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, clinical 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 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. This 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 canaliculur membranes, as described by Edwards et al. (1994). Liver homogenate and canaliculur 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-Glutamyltransferase activity was also assessed within 12 h using a commercially available diagnostic kit (Sigma Chemical Co.). Liver homogenate and canaliculur 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-glutamyltransferase 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, 1993), using a cyano column (Alltima cyano; 5 μm, 4.6 × 250 mm; Alltech Associates, Inc., Deerfield, IL) with a mobile phase consisting of 32% acetonitrile in 30 mM tetrabutylammonium 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 loaded with 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-O-β-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, 4.6 × 125 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 interassay 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 canaliculur 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 canaliculur membrane enzymes studied. Rats treated with diclofenac had 47.9, 60.4, and 51.6% lower canaliculur membrane activities of γ-glutamyltransferase, Mg2+-ATPase, and leucine aminopeptidase, respectively (p < 0.05) but showed no significant difference in canaliculur alkaline phosphatase activity compared with controls (Table 2). The lack of effect of diclofenac treatment on alkaline phosphatase activity suggests some selectivity in its action on canaliculur membrane function. Thus, it was unlikely that the lower enzyme activities were due to selective loss of canaliculur membrane during sample preparation, particularly as the results were expressed taking into account total membrane protein concentrations, which did not differ significantly between control and diclofenac-treated rats, with mean ± S.E.M. protein concentrations of 1.32 ± 0.22 and 1.84 ± 0.45 mg/ml, respectively (p > 0.05).

A number of processes could have contributed to the lower canaliculur enzyme activities in diclofenac-treated rats including: 1) redistribution of proteins from the canaliculur membrane to other intracellular domains, 2) decreased protein synthesis, or 3) decreased activity as a result of adduct formation. It has been reported that several models of cholestasis are associated with redistribution of canaliculur proteins and/or decreased synthesis (Barr and Hubbard, 1993; Stieger et al., 1994; Rost et al., 1999). For example, phallolidin-induced cholestasis in rats causes redistribution of ecto-ATPase, dipepti-

### TABLE 1

<table>
<thead>
<tr>
<th>Disease Enzyme activities</th>
<th>Control</th>
<th>Diclofenac</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Mg2+-ATPase (μmol/h/mg protein)</td>
<td>70.0 ± 13.2</td>
<td>27.7 ± 5.4*</td>
<td>6</td>
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<tr>
<td>γ-Glutamyltransferase (μmol/h/mg protein)</td>
<td>11.7 ± 2.0</td>
<td>6.1 ± 1.1*</td>
<td>6</td>
</tr>
<tr>
<td>Leucine aminopeptidase (μmol/h/mg protein)</td>
<td>10.6 ± 3.0</td>
<td>5.1 ± 1.5*</td>
<td>6</td>
</tr>
<tr>
<td>Alkaline phosphatase (μmol/mg protein)</td>
<td>12.4 ± 2.4</td>
<td>34.9 ± 14.6</td>
<td>6</td>
</tr>
</tbody>
</table>

*Relative enrichment is the ratio of specific activity in canaliculur membrane to specific activity in homogenate. p < 0.05 compared with control (Mann-Whitney U test).

### TABLE 2

<table>
<thead>
<tr>
<th>Test Results</th>
<th>Control</th>
<th>Diclofenac</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/l)</td>
<td>34.2 ± 0.5</td>
<td>27.8 ± 1.4*</td>
<td>6</td>
</tr>
<tr>
<td>Total bilirubin (μmol/l)</td>
<td>1.6 ± 1.4</td>
<td>3.2 ± 1.6</td>
<td>6</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>355 ± 33</td>
<td>217 ± 21*</td>
<td>6</td>
</tr>
<tr>
<td>Aspartate transaminase (U/l)</td>
<td>92 ± 8</td>
<td>76 ± 14</td>
<td>6</td>
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<tr>
<td>γ-Glutamyltransferase (U/l)</td>
<td>2.2 ± 1.0</td>
<td>3.5 ± 1.0</td>
<td>6</td>
</tr>
<tr>
<td>Alamine transaminase (U/l)</td>
<td>56 ± 5</td>
<td>35 ± 5*</td>
<td>6</td>
</tr>
</tbody>
</table>

*Relative enrichment is the ratio of specific activity in canaliculur membrane to specific activity in homogenate. p < 0.05 compared with control (Mann-Whitney U test).
DICLOFENAC HEPATOTOXICITY


References


