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Liver Proteome Analysis in a Rodent Model of Alcoholic Steatosis

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Abstract

Alcoholic steatosis (AS) is the initial pathology associated with early stage alcoholic liver disease (ALD) and is characterized by the accumulation of fat in the liver. AS is considered clinically benign because it is reversible, and the progression of AS to alcoholic steatohepatitis (ASH) is a key step in the development of ALD. A two-dimensional gel electrophoresis (2DE) – mass spectrometry (MS) proteomic approach was used to investigate the protein expression pattern underlying AS, as the first step towards determining liver tissue biomarkers for early-stage ALD. Several proteins involved in fatty acid and amino acid metabolism were up-regulated in 3- and 6-week ethanol-fed rats relative to isocaloric controls, which suggest a higher energy demand upon chronic exposure to ethanol. In addition, the expression of two proteins associated with alcohol-induced oxidative stress, peroxiredoxin 6 (PRDX6) and aldehyde dehydrogenase 2 (ALDH2), was down-regulated in ethanol fed rats, and suggests an increase in reactive oxygen species and oxidative stress. In order to investigate if irreversible protein modification arising from oxidative stress during AS impact protein levels, the extent of carbonylated proteins in the ethanol and isocaloric groups was identified using mass spectrometry. The detection of modified proteins involved in anti-oxidant functions further supports the notion that oxidative modification of these proteins leads to protein turnover during AS. In addition, the carbonylation of betaine-homocysteine S-methyltransferase, a protein implicated in fatty liver development, in 3-week and 6-week ethanol exposed samples suggest that this protein could be a marker for early stage AS.

Keywords

liver; steatosis; carbonylation; oxidative stress; 2DE; mass spectrometry

INTRODUCTION

Chronic ingestion of alcohol leads to a sequence of hepatic pathologies associated with alcoholic liver disease (ALD), ranging from alcoholic steatosis (AS) to alcoholic steatohepatitis (ASH), cirrhosis and liver failure ^{1–5}. Steatosis or the accumulation of fat in the liver is the initial pathology that is common to all aspects of ALD. Although AS reverts upon alcohol withdrawal ⁶, prior work indicates that the accumulation of fat leads to subsequent liver complications, with the severity of damage proportional to the extent of fat accumulation ^{7–9}. AS is generally considered clinically benign as it is reversible ^{6, 10, 11}, but the subsequent

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stages of ALD such as ASH are mostly irreversible². The progression of ALD to ASH is a limiting step in the progression of ALD, because approximately 50% of individuals with ASH go onto develop end-stage liver diseases such as fibrosis and cirrhosis. From a clinical standpoint, targeting AS for diagnosis and treatment is desirable, because once ALD progresses to ASH, only 10% of livers revert back to normal¹². Therefore, it is important to understand the mechanisms underlying AS in order to diagnose and develop therapies for treating ALD.

Proteomic approaches have been successful in identifying prognostic and diagnostic markers for several diseases, including alcohol-related diseases, cerebral palsy, severe combined immunodeficiency, and Alzheimer's disease^{13–16}. For example, two dimensional electrophoresis (2DE) based protein separation and quantification followed by matrix assisted laser desorption ionization (MALDI)-mass spectrometry (MS) identification has been used to identify differentially expressed proteins from brains of alcohol preferring and alcohol non-preferring rats¹⁷. Serum protein markers such as carbohydrate-dependent transferrin, which is known to be lower in alcoholic patients¹⁸, and ethanol glucuronide (a direct ethanol metabolite)¹⁹, have been proposed as markers for early stage ALD; however, neither of these markers accurately predicts disease progression. A major difficulty associated with identifying serum protein markers is the high abundance of a small set of proteins (e.g., albumin, transferrin) and the large dynamic concentration range over which proteins are present in serum^{20–21}, both of which complicate accurate detection and identification of low abundant putative biomarker proteins. The study of the liver proteome is an attractive alternate approach for identifying serum protein biomarkers, as the liver is the major source of several serum proteins²² and could eliminate some of the problems associated with identification of protein biomarkers for early-stage ALD.

Chronic ethanol exposure leading to AS can alter the proteome by either inducing changes in the expression levels of different proteins and/or by irreversibly modifying specific proteins²³, both of which results in cellular dysfunction. The modification of proteins is, in part, a consequence of the oxidative stress arising from cellular ethanol metabolism¹. The level of reactive oxygen species (ROS) can increase either due to ethanol-mediated decrease in the cellular ATP pool²⁴ which limits the energy available to antioxidant pathways, and/or through activation of cytochrome P450-mediated ethanol metabolism²⁵. Of the different oxidative modifications, protein carbonylation is of importance as it is not only the most common modification but is also irreversible (i.e., permanently modifies target proteins)²³.

In this study, we report the characterization of changes in the liver proteome during diet AS. Changes in the expression of liver proteins were investigated with 2DE and significantly expressed proteins were identified by mass spectrometry. In addition, protein carbonylation in liver tissue was also investigated to investigate if irreversible protein modifications arising from oxidative stress during AS impact protein levels and cellular dysfunction.

MATERIALS AND METHODS

Reagents and Supplies

All reagents and supplies for two dimensional gel electrophoresis (2DE) buffers, cell lysis buffers, and SDS-PAGE were purchased from Fisher Scientific (Hampton, NH), unless otherwise noted. Deoxyribonuclease (DNase), ribonuclease (RNase), and iodoacetamide were purchased from MP Biomedical (Irvine, CA). Cell culture media and reagents were purchased from Hyclone, (Logan, UT), unless otherwise noted.

***In vivo* rat model of AS**

A Lieber-DeCarli diet model of AS was used in this study²⁶. Briefly, Sprague-Dawley rats (SD) (220–250 g, Harlan, Houston, TX) were housed individually in cages in a temperature-controlled animal facility with a 12 h light dark cycle. Rats were utilized after a 1-week equilibration period. Weight matched male (n=8) SD rats were fed with EtOH-containing (35.5% of total calories) Lieber-DeCarli liquid diet (11.5% carbohydrate, 18% protein and 35% fat) for a period of six weeks. Control group was fed with isocaloric maltose-dextrin diet. Rats were acclimatized to EtOH treatment, following which they were divided into two groups (n=4 each): 3 week exposure and 6 week exposure groups. Control group rats were also similarly divided into two groups. All rats were weighed at the beginning of the study and weekly thereafter. At the end of the exposure, rats were sacrificed by CO₂ asphyxiation. Rats were provided humane care in compliance with the institutional guidelines (ULACC; University Laboratory Animal Care Committee) of Texas A & M University.

Preparation of protein extracts for 2DE

Liver tissue was snap frozen in liquid N₂ and stored at –80°C for further analysis. Frozen liver tissue samples (0.1mg) were homogenized in buffer containing 2M thiourea, 7M urea, 4% CHAPS, 50mM DTT, 0.5% ampholytes (BioRad, Hercules, CA), and protease inhibitors (Sigma, St. Louis, MO). Following homogenization, DNase and RNase were added to the homogenate and vortexed occasionally for 10 min, after which they were centrifuged at 13,000 × g for 15 min. The resulting supernatant was collected, and the protein concentration was measured by Bradford assay with BSA as the standard. The BioRad readyprep 2-D kit (BioRad) was further used to purify the homogenate according to the manufacturer's protocol. The protein concentration in the purified homogenates was again measured and samples were stored at –80°C until further use.

Two-dimensional gel electrophoresis

To assure 2-DE reproducibility and to prevent variations occurring due to the technique, all 2-DE gels were carried out under the same electrophoresis conditions. All gels were run in duplicate with three liver samples (experimental replicates) for each of the three experimental groups (isocaloric control, 3 week exposure, 6 week exposure), for a total of 18 gels. 2-DE was carried out using the Protean IEF cell and mini electrophoresis system (Bio-Rad, Hercules, CA). Forty µg of protein was mixed with rehydration buffer (9.5M urea, 2% CHAPS, 18mM DTT, 0.5% Ampholytes, trace amount of bromophenol blue), and loaded onto first dimension IPG strips (7cm pH 5–8, BioRad). The IPG strips were rehydrated with the samples overnight. The strips were then focused in a three-step procedure for 20,000 volt-hour (15min at 250V; 2 h with voltage ramping linearly to 4000V; finally to 20,000 V-h), and frozen at –80° C until further use. Prior to second dimension SDS-PAGE, the frozen strips were equilibrated for 15 min in DTT buffer (50mM Tris-Hcl, 6M Urea, 30% Glycerol, 2% SDS, 1% DTT, trace amount of bromophenol blue), followed by another 15 min incubation in iodoacetamide buffer (50mM Tris-Hcl, 6M Urea, 30% Glycerol, 2% SDS, 2.5% iodoacetamide, few grains of bromophenol blue) in a shaker. For second dimension separation, the IPG strips were positioned on 10% polyacrylamide gels, and the proteins were separated at 125V for 1h at room temperature. Gels were washed in ultrapure water and fixed in 30% methanol and 10% acetic acid solution for 30 min prior to staining, and washed again in methanol and water for 10 min each respectively. Gels were stained with Sypro Ruby stain (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Image analysis

Digitized images of the Sypro Ruby stained gels were analyzed using the PDQuest (ver 7.4) 2-D analysis software (Bio-Rad, Hercules, CA). The image analysis software was used for spot

detection, quantification and analysis according to the manufacturer's instructions. Briefly, the analysis scheme consisted of five steps: detection of spots, identification of landmark proteins, aligning and matching of spots in gels, quantification of matches spots and manual inspection of the spots to verify the accuracy of matching. Any errors in the spot matching procedure were manually corrected prior to the final data analysis. The spot volume was used as the analysis parameter for quantifying protein expression.

The protein spot volume was normalized to the spot volume of the entire gel (i.e., of all the protein spots). Fold-changes in protein spot levels were calculated between spot volumes in the ethanol treated group relative to that in the control gels. Statistically significant changes in protein expression were determined using two sequential data analysis criteria. First, a protein spot had to be present in a minimum of 4 out of 6 gels for each sample to be included in the analysis. Next, statistically-significant changes in expression were determined using the distribution of fold-change values in the data. Spots were determined to be statistically significant if the difference between the average intensity of a specific protein spot in the control and experimental groups was greater than one standard deviation (67% confidence limits) of the spot intensities for both groups. An absolute fold change of ± 1.3 was the lowest observed in any spot that met the above condition.

Spot excision and in-gel digestion

For MS identification of protein spots, 350 μg of liver protein was separated on a 13cm IPG strips and 10% Criterion pre-cast gels (Bio-Rad). Gels were stained with Gelcode Blue (Pierce) for visualization. We have previously utilized this approach for identifying differentially expressed protein spots 27-28. Protein spots of interest were manually excised from gels. The excised gel plugs were approximately 2 mm in diameter and ranged from 0.75 mm to 1.0 mm in thickness. Trypsin digestion was performed according to standard protocols (17) using the ProGest automated gel digestion system (Genomic Solutions, Ann Arbor, MI). Briefly, the method consisted of a series of washing and dehydrating steps using 25-mM ABC (ammonium bicarbonate) and ACN (acetonitrile), respectively. The next step was reduction via dithiothreitol at 60°C for 30 min followed by alkylation with iodoacetamide for 45 min at room temperature. The gel spots then underwent another series of washing/dehydrating steps prior to digestion with trypsin at 37°C for 4 hrs.

Trypsin digested samples were desalted, mixed with the MALDI matrix, and spotted onto a MALDI target plate using the ProMS target spotting robot (Genomic Solutions). Samples were cleaned with 70% ACN and 0.1% FA (formic acid) using C18 Ziptips (Millipore, MA) prior to spotting. The MALDI matrix consists of 5 mg mL^{-1} CHCA (α -cynao-4-hydroxy cinnamic acid, a matrix solution) prepared in 50:50 ACN:ddH₂O containing ammonium phosphate buffer (10 mM Tris HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and 0.1 % TFA (trifluoroacetic acid). Two 10 μL sample loadings were used followed by washing steps and elution with the MALDI matrix.

Mass spectrometry

All MALDI-MS and MS-MS experiments were performed using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The MS data for the MALDI plates were acquired using the reflectron detector in positive mode (700–4500 Da, 1900 Da focus mass) using 800 laser shots (40 shots per subspectrum) with internal calibration. The collision gas was air at the medium pressure setting, with 1 kV of collision energy applied across the collision cell. All MS data were searched against the Swiss-Prot protein sequence database (Release 54) using the GPS Explorer V2.1 (Applied Biosystems, Foster City, CA) software. A score of greater than 65 obtained with the Mascot search engine (Matrix Science, UK) was considered as significant²⁹. The parameters for database searching were as follows: species: *Rattus*

Norvegicus; enzyme: trypsin; maximum missed cleavages, 1; variable modifications, oxidation (Met); peptide tolerance, 85 ppm; and MS/MS fragment tolerance, 0.3 Da. Five spots were re-analyzed to confirm the generated MS data.

Biotin tagging and affinity purification of carbonylated proteins

Carbonylated proteins were linked to biotin hydrazine and separated from liver tissue protein extracts following the protocol described by Mirzaei *et al.* 30, 31. Briefly, protein extracts from liver tissue were prepared by homogenizing whole rat liver tissue with a WMP-THP115 homogenizer (White Mountain Process, Boston, Ma) in lysis buffer containing 0.1 % SDS, 0.5 % sodium deoxycholate, 1.0 % CHAPS, 0.1 M NaCl, 0.1 M sodium phosphate, 1 mM EDTA (pH 7.5), and protease inhibitor cocktail. In addition, biotin hydrazide (Pierce, Rockwell, IN) and sodium cyanoborohydride (Fisher Scientific, Hampton, NH) were added at a final concentration of 5 mM and 15 μ M, respectively. The lysate was incubated for 30 min centrifuged, and dialyzed overnight at 4°C to remove detergents and excess reagents. The concentration of carbonylated protein in the dialyzed lysate was assayed by the Bradford assay (BioRad).

Affinity purification of carbonylated proteins was done as described by Mirzaei and Regnier 30 with minor modifications. Briefly, monomeric avidin beads (Pierce, Rockford, IL) were used to capture the biotinylated carbonylated proteins at room temperature for one hour with gentle vortexing every 20 minutes. Non-specifically adsorbed proteins were removed by washing with 25 mM ammonium bicarbonate and the bound carbonylated proteins eluted from the column in 0.7 ml of elution buffer. The proteins in the eluate were lyophilized prior to resuspension for MS analysis.

Carbonylated protein detection and MS/MS analysis

Purified carbonylated protein samples were rehydrated either in 50 μ l of 25 mM ammonium bicarbonate for quantification using the Bradford assay or in 40 μ l of reducing SDS-Page buffer 32 and resolved on a 7cm 10% acrylamide gel. Gels were incubated for 15 minutes in 20% methanol and 7% acetic acid and stained with Sypro Ruby stain overnight. Stained gels were imaged using a VersaDoc imager (BioRad) and gel lanes were manually excised and cut into 35 gel slices for robotic in-gel digestion using the ProGest system (Genomic Solutions, Ann Arbor, MI). MS analysis and database searching for protein identification were performed as described above. MS and MS-MS spectra from a gel slice are shown in Supplemental Figure 1 as representative of the identification of proteins with high and low MASCOT scores.

Reverse-transcription polymerase chain reaction analysis

RNA was extracted from control and ethanol-treated rat livers using the NucleoSpin RNAII kit (Clontech, Palo Alto, CA) following the manufacturer's protocol. The expression of three genes – ALDH2, PRDX6, and ICDHP - was determined using quantitative RT-PCR. The mRNA sequence for each gene was retrieved from the Genbank database and gene specific primers designed for each transcript. RT-PCR was performed with ~ 50 ng of RNA using the Superscript II one-step RT-PCR kit (Invitrogen, CA) on a icycler real-time PCR machine (Bio-Rad, Hercules, CA). The cycle number at which the fluorescence in each amplification reaction increased beyond a threshold (in the exponential phase of amplification) was determined using the MyiQ software (Bio-Rad). Threshold cycle numbers for each gene was normalized to that of 18S rRNA (housekeeping gene). All RT-PCR experiments were done in triplicate using three different liver samples. Data reported are mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

A Lieber-DiCarli rodent alcoholic steatosis model was used to investigate changes in the rat liver proteome during alcoholic steatosis. Hematoxylin and eosin (H&E) stained liver sections from wild-type control, isocaloric control, 3-week, and 6-week experimental groups are shown in Figure 1. Accumulated fat droplets (indicated by arrows in Figures 1C & D) in the 3-week and 6-week experimental groups demonstrate the onset of AS upon ethanol consumption. Liver proteins from the control, 3-week, and 6-week experimental groups were separated by 2DE. Approximately 200 protein spots between 10 and 80 kDa were detected by 2DE. Of these, 176 protein spots were common between the three different experimental groups and were used for further analysis. Representative gel images from each experimental group is shown in Figures 2A – C. A composite master gel was generated using the PDQuest image analysis software (BioRad) in which protein spot features present in all the gels were imported onto a single synthetic gel (Figure 3).

Protein spots exhibiting a statistically significant change in expression were identified using the image analysis criteria outlined in Materials & Methods. This analysis resulted in 19 protein spots being classified as differentially expressed in ethanol-treated groups relative to the control group. Of these, 11 spots decreased in expression in the ethanol-treated group relative to control, while 8 protein spots increased in expression. Interestingly, similar trends for changes in expression were observed after 3 and 6 weeks of ethanol exposure (i.e., protein spots down-regulated at 3 weeks were also similarly down-regulated at 6-weeks).

The observation that proteins characterizing steatosis at 6 weeks are also differentially expressed at 3 weeks suggests changes in protein expression occur rapidly during the development AS and ALD. The modest changes in expression between the 3 and 6 weeks ethanol fed groups also suggests that maximum effect of ethanol on protein expression is manifested rapidly (i.e., by 3 weeks) in ALD, which is consistent with published reports showing that alcohol consumption rapidly induces steatosis ^{1, 2}. Interestingly, while protein changes are observed after 3-weeks of ethanol consumption, pronounced histopathological changes are observed only after 6-weeks of alcohol exposure (Figure 1D). This result further underscores the importance of secreted protein markers for detection of early-stage ALD, as they are likely to be detected much earlier than histopathological markers.

MALDI-TOF mass spectrometry was used to identify 17 proteins from the differentially expressed protein spots. All of the excised proteins spots resulted in a positive identification, with most Mascot ³³ protein scores over one hundred. Table 1 shows the identified differentially expressed proteins and their putative functions. The identified proteins are involved in a wide range of functions including fatty acid and amino acid metabolism, oxidative stress, and chaperone function. Several differentially expressed proteins were mitochondrial proteins (e.g., those involved in amino acid and fat metabolism), which is not surprising as the mitochondria are well established as the site for oxidative damage; therefore, any treatment that generates ‘stress’ (such as chronic ethanol exposure) is expected to affect the expression of mitochondrial proteins.

Our data show that alcohol ingestion up-regulated the expression of liver proteins involved in fat and amino acid metabolism, which likely reflects increased cellular demand for energy in AS. Acyl-CoA dehydrogenase and delta3,5-delta2, 4-dienoyl-CoA isomerase are both involved in mitochondrial beta oxidation of fatty acids ^{34, 35}, with the former enzyme catalyzing the first step in the process, while the latter is necessary for the beta oxidation of unsaturated fatty acids, namely docosahexaenoic acid ³⁵. Our data showing an increase in enzymes involved fatty acid oxidation (acyl-CoA dehydrogenase and delta3,5-delta2, 4-dienoyl-CoA isomerase) is contrary to the generally accepted view that steatosis results in

decreased fatty acid oxidation^{36, 37}. It is interesting that these enzymes are up-regulated, as the depletion of NAD⁺ from ethanol and acetaldehyde metabolism is thought to interfere with fatty acid catabolism³⁸. Similarly, 3-hydroxyisobutyrate dehydrogenase is an oxidoreductase that acts on CH-OH group of donors and is involved in branched chain amino acid catabolism³⁹, and its increase also suggests an increased energy requirement. ADP/ATP translocase 2 is increased in expression, which catalyzes the exchange of ADP and ATP across the mitochondrial membrane, and also supports the increase in ATP generation.

The expression levels of aldehyde dehydrogenase (ALDH2) and peroxiredoxin 6 (PRDX6) was reduced in the ethanol treated groups compared to the control group. In the liver, ethanol is first converted to acetaldehyde, which is thought to be more toxic than ethanol itself⁴⁰. ALDH2 is the enzyme responsible for metabolizing acetaldehyde to acetic acid; therefore, its down-regulation suggests an accumulation of acetaldehyde and increased hepatotoxicity. PRDX6 is an important antioxidant enzyme that is involved in cellular redox regulation⁴¹. Specifically, PRDX6 reduces H₂O₂ and phospholipid hydroperoxides and is an important mediator in the protection against oxidative injury. The decrease in PRDX6 expression is indicative of increased oxidative stress in the liver after 3 and 6 weeks of ethanol consumption. This data is also consistent with the increase in energy metabolism proteins as oxidative stress is one possible source of increased cellular energy requirements. Increased ATP production would likely occur in response to oxidative stress, as several studies^{42, 43} have linked ATP depletion with oxidative stress and related cellular damage; hence, leading to increased expression of proteins involved in ATP generating process such as fatty acid oxidation.

While one might expect increased oxidative stress to lead to an increase in the expression of anti-oxidant systems, several studies have actually reported that key enzymes involved in counteracting the effects of oxidative stress are actually reduced during oxidative stress, and thus their reduced expression actually indicates a state of oxidative stress^{44, 45}. Reactive oxygen species (ROS) are constantly generated in the cell during metabolism and are scavenged by anti-oxidant systems, and this equilibrium is disturbed when there is an increase in the production of ROS, which leads to oxidative stress. This, in turn, results in the down-regulation of anti-oxidant systems, and further increases ROS levels and oxidative stress. Therefore, the down-regulation of PRDX6 is indicative of hepatocellular damage and increased ROS levels in the ethanol-treated group. Together, these changes in protein expression are consistent with an increased state of oxidative stress after 3 and 6 weeks of ethanol exposure.

Moon *et al.*⁴⁴ have shown that mitochondrial proteins (e.g. ALDH2) of alcohol-fed rats are subject to oxidative stress modifications (e.g. carbonylation, nitrosylation), which led to a decrease in function (presumably due to expression) of these proteins. This is not surprising as oxidatively modified proteins have been reported to be more susceptible to ubiquitination and subsequent degradation by proteosomal/lysosomal pathways^{46, 47}. Therefore, it is possible that the decrease in PRDX6 and ALDH2 observed in our study could be a consequence of increased oxidative modification. The expression of oxidatively-modified proteins such as glutathione S-transferase A4 (GSTA4) has also been shown to decrease in obese mice⁴⁵. In order to confirm that the decrease in ALDH2 and PRDX6 was due to an increase in protein degradation and not due to decreased synthesis, we also determined their mRNA levels in ethanol-treated and control rat livers. Figure 4 shows that the mRNA levels of three proteins down-regulated in rat livers exposed to ethanol for 6 weeks – ALDH2, PRDX6, and ICDH – was either unchanged or even slightly increased in the ethanol-fed rat livers compared to controls. This result suggests that synthesis of these anti-oxidant proteins is not decreased, and further reinforces the hypothesis that the decrease in expression is likely due to increased degradation.

Based on our data showing that anti-oxidant proteins are decreased in expression in AS, we hypothesized that oxidative modification of proteins, specifically that of anti-oxidant proteins, will be significant during AS. Specifically, we focused on protein carbonylation, as it is a common irreversible protein modification under conditions of high oxidative stress. Mass spectrometry analysis showed that chaperone, antioxidant, and metabolism proteins were carbonylated in rat liver after 3-weeks and 6-weeks of ethanol exposure (Table 2). While several carbonylated proteins were detected in ethanol-fed rat livers, modified proteins were also identified in liver tissue from isocaloric control animals. Some of these proteins were unique to control liver (e.g., aldehyde dehydrogenase) while some proteins (e.g., catalase) were carbonylated in all three experimental groups. The identification of oxidatively-modified proteins in liver tissue from normal rats is not surprising as a basal level of oxidative stress exists in all cells due to normal metabolism⁴⁸. For example, Chaudhuri *et al.*⁴⁹ have reported that the carbonyl content in 4 – 6 month “young” mice was ~ 10 nmoles per mg of total protein, which was only 50% less than that observed with 22 – 23 month “old” mice. Therefore, although some proteins are carbonylated in control and ethanol-fed experimental groups, we propose that the level of carbonylation will be more significant in the ethanol-fed groups. The qualitative MALDI/MS-MS method used in this study precludes testing this hypothesis, and current work in our laboratory focuses on using iTRAQ labeling to quantify the extent of carbonylation in normal and ethanol-fed rat liver tissue. Therefore, we restrict our discussion on the significance of these proteins to the ethanol-fed experimental groups alone.

Proteins involved in protein folding and chaperone functions were carbonylated in the ethanol-fed experimental groups. This included protein disulfide-isomerase A4, 78 kDa glucose-regulated protein, and peptidyl-prolyl cis-trans isomerase A. Carbonylation of chaperone proteins is expected to cause errors in protein folding and lead to endoplasmic reticulum (ER) stress, as has been shown in alcohol fed mice⁵⁰. The observation that proteins involved in metabolism (e.g., Short chain 3-hydroxyacyl-CoA, dehydrogenase, arginase, carbamoyl-phosphate synthetase, hemoglobin) are carbonylated in AS livers is also in agreement with reports showing increased cellular dysfunction in steatosis, as carbonylated proteins have been shown to be degraded (turnover) more rapidly than non-carbonylated proteins^{46, 47}, which leads to cellular dysfunction. Betaine-homocysteine S-methyltransferase (BHMT), an enzyme which converts betaine and homocysteine to dimethylglycine and methionine, respectively and maintains homocysteine and methionine homeostasis, was also identified as a protein carbonylated in control and AS livers. Elevated homocysteine levels have been linked to arterosclerosis and to the development of fatty liver⁵¹, and mice treated with a BHMT inhibitor quickly developed elevated plasma homocysteine levels⁵². Our data suggest that elevated homocysteine and its negative effects are, in part, due to carbonylation-mediated decrease in BHMT activity. Based on our results showing carbonylation of BHMT at 3- and 6-weeks, we speculate that the extent of carbonylation of BHMT in the liver will be higher in ethanol-fed rats than control rats, and propose that BHMT could be a potential marker for AS. It should be noted that although direct measurement of BHMT expression or activity in liver tissue is not feasible, measurement of BHMT substrates (e.g., homocysteine) and/or products (e.g., dimethylglycine) in plasma can be used to non-invasively monitor changes in BHMT activity, and thereby, infer the extent of steatosis.

It has been postulated that many antioxidant proteins are especially vulnerable to oxidative modification because of their close proximity to the site of ROS generation (i.e., the mitochondrion)^{23, 48}, and the fact that they contain reactive metal centers⁵³. Glutathione S-transferase Yc-1, superoxide dismutase, and peroxiredoxin 1 were all identified as being carbonylated in the ethanol-fed groups. In addition, catalase, which also plays an important role in anti-oxidant defense, was modified in the ethanol-fed experimental and control groups. These observations support the hypothesis that anti-oxidant proteins are more susceptible to attack by ROS and oxidative modification.

Interestingly, more carbonylated proteins were identified in the 3-week ethanol fed group compared to the 6-week ethanol fed group. This result was surprising as the coomassie-stained gel image of carbonylated proteins used for MS identification (not shown) indicated the presence of more lower MW carbonylated proteins (< 40 kDa) in the 6-week sample (i.e., the gel lane corresponding to the 6-week ethanol-fed sample stained more than the control or 3-week samples). One possible explanation for this observation is that processes involved in degradation and removal of carbonylated proteins are more active in the six weeks ethanol-fed group, which will lead to higher degradation and migration of proteins at the lower MW range. Thus, although the total carbonyl content on proteins may have been higher, fewer proteins were isolated intact for MS identification. Our observation is also consistent with prior reports showing enhanced removal of oxidatively modified proteins in cells with exogenously added hydrogen peroxide or the lipid peroxidation product, 4-hydroxynonenal ^{46, 47}. Therefore, the amount of carbonylated protein after 6 weeks of ethanol exposure is likely to be impacted by the balance between the rate of formation of carbonylated proteins and their removal by degradation. Also, not all decreases in protein expression can be explained by turnover due to carbonylation, as peroxiredoxin-6, which was identified in the 2D gels as being decreased in expression, was not identified as one of the carbonylated proteins.

In summary, proteomic analysis of liver tissue from a rodent model AS model shows that sustained exposure to ethanol leads to decrease in the levels of rat liver proteins involved in anti-oxidant functions. Mass spectrometry analysis also indicates significant carbonylation of proteins, including those involved in anti-oxidant function, in the ethanol-treated groups. Our data suggests that the decrease in expression of these anti-oxidant proteins appears to be mediated mainly through degradation of carbonylated proteins rather than through a decrease in synthesis and transcription. In addition, the carbonylation of BHMT in 3-week and 6-week ethanol exposed samples, as well as its established role in fatty liver disease, suggest that BHMT could be a marker for AS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representative photomicrographs of hematoxylin and eosin (H&E) stained rat liver sections from (A) wild type control, (B) isocaloric control, (C) three week ethanol treated experimental group, and (D) six week ethanol treated experimental group. Images shown are at 40× magnification. Arrows indicate accumulated lipid droplet.

Figure 2. Representative cropped 2DE gel images of liver tissue proteins from (A) isocaloric control, (B) 3 week ethanol exposure and (C) 6 week exposure ethanol exposed rats. First dimension separation was performed using 7 cm pH 5–8 IPG strips, followed by SDS-PAGE with 10% polyacrylamide gels in the second dimension, and Sypro Ruby staining.

Figure 3. Composite master gel of liver proteins from normal and ethanol-fed rats generated using the PDQuest software. Proteins whose expression was either up-regulated or down-regulated and used for mass spectrometry identification are marked.

Figure 4. RT-PCR quantification of aldehyde dehydrogenase 2 (ALDH2), peroxiredoxin 6 (PRDX6), and isocitrate dehydrogenase (ICDH) in ethanol-treated rat livers relative to the isocaloric control. Transcript levels of each gene in the different samples were normalized to that of 18S rRNA and expressed as the ratio of transcript level in the ethanol group relative to control. The data shown represent the average of triplicate measurements from two different liver samples per experimental group.

Differentially expressed liver in alcohol and non-alcohol fed rat livers.

Table 1

SSP	3 weeks Ethanol	6 weeks Ethanol	Protein	Accession Number	Function	MASCOT score	Theoretical pI/MW	Experimental pI/MW ^a	Sequence Coverage ^b	PMF/MS-MS ^c
1201	-1.85	-1.71	Senescence maker protein-30	Q03336	Cell regulation	588	5.3/33	5.6/49	119/299	11/9
2001	-1.53	-2.01	Phosphatidylethanolamine-binding protein	P31044	Receptor	531	5.5/23	5.8/23	91/187	7/5
2403	-1.21	-1.92	Hsc70-interacting protein	P50503	Chaperone	87	5.3/41	5.8/68	114/368	9/1
8401	-2.04	-1.89	Isocitrate dehydrogenase	P41562	Metabolism	786	6.5/47	7.7/69	229/414	23/11
5101	-1.53	-1.56	Ketohexokinase	Q02974	Metabolism	364	6.2/33	7.1/44	78/298	8/5
5102	-1.57	-1.80	Endoplasmic reticulum protein ERp29	P52555	Chaperone	643	6.1/26	7.0/37	128/260	13/8
4401	-1.77	-1.28	Adenosine kinase	Q64640	Metabolism	238	5.7/40	6.7/60	117/361	11/3
3004	-1.33	-1.62	Peroxi-redoxin 6	O35244	Anti-oxidant	856	5.7/25	6.4/72	172/224	15/9
2101	-1.61	-1.50	3-hydroxyanthranilate 3,4-dehydrogenase	P46953	Amino acid metabolism	729	5.6/33	6.9/71	127/286	14/10
3502	-2.36	-1.97	Aldehyde dehydrogenase	P11884	Ethanol metabolism	218	5.7/55	6.5/84	112/519	11/5
4503	-1.49	-1.70	Aldehyde dehydrogenase	P11884	Ethanol metabolism	713	5.7/55	7.2/34	193/519	16/9
3803	1.70	1.90	Serum albumin precursor	P02770	Transport	581	5.8/66	6.3/83	152/608	15/7
6101	1.64	1.33	3-hydroxyisobutyrate dehydrogenase	P29266	Amino acid metabolism	356	6.2/32	7.0/42	157/335	10/7
6501	3.49	3.43	ADP/ATP translocase 2	Q09073	Energy production	58*	9.7/33	7.1/65	77/298	10/0
7306	1.47	2.30	Acyl-CoA dehydrogenase, short-chain specific	P15651	Fatty acid metabolism	551	6.4/42	7.6/56	132/412	19/12
6301	9.29	13.58	Acyl-CoA dehydrogenase, short-chain specific	P15651	Fatty acid metabolism	102	6.4/42	7.3/57	69/412	13/8
7101	1.69	2.07	Delta3,5-delta2,4-dienoyl-CoA isomerase	Q62651	Fatty acid metabolism	147	8.1/36	7.4/42	80/327	8/4
4402	3.48	2.77	2-oxoisovalerate dehydrogenase	P11960	Amino acid metabolism	645	5.9/46	6.9/71	194/441	15/10
7602	1.73	1.67	Glutamate dehydrogenase	P10860	Amino acid metabolism	488	6.7/56	7.6/72	211/558	18/6

^a Approximate pI and MW determined from gels

^b Sequence coverage (amino acids in peptides detected/total amino acids)

^c Number of PMF peptides and the fraction that yielded a detectable score in MS-MS analysis

* Mascot score lower than 65.

Table 2

MALDI/MS-MS identification of carbonylated proteins detected in alcohol and control rat livers.

Protein	Accession Number	Experimental Groups ^d	Function	MASCOT score ^{b, c}	Sequence Coverage ^{b, d}	PMF/MS-MS ^{b, e}
Aldehyde dehydrogenase, mitochondrial	P11884	C	Ethanol Metabolism	77	84/519	7/2
Heat shock cognate 71 kDa protein	P63018	C	Chaperone/ Folding	109	97/646	8/2
Protein disulfide-isomerase A4	P38659	C	Chaperone/ Folding	136	127/643	12/3
ATP synthase beta chain	P10719	C	Metabolism	139	162/529	11/2
Actin, cytoplasmic 2	P63259	C	Chaperone/ Folding	129	90/375	7/3
Calreticulin	P18418	C	Signaling/ Transcription	315	119/416	12/5
Superoxide dismutase, (Mn)	P07895	E3	Anti-Oxidant	243	115/222	10/4
Short chain 3-hydroxyacyl-CoA, dehydrogenase	Q9WYK7	E3	Metabolism	68	91/314	7/2
Aspartate aminotransferase, mitochondrial	P00507	E3	Metabolism	84	135/430	9/1
78 kDa glucose-regulated protein	P06761	C, E3	Chaperone/ Folding	412,412	(208, 223)/654	16/6, 17/5
Protein disulfide-isomerase precursor	P04785	C, E3	Chaperone/ Folding	379, 216	(213, 199)/509	18/7, 17/4
Arginase 1	P07824	C, E3	Metabolism	72,289	(134, 115)/323	11/6, 9/3
Carbamoyl-phosphate synthetase 1, mitochondrial	P07756	C, E3	Metabolism	153, 214	(127, 165)/1500	13/3, 16/5
Glycogen phosphorylase, liver form	P09811	C, E3	Metabolism	707, 176	(286, 146)/850	28/12, 12/3
Hemoglobin beta chain	P02091	C, E3	Metabolism	525, 351	(145, 145)/147	12/8, 11/6
Hemoglobin alpha-1/2	P01946	C, E3	Metabolism	275, 234	(78, 71)/142	6/4, 5/5
Serum albumin precursor	P02770	C, E3	Transport	657, 216	(143, 119)/608	20/3, 7/5
Malate dehydrogenase, mitochondrial	P04636	E3, E6	Metabolism	386, 185	(163, 140)/338	14/6, 10/5
Peroxisredoxin 1	Q63716	E3, E6 [*]	Anti-Oxidant	146, 62	(94, 54)/199	8/2, 3/1
Glutathione S-transferase Yc-1	P04904	E3, E6	Anti-Oxidant	146, 73	(59, 74)/221	8/2, 4/1
Peptidylprolyl isomerase A	P10111	E3, E6	Chaperone/ Folding	309, 127	(128, 62)/164	9/5, 5/2
Catalase	P04762	C, E3, E6	Anti-Oxidant	557, 216, 114	(116, 207, 82)/527	11/5, 16/5, 9/2
Betaine-homocysteine S-methyltransferase	O09171	C, E3, E6	Metabolism	723, 345, 102	(141, 151, 99)/407	20/13, 12/7, 8/2
Fructose-bisphosphate aldolase B	P00884	C, E3, E6	Metabolism	639, 680, 77	(197, 171, 103)/364	14/11, 13/12, 8/2
Lactate dehydrogenase A	P04642	C, E3, E6	Metabolism	260, 442, 154	(161, 182, 150)/332	17/6, 21/8, 16/5

^dExperimental groups (C: control; E3: 3-weeks ethanol; E6: 6-weeks ethanol)

^b For proteins present in multiple experimental groups, sequence coverage and the PMF/MS-MS ratio are given in the order in which samples are listed in the Experimental Groups column.

^c If the same protein was identified in multiple gel slices, the highest obtained score for that protein is reported.

^d Number of amino acids in peptides detected/total amino acids.

^e Number of PMF peptides and the fraction that yielded a detectable score in MS-MS analysis

^{*} Mascot score lower than 65 (p<0.05) threshold.