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THE DESTRUCTION OF CYTOCHROME P-450 BY ALCLOFENAC: POSSIBLE INVOLVEMENT OF AN EPOXIDE METABOLITE

LOUISE M. BROWN and ANTHONY W. FORD-HUTCHINSON*
Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London SE5 8RX, U.K.

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Abstract—The metabolism of alclofenac (4-allyloxy-3-chlorophenyl acetic acid) and its ability to induce destruction of cytochrome P-450 was studied in mouse hepatic microsomes obtained from control animals or animals pretreated with phenobarbitone (PB) or 3-methyl-cholanthrene (3-MC). No evidence was obtained for metabolism of alclofenac in control microsomes although in induced microsomes alclofenac was metabolised to both the dihydroxy (DHA) and phenolic (4-hydroxy-3-chlorophenyl acetic acid: HCPA) metabolites. Significant destruction of cytochrome P-450 was observed when alclofenac was incubated with microsomes from mice pretreated with PB but not from untreated or 3-MC-treated mice. This destruction is dependent on the presence of a NADPH-regenerating system and is inhibited in the presence of SKF525A, metyrapone, glutathione and cysteine. The stable metabolites DHA and HCPA caused no loss of cytochrome P-450 whereas the reactive intermediate, alclofenac epoxide, was a potent inducer of destruction in the absence of NADPH. These results suggest that destruction of cytochrome P-450 by alclofenac *in vitro* is mediated, at least in part, through the formation of a reactive epoxide metabolite.

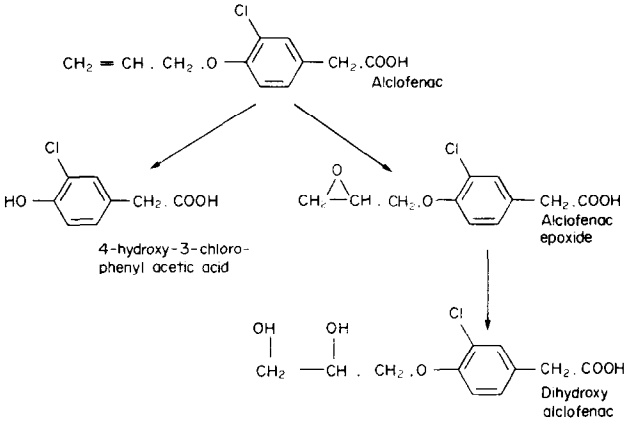

Fig. 1. The structure of alclofenac and its metabolites.

Alclofenac is an anti-inflammatory and analgesic drug containing an allyl group. It is metabolised to a dihydroxy metabolite [1] via an epoxide intermediate which has been identified in the urine of the mouse [2] and man [3] (Fig. 1). The properties of the epoxide have been investigated in a number of *in vitro* and *in vivo* tests and the compound has been shown to have mutagenic effects in the Ames test and to induce cell transformation in Syrian hamster cells [4]. Many compounds containing allyl groups, such as allylisopropylacetamide (AIA) [5], allylbarbiturates [6], fluoroxene [7] and vinyl chloride [8], have been shown to destroy cytochrome P-450 *in vitro* and *in vivo*. Destruction of cytochrome P-450 requires NADPH and molecular oxygen, is increased by PB-pretreatment and inhibited by carbon monoxide and inhibitors of drug metabolism [5–8], demonstrating that metabolism of the compounds is necessary. Destruction is also dependent on the presence of the allyl group [6, 9, 10]. Epoxides have been identified as metabolites of many compounds containing allyl groups [11, 12] and it has been postulated that these reactive metabolites mediate the destruction of cytochrome P-450. However, incubation of the microsomes with the epoxides of vinyl chloride [13], AIA [14] and some olefins [15] does not produce sufficient destruction to account for that caused by the parent compounds and it has been suggested that the epoxides are not involved. In support of this, glutathione, which is expected to conjugate with epoxides, does not inhibit the destruction caused by AIA [16]. In the present study we have examined the destruction of cytochrome P-450 by alclofenac and have studied the role of the epoxide metabolite in this process.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. Alclofenac (4-allyloxy-3-chlorophenyl acetic acid), dihydroxyalclofenac (DHA), 4-hydroxy-3-chlorophenyl acetic acid (HCPA) and 3-chloro-4(2',3'-dihydroxy)-propoxybenzoic acid were obtained from Berk Pharmaceuticals (Shalford, U.K.). Alclofenac epoxide was provided by Continental Pharma (Machelen, Belgium) and was stored under nitrogen in sealed vials at −20° until required. Flurbiprofen (2-[fluoro-4-biphenyl] propionic acid) was a gift from The Boots Co. Ltd. (Nottingham, U. K.), allylisopropylacetamide (AIA) a gift from Roche Products, Ltd. (Welwyn Garden City, U.K.) and SKF525A a gift from Smith, Kline and French Labs., Ltd. (Welwyn Garden City, U.K.). Ethereal diazomethane was prepared from nitrosotoluene sulphonamide by use of a Diazald kit obtained from Aldrich (Gillingham, U.K.). Metyrapone, bis(trimethylsilyl) trifluoroacetamide (BSTFA), NADP and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, D-glucose-6-phosphate: $NADP^+$ 1-oxido reductase), (Bakers' yeast, Type VII) were obtained from the Sigma Chemical Co. (Poole, U.K.).

Treatment of animals. Male CBA mice (25–30 g) were used in all experiments. Mice were pretreated by intraperitoneal injection with either PB (80 mg/kg) on days 1, 2 and 3 and killed on day 4 or 3-MC (80 mg/mg, suspended in arachis oil) on day 1 and killed on day 3.

Preparation of microsomes. Animals were killed by overdose of diethyl ether and the livers were removed immediately and washed in 0.9% (w/v) sodium chloride solution. The livers were sliced and a 30% (w/v) homogenate prepared in 0.25 M sucrose. The cell debris was removed by centrifugation (2000 *g* for 15 min at 4°) and the supernatant centrifuged at 9000 *g* for 20 min at 4°. The microsomes in the supernatant were aggregated with 10 mM calcium and magnesium chloride and pelletted by centrifugation (1500 *g* for 20 min at 4°) as described by Kamath, Kummerow and Narayow [17]. The microsomal pellet was washed in 0.15 M Tris buffer (pH 8.0), then resuspended in 0.1 M Tris (pH 7.4) and used immediately. Protein was determined by the method of Lowry *et al.* [18].

Incubation of microsomes with drugs. All incubations were carried out in a shaking water bath at 37° in 0.1 M Tris buffer pH 7.4 containing microsomes, 2.5 mM glucose-6-phosphate, 2.5 mM $MgCl_2$ 0.5 mM $NADP^+$ and 0.33 units/ml of glucose-6-phosphate dehydrogenase. Microsomal protein (6 mg) was suspended in a total volume of 3 ml for metabolism and 6 ml for the studies on the destruction of cytochrome P-450. Drugs were dissolved in dimethylsulphoxide (final concentration 0.1% v/v). The mixture was preincubated at 37° for 3 min prior to initiation of the reaction with $NADP^+$. In experiments carried out in the absence of $NADP^+$ the reaction was initiated by the addition of microsomes.

Identification of alclofenac and its metabolites. Alclofenac was incubated with the microsomes for 30 min and the reaction terminated by acidification to pH 1.0 with 1 M HCl. The internal standards, 200 μl of 2 mg/ml flurbiprofen and 200 μl of 1 mg/ml 3-chloro-4(2',3'-dihydroxy)-propoxybenzoic acid dissolved in methanol, were added and the microsomal suspension (3 ml) extracted twice with redistilled chloroform. The resulting emulsion was centrifuged (1000 *g*) and the chloroform layer separated and evaporated to dryness under reduced pressure at 37°. The residue was taken up in 0.5 ml methanol, transferred to a 1 ml reactivial and dried down under a stream of nitrogen. The residue was then redissolved in 50 μl of methanol and the mixture methylated by addition of an excess of diazomethane for 15 sec. After drying down, the sample was further derivatised by the addition of 100 μl BSTFA. One μl of the derivatised sample was injected onto a 25 M OV101 SCOT column installed in a Perkin–Elmer F17 gas chromatograph fitted with a flame ionisation detector and a glass-lined injector (13:1 split). Using an optimum nitrogen flow rate the column was run at an initial temperature of 210° for 10 min followed by a temperature increase to 240° (at 10°/min). Under these conditions alclofenac, DHA, HCPA and alclofenac epoxide could all be detected. Calibration curves were constructed by spiking microsomal suspensions with alclofenac and its metabolites. Flurbiprofen was used as an internal standard for alclofenac and HCPA (correlation coefficients of > 0.99 for a range of alclofenac from 100 to 1000 μM and HCPA over a range of 3–30 μM) and 3-chloro-4(2',3'-dihydroxy)-propoxybenzoic acid as an internal standard for DHA (correlation coefficients of > 0.99 for a range of 70–700 μM).

Table 1. Metabolism of alclofenac by liver microsomes obtained from rats pretreated with either phenobarbitone (PB) or 3-methylcholanthrene (3-MC)

	PB		3-MC	
Alclofenac Conc. (μM)	DHA	HCPA	DHA	HCPA
350	148±23	16±1	nd	nd
700	297±36	19±2	288±67	13±3
1000	277±32	27±2	239±34	18±4

Microsomal suspensions were incubated for 30 min with alclofenac in the presence of an NADPH regenerating system. Results are expressed as concentration of metabolite formed (μM) ± S.E.M. and are obtained from 8 determinations carried out on two separate microsomal preparations.
nd = not determined.

Table 2. The effect of SKF525A and metyrapone on the destruction of cytochrome P-450 induced by alclofenac in microsomes obtained from mice pretreated with PB

Addition to incubation mixture	% destruction
1mM alclofenac	16.4
1mM alclofenac + 1mM SKF525A	10.2 ($P < 0.01$)
1mM alclofenac + 1mM metyrapone	9.3 ($P < 0.01$)

Results are expressed as per cent destruction of cytochrome P-450 (mean of 10–15 determinations) after 30 min incubation with alclofenac + NADPH when compared to the destruction induced in the presence of NADPH alone. Statistical analysis by paired *t*-test.

Assay for cytochrome P-450. After incubation of drugs with microsomes the reaction was terminated by the addition of menadione in 100 μl methanol to produce a final concentration of 0.1 mM. The samples were then placed on ice and the cytochrome P-450 levels were determined by difference spectroscopy accoding to the method of Omuro and Sato [19]. All experiments were carried out on at least two separate preparations of microsomes.

RESULTS

Metabolism of alclofenac in mouse microsomes. No significant metabolism of alclofenac was detected in microsomes obtained from control mice. In PB- and 3-MC-pretreated mice alclofenac was converted to either DHA or HCPA (Table 1). Maximal conversion of alclofenac to the major metabolite, DHA, was observed using a substrate concentration of 700 μM. The quantities of DHA and HCPA formed accounted for > 95% of the alclofenac lost during the incubation suggesting that no significant amounts of other metabolites were produced. No alclofenac epoxide was detected at the end of the incubation procedure. Previous work has demonstrated that alclofenac epoxide is not destroyed during the extraction and derivatisation procedures used [3].

Destruction of cytochrome P-450. In the studies on the destruction of cytochrome P-450, 1 mM AIA was used as a positive control. In microsomes from control mice, both alclofenac and AIA produced a small statistically significant destruction of cytochrome P-450 (at 5 min only) when compared with microsomes incubated in the absence of drug (Fig. 2a). Similar experiments using microsomes from PB-pretreated mice showed that both alclofenac and AIA induced significant destruction of cytochrome P-450 at a concentration of 1 mM (Fig. 2b). In contrast, neither alclofenac nor AIA induced destruction in microsomes from 3-MC-pretreated mice (Fig. 2c). Further experiments designed to define the mechanisms of alclofenac induced destruction of cytochrome P-450 were all carried out using microsomes from PB-treated mice. There was no evidence for

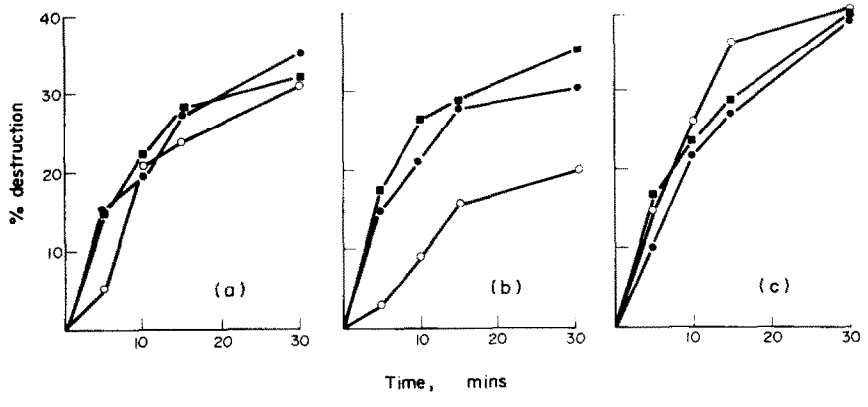

Fig. 2. The destruction of cytochrome P-450 in (a) control, (b) PB-pretreated, and (c) 3-MC pretreated mouse microsomes. Concentrations of cytochrome P-450 in the three separate preparations were 1.53 ± 0.12, 3.45 ± 0.20 and 2.29 ± 0.06 nmoles/ml. Results are expressed as percentage destruction of cytochrome P-450 in the presence of NADPH alone (○), NADPH + 1 mM alclofenac (●) and NADPH + 1 mM AIA (■). Destruction induced by either alclofenac or AIA was significantly different ($P < 0.05$) to that induced by NADPH alone at all time intervals using microsomes from PB-pretreated mice and at 5 min only using microsomes from control mice (paired *t*-test). Each point is the mean from four separate experiments using different microsomal preparations ($n = 4$–6).

Table 3. The effect of glutathione and cysteine on the destruction of cytochrome P-450 induced by alclofenac in microsomes obtained from mice pretreated with PB

Addition to incubation mixture	% destruction
1mM alclofenac	8.3
1mM alclofenac + 1mM glutathione	3.2 ($P < 0.01$)
1mM alclofenac + 1mM cysteine	0 ($P < 0.01$)

Results are expressed as per cent destruction of cytochrome P-450 (mean of 10–15 determinations) after 30 min incubation with alclofenac + NADPH when compared to the destruction induced in the presence of NADPH alone. Statistical analysis by paired *t*-test.

the formation of cytochrome P-420 during alclofenac-mediated destruction of cytochrome P-450 and the destruction was unaltered in the presence of 1.5 mM EDTA. These experiments indicate that alclofenac does not act by enhancing lipid peroxidation. Destruction of cytochrome P-450 by alclofenac was inhibited by 1 mM metyrapone and 1 mM SKF525A (43 and 39% inhibition, respectively), (Table 2). Addition of 1 mM cysteine completely blocked the destruction of cytochrome P-450 and addition of 1 mM glutathione inhibited destruction by 41% (Table 3). In the absence of an NADPH-regenerating system neither alclofenac nor its stable metabolites DHA and HCPA induced destruction of cytochrome P-450. In contrast alclofenac epoxide was a potent inducer of cytochrome P-450 destruction producing a significant effect over the concentration range 1 μM–1 mM with no formation of cytochrome P-420. This is shown in Fig. 3 where the destruction induced by the epoxide in the absence of an NADPH-regenerating system is compared to the destruction induced by the parent compound in the presence of the NADPH-regenerating system. The epoxide produces significantly more destruction of cytochrome P-450 than the parent compound at concentrations of 10 μM, 100 μM and 1 mM.

DISCUSSION

The results reported in the present work on the metabolism of alclofenac in microsomes obtained from PB- and 3-MC-pretreated mice, agree with previous *in vivo* data which has demonstrated that rodents extensively hydroxylate the drug [1]. There was little difference between either source of microsomes indicating that alclofenac may be metabolised by either cytochrome P-450 or P-448. No metabolism was detected in microsomes from control mice and one explanation may be that the extent of metabolism is so small that the amounts of metabolites are not detectable in the assay system.

The present work demonstrates that alclofenac, as has been reported for a number of other compounds containing allyl groups [5–8], destroys cytochrome P-450 *in vitro* in microsomes obtained from mice pretreated with PB. The destruction is depen-

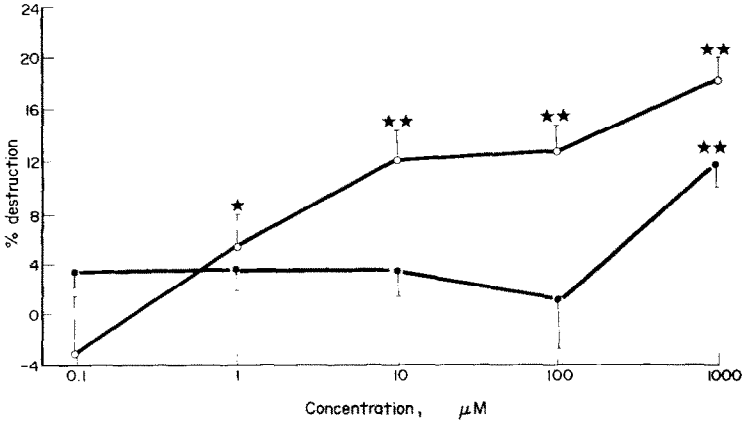

Fig. 3. The effects of varying doses of alclofenac + NADPH (●) and alclofenac epoxide in the absence of NADPH (○) on the destruction of cytochrome P-450 in microsomes from phenobarbitone-pretreated mice after 30 min incubation. Results are expressed as per cent destruction (± S.E.M.) of cytochrome P-450 induced by either alclofenac when compared with microsomes + NADPH or alclofenac epoxide compared with microsomes without NADPH. *$P < 0.05$ and **$P < 0.01$ when compared to control (paired *t*-test). Each point is the mean of experiments from two or three separate preparations of microsomes (n = 10–15).

dent upon metabolism of alclofenac because the effect is increased by PB-pretreatment, is NADPH dependent and is inhibited by SKF525A and metyrapone. No significant destruction of cytochrome was observed in microsomes obtained from 3-MC-treated mice despite the fact that alclofenac is extensively metabolised in this system. This suggests that cytochrome P-448 is not destroyed during metabolism of alclofenac. A similar result has been observed with AIA [20, 21] although fluoroxene destroys both cytochrome P-450 and P-448 [21].

One possible mechanism for the destruction of cytochrome P-450 induced by alclofenac is through the actions of a reactive epoxide metabolite. Some of the properties of the epoxide metabolite of alclofenac have been studied and it shows activities both *in vitro* and *in vivo* characteristic of an alkylating agent. Thus, it is mutagenic in the Ames test, acts as a cell transforming agent when incubated with Syrian hamster cells, forms a conjugate with cysteine, acts as a sensitising agent in the guinea-pig and irreversibly inactivates yeast alcohol dehydrogenase [4]. No epoxide was detected in the microsomal metabolism studies. This does not preclude a role for the epoxide in the destruction of cytochrome P-450 as any epoxide would be expected to be rapidly removed in one of three ways: (a) conversion to DHA by either a microsomal or cytosolic epoxide hydrolase [22], (EC 3.3.2.3 epoxide hydratase); (b) conjugation with an amino acid such as glutathione either enzymatically by glutathione-S-transferase (EC 2.5.1.18 Rx: glutathione-R-transferase) or non-enzymatically; or (c) covalent linkage to a macromolecule. It was originally suggested that destruction of cytochrome P-450 by other compounds containing allyl groups was caused by reaction with epoxide metabolites. More recently evidence has been presented to suggest that the destruction of cytochrome P-450 is not mediated by the epoxides but by a reactive species formed prior to the epoxide. The main evidence against the role of epoxides is that incubation of microsomes with the epoxides of vinyl chloride [13], AIA [14] and some olefins [15] produces little or no decrease in cytochrome P-450. This inactivity of the epoxides cannot be explained by rapid metabolism or by failure to bind to cytochrome P-450 [15]. It has been suggested that destruction of cytochrome P-450 is an inherent property of carbon–carbon double bonds and that no other substrate feature is mechanistically essential to the process [15].

The results of the present study demonstrate that an epoxide may mediate destruction of cytochrome P-450. Alclofenac epoxide was a potent inducer of cytochrome P-450 destruction and was more potent on a molar basis than the parent compound. Further evidence for the role of alclofenac epoxide in the destructive process was provided by the inhibition of alclofenac mediated destruction by cysteine and glutathione. These compounds would be expected to conjugate with the epoxide [23] and in this context a conjugate of alclofenac epoxide with cysteine has already been identified [4]. In contrast glutathione does not inhibit destruction induced by AIA [16]. These results strongly suggest that alclofenac epoxide participates in the destruction of cytochrome P-450 occurring during the metabolism of alclofenac. The possible involvement of other unknown metabolites of alclofenac cannot however be excluded. The mechanism by which the epoxide induces destruction is not clear and may involve either linkage with haem as has been demonstrated for other compounds with allyl groups [24] or by alkylation of the apoprotein moiety. In order to clarify the mechanism it will be necessary to look for the presence of 'green pigments' which have been found in microsomes treated with AIA [24] and which are due to the formation of an adduct between the substrate and the haem [24].

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