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INFLUENCE OF CARBAMAZEPINE 10,11-OXIDE ON DRUG METABOLIZING ENZYMES*

R. JUNG, P. BENTLEY and F. OESCH†
Institute of Pharmacology, University of Mainz, Obere Zahlbacher Straße 67, 6500 Mainz, F.R.G.

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Abstract—Induction studies with carbamazepine 10,11-oxide (carbamazepine oxide) were carried out with male Sprague–Dawley rats. After 3 and 7 days intraperitoneal application of carbamazepine oxide, the amount of cytochrome P-450 and the activities of monooxygenases and epoxide hydratase in liver microsomes and the glutathione *S*-transferase activity in the 100,000 *g* supernatant fraction were measured. After 3 days of pretreatment with carbamazepine oxide, the specific monooxygenase activity with 7-ethoxycoumarin as substrate was induced by 115 per cent. The specific activity of epoxide hydratase measured with styrene oxide was 46 per cent higher than in the controls. The maximal induction of glutathione *S*-transferase activity was 57 per cent. The amount of cytochrome P-450 in rat liver microsomes was not affected by carbamazepine oxide. The maximal induction of all enzyme activities measured was reached after three days of application. Seven days treatment with carbamazepine oxide resulted in no further induction. Inhibition of epoxide metabolizing enzymes by carbamazepine oxide was also investigated. The enzymatic activities of epoxide hydratase and glutathione *S*-transferases were measured in the absence and after addition of carbamazepine oxide *in vitro*. In order to allow the use of substrate concentrations far below K_m, the epoxide hydratase was measured with a sensitive radiometric assay using ^{14}C-styrene oxide of a high specific radioactivity. Under all conditions studied, carbamazepine oxide showed no significant inhibition of the epoxide hydratase. The glutathione *S*-transferase activity was measured in the 100,000 *g* supernatant fraction of rat liver homogenate with 1-chloro-2,4-dinitrobenzene and glutathione as substrates. Under all conditions tested, only the highest concentration of carbamazepine epoxide (1.0 mM) at unphysiologically low glutathione concentrations (0.25 mM, 0.10 mM) resulted in a borderline significant ($P < 0.05$) inhibition of maximally 19 per cent.

In human and animal organisms a variety of aromatic and olefinic substances are metabolized by monooxygenases to epoxides, which are further metabolized to more water-soluble derivatives by epoxide hydratase and glutathione *S*-transferases [1–8]. The detoxification of electrophilic reactive epoxides by these enzymes is of importance, since for many of these epoxides their mutagenic and/or carcinogenic effect or covalent binding to protein, DNA or RNA has been proven [1–5]. Moreover, in some cases the inactivated products may be activated again by a second epoxidation step [9–11].

Carbamazepine, a drug clinically used in the treatment of epilepsy and trigeminal neuralgia, is converted to carbamazepine oxide, one of the main metabolites in man and rat [12, 13]. It occurs at relatively high concentrations (~ 5 μM) in plasma serum of patients who are being treated with carbamazepine [14]. Previously it has been demonstrated that carbamazepine oxide itself is not mutagenic to *Salmonella typhimurium* [15]. However, epoxides which are harmless *per se* could have indirect effects by inhibition of epoxide metabolizing enzymes leading to accumulation of other epoxides. With this in mind, we have investigated the inhibition of epoxide hydratase and glutathione *S*-transferases by carbamazepine oxide. On the other hand, carbamazepine oxide may positively influence epoxide metabolizing enzymes by induction.‡ This may not only alter the rate of metabolism and thus the pharmacokinetics of the drug but also the concentration of reactive intermediates derived from other foreign compounds and even the pattern of reactive metabolites. Consequently, we have also investigated the inducing effect of carbamazepine oxide upon drug metabolizing enzymes.

MATERIALS AND METHODS

Substrates. Carbamazepine 10,11-oxide and ^{14}C-styrene oxide with a radiospecific activity of 51.6 μCi/mg were synthesized by Ciba-Geigy, Basel. 1-Chloro-2,4-dinitrobenzene and reduced glutathione were purchased from Sigma GmbH, München. 7-Ethoxycoumarin was synthesized by the standard method (Williamson's synthesis) from recrystallized 7-hydroxycoumarin (E. Merck, Darmstadt), which also served as a standard.

Induction experiments. Male Sprague–Dawley rats (180–200 g body wt) which were obtained from Versuchstierzuchtanstalt WIGA, Sulzfeld, F.R.G., were used for the induction experiments with carbamazepine oxide. The results presented were obtained in two independent experiments with 3 and 4 animals for each experimental group of animals. Carbamazepine oxide suspended in sunflower oil was injected i.p. once a day at a dose of 100 mg/kg body wt. The

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† To whom correspondence and reprint requests should be addressed.

‡ The term induction is used in this study to denote an increase in enzyme activity without any implication with respect to the underlying mechanism.

control groups received only sunflower oil. One group of the animals was treated daily for 3 days, another group daily for 7 days. The rats were killed 22 hr after the last injection between 8.00 and 9.00 a.m.

Microsomes and 100,000 *g* supernatant fractions were prepared as described [16]. Epoxide hydratase activity was measured in liver microsomes with styrene oxide as substrate, as described [17]. Monooxygenase activity was determined with a fluorimetric assay using 7-ethoxycoumarin as substrate [18].

The amount of cytochrome P-450 was measured by the method of Omura and Sato [19] with a double beam spectrophotometer Perkin–Elmer 356. Glutathione *S*-transferase activity was measured at 30° in the 100,000 *g* supernatant fraction with a photometric assay using 1-chloro-2,4-dinitrobenzene and reduced glutathione as substrates [20].

Assays for inhibition studies. Epoxide hydratase was purified to apparent homogeneity as described [16], except that the rats were pretreated on 3 consecutive days with 400 mg *trans*-stilbene oxide/kg body wt. The enzymic activity of epoxide hydratase was measured with ^{14}C-styrene oxide in a radiometric assay with an extraction procedure [17]. The amount of added enzyme was always between 1 and 64 μg/ml. For detection of enzyme activity with very low substrate concentrations, ^{14}C-styrene oxide of high specific radioactivity (51.6 μCi/mg) was used. When very low enzyme concentrations were used, styrene oxide was added in only 2 or 5 μl of CH_3CN instead of 10 μl for the standard assay to prevent a disturbing influence of the organic solvent on the enzyme. For the same reason, carbamazepine oxide was dissolved directly in the incubation buffer without organic solvents. The validity of the epoxide hydratase assay at low substrate concentrations was checked by radiochromatography and radioactive isotope dilution analysis to make sure that the radioactivity measured in the assay really corresponded to enzymically formed 1,2-dihydroxy-1-phenyl ethane.

The enzymic activity of glutathione *S*-transferases was measured in the 100,000 *g* supernatant fraction of rat liver homogenate [20]. 1-Chloro-2,4-dinitrobenzene was used since it is a good substrate for several glutathione *S*-transferases. Both 1-chloro-2,4-dinitrobenzene and glutathione concentrations were varied between 1.0 and 0.1 mM. In each case the concentration of one substrate was varied and the other substrate was at a constant concentration of 1 mM. Carbamazepine oxide was dissolved in CH_3CN and added to the total assay volume of 2.6 ml in 20 or 40 μl portions to give a final concentration of 0.5 and 1.0 mM carbamazepine epoxide. Protein concentration were measured according to Lowry *et al.* [21].

RESULTS AND DISCUSSION

Pretreatment of male Sprague–Dawley rats intraperitoneally with carbamazepine oxide resulted in an increase of the specific activities of monooxygenases, epoxide hydratase and glutathione *S*-transferases.

Table 1. Influence of pretreament with carbamazepine oxide on the activities of drug metabolizing enzymes

Pretreatment	Dose (mg/kg)	Epoxide hydratase (nmoles mg protein^{-1}min^{-1})	GSH-transferases (μmoles mg protein^{-1}min^{-1})	Monooxygenases (pmoles mg protein^{-1}min^{-1})	Cytochrome P-450 (nmoles mg protein^{-1})
Control (oil) (3 days)		7.49 ± 0.58*(100)	1.31 ± 0.17 (100)	231 ± 10 (100)	0.71 ± 0.09
Carbamazepine oxide (3 days)	100 i.p.	10.95 ± 0.91†(146)	2.05 ± 0.06†(157)	496 ± 93†(215)	0.73 ± 0.15
Control (oil) (7 days)		7.0 ± 0.9 (100)	1.40 ± 0.16 (100)	294 ± 24 (100)	0.81 ± 0.12
Carbamazepine oxide (7 days)	100 i.p.	9.6 ± 1.9 (137)	2.06 ± 0.18†(147)	591 ± 69†(201)	0.90 ± 0.18

* Specific activities are given ± S.D. The numbers in parentheses give the activity as percentage of control activity.
† Significantly different from control ($P < 0.0025$).

Table 2. Influence of carbamazepine oxide on epoxide hydratase activity with styrene oxide as substrate

	Carbamazepine oxide		
Styrene oxide	0	0.1 mM	0.45 mM
2 mM	507 ± 10*	489 ± 18	512 ± 19
0.3 mM	480 ± 25	475 ± 27	474 ± 26
0.05 mM	202 ± 17	196 ± 13	210 ± 17
0.01 mM	140 ± 16	130 ± 14	131 ± 15

* Specific acitivities in nmoles styrene glycol produced mg protein $^{-1}$min^{-1} ± S.D.

In Table 1 the influence of caramazepine oxide pretreatment on these enzymes is shown.

Three days pretreatment of the animals resulted in an increase of monooxygenase activity of 115 per cent, measured with 7-ethoxycoumarin as substrate, without influencing the amount of cytochrome P-450 in liver microsomes. The specific activity of microsomal epoxide hydratase was increased by 46 per cent. The specific activity of glutathione *S*-transferases, measured with 1-chloro-2,4-dinitrobenzene as substrate, was 57 per cent higher than in the control experiment. For all enzymes studied, the maximal induction was already observed after three days pretreatment with carbamazepine oxide. No greater inducing effect was measured after 7 days application of 100 mg carbamazepine oxide per kg body wt per day.

These results demonstrate that in male Sprague–Dawley rats carbamazepine oxide induces monooxygenase, epoxide hydratase and glutathione *S*-transferase. Thus no selective induction of epoxide metabolizing enzymes has occured. The enzyme induction caused by carbamazepine oxide seems to be similar to the enzyme induction reported to be caused by the parent compound, carbamazepine (see refs. 22–24). Salmona *et al.* [22] used carbamazepine as inducer: 6 days pretreatment of albino CD Charles River rats with a daily dose of 2 × 25 mg/kg body wt resulted in an increased epoxide hydratase activity of 77 per cent over control, measured with styrene oxide as substrate. Monooxygenase activity measured as conversion of styrene to styrene oxide showed no significant change after carbamazepine treatment nor after 3-methylcholanthrene treatment. 3-Methylcholanthrene is a well known, albeit rather selective, inducer of monooxygenases. Investigations of Pacheka *et al.* [23] show that pretreatment of rats with carbamazepine resulted in an increased oxidation of carbamazepine *in vitro* of 115 per cent.

These results suggest that carbamazepine may selectively induce some of the differing forms of monooxygenases. The monooxygenase induction by the metabolite carbamazepine oxide observed in this study may also be of a rather high degree of selectivity, since the increase in monooxygenase activity with ethoxycoumarin as substrate, chosen because it is metabolized by several monooxygenase forms [25], is not accompanied by measurable increase of the total amount of cytochrome P-450.

The induction of the monooxygenase activity was higher than that of both epoxide metabolizing enzymes measured. This could lead *in vivo* to an accumulation of metabolically synthesized reactive epoxides of foreign compounds. Such an accumulation would be potentiated if there were inhibition of epoxide metabolizing enzymes by carbamazepine oxide. Therefore we checked whether carbamazepine oxide has an influence on epoxide hydratase and glutathione *S*-transferases.

The specific activities of epoxide hydratase with and without added carbamazepine oxide are shown in Table 2. The inhibition of epoxide hydratase was never more than 7 per cent of control, which was not statistically significant under the conditions tested ($P < 0.1$). The validity and sensitivity of the assay with very low substrate concentrations was proven by isotope dilution analysis and radiochromatography. Using substrate of high specific radioactivity, we were able to detect very small amounts of enzymically synthesized product.

The enzymic activities of glutathione *S*-transferases (Table 3) were measured in the 100,000 *g* supernatant fraction with and without carbamazepine oxide. At different concentrations of 1-chloro-2,4-dinitrobenzene in the presence of 1.0 mM glutathione, no significant inhibition by carbamazepine oxide of the glutathione *S*-transferases was observed. Varying the concentration of glutathione with constant 1.0 mM 1-chloro-2,4-dinitrobenzene, a borderline significant inhibition of 13 and 19 per cent was observed ($P < 0.05$) only at the lowest concentrations of glutathione used (0.25 and 0.1 mM).

Table 3. Influence of carbamazepine oxide on glutathione *S*-transferase activity

		Carbamazepine oxide		
DNCB* (mM)	GSH† (mM)	0	0.5 mM	1.0 mM
1.0	1.0	1.48‡ ± 0.06	1.46 ± 0.03	1.40 ± 0.08
0.25	1.0	1.08 ± 0.13	0.99 ± 0.11	0.91 ± 0.06
0.1	1.0	0.71 ± 0.04	0.74 ± 0.02	0.68 ± 0.07
1.0	0.5	1.45 ± 0.12	1.40 ± 0.09	1.41 ± 0.1
1.0	0.25	1.0 ± 0.05	0.91 ± 0.04	0.87 ± 0.03§
1.0	0.10	0.53 ± 0.05	0.54 ± 0.02	0.43 ± 0.02§

* 1-Chloro-2,4-dinitrobenzene.
† Reduced glutathione.
‡ Specific activities in μmoles glutathione conjugate produced mg protein^{-1}min^{-1} ± S.D.
§ $P < 0.05$.

In the present inhibition studies we used relatively high concentrations of carbamazepine oxide which were, at maximum, more than 100 times higher than the estimated concentrations (see below) of this epoxide in human liver. After a single application and after long term treatment with carbamazepine in man at a dose of 3–20 mg carbamazepine per kg body wt per day, a plasma concentration of 5.1 ± 3.1 μM carbamazepine oxide in children and 4.9 ± 2.8 μM carbamazepine oxide in adults was measured [14]. These concentrations and the long half-life period of carbamazepine (30–45 hr after single treatment by carbamazepine) lead to the conclusion that a persistent concentration of 5 μmole/kg (5 μM) carbamazepine oxide in the human liver can realistically be assumed if there is no accumulation of the oxide in the liver. This is of course only an assumption, but it probably gives the order of magnitude of carbamazepine 10,11-oxide in the liver. Even at much higher concentrations of carbamazepine oxide used in the present *in vitro* inhibition studies, the activity of epoxide hydratase was not significantly changed. The weak inhibition of glutathione *S*-transferase activity at low glutathione concentrations *in vitro* (0.25 and 0.1 mM) seems unimportant compared with the *in vivo* situation, since physiological concentrations of glutathione in man and in animals are relatively high. In rat liver, glutathione concentrations of 3.8 – 5.6 mmole/kg were measured [26]. A detailed kinetic investigation of the weak inhibition observed is not possible in the glutathione *S*-transferases mixture (1000,000 *g* supernatant fraction), since the different glutathione *S*-transferases have different kinetic parameters.

In the present paper we could show that the relatively stable carbamazepine oxide, which is a major metabolite of carbamazepine, has no major inhibitory influence on glutathione *S*-transferases or epoxide hydratase. The enzymatic activities of glutathione-*S*-transferases, epoxide hydratase and monooxygenases were induced after pretreatment with carbamazepine oxide.

The induction of monooxygenase (ethoxycourmarin *O*-deethylase) activity was greater than that of epoxide hydratase or glutathione *S*-transferase (with 1-chloro-2,4-dinitrobenzene as substrate) activities, showing that drug metabolites may influence metabolic pathways.

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