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# Isoaspartate, carbamoyl phosphate synthase-1, and carbonic anhydrase-III as biomarkers of liver injury



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## ABSTRACT

We had previously shown that alcohol consumption can induce cellular isoaspartate protein damage via an impairment of the activity of protein isoaspartyl methyltransferase (PIMT), an enzyme that triggers repair of isoaspartate protein damage. To further investigate the mechanism of isoaspartate accumulation, hepatocytes cultured from control or 4-week ethanol-fed rats were incubated *in vitro* with tubercidin or adenosine. Both these agents, known to elevate intracellular S-adenosylhomocysteine levels, increased cellular isoaspartate damage over that recorded following ethanol consumption *in vivo*. Increased isoaspartate damage was attenuated by treatment with betaine. To characterize isoaspartate-damaged proteins that accumulate after ethanol administration, rat liver cytosolic proteins were methylated using exogenous PIMT and <sup>3</sup>H-S-adenosylmethionine and proteins resolved by gel electrophoresis. Three major protein bands of ~75–80 kDa, ~95–100 kDa, and ~155–160 kDa were identified by autoradiography. Column chromatography used to enrich isoaspartate-damaged proteins indicated that damaged proteins from ethanol-fed rats were similar to those that accrued in the livers of PIMT knockout (KO) mice. Carbamoyl phosphate synthase-1 (CPS-1) was partially purified and identified as the ~160 kDa protein target of PIMT in ethanol-fed rats and in PIMT KO mice. Analysis of the liver proteome of 4-week ethanol-fed rats and PIMT KO mice demonstrated elevated cytosolic CPS-1 and betaine homocysteine S-methyltransferase-1 when compared to their respective controls, and a significant reduction of carbonic anhydrase-III (CA-III) evident only in ethanol-fed rats. Ethanol feeding of rats for 8 weeks resulted in a larger (~2.3-fold) increase in CPS-1 levels compared to 4-week ethanol feeding indicating that CPS-1 accumulation correlated with the duration of ethanol consumption. Collectively, our results suggest that elevated isoaspartate and CPS-1, and reduced CA-III levels could serve as biomarkers of hepatocellular injury.

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## 1. Introduction

The excessive use of alcohol as a licit, recreational drug is a global healthcare problem, with enormous social and economic

impact [1–3]. Worldwide, the number of deaths attributed to alcohol is 3.3 million every year, and accounts for 5.9% of all deaths [3].

Alcohol consumption has causal linkage to a number of diseases including liver cirrhosis [2]. Since the liver is the main organ responsible for metabolizing alcohol it is particularly susceptible to alcohol's toxic effects. Research conducted over several decades has established that alcohol causes liver damage by a number of

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mechanisms including an increase of isoaspartate protein damage [4–8].

Accumulation of protein damage as isoaspartate in cellular proteins is normally restricted via enzymatic methylation by protein isoaspartyl methyltransferase (PIMT) to produce isoaspartate methyl esters (Fig. 1). Isoaspartate methyl esters are unstable at physiological pH and revert to a normal peptide backbone containing an aspartic acid via a succinimide intermediate [9,10]. This repair process and elimination of isoaspartate can be concomitant with restoration of protein function [11].

Herein we investigated the formation of isoaspartate protein damage in the livers and hepatocytes of ethanol-fed rats, and also assessed the ability of betaine supplementation to limit isoaspartate formation. We then characterized the proteins that accumulate isoaspartate damage by one dimensional polyacrylamide gel electrophoresis, employed column chromatography to partially purify a protein target of PIMT in ethanol-fed rats and PIMT KO mice, and present evidence of new biomarkers of hepatocellular injury.

## 2. Materials and methods

### 2.1. Animals and ethics statement

#### 2.1.1. Rats

Male Wistar rats of 180–200 g body weight were fed Lieber DeCarli control and ethanol liquid diets for 4 weeks as previously described [12]. Additionally, rats were fed control or ethanol diets for 8 weeks as a longer-term ethanol consumption study. Liver cytosolic proteins were prepared according to a previous publication [12]. The care, use, and procedures performed on these rats were approved by the Institutional Animal Care and Use Committee at the Omaha Veterans Affairs Medical Center, USA, under approved protocol OMA-636-00534.

#### 2.1.2. Mice

The production and breeding conditions for *Pcmt1*<sup>+/+</sup> (wild-type) and *Pcmt1*<sup>−/−</sup> (PIMT KO mice) have been reported previously [13,14]. Mice were monitored by on-site veterinarians, with all protocols undertaken in strict accordance with the recommendations for the Care and Use of Laboratory Animals, and with approval by the University of California at Los Angeles Institutional Animal Care and Use Committee, under approved protocol ARC #1993-109-

62. DNA samples from the tails of mice were genotyped by the polymerase chain reaction to confirm gene knockout [15]. Preparation of *Pcmt1*<sup>−/−</sup> liver cytosolic proteins was according to published procedures [15].

#### 2.1.3. Hepatocyte isolation and homogenisation

Rat liver hepatocytes were isolated using the collagenase-perfusion technique and cultured in 5% foetal calf serum supplemented Williams' medium E [12]. Hepatocytes were incubated with 0.75 mM adenosine or 5  $\mu$ M tubercidin in the presence or absence of 2 mM betaine for 24 h. Hepatocytes were washed twice with phosphate buffered saline (PBS), and then scraped into PBS, before centrifugation at 1400 rpm to retain cell pellets. Cell pellets were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required. Thawed hepatocyte pellets were homogenised in 100  $\mu$ l of 5 mM Tris/HCl pH 7.4, 0.25 M sucrose, 1 mM EDTA, and complete mini protease inhibitor cocktail (Roche).

#### 2.1.4. Protein concentrations

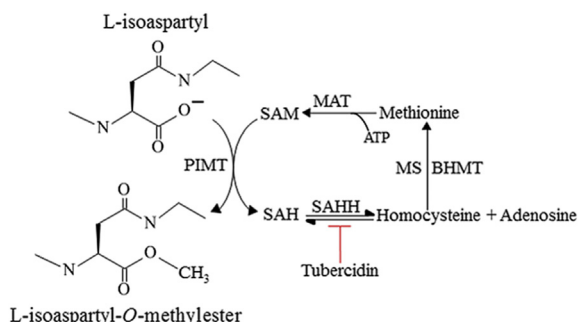
Protein concentrations of liver or hepatocyte homogenates were determined using the DC protein assay (Biorad) using a bovine serum albumin standard set (Biorad 500-0207) as protein standards.

#### 2.1.5. Isoaspartate protein damage quantitation

Isoaspartate levels in hepatocyte lysates were quantified by radiolabelled methylation based upon the method of Aswad and Deight [16]. Briefly, 100–200  $\mu$ g of protein were incubated in a final reaction volume of 50  $\mu$ l, containing 40 mM K-2(N-morpholino)ethanesulphonic acid (MES), pH 6.2, 20  $\mu$ M S-Adenosyl-L-[ $^3\text{H}$ -methyl]methionine ( $^3\text{H}$ -SAM) (Amersham Biosciences, UK, 37 MBq/ml) (2220 dpm/pmol), and 2  $\mu$ M recombinant bovine PIMT. Methylation was initiated by the addition of PIMT and incubation for 30 min at  $30^{\circ}\text{C}$ , and terminated by adding 1 ml of ice-cold 7% (w/v) trichloroacetic acid (TCA). Protein precipitated in the presence of TCA was sequentially washed and redissolved in 100  $\mu$ l of 88% (v/v) formic acid as described previously [17] to remove extraneous  $^3\text{H}$ -SAM. The protein precipitate was finally solubilised in 100  $\mu$ l of 0.5 M NaOH, 1% (v/v) methanol, 5% (v/v) Triton X-100, and transferred to 10 ml of scintillant and counted for radioactivity incorporation using a Packard Tri-Carb counter. Replicate assays were performed for all assay points from which an average value of radiolabel incorporation determined. Radiolabel incorporation into extracts in the absence of PIMT addition (buffer controls) due to endogenous methylation was subtracted from assay values. Quantitation of isoaspartate levels in hepatocytes was performed on 5–8 separate experiments for each treatment group. Isoaspartate quantitation data was analysed by three way analysis of variance (ANOVA) with all pairwise multiple comparison procedures (Holm-Sidak method) using SigmaStat. Results in Figures are expressed as means  $\pm$  SEM. A *p* value of  $<0.05$  was regarded as statistically significant.

#### 2.1.6. One dimensional polyacrylamide gel electrophoresis (1D PAGE)

Liver cytosolic proteins were methylated for 30 min at  $30^{\circ}\text{C}$  in a final volume of 20  $\mu$ l in a buffer of 50 mM K-MES buffer, pH 6.2, containing 20  $\mu$ M  $^3\text{H}$ -SAM (8250 dpm/pmol) [18]. Methylated proteins were reduced and heat-denatured and resolved on 10% Bis-Tris NuPAGE Novex pre-cast gels run with MES running buffer according to published procedures [19], in order to maintain base-labile methyl esters. For visualization, resolved proteins were either stained with colloidal Coomassie blue (Invitrogen) and then photographed using a Fujifilm digital camera, or scanned using an Odyssey laser scanner (LI-COR Biosciences, Lincoln, NE).



**Fig. 1.** Carboxyl methylation of isoaspartate protein damage by PIMT. Carboxyl methylation of L-isoaspartyl residues in proteins is catalysed by PIMT using SAM (methyl donor) to form an L-isoaspartyl-O-methylester and SAH. SAH hydrolysis to homocysteine and adenosine is catalysed by SAH hydrolase (SAHH), and can be inhibited by tubercidin. Re-methylation of homocysteine to methionine is provided by either vitamin B12 dependent methionine synthase (MS) activity or from the activity of betaine homocysteine methyltransferase (BHMT) using betaine. The conversion of methionine to SAM by methionine adenosyltransferase (MAT) utilizing ATP completes this cyclic part of the methionine metabolic pathway.

Alternatively, proteins resolved by 1D PAGE were transferred at 80 V for 2 h to a polyvinylidene difluoride (PVDF) membrane for autoradiography or Western blotting. Methylation and 1D PAGE were performed on 7 sets of control and ethanol pair-fed rats, with each set analysed 1–2 times.

### 2.1.7. Autoradiography

PVDF membranes were applied to a microchannel plate (MCP) autoradiographic imager for 48 h to visualize radiolabelled proteins [20].

### 2.1.8. Protein purification

Liver cytosolic proteins bearing isoaspartate protein damage were enriched by column chromatography using an FPLC system (GE Healthcare). A MONO Q anion exchange column (HR 5/5) was equilibrated with 50 mM Tris/HCl pH 8.0 (Buffer A) at a flow rate of 0.75 ml/min. Liver cytosolic proteins were diluted with Buffer A and then loaded onto the MONO Q column at a flow rate of 0.75 ml/min. The column was washed with Buffer A and then protein eluted with a 15 min linear gradient of 0–1 M NaCl in Buffer A collecting 0.75 ml fractions. Twenty  $\mu$ l from each fraction was resolved by 1D PAGE, proteins stained with colloidal Coomassie blue, and then laser densitometry performed using an Odyssey laser scanner. Replicate 20  $\mu$ l samples were also removed from each column fraction and the level of isoaspartate present quantified by exogenous methylation using PIMT and  $^3$ H-SAM as described above. Chromatograms from PIMT KO mice or ethanol-fed rats depicted in Figures were representative of three or four separate chromatography experiments respectively.

### 2.1.9. Mass spectrometry

Proteins were excised from gels, trypsinolysed, and purified using solid-phase extraction as described previously [21]. Peptides were analysed by matrix assisted laser-desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry using a LaserTof TT (SAI Ltd) operated in positive ion and reflectron modes. Alternatively peptides underwent microcapillary liquid chromatography tandem mass spectrometry (LC-MS/MS) using a hybrid Q-TOF instrument (Waters QToF2) equipped with a nanoelectrospray ion-source and controlled using MassLynx 4.0 software. Data-dependent product ion experiments were performed and protein identifications ascertained using the MS/MS Ion Search program in the MASCOT search engine (<http://www.matrixscience.com>). Details of the peptides identified by mass spectrometry are included as [Supplementary data section S1](#).

### 2.1.10. Western (immuno) blotting

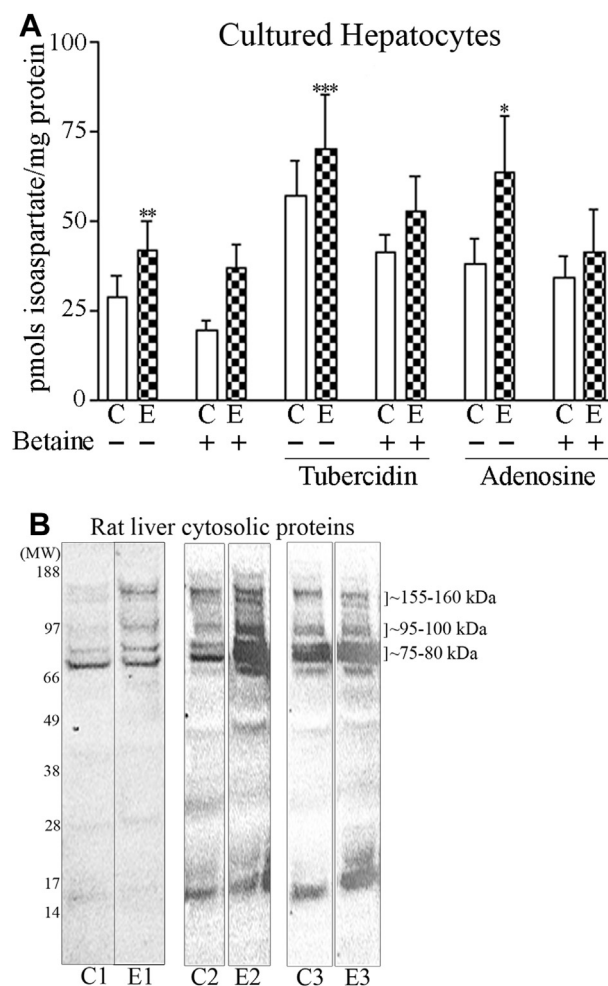
PVDF membranes were prepared according to published procedures [22], and incubated overnight at 4 °C with the primary antibody of interest: rabbit polyclonal to CPS-1 (Santa Cruz, sc-30060) at 1:500; rabbit polyclonal to betaine homocysteine S-methyltransferase (BHMT) (Abcam, ab96415) at 1:500; goat polyclonal to carbonic anhydrase-III (CA-III) (Santa Cruz, sc-50715) at 1:500; rabbit polyclonal to actin (Santa Cruz, sc-1616-R) at 1:1000; mouse monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, ab8245) at 1:3000. Blots were washed in PBS-T and then incubated for 1 h at room temperature with their corresponding horseradish peroxidase conjugated anti-species secondary antibody at 1:1000 dilution in PBS-T (Dako products P0448, P0449, P0447). Antibody immunoreactivity was visualised using SuperSignal West Pico Chemiluminescent substrate (Pierce) with the light generated captured using a Chemi Doc device (Bio-Rad). Blots were quantified using QuantityOne software intrinsic to the Chemi Doc device, or via the image processing and analysis program, Image J, developed at the National Institutes of

Health. Actin was used for normalization of rat liver protein blotting, and GAPDH used for normalization of mouse liver protein blotting. Three pairs of *Pcmt1*<sup>+/+</sup> and *Pcmt1*<sup>-/-</sup> littermates were used for blotting studies with examples of protein changes shown in Figures. For quantitation of protein bands on Western blots, statistical comparison between data means was performed with a Student's t-test using GraphPad Prism 4 software. A *p* value of <0.05 was regarded as statistically significant.

## 3. Results

### 3.1. Accumulation of isoaspartate damaged proteins in hepatocytes

Hepatocytes isolated from 4-week ethanol-fed rats exhibited a significant 29% increase (*p* = 0.006) in isoaspartate levels from those of controls (Fig. 2A). When subsequently incubated *in vitro* with tubercidin or adenosine, isoaspartate levels increased by 2.12 fold (*p* = 0.0002) and 1.60-fold respectively (*p* = 0.013) over their non-treated controls. This increase of isoaspartate was in addition to that occurring *in vivo* following ethanol-feeding. Incubation of



**Fig. 2.** Isoaspartate protein damage in cultured hepatocytes, and rat liver cytosolic proteins. (A) Hepatocytes cultured from control or ethanol-fed rats were treated with exogenous betaine, tubercidin, or adenosine, and the levels of isoaspartate damage quantified. (B) Liver cytosolic proteins from control (C) or ethanol (E) fed rats were methylated by PIMT using  $^3$ H-SAM. Radiolabelled proteins were resolved by 1D PAGE and visualised by autoradiography. Increased methylation of proteins from ethanol-fed rats was evident at molecular weights of ~75–80 kDa, ~95–100 kDa, and ~155–160 kDa. Profiles of radiolabelled proteins are shown from 3 different pairs of control and ethanol pair-fed animals.



hepatocytes with exogenous betaine produced a significant 22% reduction ( $p = 0.036$ ) of isoaspartate damage.

To characterise the liver proteins that bear isoaspartate protein damage and accumulate in ethanol-fed rats, cytosolic proteins from the livers of rats fed the control or ethanol diet were prepared and methylated with  $^3\text{H}$ -SAM using exogenous PIMT to radiolabel the isoaspartate. Radiolabelled proteins were then resolved by 1D PAGE and visualized by autoradiography. A number of methylated proteins were evident across a broad range of molecular weights (~15–170 kDa). However, more pronounced methylation was noted at ~75–80 kDa and at two doublets of ~95–100 kDa and ~155–160 kDa in proteins from ethanol-fed rats (Fig. 2B).

### 3.2. Enrichment and purification of PIMT target proteins

Liver cytosolic proteins from ethanol-fed rats and from PIMT KO mice were enriched using MONO Q column chromatography. Column fractions were resolved by 1D PAGE and stained with colloidal Coomassie – Fig. 3A, upper panel. Isoaspartate containing proteins in each fraction were quantified by methylation with  $^3\text{H}$ -SAM and recombinant PIMT, and liquid scintillation counting of methyl esters – Fig. 3A, lower panel. Peak isoaspartate containing fractions 7–10 retained a protein doublet at ~160 kDa in both PIMT KO mice and ethanol-fed rats. These column fractions were further resolved and concentrated using a microcon concentrator (Fisher) with a 100 kDa cut-off membrane; to facilitate rapid purification by removal of lower molecular weight proteins. The ~160 kDa proteins (and associated proteins) were concentrated to two fractions and resolved by 1D PAGE, with isoaspartate levels visualised by exogenous methylation with PIMT using  $^3\text{H}$ -SAM and autoradiography (Fig. 3B). Co-distribution of protein staining and isoaspartate radiolabelling was evident for the ~160 kDa protein from PIMT KO mice and ethanol-fed rats. This protein was extracted from gels and identified as Carbamoyl phosphate synthase-1 (CPS-1) by mass spectrometry (Supplementary material S1).

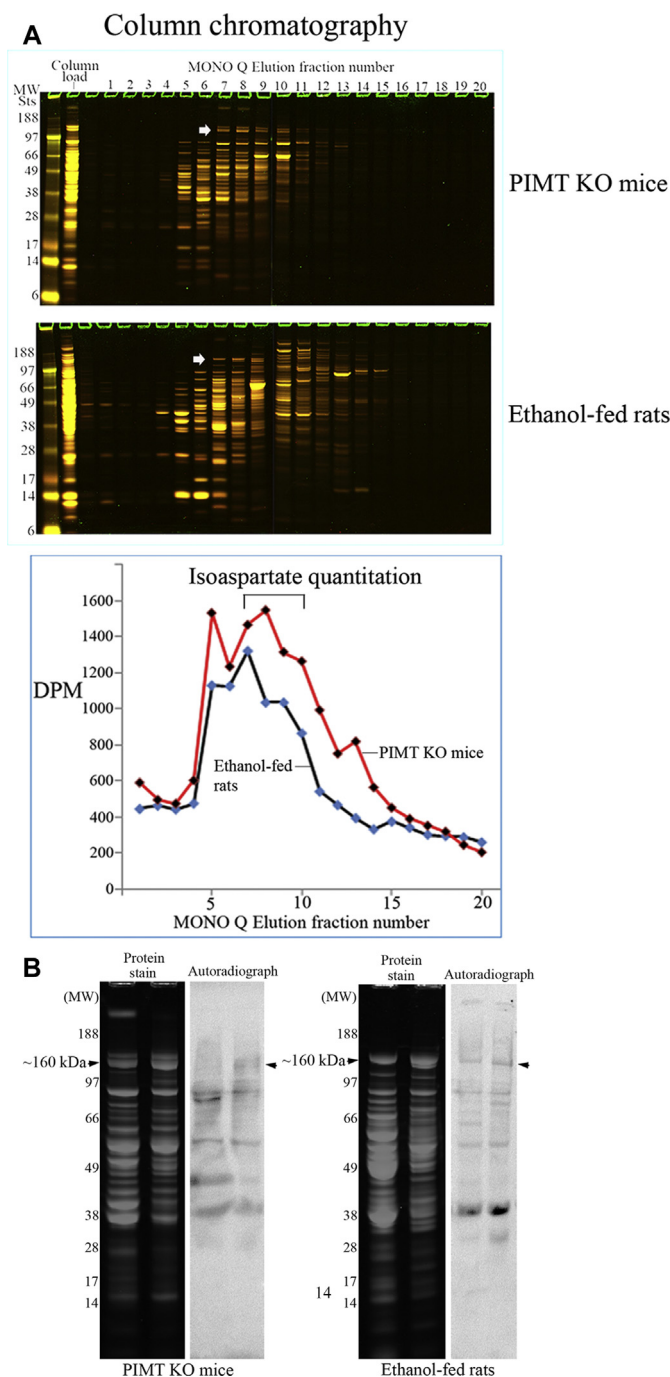
### 3.3. Overlapping changes in the liver proteome of ethanol-fed rats and PIMT knockout mice

Rats fed ethanol for 4 weeks displayed a modest (~20%) increase in CPS-1 protein levels, a significant 64% increase ( $p = 0.032$ ) in BHMT-1 and significant near complete downregulation (~98%,  $p = 0.009$ ) of CA-III (Fig. 4A). After extending the time of ethanol feeding to 8 weeks, rat CPS-1 protein levels had now increased to a significant 2.27-fold ( $p = 0.016$ ), whereas BHMT-1 levels did not change significantly (Fig. 4B). Interestingly, elevated CPS-1 (significant 53% increase ( $p = 0.044$ )) and BHMT-1 (significant 43% increase ( $p = 0.041$ )) were also the most visually prominent changes in liver cytosols from PIMT KO mice when compared to their wild-type littermates (Fig. 4B & Supplementary material S1).

## 4. Discussion

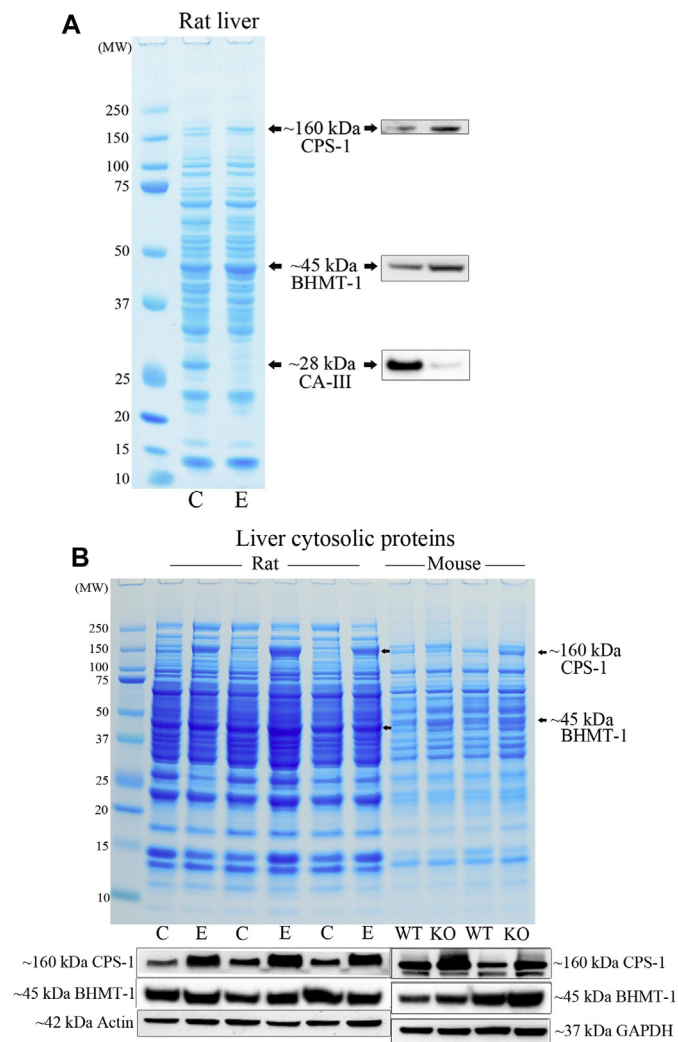
### 4.1. Ethanol-induced disruption of the methionine metabolic pathway elevates isoaspartate protein damage

We hypothesized that ethanol-induced elevation of SAH levels is responsible for inhibition of PIMT and the resultant elevation of isoaspartate protein damage. To verify this, cultured hepatocytes were incubated with tubercidin or adenosine; agents that increase cellular SAH levels via inhibition of SAHH, or promotion of SAH formation from adenosine and homocysteine, respectively. With either agent, a further increase of isoaspartate damage over that attained from ethanol feeding was observed. This may have important *in vivo* implications since it suggests that multiple steps



**Fig. 3.** Enrichment and partial purification of isoaspartate damaged proteins. **(A)** Liver cytosolic proteins from PIMT KO mice or ethanol-fed rats were enriched through binding and elution from a MONO Q column. Proteins were stained with colloidal Coomassie (upper panel). The start of elution of the ~160 kDa protein doublet in fraction 7 is marked with an arrowhead. The level of isoaspartate damage in each fraction was quantified (lower panel). **(B)** Peak column fractions 7–10 were concentrated, fractionated, and then resolved by 1D PAGE. Proteins were stained with colloidal Coomassie, or radiolabelled by PIMT using  $^3\text{H}$ -SAM, and radiolabelled proteins visualised by autoradiography. Co-distribution of protein staining and isoaspartate radiolabelling was evident for the ~160 kDa protein for both PIMT KO mice and ethanol-fed rats (marked with arrowheads).

of the methionine metabolic pathway that are impaired by ethanol exposure, can be further affected by agents that elevate intracellular SAH levels to produce an additive increase of protein damage as isoaspartate in the liver. In support of the conjecture, folate (vitamin B12) depletion can limit homocysteine remethylation via



**Fig. 4.** Comparison of the liver proteome of ethanol-fed rats and PIMT KO mice. **(A)** Liver proteome from rats fed a control (C) or ethanol (E) diet for 4 weeks were resolved by 1D PAGE and stained with colloidal Coomassie. Elevated CPS-1 and BHMT-1, and reduced CA-III protein levels were visualised by Western blotting. **(B)** Liver proteome from rats fed a control (C) or ethanol (E) diet for 8 weeks were compared with the proteome of PIMT wild-type (WT) and knockout (KO) mice (upper panel). CPS-1 and BHMT-1 protein levels were visualised by Western blotting (lower panels).

the methionine synthase-catalysed reaction, and the resultant increase in intracellular SAH levels can cause a similar increase in hepatic isoaspartate levels [23]. We had previously shown that feeding the ethanol-fed rats a diet supplemented with betaine was able to preserve a normal liver SAM:SAH ratio, and reduce isoaspartate accumulation [8]. The current study demonstrates that *in vitro* betaine treatment is also efficacious for lowering hepatocellular isoaspartate levels (Fig. 2A).

#### 4.2. CPS-1: a new liver target of PIMT

Chromatographic separation enabled us to directly compare the profile of isoaspartate-damaged proteins that accumulate in ethanol-fed rats due to impaired PIMT activity with those in mice in which PIMT is knocked out. The chromatograms showed remarkable overlap indicative of similar PIMT target proteins in these two models. We were able to purify one such target and identified it as CPS-1. The function of mitochondrial CPS-1 is to catalyse the synthesis of carbamoyl phosphate from ammonia and bicarbonate.

This is the first committed (rate-limiting) step of the urea cycle in liver, and is important in humans for nitrogen disposal via ureagenesis [24]. CPS-1 contains a number of primary canonical sequences that could form isoaspartate (Supplementary Tables 1A and 1B), but how this could impact upon protein function has yet to be determined. Our studies also revealed additional PIMT substrates, for which further chromatography and fractionation will be required to identify them.

#### 4.3. Isoaspartate, CPS-1, and CA-III as biomarkers of hepatocellular injury

An increase of liver protein damage as isoaspartate may prove a useful indication of the inhibition of PIMT activity via disruption of the methionine metabolic pathway and elevated SAH levels. An examination of the liver proteome revealed that the levels of cytosolic CPS-1 increased in parallel with the duration of alcohol consumption. Increased CPS-1 levels are presumed to reflect mitochondrial damage and redox stress [25], a process also observed in PIMT KO mice [26]. Liver BHMT-1 was also elevated in 4 week ethanol-fed rats and PIMT KO mice. BHMT-1 catalyses the remethylation of homocysteine using betaine as a methyl source to produce methionine, a precursor of SAM, as well as reducing SAH levels (Fig. 1). Hence BHMT-1 upregulation is regarded as a compensatory mechanism to restore a normal SAM:SAH ratio [27]. In contrast to CPS-1 and BHMT-1, CA-III levels are downregulated by ethanol administration or treatment with a hepatotoxicant, carbon tetrachloride [27–30].

In summary, ethanol-induced liver damage triggers an increase of protein isoaspartate levels, increased cytosolic CPS-1, and depleted CA-III levels; protein changes that may be useful biomarkers of liver injury.

#### Conflict of interest

The manuscript authors confirm that they have no commercial associations that pose a conflict of interest in connection with publication of this article.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2015.01.158>.

## Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.01.158>.

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