Biochemical Pharmacology, Vol. 42, No. 7, pp. 1367–1372, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00
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HETEROLOGOUS EXPRESSION OF HUMAN MICROSOMAL EPOXIDE HYDROLASE IN *SACCHAROMYCES CEREVISIAE*

STUDY OF THE VALPROMIDE–CARBAMAZEPINE EPOXIDE INTERACTION

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(*Received* 15 *January* 1991; *accepted* 19 *May* 1991)

Abstract—A cDNA of human microsomal epoxide hydrolase (hmEH) was constitutively and inducibly expressed in *Saccharomyces cerevisiae*. The heterologous enzyme was located mainly in the microsomal fraction of yeast cells. Yeast microsomes containing hmEH exerted styrene oxide hydrolase activity (K_m = 300 μM; V_{max} = 22 nmol/mg min) as well as carbamazepine epoxide hydrolase activity. The hmEH catalysed exclusively the formation of carbamazepine-10,11-transdihydrodiol, since no carbamazepine-10,11-cisdihydrodiol was detected. Inhibition studies using these microsomes revealed unequivocally hmEH as the target for inhibition by the antiepileptic drug valpromide. A K_i value of 27 μM was determined for the inhibitor valpromide with styrene oxide as substrate. For carbamazepine epoxide, a K_i value of 8.6 μM was obtained, which is well in line with data published for hmEH determined with human liver microsomes. Our results demonstrate the potential of heterologous gene expression in *S.cerevisiae* and its application to the *in vitro* study of pharmacological and toxicological problems.

A large number of microsomal enzymes known as cytochrome P450s are involved in the generation of reactive epoxides which can lead to the damage of cellular macromolecules. In contrast to this variety, only two cytosolic and two microsomal forms of mammalian epoxide hydrolases are known. They have been characterized biochemically and shown to differ in terms of molecular weight, substrate specificity and immunological properties [1, 2]. cDNAs for cytosolic leukotriene A_4 hydrolase and a microsomal enzyme exhibiting broad substrate specificity have been cloned, the latter from several species [3–7]. Human microsomal epoxide hydrolase (hmEH; EC 3.3.2.3) plays a crucial role in the metabolism of xenobiotic alkene and arene oxides of several polycyclic aromatic hydrocarbons as well as in the metabolism of endogenous steroids [8, 9] and some therapeutic drugs which are metabolized via epoxide intermediates [10, 11].

Clinical studies with patients co-medicated with the antiepileptic drugs carbamazepine and valpromide revealed that the plasma levels of carbamazepine epoxide were elevated significantly over the levels in patients treated with carbamazepine alone. This observation was attributed to a possible inhibition of human microsomal epoxide hydrolase by valpromide [12–14]. Further evidence for the suspected mechanism was provided in a study by Pacifici *et al.* [15] in which competitive inhibition of styrene oxide hydrolysis by valpromide was shown.

In order to obtain direct evidence for the inhibition of hmEH by valpromide we expressed the corresponding human cDNA in *S.cerevisiae* and used microsomal fractions for *in vitro* drug interaction studies. Here we show unequivocally that hmEH is strongly inhibited by valpromide. Our results also demonstrate the usefulness of heterologous gene expression in *S.cerevisiae* to address questions of pharmacological and toxicological importance. During the course of this study and consistent with our data, Kerr *et al.* [16] also reported the strong inhibition of carbamazepine epoxide hydrolysis by valpromide using either human liver microsomes or purified hmEH.

MATERIALS AND METHODS

Chemicals. Carbamazepine, carbamazepine epoxide, carbamazepine-10,11-trans- and cis-dihydrodiols, oxcarbamazepine, valpromide and [^{14}C]-styrene oxide were gifts from Ciba-Geigy (Basel, Switzerland).

Strains. Escherichia coli strain DH5αF′ (F′, *end*A1, *hsd*R17, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Φ80d*lac*ZΔM15, Δ(*lac*ZYA *arg*F) U169 was used for plasmid constructions and was transformed according to standard protocols. *S.cerevisiae* strain YHE2 (MATa/MATα, ade2-40/ade2-119, trp5-12/trp5-27, ilv1-92/ilv1-92, ura3Δ5/ura3Δ5 [17]) was transformed as described previously [18].

Media. E. coli transformants were selected on LB [19] medium containing 100 μg/mL ampicillin. Yeast

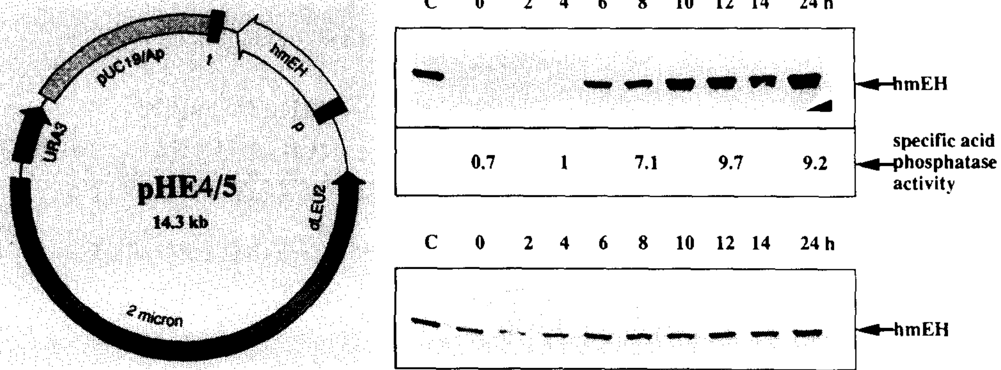

Fig. 1. Left: Map of the 2 μm based plasmids pHE4 and pHE5. They differ in the region p, representing the GAPFL promoter present in pHE5 and the PHO5 promoter in pHE4. The coding region for hmEH is indicated by an open arrow. Promoter (p) and terminator (t) are denoted by black boxes. Right: Western blots of whole cell lysates prepared from YHE2 pHE4 (upper panel) and YHE2 pHE5 cultures (lower panel) expressing hmEH. Amounts of lysed yeast cells corresponding to 0.16 O.D.$_{600}$ (7 μg protein) were applied to each lane. (C) denotes human liver microsomes (2 μg protein) as a positive control. The numbers above each panel indicate the time of sample preparation after induction in low P_i medium. The number below the upper panel give the specific acid phosphatase activity of the culture in milliunits. One unit is defined by the amount of enzyme which liberates 1 μmol *p*-nitrophenol/min/ O.D.$_{600}$ units of cells.

transformants were grown on synthetic complete medium lacking uracil (SD-ura) [20]. Induction of the PHO5 promoter was obtained by transferring the cultures to a low phosphate medium (low P_i medium) containing 2% glucose, 1% asparagine, 0.1% histidine, required amino acids, vitamins, salts and trace elements as for SD medium except that mono- and dibasic potassium phosphate was replaced by the addition of 30 μg/mL KH_2PO_4 and 1 mg/ mL KCl.

Plasmid constructions. A cDNA clone coding for hmEH [21] was mutagenized to carry a *Nar*I restriction site immediately upstream from the start codon. For that purpose the 241 bp *Sma*I/*Sac*I fragment containing the cDNAs 5′ region was subcloned into plasmid pEMBL18 [22]. Oligonucleotide mediated mutagenesis was performed according to Kunkel [23] using the 17-mer CACAT*GGCGCC*TGTACC as primer. The sequence of the *Sma*I/*Sac*I fragment containing the introduced *Nar*I restriction site was confirmed by DNA sequencing and the *Sma*I/*Sac*I fragment in the original plasmid was replaced by the mutated derivative. Subsequently, the cDNA was isolated as an *Nar*I/*Sal*I fragment and inserted into *Eco*RI/ *Xho*I digested vectors p31GAPFL-IT and p31RIT-12 (B. Meyhack, unpublished) after filling in the *Eco*RI and *Nar*I ends by Klenow polymerase. This construction placed the cDNA behind a shortened derivative (GAPFL) of the *S.cerevisiae* GAPDH promoter in p31GAPFL-IT or the PHO5 promoter in p31RIT-12 (B. Meyhack, unpublished). Faithful termination and processing of the mRNA was obtained by the presence of a PHO5 terminator on the pBR322 based vectors. The expression-cassettes were isolated as *Sal*I/*Hin*dIII and *Sal*I/*Pst*I fragments and inserted into *Sal*I/*Bam*HI sites of 2 μm vector pDP34 [24], after filling in the *Hin*dIII, *Pst*I and *Bam*HI ends by Klenow polymerase, resulting in plasmids pHE4 and pHE5 (Fig. 1).

Western blots. Yeast cell extracts were electrophoresed on 10% Lämmli slab gels and blotted onto nitrocellulose. Heterologous protein was visualized using a polyclonal rabbit antiserum recognizing hmEH. This antiserum was developed by U. M. Zanger at the Biocenter of the University of Basle. Antigenic proteins were detected using the alkaline phosphatase detection kit from Bio Rad (Munich, F.R.G.).

Enzyme assays. Epoxide hydrolase activity was determined using [^{14}C]styrene oxide as substrate in a total volume of 0.2 mL. The reaction mixture consisted of 0.1 M sodium phosphate (pH 7.4), microsomes (0.03–0.5 mg/mL) and valpromide (0–10 μM) and the reaction was started by adding [^{14}C]styrene oxide in acetonitrile (10 μL) and mixing immediately. The assays were incubated at 37° in a waterbath for 20 min and extracted as described by Oesch *et al.* [25].

The carbamazepine epoxide hydrolase assays were done in a total volume of 1 mL. The reaction mixture containing 0.1 M sodium phosphate (pH 7.4), microsomes (0.7 mg/mL) and valpromide (0–10 μM) was mixed on ice. The reaction was started after a preincubation period of 1 min at 37° by the addition of carbamazepine epoxide (0.1–1.6 mM) and vigorous mixing. The incubations were carried out at 37° in a shaking waterbath for 3 hr. The reactions were terminated by quick freezing and the metabolites were extracted and analysed by HPLC chromatography as described by Menge *et al.* [26].

Human microsomes. Microsomes from a human liver (KDL35, kidney donor liver) obtained from the human liver bank of the Department of Pharmacology, Biocenter Basel were prepared as described previously [27].

Other methods. Determination of acid phosphatase, production of yeast spheroblasts, cell disruption, fractionation and preparation of microsomes were

Table 1. Specific styrene oxide hydrolase activity of homogenate and the subcellular fractions of YHE2 pHE5 and YHE2 pDP34 and relative recovery (%) of the enzyme activity from homogenate in the cytosol and microsomal fractions

Cell fraction	YHE2 pHE5 (nmol/mg min)	YHE2 pHE5 (%)	YHE2 pDP34 (nmol/mg min)
Homogenate	7.0 ± 0.1	100	ND
Cytosol	2.2 ± 0.1	30	—
Microsomes	22.7 ± 0.6	70	ND

ND, not detectable; —, not determined.

done as described previously [17]. Protein concentrations were determined by the method of Bradford [28] using bovine serum albumin as a standard.

RESULTS

Expression of human microsomal epoxide hydrolase in S.cerevisiae

Two plasmids were constructed that allowed for constitutive and inducible expression of hmEH cDNA in yeast (Fig. 1, left). As is evident from the Western blot in the upper panel of Fig. 1, right, immunoreactive hmEH co-migrated with hmEH from a human microsomal liver sample (KDL35; kidney donor liver 35). The heterologous hmEH appeared 6 hr after transfer of a YHE2 pHE4 culture from SD-ura medium to low P_i medium and was paralleled by a simultaneous increase in the specific acid phosphatase activity, reflecting the induction of the inducible acid phosphatase gene PHO5 by inorganic phosphate starvation. In the lower panel of Fig. 1, right, constitutive expression of hmEH is shown to occur in YHE2 pHE5 during the whole growth phase, independent of PHO5-induction. Although inducible expression may provide higher yields of heterologous enzyme, constitutive expression was used for technical reasons in further experiments.

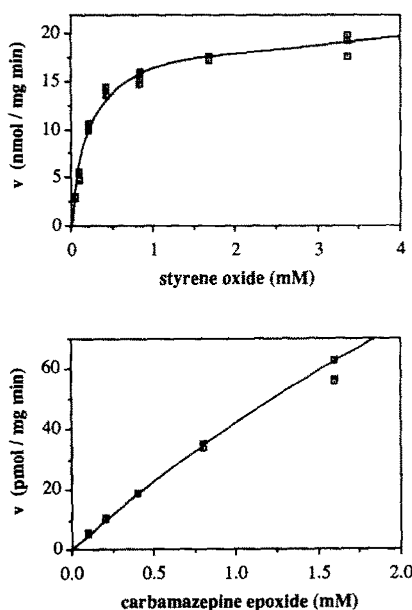

Fig. 2. Enzyme kinetics with microsomes of YHE2 pHE5 and styrene oxide (upper panel) and carbamazepine epoxide (lower panel) as substrate. The assays were performed using 0.1 and 1 mg microsomal protein with styrene oxide and carbamazepine epoxide, respectively.

Styrene oxide hydrolase activity and subcellular localization

In order to quantitate the distribution of the heterologous enzyme in different cell compartments the styrene oxide hydrolase activity of the enzyme was used as a marker. A culture of *S.cerevisiae* YHE2 pHE5 was harvested and subjected to cell fractionation as outlined in Materials and Methods. Table 1 shows the distribution of relative epoxide hydrolase activity as well as the specific epoxide hydrolase activities of homogenate and the cytosol and microsomal fractions. Of the total styrene oxide hydrolase activity, 30% remained in the cytosol and 70% co-purified with the yeast microsomal fraction which exhibited a specific styrene oxide hydrolase activity of 22.7 nmol/mg min. No styrene oxide hydrolase activity was detected in homogenate or the microsomes from the control strain transformed with vector (pDP34) alone.

Enzyme kinetics with styrene oxide and carbamazepine epoxide

The enzyme kinetic data shown in Fig. 2 were obtained under conditions of linearity with respect to incubation time and protein concentration. They demonstrate that styrene oxide hydrolysis was saturable and showed Michaelis–Menten kinetics with a V_{max} of 22.2 nmol/mg min and a K_m of 300 μM. In the case of carbamazepine epoxide as substrate, dihydrodiol formation was almost linear up to the highest possible substrate concentration. The dihydrodiol formation was highly stereospecific since carbamazepine-10,11-transdihydrodiol but apparently no carbamazepine-cisdihydrodiol was detected by HPLC chromatography (data not shown).

Inhibition of styrene oxide hydrolase by valpromide

In order to determine the inhibition constant K_i

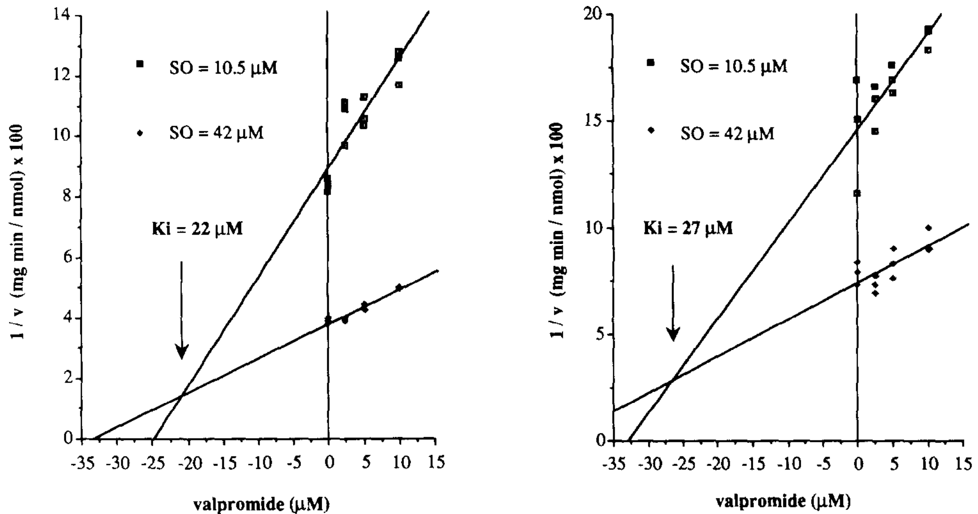

Fig. 3. Dixon plots demonstrating the inhibitory effect of valpromide on the styrene oxide (SO) hydrolase activity catalysed by YHE2 pHE5 (right) and human liver microsomes (KDL35) (left). The protein concentration in the assays was 30 μg/mL.

of valpromide obtained with authentic hmEH and with the heterologously expressed hmEH, styrene oxide hydrolysis was analysed in the presence of varying valpromide concentrations. Styrene oxide hydrolase activity present in human liver microsomes (KDL35) (Fig. 3, left), as well as in YHE2 pHE5 microsomes (Fig. 3, right), was inhibited in a dose-dependent way. Similar K_i values of 20 and 27 μM, respectively, were obtained for the two microsomal preparations. From the fact that the yeast strain used exhibited styrene oxide hydrolase activity only after transformation with plasmid pHE5 and that this enzyme activity was inhibited by valpromide, we conclude that hmEH is unequivocally the target for inhibition by valpromide.

Inhibition of carbamazepine epoxide hydrolysis by valpromide

The same YHE2 pHE5 yeast microsomes were used to study the inhibition of carbamazepine-10,11-transdihydrodiol formation from carbamazepine epoxide by valpromide. The observed dose-dependent inhibition is shown in a Dixon plot in Fig. 4. A K_i value of 8.6 μM for valpromide was determined which is similar to the value of 5.3 μM determined by Kerr *et al.* [16] using human liver microsomes.

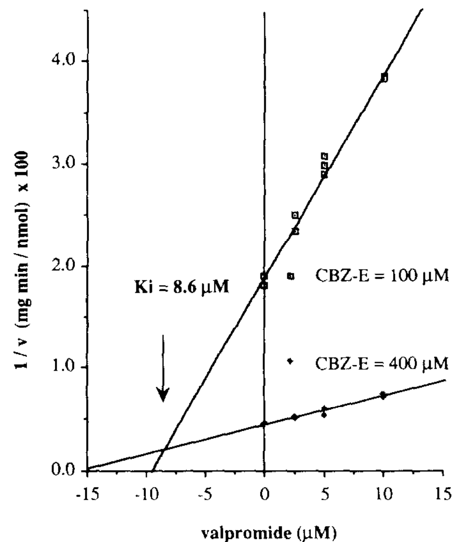

Fig. 4. Dixon plot showing the inhibitory effect of valpromide on carbamazepine transdihydrodiol formation catalysed by YHE2 pHE5 microsomes. The protein concentration in the assays was 0.8 mg/mL.

DISCUSSION

Enzymes involved in the metabolism of endogenous as well as xenobiotic compounds are usually characterized biochemically and enzymatically using tissue preparations such as subcellular fractions or purified enzymes. Today, recombinant DNA technology allows the expression of cloned cDNAs in heterologous organisms which are devoid of equivalent enzyme activities. The heterologous expression approach, therefore, represents an attractive alternative which allows the study of properties of the enzyme concerned without further need for human or animal tissue.

Several features of the lower eukaryote *Saccharomyces cerevisiae* make it an ideal candidate for stable and functional expression of human and animal xenobiotic metabolizing enzymes [24]. These features prompted us to construct strain YHE2 pHE5 which stably expressed hmEH. As expected for a typical membrane protein of the endoplasmic reticulum [29, 30], the majority of hmEH was found to be

localized in the microsomal fraction of *S.cerevisiae* YHE2 pHE5.

The enzyme kinetics for styrene oxide using YHE2 pHE5 microsomes revealed V_{max} (22 nmol/mg min) and K_m (0.3 mM) values similar to values determined for rhesus monkey liver [31] (V_{max} = 19.8 nmol/mg min, K_m = 0.23 mM) and human liver microsomes [32] (V_{max} = 18.7 nmol/mg min), [21] (V_{max} = 58.6 nmol/mg min). Considering the fact that the apparent K_m [8] and V_{max} values both depend on the relative amount of hmEH in the microsomes used as well as on the concentration of the microsomes in the assays (data not shown), the observed difference in the K_m and V_{max} values should be interpreted with caution. The enzyme kinetics determined for carbamazepine epoxide follow almost first order kinetics over the whole substrate concentration range and are comparable to the kinetics determined by Tybring *et al.* [33] using human microsomes.

Expression of hmEH has been reported previously by Davies *et al.* [34]. These authors isolated a cDNA which was identified by restriction analysis to code for hmEH and expressed it in a human lymphoblastoid cell line. They observed increased cytotoxicity and mutagenicity towards dimethylnitrosamine and benzo(*a*)pyrene in a cell line that concomitantly over-expressed hmEH with two cytochrome P450 enzymes. The combination of this cell culture system with our heterologous yeast expression system represents a powerful tool in elucidating the specific role of hmEH in xenobiotic metabolism. Due to its high sensitivity, the system of Davies *et al.* [34] may be advantageous in the detection of hmEH mediated xenobiotic induced mutations. On the other hand, the ease of obtaining large quantities of yeast microsomes containing heterologous enzymes confers a major advantage to the yeast system for a variety of *in vitro* studies related to metabolism, as outlined here for the drug interaction of carbamazepine epoxide and valpromide.

The anti-epileptic drug valpromide inhibits hmEH in a direct way. This was demonstrated by measuring the styrene oxide hydrolase activity of YHE2 and pHE5 microsomes in the presence of valpromide. The calculated inhibition constant (K_i = 27 μM) was very similar to the value determined in human microsomes (K_i = 20 μM). Furthermore, inhibition of carbamazepine-10,11-dihydrodiol formation by valpromide assayed using heterologously expressed hmEH resulted in a K_i of 8.6 μM which is well in line with the K_i of 5.3 μM reported for human liver microsomes [16]. We, therefore, conclude that the authentic and heterologous enzymes exhibit the same characteristics.

From Fig. 4, it can be deduced that a therapeutic concentration of 10 μM valpromide [35] is sufficient to inhibit carbamazepine-10,11-dihydrodiol formation by 50% at a substrate concentration of 100 μM. Considering the plasma concentration of carbamazepine epoxide, which was around 5 μM in patients receiving a chronic dosage of carbamazepine [35], it is obvious that the inhibition of carbamazepine-10,11-dihydrodiol formation *in vivo* by a therapeutic dosage of valpromide might be considerably higher than 50%. This is of importance since treatment with carbamazepine or combinations of carbamazepine with valproate, valpromide, phenytoin and phenobarbitone was related to teratogenicity and carbamazepine intoxication [36–38].

The observed *in vitro* effects add to the present data the final evidence that hmEH is the target involved in the drug interaction between carbamazepine and valpromide. Furthermore, the alternative hmEH source will allow for the prospective testing of drug interactions between arene oxide drug intermediates and drugs suitable for combination therapy without further need for human or animal tissues.

Acknowledgements—We thank Dr W. Riess, Dr G. Flesch and Dr P. Beilstein from Ciba-Geigy for providing chemicals and HPLC-facilities, and Dr B. Meyhack for providing plasmid vectors. This project was supported by grant 3.382-0.86 from the Swiss National Science Foundation.

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