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Cytochrome P450 2E1 Is a Cell Surface Autoantigen in Halothane Hepatitis

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SUMMARY

Recent studies have shown that cytochrome P450 2E1 (CYP2E1) is a major catalyst of formation of trifluoroacetylated proteins, which have been implicated as target antigens in the mechanism of halothane hepatitis. In the present investigation, trifluoroacetylated CYP2E1 was detected immunochemically in livers of rats treated with halothane. Furthermore, high levels of autoantibodies that recognized purified rat CYP2E1 but not purified rat CYP3A were detected by enzyme-linked immunosorbent assay in 14 of 20 (70%) sera from patients with halothane hepatitis. Only very low levels of such antibodies were detected in sera from healthy controls, from patients anesthetized with halothane without developing hepatitis, or from patients with other liver diseases. The intracellular distribution of CF_3CO-adducts was studied in highly differentiated FGC4 rat hepatoma cell cultures. High levels of adducts were found after 22-hr culture in the presence of halothane, and their generation was dependent on the expression of CYP2E1. Adducts were predominantly located in the endoplasmic reticulum but also, to a minor extent, on the cell surface, as detected by immunofluorescence. A very similar distribution was found for CYP2E1 in FGC4 cells, and immunoprecipitation experiments performed in cultures of FGC4-related Fao hepatoma cells suggest that surface immunoreactivity originates from a small fraction of intact CYP2E1 apoprotein. Human CYP2E1, expressed in V79 cells after cDNA transfection, was also detected to a minor extent in the plasma membrane, whereas no immunofluorescence was evident in parental V79 cells. It is suggested that immune responses to cell surface CYP2E1 could be involved in the pathogenesis of halothane hepatitis.

The anesthetic halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is associated with a severe and sometimes fatal hepatitis in approximately one in several thousand anesthetized patients and also with a mild form of liver injury that occurs in ~25% of patients (1). The great majority of patients (>80%) who develop the severe form of liver injury, termed halothane hepatitis, have been exposed to halothane on two or more occasions, and these patients frequently exhibit rash, arthralgia, eosinophilia, and a variety of autoantibodies, which are characteristics of immune-mediated drug hepatotoxicity (1). Furthermore, a series of investigations have directly implicated immune responses to novel antigens, halothane metabolite-modified protein adducts, in the mechanism of liver injury.

Initial investigations showed that patients with halothane hepatitis exhibited cellular and humoral immune sensitization to novel antigens expressed in livers of halothane-dosed rabbits. In particular, antibodies that recognized the novel antigens were shown to be highly specific for patients with halothane hepatitis and could not be detected in various control groups, including halothane-anesthetized individuals who do not develop halothane hepatitis, patients anesthetized with halothane who develop liver injury attributable to other causes (2), and patients with the mild form of halothane-induced liver dysfunction (1). The novel halothane-induced antigens have been detected on the surface of isolated rabbit hepatocytes, and it has been shown that such hepatocytes are susceptible to antibody-mediated cytotoxic killing by normal human lymphocytes *in vitro* (2). This suggests that the antibodies could be responsible for and/or contribute to liver injury in patients *in vivo*.

Halothane is oxidized by hepatic P450 to the highly reac-

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ABBREVIATIONS: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; ER, endoplasmic reticulum; PM, plasma membrane; P450 or CYP, cytochrome P450; HRP, horseradish peroxidase; PDI, protein disulfide isomerase; FITC, fluorescein isothiocyanate; DAS, diallylsulfide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; TBS, Tris-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PEST, penicillin/streptomycin; RT, room temperature; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tive metabolite trifluoroacetylchloride, which either reacts with water to yield trifluoroacetate or binds covalently to hepatic proteins and lipids to produce trifluoroacetyl adducts (CF_3CO adducts) (3). The novel antigens recognized by antibodies from patients with halothane hepatitis have been shown to comprise a range of CF_3CO-protein adducts, which are expressed in livers of halothane-exposed rabbits, rats, and humans and are concentrated in the liver microsomal fraction (1). Studies performed using the technique of SDS-PAGE and immunoblotting (4, 5) have identified at least eight distinct CF_3CO-modified polypeptide antigens, of which all except one are derived from the lumen of the ER (4–6). In addition, a second group of antigens was identified recently that could be detected by ELISA but not by immunoblotting (7). It seems that these comprise one or more CF_3CO-modified integral membrane proteins of the ER and that the epitopes recognized by the antibodies of patients are conformational (7).

Studies undertaken *in vivo* in rats and *in vitro* in rat and human liver microsomes have shown that the P450 isozyme primarily responsible for generation of the CF_3CO adducts detectable by immunoblotting is CYP2E1 (8).[1] The major CF_3CO adduct(s) formed *in vitro* exhibited a subunit molecular mass of 55 kDa, and so could constitute CYP2E1 adducts.[1] In the current report, we demonstrate expression of CF_3CO-CYP2E1 adducts in livers of rats treated with halothane *in vivo* and the presence of autoantibodies to CYP2E1 in sera from patients with halothane hepatitis. In addition, by the use of cell model systems that express CYP2E1, we demonstrate expression of a fraction of cellular CYP2E1 and CF_3CO adducts on the cell surface. These findings indicate that CYP2E1 is a cell surface autoantigen in halothane hepatitis and suggest that this enzyme plays a dual role in the mechanism of liver injury.

Materials and Methods

Chemicals

BSA (fraction V), casein (Hammarsten grade), DAS, dithio-bis-(propionic-*N*-hydroxysuccinimide ester, protein A/Sepharose CL-4B, iodoacetamide, PMSF, Nonidet P-40 (octylphenoxy polyethoxy ethanol), Tween 20 (polyoxy ethylenesorbitan monolaurate), Triton X-100 (*t*-octylphenoxy polyethoxy ethanol), *o*-phenylenediamine, and streptavidin-agarose were purchased from Sigma Chemical (Poole, UK). Halothane was purchased from Aldrich Chemical (Gillingham, UK) and distilled to eliminate thymol (a stabilizer). Imidazole was obtained from BDH Chemicals (Merck, Lutterworth, UK). Chlormethiazole was from Astra Arcus AB (Södertälje, Sweden) and was provided by Prof. Magnus Ingelman-Sundberg (Karolinska Institutet, Stockholm, Sweden). SDS and EDTA were obtained from Koch-Light (Haverhill, UK). Protein A-HRP, acrylamide/bis (40%), polymerization initiators, and molecular mass standards were from BioRad Lab (Hemel Hempstead, UK). Ham's F12 medium [including 0.86 mg of ($ZnSO_4 \times 7H_20$)/liter], Dulbecco's modified Eagle's medium (including 4500 mg of glucose/liter), fetal bovine serum, PEST (5000 IU of penicillin/ml, 5000 μg of streptomycin/ml), geneticin sulfate (G-418), and Dulbecco's PBS were from GIBCO BRL (Life Technologies, Paisley, UK). Biotinylation reagent and Sephadex G-25 was included in a protein biotinylation module from Amersham Life Sciences (Amersham, UK). Enhanced chemiluminescent HRP substrates were also obtained from Amersham Life Sciences or from Pierce & Warriner (Chester, UK). Vectashield anti-bleach was from Vector Lab (Bretton, UK).

Purified Enzymes

CYP2E1 and P450 reductase were purified to homogeneity from livers of starved and acetone-treated male Sprague-Dawley rats in the laboratory of Prof. M. Ingelman-Sundberg (Karolinska Institutet, Stockholm, Sweden) according to a published protocol (9). Purified rat CYP3A (PCNb) was a gift from Prof. J. R. Halpert (University of Arizona, Tucson, AZ) and was purified as described previously (10).

Sera

Human sera were obtained from healthy individuals (25 sera), patients anesthetized with halothane on multiple occasions who did not develop hepatitis (six sera), patients with halothane hepatitis (20 sera), patients with primary biliary cirrhosis (seven sera), or patients with other liver disorders [i.e., chronic autoimmune hepatitis (six sera), alcoholic liver disease (four sera), and chronic viral hepatitis C (seven sera)]. Rabbit anti-CF_3CO adduct antiserum, which was produced as described in detail elsewhere (11), has been shown to recognize specifically CF_3CO-modified rat and human liver proteins in immunoblots and ELISA (5).[1] Polyclonal goat anti-human IgG/HRP or anti-rabbit IgG/HRP conjugate was from Tago Immunochemicals (Serotech, Kidlington, UK). Polyclonal goat anti-rabbit IgG/FITC conjugate was from Sigma. Polyclonal rabbit anti-CYP2E1 antiserum, raised against rat liver CYP2E1 (9) but also recognizing human CYP2E1 (12), was a gift from Magnus Ingelman-Sundberg (Stockholm, Sweden). Antiserum to PDI was obtained from a rabbit that had been immunized with rat PDI, purified essentially as described previously (13). This antiserum was shown by immunoblotting to recognize purified rat PDI (subunit molecular mass, 57 kDa) and to recognize a single 57-kDa polypeptide expressed in rat liver microsomes (results not shown).

Animals

Male Sprague-Dawley rats (200–250 g) were administered halothane (10 mmol/kg) in a single dose by intraperitoneal injection of a 21.5% (v/v) solution in sesame oil. Control rats received sesame oil only. After the indicated time intervals (see Fig. 4), rats were killed, and the livers were removed, homogenized, and subjected to differential centrifugation to obtain isolated liver microsomes (5).

Cell Cultures

FGC4 rat hepatoma cells (14) were obtained from Dr. M. Weiss (Institute Pasteur, Paris, France). These cells originated from a H4IIEC3 clone of the Reuber H35 rat hepatoma (14). Batches of FGC4 cells (2×10^6/ml) were kept frozen at −70° in 1.5 ml of Ham's F12 medium, including 25% (v/v) fetal bovine serum, 16% (v/v) dimethylsulfoxide, and 2% (v/v) PEST. Before each experiment, cells were quickly thawed at +37°, diluted into 15 ml of Ham's F12 medium [including 5% (v/v) fetal bovine serum and 2% (v/v) PEST], and cultured in 75-cm^2 plastic flasks (Corning; Costar, Cambridge, MA, or Falcon; Becton & Dickinson, Parsippany, NJ) in humidified atmosphere with 5% CO_2 at 37°. Medium was renewed after 24 hr and every second day thereafter. Rat hepatoma Fao cells (15), originating from the same H4IIEC3 clone as FGC4, were obtained from M. Ingelman-Sundberg and cultured under the same conditions as FGC4 cells. V79MZ Chinese hamster cells were provided by Prof. J. Doehmer (Technische Universität München, Munich, Germany). These cells exhibited a stable expression of human CYP2E1 after transfection with an simian virus 40 early promoter-controlled expression vector, which included human CYP2E1 cDNA (12). Both the transfected, CYP2E1-expressing V79MZh2E1 cells and the non-transfected, parental V79MZ cells were cultured in Dulbecco's mod-

[1] E. Eliasson, H. Hume-Smith, I. Gardner, I. de Waziers, P. Beaune, and J. G. Kenna. Cytochrome P450 2E1-dependent generation of trifluoroacetyl protein adducts recognized by antibodies from patients with halothane hepatitis. Submitted for publication.

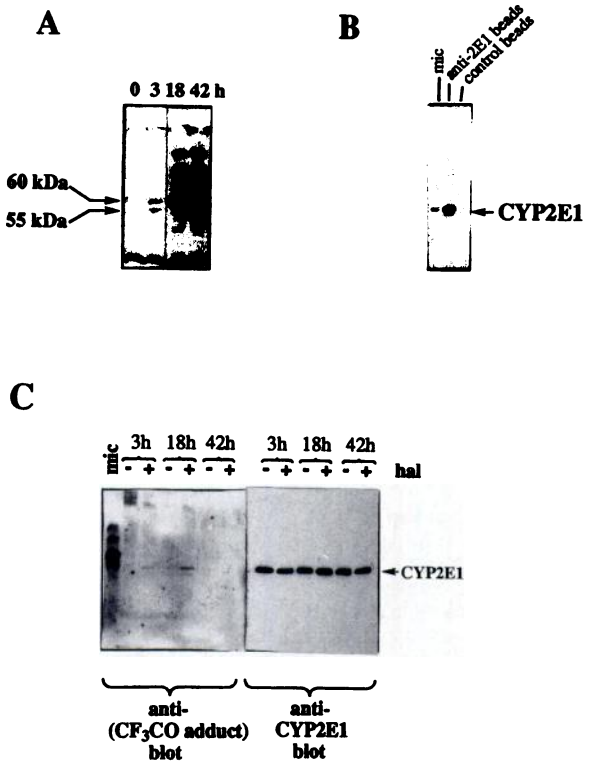

Fig. 1. Identification of CF_3CO-modified CYP2E1 in livers of rats treated with halothane. Microsomes were prepared from pooled livers of groups of four rats given an intraperitoneal dose of either halothane (10 mmol/kg) or sesame oil and killed after 3, 18, or 42 hr. A, Time-dependent formation of CF_3CO adducts. Liver microsomal proteins, corresponding to 5 μg/lane, were resolved by SDS-PAGE (8.5% gels) under reducing conditions, and immunoblots were developed using an anti-CF_3CO adduct rabbit antiserum, with final detection by peroxidase-generated chemiluminescence. B, IP of CYP2E1. Rabbit IgG specific for CYP2E1 or nonimmune IgG was cross-linked to protein A/Sepharose beads and used for precipitation of CYP2E1 from solubilized liver microsomes. Precipitates were subjected to SDS-PAGE (anti-2E1 beads and control beads, respectively), as were intact rat liver microsomes (*mic*) and CYP2E1 was detected by immunoblotting. C, Detection of CF_3CO-CYP2E1 formed in rat liver *in vivo*. CYP2E1 was immunoprecipitated from the various liver microsomes using the anti-2E1 beads; then, immunoprecipitates were resolved by SDS-PAGE (10% gels), and immunoblots were probed using anti-CYP2E1 antiserum (right) or anti-CF_3CO adduct antiserum (*left*), as was a reference sample of liver microsomes from halothane-treated animal (*mic*; 18 hr time point). Similar results were obtained in three separate experiments.

ified Eagle's medium (4500 mg/liter glucose), including 10% fetal bovine serum and 2% PEST, but geneticin sulfate (G-418, 200 μg/ml) was also included in the V79MZh2E1 cultures.

Formation of CF_3CO Adducts in FGC4 Cells

Within 5 weeks after initial thawing and reseeding, FGC4 cells were challenged with imidazole (0.5 mM, 2 days) or chlormethiazole (20 μM, 2 days) and thereafter washed twice in PBS and incubated in fresh medium for 60 min. Medium was exchanged once again, and flasks were closed air-tight with the use of silicone stoppers and parafilm. Halothane (5 μl of a 1:10 dilution in methanol) was injected through the stopper onto a strip of filter paper positioned inside the cell flask (16). This dose of halothane has been shown in a previous investigation to result in maximal generation of CF_3CO adducts in primary cultures of rat hepatocytes and has been estimated to give a final halothane concentration of ~20 μM in the culture medium (16). After the indicated incubation time (Figs. 4 and 5) at 37°, cells were washed three times in cold PBS and scraped off the plastic into Eppendorff tubes. The cells were pelleted by low-speed centrifugation and frozen in 100 μl of PBS at −20° before preparation of microsomes.

Isolation of Microsomes

Cell microsomes were isolated essentially as described previously (17) with minor modifications. The FGC4 samples (~5 × 10^6 cells) were thawed and sonicated in 1 ml of ice-cold 0.25 M sucrose, 1 mM EDTA, and 50 mM HEPES, pH 7.4. The homogenate was centrifuged at 10,000 × *g* for 10 min at +5°, and the resulting supernatant was ultracentrifuged at 109,000 × *g* for 60 min at +4° with a Beckman Optima TL ultracentrifuge with a TLA45 rotor (Beckman Instruments, Columbia, MD). Microsomal pellets were homogenized with a tight-fitting pestle in 200 μl of ice-cold centrifugation buffer. Protein concentration was determined according to the Lowry method using BSA as standard.

SDS-PAGE and Immunoblotting

Microsomal aliquots, corresponding to 150 μg (FGC4 cells) or 15 μg (rat liver), were diluted to 75 μl in water, mixed with 75 μl of SDS sample buffer [0.125 M Tris·HCl, ph 6.8, including 8% SDS (w/v), 20% glycerol (v/v), 0.002% bromphenol blue (w/v)] containing dithiotreitol (10 mg/ml), and boiled for 3 min. Thereafter, aliquots (specified in figure legends) were resolved by SDS-PAGE (using BioRad minigels with 8.5% or 10% acrylamide in the resolving gel) and transferred to nitrocellulose sheets. The sheets were incubated for 90 min at RT (20–25°) in blocking solution consisting of TBS, Tween 20 [0.05% (v/v)], fat-free dry milk [5% (w/v)], and fetal bovine serum [2% (v/v)]. Anti-CF_3CO protein antiserum was used at a 1:5000 dilution in blocking buffer and incubated for 3 hr or overnight at RT, after which blots were washed with 1% milk/TBS-Tween and incubated with goat anti-rabbit IgG/HRP conjugate at 1:5000 dilution in 1% milk/TBS-Tween for 2 hr. Primary antibody incubations with anti-CYP2E1 antiserum were performed using a 1:5000 dilution in 1% milk/TBS-Tween for 90 min at RT, followed by a thorough wash in TBS-Tween and incubation at RT for 90 min with protein A-HRP (1:3000) in 1% milk/TBS-Tween. Thereafter, sheets were washed again in TBS-Tween, and antibody reactivity was visualized using enhanced chemiluminescence HRP substrates and Hyperfilm (Amersham Life Sciences). The intensity of immunoblot signals was determined using a Molecular Dynamics scanner (Sunnyvale, CA) and ImageQuant software. The apparent molecular masses of protein adducts were estimated by comparison of their electrophoretic mobilities with those of molecular mass standards.

ELISA

Aliquots of PBS (96 μl) were added to wells of polystyrene microtiter plates (Immulon 4; Dynatech Lab, Billinghurst, UK). Subsequently, 5-μl aliquots of purified rat CYP2E1, which previously had been diluted to a final concentration of 1.8 pmol of CYP2E1/5 μl in 0.2% (w/v) sodium cholate, 50 mM HEPES, pH 7.4, were added to the wells (yielding a final protein concentration of ~1 μg/ml). Control wells received the same volumes of PBS and cholate dilutent buffer. Plates were incubated for 16 hr at +5°; then wells were washed four times in washing buffer [TBS, casein (0.5% (w/v), 0.02% (w/v) thimerosal] using a Titertek SA/12 Microplate washer (Flow Lab, ICN Biomedical, Thame, UK) at RT. Human sera (100 μl, diluted 1:100 in washing buffer) were added to the wells and plates were incubated for 3 hr at +4°. After another wash step, 100 μl of anti-human IgG/HRP conjugate (dilution 1:1000 or 1:3000 in washing buffer) was added to the wells, and plates were incubated for 2 hr at +4°. After a final wash (four times with washing buffer, then twice with TBS alone), bound HRP activity was determined by reaction with *o*-phenylenediamine (7) and determinations of absorbance at 490 nm (performed using a Titertek Multiskan Plus MkII Plate Reader; Flow Lab). The same protocol was used in ELISA studies of antibody binding to rat CYP3A and purified P450 reductase.

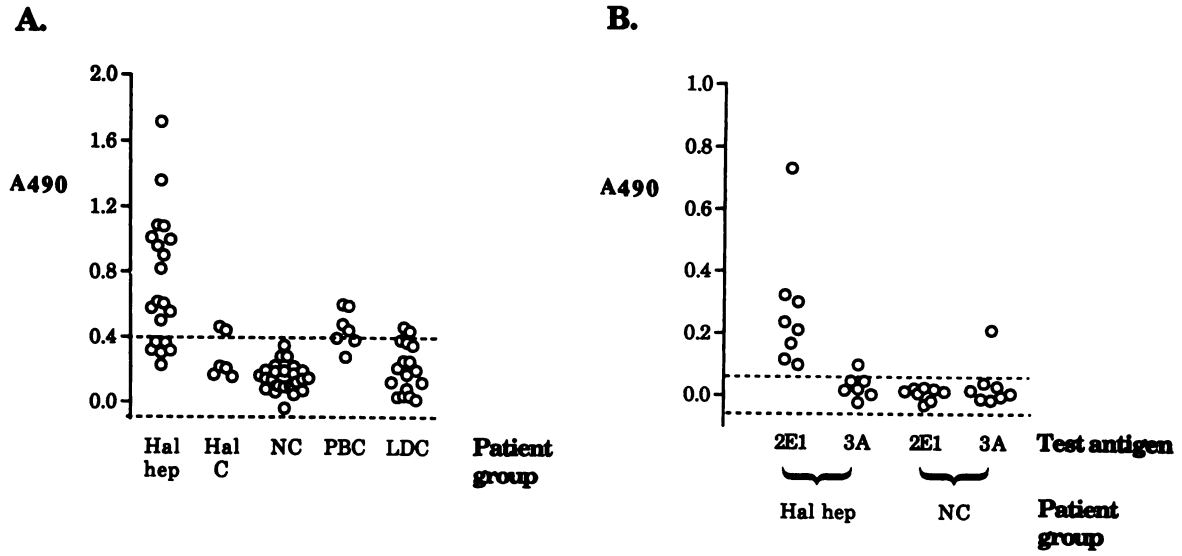

Fig. 2. Expression of anti-CYP2E1 antibodies in the sera of patients with halothane hepatitis. Binding of human antibodies (serum dilution 1:100) to purified rat CYP2E1 (A) or rat CYP2E1 and rat CYP3A (B) was investigated by ELISA. The dilutions of secondary antibody (goat anti-human IgG/HRP conjugate) were 1:1000 for A and 1:3000 for B. Sera were from patients with halothane hepatitis (*Hal hep*, 20 sera), patients undergoing multiple halothane anesthesia without hepatitis (*Hal C*, six sera), healthy controls (*NC*, 25 sera), patients with primary biliary cirrhosis (*PBC*, seven sera), or patients with other liver disorders (*LDC*, 17 sera). The background reactivity of each serum against wells coated with PBS was subtracted from each presented value. The analysis was repeated twice, with similar results.

Immunofluorescence

FGC4 cells were reseeded in 3 ml of complete F12 medium (described above) in Slide-Flasks (Nunc, Roskilde, Denmark) and grown to 40–50% confluency. The medium was replaced, and FGC4 cells were incubated with or without halothane, as described above. The cells were washed (2 × 5 min) in PBS and fixed in 2.5 ml of 5% (v/v) formaldehyde in PBS for 25 min at RT. After another wash in PBS, cells were treated by slow shaking for 20 min at RT in PBS, with or without 0.2% (v/v) Triton X-100, and then incubated without shaking for 15 hr at +5° with 10% (v/v) FBS in PBS. Subsequently, the Slide-Flasks were washed briefly in PBS and incubated (gentle shaking) for 3 hr at RT with 1 ml of primary antibody solution [either anti-CYP2E1 antiserum, diluted 1:2000 in 3% (w/v) BSA in PBS, or nonimmune rabbit antiserum or anti-CF_3CO adduct antiserum, diluted 1:400 in 1% (w/v) BSA in PBS]. Cell specimens were then washed in 3 ml of 3% (w/v) BSA (3 × 10 min) and incubated with goat anti-rabbit IgG/FITC conjugate [diluted 1:200 in 0.8 ml of 3% (w/v) BSA in PBS] for 1.5 hr at RT. Finally, specimens were washed in BSA/PBS (3 × 10 min) and once in H_2O, before being mounted in Vectashield anti-bleach under a glass coverslip. The specimens were analyzed by immunofluorescence microscopy using a Zeiss Axioskop and photographed using a Nikon UFX-DX camera, with the same exposure time for all samples. The same immunofluorescence protocol was used for V79 cells. Confocal microscopy was performed using a BioRad MRC600 with a krypton/argon laser.

IP of CYP2E1 from Liver Microsomes

Coupling of IgG to insoluble beads. Protein A/Sepharose CL-4B beads (80 mg) were equilibrated in 1 ml of IP buffer [0.15 M NaCl, 0.2% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM HEPES, pH 7.4], including 2% (w/v) BSA, to yield a 20% (v/v) suspension. This was incubated with 4 mg of anti-CYP2E1 IgG (9) or 200 μl of nonimmune rabbit serum by shaking on ice for 2 hr. Beads were pelleted and washed once in IP buffer/BSA and twice in PBS and then incubated with 1.5 ml of dithio-bis(propionic-*N*-hydroxysuccinimide ester (1 mM in PBS) for 45 min during shaking on ice. Beads were washed three times with glycine (2 mM) in PBS and stored in this buffer at +5°.

IP of CYP2E1. Liver microsomes (300 μg) were solubilized in 1 ml of ice-cold IP buffer including 0.5% (w/v) BSA, 1% (w/v) deoxycholate, 1 mM PMSF, and 1 mM iodoacetamide and then incubated with 75 μl of a 10% (v/v) suspension of IgG beads for 2.5 hr on ice, with continuous shaking. The beads were pelleted by centrifugation and washed three times in solubilization buffer and then three times in IP buffer. Beads were boiled for 15 min in 100 μl of SDS sample buffer without DTT and then analyzed by SDS-PAGE and immunoblotting. Sample loading was 10 μl/well, and immunoblotting was performed using either anti-CYP2E1 or anti-CF_3CO adduct antiserum.

IP of CYP2E1 from Hepatoma Cells

Biotinylation of IgG. Anti-CYP2E1 IgG (2.5 mg) was biotinylated in 2.5 ml of sodium bicarbonate buffer (40 mM, pH 8.6) using 60 μl of biotinylation reagent from Amersham Life Sciences, according to the manufacturer's recommendation. The biotinylated IgG was separated from free biotin on a Sephadex G-25 column, resulting in an approximate protein concentration of 0.8 mg/ml in PBS.

Precipitation of CYP2E1. Fao cells were grown to 90% confluency under the conditions described above for FGC4 cells and then washed in PBS at RT (3 × 5 ml) and cooled to +5° in 10 ml of PBS containing 5% (v/v) FBS. This buffer was replaced with a further 10 ml of FBS in PBS, with or without digitonin (50 μg/ml). Biotinylated anti-CYP2E1 IgG was added (approximate concentration, 4 or 16 μg/ml), and incubation was conducted for 75 min at +5°, with very gentle agitation. Cell integrity, or permeabilization of cells in the presence of digitonin, was verified in separate flasks by investigation of trypan blue uptake. Essentially all of digitonin-treated cells (>99%) took up trypan blue, compared with <0.5% of cells incubated in PBS. The antibody solution was then discarded, and cells were washed once in 10 ml of PBS containing 10% (v/v) FBS, once in 20 ml of PBS supplemented with 5% FBS, and once in 20 ml of PBS. Cells were lysed by shaking on ice for 15 min in 2.5 ml of 1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris, pH 7.8, including 1 mM EDTA, 1 mM PMSF, and 1 mM iodoacetamide. The lysate was further disrupted by vortex mixing, incubation on ice for 20 min, and then centrifugation at 2500 × *g* for 5 min. The supernatant was incubated on ice for 2 hr with streptavidin-agarose (60-μl beads, pre-equilibrated in lysis buffer), and then CYP2E1 bound to biotinylated IgG was pelleted by

centrifugation. The agarose pellet was washed in lysis buffer (3 × 1 ml) and once in PBS before boiling in SDS sample buffer (60 μl), without DTT, for 5 min. Aliquots (20 μl of each sample) were subjected to SDS-PAGE and immunoblotted with anti-CYP2E1 antiserum.

Results

Identification of CF_3CO-modified CYP2E1 in livers of halothane-treated rats. Liver microsomes were isolated from rats killed at 3, 18, or 42 hr after an *in vivo* dose of halothane (10 mmol/kg, in sesame oil) and from rats killed at the same time intervals after administration of the vehicle alone. Initially, formation of microsomal CF_3CO adducts was evaluated by immunoblotting, using a specific anti-CF_3CO adduct rabbit antiserum. Although only a very low of expression of two adducts (55 and 60 kDa) was evident at 3 hr after administration of halothane (Fig. 1A), much higher levels of many different CF_3CO adducts were detected after 18 hr and after 42 hr (Fig. 1A). Subsequently, IP experiments were undertaken using anti-CYP2E1 IgG antibodies coupled to insoluble beads (see Materials and Methods) to investigate expression of CF_3CO-modified CYP2E1. This procedure resulted in efficient precipitation of CYP2E1 from detergent-solubilized microsomes (Fig. 1B). Analyses of the immunoprecipitates by immunoblotting with anti-CF_3CO adduct serum revealed the presence of CF_3CO-modified CYP2E1 in livers of halothane-dosed rats but not in livers of control rats (Fig. 1C, *left*). Expression of CF_3CO-CYP2E1 could be detected in livers at 3 hr after halothane and was maximal at 18 hr (Fig. 1C). In contrast to the expression of the majority of other CF_3CO adducts (Fig. 1A), no CF_3CO-CYP2E1 was detectable after 42 hr. No significant differences between the expression of CYP2E1 apoprotein in the liver microsomes from halothane-treated and control rats were noted (Fig. 1C, *right*).

Autoantibodies to CYP2E1 in sera of patients with halothane hepatitis. The finding that CYP2E1 was modified by reactive metabolites of halothane raised the possibility that the protein could evoke an immune response in halothane hepatitis. To investigate this, purified rat CYP2E1 was used as test antigen in ELISA studies. Only low levels of antibody binding were detected using a control group of 25 sera from healthy blood donors (Fig. 2A). In contrast, levels of

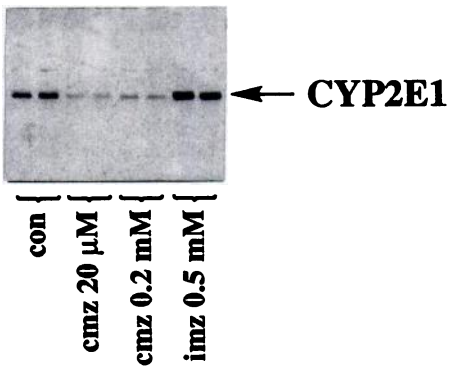

Fig. 3. Regulation of expression of CYP2E1 in FGC4 rat hepatoma cells. FGC4 cells were grown in the absence or presence of imidazole (*imz*) or chlormethiazole (*cmz*) for 2 days. Cells were harvested and microsomal CYP2E1 levels were analyzed by SDS-PAGE and immunoblotting with anti-CYP2E1 antiserum (loading 15 μg of microsomal protein/well). *con*, control.

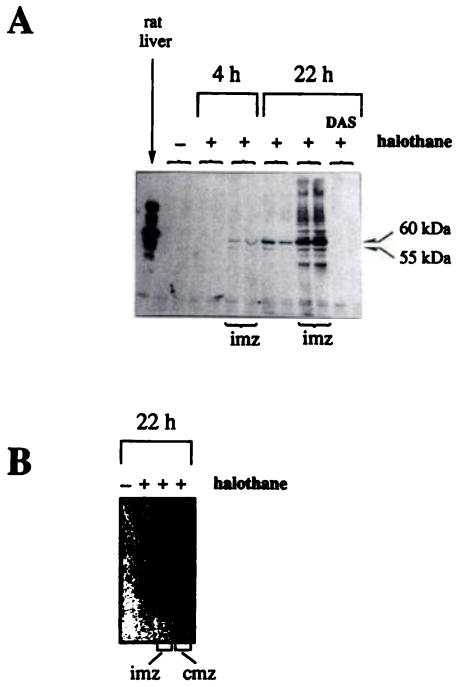

Fig. 4. Formation of microsomal CF_3CO adducts in FGC4 cells. A, Cell cultures were pretreated with or without imidazole, washed, and incubated with or without halothane. Some cells were preincubated for 1 hr with DAS (1 mM) before the addition of halothane. After 4 or 22 hr, cells were harvested in PBS, and formation of microsomal CF_3CO-protein adducts was detected by SDS-PAGE (10% gel, 40 μg of microsomal protein/well) and immunoblotting with anti-CF_3CO adduct antiserum. B, Formation of CF_3CO adducts in FGC4 cells pretreated with or without imidazole or chlormethiazole and then incubated with halothane for 22 hr. Microsomal proteins were resolved using an 8.5% gel and then developed as described above. The results were replicated two or three times.

antibody binding that exceeded the normal range (defined as the mean plus three standard deviations of the values obtained for the healthy blood donors) were detected in 14 of 20 (70%) sera from patients with halothane hepatitis (Fig. 2A, *Hal hep*). Testing of six sera from patients who had received multiple halothane anesthesia without sustaining liver injury revealed that four were negative for CYP2E1, whereas two gave results that were very slightly above the normal range (Fig. 2A, *Hal C*). Negative results were also obtained with 16 of 18 sera from patients with various liver diseases unrelated to halothane (chronic autoimmune hepatitis, viral hepatitis, and alcoholic hepatitis), whereas two were found very weakly positive (Fig. 2A, *LDC*). A low anti-CYP2E1 reactivity could be detected in four of seven sera from patients with primary biliary cirrhosis (Fig. 2A, *PBC*). However, it is notable that the levels of antibody binding to CYP2E1 were markedly higher for 9 of the 20 sera from patients with halothane hepatitis than for any of the various control sera (Fig. 2A). Further ELISA studies, undertaken using 10 of the sera from patients with halothane hepatitis that contained elevated levels of antibodies to CYP2E1, revealed that the sera did not contain antibodies to purified rat CYP3A (Fig. 2B) or purified rat NADPH-dependent P450 reductase (data not shown).

Expression of CYP2E1 and CF_3CO adducts in FGC4 cells. The highly differentiated FGC4 rat hepatoma cell line (14) was used as a model system to study the expression of

CYP2E1 in relation to the intracellular formation of CF_3CO adducts. The basal level of CYP2E1 expression in FGC4 hepatoma cells was estimated, by immunoblotting, to be ~1-2 pmol/mg of microsomal protein. Treatment of cell cultures with the CYP2E1-stabilizer imidazole [0.5 mM, (17)] for 2 days caused a 3-fold increase in CYP2E1 expression (293 ± 126% of untreated control cells, three experiments). In contrast, the expression of CYP2E1 decreased to 25 ± 3% (three experiments) after 2 days in the presence of chlormethiazole (20 μM) (Fig. 3). As shown in Fig. 1A, CF_3CO-modified microsomal proteins were formed when FGC4 cells were pretreated with or without imidazole or chlormethiazole and thereafter incubated with halothane for 4 or 22 hr at 37°. Formation of the adducts was more rapid and extensive after pretreatment of the cells with imidazole. In these cells, only two distinct adducts (55 and 60 kDa) were detectable after 4 hr, whereas many other adducts had been formed after 22 hr (Fig. 4A). The addition of the CYP2E1-selective inhibitor DAS (18) (1 mM) to the cell cultures completely blocked adduct formation (Fig. 4A), as did pretreatment of cells with chlormethiazole (Fig. 4B).

The subcellular locations of CF_3CO adducts and CYP2E1 in FGC4 cells. Studies in which expression of CF_3CO adducts in halothane-treated FGC4 cells was investigated by indirect immunofluorescence, using the anti-CF_3CO adduct antiserum, revealed no significant expression of adducts after 4 hr (Fig. 5A), whereas intense immunofluorescence was evident after 22 hr (Fig. 5B). The fluorescence was distributed in a granular pattern throughout the cytoplasm in permeabilized cells, whereas no reactivity was seen in the area corresponding to the cell nuclei (Fig. 5, B and E), which was easily identified in phase-contrast microscopy (data not shown). A very similar picture was seen when specimens were probed for the expression of PDI, an abundant ER enzyme (Fig. 6D). However, studies of adduct expression in intact cells resulted in detection of a more diffuse immunofluorescence, which was concentrated at the edges of the cell (Fig. 5, C and F). This corresponds to the pattern expected for the plasma membrane (18a). Essentially no fluorescence was detected in cells incubated without halothane (Fig. 5D) or in cells developed using preimmune serum (data not shown).

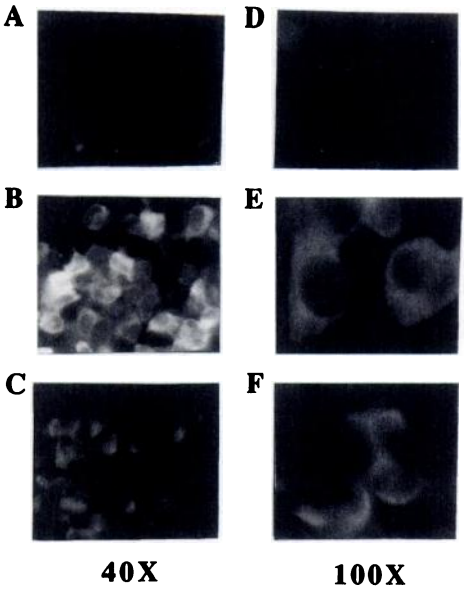

Fig. 5. Subcellular location of CF_3CO adducts in FGC4 cells. Cells were grown on Slide-Flasks and pretreated with imidazole for 2 days before washing and incubation with halothane. After 4 or 22 hr, cells were fixed in 5% formaldehyde, washed in PBS (C and F) or PBS/Triton X-100 (A, B, D, and E), and then blocked in 10% FBS and incubated with anti-CF_3CO adduct serum [1:400 in 1% (w/v) BSA/PBS] followed by anti-rabbit IgG/FITC (1:200 in 3% BSA/PBS) before analysis by immunofluorescence microscopy. A, Four hours with halothane (permeabilized cells), 40× magnification; B, 22 hr with halothane (permeabilized cells), 40×; C, 22 hr with halothane (nonpermeabilized cells), 40×; D, 22 hr without halothane (permeabilized cells), 100×; E, 22 hr with halothane (permeabilized cells), 100×; and F, 22 hr with halothane (nonpermeabilized cells), 100×.

Investigations of the cellular distribution of CYP2E1, which were undertaken using the anti-CYP2E1 antiserum, yielded a very similar pattern of results. In these studies, cells were visualized by conventional fluorescence microscopy (Fig. 6, A–C) and also by confocal microscopy (Fig. 6, D and E). In the permeabilized cells, immunofluorescence was distributed in a granular pattern all throughout the cytoplasm, whereas no fluorescence was detected in the cell nuclei (Fig. 6, B and D). Immunoreactivity was also detected to a minor extent on the surface of nonpermeabilized cells (Fig. 6, C and E). No significant fluorescence was detected in cell samples incubated without the anti-CYP2E1 antiserum (Fig. 6A).

The subcellular location of human CYP2E1 in transfected V79 cells. As a control for the specificity of the anti-CYP2E1 antibody reaction and to investigate whether human CYP2E1 is expressed on the cell surface, experiments were performed using V79 Chinese hamster fibroblast cells transfected with human CYP2E1-cDNA, which stably express human CYP2E1 (12). Immunofluorescence analysis of the transfected V79 cells after membrane permeabilization by detergent revealed that human CYP2E1 was distributed in a pattern corresponding to the ER (Fig. 7A). Immunofluorescence was also detected along the edges of cells without pretreatment with detergent (Fig. 7B). No signal was detected when nontransfected, parental V79 cells were permeabilized and incubated with the anti-CYP2E1 serum (Fig. 7C) or when immunofluorescence studies of either parental or transfected V79 cells were performed using nonimmune serum (data not shown).

Identification of intact CYP2E1 apoprotein on the surface of Fao cells. IP studies were undertaken to evaluate whether the anti-CYP2E1 reactivity evident on the surface of cultured cells corresponded to intact CYP2E1 or proteolytic fragments of the protein. Cultures of an FGC4-related cell line, Fao (15), which has a higher expression of CYP2E1 than FGC4 cells, were incubated with biotinylated anti-CYP2E1 IgG. Incubations were performed using intact cells and cells that had been permeabilized using digitonin. Subsequently, cells were washed extensively and lysed with detergent; then, the biotinylated IgG was precipitated using streptavidin-agarose beads. The precipitates were subjected to SDS-PAGE and immunoblotted using anti-CYP2E1 antiserum. This procedure showed that the CYP2E1 immunoprecipitated from the surface of nonpermeabilized cells exhibited an identical electrophoretic mobility to the intact CYP2E1 apoprotein immunoprecipitated from digitonin-permeabilized cells (Fig. 8). Furthermore, it was evident that only a very small fraction of the total cellular contents of CYP2E1 was expressed on the surface. Control experiments confirmed that no

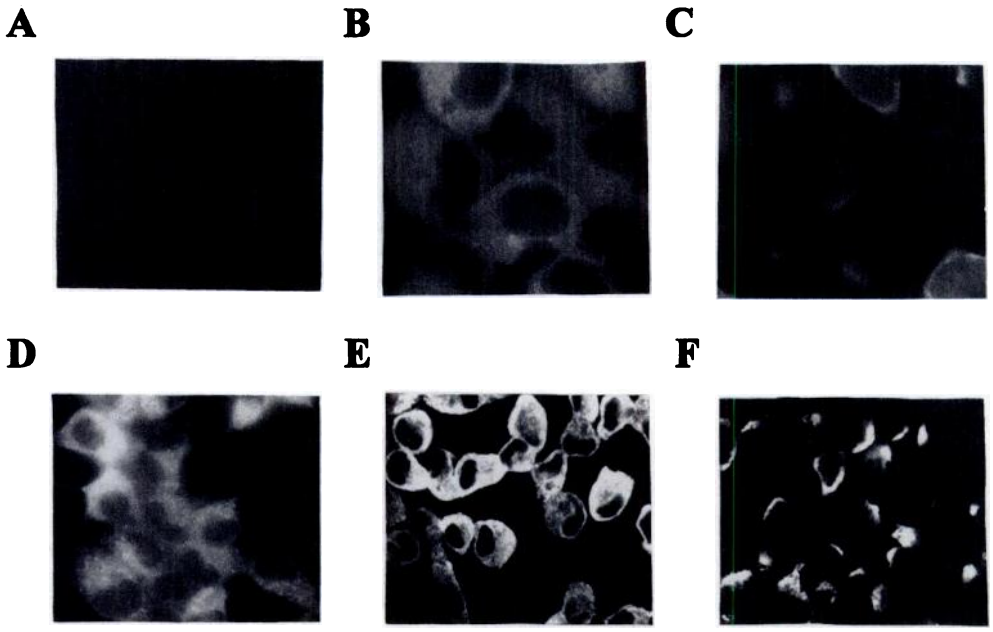

Fig. 6. Subcellular location of rat CYP2E1 in FGC4 cells. FGC4 cells were grown to 60–70% confluency in Slide-Flasks. Thereafter, cells were fixed in formaldehyde and either permeabilized by detergent or left intact, as described in Materials and Methods, and then incubated with anti-CYP2E1 antiserum (diluted 1:2000) or anti-PDI antiserum (1:2000) and, finally, FITC-coupled goat anti-rabbit IgG. The results on CYP2E1 distribution have been replicated at least five times, whereas the PDI control was performed twice. A, FITC-goat anti-rabbit IgG only (permeabilized cells), 100× magnification; B, anti-CYP2E1 antiserum (permeabilized cells), 100×; C, anti-CYP2E1 antiserum (nonpermeabilized cells), 100×; D, anti-PDI antiserum (permeabilized cells), 63×; E, confocal microscopy, anti-CYP2E1 (permeabilized cells), 40×; and F, confocal microscopy, anti-CYP2E1 (nonpermeabilized cells), 40×.

CYP2E1 was precipitated when solubilized rat liver microsomes were incubated with streptavidin-agarose in the absence of anti-CYP2E1 antibodies (data not shown).

Discussion

Identification of CF_3CO-modified CYP2E1. The IP studies of liver microsomes revealed that CF_3CO modification of CYP2E1 occurs relatively rapidly (within 3 hr) after intraperitoneal administration of a single dose of halothane to rats (Fig. 1C). The relatively rapid appearance of CF_3CO-CYP2E1, compared with other CF_3CO adducts, is consistent with the demonstration that CYP2E1 is a major catalyst of protein trifluroacetylation *in vitro* in rat and human liver microsomes[1] and *in vivo* in the rat (8). Furthermore, in agreement with previous studies (8), a 55-kDa adduct that most probably corresponds to CF_3CO-CYP2E1 was one of only two modified proteins detected at the 3-hr time point (Fig. 1A). It should also be noted that high levels of 55-kDa CF_3CO adducts have been detected after incubation of both rat and human liver microsomes with halothane *in vitro* (5, 6).[1]

It is unclear why significantly higher levels of CF_3CO-CYP2E1 were detected at 18 hr after administration of halothane than after 3 hr (Fig. 1C). One possible explanation is concentration-dependent inhibition of P450-dependent bioactivation of halothane at the early time point (16, 19).[1] Covalent modification of CYP2E1, either by cAMP-dependent phosphorylation (20) or by heme alkylation caused by reactive metabolites of carbon tetrachloride (21), has been shown to result in denaturation of the enzyme and subsequent rapid degradation. Presumably, this explains why in contrast to the many other CF_3CO adducts detectable after 18 hr, CF_3CO-modified CYP2E1 was not detectable at 42 hr (Fig. 1C).

CYP2E1 as an autoantigen in halothane hepatitis. Markedly elevated levels of antibodies that recognized rat liver CYP2E1 were detected in a high proportion (70%) of the sera from patients with halothane hepatitis, whereas only low levels of such antibodies were present in a range of control sera (Fig. 2). It is conceivable that a higher number of patient sera might recognize the human form of CYP2E1. By analogy, antibodies that recognized a purified rat liver microsomal carboxylesterase were detected by ELISA in only 2 of 10 sera from patients with halothane hepatitis (20%) (22), whereas the corresponding human enzyme, which exhibited 77% amino acid sequence identity, was recognized by 17 of 20 sera (85%) (23). Rat and human CYP2E1 are 78% homologues at the amino acid level (24), and the substrate specificities of the two forms are highly similar. However, a few minor structural differences do exist, mainly distributed on surface of the CYP2E1 molecule, as predicted by comparison with the known three-dimensional structure of bacterial CYP102 (24a). For example, it is possible that surface-located Asp92, Asp96, Pro259, and Pro262, all present only in the human enzyme, represent part of epitopes to which autoantibody binding could be reduced in ELISA assays with the rat CYP2E1 enzyme.

Sera from patients with halothane hepatitis have been shown to contain elevated levels of antibodies to numerous hepatic protein antigens other than CYP2E1 and microsomal carboxylesterase. These include protein disulfide isomerase (57 kDa), a 58-kDa protein of unknown function; calreticulin (63 kDa); ERp72 (80 kDa); and the stress proteins BiP/GRP78 (82 kDa) and endoplasmin/GRP94 (100 kDa) (reviewed in Refs. 1 and 22). Initially, immunoblotting studies revealed that the the antibodies of patients recognized unique CF_3CO-modified epitopes expressed on the adduct-modified forms of the proteins (5). However, ELISA studies undertaken using purified non-CF_3CO-modified forms of several of the proteins subsequently showed that the predominant antibody responses detectable in the sera of patients are directed against non-CF_3CO-modified epitopes, which presumably are conformational because they are not detectable by immunoblotting (1, 13, 22). It is probable that the antibody response to unmodified epitopes arises because CF_3CO adduct formation causes a specific loss of immunological tolerance and that a similar adduct-induced loss of immunological tolerance underlies the anti-CYP2E1 autoantibody response demonstrated in the present investigation. A

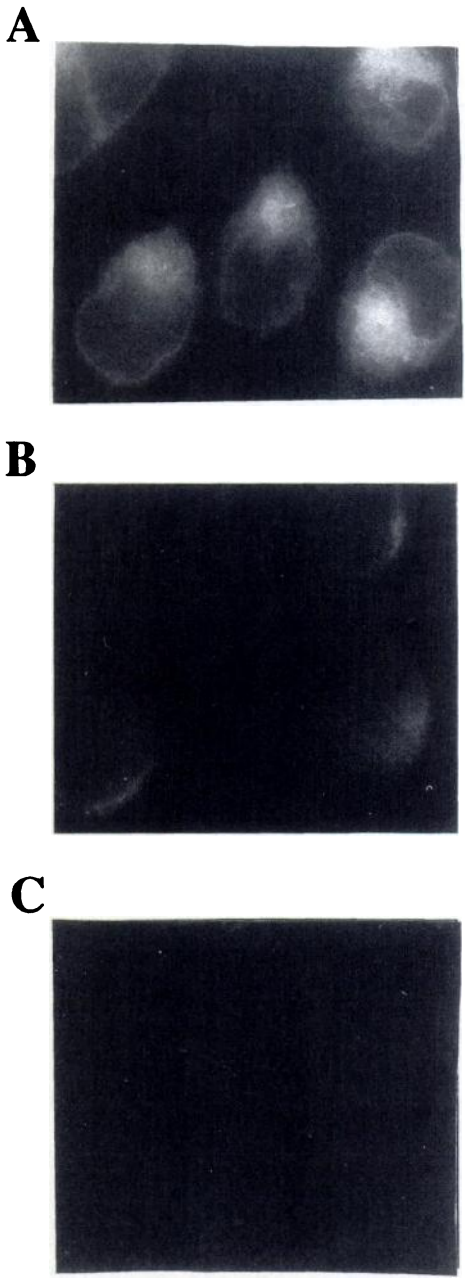

Fig. 7. Subcellular location of human CYP2E1 in V79 cells transfected with human CYP2E1-cDNA. V79 cells stably expressing human CYP2E1, or parental V79 cells, were grown to 40% confluency and fixed, washed in PBS (B) or PBS/Triton X-100 (A and C), blocked in FBS, incubated with anti-CYP2E1 antiserum and anti-rabbit IgG/FITC, and then analyzed by immunofluorescence microscopy. A, Human CYP2E1-cDNA-transfected V79 cells (permeabilized), 100× magnification; B, human CYP2E1-cDNA-transfected V79 cells (nonpermeabilized), 100×; and C, parental V79 cells (permeabilized), 100×.

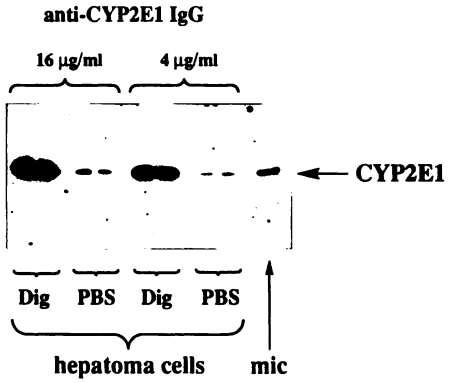

Fig. 8. IP of CYP2E1 from the surface of Fao hepatoma cells. Fao hepatoma cell cultures were permeabilized by digitonin (*Dig*) or left intact in PBS and then incubated at +5° with biotinylated anti-CYP2E1 IgG in the presence of bovine serum. Thereafter, cell cultures were washed extensively and lysed in a detergent-containing buffer including protease inhibitors. The lysate was incubated with streptavidin-agarose, which was precipitated by centrifugation and thoroughly washed in lysis buffer. Streptavidin-adsorbed proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE, and immunoblotted using anti-CYP2E1 antiserum. A reference sample of rat liver microsomes (*mic*; 2 μg) was also included in the electrophoresis. The results presented are duplicate precipitations from each culture. Similar results were obtained in two independent experiments.

nonspecific loss of immunological tolerance toward hepatic proteins seems unlikely because significant autoreactivity against another P450 (CYP3A) (Fig. 2) or against NADPH-dependent P450 reductase was not detected.

A specific loss of tolerance toward native CYP2E1, as a consequence of CF_3CO-CYP2E1 adduct formation, would be analogous with two other examples of drug-induced hepatitis, namely, hepatitis caused by tienilic acid and by dihydralazine. In both cases, P450 isozyme-specific metabolism of the causative agent to reactive metabolites that bind covalently to the same P450s was demonstrated, as was the presence in the sera of patients of autoantibodies that recognized the relevant P450s (CYP2C9 and CYP1A2) (25, 26). Furthermore, it was reported that anti-CYP3A antibodies are expressed in sera of patients who develop hypersensitivity reactions to carbamazepine, a CYP3A substrate (27). Overall, these findings prompted the speculation that autoimmune responses to P450s could be a common process underlying many allergic drug reactions. Clearly, the present findings are consistent with this hypothesis.

CYP2E1 is the first example of an integral membrane protein that has been shown to be recognized by antibodies from patients with halothane hepatitis. The other protein antigens identified to date are believed to be concentrated in the lumen of the endoplasmic reticulum and are either soluble or peripheral membrane proteins (6). A group of halothane-induced liver antigens was described recently that is recognized by antibodies from patients with halothane hepatitis in a conformation-dependent manner (7). It seems likely that a part of this group of microsomal neoantigens, shown to be integral membrane proteins (7), constitutes CF_3CO-CYP2E1 adducts.

Cell model systems for investigation of expression of CF_3CO adducts. Cultured FGC4 rat hepatoma cells provided a very useful model system for investigation of formation and trafficking of CF_3CO adducts. The FGC4 cell is highly differentiated and constitutively expresses liver-specific proteins, including albumin, phosphoenolpyruvate carboxykinase, alcohol dehydrogenase, and the P450 isozymes CYP2B (14) and CYP2E1 (28). Regulation of CYP2E1 expression in these cells shows several similarities with primary hepatocytes and with regulatory mechanisms operative *in vivo*. Thus, the cellular level of CYP2E1 is increased by imidazole (Fig. 3) and by ethanol (28), which is most probably explained by a specific ligand-dependent stabilization of the CYP2E1 holoenzyme (17). Chlormethiazole has been recog-

nized previously as a specific inhibitor of CYP2E1 expression *in vivo* during starvation of rats (29), and run-off analysis with isolated nuclei from livers of starved rats indicated that this effect was mediated at the level of transcription of the CYP2E1 gene (29). The results presented here (Fig. 3) confirm that chlormethiazole is acting directly on the liver cell and that starvation is not a prerequisite for its inhibitory action on CYP2E1 expression.

The pattern of adducts formed *in vitro* in the FGC4 cells was very similar to that detected *in vivo* in livers of halothane-treated rats. In particular, the preferential targets for CF_3CO-modification in FGC4 cells and *in vivo* seemed to be the same at early time points after halothane administration [i.e., 55- and 60-kDa proteins (compare Fig. 4A and Fig. 1A)]. Furthermore, because imidazole pretreatment of cells enhanced adduct formation, whereas chlormethiazole dramatically inhibited adduct formation (Fig. 4, A and B), it seems clear that adduct formation was dependent on the level of CYP2E1. Furthermore, an almost-complete block of adduct formation was mediated by DAS, a specific inhibitor of CYP2E1 catalysis (18). Although it cannot be excluded that some of this inhibition is due to a direct reaction between DAS and the reactive halothane metabolites, CYP2E1-dependent generation of CF_3CO adducts in FGC4 cells is consistent with the recent finding that CF_3CO adduct formation *in vitro*, in isolated rat and human liver microsomes, correlated with the microsomal content and activity of CYP2E1.[1] It is also consistent with earlier studies of adduct formation *in vivo* in livers of rats treated with halothane after pretreatment with various P450 isozyme-selective inducers (8).

Expression of CF_3CO adducts and CYP2E1 on the cell surface. Clearly, the vast majority of CF_3CO adducts and CYP2E1 were expressed intracellularly, in a morphological pattern corresponding to the ER (Fig. 5). This is consistent with the known organellar location of P450s (30), with biochemical studies that have shown that CF_3CO adducts are concentrated in microsomal fractions prepared from rabbit and rat liver (4, 5) and with the finding that the major CF_3CO adducts are modified forms of proteins believed to reside in the lumen of the ER (6). In addition, a small but significant fraction of both total cellular CF_3CO adducts and CYP2E1 were detected on the cell surface.

Earlier investigations of cell surface expression of these molecules were undertaken using cells isolated by mechanical disruption or collagenase digestion of livers. These have yielded conflicting results, which could have been affected by cell surface modification, due either to proteolysis or to incorporation of intracellular debris released from damaged cells. Immunofluorescence studies have suggested that antibodies from patients with halothane hepatitis recognize neoantigens expressed on the surface of hepatocytes isolated from livers of halothane-treated rabbits (2), whereas studies performed using anti-CF_3CO adduct antisera have indicated that such adducts are expressed on hepatocytes from rats treated with halothane *in vivo* (11) and on the surface of isolated human hepatocytes that were exposed to halothane *in vitro* (31). Similarly, CYP2E1, other P450 isozymes, and P450 reductase were found on the surface of hepatocytes isolated from rat and human liver (18a, 33). However, no expression of hepatocyte surface-located CF_3CO adducts were detected when hepatocytes isolated from livers of halothane-treated mice were analyzed by fluorescence activated flow cytometry (35). Furthermore, no CYP2B or CYP2D could be detected on the cell surface using electron microscopy (30, 36).

The investigations of the cellular location of CYP2E1 addressed two further important issues. First, studies of transfected and untransfected V79 cells (Fig. 7) verified that the cell surface immunoreactivity recognized by the anti-CYP2E1 antiserum was attributable to CYP2E1 and indicated that the cell surface form of the protein originates from the wild-type human CYP2E1 mRNA, and not a splicing variant. Second, the IP studies (Fig. 8) revealed that the cell surface immunoreactivity was due to the presence of intact CYP2E1 apoprotein, and not to any detectable proteolytic fragments. Although quantitative interpretations of the IP data should be made with great caution, the results suggest that maximally a small percentage, probably less, of the cellular contents of CYP2E1 can become expressed on the surface of the hepatoma cells studied. This implies that although there is an uncharacterized mechanism of efficient retention of P450 in the ER, leakage of P450 molecules into the secretory pathway (34) can occur. Furthermore, some epitopes of CYP2E1 must be oriented extracellularly to be recognized by antibodies on the surface of intact cells. It is interesting to note that this is inconsistent with the current models of the membrane topology of P450s in the ER, which predict that very little of the molecule is exposed on the luminal side of the lipid bilayer (37).

The role of CYP2E1 in halothane hepatitis. Our results suggest that CYP2E1 could play a dual role in halothane hepatitis: first, as a major catalyst of formation of CF_3CO-protein adducts, which elicit immune responses in susceptible patients,[1] and, second, as a cell surface target antigen that is recognized by the antibodies of patients and potentially by other immune effector mechanisms. CYP2E1 could be an especially important target antigen because it is an integral membrane protein that will be bound tightly to the cell surface. In contrast, the other CF_3CO adducts identified to date are soluble or peripheral membrane proteins that if exported from the lumen of the ER to the cell surface, could well be poorly retained and/or released from the cell. CYP2E1 is an abundant and inducible hepatic enzyme that has been shown to bioactivate many xenobiotics other than halothane (including various other volatile anesthetics and structurally related compounds) (38). In the future, it may prove to also be involved in allergic reactions to other compounds.

Acknowledgments

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