Published in final edited form as:

Free Radic Biol Med. 2015 December ; 89: 1144–1153. doi:10.1016/j.freeradbiomed.2015.10.420.

Increased hepatocellular protein carbonylation in human end-stage alcoholic cirrhosis

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Abstract

Objective—Oxidative stress is a significant contributing factor in the pathogenesis of alcoholic liver disease (ALD). In the murine models of chronic alcohol consumption, induction of oxidative stress results in increased peroxidation of polyunsaturated fatty acids to form highly reactive electrophilic α/β unsaturated aldehydes that post-translationally modify proteins altering activity. Data are presented here suggesting that oxidative stress and the resulting carbonylation of hepatic proteins is an ongoing process involved in alcohol-induced cirrhosis.

Methods—Using age-matched pooled hepatic tissue obtained from healthy humans and patients with end stage cirrhotic ALD, overall carbonylation was assessed by immunohistochemistry and LC-MS/MS of streptavidin purified hepatic whole cell extracts treated with biotin hydrazide. Identified carbonylated proteins were further evaluated using bioinformatics analyses.

Results—Using immunohistochemistry and Western blotting, protein carbonylation was increased in end stage ALD occurring primarily in hepatocytes. Mass spectrometric analysis revealed a total of 1224 carbonylated proteins in normal hepatic and end-stage alcoholic cirrhosis tissue. Of these, 411 were unique to cirrhotic ALD, 261 unique to normal hepatic tissue and 552 common to both groups. Bioinformatic pathway analysis of hepatic carbonylated proteins revealed a propensity of long term EtOH consumption to increase post-translational carbonylation of proteins involved in glutathione homeostatic, glycolytic and cytoskeletal pathways. Western analysis revealed increased expression of GSTA4 and GST π in human ALD. Using LC-MS/MS analysis, a nonenaldehyde post-translational modification was identified on Lysine 235 of the cytoskeletal protein vimentin in whole cell extracts prepared from human end stage ALD hepatic

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Conflict of Interest

The authors have no conflict of interest to report.

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tissue. Conclusions: These studies are the first to use LC-MS/MS analysis of carbonylated proteins in human ALD and begin exploring possible mechanistic links with end-stage alcoholic cirrhosis and oxidative stress.

Graphical Abstract

Background

Alcoholic liver disease (ALD) is a major contributor of liver failure in the United States today. A common phenotype of ALD is a hepatocellular environment characterized by pronounced lipid accumulation with enhanced oxidative stress^{1–3}. If chronic consumption continues, fibrosis followed by hepatic cirrhosis and death will occur^{4–6}. In this pro-oxidant environment, increased lipid peroxidation occurs resulting in the accumulation of reactive aldehydes including 4-hydroxynonenal (4-HNE)^{7,8}. These aforementioned reactive aldehydes react with DNA as well as post-translationally with Cys, Lys, and His residues (protein carbonylation) within proteins^{9–12}. Protein carbonylation has been documented in both early stage and late stage ALD. In rodent models, protein carbonylation is elevated following 6-weeks of chronic EtOH consumption^{12,13}. In late stage ALD-induced cirrhosis, increased concentrations of 4-HNE are present in serum and urine^{14,15}. Concurrently, increased malondialdehyde (MDA) protein adducts are evident in tissue sections and urine obtained from human ALD patients^{5,15}.

Using mouse models of chronic EtOH consumption, we have identified numerous proteins including the lipid phosphatase PTEN, protein kinase AMPK α , liver fatty acid binding protein and the molecular chaperone Grp78 as targets of electrophilic carbonylation by reactive aldehydes *in vivo*^{1,2,10–12,16–18}. In the aforementioned proteins, carbonylation resulted in decreased protein activity and/or function. Similarly, using mass spectrometry and a global proteomic approach, we have characterized the “lipid peroxidome” formed in murine models of early ALD¹⁹. From these reports, it was determined that during the early stages of ALD, consumption of EtOH results in increased post-translational modification of key proteins involved in cellular metabolic pathways involved in fatty acid metabolism and drug detoxification which are dysregulated in ALD^{1,17,20–26}.

To date, the effects of EtOH on production of lipid aldehydes and protein carbonylation has only been examined in animal models. The effects of a long-term alcohol abuse on post-translational carbonylation have not been examined. In this study, we used human age matched hepatic tissue isolated from healthy patients and from patients with end stage ALD-dependent cirrhosis to identify and validate hepatic proteins modified by electrophilic products of lipid peroxidation.

Materials and Methods

Human Tissue

To determine the status of protein carbonylation in human ALD, paraffin embedded and frozen hepatic tissue from normal and end stage ALD* patients (N=9 ALD, 8 normal) procured during transplantation (ages 38–59) were obtained from the University of Minnesota Liver Tissue cell Distribution Center NIH Contract #HHSN276201200017C (*patients were required to abstain from alcohol for at least 6 months prior to transplantation). Whole cell extracts (WCE) of each sample was prepared by dounce homogenization (10X) of tissue resuspended in 50mM tricine pH 8.0, 0.001M NaCl plus phosphatase and protease inhibitors (SIGMA ALDRICH, St Louis, MO) followed by sonication (3X15 seconds@ 4°C). To remove debris, samples were centrifuged at 14,000RPM (16,000g)(4°C) for 10 minutes. Supernatants were drawn off and immediately flash frozen in liquid nitrogen.

Histological Evaluation

Formalin fixed slides were analyzed following H&E or Picro-Sirius (PSR) Staining following immunohistochemical staining for 4-HNE or Cyp2E1 (Millipore, Billerica, MA) as previously described¹¹. Histologic images were captured on an Olympus BX51 microscope equipped with a four-megapixel Macrofire digital camera (Optronics; Goleta, CA) using the PictureFrame Application 2.3 (Optronics). All images were cropped and assembled using Photoshop CS2 (Adobe Systems, Inc.; Mountain View, CA). Ten polarized images were made in a “tiling” fashion across each PSR stained slide, then quantitated using the 3I Slidebook program (3I, Denver, Colorado) to arrive at the PSR stained pixels per 100x field for that slide.

Biotin hydrazide purification

Whole cell extracts (500µg) were prepared from age matched hepatic tissue obtained from both normal (N=8) and end stage ALD (N=9). Aldehyde modified proteins from each extract were derivatized using biotin hydrazide (BH)(ThermoFisher/Pierce, Waltham, MA) (5mM/2 hrs/RT/dark) followed by NaBH₄ reduction (10mM/100mM NaOH 1hr/dark) (as negative controls, non-biotin hydrazide treated wce were used). Biotin hydrazide linked carbonylated proteins were incubated overnight using monoavidin columns (ThermoFisher/Pierce). Columns were washed 5X in phosphate buffered saline (PBS) pH 7.4, 5X in PBS 0.5M NaCl (pH 7.4) and 5X in PBS/2M Urea (pH 7.4) and eluted with 6X200 microliters of 0.2M NH₄OH. Elutions were pooled and dried down using a roto-evaporator (Thermo Scientific/Savant) and boiled in 6X SDS PAGE loading buffer (15 minutes). Samples were loaded on 10% SDS PAGE gels and run at 160V for 20 min. Gels were removed and stained with Coomassie blue overnight (Imperial Stain, ThermoFisher/Pierce) followed by destaining in ddH₂O O/N. Bands containing carbonylated proteins were excised, destained, and digested with trypsin as previously described¹⁹. Following digestion, peptides were extracted, dried down, and resuspended in 0.1% formic acid in ddH₂O.

LC-MS/MS analysis

For LC-MS/MS analysis, 8µl of each peptide mixture (n=8 normal/9 ALD) was loaded on a Bruker Amazon Speed LC-MS/MS and a Bruker Maxis IMPACT LC-MS/MS. The instrument was operated using data-dependent collision-induced dissociation (CID) MS/MS or electron transfer dissociation (ETD) with a threshold of 10,000 total ion current (TIC). Data analysis was performed using ProteinScape V3.1.2 (Bruker Daltonics Inc. Billerica, MA) and Mascot (v2.1.04, Matrixscience). First a global search for proteins was conducted with a Mascot cutoff score of 80 with the following variable modifications of carbamidomethyl (C) and oxidized (M). After the initial search a second search was conducted for post-translational modification by reactive aldehydes using the masses shown in Table S1. For this 2nd iteration, peptide significance required a Mascot score higher than 40 and visual validation of spectra.

Determination of *in vivo* carbonylation of Vimentin

For determination of *in vivo* carbonylation of vimentin. 100ug of WCE was treated with 5mM biotin hydrazide for 2hr followed by dialysis overnight in PBS @4°C. Biotin hydrazide conjugated proteins were purified by incubating them with 50 µl of streptavidin (SA) beads (Pierce) for 3hrs at 4°C. Beads were washed in PBS (5X), boiled in 2X SDS PAGE buffer and vimentin carbonylation examined by Western blotting using a rabbit polyclonal vimentin antibody (Cell Signaling, Danvers MA).

Western blotting

Western blotting for protein carbonylation was performed from 10µg of biotin hydrazide treated liver extract as previously described^{9,11,27,28}. Western blotting of vimentin and GAPDH was performed using 10µg of WCE using vimentin polyclonal antibodies (cat#3932, Cell Signaling, Woburn, MA) and anti-GAPDH (cat #9484 Mouse monoclonal, ABCAM, Billerica, MA). Western blotting of GSTA4, GST π and GST μ was performed using a 12% SDS PAGE gel and antibodies directed against GSTA4 (cat#17271, Protein Tech, Chicago, IL), GST π (cat#311 rabbit polyclonal, MBL International, Woburn, MA), GST μ (cat# ab-53942 goat polyclonal, ABCAM, Cambridge, MA). Quantification of vimentin, GSTA4, GST π and GST μ expression was performed using ImageJ (NIH) and normalized to overall GAPDH expression.

Bioinformatics

UniProt IDs from proteins identified in the ALD samples, the normal samples, and in the background sample were initially converted into gene symbols using the UniProt website (UniProt Release 2015_08)²⁹. Gene symbols functionally annotated using EnrichR³⁰, KEGG 2015 pathways³¹ and PANTHER pathways³² were examined for over-representation in 5 different lists of proteins/gene symbols: 1) in 2 (50% of high yield samples) or more ALD samples and not in background; 2) in 3 (50% of high yield samples) or more normal samples and not in background; 3) in either 2 or more ALD samples or 3 or more normal samples and not in background; 4) in 2 or more ALD samples, but not in 3 or more normal samples or in the background sample; and 5) in background sample. Pathways that were nominally significant (unadjusted p-value<0.01) in at least one of the 5 lists were

retained. When no proteins from a list were found in the pathway, a p-value of 1 was assumed. Hierarchical clustering was used to describe the relationships among the enriched pathways with respect to their significance in the 5 lists, i.e., pathways that are enriched in the same groups clustered together. Hierarchical clustering was done using Euclidean distance between binary indicators of significance ($p < 0.01$ vs. $p \geq 0.01$). The hierarchical clustering analysis and the heatmap were generated using R Statistical Software (version 3.2.1).

Molecular modeling

All manipulations were performed using Discovery Studio software (Version 3.1; Accelrys Inc., San Diego, CA). The crystallographic coordinates of the 2.6Å human activated vimentin crystal structure (PDB code: 3UF1) were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org>)^{33,34}.

Statistical Analysis

The data are presented as means $\pm$ SE. Comparisons between genotype and diet was accomplished by one-way ANOVA, followed by Student Newman-Keuls *post hoc* analysis. Comparisons between two groups were accomplished using Student's T-tests. Statistical significance was set at $P < 0.05$. Prism 5 for Windows (GraphPad Software, San Diego, CA) was used to perform all statistical tests.

Results

Post-translational modification of proteins by products of lipid peroxidation has been implicated as a contributing factor in the progression of alcoholic liver disease. Although significant data have been accumulated using animal models of ALD, the effects of long-term alcohol abuse on protein carbonylation in humans has not been examined^{1,19}. In the present study we have extended murine ALD data by examining protein carbonylation occurring in hepatic tissue obtained from patients with end stage alcoholic cirrhosis.

For this study, fresh frozen human hepatic tissue and formalin fixed tissue from normal and end stage ALD was obtained prior to transplant from the University of Minnesota Liver Tissue cell Distribution Center (NIH Contract #HHSN276201200017C). For each patient relevant hepatic parameters was provided (model for end-stage liver disease (MELD)³⁵, aspartate aminotransferase (AST), international normalized ratio of prothrombin coagulation (INR), serum bilirubin, alkaline phosphatase and albumin). As shown in Table 1, all 9 patients possessed increased MELD scores indicative of severe hepatic dysfunction. Examining individual parameters, serum AST (normal range 12 – 39 U/L), INR (normal approximately 1.0), alkaline phosphatase (normal range 39 – 117 U/L), total bilirubin (adult normal range 0.1 – 1.3 mg/dL) were elevated and serum albumin (normal range 3.5– 5.7g/dL) levels suppressed.

Cirrhosis is characterized by a marked increase in fibrosis. To examine the extent of fibrosis, tissue sections obtained from human patients were stained with Hematoxylin and Eosin (H&E) and Picrosirius red. As shown in Figure 1A, tissue from healthy donors exhibited no evidence of steatosis or Picrosirius red staining characteristic of abnormal fibrotic networks

within the periportal and centrilobular regions of the liver (**upper panels**). In patients with end stage ALD, mild steatosis was observed in tissue sections with significant picosirius staining indicative of bridging fibrosis and cirrhosis. To further characterize this fibrosis, polarized light images of each picosirius red stained section were quantified¹². As shown in Figure 1B, samples analyzed from patients with end-stage ALD cirrhosis displayed significant increases in picosirius red intensity as compared to healthy controls.

Protein carbonylation is a significant factor associated with progression of early-stage ALD. Due to the limitation of animal models to study long-term alcohol ingestion on the liver, the role of protein carbonylation in alcohol-induced fibrosis has not been examined, especially in humans. However, animal studies have provided important experimental approaches for recovering carbonylated proteins from tissue such as the use of biotin hydrazide to visualize proteins modified by electrophilic products of lipid peroxidation. As shown in Figure 2A and 2B, hepatocellular protein carbonylation is significantly increased 1.6-fold in end stage ALD as compared to healthy controls. To further explore increased protein carbonylation in ALD, formalin-fixed tissues were evaluated by immunohistochemical staining using antibodies directed against 4-HNE. As shown in Figure 2C **Panels A and C**, in normal hepatic tissue, 4-HNE staining is very light and diffuse across the hepatic lobule. However, in patients with ALD-dependent cirrhosis, 4-HNE staining is significantly increased in hepatocytes (**Panels E, G**). Induction of the cytochrome P450 oxidase Cyp2E1 is mechanistically linked to the formation of reactive aldehydes in early and late stages of ALD^{5,36}. The expression of Cyp2E1 was evaluated in liver sections prepared from healthy individuals and patients with alcohol-induced cirrhosis (Figure 2C, **Panels B, D, F and H**). In livers from healthy individuals, weak Cyp2E1 staining is evident surrounding the central vein while in sections from cirrhotic end-stage ALD, Cyp2E1 staining is increased in hepatocytes panlobularly. Interestingly, Cyp2E1 expression co-localizes with 4-HNE in some hepatocytes (**Panels G and H, arrows**). Furthermore, both Cyp2E1 expression and 4-HNE modification were not evident in the surrounding fibrotic tissue which encompasses the periportal region (Figure S1).

We recently reported the use of BH derivatization followed by global LC-MS/MS analysis to identify carbonylated proteins in liver subcellular fractions isolated from ethanol-fed C57BL/6J mice¹⁹. In the present study, BH derivatization was performed on whole cell extracts prepared from pooled human normal and end stage ALD tissue. Following avidin purification, carbonylated proteins were digested with trypsin and peptides examined by LC-MS/MS. To identify background proteins, non-BH treated controls were also examined and proteins identified excluded due to the possibility of non-specific binding to the monoavidin columns. As shown in Table S2, a total of 1224 carbonylated proteins, collectively, were identified in any sample. Of these, 48% were previously identified in murine models of ALD¹⁹. Interestingly, 644 proteins presented in Table S2 have not been previously identified in murine EtOH models¹⁹. From the non-BH treated samples, a total of 59 proteins were identified as non-specifically binding the monoavidin columns and their exact carbonylation status cannot be determined using these techniques. As such these proteins were removed from our subsequent bioinformatic analysis (Table S3).

To determine proteins that were unique to each condition, Venn data analysis was performed. From the Venn diagram presented in Figure 3 and Table S4, 552 proteins were common to both normal and ALD tissue, 261 unique to tissue prepared from healthy individuals and 411 proteins were unique to samples from patients with alcohol-induced cirrhosis. To identify cellular pathways preferentially impacted by carbonylation in human ALD, proteins identified as carbonylated were subjected to bioinformatic analysis using Panther and KEGG pathways (All background proteins were excluded) ^{37,38}. From Figures 4A, 4B and Supplemental Tables S5 (PANTHER) and S6 (KEGG) long-term ingestion of alcohol resulted in increased carbonylation of proteins regulating glutathione homeostasis, glycolysis and the cellular cytoskeleton.

To identify individual peptides that are post-translationally modified by reactive aldehydes, MS-MS data was using in combination with the Mascot database. In addition to using the masses for modification by MDA, 4-HNE, 4-ONE, HHE and acrolein, Michael and Schiff base adduction of Lys, Cys and His residues by α/β unsaturated aldehydes crotonaldehyde, pentenaldehyde, hexenaldehyde, heptenaldehyde, octenaldehyde and nonenaldehyde was performed in the peptide searches (Table S1)^{39,40}. As shown in Figure 5, the cytoskeletal protein vimentin (Amino acid sequence: ²²³KVESLQEEIAFLK*²³⁶ Mascot ion score 49.2) was identified as undergoing *in vivo* post-translational modification by nonenaldehyde (2-nonenal) on Lys²³⁵ in hepatic tissue isolated from patients with end stage ALD. Furthermore from Table S2, vimentin is identified as a target of carbonylation in 9/9 patients with end-stage ALD. To confirm increased carbonylation of vimentin, quantification of aldehyde-modified vimentin was performed as previously described ^{9,27}. From Figure 6A, overall expression of vimentin expression increased by 4-fold in end stage ALD. As shown in the Western blot presented in Figure 6B and Supplemental Figure 2, carbonylation of vimentin significantly increased in tissue extracts obtained from 3/4 patients examined with end-stage ALD as compared to extracts from healthy subjects. This substantiates LC-MS/MS data (Table S2) and confirms increased carbonylation of vimentin in human ALD. Of interest, Western blotting techniques only identified vimentin carbonylation in 3 of 4 samples but LC-MS/MS in 9/9 samples demonstrating greater sensitivity using LC-MS/MS approaches towards identification of post-translationally modified proteins when compared with traditional Western blotting techniques.

The crystal structure of vimentin has revealed that it is a tetrameric protein (helices A–D) consisting of head and tail domains connected by a central rod domain with α -helical dimers forming anti-parallel coiled coil structure^{34,41}. To examine the potential effects of Lys²³⁵ 2-nonenal adduction, we examined the location of Lys²³⁵ in the crystal structure of vimentin³⁴. As shown in Figure 7A (yellow), there are Lys²³⁵ (yellow ball and stick) residues located within the central rod domains on each monomer. On 2 helices, Lys²³⁵ (Lys^{235B}, Lys^{235D}) forms an electrostatic interaction with Glu²³⁰ (Glu^{230A}, Glu^{230C}) (blue ball and stick) on the adjacent strand assisting in the association of each dimer. In addition, Arg^{158D}, Arg^{158B} (purple ball and stick) forms an additional electrostatic interaction with Glu²³⁰. On the other 2 helices, Glu^{230B}, Glu^{230D} only forms an electrostatic interaction with Lys^{235D}, Lys^{235C} due to the location of the residues (Figure 7B). From the structural data,

negation of the positive charge on Lys²³⁵ by the addition of a hydrophobic nonenaldehyde group would disrupt electrostatic interactions between Lys²³⁵ and Glu²³⁰.

From bioinformatic data, proteins regulating glutathione homeostasis are significant targets of carbonylation in human alcoholic cirrhosis. An important mechanism for removal of toxic lipid aldehydes is *via* conjugation with glutathione by Glutathione S-transferases (GSTs) ^{42–44}. During conditions of increased oxidative stress, GSTs are frequently upregulated as part of the phase 2 detoxification response ^{45,46}. In murine models of ALD, GST activity is increased in response to EtOH consumption ². To gain insight into possible mechanisms of increased carbonylation, expression of GSTA4, GST π and GST μ was investigated by Western blotting. As shown in Figure 8A, B, both GSTA4 and GST μ expression are significantly increased in end-stage ALD. Surprisingly, expression of GST π is highly variable in both normal and ALD patients but overall exhibits a downward trend in expression in ALD. Combined with the bioinformatic data presented in Figure 4A, B, these data further support the hypothesis that glutathione homeostasis is dysregulated in end stage alcoholic cirrhosis.

Discussion

Chronic ethanol consumption causes liver damage through dysregulation of a number of hepatocellular pathways. A contributor to the pathogenesis of ALD is enhanced hepatocellular oxidative stress resulting from the production of reactive species via induction of Cyp2E1 as well as xanthine and NADPH oxidases, which in turn induce lipid peroxidation of unsaturated fatty acids producing reactive aldehydes. These highly reactive lipid peroxides modify DNA as well as Lys, Cys and His residues on proteins. Following post-translational modification by reactive aldehydes, carbonylated proteins exhibit dysfunction in either enzymatic activity or cellular function. Previous studies from our laboratory have demonstrated that in early ALD, chronic ethanol consumption results in an increase in hepatic protein carbonylation by reactive aldehydes in both mice and in rats ^{1,10–12,47,48}. Recently, we reported using global BH derivatization/streptavidin purification of cellular fractions to identify a lipid peroxidome of proteins carbonylated in ALD ^{19,49}. In the present report, we extend these studies by examination of human end-stage ALD.

Few studies have been conducted examining protein carbonylation in end stage ALD. In late stage ALD-induced cirrhosis, the concentration of 4-HNE has been determined to be elevated in both serum and urine ^{14,15}. Using immunohistochemistry, increased MDA and acetaldehyde protein adducts are evident in tissue sections obtained from human patients with advanced alcoholic liver disease ⁵. In the present report, we determined that reactive aldehydes (specifically 4-HNE) are produced in hepatocytes but not in the surrounding fibrotic tissue in end stage alcoholic cirrhosis. Likewise, some 4-HNE/CypE1 co-localization occurred in hepatocytes during end-stage ALD (Figure 2, Figure S1). Cyp2E1 induction has been mechanistically linked to production of ROS and lipid peroxidation ⁵. Cyp2E1 has been reported to colocalized with 4-HNE modification in murine model ⁵⁰. Given that the samples analyzed in this study were collected at least 6 months after

abstinence from alcohol suggests that at least in end-stage ALD, Cyp2E1 induction persists independent of concomitant EtOH consumption.

It is well established that by their ability to remove reactive aldehydes using glutathione conjugation, GST's are important regulators of oxidative stress. Not surprisingly, there is considerable variability in GST μ expression but overall, GST μ expression is decreased when compared to normal hepatic tissue. In humans GSTM1-null mutations are associated with increased susceptibility to a host of human diseases and we hypothesize that this association is also present in this study. Furthermore, polymorphisms in GST μ have been identified as possible factors in fibrotic progression in ALD^{51,52}. From our data, GSTA4 and GST π expression are both increased in human ALD. By its ability to conjugate 4-HNE to GSH, GSTA4 is proposed to regulate cellular control of reactive aldehydes and increased expression suggests cellular responses to increased production of reactive aldehydes^{13,43}. Increased expression of GST π is directly linked to increased risk for hepatocellular carcinoma and polymorphisms in GST π are at a higher risk for alcoholic cirrhosis^{51,53}. We hypothesize that increased expression of GSTA4 is in response to increased carbonylation whereas the increase in GST π expression evident in human ALD signifies an increased risk for the development of HCC.

From our bioinformatic analyses, in samples from subjects with end-stage liver disease revealed a unique subset of carbonylated proteins regulating glutathione and cytoskeletal processes. Decreased glutathione homeostasis has been linked to increased hepatocellular damage in many models of ALD^{2,54-56}. Carbonylation of cytoskeletal proteins has been linked with inhibition of microtubule polymerization⁵⁷ and defects in hepatocyte microtubules and cytoskeletal elements have been identified in human ALD^{58,59}. These results suggest that carbonylation of proteins involved in the cytoskeletal integrity contributes to the accelerated and progressive death of hepatocytes during end-stage ALD.

In previous reports, the α/β unsaturated aldehyde 2-nonenal has been demonstrated to exert cytotoxic effects in *in vitro* cell culture models⁶⁰⁻⁶³. Furthermore, increased concentrations of 2-nonenal have been detected in rats following administration of carbon tetrachloride⁴⁰. Previous reports have established that post-translational aldehyde modification of cytoskeletal proteins including actin and tubulin results in cytoskeletal remodeling and decreased cell permeability^{57,64-67}. In the present study, the finding of an increase in carbonylation of the intermediate filament protein vimentin in 9/9 patients with end-stage ALD is intriguing. Aldehyde modification of vimentin on Cys³²⁸ has previously been demonstrated in THP-1 cells treated with 4-HNE which when combined with our *in vivo* data validates its susceptibility to reactive species⁶⁸. As important, regulation of vimentin on Cys³²⁸ by reactive aldehydes has recently been described as a mechanism to regulate vimentin self-association and regulation of cellular organelles^{69,70}.

In cell culture models, vimentin significantly increases in cirrhotic hepatocytes as they undergo hepatocyte epithelial mesenchymal transition⁷¹. Vimentin expression is typically low in normal hepatocytes but elevation of expression is indicative of liver undergoing repair and fibrosis. Recently, several reports have provided evidence that stellate cells are

the main source of fibrosis and are progenitor cells^{72,73}. As important, elevated vimentin expression occurs in hepatic stellate cells during fibrosis⁷³.

In a previous study, Cys³²⁸ is identified as possessing high susceptibility to oxidative modification a reaction that is prevented by intracellular zinc⁶⁹. In vitro the authors determine that zinc stimulates vimentin polymerization. Based on molecular modeling, we find that 2-nonenal adduction would provide a bulky hydrophobic group and eliminate the positive charge on Lys^{235(A-D)} of vimentin. We did not find modification of Cys³²⁸. We hypothesize that alteration of electrostatic interactions between Lys²³⁵ and Glu²³⁰ and would potentially disrupt tetramer stability and therefore intermediate filament stability. During fibrosis, increased carbonylation of hepatic cytoskeletal proteins and progressive loss of cellular structure likely contributes to the pathology of end-stage ALD⁷⁴.

In summary, this study is the first to examine and identify hepatic proteins that are post-translationally modified by reactive aldehydes in liver tissue isolated from patients diagnosed with end-stage alcoholic cirrhosis. These data support the contribution of lipid peroxidation to dysregulation of glutathione homeostatic and cytoskeletal proteins in human end-stage ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by a grant from the National Institutes of Health Institute of Alcohol Abuse and Alcoholism (NIAAA) 5R37 AA009300-18 (To D.R.P.). The authors also wish to acknowledge Joe Gomez and the Skaggs School of Pharmacy and Pharmaceutical Sciences Mass Spectrometry Core Facility for assistance in analysis of normal and end-stage ALD carbonylation.

Abbreviations

ALD	alcoholic liver disease
ALT	alanine aminotransferase
CID	collision-induced dissociation
Cyp2E1	Cytochrome P4502E1
ETD	electron transfer dissociation
EtOH	ethanol
4-HNE	4-hydroxy-2-nonenal
HHE	4-hydroxy-2-hexenal
4-ONE	4-oxononenal
MDA	malondialdehyde

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Highlights

- We demonstrate increased production of reactive aldehydes in human ALD
- Using LC-MS/MS we identify 411 carbonylated proteins unique to human ALD
- Pathway analysis revealed a propensity for adduction of GSH homeostatic proteins
- Using MS/MS, a nonenaldehyde adduct was identified on vimentin residue Lys235
- Reactive aldehyde modification of proteins contributes to pathogenesis of human ALD

Figure 1. Effects of chronic EtOH on fibrosis in humans

A. Paraffin embedded formalin fixed tissue sections were stained with hematoxylin and eosin or with PSR. Left panels; Hematoxylin eosin staining (original magnification 400X), Middle panels; PSR staining (original magnification 100X). Right panels; Polarized light exposure of PSR staining. **B.** Quantitative analysis of PSR staining (N=8 normal, 9=ALD, means $\pm$ SEM *** $p < 0.001$)

Figure 2. Effects of chronic EtOH abuse on hepatic protein carbonylation and Cyp2E1 expression

(A) Western blotting of hepatic whole cell extracts for post-translational carbonylation of proteins. (B) Quantitative analysis of carbonylated proteins (N=6/condition, means $\pm$ SEM **p<0.01, *p<0.05). (C) Immunohistochemical analysis of 4-HNE protein modification (Panels A, E-200X, Panels C, G-400X), Cyp2E1 expression (Panels B, F-200X, Panels D, H-400) and 4-HNE/Cyp2E1 colocalization (arrows). Paraffin embedded formalin fixed tissue sections were analyzed immunohistochemically using polyclonal antibodies directed against 4-HNE and Cyp2E1. Figures are representative of hepatic tissue isolated from four normal and four end-stage patients respectively.

Figure 3. VENN diagram showing the distribution of carbonylated proteins in normal and end stage ALD

Proteins identified in Supplemental Table S2 were analyzed using the VENN data analysis software at the Bioinformatics and Evolutionary Genomics (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Figure 4A.

Figure 4B

Figure 4. Bioinformatic analysis of carbonylated proteins in end-stage ALD

For enrichment analysis, five groups of proteins were identified. The ALD group includes proteins identified in the alcoholic liver disease samples, but not in the background sample. The Normal group includes proteins identified in the normal samples, but not the background sample. The ALD Only group includes proteins identified in the ALD samples but not the normal or background samples. The ALD and Normal group includes proteins identified in either the ALD or normal samples, but not in the background. The Background

group includes all proteins identified in the background sample. Proteins were functionally annotated and pathways were examined for enrichment using EnrichR. Pathways that were nominally significant ($p < 0.01$) in at least one of the 5 protein lists are included in the graphic. The colors of the heatmap range from white (unadjusted p -value > 0.01) to bright red based on the log base 10 transformation of the unadjusted p -value. A p -value of 1 was used when the pathway was not represented by any proteins in the list. **A. PANTHER Pathways Enriched For Carbonylated Proteins.** PANTHER pathways (rows) are ordered based on hierarchical clustering using the Euclidean distance and a binary indicator of significance ($p < 0.01$ vs. $p \geq 0.01$). **B. KEGG Pathways Enriched For Carbonylated Proteins.** KEGG pathways (rows) are ordered based on hierarchical clustering using the Euclidean distance and a binary indicator of significance ($p < 0.01$ vs. $p \geq 0.01$).

Figure 5. *In vivo* adduction of vimentin in human ALD

Nonenaldehyde (2-nonenal) modified peptide containing Lys²³⁵ (+140.22). MS/MS analysis was performed and the resulting b/y ion fragmentation confirmed peptide identity (KVESLQEEIAFLK*K).

Figure 6. Expression and carbonylation of vimentin in end-stage ALD

(A) Overall expression of vimentin in 10µg of whole cell extracts prepared from normal and end-stage ALD hepatic tissue (N=6/condition, means $\pm$ SEM **p<0.01, *p<0.05). Normalization was performed compared to GAPDH expression. (B) Vimentin carbonylation

is increased in end-stage ALD. 100 µg of protein isolated from human hepatic tissue from normal or end-stage ALD was incubated for 2 h with 5 mM biotin hydrazide (BH) and purified using streptavidin pulldown (SA) as described in Methods. Samples were subsequently analyzed using reducing SDS-PAGE/Western blotting with rabbit polyclonal anti-vimentin. Quantitative analysis of vimentin carbonylation (N=4/condition, means $\pm$ SEM **p<0.01, *p<0.05).

Figure 7. Global molecular modeling of Lys²³⁵ electrostatic interactions in vimentin
A. Ribbon diagram from X-Ray crystallographic structure of vimentin (3UF1³⁴) demonstrating locations of Lys²³⁵ adduct found on vimentin (Backbone-red, Lys²³⁵ residues on each domain (scaled ball and stick A,B,C,D-yellow). **B.** Ribbon diagram demonstrating locations of electrostatic interactions between Lys²³⁵ residues (scaled ball and stick, A,B,C,D-yellow) on each domain with Glu²³⁰ (scaled ball and stick A,B,C,D-blue). Also shown is the electrostatic interaction between Glu²³⁰ and Arg¹⁵⁸ (scaled ball and stick-purple).

Figure 8. Expression of hepatic GSTA4, GST π and GST μ expression in alcoholic cirrhosis (A) Western blotting of hepatic whole cell extracts for expression of GSTA4, GST π and GST μ (Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) was used as a loading control (B) Quantitative analysis of GSTA4, GST π and GST μ expression (Normalized against GAPDH, N=6/condition, means $\pm$ SEM ** p <0.01, * p <0.05).

Table 1**Serum biochemical parameters of human ALD patients**

Data was obtained from the University of Minnesota Liver Tissue cell Distribution Center NIH Contract #HHSN276201200017C. N=9, MELD (Model for End Stage Liver Disease), INR (International normalized ratio).

	Sex	Age	MELD score	INR	Bilirubin (mg/dl)	AST (U/L)	Alk. Phos. (U/L)	Albumin (g/dl)	Creatinine (mg/dl)
Patient 1	M	47	34	4.30	9.10	80	191	1.70	1.31
Patient 2	M	53	18	1.76	4.00	55	137	3.30	1.13
Patient 3	M	59	38	2.51	5.60	48	268	2.50	3.50
Patient 4	M	38	40	3.72	17.70	51	74	2.20	0.77
Patient 5	M	61	33	2.06	8	40	84	3.1	3.11
Patient 6	M	49	39	2.67	5.1	43	137	3.5	3.18
Patient 7	M	62	29	3.22	14	57	144	2	0.67
Patient 8	M	54	40	3.05	20.6	30	92	3.2	3.02
Patient 9	M	52	23	2.4	5.2	40	190	3.4	1.01
Mean $\pm$ SEM	M	52.78 $\pm$ 2.53	32.67 $\pm$ 3.33	2.85 $\pm$ 0.38	9.84 $\pm$ 2.04	49.33 $\pm$ 4.75	146.33 $\pm$ 20.73	2.77 $\pm$ 0.22	1.97 $\pm$ 0.44