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Research Paper

Chronic ethanol consumption induces mitochondrial protein acetylation and oxidative stress in the kidney

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ABSTRACT

In this study, we present the novel findings that chronic ethanol consumption induces mitochondrial protein hyperacetylation in the kidney and correlates with significantly increased renal oxidative stress. A major proteomic footprint of alcoholic liver disease (ALD) is an increase in hepatic mitochondrial protein acetylation. Protein hyperacetylation has been shown to alter enzymatic function of numerous proteins and plays a role in regulating metabolic processes. Renal mitochondrial targets of hyperacetylation include numerous metabolic and antioxidant pathways, such as lipid metabolism, oxidative phosphorylation, and amino acid metabolism, as well as glutathione and thioredoxin pathways. Disruption of protein lysine acetylation has the potential to impair renal function through metabolic dysregulation and decreased antioxidant capacity. Due to a significant elevation in ethanol-mediated renal oxidative stress, we highlight the acetylation of superoxide dismutase, peroxiredoxins, glutathione reductase, and glutathione transferase enzymes. Since oxidative stress is a known factor in ethanol-induced nephrotoxicity, we examined biochemical markers of protein hyperacetylation and oxidative stress. Our results demonstrate increased protein acetylation concurrent with depleted glutathione, altered Cys redox potential, and the presence of 4-HNE protein modifications in our 6-week model of early-stage alcoholic nephrotoxicity. These findings support the hypothesis that ethanol metabolism causes an influx of mitochondrial metabolic substrate, resulting in mitochondrial protein hyperacetylation with the potential to impact mitochondrial metabolic and antioxidant processes.

1. Introduction

A recent report by the World Health Organization found that roughly 3.3 million deaths, or 5.9% of all global deaths, were attributable to alcohol consumption [1]. While the dangers of alcohol consumption are well known, the precise molecular and cellular mechanisms of tissue injury associated with chronic alcohol use are highly complex and multifactorial [2–4]. Oxidative stress is thought to play a role in ethanol-induced renal damage [5].

The liver is a primary target organ of ethanol toxicity, causing pathologies like steatosis, inflammation, and oxidative stress [6]. The onset of alcoholic liver disease (ALD) correlates with chronic kidney disease; however, the impact of ethanol consumption on renal function remains largely unexamined [7]. Disruptions in renal function include increased oxidative stress and endothelial dysfunction [8]. While some studies suggest that chronic ethanol ingestion is not nephrotoxic, the development of acute kidney injury (AKI) associates with the mortality of ALD [5,8]. Thus, due to the fact that chronic ethanol consumption affects organ systems other than the liver, understanding the mechanisms that lead to impaired renal function is central to alleviating ethanol-related mortality.

A major proteomic footprint of ALD is a substantial increase in mitochondrial protein acetylation [9–11]. Protein hyperacetylation induces a suppressive effect on many metabolic pathways and likely plays a key role in regulating mitochondrial processes, such as antioxidant defense and energy metabolism [12]. Although enzymatic mechanisms of acetylation occur via acetyltransferase enzymes, mitochondrial protein acetylation is now hypothesized to occur mainly through non-enzymatic mechanisms [13–15]. The mitochondrial deacetylase sirtuin 3 (SIRT3) removes specific sites of acetylation; however, SIRT3 expression and activity are unchanged in a 6-week Lieber–DeCarli model of ethanol toxicity [16].

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A current hypothesis suggests that lysine acetylation serves, in part, as a covalent imprint of metabolic status, such as over-nutrition or under-nutrition [17]. Ethanol metabolism provides a hyper-influx of metabolic substrate through an overabundance of acetate. Amelioration of excess acetate generated from ethanol metabolism is primarily handled through lipid synthesis and cellular export into the bloodstream [18]. An intriguing and novel pathway for compensating with this acetate burden is through the non-enzymatic acetylation of protein lysine residues via acetyl-CoA.

Here, we report that ethanol-induced mitochondrial protein hyperacetylation occurs in the kidney. Utilizing a 6-week murine model of chronic ethanol consumption, renal mitochondrial proteins were examined by highly sensitive and specific acetylomics techniques, revealing ethanol-dependent increases in acetylated proteins. Pathway analyses demonstrated that hyperacetylated proteins from renal mitochondria are involved in a number of metabolic pathways, like the TCA cycle, oxidative phosphorylation, β-oxidation, and antioxidant defense. A key finding relating to a mechanism of alcoholic nephrotoxicity is the hyperacetylation of numerous antioxidant proteins. These hyperacetylated proteins include superoxide dismutase (SOD2), peroxiredoxins (PRX), glutathione reductase (GSR), and glutathione transferase (GST) enzymes. Given these particular targets of hyperacetylation, we examined markers of oxidative stress, including increased lipid peroxidation and altered renal cysteine and glutathione redox status.

2. Materials and methods

2.1. Animal studies

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado and were performed in accordance with published National Institutes of Health guidelines. Male, C57BL/6J mice were obtained (Jackson Laboratories) for pair-fed ethanol feeding studies ($n=12$). Mice were fed a modified Lieber–DeCarli liquid diet (Bio-Serv, Frenchtown, NJ) for 6 weeks as previously described [10,19]. Upon study completion, animals were anesthetized via intraperitoneal injection of sodium pentobarbital and euthanized via exsanguination. Kidneys were excised, weighed, and subjected to differential centrifugation using a sucrose gradient for subcellular fractionation to obtain mitochondrial-enriched fractions. Assays for plasma alanine aminotransferase (ALT) (Sekisui, Lexington, MA), hepatic and plasma triglycerides (Sekisui, Lexington, MA), plasma creatinine (Cayman, Ann Arbor, Michigan), and blood ethanol (BioAssay Systems, Hayward, CA) were performed as described elsewhere [20].

2.2. Western blotting

For immunoblotting, 25 μg of kidney mitochondrial extract were separated by standard SDS-PAGE using 12% gels run at 150 V for 1.5 h and transferred to a Hybond-PVDF membrane (GE Healthcare, Buckinghamshire, UK). Membranes were blocked for 60 min with TBS/0.1% Tween (TBS-T) and 5% non-fat dry milk (NFDM). Membranes were then probed with primary antibodies directed against acetyl-Lys (Cell Signaling, Danvers, MA). A protein assay (BCA) and paired VDAC Western using the identical conditions were performed to verify load control for the acetyl-Lys Western. Following 3 washes with TBS-T, a horseradish peroxidase conjugated secondary was applied for one hour at a 1:10,000 dilution and membrane visualization was performed via Storm Imager (GE Lifesciences, Pittsburgh, PA). Band intensities were quantified using Image J [21].

2.3. Immunohistochemistry

Sections of freshly excised kidney tissue were placed in 10% neutral buffered formalin (Sigma) for 16 h, followed by incubation in 70% ethanol overnight. Samples were processed, embedded in paraffin, cut, and sections mounted on slides by the University of Colorado Anschutz Medical Center Histology Core. Standard hematoxylin and eosin (H&E) and periodic-acid Schiff (PAS) staining was performed. Immunohistochemical characterization was performed using rabbit polyclonal antibodies directed against 4-HNE (in-house), cytochrome P450-2E1 (Cyp2E1) (Millipore, Billerica, MA) and acetyl-lysine (Cell Signaling, Woburn, MA) as described [22–24]. 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). Images in any composite were cropped and assembled in an identical way using Photoshop CS2 (Adobe Systems, Inc.; Mountain View, CA).

2.4. Determination of renal Cys and GSH redox status

Renal concentrations of cysteine (Cys), cystine (CySS), GSH, and GSSG were determined using a method by Jones et al.[49]. Briefly, a small piece of kidney tissue was extracted using 500 μL of a 5% (w/v) perchloric acid solution containing 0.2 M boric acid and 10 μM γ-Glu-Glu. The tissue was sonicated and protein was pelleted via centrifugation. 300 μL of supernatant was then incubated with iodoacetic acid for 20 min in the dark, then derivatized with dansyl chloride overnight at room temperature in the dark. Unreacted dansyl chloride was extracted using chloroform and the mixture was centrifuged. The aqueous layer (upper layer) was then analyzed by HPLC and fluorescence detection. The redox potentials for Cys (EhCySS) and GSH (EhGSSG) were calculated using the Nernst equation.

2.5. Acetylomics

Kidney mitochondrial proteins (1 mg) were trypsin-digested and incubated overnight at 4 °C with acetyl-Lys antibody conjugated to agarose (80 μL bead slurry, Immunechem, Burnaby, British Columbia, Canada) [10]. After incubation, supernatants were removed and the beads were washed 5 × with ice cold PBS. Peptides were eluted with 0.1 N HCl three times, pooled, evaporated to dryness, resuspended in 0.1% TFA in water, purified via C18 ZipTip (EMD Millipore), and resuspended in 0.1% formic acid in water for LC–MS/MS analysis. Identification of acetylated mitochondrial peptides was performed using nHPLC–MS/MS at a flow rate of 300 nL/min with a gradient of 5–40% 0.1% formic acid in acetonitrile (ACN) over 240 min on C18 trapping (20×0.1 mm^2) and analytical columns (250×0.075 mm^2). The nLC was coupled to a nano-ESI source and Impact HD Q-TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). The instrument was operated using intensity dependent CID MS/MS. MS/MS data analysis was performed using Mascot (version 2.2.04, Matrixscience) by Proteinscape (Bruker Daltonics, Billerica, MA). The final protein list included only protein IDs with a probability of 99% or greater and peptide IDs required a 95% cutoff. Manual validation of each acetylated peptide ID was performed to discard false positives and only acetylated peptides were included in our pathway analysis. The resulting gene list of acetylated proteins was analyzed by the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7) for biological process enrichment [25]. Significance was determined by fold enrichment >5.0 and Benjamini corrected $p<0.001$.