IN VIVO PERTURBATION OF RAT HEPATOCYTE CANALICULAR MEMBRANE FUNCTION BY DICLOFENAC

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ABSTRACT:
Clinical use of diclofenac is associated with a small but significant incidence of hepatotoxicity. It has been reported that in vivo diclofenac treatment results in decreased activity of the extracellular canalicular membrane protein dipeptidylpeptidase IV in rats as a consequence of protein adduct formation by its electrophilic metabolite diclofenac acyl glucuronide. The present study has investigated the effects of in vivo diclofenac treatment (15 mg/kg/day for 7 days) on the activity of another four rat extracellular canalicular membrane proteins. Animals administered diclofenac (n = 6) had 47.9, 60.4, and 51.6% lower (p < 0.05) canalicular activities of γ-glutamyltransferase, Mg2+-ATPase, and leucine aminopeptidase, respectively, compared with controls (n = 6), but there was no difference in alkaline phosphatase activity. In general, protein adduct formation by acyl glucuronides has been associated with decreased protein function, and the lower canalicular enzyme activities in diclofenac-treated rats may suggest that γ-glutamyltransferase, Mg2+-ATPase, and leucine aminopeptidase are also targets of adduct formation by acyl glucuronide metabolites of diclofenac. However, intracellular redistribution and/or decreased synthesis of these enzymes would also be consistent with our results. The ability of diclofenac acyl glucuronide (200 μg/ml) to form covalently bound adducts with γ-glutamyltransferase (10 mg/ml) was demonstrated following in vitro incubations (16 h, pH 7.4, and 37°C) in which 20.7 ± 2.1 ng of diclofenac were covalently bound per milligram of protein. In these in vitro studies, the low concentration of protein adducts formed was not associated with any significant change in γ-glutamyltransferase activity.

Like other nonsteroidal anti-inflammatory drugs, clinical use of diclofenac has been associated with a small but significant incidence of hepatotoxicity, ranging from mild, asymptomatic, reversible increases in liver function tests to jaundice and hepatitis, including several reports of fatal hepatitis (Breen et al., 1986; Helfgott et al., 1990; Purcell et al., 1991; Banks et al., 1995). In many cases, the clinical and biochemical features of diclofenac hepatotoxicity suggest the involvement of reactive or toxic metabolites (Purcell et al., 1991; Banks et al., 1995).

In humans and rats, diclofenac undergoes both oxidative and conjugative metabolism, and formation of acyl glucuronides accounts for a significant fraction of total metabolism (Degen et al., 1978). Acyl glucuronide conjugates are reactive electrophilic compounds that readily undergo a number of nonenzymatic reactions, including hydrolysis to reform the parent aglycone, intramolecular rearrangement, and formation of covalently bound adducts with endogenous proteins (Sallustio et al., 2000). Diclofenac forms covalently bound adducts with a number of rat canalicular membrane proteins in vivo (Hargus et al., 1994). A 110-kDa extracellular canalicular membrane protein, dipeptidylpeptidase IV, has been identified as one of the targets of in vivo adduct formation by diclofenac glucuronide, and in vivo adduct formation has been associated with decreased dipeptidylpeptidase IV activity (Hargus et al., 1995). Many in vitro studies indicate that, in general, acyl glucuronide-mediated adduct formation with proteins, such as albumin, tubulin, UDP-glucuronosyltransferases, and superoxide dismutase, is associated with decreased protein function (Bailey et al., 1998; Chiou et al., 1999; Terrier et al., 1999). Hence, it is likely that adduct formation with other extracellular canalicular membrane proteins may similarly result in decreased activities. In this study, we have investigated the effects of in vivo diclofenac treatment on the activities of four rat extracellular canalicular proteins, γ-glutamyltransferase, ecto-ATPase, leucine aminopeptidase, and alkaline phosphatase. In the previous report of decreased dipeptidylpeptidase IV activity (Hargus et al., 1995), the dose of diclofenac (200 mg/kg) approached its LD50 in rats (250 mg/kg) (Menasse et al., 1978) so that changes in enzyme activity might have been expected, irrespective of protein adduct formation. In this study, a diclofenac dose of 15 mg/kg was chosen since this lower dose also forms canalicular membrane protein adducts in vivo (Hargus et al., 1994) but is 17 times lower than the LD50, is below the threshold dose required to produce significant gastrointestinal bleeding in rats (Menasse et al., 1978), and is only 5-fold higher than maximum daily clinical doses. In addition, in vitro studies with diclofenac glucuronide were also carried out to investigate its reactivity and ability to bind covalently to commercially available γ-glutamyltransferase.

Materials and Methods

In Vivo Effects of Diclofenac on Canalicular Enzyme Activities. The studies were approved by the animal ethics committee of The Queen Elizabeth Hospital and were carried out in accordance with the guidelines established by...
the National Health and Medical Research Council of Australia. Diclofenac sodium salt was purchased from Sigma Chemical Co. (Castle Hill, NSW, Australia), and a 3 mg/ml diclofenac suspension was prepared in 0.5% methyl cellulose. Male Sprague-Dawley rats (200–250 g) were treated for 7 days by oral gavage with either vehicle (control group, n = 6) or 15 mg/kg diclofenac (n = 6). On day 8, the animals were sacrificed; blood was collected, and plasma separated and stored at −20°C for liver function tests. Livers were excised for the immediate preparation of canicular membranes, as described by Edwards et al. (1994). Liver homogenate and canicular membranes were stored at −80°C, and the activities of alkaline phosphatase, leucine aminopeptidase, Mg2+-ATPase, Na+/K+-ATPase, NADPH cytochrome c reductase, and succinate cytochrome c reductase were determined within 12 h, as previously described (Edwards et al., 1994). γ-Glutamyltranspeptidase activity was also assessed within 12 h using a commercially available diagnostic kit (Sigma Chemical Co.). Liver homogenate and canicular protein concentrations were assessed according to the method of Lowry et al. (1951). Measurement of plasma albumin, total bilirubin, alanine transaminase, alkaline phosphatase, aspartate transaminase, and γ-glutamyltranspeptidase was carried out by the Clinical Chemistry Unit of The Queen Elizabeth Hospital on an Axon analyzer (Technicon Instruments, Tarrytown, NY) using standard Technicon methods.

In Vitro Covalent Binding of Diclofenac Acyl Glucuronide to γ-Glutamyltransferase. Diclofenac glucuronide was prepared from bile collected during perfusion of an isolated rat liver with diclofenac. During perfusion, bile was collected into a vial containing 500 μl of 1.0 M glycine buffer, pH 3.0, and stored at −20°C until analysis. Chromatographic purification was carried out based on a previously published method (Sallustio and Fairchild, 1995) using a cyano column (Alltech cyanopropyl 5 μm, 4.6 × 250 mm; Alltech Associates, Inc., Deerfield, IL) with a mobile phase consisting of 32% acetonitrile in 30 mM tetraethylammonium hydrogen sulfate (final pH 3.5) pumped at 1.0 ml/min and UV detection at 280 nm. Aliquots of bile were injected directly onto the high-pressure liquid chromatograph, eluent corresponding to diclofenac glucuronide was collected, and the acetonitrile present evaporated under a stream of nitrogen at room temperature. Solid phase cartridges (C18 Sep-Pak; Waters Corporation, Milford, MA), which had been pretreated with 5 ml of 1% glacial acetic acid in acetonitrile and 5 ml of 0.1 M phosphate buffer, pH 2.7, were used for the concentrated high-pressure liquid chromatography eluent sample (5 ml). The cartridges were then washed with 5 ml of 0.1 M phosphate buffer, pH 2.7, and the glucuronide conjugate was eluted with 5 ml of 1% glacial acetic acid in acetonitrile, dried under a stream of nitrogen at room temperature, and stored at −20°C. The purified diclofenac glucuronide was present entirely in the 1-OH-configuration, as determined by β-glucuronidase and NaOH hydrolyses.

Diclofenac acyl glucuronide or diclofenac (200 μg/ml) were incubated with 10 mg/ml γ-glutamyltransferase (2.5 U/mg; Sigma Chemical Co.) in 2 ml of 0.1 M phosphate buffer, pH 7.4, for 16 h at either 37°C or −20°C. The extent of adduct formation with γ-glutamyltransferase was quantitated by protein precipitation and extensive washing of the protein pellet to remove any noncovalently bound drug, as previously described (Sallustio and Foster, 1995). After the washes, covalently bound diclofenac was liberated by hydrolysis with KOH and quantitated essentially according to the method of Schmitz et al. (1993) using flurbiprofen as internal standard. Chromatographic separation was achieved using an RP-Select B column (5 μm, 125 × 4 mm; Merck, Darmstadt, Germany) with mobile phase (46% acetonitrile and 0.5% glacial acetic acid in distilled water) pumped at 1.0 ml/min and UV detection at 280 nm. Under these conditions, the retention times of flurbiprofen and diclofenac were 6.5 and 8.0 min, respectively. Calibration curves (25–500 ng/ml) were linear with intersay coefficient of variation and percentage bias for the lowest calibrator of 15.7 and −3.8%, respectively (n = 5).

Statistical Analyses. All statistical analyses were carried out using the nonparametric Mann-Whitney U test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA).

Results and Discussion

As determined by relative enrichment of marker enzymes, there was negligible contamination of canicular membranes by sinusoidal, microsomal, or mitochondrial membranes (Table 1), and enzyme activities in membranes prepared from control rats (Table 2) were similar to those reported by other laboratories (Edwards et al., 1994). Diclofenac treatment had a profound effect on the activities of three of the four canicular membrane enzymes studied. Rats treated with diclofenac had 47.9, 60.4, and 51.6% lower canalicular membrane enzyme activities compared with controls (Table 2). The lack of effect of diclofenac treatment on alkaline phosphatase activity compared with controls (Table 2). The lack of effect of diclofenac treatment on alkaline phosphatase activity compared with controls (Table 2). The lack of effect of diclofenac treatment on alkaline phosphatase activity compared with controls (Table 2). The lack of effect of diclofenac treatment on alkaline phosphatase activity compared with controls (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>Diclofenac</th>
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<tbody>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>355 ± 15</td>
<td>219 ± 8</td>
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<tr>
<td>Total bilirubin (μmol/l)</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
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<tr>
<td>Albumin (g/l)</td>
<td>34.2 ± 0.5</td>
<td>32.8 ± 1.4</td>
</tr>
<tr>
<td>Aspartate transaminase (U/l)</td>
<td>535 ± 53</td>
<td>217 ± 21</td>
</tr>
<tr>
<td>Glutathione transferase (U/l)</td>
<td>35 ± 5</td>
<td>35 ± 5</td>
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* p < 0.05 compared with control (Mann-Whitney U test).
dypleptidase IV, and a number of ATP-dependent transporter proteins as a result of disruption and internalization of canaliculuar membrane fragments (Rost et al., 1999). Bile duct ligation in rats has also been associated with decreased localization of dipetidylpeptidase IV and ecto-ATPase to canaliculuar membranes and intracellular accumulation as a result of altered delivery of newly synthesized proteins to the canaliculuar domain (Barr and Hubbard, 1993; Stieger et al., 1994). In this study, a lower canaliculuar enzyme activity in the absence of a corresponding change in total homogenate activity may indicate redistribution of a canaliculuar membrane protein and would be observed as a lower relative enzyme enrichment of that protein in the canaliculuar membranes of diclofenac-treated animals compared with controls. No difference in the relative enrichment of leucine aminopeptidase or Mg$^{2+}$-ATPase, which reflects, in part, ecto-ATPase activity, was observed between the two treatment groups (Table 1). In contrast, the relative enrichment of $\gamma$-glutamyltransferase activity in canaliculuar membranes from diclofenac-treated rats was significantly lower than that in controls, suggesting redistribution as a possible mecha-nism (Table 1). However, at the diclofenac dose rate chosen for this study, there was no biochemical evidence of cholestasis, as may be reflected by raised plasma bilirubin concentrations or raised plasma alkaline phosphatase, aspartate transaminase, $\gamma$-glutamyltransferase, or alanine transaminase activities (Table 2).

Despite the use of a dose 5-fold higher than that administered clinically, the only physical sign of toxicity was a small but significant difference in weight gain between the two groups of rats, with the mean $\pm$ S.E.M. body weight of control rats increasing by 16.5 $\pm$ 0.8% compared with diclofenac-treated rats, which gained 6.0 $\pm$ 1.5% ($p < 0.05$) in weight during the study. Diclofenac-treated animals also had 18.7% lower plasma albumin concentrations compared with controls and had 38.9 and 37.5% lower plasma activities of alkaline phosphatase and aspartate transaminase, respectively, compared with controls (Table 2). Alkaline phosphatase is not a liver-specific enzyme and is present in high concentrations in osteoblasts so that plasma activity can reflect growth rate (Ringer and Dabich, 1979). Thus, compared with controls, the slightly lower growth rates of diclofenac-treated rats may have contributed to their lower plasma alkaline phosphatase activity. However, alanine transaminase and albumin are synthesized by the liver. Therefore, lower plasma concentrations of activities in diclofenac-treated animals are consistent with both decreased hepatic synthesis and/or decreased protein function, as might result from adduct formation. Interestingly, albumin adduct formation by acyl glucuronides has been well documented (Spahnh-Langguth and Benet, 1992; Sallustio et al., 2000), as has decreased function following adduct formation (Chiou et al., 1999).

To examine whether protein adduct formation by diclofenac glucuronide might directly contribute to decreased canaliculuar enzyme activities, we incubated $\gamma$-glutamyltransferase at physiological pH and temperature with diclofenac glucuronide or diclofenac. Only diclofene-ac glucuronide formed covalently bound adducts with a mean $\pm$ 2 S.E.M. of 20.7 $\pm$ 2.1 ng of diclofenac covalently bound/mg of protein (n = 5). Negligible adduct formation was found in samples stored frozen for 16 h, indicating that the extensive solvent washes effectively removed all noncovalently bound diclofenac and diclofenac glucuronide. The extent of covalently bound protein adduct formation corresponded to approximately 4 mol of adduct/mmol of protein, and not surprisingly, this low level of adduct formation had no statistically significant effect on $\gamma$-glutamyltransferase activity (data not shown). Although the concentration of diclofenac glucuronide used in the in vitro incubations was approximately 100-fold higher than the concentrations of acyl glucuronides usually attained in plasma following clinical doses (Spahn-Langguth and Benet, 1992), studies using isolated perfused rat livers demonstrate that, as a result of carrier-mediated hepatic membrane transport, concentrations of acyl glucuronides in rat bile can be up to 5000 times greater than those in circulating perfusate (Sabordo et al., 1999). Therefore, the in vitro incubation concentration of diclofenac glucuronide would have been within the range of in vivo concentrations expected in bile. However, the duration of in vitro exposure (16 h) was much less than that in vivo (7 days). Diclofenac glucuronides form covalently bound adducts with a number of rat canaliculuar membrane proteins in vivo (Hargus et al., 1994), but the degree of adduct formation has not been quantitated. Thus, despite the negative result in this pilot study, adduct formation cannot be excluded as a possible cause of decreased enzyme function in vivo.

Interestingly, the most frequent clinical manifestation of diclofenac hepatotoxicity is elevation of liver enzymes in plasma, most of which originate at the hepatocyte canaliculuar membrane. In addition, other features of clinical hepatotoxicity, such as raised plasma bilirubin, jaundice, and cholestasis, also suggest alterations to canaliculuar membrane transporter functions. Our data demonstrate that in rats diclofe-nac causes significant changes in canaliculuar enzyme activities in the absence of elevated concentrations of plasma markers typically indicative of liver dysfunction. Further studies are still required to inves-tigate the mechanism(s) underlying these changes, to determine whether inhibition of canaliculuar enzyme activity is a general property of all nonsteroidal anti-inflammatory drugs and whether it contributes to the clinical hepatotoxicity of these agents.

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