LABORATORY INVESTIGATIONS

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Immunochemical Evidence against the Involvement of Cysteine Conjugate β-lyase in Compound A Nephrotoxicity in Rats

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Background: **Compound A, a degradation product of sevoflurane, causes renal corticomedullary necrosis in rats. Although the toxicity of this compound was originally hypothesized to result from the biotransformation of its cysteine conjugates into toxic thionoacyl halide metabolites by renal cysteine conjugate β-lyase, recent evidence suggests that alternative mechanisms may be responsible for compound A nephrotoxicity. The aim of this study was to evaluate these issues by determining whether mercapturates and glutathione conjugates of compound A could produce renal corticomedullary necrosis in rats, similar to compound A, and whether renal covalent adducts of the thionacyl halide metabolite of compound A could be detected immunochemically.**

Methods: **Male Wistar rats were administered, intraperitoneally, N-acetylcysteine conjugates (mercapturates) of compound A (90 or 180 μmol/kg) or glutathione conjugates of compound A (180 μmol/kg) with or without intraperitoneal pretreatments with aminooxyacetic acid (500 μmol/kg) or acivicin (250 μmol/kg). Rats were killed after 24 h, and kidney tissues were analyzed for toxicity by histologic examination or for protein adducts by immunoblotting or immunohistochemical analysis, using antisera raised against the covalently bound thionoacyl halide metabolite of compound A.**

Results: **Mercapturates and glutathione conjugates of compound A both produced renal corticomedullary necrosis similar to that caused by compound A. Aminooxyacetic acid, an inhibitor of renal cysteine conjugate β-lyase, did not inhibit the toxicity of the mercapturates, whereas acivicin, an inhibitor of γ-glutamyltranspeptidase, potentiated the toxicity of both classes of conjugates. No immunochemical evidence for renal protein adducts of the thionacyl halide metabolite was found in rats 24 h after the administration of the mercapturates of compound A or in the kidneys of rats, obtained from a previous study, 5 and 24 h after the administration of compound A.**

Conclusion: **The results of this study are consistent with the idea that a mechanism other than the renal cysteine conjugate β-lyase pathway of metabolic activation is responsible for the nephrotoxicity of compound A and its glutathione and mercapturate conjugates in male Wistar rats. (Key words: Bioactivation; kidney; molecular toxicity.)**

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SEVOFLURANE [$FCH_2OCH(CF_3)_2$] interacts with carbon dioxide adsorbents in anesthesia delivery systems to form the vinyl ether compound A ([$F_2C = C(CF_3)OCH_2F$]).[1] Compound A administered to rats produces dose- and time-dependent renal corticomedullary necrosis.[2] It has been hypothesized that the renal toxicity may be caused by a thionacyl halide metabolite, formed from the metabolism of cysteine conjugates of compound A by renal cysteine conjugate β-lyase (renal β-lyase) (fig. 1).[3] This idea was based on the finding that the renal corticomedullary necrosis caused by other fluoroalkenes appeared to be mediated by similar toxic metabolites.[4–6] Thionoacyl halide metabolites are thought to cause toxicity, at least in part, by covalently altering renal cellular proteins.[7–10] In this regard, a preliminary immunochemical finding indicated the pres-

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ence of protein adducts of compound A in the kidneys of rats.# However, these results, using antitrifluoroacetyl sera, subsequently were found to result from nonspecific immunoreactivity (Martin *et al.,* unpublished data, December 2, 1994). Nonetheless, there is evidence to support the concept that compound A forms cysteine conjugates, which are further metabolized by renal β-lyase to produce a thionoacyl halide metabolite. Male Fischer 344 rats treated intraperitoneally with compound A excrete urinary mercapturates of compound A and urinary 3,3,3-trifluoro-2-(fluoromethoxy)propanoic acid, which would result from the reaction of the thionoacyl halide derivative of compound A with water.[11,12] It has also been shown that aminooxyacetic acid (AOAA), an inhibitor of renal β-lyase, partially blocked the nephrotoxicity of compound A in male Fischer 344 rats as measured by decreases in urine volume and proteinuria[12] and by decreases in urinary excretion of 3,3,3-trifluoro-2-(fluoromethoxy)propanoic acid.[11] Furthermore, cysteine conjugates of compound A have been shown to be substrates of rat renal β-lyase; however, the rates of reaction were several times faster than those found with human renal β-lyase, suggesting that humans may not be as susceptible to compound A nephrotoxicity as are rats.[13]

In contrast to these studies, it has been shown that pretreatment of Wistar rats with AOAA did not inhibit histologic kidney damage caused by inhalational administration of compound A.[14] This finding was confirmed in Fischer 344 rats, in which it was shown that AOAA did not inhibit the histologic renal injury or glucosuria caused by the intraperitoneal administration of compound A, even though there was improvement in biochemical markers for proximal tubular injury, namely urine volume, proteinuria, and α-glutathione-S-transferase.[15] These studies suggest that compound A–induced renal necrosis may occur by a mechanism independent of renal β-lyase.

To help resolve whether renal β-lyase has a role in compound A–induced renal necrosis, we directly studied the mechanism of renal toxicity produced by mercapturates and glutathione conjugates of compound A. We evaluated renal damage using the histologic end point of necrosis, because it is an indisputable end point of toxicity. In addition, we raised a specific antibody to detect protein adducts of compound A that would be formed by the renal β-lyase pathway of metabolism (fig. 1).

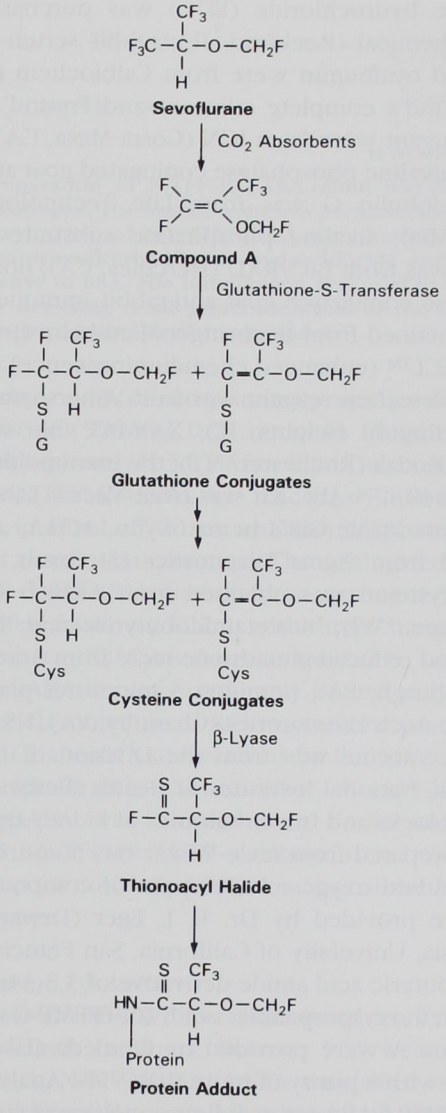

Fig. 1. A proposed pathway of compound A bioactivation and nephrotoxicity involving renal cysteine conjugate β-lyase. The first step of this mechanism involves the known dehydrofluorination of sevoflurane by carbon dioxide absorbents to form compound A. This product has been shown to form addition and addition–elimination conjugates with glutathione, in reactions catalyzed by glutathione-S-transferases. The glutathione conjugates are further metabolized to form cysteine conjugates, which are then acted on by renal cysteine conjugate β-lyase to form a thionoacyl halide reactive intermediate. The thionoacyl halide has been proposed to bind covalently to renal proteins and cause toxicity.

Materials and Methods

Chemicals and Reagents

Chemicals and reagents were obtained from the following sources. 1-Ethyl, 3-[3-(dimethylamino)propyl]car-

Martin J, Dodge M, Pohl LR: Immunochemical detection of covalently modified kidney proteins in rats treated with the sevoflurane degradation product Compound A (sevo-olefin) (abstract). ANESTHESIOLOGY 1994; 81:A436.

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bodiimide hydrochloride (EDC) was purchased from Pierce Chemical (Rockford, IL); rabbit serum albumin (RSA) and ovalbumin were from Calbiochem (La Jolla, CA); Freund's complete adjuvant and Freund's incomplete adjuvant were from ICN (Costa Mesa, CA); affinity purified alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G was from Life Technologies (Bethesda, MD); alkaline phosphatase substrate reaction mixture was from BIO/RAD (Hercules, CA); horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G was obtained from Boehringer-Mannheim (Indianapolis, IN); ECL™ (enhanced chemiluminescence) immunoblotting detection reagent was from Amersham Life Science (Arlington Heights, IL); X-OMAT film was from Eastman Kodak (Rochester, NY); the immunohistochemical Vectastain™ ABC Kit was from Vector Laboratories (Burlingame, CA); Gill's hematoxylin, AOAA, and acivicin were from Sigma Diagnostics (St. Louis, MO); N-acetyl-L-cysteine was obtained from Aldrich Chemical (Milwaukee, WI); 4-acetamidobutyric acid (N-acetyl-GABA) and reduced glutathione were from Acros Organics (Pittsburgh, PA); Immulon 4 microtiter plates were from Dynatech Laboratories (Chantilly, VA). US Standard Pertussis Vaccine was from the Division of Biological Standards, National Institutes of Health (Bethesda, MD). Paraffin blocks and frozen samples of kidney tissue (< 1 yr old) prepared from male Wistar rats 5 and 24 h after they breathed oxygen ± 300 ppm of compound A for 3 h were provided by Dr. E. I. Eger (Department of Anesthesia, University of California, San Francisco). The γ-aminobutyric acid amide derivative of 3,3,3-trifluoro-2-(fluoromethoxy)propanoic acid (N-TFFMP-GABA) and compound A were provided by Ohmeda (Liberty Corner, NJ), with a purity of more than 99%. Analytical data for N-TFFMP-GABA are as follows: melting point: 65.5 to 67.5°C; ^{1}H NMR ($CDCl_3$, tetramethylsilane standard): 1.91 (quintet, J = 7 Hz, 2H), 2.43 (triplet, J = 7 Hz, 2H), 3.42 (quartet, J = 7 Hz, 2H), 4.51 (quartet, $J_{H,F}$ = 6.7 Hz, 1H), 5.42 (ddd, $J_{H,H(geminal)}$ = 33 Hz, $J_{H,F(geminal)}$ = 51 Hz, $J_{H,H(w\text{-}coupling)}$ = 2.9 Hz, 2H), 6.85 (broad singlet, 1H); ^{19}F NMR ($CDCl_3$, fluorotrichloromethane standard): −75.00 (multiplet, 3F), −152.62 (dd, $J_{H,F}$ = 51, 57 Hz, 1F); ^{13}C NMR ($CDCl_3$, tetramethylsilane standard): 24.070, 31.084, 38.937, 77.616 (multiplet), 103.343 (doublet), 122.059 (quartet), 163.015, 178.018.

Synthesis of Compound A Mercapturates

Compound A mercapturates were synthesized by reacting compound A (23.8 mmol) with N-acetyl-L-cysteine (21.8 mmol) in methanolic sodium methoxide, according to the method of Spracklin and Kharasch.[11] ^{19}F NMR analysis revealed that the purified product consisted of approximately 54% and 46% of addition, N-acetyl-S-(1,1,3,3,3-pentafluoro-2-(fluoromethoxy)propyl)-L-cysteine (alkane), and addition–elimination, N-acetyl-S-(1-fluoro-2-(fluoromethoxy)-2-(trifluoromethyl)vinyl)-L-cysteine (alkene) products, respectively. The mercapturates were synthesized in a yield of 17% (1.23 g), and a purity of at least 98% based on thin-layer chromatographic analysis. No attempt was made to resolve the alkane and alkene isomeric products.

Synthesis of the Glutathione Conjugates of Compound A

The glutathione conjugates of compound A were synthesized by reacting reduced glutathione (6 mmol) with compound A (4 mmol), according to the methods of Jin *et al.*,[16] with the following differences: The reaction was conducted anaerobically and the high-performance liquid chromatographic method of purification was modified. The glutathione conjugates were purified by preparative high-performance liquid chromatographic analysis using a Vydac reverse-phase column (C_{18}, 10 μm, 25 cm × 2.2 cm ID, Hesparia, CA) with the absorption detector set at 220 nm. The mobile phase consisted of a mixture of solvent A (0.1% [vol/vol] trifluoroacetic acid in water) and solvent B (0.1% [vol/vol] trifluoroacetic acid in acetonitrile) delivered progressively at a flow rate of 10 ml/min using a linear gradient starting from 100% solvent A to 80% solvent B in 80 min. The glutathione conjugates were eluted as a mixture, with retention times of approximately 27 to 30 min. Solvents were removed under vacuum in a SpeedVac concentrator (Savant, Farmingdale, NY) to give 578 mg (30% yield) of product that was at least 98% pure based on high-performance liquid chromatographic analysis. ^{19}F NMR and ^{1}H NMR analyses revealed that the product was composed of approximately a 40% and 60% mixture of addition S-(1,1,3,3,3-pentafluoro-2-(fluoromethoxy)propyl)-glutathione (alkane) and addition-elimination S-(1-fluoro-2-(fluoromethoxy)-2-(trifluoromethyl)vinyl)glutathione (alkene) products, respectively. These assignments were confirmed by electrospray mass spectrometry, revealing an M+H ion at m/z 487.9 or m/z 467.8, corresponding to the alkane and alkene products, respectively.[16] No attempt was made to resolve the alkane and alkene isomeric products.

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Animal Treatments

These studies were approved by our institution's animal research committee. Male Wistar rats weighing 200–250 g (Charles River, Portage, MD) were assigned randomly to one of eight groups. All reagents were administered intraperitoneally. Group 1 (n = 5) was administered the first vehicle, 100 mM $NaHCO_3$ (*p*H 8.0); groups 2 and 3 (n = 5 each) received 90 or 180 μmol/kg of the compound A mercapturates in 100 mM $NaHCO_3$ (*p*H 8.0), respectively; group 4 (n = 5) was pretreated with 500 μmol/kg AOAA in water 1 h before the administration of the compound A mercapturates (180 μmol/kg); group 5 (n = 5) was pretreated with 250 μmol/kg acivicin in normal saline 1 h before the administration of the compound A mercapturates (180 μmol/kg); group 6 (n = 5) received the second vehicle, 0.9% normal saline; group 7 (n = 5) received 180 μmol/kg of the glutathione conjugates of compound A in normal saline; and group 8 (n = 5) was pretreated with 250 μmol/kg acivicin in normal saline 1 h before the glutathione conjugates of compound A were administered. After 24 h, animals were killed and their kidneys were removed. One kidney was cut longitudinally, fixed in buffered formalin, and embedded in paraffin by American Histolabs (Gaithersburg, MD). Slides were made (5-μm thick), stained with hematoxylin and eosin (American Histolabs), randomly ordered, and evaluated for injury by one of the investigators (L.R.P.), who was blinded to the treatment groups. Each specimen was graded for the percentage of necrotic tubular cells in the corticomedullary junction and assigned a numeric value of 1 to 5 corresponding to the degree of injury in the following manner: none, 1; minimal (up to 10%), 2; moderate (10–30%), 3; moderately severe (30–70%), 4; and severe (> 70%), 5. Sections from the paraffin blocks (5-μm thick) were also mounted on poly(L-lysine)-treated glass slides (American Histolabs). After deparaffinization, sections were stained immunohistochemically for thionacyl halide protein adducts according to a previously described procedure.[17] The remaining portions of the kidneys were used to prepare subcellular fractions according to an established method.[18] The subcellular fractions were analyzed for the presence of protein adducts of the thionoacyl halide by an immunoblotting procedure, using ECL immunoblotting detection reagent and Kodak X-OMAT™ film.[19]

Synthesis of the Immunogen

An immunogen was prepared by covalently coupling N-TFFMP-GABA to RSA using a two-step process as described by Davis and Preston[20] (fig. 2). N-TFFMP-GABA (157 mg, 0.6 mmol, in 5 ml of methanol) was reacted with 1-ethyl, 3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (576 mg, 0.75 mmol in 15 ml of 20 mM sodium phosphate, *p*H 5.0). After 2 min at room temperature, the N-TFFMP-GABA was reacted with RSA (80 mg, 1.2 μmol, in 40 ml of 200 mM sodium phosphate, *p*H 8.0) overnight at room temperature. The conjugated product (N-TFFMP-GABA-RSA) was dialyzed against 1.5 l phosphate-buffered saline, *p*H, 7.4, with three changes at 4°C and stored at −80°C until it was used. The number of N-TFFMP-GABA haptens covalently bound to lysine residues of each molecule of RSA was estimated using the method of Habeeb[21] and verified by fluorine elemental analysis (Galbraith Laboratories, Knoxville, TN). N-TFFMP-GABA was coupled in a similar manner to ovalbumin (N-TFFMP-GABA–ovalbumin) to be used as a test antigen for antibody formation against the N-TFFMP-GABA hapten.

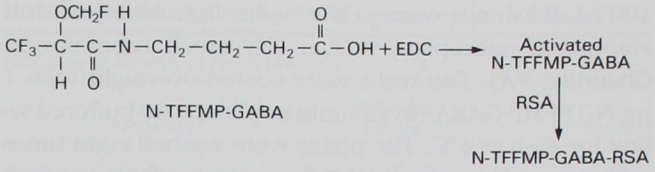

Fig. 2. Preparation of N-TFFMP-GABA-rabbit serum albumin (RSA) immunogen. The immunogen was prepared by activating the carboxyl group of N-TFFMP-GABA with 1-ethyl, 3-[3-(dimethylamino)propyl]carbodiimide hydrochloride and coupling this derivative to RSA. The immunogen was used to raise antibodies for detecting renal protein adducts of the thionoacyl halide reactive metabolite of compound A.

Preparation of Antisera

Two New Zealand White rabbits weighing 2.5 kg (Covance Research Products, Denver, PA) were injected with 750 μg N-TFFMP-GABA-RSA emulsified with two volumes of Freund's complete adjuvant at 20 sites subcutaneously along the back and at an intramuscular site in each hind limb. To augment antibody production, a total of 3 protective units of Pertussis vaccine was injected subcutaneously along six separate sites along the back. After 2 and 4 weeks, the animals were given booster doses of 750 μg N-TFFMP-GABA-RSA immunogen, emulsified in two volumes of Freund's incomplete adjuvant, by the same routes of immunization. Sera were collected after 10 days and for the ensuing 4 weeks and stored at −20°C.

Enzyme-linked Immunosorbent Assay of Rabbit Antisera

All assays were performed in triplicate in the wells of Immulon 4 microtiter plates in a total reaction volume of

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100 μl. Washings were performed using an Ultrawash II automated microplate washer (Dynatech Laboratories, Chantilly, VA). The wells were coated overnight with 1 μg N-TFFMP-GABA–ovalbumin in phosphate-buffered saline for 16 h at 4°C. The plates were washed eight times with 10 mM Tris-saline, *p*H 7.6, containing 0.5% (wt/vol) casein and 0.01% (wt/vol) Thimerosal (a washing buffer), to remove unbound material and to block unoccupied protein binding sites. Serial dilutions of preimmune and immune sera in washing buffer were added to the wells, and the plates were incubated for 3 h at room temperature. The plates were washed four times with washing buffer, followed by the addition of alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (1:1,000 in washing buffer). After incubation for 1.5 h at room temperature, the plates were washed four times with washing buffer, followed by four washes with phosphate-buffered saline. An alkaline phosphatase substrate reaction mixture was added to each well. The absorbance of the solutions in each well was determined at 405 nm after 15 min using a SpectraMax 250 automatic plate reader (Molecular Devices, Sunnyvale, CA).

To determine the specificity of the enzyme-linked immunosorbent assay for the N-TFFMP moiety, the assay was performed as described before, except that N-TFFMP-GABA antisera (1:100,000 dilution) was preincubated for 30 min with increasing concentrations of N-TFFMP-GABA or N-acetyl-GABA (0.01, 0.1, 1.0, and 10 mM) before being added to the plates.

Incubation of Compound A Mercapturates with Rat Kidney Cytosol

To show that our antisera could detect protein adducts of the thionoacyl halide metabolite of compound A in tissues, mercapturates of compound A (1 mM) and rat kidney cytosol (3 mg/ml) from untreated rats, previously dialyzed against phosphate-buffered saline overnight at 4°C to remove residual reduced glutathione, were incubated for 16 h at 37°C with gentle shaking. Samples of the reaction mixtures were analyzed for protein adducts by immunoblotting them with N-TFFMP-GABA antisera (1:1,000 dilution) before and after the antisera were preincubated for 30 min with increasing concentrations of the antisera inhibitor, N-TFFMP-GABA (0.01, 0.1, and 1.0 mM). To verify that protein adduct formation depended on β-lyase metabolism, immunoblot analysis was repeated with rat renal cytosol that had been preincubated with 1 and 10 mM AOAA for 30 min before incubation with the mercapturates of compound A.

Statistical Analysis

Three main experiments were analyzed, each consisting of a comparison of three treatments: a comparison of (1) control ($NaHCO_3$), (2) mercapturate conjugates alone, and (3) AOAA plus mercapturate conjugates; a comparison of (1) control ($NaHCO_3$), (2) mercapturate conjugates alone, and (3) acivicin plus mercapturate conjugates; and a comparison of (1) control (saline), (2) glutathione conjugates alone, and (3) acivicin plus glutathione conjugates. It was hypothesized that the mercapturate and glutathione conjugates would produce more renal necrosis than the control and that the addition of AOAA or acivicin to the conjugates would produce more renal necrosis than the conjugates alone. The Jonckheere statistic with ties[22] was used to compare the null hypothesis of no difference with the alternative hypothesis that there was an increasing trend for renal necrosis from the treatments in the order given. That is, this test determined whether treatment 1 was ≤ treatment 2, whether treatment 1 was ≤ treatment 3, and whether treatment 2 was ≤ treatment 3, with at least one strict inequality (<). When the test statistic for an experiment was significant at the 5% level, pairwise one-sided Mann–Whitney tests[22] were calculated to determine which of the three ordered pairwise comparisons showed significant differences at the 5% level.

Results

Renal Damage of Mercapturates and Glutathione Conjugates of Compound A

When rats were given a 90-μmol/kg mixture of the mercapturates of compound A, consisting of approximately 54% and 46% of addition, N-acetyl-S-(1,1,3,3,3-pentafluoro-2-(fluoromethoxy)propyl)-L-cysteine (alkane), and addition–elimination, N-acetyl-S-(1-fluoro-2-(fluoromethoxy)-2-(trifluoromethyl)vinyl)-L-cysteine (alkene) products in $NaHCO_3$ solution, respectively, no histologic renal damage was observed after 24 h (results not shown). At a dose of 180 μmol/kg of the mercapturates, renal necrosis was produced and was localized to the corticomedullary junction. The degree of necrosis ranged from minimal to severe, in which more than 70% of the corticomedullary junction tubular cells were necrotic (table 1, fig. 3B). Pretreatment with AOAA before the administration of the mercapturates did not appear to inhibit the renal necrosis produced by the mercapturate mixture (table 1, fig. 3C), whereas pretreatment with acivicin before the administration of the mercaptu-

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Table 1. Histopathology 24 h after the Administration of Mercapturates or Glutathione Conjugates of Compound A to Rats

	Incidence of Severity of Renal Necrosis*				
Treatment	None	Minimal	Moderate	Moderately Severe	Severe
Control ($NaHCO_3$)	5	0	0	0	0
Control (saline)	5	0	0	0	0
Mercapturate conjugates	0	1	2	1	1
AOAA +	0	2	1	0	2
Acivicin +	0	0	0	1	4
Glutathione conjugates	0	4	0	1	0
Acivicin +	0	1	0	2	2

* Mercapturates or glutathione conjugates (180 μmol/kg) were administered to five rats. A kidney section from each animal, which was stained with hematoxylin and eosin, was graded for percent necrotic tubular cells in the corticomedullary junction as follows: none, minimal (≤10%), moderate (10–30%), moderately severe (30–70%), and severe (>70%). A numerical value of 1 through 5, corresponding to the degree of injury where none = 1, minimal = 2, moderate = 3, moderately severe = 4, and severe = 5, was assigned to each group and used for statistical analysis of the data.

rates appeared to potentiate the severity of the corticomedullary necrosis (table 1, fig. 3D). Similarly, the administration of the 180-μmol/kg mixture of the glutathione conjugates (40:60, alkane:alkene) of compound A in saline to rats produced minimal to moderately severe corticomedullary necrosis (table 1, fig. 3E), and pretreatment with acivicin before administration of the glutathione conjugates appeared to increase the severity of the renal damage in most of the rats (table 1, fig. 3F).

The histopathologic observations were substantiated by statistical analysis. The Jonckheere statistic with ties was computed for each of the three main experiments to test the hypothesis of an increasing trend for renal necrosis, whereas the Mann–Whitney U statistic was computed for each pair of treatments to identify differences in renal necrosis. First, the comparison of (1) control ($NaHCO_3$), (2) mercapturate conjugates alone, and (3) AOAA plus mercapturate conjugates showed a highly significant trend for increasing renal necrosis for the treatments in the given order ($P = 0.0044$). The pairwise comparisons found that the control produced significantly less renal necrosis than the other two treatments ($P = 0.004$), but the one-sided Mann–Whitney U test could not reject the hypothesis that the addition of AOAA to mercapturate conjugates produced the same amount of renal necrosis as that of the mercapturate conjugates alone ($P = 0.569$). Second, the comparison of (1) control ($NaHCO_3$), (2) mercapturate conjugates alone, and (3) acivicin plus mercapturate conjugates also showed a highly significant trend for increasing renal necrosis ($P < 0.0001$). The pairwise comparisons again found that the control produced significantly less renal necrosis than the other two treatments ($P = 0.004$), and that the addition of acivicin to mercapturate conjugates produced significantly more renal necrosis than the conjugates alone ($P = 0.038$). Third, the comparison of (1) control (saline), (2) glutathione conjugates alone, and (3) acivicin plus glutathione conjugates also showed a highly significant trend for increasing renal necrosis ($P = 0.0001$). The pairwise comparisons again found that the control produced significantly less renal necrosis than the other two treatments ($P = 0.004$), and that the addition of acivicin to glutathione conjugates produced significantly more renal necrosis than the conjugates alone ($P = 0.048$).

Immunochemical Analysis of Protein Adducts of Compound A and Its Mercapturates

Antisera were raised that could be used to detect tissue protein adducts of the thionoacyl halide metabolite of compound A (fig. 1). Analyses of the free lysine and bound fluorine atom content of the immunogen used to prepare the antisera revealed that 19 N-TFFMP-GABA haptens were bound to each RSA, which represents covalent modification of 30% of the 64 lysine residues of RSA. A high titer of anti–N-TFFMP-GABA immunoglobulin G was detected in the serum of one of two rabbits immunized with N-TFFMP-GABA-RSA, as determined by enzyme-linked immunosorbent assay (fig. 4A). The finding that N-TFFMP-GABA inhibited the enzyme-linked immunosorbent assay more than 80% and N-acetyl-GABA inhibited the assay by 20% (fig. 4B) indicated that the antisera contained antibodies specifically directed against the N-TFFMP moiety and not only those directed against the GABA moiety of the immunogen and therefore could be used to detect protein covalent adducts of the thionoacyl halide of compound A. The enzyme-

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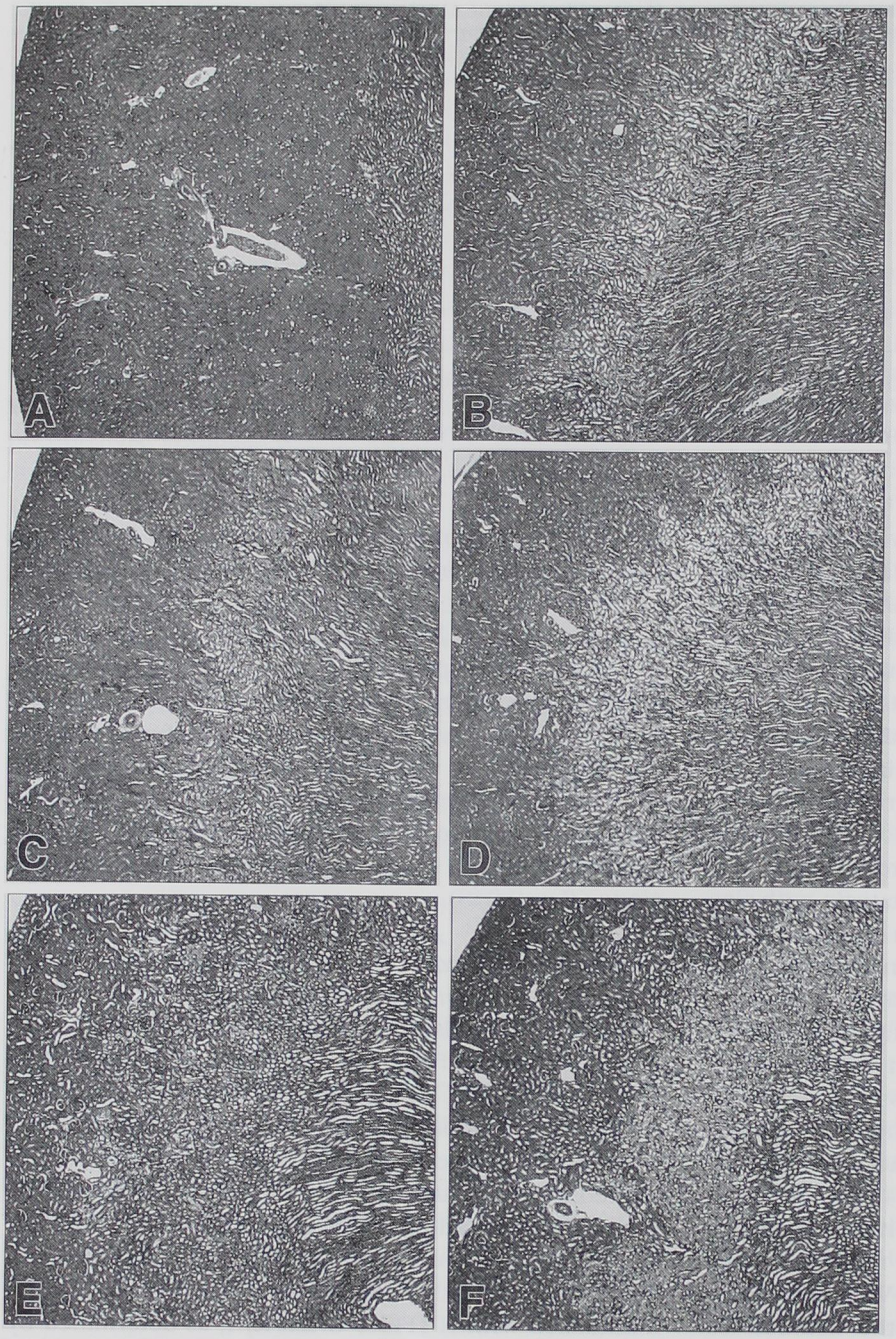

Fig. 3. Histologic evidence of corticomedullary necrosis after the administration of mercapturates or glutathione conjugates of compound A to Wistar rats. (*A*) Kidney section of a control rat. (*B*) Kidney section of a rat 24 h after intraperitoneal treatment with mercapturates of compound A (180 μmol/kg). (*C*) Same as *B*, except that the rat was pretreated with aminooxyacetic acid (500 μmol/kg given intraperitoneally) 1 h before the administration of the mercapturates. (*D*) Same as *B*, except that the rat was pretreated with acivicin (250 μmol/kg given intraperitoneally) 1 h before the administration of the mercapturates. (*E*) Kidney section of a rat 24 h after intraperitoneal treatment with glutathione conjugates of compound A (180 μmol/kg). (*F*) Same as *E*, except that the rat was pretreated with acivicin (250 μmol/kg given intraperitoneally). The sections were stained with hematoxylin and eosin.

linked immunosorbent assay results were corroborated by immunoblot analysis (fig. 5, lane 2).

When rat kidney cytosol was incubated overnight with 1 mM of the mercapturates of compound A, a major protein adduct of approximately 55 kDa was detected in the cytosol by immunoblot analysis with N-TFFMP-GABA antisera (fig. 6A, lane 2). Immunochemical detection of this protein adduct was inhibited incrementally by preincubation of the antisera with increasing concentrations of N-TFFMP-GABA before immunoblot analysis (fig. 6A, lanes 3 to 5), which indicated that the antibodies were reacting with the covalently bound thionoacyl halide metabolite of compound A. Furthermore, the antisera recognition of most of the adducts was reduced substantially by preincubation of the rat kidney cytosol with AOAA before incubation with the mercapturates

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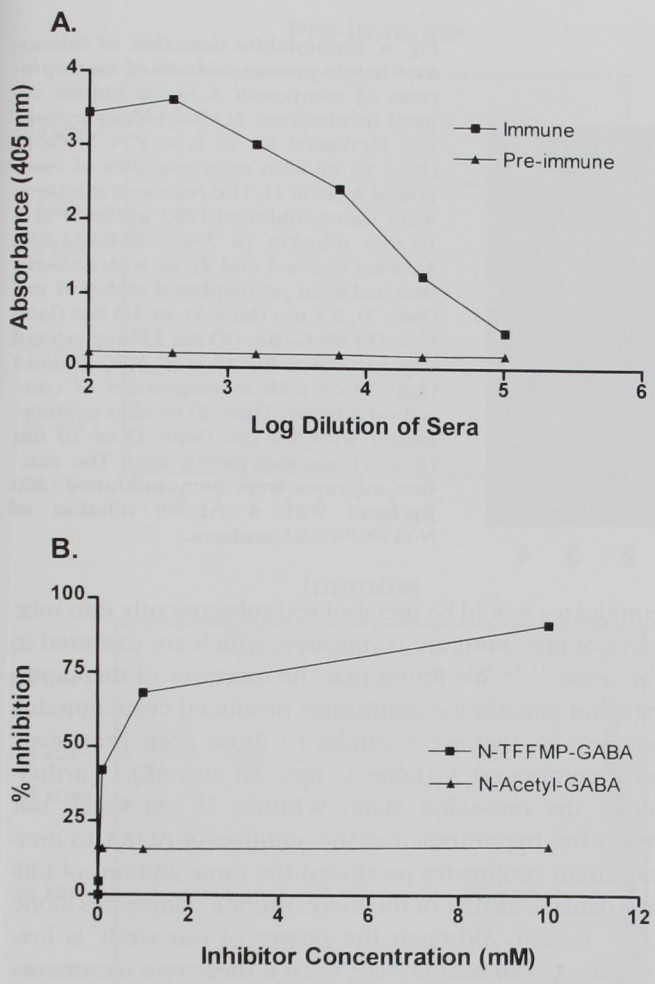

Fig. 4. Enzyme-linked immunosorbent assay determination for serum antibodies directed against the N-TFFMP moiety. A rabbit was immunized with N-TFFMP-GABA-RSA. The resulting immune sera and preimmune sera were tested for N-TFFMP antibodies in an enzyme-linked immunosorbent assay using TFFMP-GABA–ovalbumin as the test antigen (*A*). The reactivity of an immune serum sample (1:100,000 dilution) with TFFMP-GABA–ovalbumin was inhibited approximately 80% by N-TFFMP-GABA but only 20% by N-acetyl-GABA (*B*).

(fig. 6B, lanes 3 and 4). This finding indicated that the thionoacyl halide product of compound A immunochemically detected by our antisera was produced by a β-lyase enzyme, originating either from the cytosol or from bacteria present in the *in vitro* incubation solution, although there was no evidence of bacterial contamination.

No adducts of the thionoacyl halide metabolite of compound A were detected in immunoblots of kidney tissues of rats 5 and 24 h or 24 h after the administration of compound A (fig. 7) or the mercapturates, respectively (fig. 8). The only immunoreactivity seen in the blots after compound A exposure was also seen with tissues of control animals or when the preimmune sera were used in place of the immune antisera (fig. 7). Furthermore, no adducts of the thionoacyl halide metabolite of compound A were detected immunohistochemically in the kidney sections of rats 5 and 24 h or 24 h after treatment with compound A or the mercapturates, respectively (results not shown).

Discussion

The renal β-lyase pathway of metabolism has been established as an important bioactivation mechanism for several halogenated alkenes, such as tetrafluoroethylene, chlorotrifluoroethylene, hexafluoropropene, and 2-bromo-2-chloro-1,1-difluoroethylene,[23,24] which produce corticomedullary necrosis in the S3 segment of renal straight proximal tubules. This pathway of metabolic activation involves the initial formation of glutathione

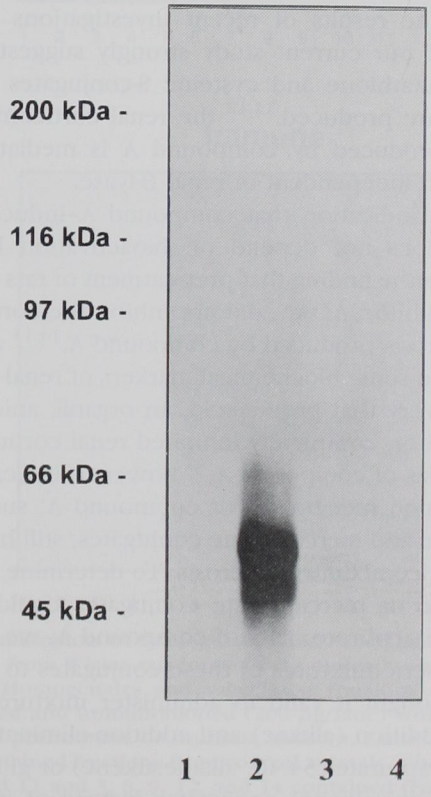

Fig. 5. Immunoblot determination of serum antibodies directed against the N-TFFMP moiety. Preimmune (lanes 3 and 4) and immune sera (lanes 1 and 2) (1:100,000 dilution) from a rabbit immunized with N-TFFMP-GABA-RSA were immunoblotted with ovalbumin (lanes 1 and 3, 1 μg) or TFFMP-GABA—ovalbumin (lanes 2 and 4, 1 μg).

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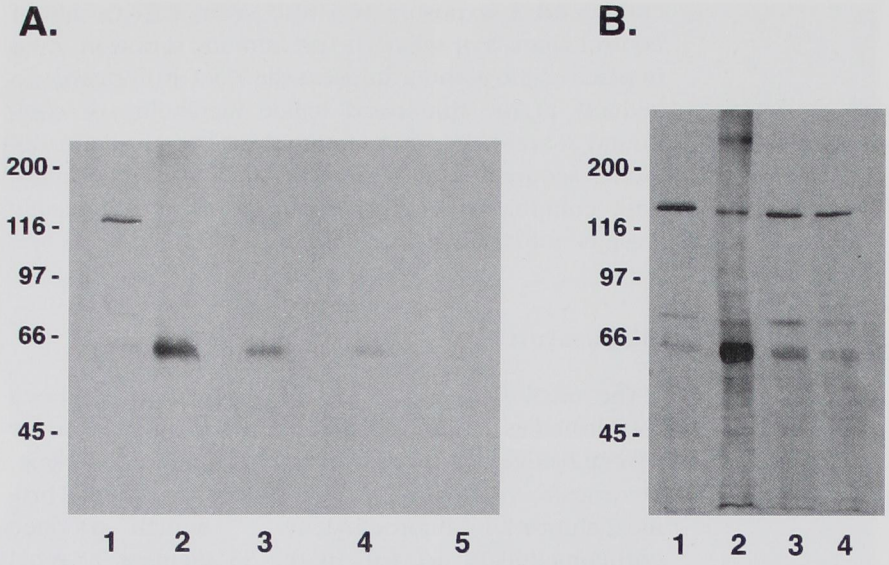

Fig. 6. Immunoblot detection of thionoacyl halide protein adducts of mercapturates of compound A in rat kidney cytosol incubations. (*A*) Rat kidney cytosol was incubated for 16 h at 37°C without (lane 1), or with mercapturates of compound A (lane 2). The reactions mixtures were immunoblotted (200 μg/lane) at a 1:1,000 dilution of N-TFFMP-GABA-RSA antisera (lanes 1 and 2), or with antisera that had been preincubated with 0.01 mM (lane 3), 0.1 mM (lane 4), or 1.0 mM (lane 5) N-TFFMP-GABA. (*B*) Rat kidney cytosol was incubated for 16 h at 37°C without (lane 1) or with mercapturates of compound A before (lane 2) or after preincubation with 1.0 mM (lane 3) or 10 mM (lane 4) aminooxyacetic acid. The reaction mixtures were immunoblotted (200 μg/lane) with a 1:1,000 dilution of N-TFFMP-GABA antisera.

conjugates, further metabolism to cysteine S-conjugates, and the renal β-lyase–dependent formation of toxic thionoacyl halide metabolites from the cysteine S-conjugates (fig. 1). The results of recent investigations and the findings of our current study strongly suggest that although glutathione and cysteine S-conjugates of compound A are produced,[11,12] the renal corticomedullary necrosis produced by compound A is mediated by a mechanism independent of renal β-lyase.

The first indication that compound A–induced renal necrosis does not depend on bioactivation by renal β-lyase was the finding that pretreatment of rats with the β-lyase inhibitor, AOAA, did not inhibit the corticomedullary necrosis produced by compound A,[14,15] although it improved some biochemical markers of renal injury.[15] The discovery that probenecid, an organic anion transport inhibitor, completely inhibited renal corticomedullary necrosis of compound A,[15] however, indicated that organic anion metabolites of compound A, such as its glutathione and mercapturate conjugates, still had a role in the corticomedullary necrosis. To determine whether glutathione or mercapturate conjugates could have a role in the nephrotoxicity of compound A, we administered isomeric mixtures of these conjugates to rats (fig. 1). We thought it valid to administer mixtures of the purified addition (alkane) and addition-elimination (alkene) mercapturate (54:46, alkane:alkene) or glutathione (40:60, alkane:alkene) conjugates instead of individual conjugates for our studies, because it had been reported that alkane and the alkene glutathione conjugates are excreted in the bile of rats treated with compound A at an approximate ratio of 1:1.[16] Furthermore, glutathione conjugates would be metabolized subsequently into mixtures of mercapturate conjugates, which are excreted in the urine.[11,12] We found that the mixtures of mercapturate and glutathione conjugates produced corticomedullary lesions that were similar to those seen previously with compound A (table 1, figs. 3B and 3E).[2] Furthermore, the one-sided Mann–Whitney U test could not reject the hypothesis that the addition of AOAA to mercapturate conjugates produced the same amount of kidney damage as that of the mercapturate conjugates alone ($P = 0.569$). Although the power of our study is low because of our sample size, even if these two treatments were distributed normally with standard deviations of 1 unit, sample sizes of 100 and 250 per treatment group would be needed to detect differences between the treatment means of 0.4 and 0.25, respectively, with 80% power. Clearly, such large sample sizes are not feasible. Nevertheless, our findings are consistent with those of previous studies of the effects of AOAA on the kidney histopathology of compound A.[14,15]

The use of the mixtures of the alkane and alkene conjugates of compound A to investigate the potential roles of these conjugates in compound A renal necrosis was validated recently. A report showed renal corticomedullary necrosis after the administration of either purified alkane or alkene glutathione conjugates to male Fischer 344 rats.[25] In the same study, the nephrotoxicity of the individual alkane and alkene cysteine S-conjugates was also studied, and the investigators found that only the alkane produced kidney damage.

Other findings indicate that renal β-lyase may have a minor, if any, role in the renal necrosis produced by

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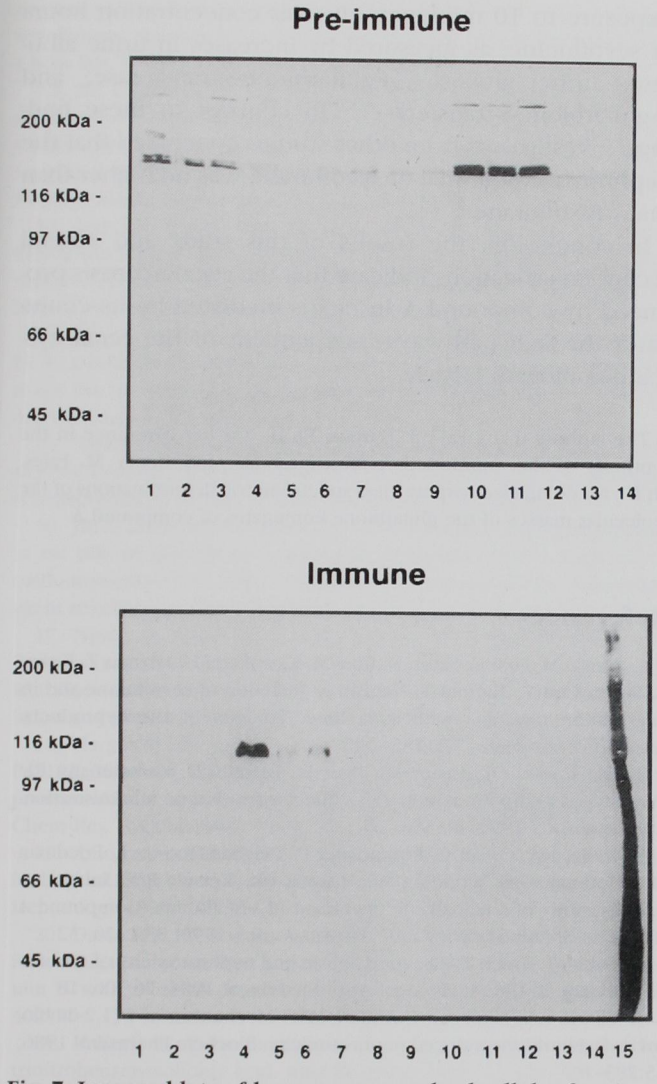

Fig. 7. Immunoblots of homogenates and subcellular fractions of kidneys from Wistar rats treated with compound A. Homogenates and subcellular fractions of kidneys were pooled and immunoblotted (200 μg/lane) with a 1:1,000 dilution of preimmune or immune antisera. Lanes 1, 4, 7, 10, and 13 contained fractions from control animals, whereas lanes 2, 5, 8, and 11 and 3, 6, 9, 12, and 14 contained fractions that were isolated 5 and 24 h, respectively, after treatment of rats for 3 h with 300 ppm compound A. The tissue fractions in each of the lanes were total homogenate (1–3), nuclear (4–6), mitochondrial (7–9), cytosolic (10–12), and microsomes (13–14). Lane 15 contained 1 μg N-FFMP-GABA–ovalbumin as a positive control.

compound A. For example, covalent adducts of the thionoacyl halide metabolite of compound A, formed from the renal cysteine conjugate β-lyase metabolism of cysteine S-conjugates of compound A (fig. 1), could not be detected in renal tissues of rats administered compound A or its mercapturates, using immunoblotting (figs. 7 and 8) or immunohistochemical techniques. Although we cannot preclude the possibility that the covalent adducts once formed *in vivo* are rapidly degraded or that the thionacyl moiety is cleaved readily from renal proteins, or both, the results of two recent reports support our immunochemical findings. First it was discovered that the cysteine conjugates of compound A are poor sub-

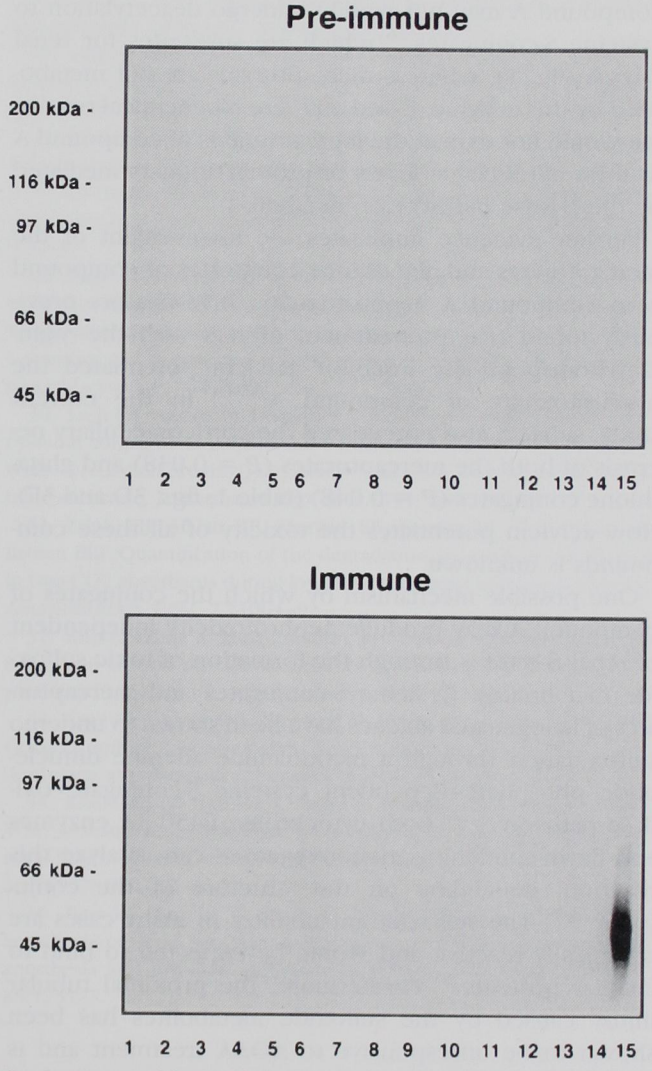

Fig. 8. Immunoblots of homogenates and subcellular fractions of kidneys from Wistar rats treated with mercapturates of compound A. Homogenates and subcellular fractions of kidneys were pooled and immunoblotted (200 μg/lane) with a 1:1,000 dilution of preimmune or immune antisera. Lanes 1, 4, 7, 10, and 13 contained fractions from control animals, whereas lanes 2, 5, 8, and 11 and 3, 6, 9, 12, and 14 contained fractions that were isolated 24 h after treatment of rats with 90 or 180 μmol/kg mercapturates of compound A, respectively. The tissue fractions in each of the lanes were total homogenate (1–3), nuclear (4–6), mitochondrial (7–9), cytosolic (10–12), and microsomes (13–14). Lane 15 contained 1 μg N-FFMP-GABA–ovalbumin as a positive control.

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strates for renal cysteine conjugate β-lyase.[13] That study showed that the cysteine conjugate of chlorotrifluoroethene, which causes nephrotoxicity by a renal β-lyase–mediated pathway,[7] is metabolized *in vitro* by cytosolic rat renal β-lyase approximately 10 times more rapidly than the cysteine conjugates of compound A. Second, it was reported that mercapturate conjugates of compound A may not readily undergo deacetylation to cysteine S-conjugates,[26] which are substrates for renal β-lyase (fig. 1), whereas mercapturates are not metabolized by this enzyme. Based on these biochemical results, we would not expect the mercapturates of compound A to form appreciable levels of protein adducts mediated by the β-lyase pathway of metabolism.

Further evidence implicates the involvement of the mercapturates and glutathione conjugates of compound A in compound A nephrotoxicity. Investigators previously found that pretreatment of rats with the γ-glutamyltranspeptidase inhibitor acivicin potentiated the nephrotoxicity of compound A.[14,15] In the current study, acivicin also potentiated the corticomedullary necrosis of both the mercapturates ($P = 0.038$) and glutathione conjugates ($P = 0.048$) (table 1, figs. 3D and 3F). How acivicin potentiates the toxicity of all these compounds is unknown.

One possible mechanism by which the conjugates of compound A may produce nephrotoxicity independent of renal β-lyase is through the formation of toxic sulfoxide metabolites. Cysteine S-conjugates and mercapturates of halogenated alkenes have been shown to undergo sulfoxidation through a nicotinamide adenine dinucleotide phosphate–dependent cysteine S-conjugate–oxidase pathway.[27,28] Both cytochrome P450 3A enzymes and flavin-containing monooxygenase can catalyze this reaction, depending on the structure of the conjugates.[28,29] The sulfoxide metabolites in many cases are chemically reactive and would be expected to bind to cellular proteins.[30] Furthermore, the proximal tubular injury caused by the sulfoxide metabolites has been shown to be unresponsive to AOAA treatment and is more severe than that caused by the corresponding cysteine S-conjugates.[28,30]

The potential for compound A nephrotoxicity in humans anesthetized with sevoflurane has been discussed repeatedly, because concentrations of this compound as high as 61 ppm have been seen in clinical practice,[31], which is near the threshold known to cause renal injury in rats (25 to 50 ppm).[32] In one study, a substantial number of healthy male volunteers showed signs of transient subclinical renal injury, peaking 3 days after exposure to 10 minimum alveolar concentration hours of sevoflurane, as measured by increases in urine albumin, urine glucose, α-glutathione-S-transferase, and π-glutathione-S-transferase.[33] In contrast to these findings, investigators from other studies concluded that the nephrotoxic potential of sevoflurane was no higher than that of isoflurane.[34,35]

In conclusion, the results of this study and several recent investigations indicate that the renal necrosis produced by compound A in rats is mediated by its conjugates through pathways independent of the renal cysteine conjugate β-lyase.

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