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Selective Protein Adducts to Membrane Proteins in Cultured Rat Hepatocytes Exposed to Diclofenac: Radiochemical and Immunochemical Analysis

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SUMMARY

The nonsteroidal anti-inflammatory drug diclofenac can be bioactivated to the reactive acyl glucuronide, which covalently binds to hepatocellular proteins in rat hepatocytes. Short term cultured rat hepatocytes were used to further study the formation and nature of protein adducts after exposure to diclofenac. Incubation of cells with [^{14}C]diclofenac (30 μM) for up to 24 hr was associated with a time-dependent increase in radioactivity bound to proteins. Upon subcellular fractionation of hepatocytes exposed to diclofenac for 2 hr, the majority of the radiolabel appeared in the microsomal fraction. By 24 hr, the specific binding had decreased by 50% in this cell compartment. In contrast, the hepatocellular plasma membrane fraction, which also was associated with high specific binding of diclofenac-derived radioactivity by 2 hr, exhibited a ~3-fold increase in adduct formation by 24 hr. Lesser amounts of radioactivity were associated with cytosolic proteins. After resolution of the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, the radioactivity was associated with a major protein band with an apparent molecular mass of 60 kDa that was present in both microsomes and plasma membranes. Further, we developed an antidiclofenac antibody against diclofenac-protein adducts by Protein A chromatography of a polyclonal antiserum raised in rabbits against a diclofenac-keyhole limpet hemocyanin adduct. The antidiclofenac antibody did recognize diclofenac-protein adducts on Western blots of homogenates of cultured rat hepatocytes exposed to diclofenac. The major detected adducts included the 60-kDa protein, which was present at all diclofenac concentrations used. In addition, the antibody recognized proteins with apparent molecular masses of 50, 80, and 126 kDa that were not evident in the radiochemical assay. There were no detectable cross-reactive epitopes of proteins recognized by the antibody on Western blots of cultured hepatocytes not treated with diclofenac. Moreover, immunoblots of liver homogenates from rats treated with diclofenac (30 mg/kg/day, intraperitoneally, for 4 days) also exhibited adducts with the 60- and 80-kDa proteins. Collectively, these results suggest that binding of diclofenac to rat hepatocyte proteins is selective and that a 60-kDa microsomal membrane protein (or protein subunit) that accumulates in the plasma membrane fraction appears to be the major target for alkylation both in cultured hepatocytes exposed to diclofenac and *in vivo*.

Long term treatment with the nonsteroidal anti-inflammatory agent diclofenac has in rare cases been associated with the development of fulminant hepatic necrosis ("diclofenac hepatitis") (2–4). The biochemical and molecular bases of these reactions remain largely unknown. Both direct toxic effects of diclofenac or of one of its metabolites (4) and hypersensitivity reactions (5, 6) have been suggested as possible mechanisms of liver injury, but evidence for either possibility awaits further clarification.

Recently we (7) and others (8) demonstrated that acute cell injury can be induced by high concentrations of diclofenac in short term cultured rat hepatocytes and that these cytotoxic effects are mediated via activation of the drug by cytochrome P450, probably by isoforms of the CYP2C subfamily (7, 9). As an alternative pathway, diclofenac can be detoxified by glucuroconjugation in the rat liver (10). The resulting acyl glucuronides are, however, unstable and may react with proteins in the hepatocytes, resulting in the formation of covalent protein adducts (1, 7, 11–13).[1]

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[1] Covalent protein binding is defined as irreversibly bound drug equivalents, dependent on drug metabolism, that remain associated with proteins after stringent washing with polar and nonpolar solvents and after protein resolution by SDS-PAGE.

ABBREVIATIONS: TPHU, 7,7,7-triphenylheptyl-UDP; BSA, bovine serum albumin; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; UDPGT, UDP-glucuronosyltransferase; WME, Williams' medium E; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence.

Covalent protein adducts not only may reflect bioactivation of a drug to a reactive intermediate, and could thus be used as a cumulative measure of the net activation (14, 15), but also may, under certain conditions, become neoantigens and elicit an immune response directed against the liver. For a number of compounds, including halothane (16, 17), dihydralazine (18), tienilic acid (19), and ethanol (20, 21), neoantigens have been characterized and identified, although the pathomechanism of immune-mediated hepatocyte injury remains enigmatic. The first step toward an understanding of possible links between alkylation of specific proteins and the possible role of the protein adducts as immunogens or in metabolic idosyncrasies is the identification of the target selectivity, of the molecular mass and subcellular localization of the adducts, and of the time course of covalent binding.

Therefore, the present study was aimed at further characterizing the diclofenac-protein adducts in hepatocytes. By using two independent approaches, i.e., radiochemical detection of the radiolabeled protein-bound drug and immunochemical detection of the adducts using a polyclonal antidiclofenac antibody, the results of this study indicate that diclofenac selectively binds to few proteins or protein subunits. The main protein target appears to be of an apparent molecular mass of 60 kDa, and the adducts initially appear in the microsomes and later shift to the plasma membrane.

Experimental Procedures

Chemicals. [*phenylacetic acid ring*-U-^{14}C]Diclofenac (6.4 Ci/mol; radiochemical purity, >98%), 4′-methoxydiclofenac, and sulfaphenazole were generously provided by Ciba-Geigy, Ltd. (Basel, Switzerland). TPHU was a generous gift from Dr. G. Mulder, Center for Bio-Pharmaceutical Sciences, University of Leiden (Leiden, The Netherlands). BSA, dexamethasone, insulin, (−)-borneol, and diclofenac sodium were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum, penicillin/streptomycin, and Hanks' balanced salt solution were from Amimed (Basel, Switzerland). WME without phenol red was obtained from Tecnomara (Zurich, Switzerland). Collagenase from *Clostridium histolyticum* was purchased from Boehringer (Mannheim, Germany), and horseradish peroxidase-conjugated anti-rabbit IgG and hyperfilms were from Amersham (Buckinghamshire, England).

Animals. Permission for animal studies was obtained from the Kantonales Veterinäramt Zurich, and all study protocols were in compliance with institutional guidelines. Male Sprague-Dawley rats (Iva:SIV-50) were obtained from SAVO (Kisslegg im Allgäu, Germany). They were adapted to laboratory conditions for 10 days and were 7 weeks of age at the start of the experiments. They received Nafag 890 rat pellets (Nafag, Gossau, Switzerland) and water *ad libitum*. The animals were housed in groups of two in Macrolone cages with wood shavings as bedding, under controlled environmental conditions. Some rats were administered diclofenac sodium by intraperitoneal injection (30 mg/kg/day) for 4 consecutive days. Control rats received saline only. Two hours after the last injection, the rats were euthanized with pentobarbital, and the livers were removed for protein analysis. For immunization and preparation of polyclonal antibodies, male New Zealand White rabbits (3 kg) kept under standardized conditions were used.

Hepatocyte isolation and short term culture. Hepatocytes were isolated from fed untreated rats by a two-step collagenase perfusion method, as described (22). Cells (2×10^6) were seeded into 60-mm collagen-coated culture dishes (Primaria; Becton-Dickinson, Oxnard, CA) in 3 ml of WME in the presence of 10% fetal calf serum. For the isolation of plasma membrane fractions, 5×10^6 cells were seeded into 100-mm dishes in 7 ml of WME. After a 3-hr attachment period at 37° in a 5% CO_2/95% air atmosphere, the medium was replaced by serum-free WME supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 100 nM insulin, and 100 nM dexamethasone, and the cells were precultured for at least 24 hr. Typically, the protein yield was 1 mg/10^6 hepatocytes. All experiments were done in serum-free WME in the absence of hormones or antibiotics. All data points for each cell preparation were measured in triplicate.

Isolation of subcellular fractions of cultured hepatocytes. Subcellular fractions were prepared from hepatocytes that had been precultured for 24 hr and subsequently exposed to diclofenac. For the preparation of microsomes and cytosol, the hepatocyte cultures were extensively washed with ice-cold PBS, pH 7.4, harvested in homogenization buffer (75 mM KCl, 100 mM phosphate buffer, pH 7.4), and ultrasonicated. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min at 4°. The microsomal pellet was washed in homogenization buffer and recentrifuged, and the supernatant (cytosol fraction) was saved.

Plasma membranes were isolated according to the method of Nicotera *et al.* (23), as modified by Jacobson (24), with slight modifications. Briefly, Cytodex 3 beads (Pharmacia, Dübendorf, Switzerland) were swollen in PBS and washed three times with sucrose/acetate buffer, pH 5.0 (7 volumes of 310 mM sucrose plus 3 volumes of 49 mM sodium acetate). Cultured hepatocytes were washed with ice-cold PBS and incubated with 3 ml of collagenase/5×10^6 cells (100 units/ml) for 10 min at 37°, to collect the cells. All of the following steps were performed in the presence of 0.1 M phenylmethylsulfonyl fluoride and 1 μM leupeptin. The hepatocytes were washed twice with Krebs-Henseleit buffer, pH 7.4, and after 15-min preincubation at 37° the cells were washed with sucrose/acetate buffer, pH 5.0, containing 1 mM Ca^{2+}/Mg^{2+}. One-milliliter portions of the bead suspension were added dropwise, with gentle stirring, to the same volume of hepatocyte suspensions. After 10–20 min at room temperature, an equal volume of poly-L-glutamic acid (1 mg/ml in Tris, pH 8) was added. The bead/cell suspension was washed several times with 10 mM Tris buffer, pH 7.4, until all unbound cells were removed. The beads were then resuspended in a small volume of Tris buffer and vortexed for four periods of 5 sec each. After several washes with Tris buffer, the membranes attached to the beads were removed by addition of 1 M Na_2HPO_4.

Incubation with [^{14}C]diclofenac and assessment of radioactivity covalently bound to proteins. Cultured hepatocytes were incubated with various concentrations of diclofenac containing trace amounts of radiolabeled diclofenac (typically 80 nCi of [^{14}C]diclofenac/ml of culture medium). After 2-hr or 24-hr exposure, the cultures were extensively washed with ice-cold PBS, and the cells were homogenized or the subcellular fractions were isolated. The proteins were precipitated by the addition of 4 volumes of ice-cold methanol and were filtered on glass fiber filters (Whatman, Maidstone, England), followed by several washing steps with methanol and ethanol until the radioactivity in the eluate had reached control levels. The filters were then transferred into scintillation vials and incubated overnight in 2.5 ml of 1 M NaOH. Finally, 10 ml of Ultima Gold scintillation cocktail (Packard) were added, and the total cell-associated radioactivity was determined by liquid scintillation counting. For calculation of the data, the background values (zero-time incubation) were subtracted.

SDS-PAGE and fluorography. Hepatocyte homogenates or subcellular fractions were mixed at a ratio of 3:1 (v/v) with lysis buffer [150 mM Tris·HCl, pH 6.8, 300 mM DTT, 6% (w/v) SDS, 0.3% (w/v) bromphenol blue, 30% (v/v) glycerol] and heated to 95° for 5 min. SDS-PAGE was performed according to the method of Laemmli (25), using a 4% stacking gel and a 10% separating gel. Electrophoresis was typically run at 30 mA/gel for 1 hr (minigels) or 4 hr (large gels). The gel was incubated for 30 min with fixing solution [40% (v/v) methanol, 10% (v/v) acetic acid] and subsequently placed into Amplify (Amersham) for 30 min. The gel was then dried under vacuum and exposed on hypersensitive autoradiography films for 2 months. In all experiments, the apparent molecular masses of proteins were estimated by

comparing their relative mobilities with those of marker proteins of known molecular masses.

Production of polyclonal antidiclofenac antisera. Diclofenac was conjugated to KLH and BSA with EDC according to the instructions of the Pierce Imject Immunogen EDC conjugation kit. Specifically, 500 μg of diclofenac were dissolved in 500 μl of conjugation buffer (0.1 M 2-morpholinoethanesulfonic acid monohydrate, 0.9 M NaCl, 0.002% NaN_3, pH 7.4) and added to 2 mg of KLH reconstituted in 200 μl of deionized water. EDC (500 μg) in 50 μl of deionized water was mixed with the diclofenac/KLH solution. Conjugation was carried out for 2 hr at room temperature. The conjugate was purified by gel filtration.

For the immunization, two rabbits were immunized subcutaneously with 50 μg of the diclofenac-KLH conjugate emulsified in 1 ml of Freund's complete adjuvant/saline (1:1). Rabbits were immunized every 10 days until the diclofenac antibody titer was high. Blood was collected from the ear vein, serum was prepared, and the antibody titers were determined. The sera were stored at −30°. Titers were maintained by booster injections of the immunogen every 2 months.

Preparation of affinity-purified antidiclofenac IgG. To obtain the IgG fraction of the antidiclofenac serum, the serum was purified by chromatography using a Protein A bead column. The pH of the serum was adjusted to 8.0 with 1 M Tris·HCl, pH 8.0. The serum was subsequently passed through a 10-ml Protein A column. The column was washed with PBS, pH 8.0, until the absorbance of the wash fractions at 280 nm reached the base-line value. The bound antibody was eluted with 0.1 M glycine, pH 3.0. One-milliliter fractions were collected and neutralized with 1 M Tris, pH 8.0. The IgG-containing fractions were identified by their absorbance at 280 nm.

Alkaline phosphatase ELISA. The titers and the specificity of antibodies for recognized diclofenac-protein adducts were assayed using an ELISA with alkaline phosphatase-coupled anti-rabbit IgG. ELISA plates were coated with 300 ng of BSA-diclofenac adduct/well in 100 μl of PBS, pH 7.4, for 2 hr at 37°. The wells were blocked with 200 μl of PBS, pH 7.4, containing 2% (w/v) BSA, for 1 hr at 37°. Three washes with PBS, pH 7.4, were followed by the addition of 100 μl/well of serum diluted (1/1000 to 1/20,000) in PBS/1% BSA and further incubation for 2 hr at 37°. The wells were washed once with PBS/0.25% Tween-20 and three times with PBS. Subsequently, 100 μl of alkaline phosphatase-coupled anti-rabbit IgG were added and incubated for 2 hr at 37°. After one wash with PBS/0.25% Tween-20 and three washes with PBS, 100 μl of substrate (two 5-mg tablets of Sigma 104 substrate in 10 ml of substrate buffer containing 9.6% diethanolamine and 240 mM $MgCl_2$, pH 9.8) were added, and readings with a test wavelength of 405 nm and a reference wavelength of 492 nm were taken after 5, 15, and 30 min. For the competitive inhibition experiments, ELISAs were performed as described above, except that the incubation solution (serum diluted 1/2000) contained various concentrations of inhibitors.

Immunoblotting. SDS-PAGE was performed as described above. The resolved proteins were transferred electrophoretically to ECL-nitrocellulose (26), using a transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% (v/v) methanol. After transfer for 1 hr at 100 V and 250 mA, the nitrocellulose was blocked overnight at 4° with blocking buffer consisting of 5% (w/v) milk powder and 0.25% (v/v) Tween-20, in PBS, pH 7.4. The blot was subsequently washed with one 15-min and two 5-min washes in washing buffer [0.25% (v/v) Tween-20 in PBS, pH 7.4] and incubated with the antidiclofenac antibody (1.5 μg/ml in blocking buffer) for 1 hr at room temperature. After washing for 15, 5, and 5 min in washing solution, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit F(ab)′ for 1 hr at room temperature. After washing for 15, 5, and 5 min in washing buffer, the ECL detection reagents (Amersham) were added for 1 min. The liquid was discarded, and the membranes were incubated on ECL films for 1–10 min. After exposure to ECL films, some membranes were stained with Ponceau S [0.2% (w/v) in 3% (v/v) acetic acid] for 5 min. In all experiments, the apparent molecular masses of proteins were estimated by comparing their relative mobilities with marker proteins of known molecular mass.

Other analytical methods. Na^+/K^+-ATPase activity was determined according to the method of Scharschmidt *et al.* (27), and NADPH-cytochrome *c* reductase activity was determined according to the method of Lake (28). Lactate dehydrogenase activity was measured with a test kit from Boehringer. Total protein was determined according to the method of Bradford (29), using BSA as standard protein.

Results

Time-dependent covalent binding profile of [^{14}C]diclofenac in subcellular compartments of cultured hepatocytes. Cultured rat hepatocytes were exposed to diclofenac at concentrations of 10 nM to 100 μM for up to 24 hr. Previous experiments demonstrated that these concentrations are not cytotoxic and that diclofenac undergoes acyl glucuronidation, resulting in the formation of a reactive intermediate that can covalently bind to hepatocellular proteins (7). Alkylation of hepatocellular proteins was concentration dependent and the adducts accumulated over the incubation period, reaching maximal binding at 24 hr.

To quantitatively determine the amount of covalently bound diclofenac equivalents in various subcellular compartments, cultured hepatocytes were exposed to [^{14}C]diclofenac and subsequently fractionated into microsomal, cytosolic, and plasma membrane fractions, with a high degree of purification. To exclude possible contamination with proteins from other subcellular compartments, the determination of marker enzymes was included. As summarized in Table 1, microsomes, cytosol, and plasma membranes exhibited minimal contamination, and marker enzyme activities were enriched over the whole-cell homogenate by factors of 3, 4, and 32, respectively. Protein recovery in these subcellular fractions varied depending on the isolation method used; particularly, the recovery of plasma membrane proteins was low (Table 1). Next, to assess the time course and stability of the formed adducts over 24 hr, the radioactivity associated with these subcellular fractions prepared from hepatocyte cultures exposed to [^{14}C]diclofenac was analyzed by liquid scintillation counting. As depicted in Fig. 1, the greater part of total radioactivity (calculated on a per milligram basis for total radioactivity in microsomes, plasma membranes, and cytosol) was recovered in the microsomal fraction after 2 hr, whereas a small amount of the total radiolabel, but nevertheless featuring a high specific activity, was present in the plasma membrane fraction. In contrast, after 24 hr the radioactivity in the microsomal fraction had decreased by ~50%, whereas that in the plasma membranes had increased approximately 3-fold. Thus, when the relative distribution of total protein in the subcellular hepatocyte fractions was taken into account, the bulk of the radioactivity in hepatocytes was still associated with the microsomes after 24 hr (9.3% of total bound drug equivalents, compared with 25% after 2 hr). Nevertheless, the specific activity of plasma membrane proteins was high at 24 hr, although only approximately 2.5% of the total bound radioactivity was present in the plasma membranes (compared with 0.9% after 2 hr). Finally, the radioactivity in the cytosolic fraction featured a low specific activity, accounted for only a small portion of the total radioactivity, due to the great abundance of cytosolic proteins, and only marginally increased from 2 to 24 hr of incubation. The apparent recovered radioactivity in these three subcellular fractions accounted for

TABLE 1

Marker enzyme activities in the subcellular fractions prepared from cultured rat hepatocytes

Microsomes, plasma membranes, and cytosolic fractions of 24-hr-precultured rat hepatocytes were prepared as described in Experimental Procedures. The activities of Na^+/K^+-ATPase, NADPH-cytochrome *c* reductase, and lactate dehydrogenase (LDH) were determined in these fractions and in the whole-cell homogenate, to assess the degree of purification. Numbers in parentheses indicate fold enrichment over the homogenate. Data are means ± standard deviations of three independent experiments.

Marker enzyme	Whole-cell homogenate	Plasma membranes	Microsomes	Cytosol
Na^+/K^+-ATPase (μmol/hr/mg of protein)	0.15 ± 0.01	4.81 ± 0.88 (32.1)	0.18 ± 0.04 (1.2)	ND[a]
NADPH-cytochrome *c* reductase (μmol/hr/mg of protein)	4.14 ± 0.30	0.62 ± 0.01 (0.15)	12.96 ± 0.83 (3.13)	1.66 ± 0.12 (0.4)
LDH (units/mg of protein)	1200 ± 165	12 ± 2 (0.01)	24 ± 12 (0.02)	4800 ± 960 (4.0)
Protein recovery (mg/10^7 hepatocytes)	10.0 ± 2.7	0.016 ± 0.005	1.50 ± 0.24	5.0 ± 1.1

[a] ND, not detectable.

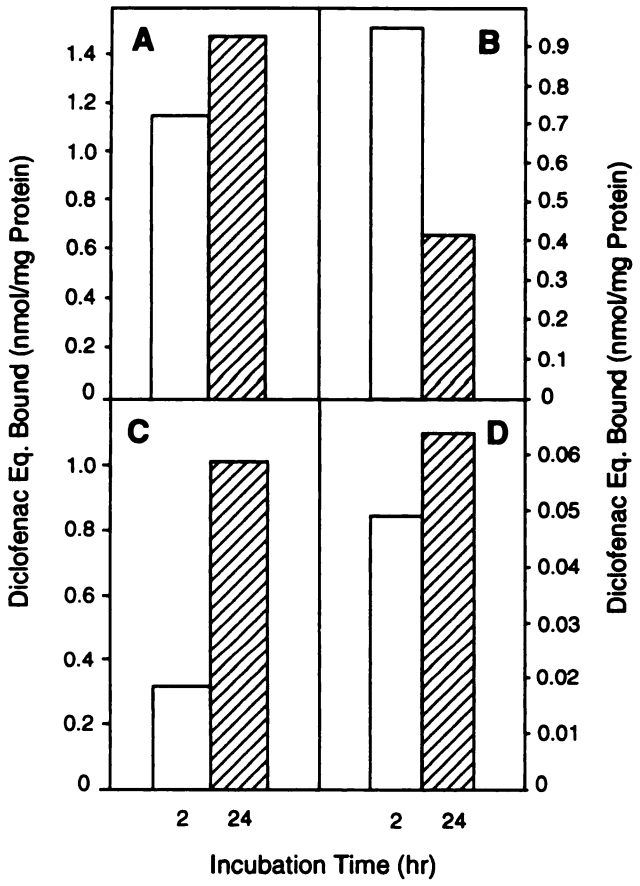

Fig. 1. Time-dependent distribution of diclofenac-protein adducts in subcellular fractions of cultured rat hepatocytes exposed to [^{14}C]diclofenac. Two hours (□) or 24 hr (■) after treatment of the cells with 30 μM diclofenac sodium containing 80 nCi/ml radiolabeled diclofenac, the microsomal, plasma membrane, and cytosolic fractions were prepared as described in Experimental Procedures, and the amount of covalently bound radiolabel was determined by liquid scintillation counting. A, Cell homogenate; B, microsomes; C, plasma membranes; D, cytosol. Values are shown as specific radioactivity/mg of protein and are expressed as mean of two experiments (variation, <10%).

only a fraction (approximately one fourth) of the total protein-associated radioactivity, which leaves unresolved the question of the fate or distribution of the remaining radioactivity, because we did not analyze the 10,000 × *g* pellet or other subcellular compartments. Taken together, these results indicate that there was a time-dependent shift in the distribution of protein adducts, with losses of radiolabel in the microsomes and accumulation of label in the plasma membranes.

Radiochemical analysis of radioactivity covalently bound to rat hepatocyte proteins resolved by SDS-PAGE. Hepatocellular proteins from homogenates and from subcellular fractions of hepatocyte cultures exposed to [^{14}C] diclofenac were electrophoretically resolved by SDS-PAGE under both reducing and nonreducing (omission of DTT) conditions. Fluorographic analysis of the cell homogenates revealed that the radioactivity was consistently associated with a major protein band with an apparent molecular mass of 60 kDa. Furthermore, fluorographic analysis of the subcellular fractions revealed that the radiolabeled 60-kDa proteins were present both in the microsomal fraction and in the plasma membrane fraction but were not detectable in the cytosolic fraction (Fig. 2). These data corroborate and extend the findings obtained by scintillation counting of the total radiolabeled adducts (Fig. 1). Moreover, this indicates that the proteins targeted by [^{14}C] diclofenac intermediates, presumably by its acyl glucuronide, are membrane-bound proteins.

In initial preparations, run under identical conditions except for the inclusion of leupeptin (1 μM) and aprotinin (0.1 μM),

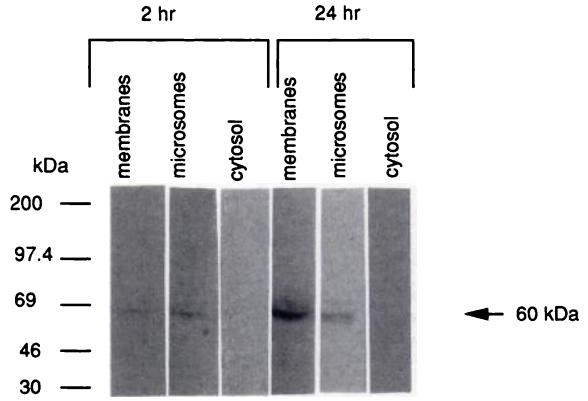

Fig. 2. Fluorographic analysis of plasma membrane, microsomal, and cytosolic proteins from cultured rat hepatocytes exposed to [^{14}C]diclofenac. Two hours or 24 hr after treatment of the cells with 30 μM diclofenac sodium containing 80 nCi/ml radiolabeled diclofenac, the proteins in the homogenate and in the subcellular fractions were resolved by SDS-PAGE (90 μg/lane), followed by fluorography. The fluorogram demonstrates the presence of covalently bound radioactivity co-migrating with a protein with apparent molecular mass of 60 kDa that is present in both the plasma membrane and microsomal fractions but is not detectable in the cytosol. On the *left*, the migration pattern of the marker proteins in the SDS-PAGE gel is indicated. The calculated radioactivity loaded in the lanes for electrophoresis was as follows: at 2 hr: plasma membranes (*membranes*), 320 dpm; microsomes, 1325 dpm; cytosol, 91 dpm; at 24 hr: plasma membranes, 1350 dpm; microsomes, 335 dpm; cytosol, 91 dpm.

the radiolabel was irreversibly bound to a protein(s) of approximately 30 kDa (1), in the absence of the 60-kDa protein. It is not unreasonable to suggest that the low molecular mass proteins may represent subunits or degradation products. Furthermore, identical qualitative or quantitative radiolabel distribution was seen in the presence and absence of DTT during SDS-PAGE, indicating that disulfide bond cleavage may not be a critical factor during protein resolution. The possibility cannot be excluded that other proteins may also be minor potential targets of diclofenac adduct formation, but these adducts were below the level of detection. These results indicate that binding is selective and that the described 60-kDa protein or protein subunit may constitute the major target for the reactive diclofenac acyl glucuronide formed in the hepatocytes upon exposure to diclofenac.

Characterization of the antidiclofenac antibody and specificity of binding. To detect the presence of antibodies in rabbit serum and to determine the specificity of the rabbit antidiclofenac antibodies raised against the diclofenac-KLH adduct, the antibodies were tested by ELISA against both the diclofenac-BSA conjugate and BSA alone. Fig. 3 demonstrates that the Protein A-purified polyclonal IgG recognized the solid-phase antigen of diclofenac coupled to BSA, whereas BSA alone was not antigenic. As expected, there was some cross-reactivity with KLH, which had served as the carrier protein for immunization (data not shown). Antidiclofenac activity was apparent even at an antiserum dilution of 1/40,000.

To further characterize the specificity of the antidiclofenac antibody, we performed a competitive ELISA. Fig. 4 demonstrates that diclofenac was approximately 2 orders of magnitude more effective as an inhibitor of the immunochemical reaction than was its 4′-methoxy derivative and that *N*-acetyl-*p*-aminophenol was a poor inhibitor.

Immunochemical analysis of rat hepatocyte proteins after exposure of cultured cells to diclofenac. To detect electrophoretically resolved diclofenac-protein adducts, Western blots using antidiclofenac antibodies were used, as a complementary method to the radiochemical analysis of covalently bound diclofenac. Diclofenac-protein adducts were immunochemically analyzed in homogenates of cultured rat hepatocytes exposed to 30 μM diclofenac for 2 or 24 hr (Fig. 5). The results demonstrate that diclofenac binding to proteins in intact cells occurred in a selective manner and that proteins or protein subunits with apparent molecular masses of 50 and 60 kDa were major targets for alkylation. In contrast, in hepatocytes not exposed to diclofenac detectable binding was absent, indicating that there was negligible nonspecific binding.

To provide additional evidence for our hypothesis that binding was related to diclofenac metabolism to its acyl glucuronide,

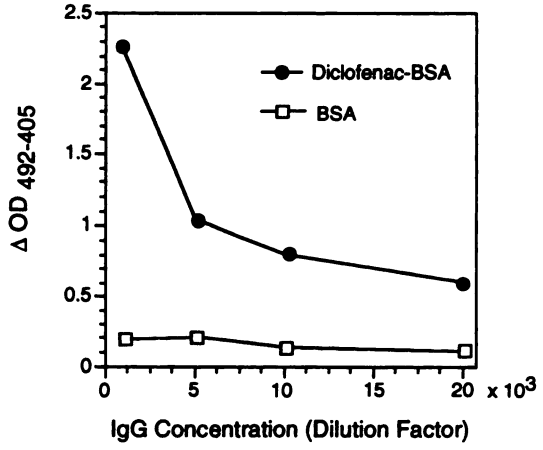

Fig. 3. Detection of antibodies raised against diclofenac-protein adducts, using ELISA. Wells of microtiter plates were coated with either 0.3 μg of diclofenac-BSA conjugate (●) or native BSA (□). Serum from rabbits immunized with diclofenac-KLH was partially purified, serially diluted, and added to the wells. Bound rabbit antibody was detected using alkaline phosphatase-conjugated anti-rabbit IgG. Alkaline phosphatase activity is expressed as absorbance (*OD*)/15 min.

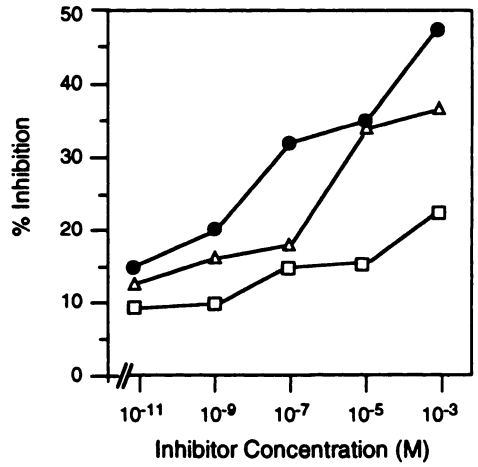

Fig. 4. Competitive inhibition of binding of antidiclofenac antibodies to solid-phase diclofenac-BSA conjugate. ELISAs were performed as described in the legend to Fig. 3, and the following inhibitors were used at the indicated concentrations: diclofenac (●), 4′-methoxydiclofenac (Δ), and *N*-acetyl-*p*-aminophenol (□).

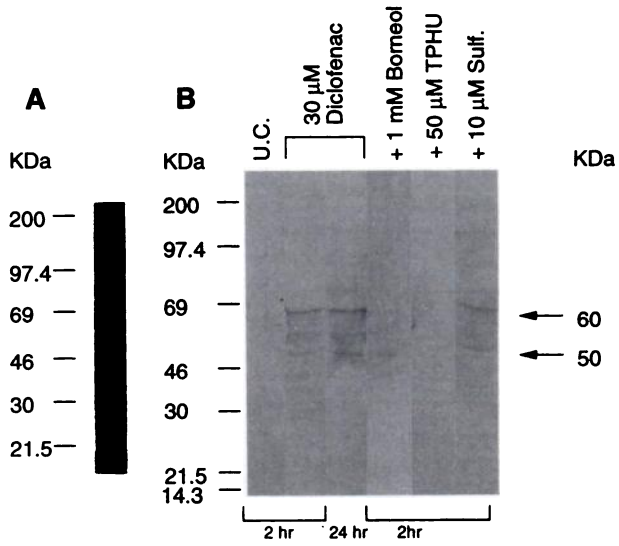

Fig. 5. Western blot analysis of electrophoretically resolved proteins in homogenates of cultured rat hepatocytes exposed to diclofenac. A, Coomassie Blue staining of hepatocellular proteins; B, immunoblot. Two hours or 24 hr after treatment of the cells with 30 μM diclofenac, or in untreated control hepatocytes (*U.C.*), the proteins were resolved by SDS-PAGE (100 μg/lane), transferred to nitrocellulose, and probed with antidiclofenac IgG (dilution, 1/2000). Incubation of the cells in the presence of the UDPGT inhibitors borneol or TPHU greatly diminished or abolished the appearance of two proteins with apparent molecular masses of 60 and 50 kDa, whereas the CYP2C inhibitor sulfophenazole (*Sulf.*) did not affect the immunostaining of these proteins. On the *left*, the migration patterns of the marker proteins in the SDS-PAGE gel are indicated.

we included the specific UDPGT inhibitor TPHU in the incubation medium containing diclofenac. Immunochemical analysis of these cultures revealed that the antibody did not detect the proteins described above (Fig. 5). Similarly, concurrent experiments with the less specific UDPGT inhibitor borneol resulted in a markedly reduced staining profile with the antibody probe. In contrast, exposure of the hepatocytes to diclofenac in the presence of sulfophenazole, a specific inhibitor of the cytochrome P450 (CYP2C) subfamily, was not associated with alterations in the pattern or extent of the protein bands recognized by the antibody, indicating that this cytochrome P450 isoform was not involved in the generation of diclofenac-protein adducts.

To study the formation of diclofenac adducts as a function of the diclofenac concentrations, hepatocyte cultures were exposed to high (100 and 300 μM) but not cytotoxic (7) concentrations of the drug. Fig. 6 illustrates that, in comparison with low diclofenac concentrations used (Fig. 5), additional proteins were recognized by the antidiclofenac antibodies in immunoblots, i.e., a 80-kDa protein (at 100 μM) and a 126-kDa protein (at 300 μM). This indicates that different proteins may be targeted progressively with increasing drug concentrations.

Immunochemical detection of diclofenac adducts in the liver of rats treated with diclofenac. To compare the results obtained *in vitro* with those from *in vivo* exposure, rats were administered diclofenac or saline intraperitoneally for 4 days (30 mg/kg/day). Immunoblot analysis of liver homogenates from the treated rats, but not from control rats, revealed that the antidiclofenac antibody recognized at least two of the proteins detected *in vitro*. The most prominent adducts were associated with proteins with apparent molecular masses of 60 and 80 kDa (Fig. 7).

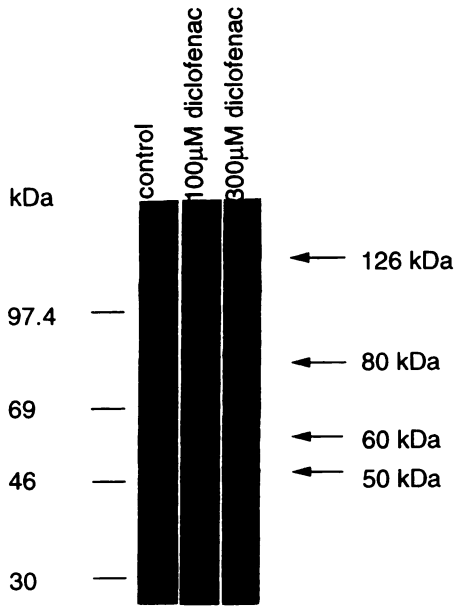

Fig. 6. Western blot analysis of diclofenac-protein adducts in homogenates of cultured rat hepatocytes exposed to high concentrations of diclofenac. Seven hours after treatment of the cells with 100 or 300 μM diclofenac, the proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antidiclofenac IgG as described in the legend to Fig. 5. The immunoblot analysis demonstrates the concentration-dependent presence of four major proteins recognized by the antidiclofenac antibody. On the *left*, the migration pattern of the marker proteins in the SDS-PAGE gel is indicated.

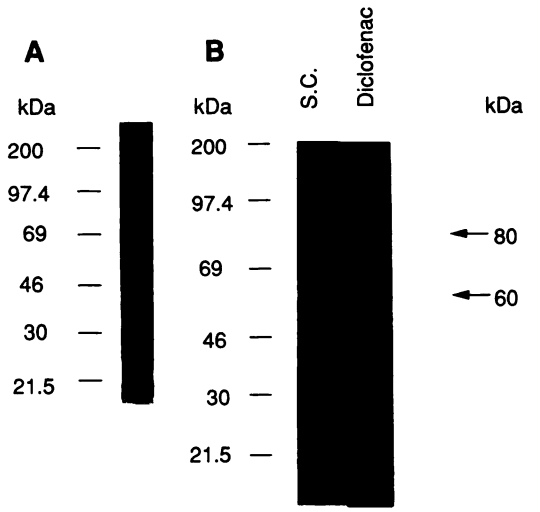

Fig. 7. Western blot analysis of diclofenac-protein adducts in livers of rats treated with diclofenac *in vivo*. Livers from saline-treated control rats (*S.C.*) or from rats injected intraperitoneally with diclofenac sodium (30 mg/kg/day, for 4 consecutive days) were homogenized, and proteins were resolved by SDS-PAGE (100 μg/lane). A, Ponceau S staining of blotted liver proteins; B, immunoblot analysis. The Western blot demonstrates the presence of two major proteins, with apparent molecular masses of 80 and 60 kDa, recognized by the antidiclofenac antibody. On the *left*, the migration pattern of the marker proteins in the SDS-PAGE gel is indicated.

Discussion

In this investigation, we have detected covalent protein adducts of the putative reactive metabolite of diclofenac, its acyl glucuronide, in subcellular fractions of cultured rat hepatocytes exposed to the drug, as well as in liver homogenates of rats treated with diclofenac. The results indicate that bioactivated diclofenac selectively reacts with a small number of hepatocellular protein targets. The proteins that are predominantly alkylated by diclofenac were found to be membrane proteins located in both the endoplasmic reticulum and plasma membranes of cultured hepatocytes.

The apparent high selectivity of protein targeting by activated diclofenac may be based on several possible mechanisms. For example, the drug intermediate may be highly reactive and cause alkylation of specific proteins during pharmacological drug-protein interactions in the cell. A similar mechanism has been proposed for drug-induced hepatitis, where autoantibodies were directed against hepatic cytochrome P450 forms that bioactivate the drug (30). Alternatively, a potential target protein may be abundant in the vicinity of the site of bioactivation. Finally, several other proteins may have become alkylated less selectively but were, however, not detectable in our system.

Two different approaches were used to quantitatively and qualitatively estimate the covalent drug-protein adducts. First, with the use of [*ring*-^{14}C]diclofenac we recently described the quantitative determination of covalent drug binding to hepatocellular proteins in cultured rat hepatocytes occurring via glucuronide formation (7). Moreover, in the present study the radiochemical approach allowed us to determine the subcellular targets and the time course of binding, as well as the selectivity of adduct formation with specific cellular proteins. Second, we have developed an immunochemical approach, using a Protein A-purified IgG fraction from a polyclonal antidiclofenac antiserum raised against a diclofenac-KLH adduct, which enabled

us to characterize the epitopes recognized by the antibody resulting from covalent interactions between diclofenac and proteins in intact rat hepatocytes and *in vivo*.

In the present study, a protein or protein subunit with an apparent molecular mass of 60 kDa was demonstrated to be the major target that was invariably alkylated by low and high concentrations of diclofenac and that was detected with both the radiochemical and immunochemical methods. Furthermore, in immunoblots of cells exposed to diclofenac, the antidiclofenac antibodies recognized at least three additional proteins that were not detected by fluorography. It is unlikely that these proteins represent native epitopes and are unrelated to drug alkylation, because they were not detected in cells that were not exposed to diclofenac. The reason why the 50-, 80-, and 126-kDa proteins were not found in the radiochemical/fluorography analysis might be a higher sensitivity of the ECL system, compared with fluorography; alternatively, one could speculate that these antigens may represent haptens generated from metabolites different from those carrying the radiolabel, or from secondary reactions. It seems, however, that the formation of all detected adducts was dependent on the formation of UDPGT-mediated acyl glucuronidation, as evidenced by the clear inhibition of covalent binding (7) and of the immunochemical reaction (Fig. 5) by UDPGT inhibitors. Collectively, these findings illustrate the value of using both radiochemical and immunochemical approaches for the detection of specific adducts.

The time course of analysis after exposure of hepatocytes to diclofenac may be important for the detection of protein adducts. Different alkylated proteins may exhibit different half-lives in hepatocytes, and protein adducts may become differentially degraded (31–33) or may be transported within or between subcellular compartments. For example, alkylated membrane proteins in the endoplasmic reticulum may be processed and transported via different transcellular pathways to the plasma membrane. In this study, a consistent feature was the marked time-dependent shift in the distribution of the diclofenac adducts (Figs. 1 and 2). The possibility cannot be excluded that two different proteins of similar molecular masses were alkylated in either the endoplasmic reticulum or the plasma membranes. Alternatively, it can be surmised that adducts to the same protein are formed in both subcellular compartments or that the protein adducts are transported to the plasma membrane. Drug-altered proteins in the plasma membranes may become particularly important in immune recognition and processing. For example, the presence of protein adducts of reactive metabolites of isaxonine, a drug that is associated with immune-mediated liver injury in humans, has been demonstrated in isolated hepatocyte plasma membranes (34). Furthermore, functional cytochrome P450 forms that were recognized by several autoantibodies have recently been demonstrated to be present in rat and human hepatocyte plasma membranes (35, 36).

The presence of at least two of the major drug-altered protein species (60 and 80 kDa) both in cultured hepatocytes and in the livers of rats exposed to diclofenac *in vivo* lends further support to the validity of the binding studies in cell cultures but also emphasizes the necessity of analyzing drug adducts formed *in vivo* and in various species. In fact, comparison of these results with those from an *in vivo* mouse study (12) demonstrates that, although in both species a small number of major proteins appear to be recognized by the antibody, the apparent molecular sizes of the targeted proteins differ, with the exception of a 50-kDa protein. It remains to be determined whether similar hepatocellular proteins may be targeted in humans, in spite of the relatively low therapeutic diclofenac concentrations (plasma C_{max} in the low micromolar range) (37) and low rates of acyl glucuronide formation in human liver (10). Eventually, these findings might provide a clue for the elucidation of the rare but severe idiosyncratic liver reactions that occur in patients after long term treatment with a number of nonsteroidal anti-inflammatory drugs that can form reactive acyl glucuronides (33). Covalent binding of drugs to hepatocellular proteins has often been implicated in eliciting hypersensitvitiy reactions in the liver, via the formation of neoantigens. A crucial step in understanding the mechanisms of drug hypersensitivity or metabolic idiosyncrasy is the detection and characterization of the specific proteins that are targeted by the bioactivated drugs and eventually become immunogenic after processing and presentation to the immune system or, alternatively, may play a crucial role in cell homeostasis. Thus, further characterization and identification of the proteins targeted by diclofenac metabolites and the demonstration of diclofenac protein adducts in patients with diclofenac hepatitis remain goals for the future.

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