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Immunochemical Evidence of Trifluoroacetylated Cytochrome P-450 in the Liver of Halothane-Treated Rats

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SUMMARY

Four hours after the administration of halothane to phenobarbital-pretreated rats, subcellular fractions of liver were isolated and the proteins in the fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets, and immunochemically stained with anti-trifluoroacetylated antibodies. The microsomal fraction contained the highest level of trifluoroacetylated adducts. Its major trifluoroacetylated component was immunochemically identified as a phenobarbital-inducible form of cytochrome P-450 (54 kDa), whereas the other observed trifluoroacetylated protein fraction (59 kDa) was not identified. The plasma membrane fraction also contained a 54-kDa trifluoroacetylated adduct, which was immunochemically related to the 54-kDa cytochrome P-450. Microsomes from untreated rats that were administered halothane contained only the 59-kDa trifluoroacetylated protein fraction. The specificity of the immunochemical staining for the bound oxidative metabolite of halothane was confirmed by the finding that rats treated with deuterated halothane had considerably less stained liver proteins than did those treated with halothane. These results suggest that the CF_3COX oxidative metabolite of halothane is so reactive that it binds predominantly to the cytochrome P-450 that produced it.

INTRODUCTION

The fulminant hepatotoxicity caused by halothane has been thought to have an immunological basis, because this toxicity occurs most often after repeated administration of halothane and because sera from several patients recovering from severe halothane hepatotoxicity contained specific antibodies and lymphocytes which interacted with hepatocytes from halothane-treated rabbits (1–9). It therefore seemed possible that a reactive metabolite may alter the surface structure of hepatocytes and, in susceptible individuals, induce an immune response, which in turn leads to fulminant hepatotoxicity.

In accord with this view, cytochrome P-450 in liver microsomes converts halothane to at least two different reactive metabolites that conceivably could cause alterations on the surface of hepatocyte plasma membranes. Under anaerobic conditions, halothane ($CF_3CHClBr$) is reduced by liver microsomes to produce the reactive radical intermediate, 1-chloro-2,2,2-trifluoroethyl radical (CF_3CHCl). This product can react with liver microsomal protein, lipid, and presumably other unidentified target substances in the liver (10–13). In contrast, liver microsomes in air catalyze the oxidation of halothane to a trifluoroacetyl halide (CF_3COX) intermediate that either acylates tissue molecules to form TFA[1] adducts or reacts with water to form trifluoroacetic acid (10, 14–17).

Neuberger *et al.* (8) provided evidence suggesting that the oxidative pathway may be more important than the reductive pathway for the generation of halothane-altered plasma membranes. To evaluate this possibility, we recently developed immunochemical procedures for detecting TFA adducts in hepatocytes (18). After the administration of halothane to rats, TFA adducts were preferentially localized in hepatocytes in the centrilobular region of the liver. Moreover, TFA adducts also appeared to be present in the plasma membranes of the hepatocytes. In the present investigation, we have extended these studies by immunochemically identifying a major target of CF_3COX in hepatocytes from halothane-treated rats.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from the following sources: Dextran 500 and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, NJ); sodium cholate, Lubrol PX, and polyethylene glycol (approximate molecular weight 8000) from Sigma Chemical Company (St. Louis, MO); DEAE Affi-Gel Blue resin, Bio-Gel HTP

[1] The abbreviations used are: TFA, trifluoroacetyl group; IP, intraperitoneal; EDTA, ethylenediaminetetraacetic acid; halothane-d, deuterium-labeled halothane; SDS/PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-HCl-buffered saline, pH 7.4; SBR, swinging bucket rotor; BSA-TBS, 3% bovine serum albumin in Tris-HCl-buffered saline, pH 7.4; PBS, phosphate-buffered saline.

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hydroxylapatite resin, and 4-chloro-1-naphthol from Bio-Rad Laboratories (Richmond, CA); Freund's complete and incomplete adjuvant, agarose, normal rabbit IgG, goat antirabbit IgG, and peroxidase-rabbit antiperoxidase complex from Miles Laboratories (Naperville, IL); DE-52 anion exchange resin from Whatman, Inc. (Clifton, NJ). Emulgen 911 detergent was a gift from Kao Corporation (Tokyo, Japan); rabbit anti-TFA was prepared as previously described (18); and halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) was purified by distillation before it was used. Deuterium-labeled halothane (halothane-d) was prepared as previously described (16).

Purification of two forms of cytochrome P-450 from phenobarbital-treated rats. Two forms of cytochrome P-450 were purified from phenobarbital-treated rats by modifications of the methods of Imai and Sato (19) and Guengerich *et al.* (20). Male Sprague-Dawley rats (60–80 g) were obtained from Taconic Farms (Germantown, NY), treated with sodium phenobarbital (80 mg/kg in normal saline, IP) for 4 days, and killed 24 hr after the last treatment. Microsomes were prepared as previously described (21) (130 ml, 32.6 mg/ml, and 2.45 nmol of cytochrome P-450/ml) and were solubilized by stirring in 930 ml of 0.1 M potassium phosphate (pH 7.25) containing 1 mM EDTA, 1 mM dithiothreitol, 0.6% (w/v) sodium cholate, and 20% (v/v) glycerol (buffer A) for 30 min at 4°. The mixture was centrifuged at 100,000 × *g* for 65 min at 4°, and the supernatant (1030 ml) was divided in half and applied to each of two octylamine Sepharose columns (2.5 × 40 cm), which had been equilibrated with 1000 ml of buffer A at 1 ml/min at 4°. The columns were washed with 800 ml of buffer B (same as buffer A but containing 0.5% (w/v) sodium cholate), and the cytochromes P-450 were eluted from the columns with buffer C (same as buffer A, but containing 0.4% (w/v) sodium cholate and 0.08% (v/v) Emulgen 911). After 500 ml, the cytochromes P-450 began to elute from the columns. The fractions containing the cytochromes P-450 from each column were pooled (224 ml), diluted with 2 vol of a solution containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% (v/v) Emulgen 911, and divided into two equal portions. Each of these was applied to a column of hydroxylapatite (5 × 14 cm) which had been equilibrated with 300 ml of 35 mM potassium phosphate (pH 7.25) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% (v/v) glycerol, and 0.2% Emulgen 911 (buffer D) at 1 ml/min at 4°. The columns were washed with 150 ml of buffer D and the cytochromes P-450 were eluted from columns first with buffer E (same as buffer D, but containing 90 mM potassium phosphate) and then with buffer F (same as buffer D, but containing 150 mM potassium phosphate). The 90 mM and 150 mM potassium phosphate fractions from each column contained mainly proteins of molecular weights 54,000 and ≤52,000, respectively, as determined by SDS/PAGE following the method of Laemmli (22) as modified by Guengerich (23). The respective fractions were pooled (approximately 100 ml total volume for each pooled fraction) and dialyzed for 48 hr at 4° against 50 vol of 5 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% (v/v) glycerol, 0.2% (v/v) Emulgen 911, and 0.5% (w/v) sodium cholate (buffer G), with one change.

The 54-kDa cytochrome P-450 was isolated from the dialyzed 90 mM hydroxylapatite column fraction by chromatography on a column of DE-52 anion exchange resin (2.5 × 20 cm) which had been equilibrated with 200 ml of buffer G at 1 ml/min at room temperature. After the sample was loaded, the column was washed with 60 ml of buffer G and then eluted with a 500-ml linear gradient of 0 to 0.2 M potassium chloride in buffer G. The 54-kDa form of cytochrome P-450 began eluting from the column at approximately 50% through the salt gradient. The fractions containing the apparently homogeneous protein were combined and dialyzed for 48 hr at 4° against 50 vol of 10 mM potassium phosphate (pH 7.25) containing 0.1 mM EDTA and 20% glycerol (buffer H) with one change. The detergent was removed and the enzyme was concentrated by applying the dialyzed sample to a hydroxylapatite column (1 × 7 cm) which had been equilibrated with 25 ml of buffer H at 1 ml/min at 4°. The column was washed with 35 mM potassium phosphate (pH 7.25) containing 20% glycerol until the absorbance (at 276 nm) of the eluent, due to Emulgen 911, was less than 0.01. The cytochrome P-450 was then eluted from the column with 300 mM potassium phosphate (pH 7.25) containing 20% glycerol, dialyzed for 24 hr at 4° against 50 mM potassium phosphate (pH 7.25) containing 25% glycerol with one change, and stored at −80° until used. The 54-kDa cytochrome P-450 was purified with a yield of 2.8% and had a specific content of 17.0 nmol/mg of protein, an absolute oxidized spectrum with a Soret maximum at 417.0 nm, an absolute reduced spectrum with a Soret maximum at 413.5 nm, a carbon monoxide-reduced difference spectrum with a Soret maximum at 449.4 nm, and a metyrapone binding spectrum to ferrous cytochrome P-450 with a maximum at 444.8 nm. Based upon the physical properties of this enzyme and the way it was purified, it most likely corresponds to cytochromes PB-B of Guengerich *et al.* (20, 24) and PB-4 of Waxman and Walsh (25).

The 52-kDa cytochrome P-450 was isolated from the dialyzed 150 mM hydroxylapatite column fraction on a DE-52 anion exchange column as described above. The fractions containing apparently homogeneous 52-kDa cytochrome P-450 eluted from the column at approximately 40% through the potassium chloride gradient. They were combined, the detergent was removed, and the enzyme was concentrated as described above. The 52-kDa cytochrome P-450 was purified with a yield of 1.5% and had a specific content of 8.8 nmol/mg of protein, an absolute oxidized spectrum with a Soret maximum at 417.7 nm, an absolute reduced spectrum with a Soret maximum at 413.5 nm, a carbon monoxide-reduced difference spectrum with a Soret maximum at 449.3 nm, and a metyrapone binding spectrum to ferrous cytochrome P-450 with a maximum at 444.8 nm. At the present time, it is not clear whether this enzyme corresponds to any form purified by other investigators.

Antiserum to purified 52- and 54-kDa cytochromes P-450 were produced in female New Zealand White rabbits essentially by the method of Kamataki *et al.* (26). The antiserum to the two forms of cytochrome P-450 did not cross-react when analyzed by Ouchterlony double immunodiffusion.

Preparation of subcellular fractions of liver after the administration of halothane or halothane-d. Male Sprague-Dawley rats (150–250 g) were obtained from Taconic Farms and maintained on Purina Rat Chow and water *ad libitum*. Animals were treated daily with sodium phenobarbital (80 mg/kg in normal saline, IP) for 3 days. At 24 hr after the last treatment, the rats were administered halothane, halothane-d (10 mmol/kg in 50% sesame oil solution, v/v, IP) or sesame oil (IP). After 4, 12, or 24 hr, the animals were killed by decapitation and their livers were removed and placed on ice.

The total homogenate, cytosol, and microsomal fractions were isolated in the following manner. Livers were homogenized in 3.3 vol of ice-cooled 50 mM TBS. A portion of this total homogenate was saved and the remainder was centrifuged at 9,000 × *g* for 20 min at 4°. The supernatant was collected and centrifuged at 105,000 × *g* for 60 min at 4°. The resultant supernatant (cytosol fraction) was saved and the microsomal pellet was resuspended in the same volume of TBS and recentrifuged at 105,000 × *g* for 60 min. The microsomal pellet was resuspended in TBS to a protein concentration of approximately 30 mg/ml (microsomal fraction).

A plasma membrane fraction was isolated by a modification of the procedure of Lesko *et al.* (27). An aqueous two-phase system was prepared by adding solutions of Dextran 500 (200 g of a 20% aqueous solution, w/w), polyethylene glycol (103 g of a 30% aqueous solution, w/w), 0.22 M sodium phosphate (333 ml, pH 6.5), and water (179 ml) to a separatory funnel. The mixture was shaken and allowed to separate into two phases for 48 hr at 4°. Portions of liver (15 g) were homogenized in 200 ml of ice-cooled 0.5 mM calcium chloride containing 1 mM sodium bicarbonate (pH 7.5, buffer A) with a Dounce homogenizer (15 excursions with a loosely fitting pestle). The homogenate was diluted with 1500 ml of buffer A, passed through four layers of cheesecloth, and centrifuged in an SBR at 1350 × *g* for 30 min at 4°. The pellet was suspended in 53 ml of the top phase of the polymer mixture, mixed

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gently with 53 ml of the bottom phase of the polymer mixture, and centrifuged (SBR) at 1100 × *g* for 15 min at 4°. The plasma membrane fraction, which was located at the interface of the two polymer solutions, was collected, resuspended in 53 ml of the top phase, mixed with 53 ml of the bottom phase, and recentrifuged (SBR) at 1100 × *g* for 15 min at 4°. The plasma membrane fraction was further purified by repeating the latter isolation procedure an additional two times. It was then resuspended in 70 ml of buffer A, centrifuged (SBR) at 1100 × *g* for 15 min at 4°, and resuspended in 2 ml of TBS.

All isolated cellular fractions were rapidly frozen in a dry-ice acetone bath and stored at −20° until used.

Immunoblotting of components of the liver subcellular fractions with anti-TFA or anti-cytochrome P-450 (52 and 54 kDa) serum. The total homogenate and the cytosol, microsomal, and plasma membrane fractions were solubilized by sonication for 30 min at 20° with an equal volume of 25% (w/v) SDS containing 10% (v/v) mercaptoethanol, followed by the addition of 1 vol of 40% (v/v) glycerol. SDS/PAGE separation of the components of the solubilized samples was performed essentially by the method of Laemmli (22) as modified by Guengerich (23) in a separating gel (7.5% acrylamide) 1.5 mm thick and 12 cm long; stacking was done at 25 mamp/gel and the separation was performed at 50 mamp/gel. The separated proteins were transferred electrophoretically from the polyacrylamide gels to nitrocellulose sheets following the general procedure of Towbin *et al.* (28). The transfer was performed at a current of 150 mamp for 12 hr in a transferring buffer composed of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3. The blots were either stained for protein with amido black (29) or prepared for immunoperoxidase staining by initially shaking gently in 3% (w/v) BSA-TBS for 3 hr at room temperature to block any reactive sites of the nitrocellulose. After removing the excess bovine serum albumin solution, the blots were incubated with either anti-TFA serum (diluted 1:1,000 in BSA-TBS) overnight at 4° or anti-cytochrome P-450 (52 or 54 kDa) serum (diluted 1:5,000 or 1:10,000, respectively, in BSA-TBS) for 1 hr at room temperature. They were washed three times with 0.05% (v/v) Tween-20 in TBS (10 min each wash, with gentle shaking at room temperature) and incubated with goat antirabbit IgG solution (diluted 1:100 in BSA-TBS) for 1 hr at room temperature. The blots were washed three times with 0.05% (v/v) Tween-20 in TBS and incubated with peroxidase-rabbit antiperoxidase complex (diluted 1:1,000 in 0.25% (w/v) gelatin in TBS) for 1 hr at room temperature. After being washed three times with 0.05% (v/v) Tween-20 in TBS, the blots were incubated with a fresh peroxidase substrate mixture composed of 4-chloro-1-naphthol (60 mg in 20 ml of methanol), TBS (100 ml), and 30% (v/v) hydrogen peroxide (60 μl) for 30 min at room temperature to stain the gels. The reactions were stopped by washing the stained blots several times in water.

Removal of TFA adduct from liver microsomes with Sepharose-bound anti-cytochrome P-450 (54 kDa) IgG. The IgG fraction of the anti-cytochrome P-450 (54 kDa) serum was isolated on a DEAE Affi-Gel Blue column following the directions given by Bio-Rad Laboratories.

Anti-cytochrome P-450 (54 kDa) IgG or normal rabbit IgG was coupled to CNBr-activated Sepharose 4B in the following manner. The CNBr-activated Sepharose 4B (1 g) was washed with 1 mM HCl, suspended in 6 ml of 0.1 M $NaCO_3$ (pH 8.3) containing 0.5 M NaCl (buffer A), divided into two portions, and centrifuged at 1,500 × *g* for 4 min in order to remove excess buffer A. Each IgG solution (1.5 ml of 5 mg/ml in buffer A), followed by 1 ml of buffer A was added to a portion of the sedimented gel and the mixtures were incubated with gentle rotation for 2 hr at room temperature. The gels were reisolated by centrifugation and the remaining reactive groups were blocked by treatment with 0.2 M glycine in 10 ml of buffer A with gentle mixing overnight at 4°. The gels were centrifuged and washed alternately with buffer A (10 ml) with a mixture of 0.1 M acetic acid and 0.5 M NaCl at least 10 times. They were then resuspended in PBS, packed into a Pasteur pipet (600-μl bed volume), and equilibrated with a solution of 0.4% (w/v) sodium cholate, 0.2% (v/v) Lubrol PX, 6.7% (v/v) glycerol, 1.3 mM EDTA, and 0.5 M NaCl in PBS. Microsomes (1 ml of a 31.5-mg/ml suspension), isolated 12 hr after halothane treatment of phenobarbital-pretreated rats, were solubilized by sonication for 30 min at room temperature in a mixture of 6% (w/v) sodium cholate in PBS (100 μl), 3% (v/v) Lubrol PX in PBS (100 μl), 2 mM EDTA in PBS (100 μl), glycerol (100 μl), and PBS (100 μl) and centrifuged at 100,000 × *g* for 60 min at 4°. An aliquot (10 μl) of the supernatant was applied to each column, followed by the addition of buffer A (20 μl), incubation for 36 hr at room temperature, and elution with buffer A. Fractions of approximately 65 μl (3 drops of eluent) were collected, diluted with a mixture of 10% (w/v) SDS, 2% (v/v) mercaptoethanol, 16% (v/v) glycerol, and 0.01% (w/v) bromophenol blue, and the constituent polypeptides were separated by SDS/PAGE. They were transferred to nitrocellulose and probed with either anti-TFA or anti-cytochrome P-450 (54 kDa) serum following the same procedures described above.

Other methods. Protein was determined by the method of Lowry *et al.* (30) with bovine serum albumin as standard. Cytochrome P-450 content was calculated according to the method of Omura and Sato (31) from the CO-reduced difference spectrum based on an extinction coefficient of 91 mM^{-1} cm^{-1}.

RESULTS

Immunoperoxidase detection of TFA adducts in various subcellular fractions of rat liver. Four hours after halothane administration to phenobarbital-pretreated rats, TFA adducts were detected in various subcellular fractions of the liver by immunoperoxidase staining with anti-TFA serum of an SDS/PAGE blot (Fig. 1). Although the total cellular homogenate contained several TFA components, one major product was present with an apparent molecular weight of 54,000. This TFA adduct was localized in the microsomal fraction and not in the cytosolic fraction of the cell. A TFA component with identical electrophoretic properties was also detected in the plasma membrane fraction of the cell.

The total homogenate from rats treated with halothane-d contained significantly smaller amounts of TFA adducts than did the homogenates of halothane-treated rats. This finding confirmed that the staining was predominantly due to the presence of bound TFA groups and not reductive metabolites, since substitution of the hydrogen atom of halothane by a deuterium atom decreases the rate of oxidation but does not alter the rate of reduction of halothane (16). Moreover, no staining of cellular components was observed when normal serum was used in place of the anti-TFA serum or when anti-TFA serum was used to assess covalently bound material in the total homogenates of untreated rats.

The 54-kDa microsomal component was the major anti-TFA staining fraction in the microsomes of phenobarbital-pretreated rats 4, 12, and 24 hr after the administration of halothane (Fig. 2). A less intensely stained TFA component was also detected in these microsomes. This fraction had an apparent molecular weight of 59,000 and corresponded to a weakly stained protein component of the total homogenate (Fig. 1). A fraction with the same apparent molecular weight was the only TFA adduct detected in the microsomes of normal rats 12 and 24 hr after halothane treatment but was not observed in the microsomes 4 hr after halothane treatment.

Immunochemical identification of the microsomal TFA adducts with anti-cytochrome P-450 antibodies. In order to determine whether the 54-kDa anti-TFA staining component in the microsomes of phenobarbital-pre-

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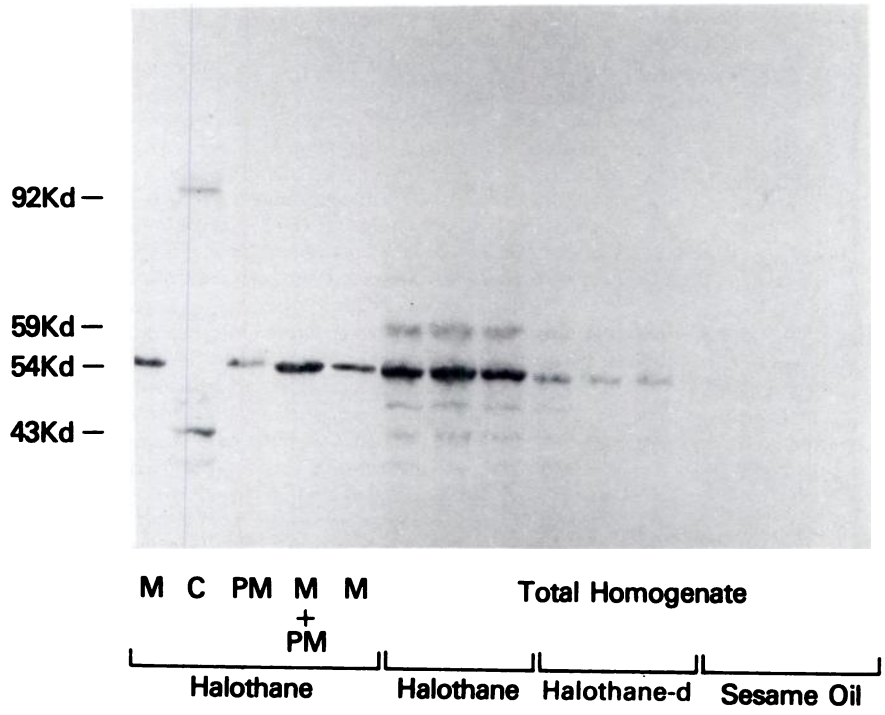

FIG. 1. *Immunoperoxidase staining with anti-TFA serum of SDS/PAGE blots of liver subcellular fractions 4 hr after halothane or halothane-d treatment of phenobarbital-pretreated rats*

Rats were pretreated with phenobarbital and then administered halothane, halothane-d (10 mmol/kg in sesame oil solution), or sesame oil (control group). After 4 hr, proteins from various liver subcellular fractions were separated by SDS/PAGE, transferred to nitrocellulose, and indirectly immunoperoxidase stained with anti-TFA serum as described in Experimental Procedures. The following amounts of subcellular fractions were applied to the wells of the gel: *M* (microsomes), 27 μg; *C* (cytosol), 160 μg; *PM* (plasma membrane), 27 μg; *M + PM*, 27 μg each; *Total Homogenate*, 100 μg. The molecular weight designations in kilodaltons (Kd) were determined by applying a mixture of protein standards containing 0.5 μg each of ovalbumin (45 Kd), glutamate dehydrogenase (53 Kd), bovine serum albumin (66.2 Kd), and phosphorylase B (92.5 Kd) to a well and detecting their positions on the nitrocellulose sheet by staining with amido black.

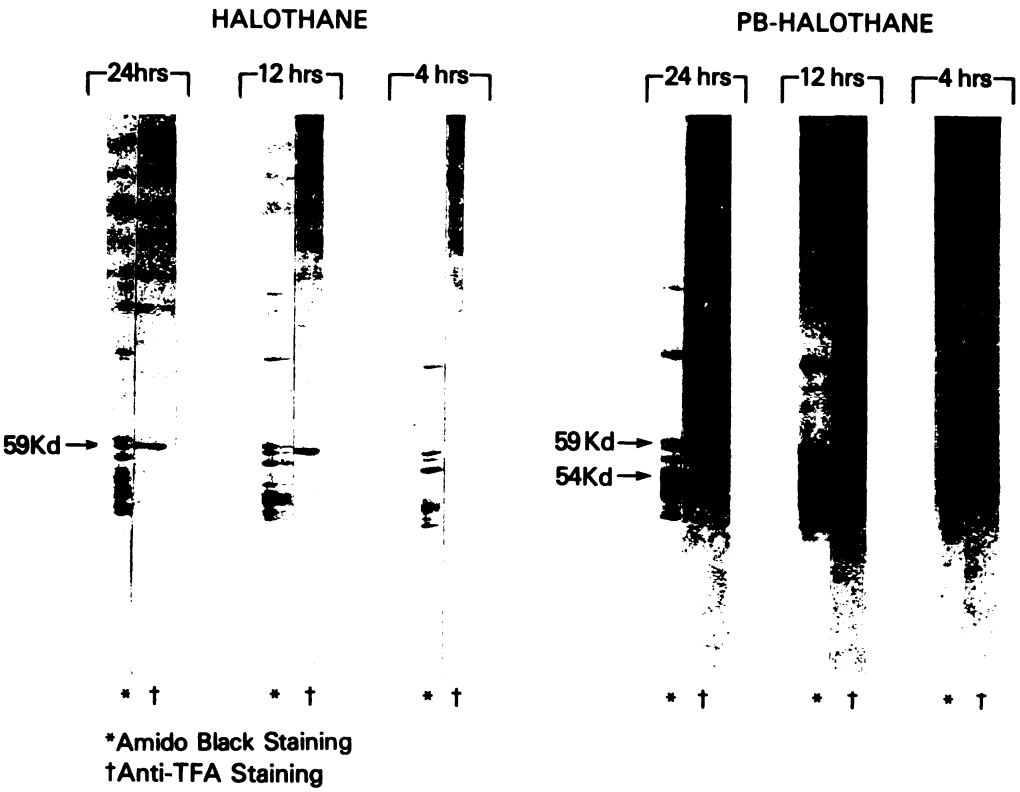

FIG. 2. *Immunoperoxidase staining with anti-TFA serum and protein staining with amido black of SDS/PAGE blots of liver microsomes 4, 12, and 24 hr after halothane treatment of control or phenobarbital-pretreated rats*

Rats were pretreated with phenobarbital and then administered halothane (10 mmol/kg in sesame oil solution). After 4, 12, and 24 hr, liver microsomes were isolated and samples (10 μg) were applied to each well of the gel; then, the resulting blots were stained with either anti-TFA serum or amido black as described in Experimental Procedures. The molecular weights were determined as described in the legend to Fig. 1.

treated rats might be cytochrome P-450, the immunoperoxidase staining of the microsomes was repeated with both anti-TFA and anti-cytochrome P-450 (Fig. 3). The staining patterns of microsomes 4 hr after the administration of halothane showed that the 54-kDa TFA component and the 54-kDa form of cytochrome P-450 had the same apparent molecular weight and suggested that the TFA moiety was bound to the 54-kDa form of cytochrome P-450. This idea was supported by passing detergent-solubilized microsomes, isolated 12 hr after the administration of halothane to phenobarbital-pretreated rats, through an affinity column of anti-cytochrome P-450 (54 kDa) IgG and showing that this treatment simultaneously removed the 54-kDa protein that reacted with anti-TFA and anti-cytochrome P-450 (54 kDa) serum (Fig. 4). The immunoperoxidase-stained 59-kDa fraction, however, passed through this affinity column, indicating that it was not immunochemically related to

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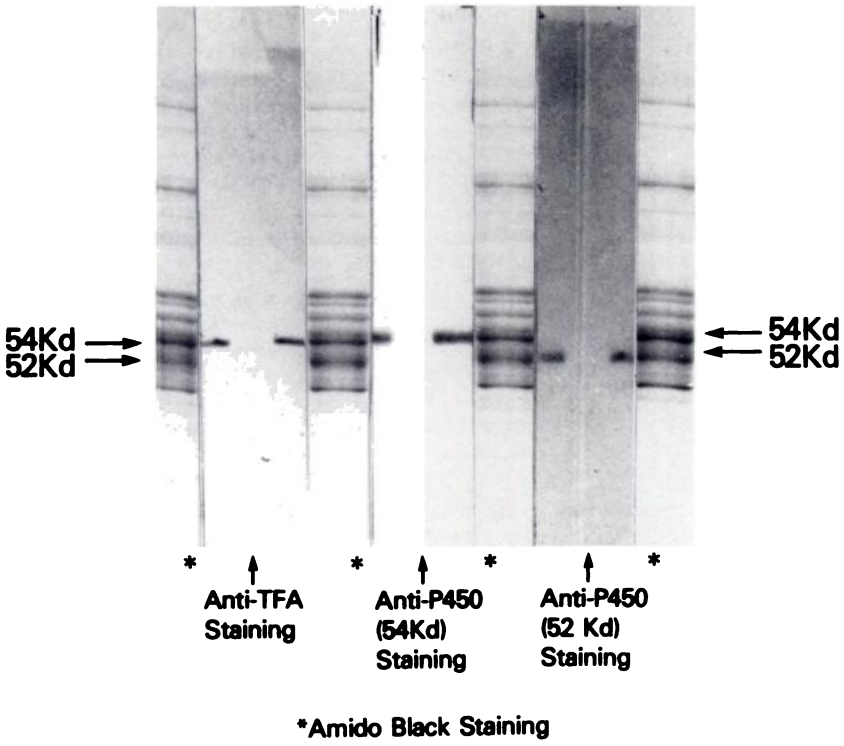

FIG. 3. *Immunoperoxidase staining with anti-TFA, or anti-cytochrome P-450 (52 or 54 Kd) serum and protein staining with amido black of SDS/PAGE blots of liver microsomes 4 hr after halothane treatment of phenobarbital-pretreated rats*

Rats were pretreated with phenobarbital and then administered halothane (10 mmol/kg in sesame oil solution). After 4 hr, liver microsomes were isolated and samples (10 μg) were applied to each well of the gel; then, the resulting blots were stained with anti-TFA, anti-cytochrome P-450 (52 or 54 Kd) serum or amido black as described in Experimental Procedures. The molecular weights were determined as described in the legend to Fig. 1.

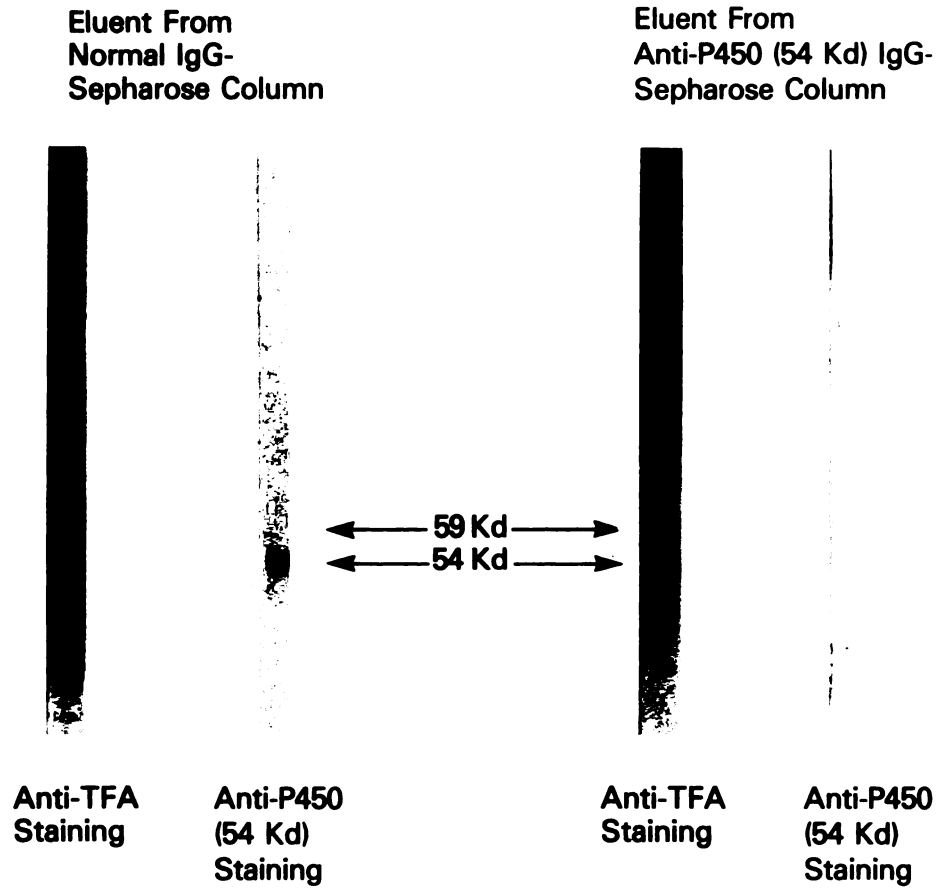

FIG. 4. *Specific removal of 54-Kd TFA adduct from rat liver microsomes with Sepharose-bound anti-cytochrome P-450 (54 Kd) IgG*

Rats were pretreated with phenobarbital and then administered halothane (10 mmol/kg in sesame oil solution). After 12 hr, liver microsomes were isolated, solubilized in detergent, and passed through a column of either Sepharose-bound normal rabbit IgG or Sepharose-bound anti-cytochrome P-450 (54-Kd) IgG. The protein components in the eluents from the columns were applied to wells of a gel and the resulting blots were stained with either anti-TFA or anti-cytochrome P-450 (54 Kd) serum as described in Experimental Procedures. The molecular weights were determined as described in the legend to Fig. 1.

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the TFA cytochrome P-450 (54 kDa) protein. In contrast, the protein that reacted with anti-TFA and anti-cytochrome P-450 (54 kDa) serum passed through a Sepharose column containing bound normal IgG in place of anti-cytochrome P-450 (54 kDa) IgG.

DISCUSSION

In this investigation, we have used specific immunoperoxidase staining methods to detect protein adducts of the reactive oxidative metabolite of halothane, CF_3COX, in subcellular fractions of the rat liver. The results indicate that CF_3COX reacts covalently with a relatively small number of proteins in the cell. In phenobarbital-pretreated rats, cytochrome P-450 (54 kDa), which is apparently equivalent to cytochrome PB-B of Guengerich *et al.* (20, 24) and cytochrome PB-4 of Waxman and Walsh (25), is either identical to or immunochemically closely related to the protein in the endoplasmic reticulum of the cell that reacts predominantly with this metabolite (Fig. 4).

The specificity of the reaction is probably due to the high reactivity of CF_3COX. Consequently, most of this species is probably trapped by the molecule of cytochrome P-450 that has produced it before it has had time to diffuse away and react with other proteins. The binding of the TFA group to cytochrome P-450, however, does not appear to inhibit its catalytic activity, since treatment of rats with halothane-d in place of halothane leads to less TFA cytochrome P-450 (54 kDa) (Fig. 1), but does produce a greater decrease in benzphetamine demethylation activity in liver microsomes than that produced by halothane treatment (32). This finding is consistent with *in vitro* studies which have indicated that the reductive rather than the oxidative pathway of halothane metabolism is predominantly responsible for the cytochrome P-450-inhibitory properties of halothane (33, 34). In contrast, cytochromes P-450 in rat liver are inactivated by the reactive oxamyl chloride metabolite of the antibiotic chloramphenicol (35, 36).

Although the identity of the 59-kDa TFA protein detected in microsomes of both phenobarbital-pretreated and normal rats (Fig. 3) has not been determined, it is possibly another TFA isozyme of cytochrome P-450. This idea seems reasonable based upon the high degree of reaction specificity of CF_3COX found in the livers of phenobarbital-pretreated rats and the fact that only one TFA protein was detected in the microsomes of the untreated rats that were administered halothane.

The 54-kDa TFA adduct detected in the plasma membrane fraction of the phenobarbital-pretreated rats that had been administered halothane (Fig. 1) is potentially the most interesting finding of this study. Preliminary results indicate that it is immunochemically related to cytochrome P-450 (54 kDa) (results not shown). If this is found not to be due to contamination by the microsomal fraction of the cell (27), which cannot be ruled out at this time, it would suggest that either a microsomal cytochrome P-450 TFA adduct has been incorporated into the plasma membrane of the cell or that CF_3COX has been formed by cytochrome P-450 in the plasma membrane and has reacted covalently with it. Clearly, more work is needed to elucidate the identity and origin of this plasma membrane TFA adduct and to determine its relevance to halothane-induced immunotoxicity.

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