Generation of Antibodies to Di- and Trichloroacetylated Proteins and Immunochemical Detection of Protein Adducts in Rats Treated with Perchloroethene

Axel Pähler,[†] Gerhard Birner,[†] Jean Parker,[‡] and Wolfgang Dekant*,[†]

Institut für Toxikologie, Universität Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany, and United States Environmental Protection Agency, NCEA, Washington, D.C. 20460

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Antibodies directed against chemical specific protein modifications are valuable tools to detect and comparatively quantify protein modifications. Both N^{ϵ}-(dichloroacetyl)-L-lysine and N^{ϵ}-(trichloroacety)l-L-lysine have been detected as modified amino acids in liver and kidneys of rats treated with perchloroethene (PER) after proteolysis. These protein modifications are formed by the interaction of reactive metabolites formed from PER with proteins. In this study we developed monospecific antibodies to dichloroacetylated and to trichloroacetylated amino acids to detect modified proteins in the target organs of PER toxicity. These antibodies were prepared by immunization of rabbits with modified keyhole limpet hemocyanin (KLH) coupled with either the dichloroacetyl or trichloroacetyl moiety. Enzyme-linked immunosorbent assays (ELISA) indicated that the polyclonal rabbit sera recognized dichloroacetylated or trichloroacetylated rabbit serum albumin (RSA), but not unmodified protein. Therefore, we further purified rabbit antisera on either N^{ϵ}-(dichloroacetyl)-L-lysine or N^{ϵ}-(trichloroacetyl)-L-lysine immobilized to immunoaffinity columns to obtain monospecific antibodies. The potential of these antibodies in the detection of di- and trichloroacetylated proteins and their selectivity for the desired dichloroacetyl or trichloroacetyl group was demonstrated in competitive enzme-linked immunosorbent assays with several structurally related compounds. Anti-dichloroacetyl (anti-DCA) antibody binding to dichloroacetylated RSA was inhibited by N^{ϵ}-(dichloroacetyl)-L-lysine with an IC_{50} value of 150 μM whereas inhibition by N^{ϵ}-(monochloroacetyl)-L-lysine and N^{ϵ}-(trichloroacetyl)-L-lysine showed an IC_{50} value of 100 mM. The binding of the anti-trichloroacetyl (anti-TCA) antibody to trichloroacetylated RSA was inhibited by N^{ϵ}-(dichloroacetyl)-L-lysine with an IC_{50} value of 80 mM. The inhibition by N^{ϵ}-(trichloroacetyl)-L-lysine was again 3 orders of magnitude stronger resulting in an IC_{50} value of 90 μM. N^{ϵ}-(acetyl)-L-lysine and unmodified RSA did not effect antibody binding to the chemically modified antigen. The antibodies were also successfully applied to detect modified proteins in subcellular fractions of liver and kidney from PER treated rats demonstrated in immunoblot. Protein adduct formation from different PER metabolism pathways was confirmed by the observation that the majority of dichloroacetylated proteins were located in kidney mitochondria and trichloroacetylated proteins were located in liver microsomes.

Introduction

Perchloroethene (tetrachloroethene, PER) is extensively used in industry as a metal-degreasing solvent and as dry cleaning agent. Due to its volatility and resistance to degradation, it is a widely distributed environmental air pollutant and groundwater contaminant (*1*).

Long-term exposure of rodents to PER has been shown to increase the incidence of liver tumors in male mice and to result in a small but significant increase in the incidence of renal tumors in male rats (*2*). The chronic toxicity of PER is most likely mediated by bioactivation reactions. PER is known to be metabolized by both cytochrome P450 and glutathione dependent biotransformation pathways leading to the generation of reactive metabolites which may covalently bind to cellular macromolecules (Figure 1). Cytochrome P450 oxidation of PER results in formation of trichloroacetyl chloride which reacts with amino groups in macromolecules (*3*, *4*). In addition, glutathione conjugation of PER followed by cysteine conjugate β-lyase-mediated activation of *S*-(1,2,2-trichlorovinyl)-L-cysteine is likely responsible for the nephrotoxicity and possible renal tumorgenicity of PER (*5*−*7*). Dichlorothioketene formed by the β-lyase mediated cleavage of *S*-(1,2,2-trichlorovinyl)-L-cysteine is presumed to be the ultimate metabolite responsible for the mutagenic and nephrotoxic effects (*8*−*10*). Both N^{ϵ}-(dichloroacetyl)-L-lysine and N^{ϵ}-(trichloroacetyl)-L-lysine have been identified as modified amino acids in proteins by GC/MS in the liver and kidneys of rats treated with PER. Formation of protein adducts in rats has also been shown by detection of covalently bound [^{14}C]-derived radioactivity after administration of [^{14}C]-PER comigrating in SDS−PAGE of rat liver and kidney subcellular fractions with proteins detected by an rabbit anti-trifluoroacetyl antiserum having cross reactivity to di-

* Corresponding author. Tel.: +49(931)201 3449. Fax: +49(931)-201 3446. e-mail: dekant@toxi.uni-wuerzburg.de.
[†] Universität Würzburg.
[‡] US EPA.

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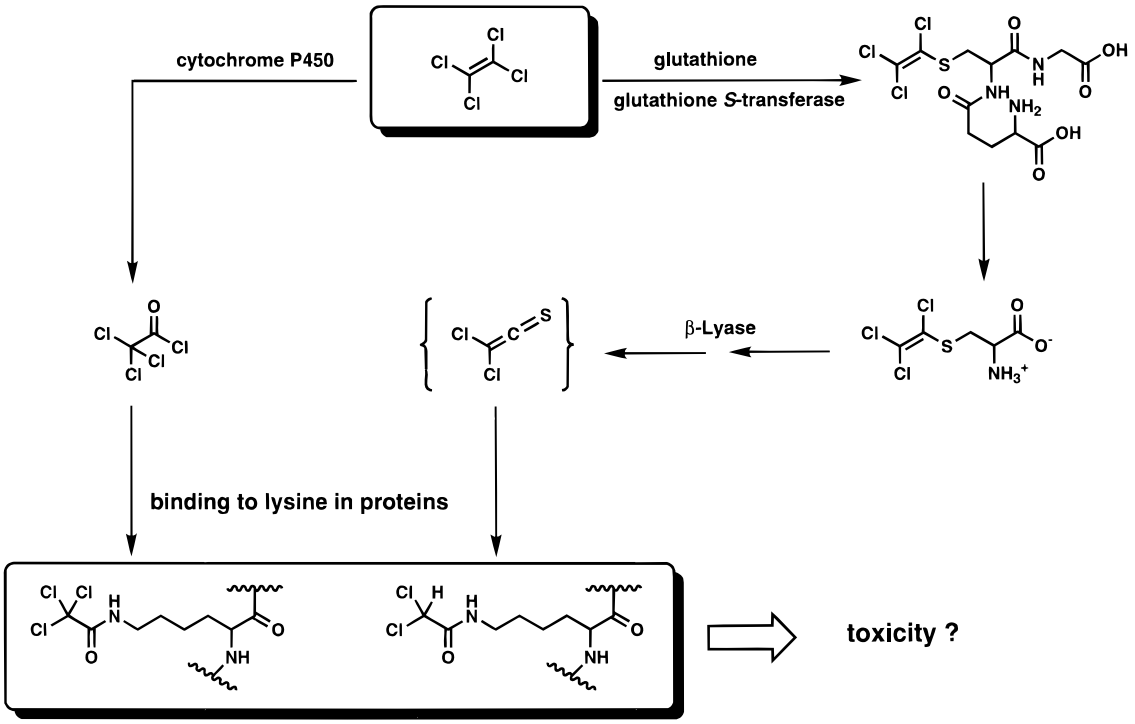

Figure 1. Pathways for protein adduct formation in PER metabolism.

and trichloroacetylated amino acids (*4*). Due to the cross-reactivity of these antibodies, the contribution of the different pathways of PER biotransformation to the formation of protein modifications could not be evaluated. We therefore developed monospecific antibodies to dichloroacetylated and to trichloroacetylated proteins. These were produced using keyhole limpet hemocyanin (KLH) as the carrier protein by coupling the desired functional group via a bifunctional spacer molecule as glycine or *p*-aminobenzoic acid. By this procedure, a better immunological recognition of the chemically modified epitopes of the protein was obtained. After purification of the antibodies by immunoaffinity chromatography from polyclonal rabbit sera, antibodies with high specificity for dichloro- and trichloroacetyl-modified proteins were obtained.

Experimental Procedures

Materials. PER (99% purity) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride were obtained from Aldrich Chemical Co. (Deisenhofen, Germany). KLH, rabbit serum albumin (RSA), N^{ϵ}-(acetyl)-L-lysine hydrochloride, and lipoic acid were from Sigma Chemical Co. (Deisenhofen, Germany). HiTrap *N*-hydroxysuccinimide-activated agarose columns and Sephadex PD-10 columns were obtained from Pharmacia Biotech (Freiburg, Germany). Alkaline phosphatase conjugated goat anti-rabitt IgG was from BioRad (München, Germany). Horseradish peroxidase conjugated goat anti-rabbit IgG and streptavidin-horseradish peroxidase were from Amersham Life Science (Braunschweig, Germany). Enhanced chemiluminescence reagents were purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals used were reagent, electrophoresis, or gradient grade as commercially available.

Synthesis of Haptens and Antibody Inhibitors. N^{ϵ}-(Monochloroacetyl)-L-lysine, N^{ϵ}-(dichloroacetyl)-L-lysine, N^{ϵ}-(trichloroacetyl)-L-lysine, and N^{ϵ}-(trifluoroacetyl)-L-lysine were synthesized as described previously (*4*). *N*-Dichloroacetyl-*p*-aminobenzoic acid and *N*-(trichloroacetyl)glycine were prepared referring to a method by Lautsch and Heinicke (*11*). Briefly, dichloroacetyl chloride or trichloroacetyl chloride (22 mmol) were directly added to *p*-aminobenzoic acid or glycine (20 mmol) at 0 °C with vigorous stirring. A 50 mL volume of diethyl ether was then added to the colorless solution, and the mixture was heated at flux for 30 min. After evaporation of the ether, petroleum ether (bp 60−80 °C) was added to the remaining oil to precipitate the desired products. The precipitates were filtered off in vacuo and washed twice with petroleum ether. *N*-Dichloroacetyl-*p*-aminobenzoic acid and *N*-(trichloroacetyl)glycine were isolated with >98% purity as checked by analytical HPLC. Electrospray mass spectrometry of the synthetic chloroacetylated amino acids was performed on a Fisons TRIO 2000 mass spectrometer equipped with an electrospray probe allowing sample introduction at a flow rate of 10 μL/min. Approximately 10 μg of each compound were injected in the bypass mode dissolved in water/acetonitrile (50/50, v/v) using 70% acetonitrile containing 0.1% formic acid as mobile phase.

NMR Data and Mass Spectrometry. NMR spectra of fully protonated haptens (trifluoroacetate salts) dissolved in Me_2SO-d_6 were acquired at the Institute of Organic Chemistry on a Bruker AMX 250 NMR spectrometer. Chemical shifts were calculated relative to tetramethylsilane ($\delta = 0$ ppm).

Mass spectra in electron impact (EI) mode at 70 eV were acquired on a Fisons MD 800 instrument. Mass spectra using chemical ionization and negative ion (NCI) detection were aquired on a Fisons Trio 2000 mass spectrometer (Mainz, Germany) with methane as the reactant gas. The ion source was kept as 200 °C for EI and at 150 °C for NCI. Both mass spectrometers were equipped with a Carlo Erba 8000 gas chromatograph. Samples were injected in the splitless mode and linear temperature gradients from 50 to 280 °C with 15 °C/min were applied for separation. The injector and the transfer line were held at 280 °C.

***N*-(Dichloroacetyl)-*p*-aminobenzoic acid:** ^{13}C NMR (63 MHz, DMSO-d_6): δ [ppm] = 67.29 (CCl_2H); 119.33, 126.70, 130.61, 141.68 (C_6H_4); 162.19 (HN*C*O); 166.83 (CO_2H). ^{1}H NMR (250 MHz, DMSO-d_6): δ [ppm] = 6.66, s (1H), CCl_2H; 7.74 d (2H), 7.76, d (2H), C_6H_4; 10.98, s (1H), N-*H*; 12.20, s (1H), COO*H*.

Mass Spectra of *O*-Methyl Esters after BF_3/Methanol Derivatization. *N*-(Dichloroacetyl)-*p*-aminobenzoic acid, EI: *m*/*z* (^{35}Cl) (%) = 261 (23) (2 Cl) [M^+], 230 (18) (2 Cl) [$M^+ - OCH_3$], 178 (100) [$M^+ - CCl_2H$], 150 (41) [$M^+ - CCl_2H - CO$].

NCI: *m/z* (^{35}Cl) = 260 (2 Cl) [M^+ - H], 225 (1 Cl) [M^+ - HCl], 189 [M^+ - H − 2 Cl].

***N*-(Trichloroacetyl)glycine:** ^{13}C NMR (63 MHz, DMSO-d_6): δ [ppm] = 42.37 (CH_2); 92.43 (CCl_3); 162.05 (HN*C*O), 169.93 (CO_2H). ^{1}H NMR (250 MHz, DMSO-d_6): δ [ppm] = 3.93, d (2H), NHCH_2CO; 9.36, t (1H); N-*H*; 9.99, s (1H), COO*H*.

***N*-(Trichloroacetyl)glycine:** EI: *m/z* (^{35}Cl) (%) = 233 (6) (3 Cl) [M^+], 202 (3) (3 Cl) [$M^+ - OCH_3$], 174 (13) (3 Cl), [$M^+ -$ CO, $- OCH_3$], 88 (100) [$M^+ - C_2Cl_3O$]. NCI: *m/z* (^{35}Cl) = 232 (3 Cl) [M^- - H], 197 (2 Cl) [$M^- -$ Hcl].

Protein Determination. Protein concentrations in liquid samples were determined by the method described by Bradford (*12*) basing on the formation of a protein−dye complex with coomassie blue G-250.

Antigen Synthesis and Rabbit Immunization Protocol. KLH used as the carrier protein in rabbit immunization procedures and RSA as nonrelated carrier protein used as a solid-phase antigen in enzyme-linked immunosorbent assay (ELISA) experiments were coupled with the desired haptens by the carbodiimide method using a two-step conjugation reaction (*13*). Briefely, 2.5 mL of a methanolic solution of *N*-(trichloroacetyl)glycine or *N*-(dichloroacetyl)-*p*-aminobenzoic acid (30 mmol) were added to 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (288 mg, 1.5 mmol) in 7.5 mL of 20 mM sodium phosphate buffer (pH 5.0). After 2 min, the reaction mixture was mixed with a solution of 40 mg of KLH in 20 mL of 160 mM sodium phosphate buffer (pH 8.0) and incubated overnight at room temperature. The obtained conjugate was dialyzed three times against 3 L of bidistilled water. Coupling to RSA was performed in a similar manner, with the exception of using 60 mmol of the hapten. Free amino groups in treated proteins were determined by a spectrophotometric method (*14*). The solid protein sample (2 mg each) was placed in a 15 mL screw cap test tube, and 1 mL of $NaHCO_3$ (4%, pH 8.5) and 1 mL of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (0.5% in water) solution were added. The reaction mixture was heated at 40 °C for 4 h with gentle agitation. Then, 3 mL of 6 N HCl were added, and the mixture was autoclaved at 120 °C and 15 psi for 1 h to dissolve and hydrolyze any insoluble material. The hydrolysate was diluted with 5 mL of bidistilled water and the aqueous solution extracted five times with 20 mL of diethyl ether each for removal of unbound TNBS. An aliquot of the aqueous phase (5 mL) was heated for 15 min at 70 °C to evaporate residual ether. The samples were then analyzed at 346 nm in a Ultrospec 2000 spectrophotometer (Pharmacia, Germany). All samples were read against a blank of reagent prepared by the same procedure except HCl was added prior to TNBS addition to prevent reaction with protein amino groups. All samples were analyzed as triplicates. Reaction of free ϵ-amino groups with TNBS occurs only under alkaline conditions and results in the formation of a product stable to acid. As the UV-absorbance at 346 nm is directly proportional to the concentration of ϵ-amino groups in the protein, the percentage of conjugation can be calculated on the concentration of ϵ-amino groups in the carrier protein and the concentration of ϵ-amino groups in the haptene−protein conjugate.

Female New Zealand White rabbits were immunized at 10 sites along the back sc and im in the right and left hindquarters with a total amount of 500 μg of either lyophilized *N*-(trichloroacetyl)glycine modified KLH or *N*-(dichloroacetyl)-*p*-aminobenzoic acid modified KLH suspended in 1 mL of Freund's complete adjuvant. Animals were boosted with 500 μg of each compound in 1 mL of Freund's incomplete adjuvant in 4 week intervals after the first injection.

Enzyme-Linked Immunosorbent Assay (ELISA). Anti-trichloroacetyl (anti-TCA) and anti-dichloroacetyl (anti-DCA) serum antibodies were determined by indirect ELISA as described (*15*) by using either *N*-(trichloroacetyl)glycine modified RSA or *N*-(dichloroacetyl)-*p*-aminobenzoic acid modified RSA as the solid-phase antigen. Determination of working concentrations were performed with serial dilutions (1 μg/mL to 10 μg/mL) of antigens in carbonate buffer (100 μL) (pH 9.6) applied to polystyrene microtiter plate wells (Greiner, Germany) and incubated overnight at 4 °C. The wells were washed with NaCl (0.9%, w/v containing 0.05%, v/v Tween-20) and rabbit serum (100 μL, added in dilutions of 1:50 to 1:10 000 in PBS-T) was then incubated for 1 h at room temperature. After additional washing steps, specific antibody binding was detected using *p*-nitrophenyl phosphate as substrate for an alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:1 000 dilution). The optical density of each well was measured after 30 min and addition of 50 μL of 3 M NaOH with a Dynatech MRX microplate reader at 405 nm. Antibody characterization by competitive ELISA of affinity purified antibodies was carried out in the same manner, except that 2 μg/mL of antigen was used for coating. Purified antibodies (50 μL, diluted 1:500 in PBS-T) were incubated after plate washing in the presence of an equal volume of various inhibitory substances in the range from 10000 μM to 1 μM for 1 h at room temperature. The assays were carried out as described above.

Affinity Purification of Antibodies from Polyclonal Rabbit Antisera. Monospecific antibodies to dichloroacetyl and trichloroacetyl groups were obtained from polyclonal rabbit anti-*N*-(trichloroacetyl)glycine modified KLH and anti-*N*-dichloroacetyl-*p*-aminobenzoic acid modified KLH antisera by affinity chromatography. For antisera purification either N^ϵ-(trichloroacetyl)-L-lysine or N^ϵ-(dichloroacetyl)-L-lysine were attached to *N*-hydroxysuccinimide-activated agarose columns (HiTrap *N*-hydroxysuccinimide-activated, Pharmacia, Germany). Columns were prepared for antibody purification by rinsing with 20 volumes of 20 mM Tris-HCl at pH 8. Exchange of buffers for the rabbit immune serum to the starting conditions for column chromatography (20 mM TRIS at pH 8) was performed by gel filtration chromatography on Sephadex PD-10 columns. Therefore, 2.5 mL of rabbit serum was applied to a PD-10 column rinsed with Tris-buffer and eluted with 3.1 mL of this buffer. Rabbit sera were then recirculated on affinity columns at 4 °C overnight. Unbound and nonspecifically bound components were eluted, rinsing the column with 10 volumes of starting buffer. Elution of hapten specific antibody fractions was achieved by elution with 20 mM glycine buffer containing 0.5 M NaCl at pH 2.7 which was immediately neutralized with 1 M Tris followed by desalting on PD-10 columns using 10 mM sodium phosphate buffer pH 7.4 containing 0.01% sodium azide. Aliquots of the residual preparation (0.1 mg of antibody/mL) were stored at −70 °C and only thawed once.

SDS−PAGE and Immunoblotting. Stock solutions of reference proteins (chemically modified RSA, 0.1 mg/mL) were diluted with SDS sample buffer (0.125 M Tris-HCl, pH 6.8, containing 10% (w/v) SDS, 20% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and 10% (v/v) 2-mercaptoethanol) to a final concentration of 100 ng/mL. Mitochondria, cytosol, and microsomes were diluted to a final protein concentration of 1 μg/μL. Samples were heated to 95 °C for 10 min prior to redisolving aliquots (as indicated in figure legends) by SDS−PAGE as described by Laemmli with 12% and 4.5% polyacrylamide gels at room temperature (*16*). Proteins were separated for 90 min at 30 mA from 8.4 × 7.0 mm minigels of 0.75 mm thickness using BioRad MiniProtean II gel electrophoresis equipment. Running buffer consisted of 438 mM glycine, 57 mM Tris, 4 mM SDS, and 2 mM ethylenediaminotetraacetic acid in water without pH adjustment. Separated proteins were transferred to nitrocellulose sheets (BioRad, TransBlot pure nitrocellulose 0.45 μm) by tank blotting using 25 mM Tris buffer containing 192 mM glycine and 10% (v/v) methanol at pH 8.3 for 200 V·h. After protein transfer, the sheets were blocked for 1 h at room temperature with phosphate-buffered saline (PBS; containing 10 mM Na_2HPO_4, 3 mM KH_2PO_4, and 137 mM NaCl, pH 7.4), containing 1% (w/v) casein and 0.02% (w/v) thiomersal. After washing the sheets with three quick changes of PBS-T (PBS, containing 0.05% Tween 20) and then once for 15 min and twice for 5 min with PBS-T, sheets were incubated with anti-DCA or anti-TCA antibody (1:1 000 in PBS-T) overnight at 4 °C. After additional washing steps as described above the goat anti-rabbit

Table 1. Determination of Modified Amino Groups in Synthetic Hapten/Protein Conjugates by 2,4,6-Trinitrobenzenesulfonic Acid Derivatization Expressed as Modification [%] of Free Amino Groups in Unmodified Proteins

hapten/protein conjugate	extent of protein modification [%] by spectrophotometric TNBS method
N-(trichloroacetyl)-glycine modified RSA	76 ± 1.2
N-(dichloroacetyl)-*p*-aminobenzoic acid modified RSA	83 ± 0.9
N-(trichloroacetyl)-glycine modified KLH	99 ± 0.6
N-(dichloroacetyl)-*p*-aminobenzoic acid modified KLH	98 ± 1.4

horseradish peroxidase-conjugated secondary antibody was incubated (1:5000 in PBS-T) for 1 h at room temperature. After washing, visualization of recognized bands was performed by enhanced chemiluminescence detection on Hybond enhanced chemiluminescence (ECL) film using Pierce luminescence reagents.

Animals and Treatment. Male Wistar rats were obtained from Harlan Winkelmann (Borchen, Germany). Animals were kept at constant humidity and temperature (21 °C) in the animal facility of the department with a 12 h light/dark cycle. Before the experiments, the animals were acclimated to the metabolic cages for 3 days, and control urine was collected during this time for 12 h before the exposure. PER (1 000 mg/kg body weight) was administered to male Wistar rats ($n = 2$) by gavage (dissolved in 500 μL of corn oil) at 10 a.m, and the animals were transferred to metabolic cages. After 24 h the rats were sacrificed by cervical dislocation and subcellular fractions were prepared as described elsewhere (*4*). A male rat given corn oil alone served as a control.

Results

Synthesis of Protein Conjugates and Amino Acid Derivatives. Generation of antibodies directed to dichloro- or trichloroacetyl moieties attached to lysine residues in proteins was achieved by coupling of *N*-dichloroacetyl-*p*-aminobenzoic acid or *N*-(trichloroacetyl)glycine to KLH and RSA. Extent of modification of free amino residues in KLH used in rabbit immunization procedures and RSA used as solid-phase antigens in ELISA experiments was measured by determination of the free ϵ-amino groups in the modified proteins. Derivatization of amino groups by TNBS gives the percentage of modified lysine residues as compared to the native proteins. Whereas almost all ϵ-amino groups were modified in dichloro- and trichloroacetylated KLH, only approximately 80% of free amino groups were modified in RSA (Table 1). In addition, the introduced modifications resulted in a significant alteration of proteins isoelectric points of the proteins as determined by capillary isoelectric focusing as indicated by the appearance of additional or shifted signals for the modified proteins as compared to the starting materials (data not shown).

Epitope specifity of rabbit antisera and affinity purified antibodies was tested against several N^{ϵ}-(haloacetyl)-L-lysine derivatives synthesized as described previously (*4*) and lipoic acid which has been reported to be recognized by antibodies against N^{ϵ}-(trifluoroacetyl)-L-lysine (*17*−*19*). Purity and structure of synthesized inhibitors was confirmed by HPLC and mass spectrometric analysis. Electrospray mass spectral analysis of N^{ϵ}-(chloroacteyl)-L-lysine derivatives and N^{ϵ}-(trifluororoacteyl)-L-lysine showed molecular ions $[M + H]^+$ at m/z (^{35}Cl) 223 (1 Cl, N^{ϵ}-(chloroacetyl)-L-lysine), 257 (2 Cl, N^{ϵ}-(dichloroacetyl)-L-lysine), 291 (3 Cl, N^{ϵ}-(trichloroacetyl)-L-lysine) and 241 (N^{ϵ}-(trifluoroacetyl)-L-lysine) with single peaks detected at 214 nm in reversed phase HPLC. The structure of the lysine conjugates were further confirmed by GC/MS with chemical ionization and negative ion detection after derivatization with 1,3-dichloro-1,1,3,3-tetrafluoroacetone (*4*). Identification of the 2,2-bis(chlorodifluoromethyl)-4-substituted-1,3-oxazolidin-5-one products formed by condensation of 1,3-dichloro-1,1,3,3-tetrafluoroacetone with the carboxy- and the α-amino-group of lysine confirmed the modification of the ϵ-amino group of lysine in all reference compounds (Table 2).

Characterization of Anti-DCA and Anti-TCA Antibody Specifity. Rabbit antisera from *N*-(dichloroacetyl)-*p*-aminobenzoic acid modified KLH or *N*-(trichloroacetyl)glycine modified KLH immunized rabbits revealed increased reactivity against the solid-phase antigens *N*-(dichloroacetyl)-*p*-aminobenzoic acid modified RSA or *N*-(trichloroacetyl)glycine modified RSA 4−12 weeks after the first antigen injection in indirect ELISA experiments. Immune sera revealed much higher activity against this antigen than against dichloro- or trichloroacetylated RSA without spacer (Figure 2). Preimmune sera of these animals did not react with these antigens. In addition, antibody-containing sera did not bind to unmodified RSA. Immunoaffinity purification of rabbit sera on N^{ϵ}-(dichloroacetyl)-L-lysine or N^{ϵ}-(trichloroacetyl)-L-lysine coupled to activated agarose columns yielded monospecific antibodies. Structural specifity of the purified antibodies was tested in competitive ELISA experiments by determination of IC_{50} values for inhibition of anti-DCA or anti-TCA antibody binding to the corresponding solid-phase antigens (dichloroacetylated RSA or trichloroacetylated RSA) by several inhibitor substrates (Figure 3). N^{ϵ}-(Acetyl)-L-lysine and unmodified RSA did not effect antibody binding to the solid-phase antigen up to the highest inhibitor concentrations used. About 25% of the unchallenged antibody binding values were inhibited by these substrates at 100 mM. The highest concentration for RSA used was 1 mM because of its limited solubility. Binding of the anti-DCA antibody was inhibited by the addition of N^{ϵ}-(dichloroacteyl)-L-lysine with an IC_{50} value of 150 μM. N^{ϵ}-(trichloroacetyl)-L-lysine and N^{ϵ}-(monochloroacetyl)-L-lysine inhibited 50% of antibody binding at concentrations of approximately 100 mM (Figure 3A). Anti-TCA antibody binding to trichloroacetylated RSA was inhibited in the presence of N^{ϵ}-(trichloroacetyl)-L-lysine with an IC_{50} of 90 μM. Only weak interactions with an IC_{50} of 65 mM for N^{ϵ}-(dichloroacetyl)-L-lysine and 80 mM for N^{ϵ}-(monochloroacetyl)-L-lysine were observed (Figure 3B). N^{ϵ}-(trifluoroacetyl)-L-lysine and lipoic acid had minimal effects on antibody binding for anti-DCA- or anti-TCA antibody at concentrations below 1 M where nonspecific denaturating effects resulted in antibody inactivation. Again purified antibodies revealed higher activity against *N*-(dichloroacetyl)-*p*-aminobenzoic acid modified RSA or *N*-(trichloroacetyl)glycine modified RSA than against dichloro- or trichloroacetylated RSA. However, the inhibition of (dichloroacetyl)-*p*-aminobenzoate and trichloroacetyl−glycine was not significantly higher than (dichloroacetyl)- or (trichloroacetyl)lysine. Modified *p*-aminobenzoic acids do not inhibit more efficiently than other amino acids.

Immunoblot detection of chemically dichloroacetylated and trichloroacetylated RSA using monospecific anti-

Table 2. Characteristic Fragments (*m/z*) of 2,2-Bis(chlorodifluoromethyl)-4-substituted-1,3-oxazolidin-5-one Products after 1,3-Dichloro-1,1,3,3-tetrafluoroacetone Derivatization of L-lysine Derivatives in Chemical Ionization Mode Using Negative Ion Detection

	L-lysine derivatives			
fragment	N^{ϵ}-(monochloroacetyl)-L-lysine	N^{ϵ}-(dichloroacetyl)-L-lysine	N^{ϵ}-(trichloroacetyl)-L-lysine	N^{ϵ}-(trifluoroacetyl)-L-lysine
$[M]^-$	402 (3 Cl)	436 (4 Cl)	470 (5 Cl)	422 (2 Cl)
$[M - CO_2 - HCl]^-$	322 (2 Cl)	356 (3 Cl)	390 (4 Cl)	342 (1 Cl)
$[M - C(CF_2Cl)_2 - CO_2 - H]^-$	175 (1 Cl)	209 (2 Cl)	243 (3 Cl)	–

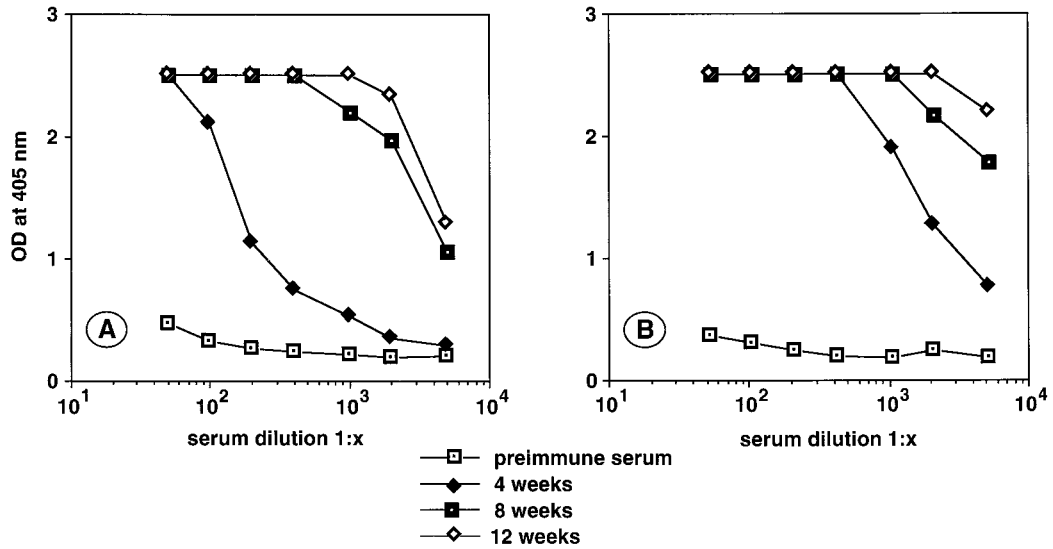

Figure 2. Immune response to *N*-(dichloroacetyl)-*p*-aminobenzoic acid and *N*-(trichloroacetyl)glycine modified KLH as shown by indirect ELISA to the solid phase andigens dichloroacetylated RSA (A) and trichloroacetylated RSA (B).

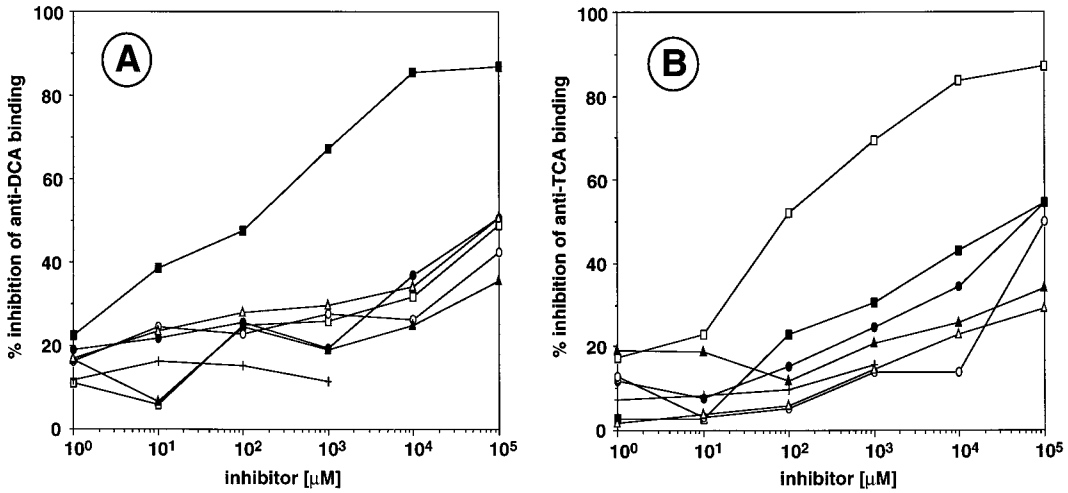

Figure 3. Inhibition of anti-DCA (A) and anti-TCA (B) antibody binding to dichloroacetylated RSA and trichloroacetylated RSA used as solid-phase antigens (2.0 μg/well each). Competitive ELISA was carried out by incubation of the affinity purified antibodies (1:500, v/v of 0.1 mg/mL) with serial dilution of the inhibitors N^{ϵ}-(dichloroacetyl)-L-lysine (■), N^{ϵ}-(trichloroacetyl)-L-lysine (□), N^{ϵ}-(monochloroacetyl)-L-lysine (●), N^{ϵ}-(trifluoroacetyl)-L-lysine (○),N–$^{\epsilon}$-(acetyl)-L-lysine, (▲), RSA (+) and lipoic acid (△).

DCA and anti-TCA antibodies confirmed the results from competitive ELISA experiments on epitope specifity. Affinity-purified antibodies were incubated with blotted proteins in the presence of several inhibitory substances. Antibody binding to the desired reference protein was compared to the response of unchallenged antibody binding. N^{ϵ}-(Acetyl)-L-lysine and N^{ϵ}-(trifluoroacetyl)-L-lysine did not effect anti-DCA or anti-TCA antibody binding to the reference proteins dichloroacetylated RSA or trichloroacetylated RSA blotted to nitrocellulose in concentrations of 50 and 500 μM. N^{ϵ}-(Dichloroacetyl)-L-lysine completely inhibited anti-DCA antibody binding to dichloroacetylated RSA (Figure 4A) at 50 and 500 μM whereas antibody binding was not affected by N^{ϵ}-(trichloroacetyl)-L-lysine at these concentrations. Binding of the anti-TCA antibody to trichloroacetylated RSA was inhibited in the same way by N^{ϵ}-(trichloroacetyl)-L-lysine (Figure 4B). N^{ϵ}-(Dichloroacetyl)-L-lysine inhibited anti-TCA antibody binding to give approximately 10% of the unchallenged response at 500 μM as compared by the integrated optical density of the specific band but did not affect binding at 50 μM. Whereas N^{ϵ}-(chloroacetyl)-L-lysine did not affect binding of anti-TCA antibody to trichloroacetylated RSA at 50 and 500 μM, binding of

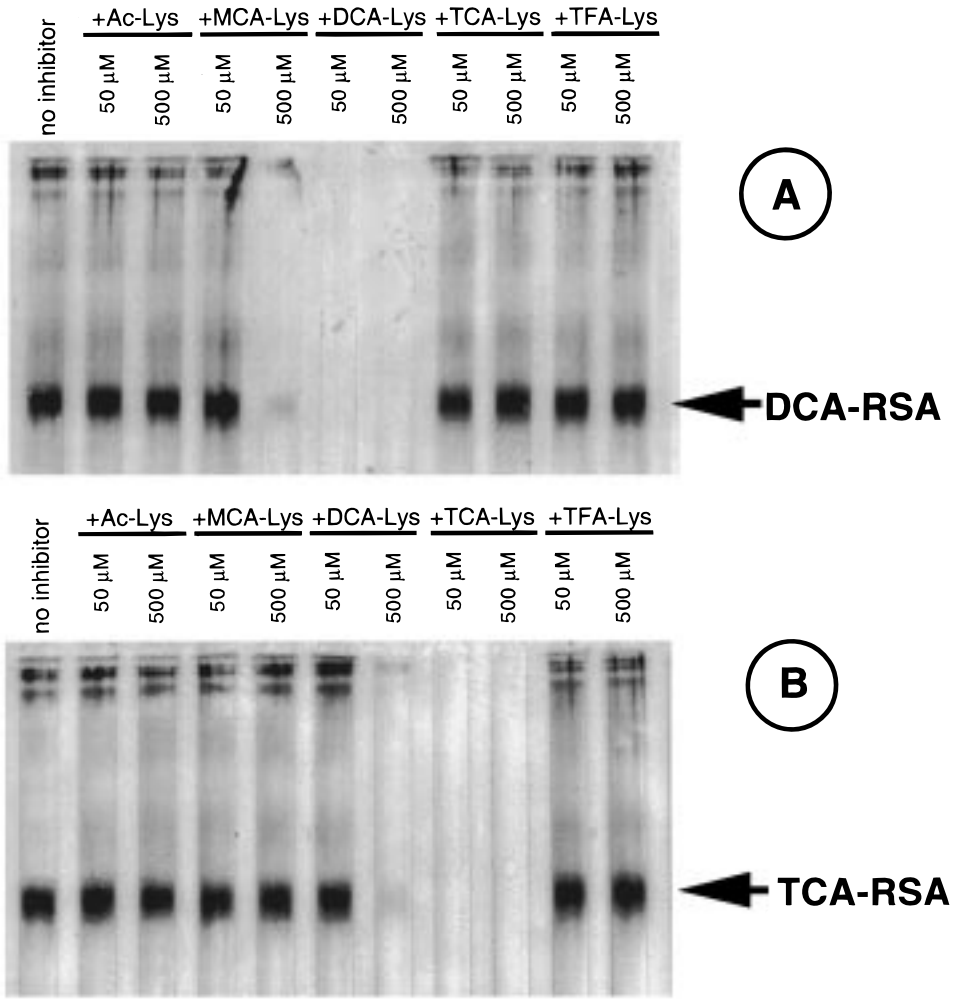

Figure 4. Inhibition of immunoblot detection of the synthetic reference proteins (approximately 10 ng/lane) dichloroacetylated RSA by anti-DCA antibody (A) and trichloroacetylated RSA anti-TCA antibody (B) by several structurally related compounds.

anti-DCA antibody to dichloroacetylated RSA was significantly inhibited at 500 μM.

Immunochemical Detection of PER-Derived Protein Adducts in Vivo. The formation of PER-derived protein adducts in vivo was confirmed after a single dose gavage of PER (1000 mg/kg body weight by gavage) to male Wistar rats. Subcellular fractions of liver and kidney were isolated 24 h later. Di- and trichloroacetylated RSA were used as reference proteins in all experiments analyzing mitochondria, cytosol, and microsomes from liver and kidney in parallel. The majority of the modified proteins recognized by anti-DCA antibody were present in kidney mitochondria and kidney cytosol. Only weak bands were detected in kidney microsomes; the intensity of these bands was only little above above background when compared to untreated controls. Modified proteins were not observed in kidney subcellular fractions of control rats by anti-DCA and in liver by anti-TCA antibody (Figure 5A, B). Detection of trichloroacetylated proteins using anti-TCA antibody (Figure 6B) revealed the most intensive response in liver microsomes and liver cytosol. Liver mitochondria, kidney mitochondria, and kidney microsomes showed only low concentrations of modified proteins whereas kidney cytosol showed a small but significant response in the detection of trichloroacetylated proteins. No protein adduct formation could be detected in liver subcellular fractions of an untreated control by anti-DCA and in kidney by anti-TCA antibody (data not shown).

Discussion

This study presents data on the preparation of rabbit polyclonal antisera to dichloroacetylated and trichloroacetylated proteins which were affinity purified to yield specific antibodies to dichloroacetylated or trichloroacetylated lysine residues as shown by competitive ELISA characterization (Figure 3). Anti-DCA and anti-TCA antibodies were monospecific toward the desired haloacetylated proteins (chemically modified RSA) and showed only a weak interaction with the other related moiety. This specificity was possibly due to the introduction of an amino acid spacer between the chemical moiety attached to the ϵ-amino group of lysine residues in proteins used for rabbit immunization. This method in antigen synthesis provides a better expression of the desired protein modification more distant from nonspecific protein epitopes. Since some cross reactivity of the anti-TCA antibody to dichloroacetyl groups is observed using glycine as a spacer molecule, the introduction of the more space-filling amino acid *p*-aminobenzoic acid may cause the higher specificity in case of the anti-DCA antibody.

Application of anti-DCA and anti-TCA antibodies to detect protein modifications in liver and kidney subcel-

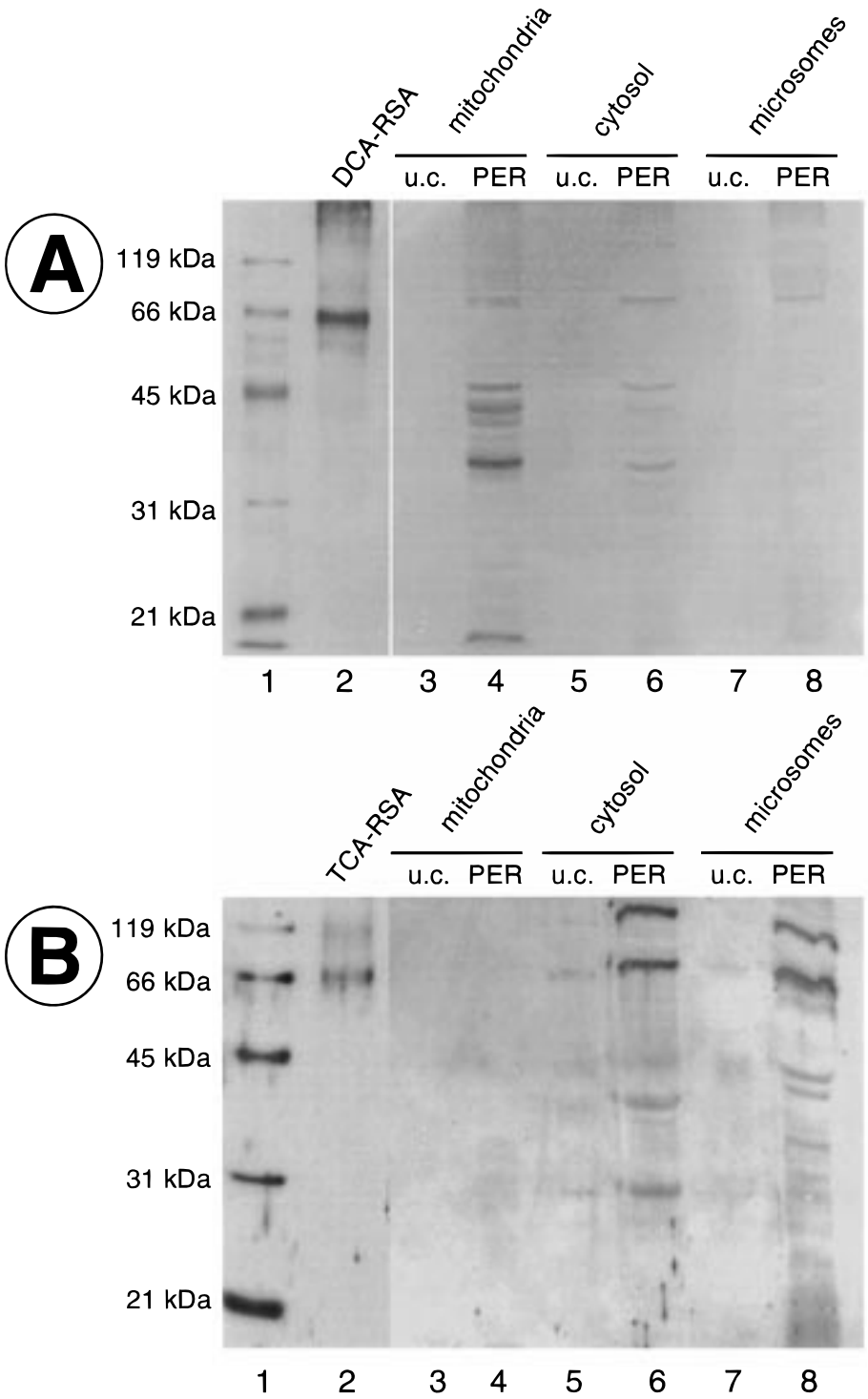

Figure 5. Immunoblot detection of dichloroacetylated proteins by anti-DCA antibody in kidney subcellular fractions (10 μg of total protein per lane) of PER (1000 mg/kg body weight) treated male Wistar rats (PER, lane 4: mitochondria, lane 6: cytosol; lane 8: microsomes) compared to a untreated control rat (A); lane 3: mitochondria; lane 5: cytosol; lane 7: microsomes. Panel B shows detection of trichloroacetylated proteins in liver subcellular fraction by anti-TCA antibody compared to the untreated control (u.c.). Lane 1: molecular weight marker; lane 2: 100 ng of dichloroacetylated RSA (A), 100 ng trichloroacetylated RSA (B).

lular fractions of PER treated rats showed significant protein adduct formation compared to the untreated control.

The use of monospecific antibodies to detect immunoreactive proteins in the livers and kidneys of PER-treated rats reveals distinct patterns of modified proteins in the different organs and subcellular fractions. Possible reasons for these differences are most likely due to the chemistry of the reactive intermediates formed, the subcellular distribution of the enzymes catalyzing the formation of the reactive intermediates, the structures of the proteins present in these subcellular fractions, and substrate availability in the respective organ.

The appearance of dichloroacetylated proteins recognized by the anti-DCA antibody was most intensive in kidney mitochondria. Dichloroacetylated proteins were found in liver cytosol in approximately 30% of the relative intensities of the protein bands detected in kidney cytosol

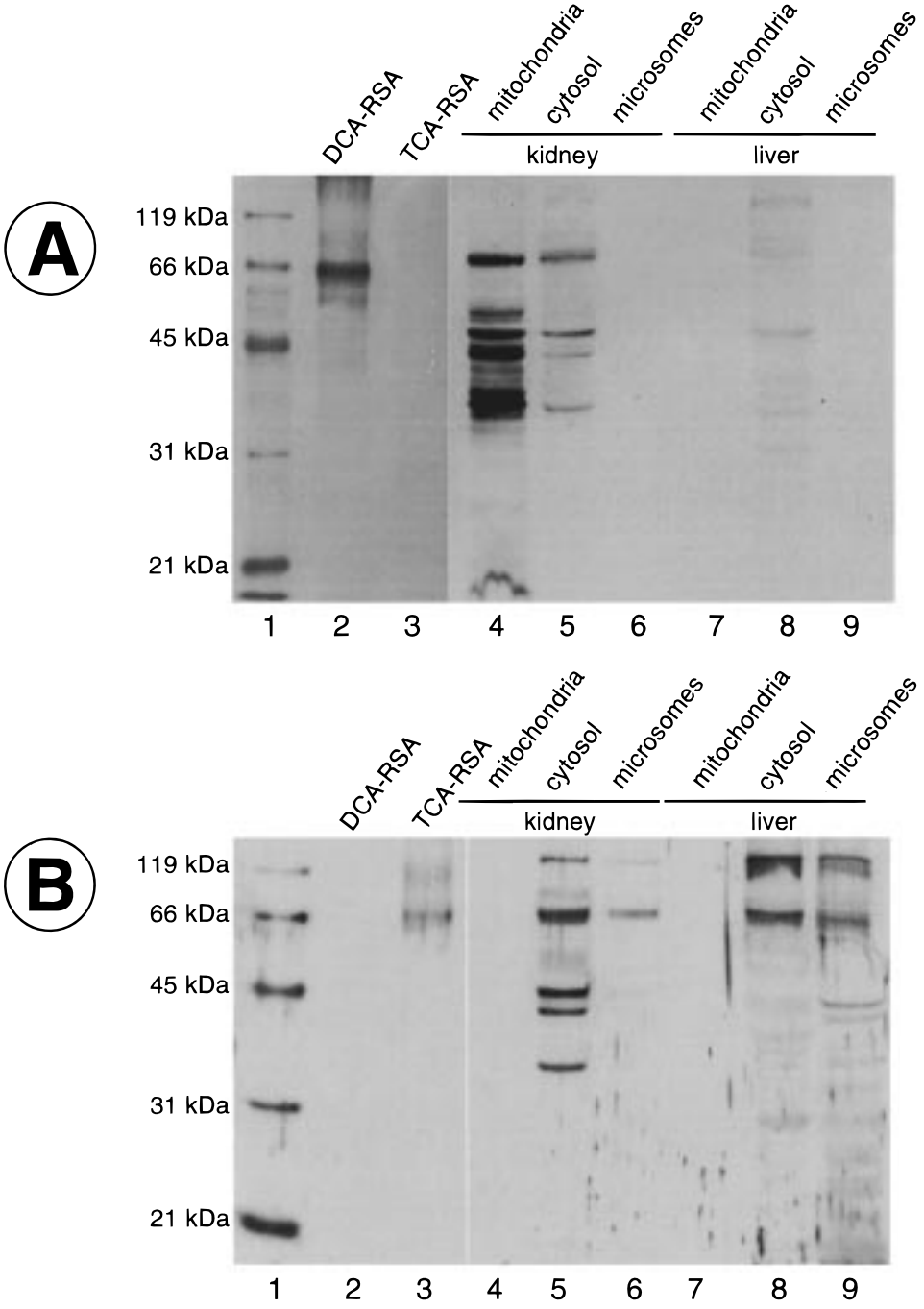

Figure 6. Immunoblot detection of protein adducts in kidney and liver subcellular fractions using affinity purified rabbit anti-DCA (A) or anti-TCA antibody (B). Male Wistar rats were given PER (1000 mg/kg b.w.) and were sacrificed 24 h after dosing. Lanes 1: molecular weight marker; lane 2: 100 ng of dichloroacetylated RSA; lane 3: 100 ng of trichloroacetylated RSA; lane 4: 10 μg of kidney mitochondria; lane 5: 10 μg of kidney cytosol; lane 6: 10 μg of kidney microsomes; lane 7: 10 μg of liver mitochondria; lane 8: 10 μg of liver cytosol; lane 9: 10 μg of liver microsomes.

as determined by comparison of the total integrated optical densities. In liver microsomes, modified proteins detected by the anti-DCA antibody were not present (Figure 6A). This finding is consistent with the observation that renal mitochondria contain the highest activity of β-lyases compared to cytosol and microsomes most likely responsible for the activation of the PER metabolite *S*-(1,2,2-trichlorovinyl)cysteine to the dichloroacetylating agent dichlorothioketene leading to dichloroacetylated proteins (*20*−*23*). Cysteine conjugate β-lyase activity is also present in liver cytosol, thus explaining the detection of dichloroacetylated proteins in the liver of PER-treated rats. However, due to a presumed lower availability of *S*-(1,2,2-trichlorovinyl)-L-cysteine in the liver because of its accumulation in the kidney (*24*) for bioactivation by β-lyases, protein modification is less intensive in the liver (*25*). Since the formed dichlorothioketene has only a very short half-life, it most likely reacts with proteins close to the site of its formation. In the modified proteins, lysine may be the only amino acid forming stable adducts, and mass spectrometric evidence for the formation of N^{ϵ}-(dichloroacetyl)-L-lysine in kidney subcellular fractions has been presented (*4*). Dichlorothioketene may also react with the thiol group in cysteine residues in proteins;

however, the formed products are not stable and may not be detected by the antibody or by mass spectrometry. Intermediate formation of protein adducts from fluoroalkyl *S*-conjugates at the cysteine sulfur atom has been observed (*26*).

The most intensive response of anti-TCA antibody was observed in liver microsomes and cytosol of PER treated rats. This may be due to the oxidative bioactivation of PER by microsomal enzymes to trichloroacetyl chloride which is capable of acylating cellular proteins. Like dichlorothioketene, the acyl halide formed by oxidative metabolism of PER is highly reactive and is expected to react with amino acids close to the site of formation. In addition to the reaction of the acyl halide with proteins, reaction with lipids may also occur (*3*).

In liver microsomes, immunoreactive proteins with molecular weights of approximately 40−45 kDa were detected. Since cytochromes P450 are involved in the oxidation of PER to trichloroacetyl chloride (*27*), a reaction of the acyl halide with this protein is likely. Several cytochrome P450 enzymes have molecular weights of approximately 50 kDa; moreover, acylation of a specific cytochrome P450, cytochrome P450 2E1, has been demonstrated by acylating agents formed metabolically from trichloroethene (*28*) and halothane. In liver cytosol, one of the major immunoreactive proteins likely represents albumin (66 kDa) detected as well by the anti-DCA antibody in kidney cytosol. The identity of the lower molecular weight proteins in liver cytosol may represent glutathione *S*-transferases. Cytosolic glutathione *S*-transferases have been identified as proteins modified during the oxidative metabolism of halothane (*29*). The reaction likely involves glutathione conjugate formation of trichloroacetyl chloride. The formed thioester is also an acylating agent and may acylate lysine in the vicinity of the glutathione binding site of the glutathione *S*-transferases. In kidney subcellular fractions, the anti-DCA antibody recognized major immunoreactive proteins in the molecular mass range of 28−47 kDa. Some of these proteins may represent cysteine conjugate β-lyases, which were reported to have molecular mass ranges of 43−55 kDa (*21*). In addition, enzyme subunits of dehydrogenase complexes such as pyruvate and 2-oxoglutarate dehydrogenase have the appropriate molecular masses to be considered as possible candidates for adduct formation. Cysteine *S*-conjugates of haloalkenes have been demonstrated to inhibit these enzymes (*30*−*32*).

In summary, antibodies were developed with high specificity for protein modifications induced by PER metabolites. These antibodies may have broad utility to study toxic effects of PER, but also for any other haloalkenes metabolized to identical reactive intermediates. Moreover, the high sensitivity of this method for the detection of modified proteins may be used to detect and quantify modified proteins as a biochemical effect marker in comparative studies on PER metabolism and toxicity.

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