCi), dissolved in DMSO, for 30 min at 37°C in a shaking water bath. The use of 5% ethanol (v/v) as a solvent decreased enzyme activity, and thus, 5% DMSO (v/v) was used as the solvent. The incubations were terminated by the addition of ice-cold methanol (100 μL) and left overnight at 4°C to allow the protein to precipitate. Water (40 μL) was then added to each incubation, mixed thoroughly by vortexing, and centrifuged (2000 *g* for 15 min). The supernatant was used for chromatographic analysis. Each incubation was performed in duplicate. To measure the extent of non-enzymatic hydrolysis under the experimental conditions employed, a non-protein control incubation containing only CSO and sodium phosphate buffer was also performed. All incubations were protected from direct exposure to light. The protein concentration was determined by the method of Lowry *et al.* [20].

Inhibition of microsomal epoxide hydrolase by 1,1,1-trichloropropene oxide

Initial experiments using the above method failed to show any inhibition of lymphocyte HYL1 by TCPO. This was thought to be because of a possible chemical interaction between TCPO and DMSO. Therefore, the method was modified by substituting 1% ethanol (v/v) for the DMSO, at which concentration it had no effect on enzyme activity. The lysate from lymphocytes (8×10^6 in phosphate buffer, pH 7.4) was incubated either with or without TCPO (90 μM) at 37°C for 1 min before the addition of ^{3}H-CSO (30 μM; 1.5 μCi) in 1 μL of ethanol to give a final incubation volume of 100 μL. TCPO and/or cells were omitted from control incubations. The incubations were performed at 37°C for 30 min in a shaking water bath and terminated with methanol, as outlined above. All incubations were performed in duplicate.

HPLC analysis of microsomal epoxide hydrolase activity

The supernatants from the lymphocyte incubations were analysed for unchanged CSO, and the *trans*-dihydrodiol metabolite of CSO by HPLC linked to a radiometric detector (Flo-one Beta, Canberra Packard, Berk-

Table 1. Clinical characteristics of the carbamazepine-hypersensitive patients

Patient	Sex	Age	Time to adverse reaction	Manifestations
1	Female	22	2 weeks	Fever, toxic epidermal necrolysis, leucopenia, abnormal liver function tests
2	Male	18	5 weeks	Fever, desquamating rash, hepatitis, jaundice, eosinophilia
3	Female	49	1 week	Fever, Stevens-Johnson syndrome, abnormal liver function tests
4	Male	28	3 weeks	Fever, extensive erythematous rash, eosinophilia
5	Female	75	4 weeks	Fever, toxic erythema, eosinophilia
6	Male	37	1 week	Fever, erythematous rash, hepatitis, renal failure

shire, U.K.) and to a UV spectrophotometric detector (Spectra-Physics SP8800). Supernatant (50 μL) was injected onto a reversed phase silica column (Techopak $10C_{18}$; 30 cm × 3.9 mm; HPLC Technology, Macclesfield, U.K.), and eluted with a 55–75% methanol:water gradient for 10 min and at 75% methanol for a further 5 min, at a flow rate of 1 mL/min. The absorbance of the eluant was monitored at 254 nm. Unreacted CSO and its diol metabolite were identified by comparison of their retention times (7 min and 11 min for the *trans*-dihydrodiol and CSO, respectively) with those of co-injected authentic standards (Fig. 1). The percentage conversion of CSO to its dihydrodiol metabolite was quantified by integration of the total radioactivity eluted from the column to determine HYL1 activity. The intra-assay coefficient of variation was 8%.

Statistical analysis

The apparent Michaelis-Menten kinetic parameters, K_m and V_{max}, were estimated by nonlinear least squares regression of the velocity vs concentration curve. Intrinsic clearance (CL_{int}) was derived from the ratio of V_{max} to K_m. The results are presented as the mean ± SEM. Statistical analysis was performed with the Mann Whitney U-test using the Arcus statistical software package (Dr. I. Buchan, The University of Liverpool), accepting $P < 0.05$ as statistically significant.

RESULTS

Using HPLC analysis (Fig. 1), a clear separation between the diol and unreacted CSO was obtained, allowing better chemical characterisation of the products formed than was possible with the previously used extraction assay [19]. There was no spontaneous hydrolysis of the ^{3}H-CSO in control incubations (i.e. in the absence of lymphocytes). This compares with the extraction assay used in our previous study [19], which had a signal-to-noise ratio of 2:1. A major advantage of the present assay was that only 1.6×10^6 cells were required per incubation, compared to our previous study [19], where 8×10^6 cells were needed in each incubation to measure activity. This allowed the determination of Michaelis-Menten kinetics of the HYL1 enzyme in lymphocytes using less than 40 mL of blood.

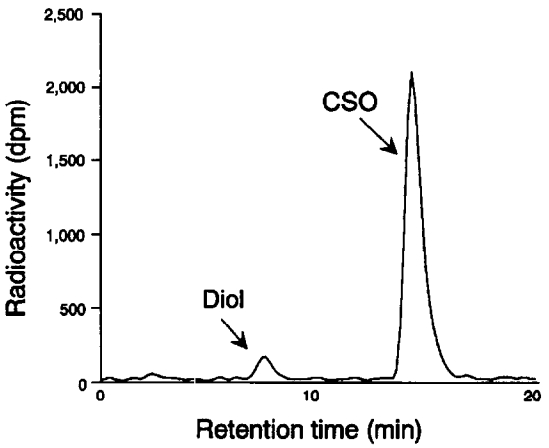

Fig. 1. Typical radiometric HPLC chromatogram obtained from the incubation of lymphocytes (1.6×10^6 cells/incubation) with ^{3}H-*cis* stilbene oxide at 37°C for 30 min. The lymphocytes in this example were obtained from a healthy volunteer. The rate of hydrolysis was calculated directly from the percent turnover of *cis*-stilbene oxide to the dihydrodiol.

The kinetic parameters of lymphocyte HYL1 in CBZ-hypersensitive patients were compared to those of patients on CBZ without adverse effects and healthy volunteers. In the two control groups, there was a small degree of inter-individual variability in the K_m (2–4-fold), V_{max} (2–5-fold), and a clearance (2-fold). In the hypersensitive patients, K_m (15-fold) and V_{max} (8-fold), but not clearance (3-fold), showed a greater degree of variability than in the control groups. However, there was no significant difference in the kinetic parameters of HYL1 between the hypersensitive patients and both control groups (Fig. 2). There was also no difference between patients currently on CBZ and healthy volunteers, indicating a lack of induction of HYL1 in lymphocytes by CBZ. Furthermore, in the healthy volunteers, the GSTM1 genotype did not influence the kinetic parameters.

A previous study using the extraction assay with CSO as a substrate failed to show any inhibition of the HYL1 in lymphocytes by TCPO [8], a known inhibitor of liver HYL1 [9]. However, using radiometric HPLC analysis, we found that TCPO (90 μM) produced 82% inhibition of the HYL1 in lymphocytes, which was comparable to that observed in liver microsomes in our laboratory using the same assay conditions (unpublished data).

DISCUSSION

In the present study, the apparent Michaelis-Menten kinetics of lymphocyte HYL1 have been measured by radiometric HPLC using ^{3}H-CSO as a substrate. A comparison with our previous assay, which was based on differential extraction [19], shows that we were able to reduce the number of lymphocytes per incubation by 5-fold, which allowed full kinetic analysis of the lymphocyte enzyme using less than 40 mL of blood. An improvement of the signal-to-noise ratio was also achieved when compared to our previous assay [19]. Seidegard and co-workers [8] have previously measured the kinetics of lymphocyte HYL1 using CSO as a substrate in an extraction assay. However, there are several important methodological differences that make a direct comparison of the values obtained in the two assays difficult: First, CSO with a much lower specific activity was used to measure the kinetic parameters; secondly, the assay was performed at pH 9.8 rather than the physiological pH used in the present study; thirdly, a much larger number of cells (10^7) was used in each incubation; and finally, a longer incubation time (60 min) was used.

It has been postulated that a deficiency of HYL1 may predispose to anticonvulsant hypersensitivity [4, 5] and teratogenicity [21]. Evidence supporting this has been presented by Buehler *et al.* [22], who showed that there was trimodal distribution of HYL1 activity in amniocytes, only infants with the lowest activity being affected by the foetal hydantoin syndrome. The observation of increased lymphocyte sensitivity in CBZ-hypersensitive patients using the *in vitro* cytotoxicity assay [4, 5] would suggest that the defect is expressed in lymphocytes despite the fact that these cells were not the primary tissue affected by the toxicity. We have previously demonstrated HYL1 activity in the lymphocytes of one hyper-

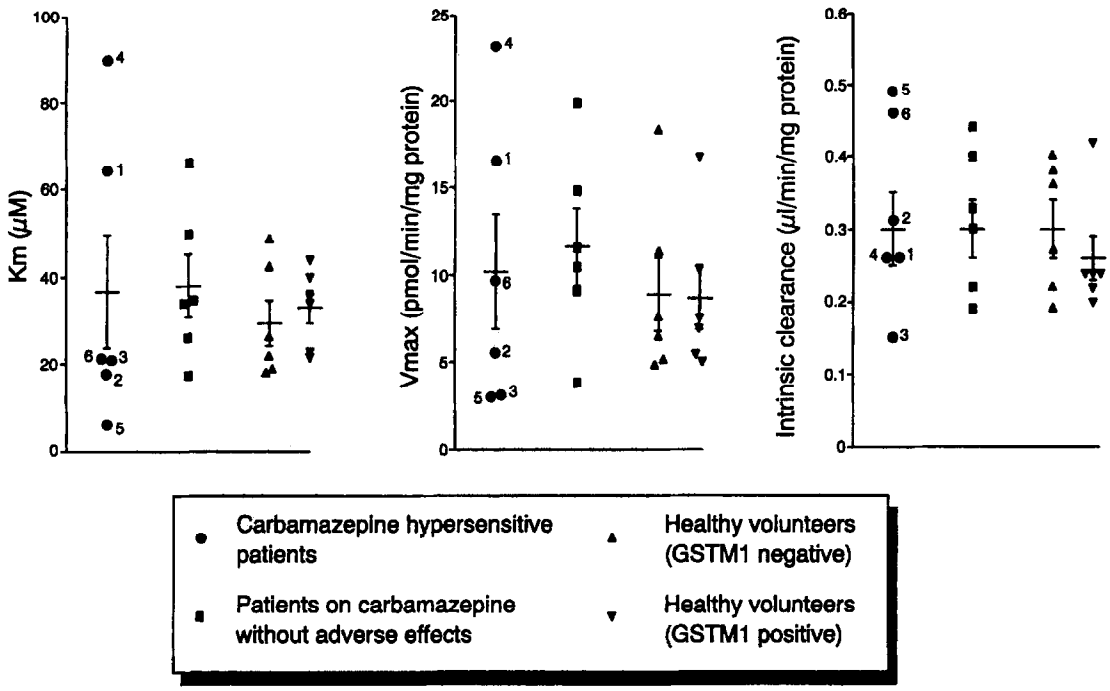

Fig. 2. The kinetic parameters of lymphocyte microsomal epoxide hydrolase (HYL1), expressed as K_m, V_{max}, and intrinsic clearance, in carbamazepine hypersensitive patients ($n = 6$), patients on carbamazepine without adverse effects ($n = 6$), and normal, healthy volunteers ($n = 12$). Six of the healthy volunteers were genotyped as being GSTM1 positive; the other six were GSTM1 negative. The carbamazepine hypersensitive patients are numbered as listed in Table 1. The horizontal bars represent the mean; the vertical bars represent the SEM. Statistical analysis was performed using the Mann-Whitney test by comparing the kinetic parameters of the different groups.

sensitive patient [14]; however, a single time point measurement of activity would not have detected alterations in substrate affinity or specificity of the enzyme. Therefore, in this study we have measured the kinetics of lymphocyte HYL1 in CBZ hypersensitive patients. We found no difference in the K_m, V_{max}, and apparent clearance in these patients when compared with either non-hypersensitive patients or healthy volunteers (Fig. 2), although there was greater inter-individual variability of the K_m and V_{max} values in hypersensitive patients than in other individuals. In view of this variability and the fact that only six patients were studied, it is possible that our study lacks the statistical power to detect a small difference. However, it should be noted that there was no hypersensitive patient who completely lacked activity. Furthermore, the results of this study are in accordance with our previous study using radioimmunoassay [23], where again no difference was detected in HYL1 protein levels between hypersensitive patients and controls. Genetic analysis of the *HYL1* gene performed by Gaedigk *et al.* [24] and ourselves (Green *et al;* this issue) also supports this conclusion, since specific mutations in the exonic regions of the gene that correlate with the occurrence of anticonvulsant hypersensitivity have not been demonstrated. The variability observed in K_m and V_{max} values, particularly in the hypersensitive patients, may be genetically determined, although in patients 1–5 who have been genotyped (see Green *et al;* this issue), there was no consistent pattern of mutations. Indeed, the K_m varied widely despite the same genotype in patients 2 and 4 (Fig. 2). The variation in K_m may also be a result of individual differences in lipid content in the cell lysate, as has been shown for benzo[a]pyrene-4,5-oxide hydrolysis by HYL1 [25]. Despite the negative findings of the study, a role for HYL1 in predisposition to CBZ hypersensitivity cannot be totally excluded, since minor differences in enzyme structure may have lead not only to changes in enzyme activity, but also to changes that may have been substrate-specific, and thus would be undetectable using CSO as a substrate. With respect to the latter, such a situation is known to exist with the enzyme butyrylcholinesterase (pseudocholinesterase) [26], where different mutations lead to marked differences in activity towards different substrates.

It is possible that the enzyme in lymphocytes is not representative of the HYL1 present in the tissues affected by hypersensitivity such as the skin and liver. Indeed, on the basis of differences in substrate selectivity and inhibition of enzyme activity, it has recently been suggested that HYL1 in the skin is different from that in the liver [27]. Furthermore Seidegard *et al.* [8] have reported that TCPO, which is known to inhibit the hepatic enzyme, had very little effect on the lymphocyte enzyme. However, in the present study, radiometric analysis has shown TCPO to inhibit lymphocyte HYL1 by over 80%, a finding comparable to that observed with human liver in our laboratory (unpublished data). The discrepancy between the two studies may be the consequence of the use of DMSO as a solvent in the study of Seidegard *et al.* [8], which we have found to inactivate TCPO. The observation that the lymphocyte can be used as a sentinel cell for hepatic and pulmonary HYL1 ac-

tivity provides further evidence that the enzyme in different tissues is the same [10]. It is also consistent with molecular studies that have shown that there is only one gene for HYL1 located on chromosome 1 [28].

Recently, we showed that at CBZ doses that produced marked induction of hepatic CYP3A, there was only a minimal increase in lymphocyte HYL1 activity [19]. A lack of induction of HYL1 in lymphocytes by CBZ is confirmed by the V_{max} values obtained in the present study, there being no difference between patients on CBZ and the healthy volunteers (Fig. 2). In contrast, induction of HYL1 has been demonstrated in liver, suggesting tissue-specific differences in regulation of the enzyme [29–31]. The lack of induction of the lymphocyte enzyme by CBZ also rules out the possibility that enzyme induction may have been responsible for the differences between hypersensitive and non-sensitive patients in cell death observed in the *in vitro* cytotoxicity assay.

GSTM1, like HYL1, metabolises epoxides [11], but is also polymorphically expressed [12, 13]. In the present study, there was no relationship between GSTM1 genotype and HYL1 activity, suggesting that there is a large reserve of HYL1 activity in tissues that does not necessitate its reciprocal elevation in the absence of another enzyme that metabolises epoxides. It is also in accordance with our finding that there was no association between the GSTM1 genotype and the occurrence of CBZ hypersensitivity [32].

In summary, using high specific activity ^{3}H-CSO as a substrate, we have developed an assay for HYL1 incorporating chromatographic analysis of the products formed, which is sensitive enough to determine the kinetic parameters in a peripheral accessible cell with low enzyme activity, such as the lymphocyte, using less than 40 mL of blood. Using this assay, we failed to show any difference in the kinetics of lymphocyte HYL1 in CBZ-hypersensitive patients compared with non-sensitive patients, and individuals not exposed to the drug.

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