Identification of oxidized mitochondrial proteins in alcohol-exposed human hepatoma cells and mouse liver

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Heavy alcohol consumption can damage various cells and organs partly through production of reactive oxygen species (ROS) and mitochondrial dysfunction. Treatment with antioxidants can significantly reduce the degree of damage. Despite well established roles of ROS in alcohol-induced cell injury, the proteins that are selectively oxidized by ROS are poorly characterized. We hypothesized that certain cysteinyl residues of target proteins are oxidized by ROS upon alcohol exposure, and these modified proteins may play roles in mitochondrial dysfunction. A targeted proteomics approach utilizing biotin-*N*-maleimide (biotin-NM) as a specific probe to label oxidized cysteinyl residues was employed to investigate which mitochondrial proteins are modified during and after alcohol exposure. Human hepatoma HepG2 cells with transduced CYP2E1 (E47 cells) were used as a model to generate ROS through CYP2E1-mediated ethanol metabolism. Following exposure to 100 mM ethanol for 4 and 8 h, the biotin-NM-labeled oxidized proteins were purified with agarose coupled to either streptavidin or monoclonal antibody against biotin. The purified proteins were resolved by two-dimensional gel electrophoresis and protein spots that displayed differential abundances were excised from the gel, in-gel digested with trypsin and analyzed for identity utilizing either matrix-assisted laser desorption-time of flight mass spectrometry or microcapillary reversed-phase liquid chromatography-tandem mass spectrometry. The results demonstrate that heat shock protein 60, protein disulfide isomerase, mitochondrial aldehyde dehydrogenases, prohibitin, and other proteins were oxidized after alcohol exposure. The identity of some of the proteins purified with streptavidin-agarose was also confirmed by immunoblot analyses using the specific antibody to each target protein. This method was also used to identify oxidized mitochondrial proteins in the alcohol-fed mouse liver. These results suggest that exposure to ethanol causes oxidation of various mitochondrial proteins that may negatively affect their function and contribute to alcohol-induced mitochondrial dysfunction and cellular injury.

Keywords: Alcohol / CYP2E1 / Cysteine oxidation / Reactive oxygen species

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Abbreviations: ALDH, aldehyde dehydrogenase; **BIAM**, biotin-iodoacetamide; **biotin-NM**, biotin-*N*-maleimide; **GRP**, glucose-regulated protein; **HRP**, horse radish peroxidase; **HSP**, heat shock protein; **NEM**, *N*-ethylmaleimide

1 Introduction

Long-term alcohol (ethanol) abuse can damage various cells and organs including the liver, brain, heart, pancreas, and testis [1–4]. Alcohol-mediated damage can be attributed to many underlying causes consisting of environmental factors and host genetic makeup. Reactive oxygen species (ROS) and reactive nitrogen species play an important role in alcohol-mediated tissue injury. Some alcohol-mediated tissue injuries can be partially reversed

especially at the early stages of disease by termination of alcohol usage. In addition, the severity of these pathological states can be attenuated or prevented by treatment with various antioxidants. A variety of mechanisms by which ethanol may induce oxidative stress in the liver and other tissues has been reviewed [3–5]. The role of CYP2E1, the major isoform of the ethanol-inducible cytochrome P450, in ROS production has been actively studied by numerous investigators, due to its induction in many types of cells and tissues by its substrates such as alcohol or acetone and under pathophysiological conditions [6–9], its ability to function as an active NADPH oxidase [10] even in the absence of its substrates [11], and its role in the metabolism of many small molecular toxic compounds and long chain fatty acids [12, 13]. In fact, a positive corelationship between elevated levels of CYP2E1 and tissue injury was demonstrated in a cell culture model [14] as well as animal models [15, 16].

Through ROS production, the CYP2E1-mediated reaction can produce a potent cytotoxic ·OH radical, which can damage DNA [17, 18] and proteins [19]. In fact, ethanol-mediated apoptotic and necrotic cell damage can be prevented by antibodies to CYP2E1, chemical inhibitors of CYP2E1, ·OH scavengers such as DMSO, and transfection of the respective cDNA for superoxide dismutase [14, 20–23]. Conversely, simultaneous exposure to two toxic substrates of CYP2E1, such as ethanol and arachidonic acid or pyrazole, exacerbates the damaging conditions often in the absence of glutathione, as demonstrated in animal models [15, 22] and E9 or E47 HepG2 hepatoma cells with stable transfection of human CYP2E1 [14, 20, 21]. The cells with transduced CYP2E1 were more susceptible to toxic cell damage caused by exposure to ethanol [14, 20], polyunsaturated fatty acids [21], and acetaminophen [24] than their counterparts such as HepG2 cells without transduced CYP2E1 or parental cells. Furthermore, hepatoma cells with transduced CYP3A, another P450 isozyme used as a negative control, were not damaged when exposed to ethanol and other CYP2E1 substrates. These results strongly support the important role of CYP2E1 in ROS production that often leads to apoptotic and necrotic cell or tissue damage.

Mitochondria are the vital organelle responsible for providing cellular energy through the tricarboxylic acid cycle linked with oxidative phosphorylation, where the end result is generation of a chemical potential across the membrane that drives ATP synthesis. Reports over the past decade have revealed that mitochondria are directly involved in apoptosis processes through translocation of various proteins associated with the apoptotic process. The proteins that are released from mitochondria include cytochrome c, smac/diablo, and apoptosis inducing factor, which amplifies the execution of apoptosis [25, 26]. In contrast, proapoptotic proteins such as Bax and Bad are translocated to the mitochondria from the cytoplasm to initiate the actual cell death process through formation of an open channel for cytochrome c release to the cytoplasm to produce an amplification of the cell death pathway. It has been estimated that under normal states, up to 2% of oxygen is leaked from the mitochondrial respiratory chain [27] while more ROS are released after alcohol exposure partly due to inhibition of complex I and III [4, 5] and possibly through ROS production catalyzed by CYP2E1 located in the endoplasmic reticulum [3, 14, 20–23] and mitochondria [28]. Despite the well established effect of alcohol on ROS production and mitochondrial dysfunction [4, 5], which mitochondrial proteins are oxidatively modified and whether their biological functions are altered is poorly understood. Therefore, the aim of this work was to identify mitochondrial proteins whose cysteinyl residues are susceptible to oxidation in E47 HepG2 hepatoma cells upon exposure to ethanol.

2 Materials and methods

2.1 Chemicals and other materials

N-ethyl-maleimide (NEM), biotin-conjugated *N*-maleimide (biotin-NM), DTT, and agarose-conjugated mAb against biotin (agarose-mAb-biotin) were obtained from Sigma Chemical (St. Louis, MO, USA) in highest purity. Biotin-iodoacetamide (BIAM), 2',7'-dichlorofluoroscein diacetate, horse radish peroxidase (HRP)-conjugated streptavidin, and streptavidin-agarose were purchased from Molecular Probe (Eugene, OR, USA). Protease inhibitor cocktail and polyclonal antibody to prohibitin were purchased from Calbiochem (San Diego, CA, USA). Sephadex G25 spin columns were obtained from Amersham Biosciences (Uppsala, Sweden). MS compatible Silver-stain was from Bio-Rad (Silver Stain Plus; Hercules, CA, USA). Porcine sequencing grade modified trypsin was from Promega (Madison, WI, USA). All tissue culture media including fetal bovine serum, antibiotics, and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Specific antibodies to the proteins analyzed were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) or Sigma Chemicals, as indicated. Polyclonal antibody to mitochondrial aldehyde dehydrogenase 2 (ALDH2) was prepared in rabbits as previously described [23]. Rabbit polyclonal antibody against mitofilin was kindly provided by Dr. Paul R. Odgren (University of Massachusetts Medical School, North Worcester, MA, USA) [29].

2.2 Cell lines and cell culture

E47 HepG2 human hepatoma cells with transduced human CYP2E1 and control C34 HepG2 cells transfected with vector alone [20] were kindly provided by Dr. Arthur I. Cederbaum (Mount Sinai Medical School, New York, NY, USA) and maintained in minimal essential medium with Earl's salts (MEM), 10% v/v fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL G418 in a humidified incubator under 5% CO_2/95% air at 37°C. E47 and C34 HepG2 cells, grown in MEM with 2% fetal bovine serum on 150 mm plastic culture dishes, similar to the conditions described previously [20], were incubated with 100 mM ethanol (diluted in normal growth media) for different periods of time. To prevent ethanol evaporation during exposure, each culture dish was tightly wrapped with Parafilm. After exposure to ethanol for the indicated times, the culture medium was aspirated and attached cells were washed twice with cold 1 × PBS containing 50 mM NEM and then incubated with the same buffer for an additional 10 min before being scraped with a plastic scraper, and harvested by centrifugation at 2500 × *g* for 5 min. The harvested cells were homogenized at 4°C with mitochondria extraction buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 250 mM sucrose with protease inhibitors) to obtain mitochondrial pellets by differential centrifugation [30], and washed twice with PBS before being rapidly frozen in dry ice and stored at −80°C until use.

2.3 Labeling of oxidized cysteine residues of mitochondrial proteins with biotin-NM

All buffers used in this study were freshly pre-equilibrated with helium or nitrogen gas for at least 30 min to remove dissolved oxygen. To prepare solubilized mitochondrial proteins, frozen mitochondrial pellets of E47 and C34 HepG2 cells were quickly thawed and incubated in mitochondria extraction buffer with 1% CHAPS for 5 min. The concentration of the solubilized mitochondrial proteins was determined using the Bio-Rad protein assay kit, as described previously [31, 32]. Labeling of oxidized protein thiols with biotin-NM was performed by the method of Lind *et al.* [33] with the following modifications. After the second PBS wash, the cells were resuspended in HEPES buffer containing NEM (40 mM HEPES, pH 7.4, 50 mM NEM, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors) and incubated at room temperature for 20 min to block reduced thiols. CHAPS was added to a final concentration of 1% w/v and the mixture was incubated for another 15 min. To remove excess NEM, the protein mixture was passed through a Sephadex G25 spin column that had been equilibrated with elution buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, pH 7.4 and 1% CHAPS). DTT was added to the protein eluates at a final concentration of 5 mM and the solution was incubated for 20 min at room temperature to reduce the oxidized cysteinyl residues. A stock solution of 2 M biotin-NM in DMSO was added to the protein eluates at a final concentration of 6 mM. After determining the protein concentration of the eluates from the first Sephadex G25 spin columns, equal amounts of biotin-NM labeled protein extracts were passed through a second Sephadex G25 spin column pre-equilibrated with elution buffer to remove excess biotin-NM and salts. The biotin-NM labeled proteins were affinity purified with streptavidin-agarose beads. Alternatively, biotin-NM labeled proteins were also purified with agarose-mAb-biotin. The biotinylated samples were washed twice with cell lysis buffer with 1% CHAPS to remove any nonspecifically bound proteins. The biotin-NM labeled proteins bound to the agarose beads were dissolved in 2-D PAGE buffer (8 M urea, 20 mM DTT, 2% CHAPS, 0.5% IPG buffer, pH 3–10) for 30 min before IEF on dry IPG strips (nonlinear pH gradient of 3–10; Amersham Biosciences) for 24 h at 50 000 Vh, as recommended by the manufacturer.

2.4 Electrophoresis and immunoblot analyses

Part of the purified or unpurified biotin-NM labeled mitochondrial proteins were dissolved in Laemmli's buffer for immunoblot analysis using the specific antibody against each target protein as indicated in the text. The antigen detected by the primary antibody was visualized with the appropriate secondary antibody conjugated with HRP for ECL detection, as described [31, 32].

2.5 Analysis of 2D-PAGE protein bands by MS

2.5.1 2-D PAGE

Purified proteins were analyzed by 2-D PAGE, which typically consisted of a broad range IEF step (pH gradient of 3–10) followed by electrophoresis in a 10% gel. Gels were stained with Silver Stain Plus according to the manufacturer's protocol. Each gel was scanned with a Molecular Dynamics Personal Densitomer SI and analyzed with ImageQuant software (Amersham Biosciences). Protein spots of interest were excised from the gels with a razor blade and quickly frozen in dry ice until further analysis.

2.5.2 Protein digestion

Silver-stained protein gel spots were digested as described by Wilm *et al.* [34]. Samples were desalted with C18 Zip Tips (Millipore, Bedford, MA, USA) according to the manufacturer's protocols and stored at −20°C until MS analysis.

2.5.3 MALDI-TOF MS analysis

One microliter of sample was cocrystallized with 1 μL of a 20% solution of CHCA in 50% ACN, 1% TFA, and spotted directly onto a stainless steel MALDI target plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) using a laser frequency of 200 Hz. MALDI spectra were internally calibrated ($<$ 20 ppm) using known trypsin autolysis peptides. Post-acquisition baseline correction and smoothing were carried out using software provided with the TOF/TOF instrument. The peptides were identified *via* PMF using GPS Explorer software (Applied Biosystems).

2.5.4 Microcapillary RPLC-MS/MS analysis

Microcapillary (μ) RPLC was performed using an Agilent 1100 capillary LC system (Agilent Technologies, Palo Alto, CA, USA) coupled online to an IT mass spectrometer (LCQ DecaXP; Thermo Electron, San Jose, CA, USA) with the nanoelectrospray interface supplied by the manufacturer. μRPLC separations of each sample were performed using 75 μm i.d. $\times$ 360 o.d. $\times$ 10 cm long fused silica microcapillary columns (Polymicro Technologies, Phoenix, AZ, USA) that were slurry packed in house with 3 μm, 300 Å pore size C-18 silica-bonded stationary phase (Vydac, Hysperia, CA, USA). After injecting 7 μL of sample, the μRPLC column was washed for 20 min with 98% solvent A (0.1% formic acid in water, v/v) and peptides were eluted using a linear gradient of 2% solvent B (0.1% formic acid in 100% ACN, v/v) to 42% solvent B in 40 min, then to 98% solvent B in an additional 10 min, all at a constant flow rate of 500 nL/min. The IT-MS was operated in data-dependent mode in which each full MS scan was followed by three MS/MS scans where the three most abundant peptide molecular ions were dynamically selected CID using a normalized collision energy of 38%. Dynamic exclusion was utilized to prevent redundant acquisition of peptides previously selected for MS/MS. The temperature of the heated capillary and electrospray voltage were 160°C and 1.7 kV, respectively.

2.5.5 Bioinformatic analysis

Tandem MS spectra from the μRPLC-MS/MS analyses were searched against the Swiss Protein human proteomic database from the Expert Protein Analysis System (http://www.expasy.org) with SEQUEST operating on an 18 node Beowulf cluster. For a peptide to be considered legitimately identified, it had to achieve stringent charge state and proteolytic cleavage-dependent cross correlation (X_{corr}) and a minimum correlation (ΔC_n) score of 0.08, similar to those previously reported [35–37].

3 Results

3.1 Alcohol-dependent oxidation of mitochondrial proteins

It is well established that alcohol produces ROS through inhibition of the mitochondrial electron transport system [4, 5] and *via* CYP2E1-related alcohol metabolism [3, 14, 17, 20]. Marked elevation of dichlorofluorescein fluorescence, a well-established indicator of ROS [38], was observed under fluorescence microscopy after E47 cells were exposed to 50 and 100 mM ethanol for 4 and 8 h as compared to an untreated control (data not shown), confirming the previous results of ethanol-mediated ROS production [14, 20, 39]. Based on ROS production through CYP2E1-mediated ethanol metabolism in E47 cells, mitochondrial proteins from untreated and 8 h ethanol exposed cells were labeled with biotin-NM as described in Section 2.3. To determine whether mitochondrial proteins are oxidized after alcohol exposure, immunoblot analysis was performed to detect the biotin-NM labeled proteins using streptavidin-HRP or mAb-biotin-HRP as a probe. Figure 1A shows a typical pattern of Coomassie blue stained mitochondrial proteins in 1-D SDS polyacrylamide gels, verifying that similar amounts of protein were loaded for each lane. Only a small number of proteins in the untreated control sample were recognized with streptavidin-HRP (Fig. 1B, lane 1). However, the intensity and the number of oxidized protein thiols

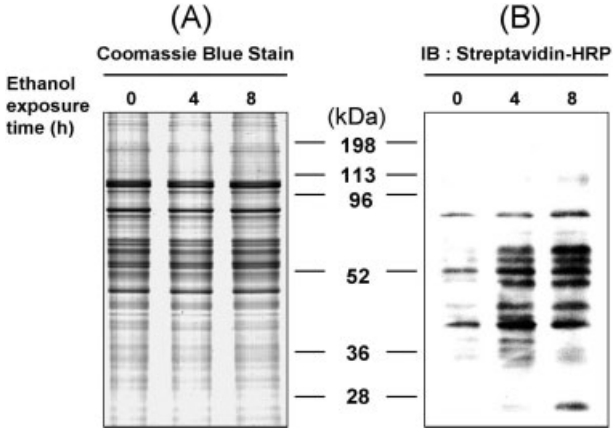

Figure 1. Increased oxidation of mitochondrial proteins in E47 HepG2 hepatoma cells after alcohol exposure. (A) Equal amounts (20 μg/lane) of biotin-NM labeled mitochondrial proteins isolated from untreated and ethanol exposed E47 HepG2 cells were separated on 10% SDS-PAGE gels and stained with Coomassie blue. (B) Another set of the same protein samples was electroblotted to a PVDF-immobilon membrane and subjected to immunoblot analysis using streptavidin-HRP. Detection of the antigens was performed using the ECL method. This figure represents a typical result of at least three different experiments.

detected by streptavidin-HRP (Fig. 1B, lanes 2 and 3) were greatly increased in a time-dependent manner after exposure to 100 mM ethanol, which was used in previous reports [14, 20] and which is within the concentration range observed in human alcoholics. A corresponding increase in the level of biotin-NM labeled proteins was also observed with mAb-biotin-HRP (data not shown). Increased oxidation of mitochondrial proteins was observed after 8 h of exposure to 50 and 100 mM ethanol, respectively, in a concentration-dependent manner (Fig. 2). Several mitochondrial proteins from control C34 cells (without transduced CYP2E1) were also oxidized after alcohol exposure, although the band intensities were approximately 40 to 50% less than those of the corresponding E47 cells exposed to the same ethanol concentration (Fig. 2). Due to stronger signal intensities observed in the samples exposed to 100 mM ethanol than those treated with 25 or 50 mM ethanol, we used 100 mM ethanol in the subsequent experiments. These data suggest that ethanol alone could produce considerable amounts of ROS in C34 HepG2 cells without transduced CYP2E1 possibly through inhibition of mitochondrial complex I and III [4, 5], although additional ROS were produced through CYP2E1-mediated ethanol metabolism as in E47 HepG2 cells. Furthermore, these results indicate that ethanol exposure caused oxidation of mitochondrial proteins, some of which were labeled with biotin-NM.

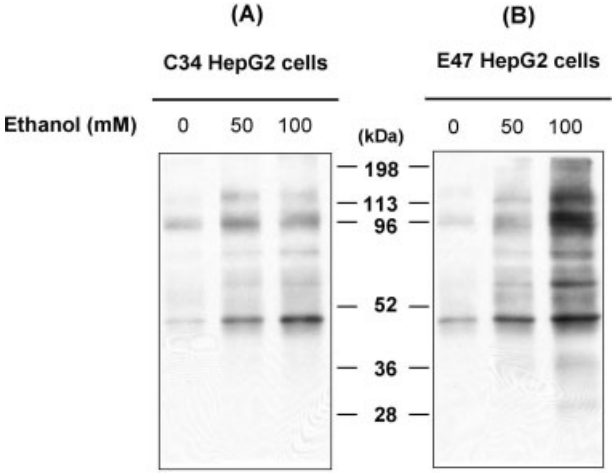

Figure 2. Ethanol concentration dependent increases in oxidized mitochondrial proteins in C34 and E47 HepG2 hepatoma cells. C34 (A) and E47 HepG2 (B) cells were exposed to 50 or 100 mM ethanol for 8 h before cell harvest. Equal amounts (20 μg/lane) of biotin-NM labeled mitochondrial proteins isolated from untreated and ethanol exposed C34 and E47 HepG2 cells were separated on 10% SDS-PAGE gels, electroblotted to a PVDF-immobilon membrane and subjected to immunoblot analysis using streptavidin-HRP. This figure represents a typical result of two different experiments.

3.2 Similarity of 2-D gel patterns of biotin-NM labeled proteins purified with streptavidin-agarose and mAb to biotin-agarose

Because of the increased levels of biotin-NM labeled proteins after alcohol exposure (Fig. 1), the 2-D gel patterns of biotin-NM labeled proteins after affinity purification with streptavidin-agarose beads or immunoprecipitation with mAb-biotin-agarose beads were investigated. We hypothesized that biotin-NM labeled oxidized proteins should exhibit similar patterns of protein spots on Silver-stained 2-D gels regardless of the purification method utilized. Our results showed similar patterns of protein spots on 2-D gels of biotin-NM labeled proteins after purification with streptavidin-agarose (Fig. 3A) or immunoprecipitation with mAb-biotin-agarose (Fig. 3B), except for the spots of the heavy (52 kDa) and light (26 kDa) chains of the mAb to biotin (Fig. 3B). In addition, similar Silver-stained 2-D gel patterns were observed after mitochondrial proteins were labeled with BIAM followed by purification with streptavidin-agarose, although intensities of some protein spots in the 8 h treated samples were significantly lower than those of untreated samples (data not shown) as indicated [40]. These results establish that alcohol exposure results in the oxidization of certain mitochondrial proteins and those biotin-NM-labeled, oxidized mitochondrial proteins were successfully recognized by either streptavidin or mAb to biotin. Furthermore, these data demonstrate the utility of the present approach to detect oxidized cysteinyl residues in proteins.

3.3 Increased oxidative modification of mitochondrial proteins after alcohol exposure

Because of the oxidative modification of several mitochondrial proteins after alcohol exposure, the pattern of protein separation on 2-D gels was studied and the identity of those proteins whose cysteinyl residues were oxidized and labeled with biotin-NM was determined. Figure 4 represents typical patterns of Silver-stained 2-D gels for untreated and 8 h ethanol exposed samples that were purified using streptavidin-agarose beads. Only a few proteins labeled with biotin-NM were detected in the untreated sample (Fig. 4A). This result is most likely due to ROS generated from CYP2E1 contained in E47 HepG2 cells even in the absence of its substrates, as reported [11]. However, the number of labeled proteins and their spot intensities were significantly increased after exposure to alcohol for 8 h (Fig. 4B).

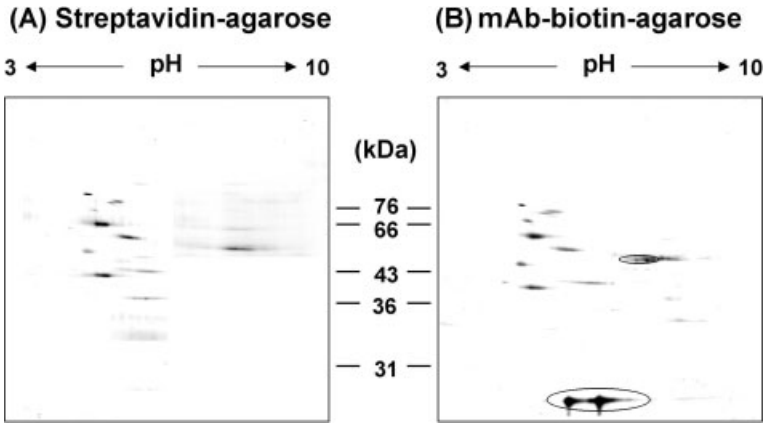

Figure 3. Similar 2-D gel patterns of biotin-NM labeled proteins purified with streptavidin-agarose and mAb-biotin-agarose. Biotin-NM labeled mitochondrial proteins from 8 h ethanol exposed cells were purified with streptavidin-agarose (A) or mAb-biotin-agarose (B), analyzed by 2-D PAGE, and then visualized with Silver-stain. Potential spots for the heavy and light chains of immunoglobulin of mAb to biotin are marked with circles (B). This figure represents a typical result from three different experiments.

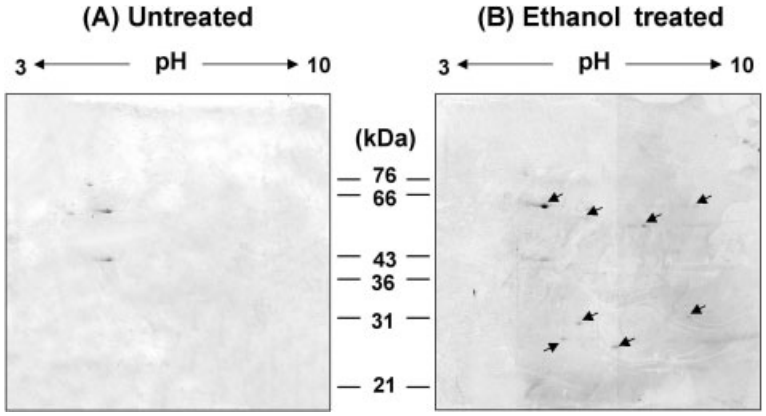

Figure 4. Increased oxidative modification of mitochondrial proteins after alcohol exposure. Biotin-NM labeled mitochondrial protein samples from untreated (A) or 8 h ethanol exposed cells (B) were purified with streptavidin-agarose beads, analyzed by 2-D PAGE, and then visualized with Silver-stain. Protein spots with statistically significantly ($p < 0.05$) increased intensities after ethanol exposure are indicated with arrows. This figure represents a typical result from at least four different experiments.

3.4 Summary of protein sequencing analyses

To identify each protein spot, the 2-D gel analysis was scaled up to increase the amount of streptavidin-agarose purified proteins. Many mitochondrial proteins existed as aggregated spots in the pH 8–10 range (data not shown). In contrast, other proteins were present as isolated spots in the pH 4–8 range and thus excised from the 2-D gel (Fig. 5), in-gel digested with trypsin, and analyzed by either MALDI-TOF/TOF or μRPLC-MS/MS. The results of this analysis are presented in Table 1, which summarizes the identity and property of a few of the mitochondrial protein spots identified. This analysis established that, among others, glucose-regulated protein 78 (GRP78), heat shock protein 60 (Hsp60), protein disulfide isomerase, mitochondrial ALDH2 and ALDH5, and prohibitin were oxidized after ethanol exposure. In addition, peptide sequences of other mitochondrial preparations, that were not shown in Fig. 5 or summarized in Table 1, were obtained after protein labeling with BIAM followed by purification with streptavidin-agarose (data not shown). These proteins, some of which were detected in small quantities, include mitofilin, GRP75, Huntington interact-

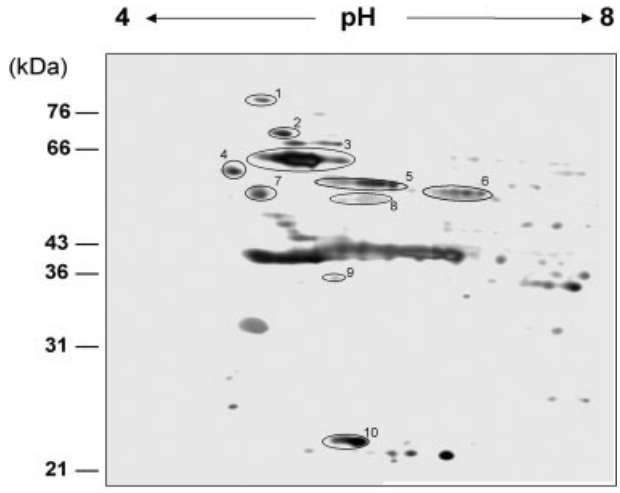

Figure 5. Separation of biotin-NM labeled proteins on a 2-D gel for MS analysis. Biotin-NM labeled mitochondrial proteins were purified with streptavidin-agarose beads, analyzed on a 2-D gel, and Silver-stained. Clearly separated protein spots from this particular gel (pH range 4–8) were excised and subjected to MS analysis following in-gel tryptic digestion. A number (spots 1–10) is shown near each mitochondrial protein spot.

Table 1. Summary of LC-MS/MS peptide sequence analyses for protein identification

Spot no.	Protein	Peptide[a)]	Mass (kDa)[b)]	Swiss-Prot Accession[c)]	Xcorr[d)]	Charge State[e)]	Delta Cn[f)]
1	GRP 78		79	P11021			
		R.VTAEDK.G			2.03	1	0.12
		R.ALSSQHQAR.I			2.26	2	0.29
		K.ITITNDQNR.L			2.57	2	0.32
		K.VYEGERPLTK.D			2.80	2	0.54
		R.VEIIANDQGNR.I			3.55	2	0.41
		K.NQLTSNPENTVFDAK.R			3.73	2	0.60
		K.SQIFSTASDNQPTVTIK.V			5.06	2	0.52
		R.ITPSYVAFTPEGER.L			3.53	2	0.50
		K.VTHAVVTVPAYFNDAQR.Q			2.93	2	0.48
		R.NELESYAYSLK.N			3.25	2	0.22
		K.ELEEIVQPIISK.L			3.86	2	0.40
		R.IINEPTAAAIAYGLDK.R			4.63	2	0.58
2	GRP 78		72	P11021			
		K.NQLTSNPENTVFDAK.R			4.03	2	0.59
		K.SQIFSTASDNQPTVTIK.V			4.58	2	0.51
		R.IINEPTAAAIAYGLDK.R			2.78	2	0.21
3	Hsp60		61	P10809			
		K.VGGTSDVEVNEK.K			3.26	2	0.42
		R.VTDALNATR.A			3.60	2	0.41
		K.VGEVIVTK.D			2.21	1	0.33
		K.NAGVEGSLIVEK.I			3.48	2	0.54
		K.LVQDVANNTNEEAGDGTTTATVLAR.S			4.61	3	0.49
		R.TVIIEQSWGSPK.V			2.36	1	0.39
		R.GYISPYFINTSK.G			3.50	2	0.49
		R.IQEIIEQLDVTTSEYEKEK.L			4.76	2	0.52
		K.ISSIQSIVPALEIANAHR.K			3.85	2	0.54
		K.TLNDELEIIEGMK.F			4.59	2	0.55
		R.KPLVIIAEDVDGEALSTLVLNR.L			3.03	2	0.59
		R.TALLDAAGVASLLTTAEVVVTEIPKEEK.D			5.62	3	0.53
		R.TALLDAAGVASLLTTAEVVVTEIPK.E			4.20	3	0.48
4	Protein disulfide isomerase		58	P07237			
		K.DHENIVIAK.M			2.37	2	0.23
		K.YKPESEELTAER.I			3.87	3	0.34
		K.LGETYKDHENIVIAK.M			4.38	3	0.57
		K.VDATEESDLAQQYGVR.G			5.70	2	0.65
		K.YQLDKDGVVLFK.K			3.98	2	0.49
		K.THILLFLPK.S			2.85	2	0.46
		K.HNQLPLVIEFTEQTAPK.I			4.84	2	0.52
		K.ENLLDFIK.H			2.52	2	0.25
		R.TGPAATTLPDGAAAESLVESSEVAVIGFFK.D			3.92	2	0.65
5	Protein disulfide isomerase A3		57	P30101			
		K.TVAYTEQK.M			2.11	1	0.39
		R.DGEEAGAYDGPR.T			4.16	2	0.56
		R.EATNPPVIQEEKPK.K			3.17	2	0.55
		R.LAPEYEAAATR.L			3.48	2	0.37
		K.QAGPASVPLR.T			2.62	2	0.45

Table 1. Continued

Spot no.	Protein	Peptide[a)]	Mass (kDa)[b)]	Swiss-Prot Accession[c)]	Xcorr[d)]	Charge State[e)]	Delta Cn[f)]
		K.DPNIVIAK.M			1.93	1	0.29
		R.TADGIVSHLK.K			2.51	2	0.40
		K.LNFAVASR.K			2.12	1	0.23
		K.YGVSGYPTLK.I			3.13	2	0.59
		K.MDATANDVPSPYEVR.G			4.46	2	0.49
		K.FVMQEEFSR.D			3.31	2	0.49
		R.FLQDYFDGNLKR.Y			3.61	2	0.41
		R.GFPTIYFSPANK.K			3.10	2	0.53
		R.FLQDYFDGNLK.R			3.74	2	0.42
		K.DLLIAYYDVDYEK.N			4.71	2	0.52
		R.ELSDFISYLQR.E			3.80	2	0.51
		K.TFSHELSDFGLESTAGEIPVVAIR.T			3.93	3	0.51
6	Aldehyde dehydrogenase 5		55	P30837			
		K.KIEEVVER.A			2.66	2	0.17
		R.VLGYIQLGQK.E			3.73	2	0.50
		K.VGNPFELDTQQGPQVDK.E			2.74	2	0.50
		R.YGLAAAVFTR.D			4.26	2	0.54
		R.IAKEEIFGPVQPLFK.F			3.74	3	0.43
		K.VAEQTPLSALYLASLIK.E			5.25	2	0.59
7	Aldehyde dehydrogenase 2		54	P05091			
		K.VAEQTPLTALYVANLIK.E			3.70	2	0.52
8	Aldehyde dehydrogenase 5		52	P30837			
		K.VAEQTPLSALYLASLIK.E			3.98	2	0.47
9	Aldehyde dehydrogenase 5		36	P30837			
		K.EEIFGPVMQILK.F			2.42	2	0.45
10	Prohibitin		25	P35232			
		K.AAIISAEGDSK.A			2.85	2	0.53
		R.FDAGELITQR.E			4.26	2	0.47
		R.ILFRPVASQLPR.I			3.05	2	0.29
		R.KLEAAEDIAYQLSR.S			4.18	3	0.27
		R.NITYLPAGQSVLLQLPQ.-			4.08	2	0.45
		R.AATFGLILDDVSLTHLTFGK.E			4.19	2	0.59
		K.FGLALAVAGGVVNSALYNVDAGHR.A			3.95	2	0.63

a) Sequences of the peptides identified by MS/MS are bracketed by periods, outside of which are the residues immediately *N*- and *C*-terminal to the identified peptide. Because of the very low abundance of certain protein spots in the 2-D gel and contamination with other proteins, we might have missed certain protein spots for other ALDH isozymes. Despite the small number of peptides identified, we are confident that those peptides that were identified by MS analysis represent the peptide sequences of mitochondrial ALDH isozymes listed. The presence of ALDH2 in the streptavidin-agarose purified proteins was also confirmed by immunoblot analysis using a specific antibody to ALDH2 (Fig. 6B). However, we could not accurately estimate the magnitude of ethanol-mediated changes in spot intensity, due to very low spot intensities of some proteins in the untreated control sample

b) The apparent mass listed in the Table represents that observed on 2-D gels

c) Swiss-Prot protein accession references

d) SEQUEST cross correlation score

e) Charge state of the peptide molecular ion chosen for MS/MS

f) SEQUEST delta correlation score

ing protein 1, and porin. Our results also indicate that certain cytosolic or nuclear proteins, present as contaminants in this particular mitochondrial preparation, were also oxidized by ethanol mediated ROS.

3.5 Confirmation of oxidized proteins by immunoblot analysis for streptavidin-agarose purified proteins

To confirm the identity of each protein summarized in Table 1, immunoblot analyses were performed using a specific antibody against some of the biotin-NM labeled oxidized proteins after they were purified with streptavidin-agarose beads. Immunoblot analyses revealed that Hsp60 (Fig. 6A), ALDH2 (Fig. 6B), and prohibitin (Fig. 6C) were detected and that ethanol exposure did not appear to change their levels, although each of these proteins might contain an increased number of biotin-NM labeled thiols after alcohol exposure. The presence of other mitochondrial proteins such as mitofilin (Fig. 6D), Hsp70 (Fig. 6E), $NADP^+$-dependent isocitrate dehydrogenase (Fig. 6F) and porin (not shown) were also detected by immunoblots using the specific antibody to each protein. These immunoblot data with streptavidin-agarose purified mitochondrial proteins clearly support that mitochondrial proteins, even those detected in minor quantities by the MS identifications, were oxidized by alcohol exposure.

3.6 Increased oxidation of mitochondrial proteins in mouse livers after chronic alcohol exposure

The results shown above (Figs. 1–6) demonstrate the utility of our method to identify oxidatively modified mitochondrial proteins from cultured hepatoma cells that were acutely exposed to various ethanol concentrations up to 100 mM. To determine whether this method can be applied to detect oxidized proteins from animals that were chronically treated with a low dose of alcohol in a pair-feeding regimen [3, 23], fresh livers prepared from pair-fed control and alcohol-fed mice (n = 4 *per* group) were subjected to biotin-NM labeling followed by detection with streptavidin-HRP or mAb-biotin-HRP. Figure 7A shows a Coomassie blue stained gel for similar loading of mitochondrial proteins for control and alcohol exposed groups, respectively. Several proteins in the pair-fed control mouse livers were oxidized and thus detected with mAb-biotin-HRP (Fig. 7B, lanes 1 and 2). However, the intensity and the number of oxidized proteins greatly increased in the alcohol treated counterparts (Fig. 7B, lanes 3 and 4). Similar increases in the level of oxidized proteins, detected with streptavidin-HRP, were observed in alcohol-fed mouse livers (Fig. 7C, lanes 3 and 4) compared to pair-fed controls (Fig. 7C, lanes 1 and 2). Therefore, these results support previous results [1–6, 19] that chronic alcohol exposure can produce oxidative stress leading to oxidation of certain proteins. These data also establish that the biotin-NM labeling method can be successfully used to identify oxidized mitochondrial proteins from *in vitro* cultured cells as well as *in vivo* animals.

4 Discussion

High doses of alcohol can damage many tissues and cells through changes in signal transduction pathways while promoting ROS production and mitochondrial dysfunction. Although alcohol-mediated DNA damage can take place in various cells or tissues, as reported [41, 42], the role of DNA damage in alcohol mediated tissue damage is still poorly enumerated. In addition, various proteins can

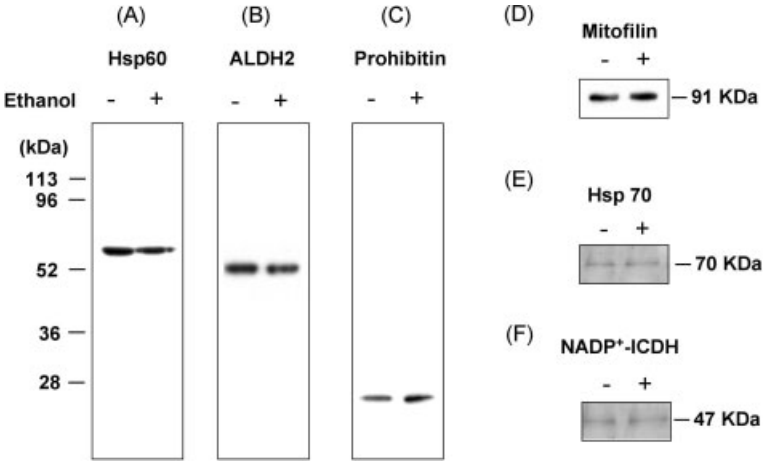

Figure 6. Confirmation of the presence of biotin-NM labeled oxidized proteins by immunoblot analyses. Biotin-NM labeled mitochondrial protein samples of untreated or 8 h ethanol exposed cells were purified with streptavidin-agarose beads, analyzed by 1-D SDS-PAGE, and then subjected to immunoblot analysis using the specific antibody against each target protein: Hsp60 (A), ALDH2 (B), prohibitin (C), mitofilin (D), Hsp70 (E), and $NADP^+$-dependent isocitrate dehydrogenase (ICDH) [46] (F). This figure represents a typical result from at least two different experiments.

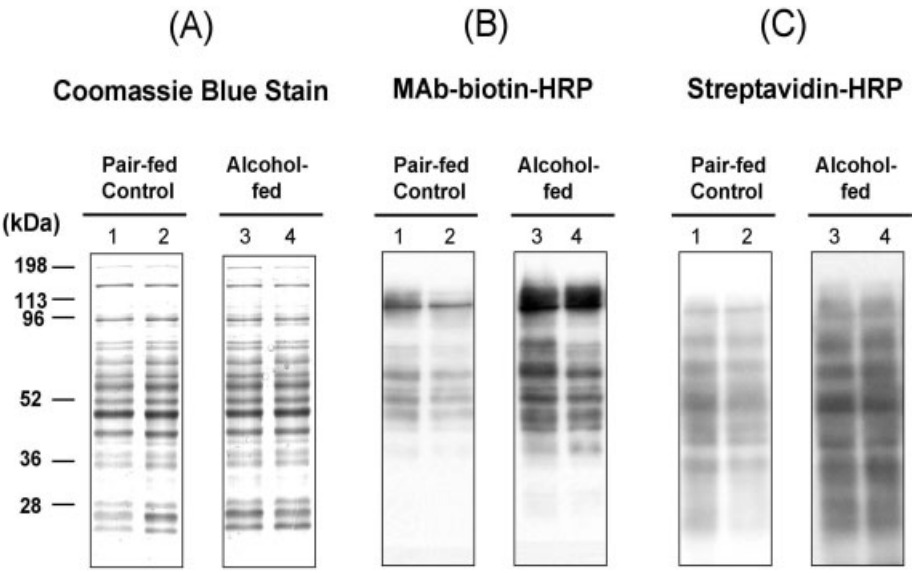

Figure 7. Increased oxidation of mitochondrial proteins in mouse livers after alcohol exposure. (A) Equal amounts (15 μg/lane) of biotin-NM labeled liver mitochondrial proteins (*n* = 2 *per* lane), freshly isolated from pair-fed control and ethanol-fed mice for 8 weeks as described [23], were separated on 10% SDS-PAGE gels and stained with Coomassie blue. Another set of the same protein samples was transferred to PVDF-immobilon membranes and subjected to immunoblot analysis using mAb-biotin-HRP (B) and streptavidin-HRP (C), respectively. This figure represents a typical result from two separate experiments.

be oxidized after alcohol exposure and oxidation of these proteins may lead to alcohol mediated cell damage through their inactivation or alteration of their normal function. This situation is particularly true in the mitochondria, since mitochondria are the main source of ROS production and the target organelle for ROS-related damage. In fact, alcohol was shown to directly interfere with the function of the mitochondrial electron transport chain especially at the levels of complex I and III [4, 5], leading to markedly increased levels of ROS production in many cells, including hepatocytes. In addition, it is likely that ROS in E47 HepG2 cells is produced through CYP2E1 mediated ethanol metabolism, as previously demonstrated [14, 20], where treatment with diallyl sulfide, a chemical inhibitor of CYP2E1, or antioxidants including vitamin C and trolox, a water-soluble vitamin E analog, prevented or significantly reduced alcohol-induced damage to these cells. Taken together, these results suggest an important role of alcohol-related oxidative cell damage. However, which proteins are oxidized and whether oxidation of these proteins results in altered cell physiology is not known. In this report, we sought to identify mitochondrial proteins whose cysteinyl residues are oxidized after exposure to a 100 mM dose of alcohol, which has been previously reported to damage cells [14, 20].

Many laboratories have reported various, sensitive methods to detect oxidized protein thiols after exposure to alcohol and other pro-oxidant compounds. These include (4-iodobutyl) triphenylphosphonium (IBTP) [19], biotin-NM [33], BIAM [40], isotope-coded affinity tags (ICAT) [43, 44], and 5-iodoacetamidofluorescein [45]. Oxidized cysteinyl residues do not react with the sulfhydryl reagents such as BIAM [40] and ICAT [43]. Therefore decreased efficiencies of labeling of oxidized protein thiols with these methods would be expected. Similar decreases in mitochondrial protein labeling with IBTP are expected, as recently demonstrated in the livers from chronically ethanol treated rats [19]. In addition, IBTP may possess another disadvantage with its dependence on mitochondrial membrane potential [19], which can be altered after exposure to pro-oxidants. Unlike these methods, the current method employing biotin-NM possesses a significant advantage of detecting the increased density of oxidized protein labeling, which is easier than detecting decreased amounts of protein labeling, especially in cases of subtle changes. Despite the differences in these methods, oxidation of certain cysteinyl residues of GRP78, Hsp60, protein disulfide isomerase and mitochondrial ALDH2 was consistently observed following exposure to alcohol. It is possible that many other oxidized mitochondrial proteins aggregated in the pH 8–10 range could also be identified. These results substantiate the notion that modification of these proteins may be directly or indirectly related to alcohol-mediated mitochondrial dysfunction followed by cell damage.

We also noticed that some mitochondrial proteins oxidized after alcohol exposure were detected by one method but not recognized by another method, while some proteins were identified by both methods. For instance, as indicated by the current study, the proteins that were only detected by biotin-NM included ALDH5 and prohibitin. The presence of these proteins was also confirmed by immunoblot analyses using the specific

antibody to each protein after biotin-NM labeled proteins were purified with streptavidin-agarose beads. In contrast, the results with the IBTP method demonstrate that pyruvate carboxylase, glutamate dehydrogenase, and acetyl-coenzyme A acyl transferase 2 are also oxidized after alcohol exposure [19]. The reason for the differential detection of some of the mitochondrial proteins is not known. Selective detection by two different methods could result from different treatment protocols (acute *versus* chronic alcohol exposure), different concentrations of ethanol, *in vitro versus in vivo* models, and other unknown factors. In addition, the difference could result from the fact that many oxidized proteins aggregated in the pH 8–10 range were not excised from the 2-D gel for MS analysis. The reason for differential detection and the role of those oxidized mitochondrial proteins in alcohol-mediated mitochondrial dysfunction remains to be studied.

The results of this study with biotin-NM and BIAM show that molecular chaperone proteins (GRP78, GRP75, Hsp70, Hsp60, protein disulfide isomerase and prohibitin), structural proteins (mitofilin and porin), and cellular defensive enzymes (mitochondrial ALDH isozymes, protein disulfide isomerase and mitochondrial $NADP^+$-dependent isocitrate dehydrogenase [46]) were oxidized by ROS produced during and after CYP2E1-mediated ethanol metabolism. Oxidation of these proteins were observed at 4 and 8 h, long before the majority of cell death was observed at 48 h after ethanol exposure of E47 HepG2 cells (our results and [14, 15]). In fact, overexpression of some of these proteins was protective against cellular injury caused by ischemia and reperfusion [47, 48] or lipid peroxidation and other stressful insults [46, 49–51]. Therefore, it is possible that oxidation of these proteins may lead to reduction in their cellular functions, as recently suggested [4, 5, 19]. The functional consequence of oxidation of some of these proteins is presently being investigated in our laboratory.

5 Concluding remarks

In conclusion, using the targeted proteomic approaches described, several mitochondrial proteins whose cysteinyl residues are sensitive to oxidation after alcohol exposure were unambiguously identified. Labeling of these oxidized proteins with biotin-NM permits detailed analysis of the mechanism by which oxidatively modified protein thiols are related to alcohol mediated mitochondrial dysfunction. This technique can also be applied to identify oxidized protein thiols in the cytoplasm of other types of cells or tissues under various pathophysiological conditions and after exposure to other toxic substances known to produce ROS.

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