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RAPID COMMUNICATION

IMMUNOCHEMICAL DETECTION OF COVALENTLY MODIFIED KIDNEY PROTEINS IN *S*-(1,1,2,2-TETRAFLUOROETHYL)-L-CYSTEINE-TREATED RATS

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The nephrotoxicity of several haloalkenes is associated with glutathione *S*-conjugate formation, metabolism of the glutathione *S*-conjugate to the corresponding cysteine *S*-conjugate and translocation to the kidney, and metabolism by renal cysteine conjugate β-lyase (β-lyase, EC 4.4.1.13) to electrophilic metabolites that cause cell damage and death in proximal tubular cells (1,2). *S*-(1,1,2,2-Tetrafluoroethyl)-L-cysteine (TFEC), the cysteine *S*-conjugate of tetrafluoroethene, is nephrotoxic (3), and covalent modification of cellular macromolecules has been implicated in the toxicity of *S*-conjugates (4,5) and other xenobiotics (6,7).

The present study utilized immunochemical methods to demonstrate selective binding of the TFEC metabolites to renal proteins in TFEC-treated rats. Previous studies indicated that mitochondria are a primary target of toxic *S*-conjugates (8,9) and that β-lyase activity is present in both renal cytosolic and mitochondrial fractions (10). Therefore, renal mitochondrial and cytosolic fractions were examined for the presence of modified proteins, which were detected with polyclonal antibodies to trifluoroacetylated proteins that cross-react with protein-bound metabolites of TFEC.

MATERIALS AND METHODS

TFEC was obtained by reaction of tetrafluoroethene (PCR, Gainesville, FL) with cysteine under the conditions described by Dohn et al. (11). The proton NMR and fast atom bombardment-mass spectrometry of the product were in agreement with published values (5). Reagents for electrophoresis and immunoblots were purchased from BioRad (Richmond, CA). Anti-trifluoroacetyl (TFA) sera was a gift from Dr. Lance R. Pohl (NIH). Male Fischer 344 rats (200-300 g) were purchased from Charles River Laboratories, Wilmington, MA.

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) and then injected i.p. with saline, with TFEC, or with aminooxyacetic acid (AOAA) and TFEC; AOAA was given 1 hr before TFEC. Kidney mitochondrial and cytosolic fractions were prepared as described previously (12).

Electrophoresis and immunoblots were performed according to the method of Christ et al. (13) with these modifications: 7.5% polyacrylamide gels were used for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and alkaline phosphatase-conjugated goat-anti-rabbit IgG was used as the second antibody. Immunoblots were incubated with anti-TFA sera for 24 hr at 4°, washed as described (13), incubated with a second antibody for 24 hr at 4°, washed (13), and incubated with substrates for alkaline phosphatase for 30 min. In the preadsorption control studies, anti-TFA serum (diluted 1:1000 in 0.5% casein/0.01 M

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Tris/0.15 M NaCl/0.02% Thimerosol) was incubated with 500 μM N^{ϵ}-TFA-lysine (4 hr, room temp.) and was then used in the standard manner for the immunoblot. Immunoblots were photographed with a Canon AT-1 camera (50 mm Macro lens, Kodak Tmax-100 film). Prints were made with Polycontrast III paper (Kodak) and a 4.5 gelatin filter.

RESULTS AND DISCUSSION

Dose-response studies. Rats were given 0, 0.1, 0.25, 0.5, or 1 mmol/kg TFEC; after 1 hr the anesthetized animals were killed by severing the aorta, and renal mitochondrial, microsomal, and cytosolic fractions were obtained by differential centrifugation. Proteins from the subcellular fractions were separated by SDS-PAGE and immunoblotted as described above. Figure 1 shows that TFEC-derived adducts were detectable in renal mitochondrial and cytosolic proteins from rats given 0.25 mmol TFEC/kg and showed increased intensity at higher doses. Two mitochondrial proteins with estimated M_r of 80 and 40 kDa were preferentially labeled 1 hr post-injection; additional labeled proteins were detected after giving 1 mmol/kg TFEC (61, 85, 97, 130, 155 kDa). A band of low intensity at about 66 kDa was also detected on immunoblots of cytosolic protein from TFEC-treated rats. Labeled proteins were not detected in subcellular fractions from control rats or in microsomal fractions (data not shown).

Time-course studies. Rats were given TFEC (0.25 mmol/kg) and killed 0, 0.5, 1, 3, 6, or 24 hr later. Samples of mitochondrial and cytosolic proteins were separated by SDS-PAGE and immunoblotted as described above. Modified proteins were detectable at 0.5 hr in both mitochondrial and cytosolic fractions of TFEC-treated rats (Fig. 2). In mitochondrial fractions, the 80 kDa protein was labeled at all times from 0.5 to 24 hr and was most intensely labeled at 3 hr. In cytosolic fractions, a band with an estimated molecular mass of 66 kDa was observed and was intensely labeled at 3 and 6 hr.

Aminooxyacetic acid inhibition of covalent binding. Rats were given AOAA (0.5 mmol/kg) 1 hr before TFEC (0.25 mmol/kg) or were given TFEC (0.25 mmol/kg) alone. One hour after giving TFEC, subcellular fractions were prepared and proteins were separated by SDS-PAGE and immunoblotted with anti-TFA sera, as described above. Figure 1 shows that AOAA administration decreased the intensity of protein labeling in TFEC-treated rats compared with rats given TFEC (0.25 mmol/kg) alone. Reduction of labeling by AOAA, a known inhibitor of β-lyase (14), indicates that metabolism is required for binding of TFEC metabolites to proteins.

To determine the selectivity of the response, the anti-TFA serum that was used for detection of labeled proteins was preadsorbed with N^{ϵ}-TFA-lysine (500 μM) and was then used to probe labeled proteins (13). No labeled protein was detected with preadsorbed serum, indicating that the anti-TFA serum recognized covalently bound TFEC metabolites (data not shown).

These results show that covalently modified renal mitochondrial and cytosolic proteins are present in TFEC-treated rats and that covalent binding occurs in a time- and dose-dependent manner. These findings provide evidence for a role for protein adduct formation in TFEC-induced toxicity and are the first immunochemical demonstration of covalently modified target-organelle proteins in *S*-conjugate-induced toxicity. Previous work on TFEC and other nephrotoxic *S*-conjugates also indicates a possible role for chemically modified proteins in the expression of toxicity (4,5,8,9). Five mitochondrial sites have been identified as possible targets, based on inhibition of oxygen consumption and inhibition of metabolism. These putative targets are multienzyme complexes made up of several component enzymes: succinate:coenzyme Q reductase (contains 7-8 nonidentical subunits with M_r ranging from 70 to 12 kDa), isocitrate dehydrogenase (8 identical 41 kDa subunits),

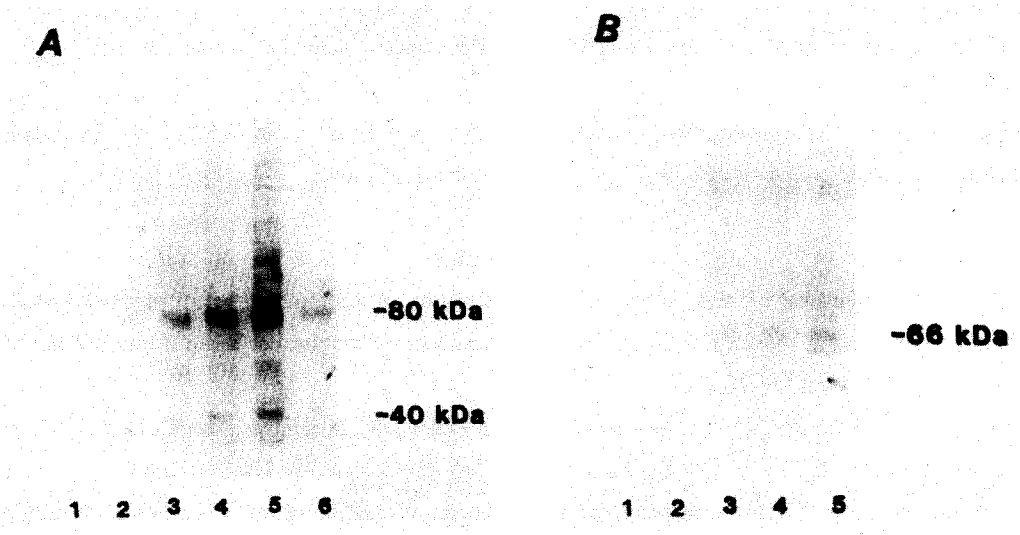

Fig. 1. Dose-dependent appearance of labeled kidney proteins from TFEC-treated rats and inhibition of labeling by AOAA. Kidneys were removed from rats exposed to TFEC for 1 hr at the doses indicated below. Mitochondrial and cytosolic fractions were isolated as described in Materials and Methods. Proteins (200 μg/lane) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-TFA serum. Panel A: mitochondrial proteins; lane 1, control (saline); lane 2, 0.1 mmol/kg TFEC; lane 3, 0.25 mmol/kg TFEC; lane 4, 0.5 mmol/kg TFEC; lane 5, 1.0 mmol/kg TFEC. Lane 6: rats were given AOAA (0.5 mmol/kg) 1 hr before 0.25 mmol/kg TFEC. B: cytosolic proteins; lane 1, control (saline); lane 2, 0.1 mmol/kg TFEC; lane 3, 0.25 mmol/kg TFEC; lane 4, 0.5 mmol/kg TFEC; lane 5, 1.0 mmol/kg TFEC.

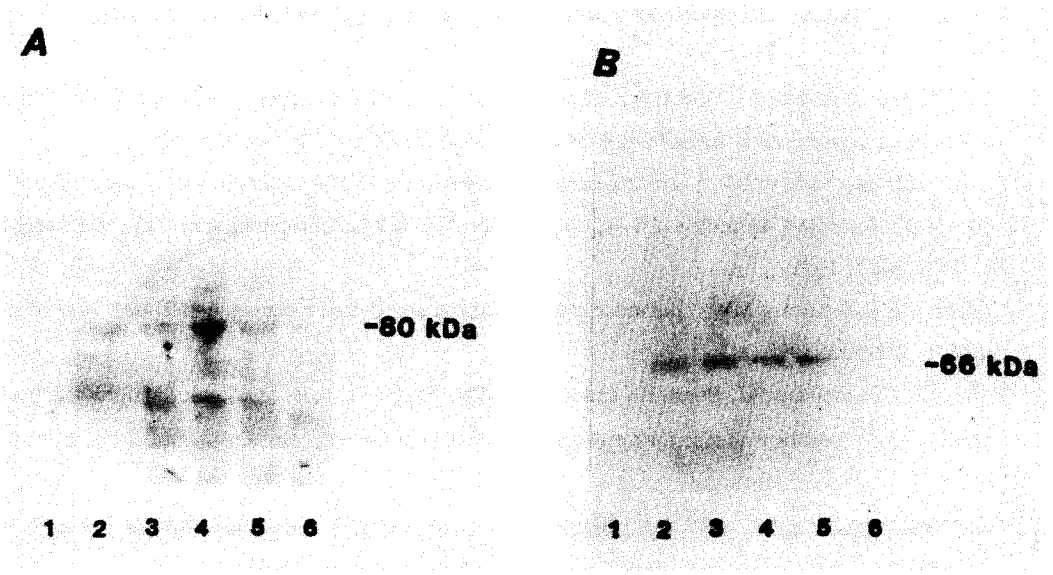

Fig. 2. Time-dependent appearance of labeled kidney proteins from TFEC-treated rats. Kidneys were removed from rats exposed to 0.25 mmol/kg TFEC for the time periods indicated below. Mitochondrial and cytosolic fractions were isolated as described in Materials and Methods. Proteins were separated by SDS-PAGE (300 μg/lane), transferred to nitrocellulose membranes, and immunoblotted with anti-TFA serum. Panel A: mitochondrial proteins; lane 1, 0 hr; lane 2, 0.5 hr; lane 3, 1 hr; lane 4, 3 hr; lane 5, 6 hr; lane 6, 24 hr. B: cytosolic proteins; lane 1, 0 hr; lane 2, 0.5 hr; lane 3, 1 hr; lane 4, 3 hr; lane 5, 6 hr; lane 6, 24 hr.

coenzyme QH_2:cytochrome c reductase (8 nonidentical subunits with M_r of 8 to 46 kDa) (8), pyruvate dehydrogenase (5 subunits with M_r of 41, 36, 52, 55, and 50 kDa) and α-ketoglutarate dehydrogenase (3 subunits with M_r of 48, 55, and 113 kDa) (9). Identification of the altered proteins detected in this and previous studies may provide information about the mechanism of *S*-conjugate-induced cell damage and death.

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