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THE EFFECT OF ENZYME INDUCTION ON THE CYTOCHROME P450-MEDIATED BIOACTIVATION OF CARBAMAZEPINE BY MOUSE LIVER MICROSOMES

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Abstract—Predisposition to idiosyncratic toxicity with carbamazepine is thought to be due to a deficiency of the detoxication enzyme, microsomal epoxide hydrolase, although in some cases, concurrent administration of enzyme inducers might be a contributory risk factor, by altering the critical balance between bioactivation and detoxication. In this study, a mouse model has been used to determine the factors affecting carbamazepine bioactivation, using covalent binding and cytotoxicity as markers of bioactivation *in vitro*. Microsomes prepared from mice pre-treated with phenobarbitone increased (relative to the control microsomes) the formation of cytotoxic (12.3% vs 3.2%), protein-reactive (3.0% vs 2.0%) and stable (33.8% vs 18.1%) metabolites of carbamazepine. Similarly, pre-treatment with dexamethasone also increased the formation of the cytotoxic (24.8% vs 6.7%), protein-reactive (2.8% vs 1.5%) and stable (38% vs 19.8%) metabolites of carbamazepine, while β-naphthoflavone pre-treatment did not increase the formation of either the toxic or stable metabolites of carbamazepine when compared with its control microsomes. Co-incubation with gestodene (10–250 μM) resulted in a dose-dependent inhibition of both the bioactivation of carbamazepine and the formation of its stable 10,11-epoxide. SDS–PAGE and immunoblotting of the microsomes with anti-CYP3A antibody revealed the presence of a 52 kDa protein band in each preparation of microsomes, but the relative intensities of the bands, as measured by laser densitometry, were highest with the phenobarbitone and dexamethasone microsomes. The microsomal oxidation of cortisol to 6β-hydroxycortisol was also enhanced by pre-treatment of mice with phenobarbitone (6.5% vs 2.7%) and dexamethasone (8.2% vs 4.3%), but not β-naphthoflavone (2.2% vs 1.6%), when compared with their respective control microsomes, and was inhibited (range 25–68% inhibition), with all the microsomes by gestodene (50 μM). Taken collectively, the data in this study demonstrate that in the mouse, induction of the CYP3A subfamily significantly increases carbamazepine bioactivation. It is likely that in humans inducers of the orthologous form of this enzyme, most notably anticonvulsants, may increase the bioactivation of carbamazepine.

Carbamazepine (CBZ†) is widely used for the treatment of epilepsy, trigeminal neuralgia and manic depression. Although generally well tolerated, a minority of patients prescribed CBZ can develop severe, potentially life-threatening idiosyncratic reactions [1–3] such as agranulocytosis, hepatitis and Stevens–Johnson syndrome. The reasons why only a minority of individuals are affected are poorly understood.

In general, idiosyncratic drug reactions are thought to be caused by the formation of toxic, chemically reactive metabolites by the cytochrome P450 enzymes, which then cause toxicity either directly or indirectly by initiating an immune reaction [4, 5]. We have recently shown that CBZ can be bioactivated *in vitro* to cytotoxic and protein-reactive metabolites by hepatic microsomes, the formation of which can be inhibited by ketoconazole, a cytochrome P450 inhibitor [6], indicating that the bioactivation of CBZ is cytochrome P450 dependent [7]. Furthermore, the metabolism-dependent cytotoxicity of CBZ can be enhanced by co-incubation with trichloropropene oxide [2, 8], an epoxide hydrolase inhibitor, and mitigated by exogenous microsomal epoxide hydrolase [7], suggesting that the reactive metabolite is an arene oxide. Therefore, an imbalance between activation of CBZ to its chemically reactive epoxide metabolite and its detoxification by microsomal epoxide hydrolase may be responsible for the sensitivity of certain individuals to CBZ idiosyncratic toxicity.

Using an *in vitro* cytotoxicity assay, cells from CBZ-hypersensitive patients have been found to be more sensitive to CBZ metabolites generated *in situ* than appropriate controls suggesting a detoxication deficiency, i.e. a deficiency of the enzyme microsomal epoxide hydrolase [1, 2]. However, enzyme induction by enhancing bioactivation and thus further altering the balance between activation and detoxication, may also be an important contributory risk factor [7]. This may be particularly relevant for epileptic patients since some of the most commonly used anticonvulsants (phenytoin, CBZ and phenobarbitone) are potent enzyme inducers [9–11]. Furthermore, at least 10% of epileptics are on

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† Abbreviations: BNF, β-naphthoflavone; CBZ, carbamazepine; CBZ-10,11-E, carbamazepine-10,11-epoxide; DEX, dexamethasone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HSA, human serum albumin; MNL, mononuclear leucocytes; PB, phenobarbitone; PEG, polyethylene glycol.

multiple anticonvulsants [11] which may have additive effects on the bioactivation of CBZ.

In previous studies of the mechanisms of CBZ hypersensitivity, mouse microsomes have been used to generate the toxic metabolite of CBZ [1, 2, 8]. In addition, a mouse model has also been used to investigate the mechanisms of anticonvulsant teratogenicity, particularly with phenytoin [12]. In this study, in order to elicit the factors affecting CBZ bioactivation, we have used mouse liver microsomes to investigate the effects of three different types of model enzyme inducing agents, phenobarbitone (PB), dexamethasone (DEX) and β-naphthoflavone (BNF).

MATERIALS AND METHODS

Chemicals. CBZ, human serum albumin (HSA, fraction V), PB, DEX, BNF, NADPH (tetrasodium salt), glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co. (Poole, U.K.). [10,11-^{14}C]CBZ (radiochemical purity 99%) and carbamazepine-10,11-epoxide (CBZ-10,11-E) were gifts from Ciba-Geigy Pharmaceuticals (Basle, Switzerland). [1,2,6,7-^{3}H]Cortisol (radiochemical purity 98%) was obtained from Amersham International (Amersham, U.K.) while 6β-hydroxycortisol was synthesized by Dr J. H. K. Yeung (Chinese University of Hong Kong). All solvents were of HPLC grade and were products of Fisons plc (Loughborough, U.K.).

Induction and preparation of mouse liver microsomes. Groups of six male CBA/ca mice (25–30 g) were induced with PB (60 mg/kg i.p. in 0.9% saline for 3 days), DEX [100 mg/kg i.p. in polyethylene glycol (PEG)/saline (75:25, v/v) for 3 days] and BNF (60 mg/kg i.p. in corn oil for 3 days), the control mice (N = 6 per group) receiving equivalent volumes of vehicle only. The mice were fasted for 24 hr and then killed by cervical dislocation. The livers were removed and microsomes prepared by differential centrifugation [13]. Pooled microsomes from each group of animals were then stored at −80° until used. Protein content was estimated by the method of Lowry *et al.* [14] and cytochrome P450 content by the method of Omura and Sato [15]. The total cytochrome P450 content increased (when compared with microsomes prepared from control animals) after induction with both PB (1.3 vs 1.0 nmol/mg protein) and DEX (1.6 vs 0.8 nmol/mg protein), but not with BNF pre-treatment (0.6 vs 0.5 nmol/mg protein). However, immunoblotting of the microsomes using monoclonal antibodies directed against CYP1A2 (provided by Dr R. Riley, Fisons, Loughborough, U.K.) showed induction of this P450 isozyme only after pre-treatment with BNF (data not shown).

Determination of the metabolism-dependent cytotoxicity of CBZ. Peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinized venous blood as described previously [16]. Their viability upon isolation was >95%. To eliminate inter-individual variability in detoxication enzymes, MNL used in these experiments were isolated from the same donor.

Isolated MNL (1×10^6) in HEPES-buffered balanced salt medium (1 mL) [17] were incubated with CBZ [50 μM; dissolved in methanol, 1% (v/v) final concentration] and either induced or control murine liver microsomes (1 mg) in the presence or absence of NADPH (1 mM) for 2 hr at 37°. The concentration of CBZ used was not directly cytotoxic. After 2 hr, the cells were sedimented and resuspended in drug-free medium (HEPES-buffered medium containing 5 mg/mL HSA). After a further 16 hr incubation at 37°, cell viability was determined by trypan blue dye exclusion as reported previously [16]. All incubations were performed in quadruplicate.

Determination of the metabolism of CBZ to protein-reactive and stable metabolites by murine hepatic microsomes. [^{14}C]CBZ (50 μM; 0.15 μCi) was incubated with the different sets of murine microsomes (1 mg protein) in HEPES-buffered medium (pH 7.4; final volume 1 mL), the reaction being initiated by the addition of NADPH (1 mM, omitted from control incubation) and terminated after 2 hr at 37° by the addition of 3 mL acetonitrile. The incubations were left overnight at 4° to precipitate the protein and irreversible binding of the radiolabelled material was determined after exhaustive solvent extraction of the protein as described previously [7]. All incubations were performed in quadruplicate. Irreversible binding of [^{14}C]CBZ is expressed as a percentage of the initial radioactivity bound to the incubated microsomal protein.

The effect of gestodene on the covalent binding of CBZ was determined by the addition of gestodene (10–250 μM; dissolved in 10 μL methanol) to the above incubations prior to the initiation of the reaction by NADPH.

The supernatants from the incubations with [^{14}C]-CBZ were analysed for unchanged CBZ and its 10,11-epoxide by radiometric HPLC using a previously published procedure [18] with minor modifications [7], the radioactivity being monitored throughout the run and the peaks being integrated and expressed as percentage radioactivity eluting from the column.

Determination of the in vitro *metabolism of cortisol to 6β-hydroxycortisol by murine hepatic microsomes.* This was performed according to the method of Abel *et al.* [19] with minor modifications. Briefly, hepatic microsomes (1 mg protein) from control or induced mice were incubated with [^{3}H]cortisol (0.1 μCi) and cortisol (1 μM) in 0.067 M phosphate buffer (pH 7.4; final incubation volume 0.5 mL) for 2 hr at 37°. The reaction was initiated by the addition of a NADPH-regenerating system (10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 5 mM NADP and 2 U glucose-6-phosphate dehydrogenase). Some incubations also contained gestodene (50 μM). The reaction was terminated by cooling in crushed ice and followed by extraction with ethyl acetate (2 × 2 mL). After centrifugation to sediment protein, the organic layer was transferred to a 10-mL glass tube and evaporated under nitrogen. The samples were then reconstituted in 100 μL methanol.

HPLC analysis was performed on 25-μL aliquots of the sample using a reversed phase C_8 column (Spherisorb, 5 μm, 25 cm × 4.6 mm i.d.; HPLC Technology, Macclesfield, U.K.). The solvent system

(75% 0.4 mM ammonium phosphate buffer: 25% acetonitrile) was delivered at a flow rate of 0.7 mL/min (Altex, Anachem, Luton, U.K.) and the absorbance of the eluant was monitored at 220 nm (Kratos Spectraflow 773) for 50 min. Cortisol and 6β-hydroxycortisol were identified by co-injection of authentic compounds. Quantification of cortisol and 6β-hydroxycortisol was accomplished by collecting 1-mL fractions of eluant to which 4 mL of scintillant were added before determination of radioactivity using liquid scintillation spectroscopy for 4 min. 6β-Hydroxycortisol was expressed as percentage of the total radioactivity eluting from the column.

SDS–PAGE and immunoblotting of the murine microsomal proteins. Murine hepatic microsomes (50 μg) were separated on a 10% gel by SDS–PAGE according to the method of Laemmli [20]. The separated proteins were transferred to nitrocellulose electrophoretically. Immunoblotting was performed by the method of Towbin and Gordon [21]. Briefly, following blocking of non-specific binding sites, the nitrocellulose was incubated with antiserum containing polyclonal antibodies to rat CYP3A (1:2500 dilution, Oxygene, Dallas, TX, U.S.A.). A horseradish peroxidase-labelled second antibody (dilution 1:5000) was used to reveal the immunoreactive polypeptides, the sites of antibody binding being visualized by enhanced chemiluminescence (Amersham International). The relative intensities of the bands in the different sets of microsomes were quantified by laser densitometry (LKB Ultroscan XL, Brømma, Sweden) and integration of the absorbance peak associated with each band.

Statistical analysis. All the results are presented as mean ± SEM. Statistical analysis was performed by ANOVA, accepting $P < 0.05$ as significant. Correlation coefficients were calculated by linear regression analysis.

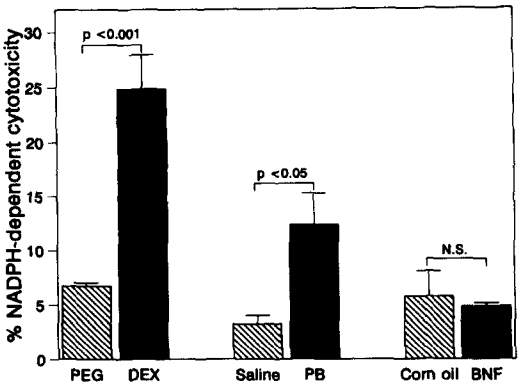

Fig. 1. The effect of induction with DEX, PB and BNF on the metabolism (NADPH)-dependent cytotoxicity of CBZ (50 μM). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by ANOVA comparing the induced microsomes with their respective controls: PEG, saline and corn oil for DEX, PB and BNF microsomes, respectively.

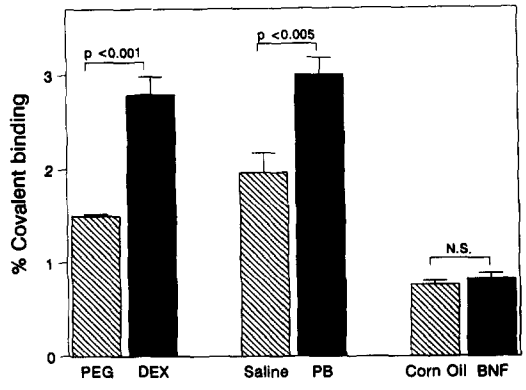

Fig. 2. The effect of induction with DEX, PB and BNF on the irreversible binding of [^{14}C]CBZ (0.15 μCi; 50 μM). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by ANOVA comparing the induced microsomes with their respective controls: PEG, saline and corn oil for DEX, PB and BNF microsomes, respectively.

RESULTS

Effect of enzyme induction on the formation of cytotoxic, protein-reactive and stable metabolites of CBZ

Enzyme induction with both PB and DEX increased the bioactivation of CBZ to cytotoxic (Fig. 1) and protein-reactive species (Fig. 2) when compared with their respective control microsomes. In contrast, BNF pre-treatment did not increase the bioactivation of CBZ relative to the corn oil (control) pre-treated mice (Figs 1 and 2).

The metabolic conversion of CBZ to the stable 10,11-epoxide was increased (relative to the respective control microsomes) after induction with DEX (38.0 ± 1.3% vs 19.8 ± 0.8%; $P < 0.001$) and PB (33.8 ± 1.1% vs 18.1 ± 0.9%; $P < 0.001$) but not BNF (5.0 ± 0.4% vs 5.6 ± 0.5%; not significant).

Effect of gestodene on the formation of protein-reactive and stable metabolites of CBZ

Gestodene, a specific inhibitor of CYP3A4 [22, 23], could not be used as an inhibitor of CBZ bioactivation in the lymphocyte cytotoxicity assay because it was cytotoxic itself. However, co-incubation of gestodene with radiolabelled CBZ resulted in a dose-dependent inhibition of covalent binding with PB and DEX microsomes, and with their respective control microsomes (Fig. 3). With BNF and corn oil microsomes, gestodene (50 μM) also inhibited covalent binding by 30% ($P < 0.05$) and 41% ($P < 0.01$), respectively. The conversion of CBZ to CBZ-10,11-E was also inhibited in a dose-dependent manner by gestodene, resulting in a highly significant correlation between covalent binding and CBZ-10,11-E formation (Fig. 4).

Effect of enzyme induction and enzyme inhibition on 6β-hydroxycortisol formation

Microsomal induction with PB and DEX, but not BNF, increased 6β-hydroxycortisol formation (Table 1). In addition, with all sets of microsomes, co-

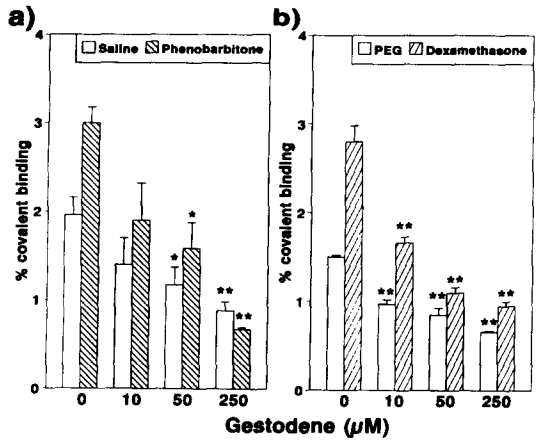

Fig. 3. The effect of gestodene (10, 50 and 250 μM) on the bioactivation of [^{14}C]CBZ to a protein-reactive metabolite in the presence of hepatic microsomes prepared from mice pretreated with (a) PB or its vehicle (saline) and (b) DEX or its vehicle PEG. The results represent the mean of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without gestodene: *P < 0.05, **P < 0.001.

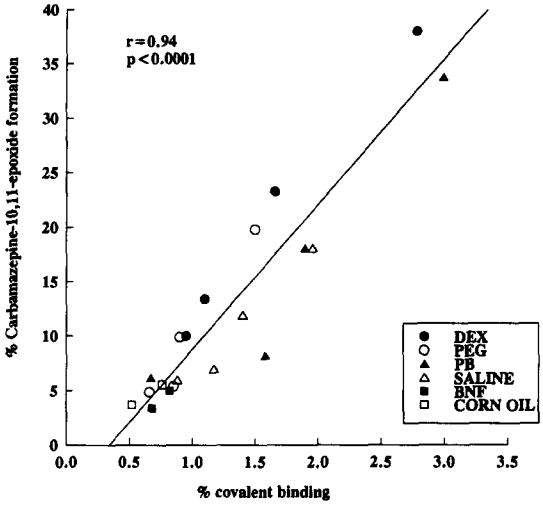

Fig. 4. The correlation between irreversible binding of [^{14}C]CBZ and the formation of the stable CBZ-10,11-E in the same incubations with the six different sets of microsomes. Each data point represents the mean of quadruplicate incubations.

incubation with gestodene (50 μM) inhibited 6β-hydroxycortisol formation (Table 1). Linear regression analysis showed that the formation of 6β-hydroxycortisol correlated with both cytotoxicity ($r = 0.91$, $P < 0.01$) and covalent binding ($r = 0.91$, $P < 0.001$) of CBZ.

Immunoblotting of murine hepatic microsomes

The polyclonal anti-CYP3A antibodies recognised a protein band with an apparent molecular mass of 52 kDa in all the murine hepatic microsomes. However, the relative intensity of the bands, as determined by laser densitometry, was variable between the microsomes, being highest with the DEX and PB pre-treated microsomes (Fig. 5). A cross-reacting band of lower molecular mass was seen with the DEX-pre-treated microsomes (Fig. 5), however, the identity of this protein was unknown. There was a significant correlation between the absorbance values of the bands and both the cytotoxicity ($r = 0.86$, $P < 0.03$) and covalent binding ($r = 0.89$, $P < 0.02$) of CBZ.

Table 1. The effect of induction with DEX, PB and BNF on the formation of 6β-hydroxycortisol by mouse liver microsomes *in vitro*

Microsomes	6β-Hydroxycortisol formation (%)	
	Without gestodene	Gestodene (50 μM)
PEG	4.3 ± 0.9	1.9 ± 0.2‡
DEX	8.2 ± 0.7*	2.6 ± 0.5‡
Saline	2.7 ± 0.3	1.8 ± 0.2‡
PB	6.5 ± 0.2†	2.7 ± 0.7‡
Corn oil	1.6 ± 0.1	1.2 ± 0.1‡
BNF	2.2 ± 0.2	1.6 ± 0.1

The results represent the mean ± SEM of quadruplicate incubations.

Statistical analysis performed by comparing induced microsomes with their respective controls: *P < 0.05, †P < 0.005, and by comparing incubations with and without gestodene (50 μM): ‡P < 0.05.

DISCUSSION

The cytochrome P450 enzymes, located mainly in the endoplasmic reticulum, are a superfamily of haemoprotein enzymes [24–27] with diverse, but often overlapping substrate specificities with respect to the oxidative metabolism of drugs and endobiotics such as steroids [4, 27–29]. In certain circumstances, cytochrome P450 enzymes can bioactivate drugs to toxic, chemically reactive intermediates, which, if not adequately inactivated by detoxication processes such as the glutathione enzyme system and epoxide hydrolase, can lead to various forms of toxicity including carcinogenicity, tissue necrosis and immune-mediated drug toxicity [4, 5, 30]. Factors affecting either activation and/or detoxication may therefore be responsible for predisposition of certain individuals to idiosyncratic drug toxicity. Thus, induction of the cytochrome P450 enzymes may selectively enhance the formation of chemically reactive metabolites, and thereby overwhelm detoxication processes [31]. In addition, induction of one enzyme is often accompanied by a decrease in the other cytochrome P450 isozymes [32], which may also perturb the metabolism of drugs leading to toxicity [31].

The purpose of this study was to determine the

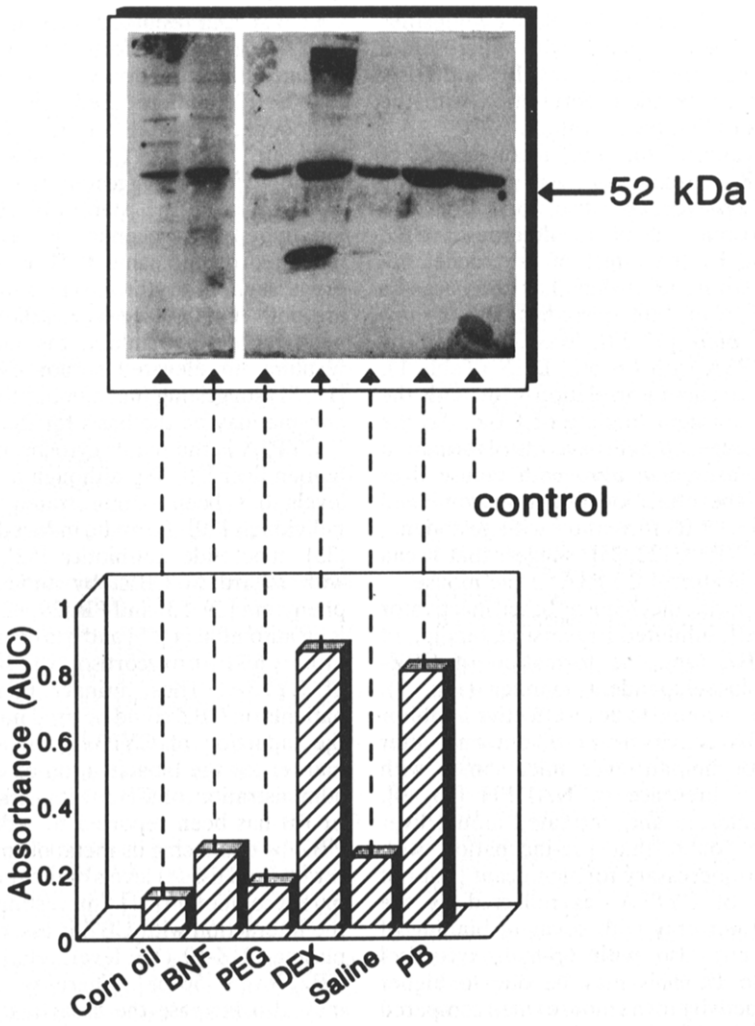

Fig. 5. Immunoblots of murine liver microsomal proteins exposed to polyclonal antibodies raised against rat CYP3A. Microsomal protein (50 μg) prepared from mice pretreated with corn oil, BNF, PEG, DEX, saline and PB were separated on a 10% polyacrylamide gel and transferred to the nitrocellulose support electrophoretically. Immunoblotting was carried out as described in Materials and Methods, with the sites of antibody binding being visualized by enhanced chemiluminescence. The "control" refers to microsomes prepared from rats pretreated with pregnenolone-16α-carbonitrile (supplied by the manufacturer as a positive control). The BNF and corn oil microsomes were run on a separate gel from the other four sets of microsomes; the "control" microsomes were run on both gels, the relative intensities of the bands on the two gels being similar (0.95 and 1.0 absorbance units as assessed by laser densitometry). The indicated size (52 kDa) was determined by interpolation of the mobility of molecular mass standards. The bottom half of the figure is a graphical representation of the relative intensities of the bands with the different microsomes, as determined by laser densitometry.

effect of enzyme induction on the bioactivation of CBZ, using two indirect markers for the formation of toxic metabolites, namely cytotoxicity and covalent binding. Thus, it was found that enzyme induction with PB and DEX, but not BNF, increased the formation of stable and toxic metabolites of CBZ (Figs 1 and 2) relative to their respective control microsomes, which had been treated with vehicle only. Such treatment of control animals was considered to be important because vehicles such as corn oil can themselves have an effect on the P450 isozyme profile [33]. The three model enzyme inducing agents used in this study are selective enzyme inducers in that PB is an inducer of CYP2B, CYP2C and CYP3A [32] while DEX and BNF are inducers of CYP3A [32, 34, 35] and CYP1A [36], respectively. Therefore, the increase in the bioactivation of CBZ observed after induction with DEX and PB would suggest that the CYP3A family is involved in its bioactivation. In accordance with this, immunoblotting performed with antibodies raised against rat CYP3A (which have been shown to cross-react with the mouse orthologue [35]) not only recognised a protein in all the mouse liver

microsomes (Fig. 5) but also the relative intensities of these bands were highest with microsomes prepared from mice pre-treated with PB and DEX (Fig. 5) showing an excellent correlation with the two parameters of CBZ bioactivation.

Supportive evidence for the involvement of CYP3A in CBZ bioactivation was provided by measurement of 6β-hydroxycortisol formation with the same microsomes used to determine CBZ bioactivation and by the effect of gestodene. 6β-Hydroxycortisol formation, which has been used as an index of CYP3A for human studies both *in vitro* [19, 37] and *in vivo* [37–39], was increased by induction of CYP3A with PB and DEX (Table 1), and showed an excellent correlation with both the cytotoxicity and covalent binding of CBZ. To the best of our knowledge, 6β-hydroxycortisol formation has not been measured *in vitro* with mouse liver microsomes but the effect of induction combined with the inhibition of its formation with gestodene, an inhibitor of CYP3A [22, 23], suggest that it can be used as an indicator of CYP3A in the mouse.

Gestodene, a specific mechanism-based inactivator of CYP3A [22, 23], inhibited irreversible binding of radiolabelled CBZ (and the formation of CBZ-10,11-E) in a dose-dependent manner (Fig. 3). Gestodene has been found to be an effective inhibitor of human CYP3A activity *in vitro*, but only after pre-incubation of human liver microsomes with gestodene in the presence of NADPH [23, 40]. However, with induced and untreated mouse liver microsomes, we found that pre-incubation with gestodene was not necessary for significant (but not total) inhibition of CYP3A, as reflected by the results observed not only with covalent binding of CBZ (Fig. 3) but also with 6β-hydroxycortisol formation (Table 1). This may be due to higher relative CYP3A activity in the mouse when compared with man [41]. We have previously shown that ketoconazole, like gestodene, can also inhibit CBZ bioactivation [7]. Although ketoconazole can inhibit multiple cytochrome P450 isozymes at higher concentrations [42], at concentrations less than 5 μM it is a relatively selective inhibitor of CYP3A [42]. Thus, in agreement with the findings of this study, it was possible to inhibit CBZ bioactivation by 1 μM ketoconazole [7], consistent with the involvement of CYP3A. In order to define the specific isozyme of CYP3A in the mouse responsible for bioactivation of CBZ, particularly when using different enzyme inducers such as PB and DEX, a more selective inhibitor such as cannabidiol [43] would be required.

How do these results with mouse microsomes relate to humans? It is well known that there are cross-species differences in the metabolism of many compounds [44], and therefore it is not always possible to extrapolate directly from data obtained in animals to humans [45]. However, the CYP3A family is highly conserved in mammalian species [35, 43, 46] and immunochemical similarity has been observed between the human orthologue and the mouse CYP3A enzyme [43] suggesting that in humans CBZ may also be metabolized by the CYP3A enzyme. In support of this, Kerr *et al.* [47] have recently shown that the epoxidation of CBZ to the stable 10,11-epoxide in humans is mediated by CYP3A4. Our results in the mouse are in accordance with this in that the formation of CBZ-10,11-E was enhanced by induction with both PB and DEX but not BNF, and reduced by co-incubation with gestodene, resulting in an excellent correlation between CBZ-10,11-E formation and covalent binding (Fig. 4), suggesting that the epoxidation of CBZ to both the stable 10,11-epoxide and the putative toxic, chemically reactive epoxide is mediated by the same P450 isozyme. In addition, drugs such as erythromycin and verapamil, which are both metabolized by CYP3A [35, 48–50], have been reported to inhibit the metabolism of CBZ resulting in elevated serum CBZ concentrations [11, 51] suggesting that competitive inhibition of this enzyme may be the basis for the interaction.

CYP3A is the major cytochrome P450 enzyme in human liver [29, 48], although a 26-fold variation in levels has been demonstrated between different individuals [29]. It can be induced by glucocorticoids [35], macrolide antibiotics [52], and importantly, with regard to CBZ, by anticonvulsants such as phenytoin [29, 53] and PB [29, 32, 54]. Indeed, CBZ is an autoinducer [55] and has been shown to increase urinary 6β-hydroxycortisol [10], suggesting induction of CYP3A. Thus, urinary 6β-hydroxycortisol in patients on CBZ could be used not only to determine the induction of CYP3A, but also as an indirect marker for the bioactivation of CBZ. Concomitant administration of CBZ with either phenytoin and/or PB has been reported to reduce the efficacy of CBZ by enhancing its metabolism and thus reducing serum (and tissue) levels of the therapeutically active parent compound [11]. Interestingly, with phenytoin, the interaction with CBZ is associated with a rise in plasma CBZ-10,11-E levels relative to the decreased CBZ levels [56–58]. Therefore, enzyme induction may also increase the bioactivation of CBZ to the toxic epoxide leading to idiosyncratic toxicity in certain individuals, particularly in those who have low activity of microsomal epoxide hydrolase, which may be either genetically determined [1, 2] or by concurrent administration of anticonvulsants such as valproic acid [59, 60], valpromide [59, 60] or progabide [61] which are known to be inhibitors of this enzyme. It is interesting to note that the detoxication pathway, i.e. microsomal epoxide hydrolase, can also be induced by the aromatic anticonvulsants (CBZ, phenytoin, PB); however, several studies have shown that the epoxidation of CBZ is induced to a greater extent than the hydration pathway [55, 62, 63], thus creating (or further exacerbating) the imbalance between bioactivation and detoxication.

In conclusion, the results of this study suggest that bioactivation of CBZ in the mouse is dependent on CYP3A, induction and inhibition of this enzyme increasing and reducing bioactivation, respectively. Furthermore, given the high degree of conservation across species among the CYP3A sub-family of enzymes, the mouse provides a useful model for investigating the effects of enzyme induction not only on CBZ idiosyncratic toxicity, but also on the teratogenicity of CBZ. In humans, inducers of the orthologous form of this enzyme, most notably the aromatic anticonvulsants, may create an imbalance

between bioactivation and detoxication of CBZ resulting in idiosyncratic toxicity.

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REFERENCES

1. Shear NH, Spielberg SP, Cannon M and Miller M, Anticonvulsant hypersensitivity syndrome: *in vitro* risk assessment. *J Clin Invest* **82**: 1826–1832, 1988.
2. Pirmohamed M, Graham A, Roberts P, Smith D, Chadwick D, Breckenridge AM and Park BK, Carbamazepine hypersensitivity: assessment of clinical and *in vitro* chemical cross-reactivity with phenytoin and oxcarbazepine. *Br J Clin Pharmacol* **32**: 741–749, 1991.
3. Pellock JM, Carbamazepine side effects in children and adults. *Epilepsia* **28** (Suppl 3): S64–S70, 1987.
4. Park BK, Metabolic basis of adverse drug reactions. *J R Coll Physicians Lond* **20**: 195–200, 1986.
5. Park BK, Coleman JW and Kitteringham NR, Drug disposition and drug hypersensitivity. *Biochem Pharmacol* **36**: 581–590, 1987.
6. Sheets JJ and Mason JI, Ketoconazole: a potent inhibitor of cytochrome P-450 dependent drug metabolism in rat liver. *Drug Metab Dispos* **12**: 603–608, 1984.
7. Pirmohamed M, Kitteringham NR, Guenthner TM, Breckenridge AM and Park BK, Investigation into the formation of cytotoxic, protein reactive and stable metabolites from carbamazepine *in vitro*. *Biochem Pharmacol* **43**: 1675–1682, 1992.
8. Riley RJ, Kitteringham NR and Park BK, Structural requirements for bioactivation of anticonvulsants to cytotoxic metabolites *in vitro*. *Br J Clin Pharmacol* **28**: 482–487, 1989.
9. Conney AH, Pharmacological implications of microsomal enzyme induction. *Pharmacol Rev* **19**: 317–366, 1967.
10. Park BK and Breckenridge AM, Clinical implications of enzyme induction and enzyme inhibition. *Clin Pharmacokinet* **6**: 1–24, 1981.
11. Brodie MJ, Established anticonvulsants and the treatment of refractory epilepsy *Lancet* **ii**: 350–354, 1990.
12. Finnell, RH, Genetic differences in susceptibility to anticonvulsant drug-induced developmental defects. *Pharmacol Toxicol* **69**: 223–227, 1991.
13. Purba HS, Maggs JL, Orme ML'E, Back DJ and Park BK, The metabolism of 17α-ethinyloestradiol by human liver microsomes: formation of catechol and chemically reactive metabolites. *Br J Clin Pharmacol* **23**: 447–453, 1987.
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
15. Omura T and Sato R, The carbon monoxide binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
16. Riley RJ, Lambert C, Maggs JL, Kitteringham NR and Park BK, An *in vitro* study of the microsomal metabolism and cellular toxicity of phenytoin, sorbinil, and mianserin. *Br J Clin Pharmacol* **26**: 577–588, 1988.
17. Spielberg SP, Acetaminophen toxicity in human lymphocytes *in vitro*. *J Pharmacol Exp Ther* **213**: 395–398, 1980.
18. Regnaud L, Sirois G and Chakrabarti S, Effect of four-day treatment with carbamazepine at different dose levels on microsomal enzyme induction, drug metabolism and drug toxicity. *Pharmacol Toxicol* **62**: 3–6, 1988.
19. Abel SM, Maggs JL, Back DJ, Newby S, Colbert J and Park BK, Cortisol metabolism by human liver *in vitro*. *Br J Clin Pharmacol* **33**: 231P, 1992.
20. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
21. Towbin H and Gordon J, Immunoblotting and dot immunobinding—current status and outlook. *J Immunol Methods* **72**: 313–340, 1984.
22. Guengerich FP, Inhibition of oral contraceptive steroid-metabolizing enzymes by steroids and drugs. *Am J Obstet Gynecol* **163**: 2159–2163, 1990.
23. Guengerich FP, Mechanism-based inactivation of human liver microsomal cytochrome P-450 IIIA4 by gestodene. *Chem Res Toxicol* **3**: 363–371, 1990.
24. Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991.
25. Ryan DE and Levin W, Purification and characterization of hepatic microsomal cytochrome P450. *Pharmacol Ther* **45**: 153–239, 1990.
26. Guengerich FP, Characterization of human cytochrome P450 enzymes. *FASEB J6*: 745–748, 1992.
27. Gonzalez FJ, The molecular biology of cytachrome P450s. *Pharmacol Rev* **40**: 243–288, 1989.
28. Simmons DL, Lalley PA and Kasper CB, Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice. *J Biol Chem* **260**: 515–521, 1985.
29. Shaw PM, Barnes TS, Cameron D, Engeset J, Melvin WT, Omar G, Petrie JC, Rush WR, Snyder CP, Whiting PH, Wolf CR and Burke MD, Purification and characterization of an anticonvulsant-induced human cytochrome P-450 catalysing cyclosporin metabolism. *Biochem J* **263**: 653–663, 1989.
30. Pohl RL, Satoh H, Christ DD and Kenna JG, Immunologic and metabolic basis of drug hypersensitivities *Annu Rev Pharmacol* **28**: 367–387, 1988.
31. Park BK and Kitteringham NR, Assessment of enzyme induction and enzyme inhibition in humans: toxicological implications. *Xenobiotica* **20**: 1171–1185, 1990.
32. Waxman DJ and Azaroff L, Phenobarbital induction of cytochrome P-450 gene expression. *Biochem J* **281**: 577–592, 1992.
33. Yoo J-SH, Hong J-Y, Ning SM and Yang CS, Roles of dietary corn oil in the regulation of cytochromes P450 and glutathione *S*-transferases in rat liver. *J Nutr* **120**: 1718–1726, 1990.
34. Watkins PB, Wrighton SA, Maurel P, Schuetz EG, Mendez-Picon G, Parker GA and Guzelian PS, Identification of an inducible form of cytochrome P-450 in human liver. *Proc Natl Acad Sci USA* **82**: 6310–6317, 1985.
35. Wrighton SA, Schuetz EG, Watkins PB, Maurel P, Barwick J, Bailey B, Hartle HT, Young B and Guzelian P, Demonstration in multiple species of inducible hepatic cytochromes P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. *Mol Pharmacol* **28**: 312–321, 1985.
36. Okey AB, Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther* **45**: 241–298, 1990.
37. Ged C, Rouillon JM, Pichard L, Combalbert J, Bressot N, Bories P, Michel H, Beaune P and Maurel P, The

increase in urinary excretion of 6 beta-hydroxycortisol as a marker of human hepatic cytochrome P450IIIA induction. *Br J Clin Pharmacol* **28**: 373–387, 1989.
38. Park BK and Ohnhaus EE, 6β-Hydroxycortisol. A simple, non-invasive index of enzyme induction. *Arztl Lab* **24**: 1–6, 1983.
39. Ohnhaus EE, Breckenridge AM and Park BK, Urinary excretion of 6β-hydroxycortisol and the time course measurement of enzyme induction in man. *Eur J Clin Pharmacol* **36**: 39–46, 1989.
40. Fleming CM, Branch RA, Wilkinson GR and Guengerich FP, Human liver microsomal N-hydroxylation of dapsone by cytochrome P-4503A4. *Mol Pharmacol* **41**: 975–980, 1992.
41. Eberhardt DC, Gemzik B, Halvorson MR and Parkinson A, Species differences in the toxicity and cytochrome P450 IIIA-dependent metabolism of digitoxin. *Mol Pharmacol* **40**: 859–867, 1991.
42. Maurice M, Pichard L, Daujat M, Fabre I, Joyeux H, Domergue J and Maurel P, Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASEB J* **6**: 752–758, 1992.
43. Bornheim LM and Correia MA, Purification and characterisation of the major hepatic cannabinoid hydroxylase in the mouse: a possible member of the cytochrome P-450IIC subfamily. *Mol Pharmacol* **40**: 228–234, 1991.
44. Smith DA, Species differences in metabolism and pharmacokinetics: are we close to an understanding? *Drug Metab Rev* **23**: 355–373, 1991.
45. Gonzalez FJ, Crespi CL and Gelboin HV, cDNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. *Mutat Res* **247**: 113–27, 1991.
46. Ciaccio PJ and Halpert JR, Characterization of a phenobarbital-inducible dog liver cytochrome P450 structurally related to rat and human enzymes of the P450IIIA (steroid-inducible) gene subfamily. *Arch Biochem Biophys* **271**: 284–299, 1989.
47. Kerr BM, Sanins SM, Levy RH and Thummel KE, Role of P450 3A4 in the 10,11-epoxidation of carbamazepine. *ISSX Abstract (Amsterdam)*: 196, 1991.
48. Wrighton SA, Ring BJ, Watkins PB and VandenBranden M, Identification of a polymorphically expressed member of the human cytochrome P-450III family. *Mol Pharmacol* **36**: 97–105, 1989.
49. Kroemer HK, Beaune P, Henderson CJ, Wolf CR and Heidemann H, Identification of cytochrome P-450 isozymes involved in the metabolism of verapamil. *Naunyn Schmiedebergs Arch Pharmacol* **343** (suppl): R124, 1991.
50. Kroemer HK, Echizen H, Heidemann H and Eichelbaum M, Predictability of the *in vivo* metabolism of verapamil from *in vitro* data: contribution of individual metabolic pathways and stereoselective aspects. *J Pharmacol Exp Ther* **260**: 1052–1057, 1992.
51. Macphee G, McInnes G, Thompson G and Brodie M, Verapamil potentiates carbamazepine neurotoxicity: a clinically important inhibitory interaction. *Lancet* **i**: 700–703, 1986.
52. Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS, Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**: 2171–2178, 1985.
53. Werk EE, MacGee J and Sholiton LJ, Effect of diphenylhydantoin on cortisol metabolism in man. *J Clin Invest* **43**: 1824–1834, 1964.
54. Burstein S and Klaiber EL, Phenobarbital-induced increase in 6-beta-hydroxycortisol excretion: clue to its significance in urine. *J Clin Endocrinol Metab* **25**: 293–296, 1965.
55. Kudriakova TB, Sirota LA, Rozova GI and Gorkov VA, Autoinduction and steady-state pharmacokinetics of carbamazepine and its major metabolites. *Br J Clin Pharmacol* **33**: 611–615, 1992.
56. Dam M, Jensen A and Christiansen J, Plasma level and effect of carbamazepine in grand mal and psychomotor epilepsy. *Acta Neurol Scand* **60** (suppl): 33–38, 1975.
57. Brodie MJ, Forrest G and Rapeport WG, Carbamazepine-10,11-epoxide concentrations in epileptics on carbamazepine alone and in combination with other anticonvulsants. *Br Med J* **16**: 747–749, 1983.
58. Westenberg HGM, Van Der Kleijn E, Oei TT and De Zeeuw RA, Kinetics of carbamazepine and carbamazepine-epoxide determined by the use of plasma and saliva. *Clin Pharmacol Ther* **23**: 320–328, 1978.
59. Kerr BM, Rettie AE, Eddy C, Loiseau P, Guyot M, Wilensky AJ and Levy RH, Inhibition of human liver microsomal expoxide hydrolase by valproate and valpromide: *in vitro/in vivo* correlation. *Clin Pharmacol Ther* **46**: 82–93, 1989.
60. Kerr BM and Levy RH, Unsubstituted amides: a new class of potent inhibitors of human microsomal epoxide hydrolase. *Drug Metab Dispos* **18**: 540–542, 1990.
61. Kutt H, Solomon GE, Dhar AK, Resor SR Jr, Krall RL and Morselli PL, Effects of progabide on carbamazepine epoxide and carbamazepine concentrations in plasma. *Epilepsia* **25**: 674, 1984.
62. Eichelbaum M, Tomson T, Tybring G and Bertilsson L, Carbamazepine metabolism in man. Induction and pharmacogenetic aspects. *Clin Pharmacokinet* **10**: 80–90, 1985.
63. Rane A, Hojer B and Wilson JT, Kinetics of carbamazepine and its 10,11-epoxide in children. *Clin Pharmacol Ther* **19**: 276–283, 1976.