Published in final edited form as:

Toxicol Mech Methods. 2014 January ; 24(1): . doi:10.3109/15376516.2013.843112.

Proteomic identification of carbonylated proteins in the kidney of trichloroethene-exposed MRL+/+ mice

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Abstract

Trichloroethene (TCE), a common environmental and occupational pollutant, is associated with multi-organ toxicity. Kidney is one of major target organs affected as a result of TCE exposure. Our previous studies have shown that exposure to TCE causes increased protein oxidation (protein carbonylation) in the kidneys of autoimmune-prone MRL +/+ mice, and suggested a potential role of protein oxidation in TCE-mediated nephrotoxicity. To assess the impact of chronic TCE exposure on protein oxidation, particularly to identify the carbonylated proteins in kidneys, female MRL+/+ mice were treated with TCE at the dose of 2 mg/ml via drinking water for 36 weeks and kidney protein extracts were analyzed for protein carbonyls and carbonylated proteins identified using proteomic approaches (2D gel, Western blot, MALDI TOF/TOF MS/MS, etc.). TCE treatment led to significantly increased protein carbonyls in the kidney protein extracts (20,000g pellet fraction). Interestingly, among 18 identified carbonylated proteins, 10 were found only in the kidneys of TCE-treated mice, whereas other 8 were present in the kidneys of both control and TCE-treated mice. The identified carbonylated proteins represent skeletal proteins, chaperones, stress proteins, enzymes, plasma protein, and proteins involved in signaling pathways. The findings provide a map for further exploring the role of carbonylated proteins in TCE-mediated nephrotoxicity.

Keywords

trichloroethene; oxidative stress; proteomics; protein carbonylation; autoimmune nephritis

Introduction

Trichloroethene (TCE, C₂HCl₃), an extensively used industrial agent, is also a well-known environmental pollutant (Tabrez et al., 2009). TCE exposure has been associated with various pathological changes, including accelerated autoimmune response, heart defects, carcinogenicity in animal models and humans, and toxic responses in nervous system, liver and kidney (Khan et al., 1995, 2001; Caldwell and Keshava et al., 2006; Chiu et al., 2006; Cooper et al., 2009; Gilbert et al., 2009). Even though TCE is one of the most widespread environmental contaminants and a notable multi-system and multi-organ toxic agent, molecular mechanisms by which TCE leads to toxic/pathogenic response, especially an autoimmune response, are not well understood.

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Previously, we have shown that TCE exposure in MRL +/+ mice induces/exacerbates an autoimmune response (Khan et al., 1995, 2001; Gilbert et al., 2009; Wang et al., 2007; Cai et al., 2008). Increased perivascular inflammatory infiltrates in kidneys and a strong deposition of immunoglobulins in the glomeruli were observed in MRL +/+ mice chronically exposed to a low dose of TCE – findings consistent with nephritis (Cai et al., 2008). Earlier studies have also shown that TCE exposure is associated with oxidative stress, which was evidenced by increased oxidative DNA damage in liver (Toraason et al., 1999; Hu et al., 2008) and increased protein oxidation and lipid peroxidation in serum, liver, kidney and lung (Cojocel et al., 1989; Channel et al., 1998; Khan et al., 2001; Wang et al., 2007, 2008, 2009, 2012). Oxidative stress has been implicated in the pathogenesis of various human diseases (Sayre et al., 2001; Hadjigogos, 2003). However, specific pathways by which oxidative stress may contribute to TCE-mediated nephrotoxicity remains largely unknown.

Protein carbonylation, a marker of protein oxidation, is mediated by reactive oxygen species (ROS), which are generated by aerobic metabolism, environmental stressors and/or during disease pathogenesis (Sayre et al., 2001; Nystrom, 2005). ROS can directly attack the side chains of proline, arginine, lysine and threonine to form protein carbonyls or indirectly modify the side chains of cysteine, histidine or lysine residues by lipid peroxidation and carbohydrate oxidation products leading to increased protein carbonylation (Nystrom, 2005). Carbonylation of proteins often results in their fragmentation, aggregation, and enhanced susceptibility to proteolytic digestion (Yu, 1994), leading to loss in their functions. Protein carbonylation is associated with various diseases, such as chronic renal failure, diabetes, Alzheimer's disease, rheumatoid arthritis and system lupus erythematosus (SLE; Dalle-Donne et al., 2003a, 2006; Morgan et al., 2005; Wang et al., 2009). In an earlier study, TCE exposure in an autoimmune-prone MRL+/+ mice (a model for SLE) led to increased protein oxidation (protein carbonylation) in the kidneys (Wang et al., 2009). Increased carbonylation in the kidney was associated with increases in serum autoantibodies, suggesting a potential role of protein oxidation in TCE-mediated autoimmunity and/or kidney damage (Wang et al., 2009). These findings necessitated the identification and characterization of specific carbonylated proteins in the kidneys to further elucidate the role of protein oxidation in TCE-induced nephritis.

The current study, using proteomic approaches, is focused on identifying carbonylated proteins in the protein extracts of kidney from TCE-treated MLR +/+ mice. We uncovered a total of 18 carbonylated proteins that were present in control and/or TCE-treated mice, but 10 of the 18 carbonylated proteins were found only in TCE-treated mice.

Materials and methods

Materials

TCE (99.5%), dinitrophenyl hydrazine (DNPH) and other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). Precast immobilized DryStrips (pH 3–11NL, 11 cm) and IPG buffer (pH 3–11NL) were purchased from GE Healthcare (Uppsala, Sweden). 2D protein extraction buffer-III was purchased from GE Healthcare (Piscataway, NJ). SDS-Tris-glycine gradient gel (10–20%), coomassie blue G250 stain solution, PVDF membrane and fat-free milk were purchased from Bio-Rad (Hercules, CA). Goat anti-dinitrophenol (DNP) polyclonal antibody and HRP-conjugated donkey anti-goat IgG were purchased from Oxford Biomedical Research (Oxford, MI). Amersham ECL plus Western blotting detection kit was purchased from GE Healthcare (Buckinghamshire, UK).

Animal treatment

Five-week-old female autoimmune-prone MRL +/+ mice, a model for SLE (Khan et al., 1995, 2001; Wang et al., 2007, 2009; Cai et al., 2008; Gilbert et al., 2009) - a disease which is more common in females (Jacobson et al., 1997; Walsh and Rau, 2000; Danchenko et al., 2006), were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a controlled-environment animal room (temperature, 22 °C; relative humidity, 50%; photoperiod, 12 h light/dark cycle) for one week prior to the treatment. The mice grouped in six each, were treated with TCE [2 mg/ml in drinking water containing 1% (V/V) Alkamuls EL-620 emulsifier (Rhodia Chemicals, Cranbury, NJ)] (Griffin et al., 2000; Gilbert et al., 2006; Blossom and Doss, 2007; Wang et al., 2007, 2012; Cai et al., 2008), while the control group received water containing emulsifier only. The solutions were changed twice a week. The average TCE intake during the treatment period was ~260 µg/g/day. After 36 weeks of treatment, the mice were euthanized under nembutal (sodium pentobarbital) anesthesia, and the kidneys were removed and stored at -80 °C until further analysis. The experiments were performed in accordance with the guidelines of National Institutes of Health and approved by the Institutional Animal Care and Use Committee of University of Texas Medical Branch at Galveston.

Protein extraction

Kidneys from control and TCE-treated mice were first washed with ice cold phosphate buffered saline (PBS) and then homogenized in a buffer [PBS (pH 7.4), 1% Igepal-CA630 (NP-40), 1X protease inhibitor cocktail, 10 mM DTT and 10 mM EDTA] on ice and then centrifuged at 750 g for 20 min at 4°C to remove the cell debris. The top fraction was transferred to new tubes and centrifuged at 20,000 g for 20 min at 4°C. The supernatant was taken out and mixed with 1% streptomycin sulfate to remove DNA contaminants (Fraction #1; Blackburn et al., 1999), while the pellet was dissolved in a buffer containing 6M urea, 1% NP-40, 20 mM Tris-HCl pH 7.4, and 10 mM EDTA (Fraction #2) in a volume same as the supernatant. The protein extract of fraction #2 (comprised of membrane proteins, mitochondria, lysosomes and peroxisomes; Harshman et al., 1991; Bizzozero et al., 2007) alone, and the mixture of fraction #1 and #2 (1:1, V/V) were subjected to further analysis.

Detection of protein carbonyls by spectrophotometric assay

The carbonyls in the total proteins (fraction #1 and #2) or protein extract of fraction #2 of TCE-treated and control mice were derivatized with DNPH and then analyzed as its DNP adducts (DNP-derivatives), as described earlier (Fagan et al., 1999; Dalle-Donne et al., 2003b) with slight modifications. The total protein (mixture of fraction #1 and #2, 200 µg) or protein extract of fraction #2 (500 µg) was treated with acetone:HCl (100:3, V/V) in 1:9 ratio ($V_{\text{protein}}:V_{\text{acetone-HCl}}$) to remove heme contaminant. The mixture was then centrifuged at 10,000 g for 15 min at 4°C. The pellet was washed twice with 10% TCA, and then DNPH (10 mM stock, dissolved in 2.0 N HCl) was added to a final concentration of 8 mM in 1.5 N HCl for reaction with the carbonyl groups of proteins. The reaction was carried out in dark at room temperature in dark for 2 h. Then, an equal volume of 20% TCA was added to the reaction mixture, and incubated for 20 min on ice for the precipitation of the proteins. After centrifugation at 10,000 g for 15 min at 4°C, the pellet was washed with 500 µl of ethanol:ethylacetate (1:1, V/V) three times, and centrifuged at 20,000 g for 20 min at 4°C to remove the unreacted DNPH. The pellet was then dissolved in 6 M guanidine hydrochloride in 10 mM potassium phosphate buffer (pH 3.5). The absorbance was measured at 370 nm. The protein carbonyl content was calculated according to the molar extinction coefficient of DNPH at 370 nm, which is 22,000 M⁻¹cm⁻¹.

Detection of carbonylated proteins by 2D-gel and Western blotting

The protein carbonyls in the protein extract of fraction #2 of TCE-treated mice showed a significant increase compared to the controls, and were subjected to 2D gel and Western blot analyses. Five hundred microgram of protein from fraction #2 was added in 200 μ l of 2D protein extraction buffer-III containing 100 mM DTT, trace amount of bromophenol blue and 1% IPG buffer, pH 3–11 NL, and incubated at 21°C for 1 h. The proteins were then rehydrated to the DryStrip (11 cm, pH 3–11 NL) overnight at the same temperature. For each sample, IPG strips were prepared in duplicate. For the first dimension, isoelectric focusing (IEF) was performed at 20°C on Ettan IPGphor3 (GE Healthcare, Sweden) in the following steps: 200 V for 30 min, 500 V for 1.5 h, 1000 V for 1.5 h, 8000 V for 2 h and 8000 V for 24000 Vhr. The strips were then equilibrated for 1 hr in equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 20 mM iodoacetamide, 2% SDS, 20% glycerol]. After rinsing two times with SDS-PAGE running buffer, the strips were loaded on to 10–20% SDS-Tris-glycine gradient gel (13.3 $\times$ 8.7 cm) and were then run at 150 V for 2 h and 20 min at room temperature (RT) for the 2nd dimension separation. Following the electrophoresis, one of the duplicate gels of each sample was stained with coomassie blue G250 (CBB G250), while the other one was used for Western blotting by transferring the proteins to PVDF membrane. The PVDF membrane was then sequentially incubated with TBS (pH 7.4) with 20% methanol, 1 h; 1.5 N HCl, 5 min; DNPH (100 μ g/ml), 5 min; 1.5 N HCl, 5 min. for three times; and finally 100% methanol for 30 min (Banan et al., 2000). All these steps were performed at 21°C. The PVDF membrane was blocked with 5% fat-free milk in TBST (pH 7.4) for 30 min at RT and then incubated with anti-DNP IgG (1:1000 dilution in TBST containing 5% fat-free milk, pH 7.4) at 21°C for 2 hr. The membrane was washed and incubated with HRP conjugated secondary antibody (1:5000 dilution in TBST containing 5% fat-free milk) for 1 h at 21°C. The signal was visualized by enhanced chemiluminescence detection.

SameSpots analysis of protein expression variation

2D gel images (CBB G250 stained) were acquired by 2D Proteomic Imaging System (ProXPRESS, PerkinElmer, Inc.). The scanning resolution of CCD camera (software, ProScan V4.0.0.10) was 100 μ m with white light and the exposure time was 600 msec. The 2D gel images were then analyzed by the software Progenesis SameSpots (nonlinear dynamics, version 4.0) which has been judged to be much improved in reproducibility compared to previous generations of 2D gel analysis software (Silva et al., 2010). To match the same spot (same protein) between overlapped gel images, one of the six gels was chosen as reference. With the help of manually drawn and automatically added vectors from each image to the reference, images were aligned at the pixel level. The program then performed automatic spot detection and background subtraction. The software assigns the same spot on every gel in the analysis identical shape (spot outline) and spot number. Spot volumes were normalized to those of the reference gel to obtain normalized volumes that are comparable across gels. Protein expression fold changes between controls and TCE-treated mice were determined as described below in the statistical analysis.

Trypsin digestion and MALDI-TOF-TOF MS analysis

The carbonylated protein spots were manually picked up from the 2D gel. The protein was digested with trypsin (0.1 μ g per spot, Promega) in 10 μ l of 25 mM ammonium bicarbonate (pH 8.0) for 6 h at 37°C. One μ l of digested sample solution was used for MALDI TOF/TOF MS. Alpha-cyano-4-hydroxycinnamic acid was used as matrix for mass analysis. The data was collected from an Applied Biosystems 4800 MALDI TOF/TOF proteomics analyzer. The instrument was operated in a positive ion reflection mode with mass range from 850 to 3000 Da. The focus mass was set at 1700 Da. For MS data, 2000–4000 laser shots were acquired and averaged from each sample spot. Following MALDI MS analysis, MALDI

MS/MS was performed on several (5–10) abundant ions from each sample spot. A 1 kV positive ion MS/MS method was used to acquire data under post-source decay (PSD) conditions. The instrument precursor selection window was ± 3 Da. For MS/MS data, 2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1056.475, and 1441.635 (from precursor mass 1570.700).

Identification of proteins

For protein identification, Applied Biosystems GPS Explorer™ (Version 3.6) software was employed for searching the respective protein database using both MS and MS/MS spectral data. Protein match probabilities were determined by using MASCOT scores. The MASCOT score of more than 64 was considered significance ($p < 0.05$). MS peak filtering included the following parameters: mass range 800 Da to 4000 Da, minimum S/N filter = 10, mass exclusion list tolerance = 0.5 Da, and mass exclusion list (for some trypsin and keratin-containing compounds) included masses 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, and 2211.1. For MS/MS peak filtering, the minimum S/N filter = 10. The mass data was matched to the NCBI protein database. Precursor tolerance was set at 0.2 Da; MS/MS fragment tolerance was set at 0.3 Da; mass = monoisotopic; and peptide charges were only considered as +1.

Statistical analysis

The significance of changes in carbonyl content between control and TCE-treated mice was evaluated by Student's *t*-test. Similarly, the intensities of same protein spots in 2D gels of control and TCE-treated mice were also compared on log-transformed normalized volumes by Student's *t*-test. *p* values < 0.05 were considered significant.

Results

Protein carbonyls in the kidney extracts of TCE-treated mice

Protein carbonyls in the kidneys extracts (both total protein extracts and fraction #2 protein extracts) of control and TCE-treated mice were analyzed by spectrophotometry. Compared to the controls, the protein carbonyls in fraction #2 protein extracts of kidneys from TCE-treated mice increased significantly ($p < 0.05$, Fig. 1), whereas carbonyl content in the total protein extracts did not show statistically significant elevation (data not shown). These findings, thus, provided the basis for the identification of specific carbonylated proteins in the fraction #2 protein extracts of kidneys.

Carbonylated protein spots identified by 2D immunoblotting

Kidney protein extracts of fraction #2 from control and TCE-treated mice were separated by 2D gel electrophoresis. Each sample was run in duplicate – one for CBB G250 stain and the other one for Western blot analysis. The 2D gel protein profile of a representative control sample is shown in Fig. 2A, while Fig. 2B is the corresponding Western blot map with specific anti-DNP antibody recognizable carbonylated proteins of the same sample. The carbonylated proteins, shown in black circles (Fig. 2B), were matched to the 2D gel protein profiles (Fig. 2A). Similarly, the 2D gel protein profile of a kidney extract from TCE-treated mice is shown in Fig. 3A, whereas Fig. 3B shows the corresponding Western blot map of carbonylated protein spots of the same sample. The carbonylated protein spots, shown in black circles (Fig. 3B), were also matched to the 2D gel protein profiles (Fig. 3A).

The 2D gel protein profiles of controls and TCE-treated mice (3 each) matched well when analyzed by using Samespots program (data not shown). Among analyzed controls, we

identified 12 carbonylated protein spots (Fig. 2A, 2B), which were also found in TCE-treated protein extracts. Carbonylated proteins increased remarkably in the protein extracts of TCE-treated mice. We identified a total of 32 carbonylated protein spots among the TCE-treated samples, out of which 20 were found only in the TCE-treated protein extracts (Fig. 3A, 3B). The carbonylated protein spots (same protein spots present in at least two samples) were picked up and subjected to MALDI TOF/TOF MS and MS/MS analyses.

Identified carbonylated proteins in the kidneys

The carbonylated protein spots were analyzed by MALDI TOF/TOF MS and MS/MS, and matched in the protein database. Probability-based MASCOT score was used to evaluate their identifications. The MASCOT score of more than 64 represents the statistical confidence >95%. A total of 18 carbonylated proteins were identified, of which 10 were found in the protein extracts of TCE-treated mice only (Table 1), whereas 8 were found in both control and TCE-treated mice (Table 2). The carbonylated proteins represented skeleton proteins, villin2 (ezrin; Nos. 11 and 22), moesin (No. 26), tropomyosin 3 gamma (No. 1), chaperone and stress proteins, HSP70 protein 5 (GRP78; No. 14), HSP70 protein 9 (GRP 75; No. 17), chaperone complex chain A (No. 16); plasma protein, albumin precursor (No. 18); enzymes, aldehyde dehydrogenase 7A1 (ALDH7A1; No. 8), dihydroxyacetone kinase 2 (No. 12), 3-methylcrotonyl-CoA carboxylase, beta chain (No. 13), glucose regulated protein, putative (ERp57; protein disulfide isomerase A3; Pdia3; No.7), protein disulfide isomerase A4 (ERp72; Pdia4; No.15), propionyl CoA-carboxylase alpha (No. 23), aconitase 2 (No. 28) and nonselenium glutathione peroxidase (No. 31). The identified carbonylated proteins also included signaling pathway proteins, annexin A5 (No. 2), transformation/transcription domain-associated protein, (TRRAP; Tra1 homolog; No. 9), and Immt protein (mitofilin; mitochondrial inner membrane protein; Nos. 10, 19, 20, 21, 24 and 25).

Protein expression variation in 2D gel profiles

The expression of carbonylated proteins was analyzed by Progenesis SameSpots (version 4.0) by comparing the normalized mean intensity of same protein spot in 2D gel of TCE-treated mice with the controls. The results show that the expression of only 3 of the 18 carbonylated proteins changed significantly ($p < 0.05$), and those proteins are Annexin A5 (No. 2), villin2 (No. 11) and Immt protein (No. 21). The expression of other proteins showed no significant difference between the control and TCE-treated mice ($p > 0.05$, Table 3).

Discussion

Increasing attention is being focused on the role of oxidative stress in the pathogenesis of SLE (Hadjigogos, 2003; Morgan et al., 2005, 2007, 2009; Wang et al., 2010). Lupus glomerulonephritis is one of the most common and most threatening manifestations of SLE (Doria and Gatto, 2012). Studies also showed that ROS play a major role in many glomerular diseases (Gwinner and Grone, 2000; Bülbül et al., 2008). Carbonylated proteins, generated as a consequence of ROS-mediated protein oxidation, have been found in inflammation, chronic renal failure and SLE (Dalle-Donne et al., 2003a; Morgan et al., 2005; 2007; Zhang et al., 2008; Wang et al., 2009). The increased protein carbonylation is not only one of the markers of oxidative damage, but also associated with the progression and severity of disease activity including SLE (Dalle-Donne et al., 2006; Zhang et al., 2008), which imply that protein carbonylation could play an important role in the disease pathogenesis and progression. In an earlier study, even though we reported increased protein carbonyls in the kidneys of TCE-treated mice (Wang et al., 2009), the carbonylated proteins were not identified. Thus, in the current study, we combined proteomic and immunoblotting

approaches to target proteins susceptible to oxidation in the kidney protein extracts, in an attempt to understand their roles in TCE-induced kidney damage and/or autoimmune nephritis. Our proteomic data show that out of 18 carbonylated proteins identified in kidney protein extracts, 10 were found only in TCE-treated MRL +/+ mice, whereas 8 carbonylated proteins were found in both control and TCE-treated mice.

Among the identified carbonylated skeletal proteins, villin 2 (ezrin) has been suggested as a glomerular epithelial cell marker of podocyte injury, and its down-regulation could alter the permeability of the filtration barrier in steroid-resistant and proteinuric glomerulopathies (Lai et al., 2009). Progressive deregulation of ezrin was found in nephrotic syndrome glomerulopathies from Minimal change disease to diffuse mesangial proliferation-sensitivity in children (Ostalska-Nowicka et al., 2006). Studies have also shown that ezrin is involved in infection and autoimmune diseases. For example, ezrin increased more than two fold in *Helicobacter pylori*-infected gastric epithelial AGS (gastric adenocarcinoma) cells (Lim et al., 2004), and was found as an autoantigen in *streptococcal*-induced autoimmune response of psoriasis (Besgen et al., 2010). Ezrin and other skeletal protein, moesin, are novel molecules involved in T cell activation signaling, and controlling the localization of CD43 and other ERM (ezrin/radixin/moesin) binding proteins to the distal pole complex (DPC) (Chu et al., 2003; Burkhardt et al., 2008). ERM functional defect led to reduced expression of cytokines IL-2 and γ -interferon (Allenspach et al., 2001; Cullinan et al., 2002; Neisch et al., 2010). Moesin was also found as an autoantigen in patients with acquired aplastic anemia (Takamatsu et al., 2007) and as a carbonylated protein in tumor cells in photodynamic treatment-induced oxidative stress (Tsaytler et al., 2008). All the evidences suggest the multiple roles of ezrin and moesin in cell immunity, inflammation and kidney diseases. Although the functional consequences of ezrin and moesin carbonylation are still unknown in the kidneys of TCE-exposed mice, exploring such functional consequences is highly desirable.

Among the carbonylated enzymes, endoplasmic reticulum protein 57 (ERp57; Pdia3; Grp58) is a chaperon protein and a protein folding catalyst, and is responsible for inhibiting misfolded protein aggregation and disulfide bond formation through reduction and isomerization of incorrect disulfide bond to maintain the new synthesized protein native structure (Wilkinson and Gilbert, 2004; Jordan and Gibbins, 2006). ERp57 plays diverse functions (Coe and Michalak, 2010), and one of the functions of ERp57 is to recruit the MHC class I to the antigenic peptide-loading complex and maintain the complex in a steady state, and defects in ERp57 diminishes the ability of MHC class I antigen presentation in B cells (Ni and Lee, 2007). In addition, carbonylated protein disulfide isomerase was found in H₂O₂-treated HL-60 cells (England and Cotter, 2004) and in aged mouse (Rabek et al., 2003), suggesting that ERp57 is a target protein susceptible to oxidative modification. The other family member, ERp72 (pdia4), has also shown the chaperon and disulfide isomerase activities in nascent protein folding through forming native disulfide bond, and active site motif of the enzyme is similar to ERp57 (C X X C) (Satoh et al., 2005; Kozlov et al., 2009). Studies reveal that the cysteine residue at the enzyme active site motif C X X C (X could be any residue) plays a key role in the enzyme activity; mutation in either of the residues abolishes its enzyme activity (Beynon-Jones et al., 2006). Elevated carbonyls due to TCE-induced lipid peroxidation-derived aldehydes (Wang et al., 2009), such as HNE and MDA, could modify the cysteines in the enzyme's active site leading to structural changes in the active site, and thus may result in the loss of their reductase and isomerase activities.

Aldehyde dehydrogenase 7A1 (ALDH7A1), which was found carbonylated only in TCE-treated mice in this study, is highly expressed in both animal and human kidneys. ALDH7A1, a NAD⁺-specific cofactor enzyme, catalyzes a variety of aldehydes to their corresponding acids to detoxify the aldehydes (Marchitti et al., 2008). Structural studies

reveal that arginine residue 300 of ALDH7A1 is crucial for its catalytic activity, and R300A mutation leads to remarkably decreased enzyme activity (Tang et al., 2008). TCE-induced elevation in ROS could lead to reduction in ALDH7A1 function and result in the accumulation of aldehydes. Studies also found that ALDH7A1 acted as an autoantigen in unexplained infertility and premature ovarian failure in women (Edassery et al., 2010), and was carbonylated in Huntington disease (Sorolla et al., 2010) and in kidney medulla of hypertensive rat (Tyther et al., 2009). The evidences thus suggest a role of ALDH7A1 in autoimmunity and oxidative stress-related pathologies.

The other identified enzymes, propionyl CoA-carboxylase (PCC) and 3-methylcrotonyl-CoA carboxylase (MCCB) are biotin cofactor enzymes. Their function involves the catabolism of branched chain amino acids, odd-chain length fatty acids and cholesterol (Jitrapakdee and Wallace, 2003). The dihydroxyacetone kinase (DhaK) converts the dihydroxyacetone (DHA) into the glycolytic intermediate DHA-phosphate, which detoxifies the DHA-induced toxicity (Molin et al., 2007). DhaK also plays roles in the catabolism of glycerol and methanol assimilation (Erni et al., 2006). Although the functional alteration in the carbonylated forms of these proteins following TCE exposure is still unclear, it would be worthwhile to explore the consequences of carbonylation of these proteins in TCE-induced autoimmune nephritis.

Among the identified carbonylated signal pathway proteins, transformation/transcription domain-associated protein (TRRAP) is highly conserved protein in evolution. TRRAP is a common component of many histone acetyltransferase (HAT) complexes, which is responsible for the recruitment of these complexes to chromatin during transcription, replication and DNA repair, and mediates diverse cellular functions (Murr et al., 2007). Therefore, functional consequences of TRRAP carbonylation will be worth further investigation in TCE-induced pathological response.

Mitofilin (Immt protein) is a mitochondrial inner membrane protein, which plays an important role in normal mitochondrial function (Wang et al., 2008). Studies have shown that mitofilin knockdown resulted in reduced cell proliferation and increased apoptosis (Mun et al., 2010). Mitofilin depletion is also associated with the mitochondrial DNA damage (Rossi et al., 2009). Mitofilin, as an autoantigen, is present in the Melanoma-associated retinopathy (Pfähler et al., 2007), and one of the functions of mitofilin is its involvement in B cell receptor signal transduction (Mielenz et al., 2005). All the findings suggest that mitofilin functional abnormality could result in a variety of pathologies, including autoimmunity. Carbonylation of mitofilin, as observed in this study following TCE exposure, could thus contribute to the pathological changes in the kidneys.

Annexin A5, a Ca^{2+} and phospholipid binding protein of Annexins family, may play a role in autoimmune diseases. It was found to be an autoimmunogen in patients with autoimmune rheumatic disease, such as SLE, primary antiphospholipid syndrome, or systemic sclerosis (Iaccarino et al., 2011). Study also reveals that patients with kidney and other diseases which result in renal malfunction show increased urinary Annexin A5 level (Markoff ND Gerke, 2005). Increased expression of Annexin A5 in the kidneys of TCE-treated mice, as observed in this study, could be important in TCE-induced renal pathogenesis.

In conclusion, the current study provides further evidence that TCE exposure leads to significantly increased protein carbonylation in mouse kidneys. Substantial conformational changes due to oxidative modification of proteins could be a potential source of neoantigens, generating an autoimmune response. The identified carbonylated proteins in this study represent skeletal proteins, chaperones, enzymes, and signaling pathways. Their identification provides a map of increased carbonylated proteins in TCE-induced oxidative

stress, and opens a new avenue for the study of molecular mechanism of TCE-induced autoimmunity and/or autoimmune nephritis.

Acknowledgments

Declaration of Interest

The authors declare that there is no conflict of interest.

The work was supported by grant ES016302 from the National Institute of Environmental Health Sciences (NIEHS), NIH, and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH. Authors also thank Dr. Kizhake V. Soman in the Biomolecular Resource Facility of UTMB (Supported by NHLBI Proteomics Center Grant N01-HV-00245 to Dr. A. Kurosky) for assistance with Progenesis SameSpots software.

References

- Allenspach EJ, Cullinan P, Tong J, Tang Q, Tesciuba AG, Cannon JL, Takahashi SM, Morgan R, Burkhardt JK, Sperling AI. ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. *Immunity*. 2001; 15:739–750. [PubMed: 11728336]
- Banan A, Zhang Y, Losurdo J, Keshavarzian A. Carbonylation and disassembly of the F-actin cytoskeleton in oxidant induced barrier dysfunction and its prevention by epidermal growth factor and transforming growth factor α in a human colonic cell line. *Gut*. 2000; 46:830–837. [PubMed: 10807896]
- Besgen P, Trommler P, Vollmer S, Prinz JC, Ezrin, maspin, peroxiredoxin 2 and heat shock protein 27: potential targets of a streptococcal-induced autoimmune response in psoriasis. *J Immunol*. 2010; 184:5392–5402. [PubMed: 20363977]
- Beynon-Jones SM, Antoniou AN, Powis SJ. Mutational analysis of the oxidoreductase ERp57 reveals the importance of the two central residues in the redox motif. *FEBS Lett*. 2006; 580:1897–1902. [PubMed: 16516209]
- Bizzozero OA, Reyes S, Ziegler J, Smerjac S. Lipid peroxidation scavengers prevent the carbonylation of cytoskeletal brain proteins induced by glutathione depletion. *Neurochem Res*. 2007; 32:2114–2122. [PubMed: 17551832]
- Blackburn AC, Doe WF, Buffinton GD. Protein carbonyl formation on mucosal proteins in vitro and in dextran sulfate-induced colitis. *Free Radic Biol Med*. 1999; 27:262–270. [PubMed: 10468197]
- Blossom SJ, Doss JC. Trichloroethylene alters central and peripheral immune function in autoimmune-prone MRL(+/+) mice following continuous developmental and early life exposure. *J Immunotoxicol*. 2007; 4:129–141. [PubMed: 18958721]
- Bülbül M, Oner A, Demircin G, Erdoğan O. Oxidative stress in children with acute glomerulonephritis. *Ren Fail*. 2008; 30:209–214. [PubMed: 18300123]
- Burkhardt JK, Carrizosa E, Shaffer MH. The actin cytoskeleton in T cell activation. *Annu Rev Immunol*. 2008; 26:233–259. [PubMed: 18304005]
- Cai P, König R, Boor PJ, Kondraganti S, Kaphalia BS, Khan MF, Ansari GAS. Chronic exposure to trichloroethylene causes early onset of SLE-like disease in female MRL +/+ mice. *Toxicol Appl Pharmacol*. 2008; 228:68–75. [PubMed: 18234256]
- Caldwell JC, Keshava N. Key issues in the modes of action and effects of trichloroethylene metabolites for liver and kidney tumorigenesis. *Environ Health Perspect*. 2006; 114:1457–1463. [PubMed: 16966105]
- Channel SR, Latendresse JR, Kidney JK, Grabau JH, Lane JW, Steel-Goodwin L, Gothaus MC. A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. *Toxicol Sci*. 1998; 43:145–154. [PubMed: 9710956]
- Chiu WA, Caldwell JC, Keshava N, Scott CS. Key scientific issues in the health risk assessment of trichloroethylene. *Environ Health Perspect*. 2006; 114:1445–1449. [PubMed: 16966103]
- Chu P, Pardo J, Zhao H, Li CC, Pali E, Shen MM, Qu K, Yu SX, Huang BCB, Yu P, Masuda ES, Molineaux SM, Kolbinger F, Aversa G, Vries J, Payan D G, Liao XC. Systematic identification of regulatory proteins critical for T-cell activation. *J Biol*. 2003; 2:211–216.

- Coe H, Michalak M. ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. *Int J Biochem Cell Biol.* 2010; 42:796–799. [PubMed: 20079872]
- Cojocel C, Beuter W, Muller W, Mayer D. Lipid peroxidation: a possible mechanism of trichloroethylene-induced nephrotoxicity. *Toxicology.* 1989; 55:131–141. [PubMed: 2711399]
- Cooper GS, Makris SL, Nietert PJ, Jinot J. Evidence of autoimmune-related effects of trichloroethylene exposure from studies in mice and humans. *Environ Health Perspect.* 2009; 117:696–702. [PubMed: 19479009]
- Cullinan P, Sperling AI, Burkhardt JK. The distal pole complex: a novel membrane domain distal to the immunological synapse. *Immunol Rev.* 2002; 189:111–122. [PubMed: 12445269]
- Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med.* 2006; 10:389–406. [PubMed: 16796807]
- Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. *Trends Mol Med.* 2003a; 9:169–176. [PubMed: 12727143]
- Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta.* 2003b; 329:23–38. [PubMed: 12589963]
- Danchenko N, Satia JA, Anthony MS. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. *Lupus.* 2006; 15:308–318. [PubMed: 16761508]
- Doria A, Gatto M. Nephritogenic-antinephritogenic antibody network in lupus glomerulonephritis. *Lupus.* 2012; 21:1492–1496. [PubMed: 23042821]
- Edassery SL, Shatavi SV, Kunkel JP, Hauer C, Brucker C, Penumatsa K, Yu Y, Dias JA, Luborsky JL. Autoantigens in ovarian autoimmunity associated with unexplained infertility and premature ovarian failure. *Fertil Steril.* 2010; 94:2636–2641. [PubMed: 20522323]
- England K, Cotter T. Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER. *Biochem Biophys Res Commun.* 2004; 320:123–30. [PubMed: 15207711]
- Erni B, Siebold C, Christen S, Srinivas A, Oberholzer A, Baumann U. Small substrate, big surprise: fold, function and phylogeny of dihydroxyacetone kinases. *Cell Mol Life Sci.* 2006; 63:890–900. [PubMed: 16505971]
- Fagan JM, Slecza BG, Sohar I. Quantitation of oxidative damage to tissue proteins. *Int J Biochem Cell Biol.* 1999; 31:751–757. [PubMed: 10467731]
- Gilbert KM, Przybyla B, Pumford NR, Han T, Fuscoe J, Schnackenberg LK, Holland RD, Doss JC, MacMillan-Crow LA, Blossom SJ. Delineating liver events in trichloroethylene induced autoimmune hepatitis. *Chem Res Toxicol.* 2009; 22:626–632. [PubMed: 19254012]
- Gilbert KM, Pumford NR, Blossom SJ. Environmental contaminant trichloroethylene promotes autoimmune disease and inhibits T-cell apoptosis in MRL(+/+) mice. *J Immunotoxicol.* 2006; 3:263–267. [PubMed: 18958707]
- Griffin JM, Gilbert KM, Lamps LW, Pumford NR. CD4(+) T-cell activation and induction of autoimmune hepatitis following trichloroethylene treatment in MRL+/+ mice. *Toxicol Sci.* 2000; 57:345–352. [PubMed: 11006364]
- Gwinner W, Grone HJ. Role of reactive oxygen species in glomerulonephritis. *Nephrol Dial Transplant.* 2000; 15:1127–1132. [PubMed: 10910434]
- Hadjigogos K. The role of free radicals in the pathogenesis of rheumatoid arthritis. *Painminerva Med.* 2003; 45:7–13. [PubMed: 12682616]
- Harshman LG, Casas J, Dietze EC, Hammock BD. Epoxide hydrolase activities in *Drosophila melanogaster*. *Insect Biochem.* 1991; 21:887–894.
- Hu C, Jiang L, Geng C, Zhang X, Cao J, Zhong L. Possible involvement of oxidative stress in trichloroethylene-induced genotoxicity in human HepG2 cells. *Mutat Res.* 2008; 652:88–94. [PubMed: 18289923]
- Iaccarino L, Ghirardello A, Canova M, Zen M, Bettio S, Nalotto L, Punzi L, Doria A. Anti-annexins autoantibodies: their role as biomarkers of autoimmune diseases. *Autoimmun Rev.* 2011; 10:553–558. [PubMed: 21527362]
- Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol.* 1997; 84:223–243. [PubMed: 9281381]

- Jitrapakdee S, Wallace JC. The biotin enzyme family: conserved structural motifs and domain rearrangements. *Curr Protein Pept Sci*. 2003; 4:217–229. [PubMed: 12769720]
- Jordan PA, Gibbins JM. Extracellular disulfide exchange and the regulation of cellular function. *Antioxid Redox Signal*. 2006; 8:312–324. [PubMed: 16677077]
- Khan MF, Kaphalia BS, Prabhakar BS, Kanz MF, Ansari GA. Trichloroethene-induced autoimmune response in female MRL $+/+$ mice. *Toxicol Appl Pharmacol*. 1995; 134:155–160. [PubMed: 7676450]
- Khan MF, Wu X, Ansari GA. Anti-malondialdehyde antibodies in MRL $+/+$ mice treated with trichloroethene and dichloroacetyl chloride: possible role of lipid peroxidation in autoimmunity. *Toxicol Appl Pharmacol*. 2001; 170:88–92. [PubMed: 11162772]
- Kozlov G, Maattanen P, Schrag JD, Hura GL, Gabrielli L, Cygler M, Thomas DY, Gehring K. Structure of the noncatalytic domains and global fold of the protein disulfide isomerase ERp72. *Structure*. 2009; 17:651–659. [PubMed: 19446521]
- Lai KN, Leung JC, Chan LY, Saleem MA, Mathieson PW, Tam KY, Xiao J, Lai FM, Tang SCW. Podocyte injury induced by mesangial-derived cytokines in IgA nephropathy. *Nephrol Dial Transplant*. 2009; 24:62–72. [PubMed: 18685143]
- Lim JW, Kim H, Kim JM, Kim JS, Jung HC, Kim KH. Cellular stress-related protein expression in *Helicobacter pylori* infected gastric epithelial AGS cells. *Int J Biochem Cell Biol*. 2004; 36:1624–1634. [PubMed: 15147740]
- Marchitti SA, Brocker C, Stagos D, Vasiliou V. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin Drug Metab Toxicol*. 2008; 4:697–720. [PubMed: 18611112]
- Markoff A, Gerke V. Expression and functions of annexins in the kidney. *Am J Physiol Renal Physiol*. 2005; 289:F949–956. [PubMed: 16210453]
- Mielenz D, Vettermann C, Hampel M, Lang C, Avramidou A, Karas M, Jäck HM. Lipid rafts associate with intracellular B cell receptors and exhibit a Bcell stage-specific protein composition. *J Immunol*. 2005; 174:3508–3517. [PubMed: 15749887]
- Molin M, Pilon M, Blomberg A. Dihydroxyacetone-induced death is accompanied by advanced glycation endproduct formation in selected proteins of *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. *Proteomics*. 2007; 7:3764–3774. [PubMed: 17890650]
- Morgan PE, Sturgess AD, Davies MJ. Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus. *Arthritis Rheum*. 2005; 52:2069–2079. [PubMed: 15986354]
- Morgan PE, Sturgess AD, Hennessy A, Davies MJ. Serum protein oxidation and apolipoprotein CIII levels in people with systemic lupus erythematosus with and without nephritis. *Free Radic Res*. 2007; 41:1301–1312. [PubMed: 17957542]
- Morgan PE, Sturgess AD, Davies MJ. Evidence for chronically elevated serum protein oxidation in systemic lupus erythematosus patients. *Free Radic Res*. 2009; 43:117–127. [PubMed: 19096973]
- Mun JY, Lee TH, Kim JH, Yoo BH, Bahk YY, Koo HS, Han SS. *Caenorhabditis elegans* mitofilin homologs control the morphology of mitochondrial cristae and influence reproduction and physiology. *J Cell Physiol*. 2010; 224:748–756. [PubMed: 20578245]
- Murr R, Vaissière T, Sawan C, Shukla V, Herceg Z. Orchestration of chromatin-based processes: mind the TRRAP. *Oncogene*. 2007; 26:5358–5372. [PubMed: 17694078]
- Neisch AL, Fehon RG. Ezrin, Radixin and Moesin: key regulators of membrane–cortex interactions and signaling. *Curr Opin Cell Biol*. 2011; 23:1–6. [PubMed: 21190823]
- Ni M, Lee AS. ER chaperones in mammalian development and human diseases. *FEBS Lett*. 2007; 581:3641–3651. [PubMed: 17481612]
- Nystrom T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J*. 2005; 24:1311–1317. [PubMed: 15775985]
- Ostalska-Nowicka D, Zachwieja J, Nowicki M, Kaczmarek E, Siwinska A, Witt M. Ezrin – a useful factor in the prognosis of nephrotic syndrome in children: an immunohistochemical approach. *J Clin Pathol*. 2006; 59:916–920. [PubMed: 16522749]

- Pföhler C, Preuss KD, Tilgen W, Stark A, Regitz E, Fadle N, Pfreundschuh M. Mitofilin and titin as target antigens in melanoma-associated retinopathy. *Int J Cancer*. 2007; 120:788–795. [PubMed: 17131336]
- Rabek JP, Boylston WH III, Papaconstantinou J. Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem Biophys Res Commun*. 2003; 305:566–572. [PubMed: 12763031]
- Rossi MN, Carbone M, Mostocotto C, Mancone C, Tripodi M, Maione R, Amati P. Mitochondrial localization of PARP-1 requires interaction with mitofilin and is involved in the maintenance of mitochondrial DNA integrity. *J Biol Chem*. 2009; 284:31616–31624. [PubMed: 19762472]
- Satoh M, Shimada A, Keino H, Kashiwai A, Nagai N, Saga S, Hosokawa M. Functional characterization of 3 thioredoxin homology domains of ERp72. *Cell Stress Chaperones*. 2005; 10:278–284. [PubMed: 16333982]
- Sayre LM, Smith MA, Perry G. Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem*. 2001; 8:721–738. [PubMed: 11375746]
- Silva E, O’Gorman M, Becker S, Auer G, Eklund A, Grunewald J, Wheelock A. In the eye of the beholder: does the master see the SameSpots as the novice? *J Proteome*. 2010; 9:1522–1532.
- Sorolla MA, Rodríguez-Colman MJ, Tamarit J, Ortega Z, Lucas JJ, Ferrer I, Ros J, Cabiscol E. Protein oxidation in Huntington disease affects energy production and vitamin B6 metabolism. *Free Radic Biol Med*. 2010; 49:612–621. [PubMed: 20639122]
- Tabrez S, Ahmad M. Toxicity, biomarkers, genotoxicity, and carcinogenicity of trichloroethylene and its metabolites: a review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2009; 27:178–196. [PubMed: 19657920]
- Takamatsu H, Feng X, Chuhjo T, Lu X, Sugimori C, Okawa K, Yamamoto M, Iseki S, Nakao S. Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia. *Blood*. 2007; 109:2514–2520. [PubMed: 17110458]
- Tang WK, Wong KB, Lam YM, Cha SS, Cheng CH, Fong WP. The crystal structure of seabream antequitin reveals the structural basis of its substrate specificity. *FEBS Lett*. 2008; 582:3090–3096. [PubMed: 18694748]
- Toraason M, Clark J, Dankovic D, Mathias P, Skaggs S, Walker C, Werren D. Oxidative stress and DNA damage in Fischer rats following acute exposure to trichloroethylene or perchloroethylene. *Toxicology*. 1999; 138:43–53. [PubMed: 10566590]
- Tsaytler PA, O’Flaherty MC, Sakharov DV, Krijgsveld J, Egmond MR. Immediate protein targets of photodynamic treatment in carcinoma cells. *J Proteome Res*. 2008; 7:3868–3878. [PubMed: 18652502]
- Tyther R, Ahmeda A, Johns EJ, Sheehan D. Protein carbonylation in kidney medulla of the spontaneously hypertensive rat. *Proteomics Clin Appl*. 2009; 3:338–346.
- Walsh SJ, Rau LM. Autoimmune diseases: a leading cause of death among young and middle-aged women in the United States. *Am J Public Health*. 2000; 90:1463–1466. [PubMed: 10983209]
- Wang G, Cai P, Ansari GA, Khan MF. Oxidative and nitrosative stress in trichloroethene-mediated autoimmune response. *Toxicology*. 2007; 229:186–193. [PubMed: 17123686]
- Wang G, König R, Ansari GA, Khan MF. Lipid peroxidation-derived aldehyde-protein adducts contribute to trichloroethene-mediated autoimmunity via activation of CD4+ T cells. *Free Radic Biol Med*. 2008; 44:1475–1482. [PubMed: 18267128]
- Wang G, Wang J, Ma H, Khan MF. Increased nitration and carbonylation of proteins in MRL+/+ mice exposed to trichloroethene: potential role of protein oxidation in autoimmunity. *Toxicol Appl Pharmacol*. 2009; 237:188–195. [PubMed: 19332086]
- Wang G, Pierangeli SS, Papalardo E, Ansari GAS, Khan MF. Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity. *Arthritis Rheum*. 2010; 62:2064–2072. [PubMed: 20201076]
- Wang G, Wang J, Fan X, Ansari GAS, Khan MF. Protein adducts of malondialdehyde and 4-hydroxynonenal contribute to trichloroethene-mediated autoimmunity via activating Th17 cells: dose- and time-response studies in female MRL+/+ mice. *Toxicology*. 2012; 292:113–122. [PubMed: 22178267]
- Wilkinson B, Gilbert HF. Protein disulfide isomerase. *Biochim Biophys Acta*. 2004; 1699:35–44. [PubMed: 15158710]

- Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev.* 1994; 74:137–161.
- Zhang Q, Ye DQ, Chen GP. Study on the relationship between protein oxidation and disease activity in systemic lupus erythematosus. *Chin J Epidemiol.* 2008; 29:181–184.

Figure 1. Carbonyl content in the kidney protein extracts (fraction #2) of control and TCE-treated mice. The data is expressed as mean $\pm$ SEM (n=6). * $p < 0.05$.

Figure 2.

2D gel profile of kidney proteins (fraction #2) of a control mouse (CBB G250 stained) is shown in Fig. 2A, whereas carbonylated proteins identified by Western blot are shown in Fig. 2B (black circles) which were also matched to the 2D gel protein profile (Fig. 2A). The numbers used for the spots are the same as in Tables 2 and 3.

Figure 3.

2D gel profile of kidney proteins (fraction #2) of a TCE-treated mouse (CBB G250 stained) shown in Fig. 3A. The carbonylated protein, identified by Western blot, is shown in Fig. 3B (black circles), which were also matched to the 2D gel protein profile (Fig. 3A). The numbers used for the spots are the same as in Tables 1–3.

TABLE 1
Summary of carbonylated proteins present only in kidney extracts of TCE-treated mice

Spot no. ^a	Protein name	Accession no. ^b	MW.(Da)	Identified peptide no.	Identified peptide coverage %	MASCOT score ^c	Theo. pI
7	Unnamed protein (glucose regulated protein, putative)	gi 74143892	55392.9	11	22	90	5.53
8	Unnamed protein (aldehyde dehydrogenase 7 family, member A1 isoform)	gi 74213681	55523.5	7	17	74	6.11
9	Transformation/transcription domain- associated protein, Tral homolog	gi 38605208	291369.9	21	10	72	8.76
10	Immt protein	gi 82568928	50124.8	14	36	96	5.72
11	Unnamed protein (villin 2)	gi 26330558	50242.7	12	22	186	5.62
12	Dihydroxyacetone kinase 2	gi 21703976	59653.4	14	32	149	6.44
13	Unnamed protein (3-Methylcrotonyl- CoA carboxylase 2)	gi 74226278	61399.3	15	32	192	8.38
15	Pdia4 protein	gi 45219865	65046.2	11	20	67	5.91
19	Immt protein	gi 82568928	50124.8	8	19	127	5.72
20	Immt protein	gi 82568928	50124.8	19	49	499	5.72
21	Inner membrane protein, mitochondrial, isoform CRA_b	gi 148666539	86241.5	23	33	348	7.02
22	Unnamed protein (villin 2, putative)	gi 74179741	69349.6	22	30	398	5.83
23	Propionyl CoA-carboxylase alpha- subunit	gi 15667251	79610	23	37	509	7.02
24	Inner membrane protein, mitochondrial	gi 70608131	83848.2	22	33	462	6.18
25	Immt protein	gi 82568928	50124.8	21	53	154	5.72
26	Moesin	gi 199765	66440.1	4	6	71	5.94
30	pORF2	gi 16508047	149458.5	16	14	46	9.7
4	Nonseleum glutathione peroxidase	gi 149241041	12496.5	3	48	34	8.62
5	Laminin receptor	gi 293694	32698.4	5	21	54	4.74
6	Interferon activated gene 204	gi 118130979	69394.3	7	19	41	8.91

^a Spot numbers are the same as shown in Fig. 3(A, B).

^b The accession numbers in NCBI database.

^c A MASCOT score >64 was considered significant ($p < 0.05$) for protein identified from the database.

TABLE 2
Summary of carbonylated proteins present in the kidney extracts of both control and TCE-treated mice

Spot no. ^a	Protein name	Accession no. ^b	MW. (Da)	Identified Peptide no.	Identified peptide coverage %	MASCOT score ^c	Theo. pI
1	Tropomyosin 3, gamma	gi 40254525	33128.8	14	32	178	4.73
2	Unnamed protein (Annexin A5, putative)	gi 74151976	35760.2	15	48	175	4.83
3	Tropomyosin 1 alpha	gi 256000796	28326.5	10	34	62	4.74
14	Unnamed protein (HSP 70 kD protein 5, putative)	gi 74143673	56857.7	14	33	115	5.43
16	Chaperone Complex, Chain A	gi 215261181	42316.8	10	30	85	6.69
17	Stress-70 protein, mitochondrial (HSP70 protein 9, GRP-75)	gi 14917005	73482.7	18	35	658	5.91
18	Albumin precursor	gi 163310765	68647.7	16	30	106	5.75
27	Unnamed protein (villin 2, putative)	gi 26330558	50242.7	5	9	62	5.62
28	Aconitase 2, mitochondrial precursor	gi 18079339	85410	23	32	175	8.08
31	Nonelenium glutathione peroxidase	gi 149241041	12496.5	5	52	65	8.62
32	Nonelenium glutathione peroxidase	gi 149241041	12496.5	5	52	58	8.62
33	Nonelenium glutathione peroxidase	gi 149241041	12496.5	5	52	59	8.62

^aThe numbers are same as shown in Fig. 2 (A, B) and Fig. 3 (A, B).

^bThe accession numbers in NCBI database.

^c A MASCOT score >64 was considered significant ($p < 0.05$) for protein identified from the database.

TABLE 3

Summary of carbonylated protein expression variation in 2D gel profiles of TCE-treated vs. control mice.

Spot no. ^a	Protein name	Significance expression variation (p value ^d)	Expression variation of fold change ^c
1	Tropomyosin 3, gamma	0.963	1.0
2	Unnamed protein (Annexin A5, putative)	0.031	2.2
3	Tropomyosin 1 alpha	0.913	1.0
14	Unnamed protein (HSP 70 kD protein 5, putative)	0.348	1.3
16	Chaperone Complex, Chain A	0.232	1.2
17	Stress-70 protein, (HSP70 protein 9, GRP75)	0.319	1.3
18	Albumin precursor	0.155	1.4
27	Unnamed protein (villin 2, putative)	0.923	1.0
28	Aconitase 2, mitochondrial precursor	0.855	1.0
31	Nonselenium glutathione peroxidase	0.275	1.4
32	Nonselenium glutathione peroxidase	0.505	1.3
33	Nonselenium glutathione peroxidase	0.823	1.0

Spot no. ^b	Protein name	Significance expression variation (p value ^d)	Expression variation of fold change ^c
7	Unnamed protein (glucose regulated protein, putative)	0.407	1.1
8	Unnamed protein (aldehyde dehydrogenase 7 family, member A1 isoform)	0.215	1.3
9	Transformation/transcription domain-associated protein, Tra1 homolog	0.481	1.1
10	Immt protein	0.832	1.0
11	Unnamed protein (villin 2)	0.015	1.3
12	Dihydroxyacetone kinase 2	0.387	1.1
13	Unnamed protein (3- Methylcrotonyl-CoA carboxylase 2)	0.756	1.1
15	Pdia4 protein	0.798	1.1
19	Immt protein	0.236	1.3
20	Immt protein	0.921	1.1
21	Inner membrane protein (Immt), mitochondrial	0.008	1.3
22	Unnamed protein (villin 2, putative)	0.789	1.0
23	Propionyl CoA-carboxylase alpha-subunit	0.631	1.1
24	Inner membrane protein, mitochondrial	0.150	1.3
25	Immt protein	0.441	1.1
26	Moesin	0.151	1.2
30	pORF2	0.863	1.1

^a Carbonylated proteins found in both control and TCE-treated mice.^b Carbonylated proteins found only in TCE-treated mice^c Protein expression fold changes between the two groups were analyzed on CBB G250 stained protein spots in 2D gels.

^dThe protein expression variation between two groups were analyzed by student t-test, and $p < 0.05$ was considered significant. The protein spot nos. 11 and 21 show significant increases, whereas spot no. 2 shows significant decrease.