ELSEVIER

Biochimica et Biophysica Acta 1291 (1996) 45–52

BBA Biochimica et Biophysica Acta

Ethanol consumption alters trafficking of lysosomal enzymes and affects the processing of procathepsin L in rat liver

Kusum K. Kharbanda, Daniel L. McVicker, Rowen K. Zetterman, Terrence M. Donohue Jr. *

Liver Study Unit, Department of Veterans Affairs (VA) Medical Center, and Departments of Medicine and Biochemistry / Molecular Biology, University of Nebraska College of Medicine, 4101 Woolworth Avenue, Omaha, NE 68105, USA

Received 12 March 1996; accepted 17 April 1996

Abstract

In order to determine whether ethanol consumption alters the targeting of hepatic lysosomal enzymes to their organelles, we examined the sedimentation properties of lysosomal hydrolases in ethanol-fed rats and their pair-fed controls. Rats were fed a liquid diet containing either ethanol (36% of calories) or isocaloric maltose dextrin for one to five wk. Liver extracts were fractionated by Percoll density gradient centrifugation and fractions obtained were analyzed for the distribution of lysosomal marker enzymes. Heavy lysosomes were further purified from these gradients and the activity of specific hydrolases was determined. Compared with those from controls, isolated lysosomes from ethanol-fed rats showed a 20–50% reduction in the activity of lysosomal acid phosphatase and β-galactosidase. Decreased intralysosomal hydrolase activity in ethanol-fed rats was associated with a significant redistribution of these enzymes as well as those of cathepsins B and L to lighter fractions of Percoll density gradients. This indicated an ethanol-elicited shift of these enzymes to lower density cellular compartments. In order to determine whether ethanol administration affects the synthesis and proteolytic maturation of hepatic procathepsin L, we conducted immunoblot analyses to quantify the steady-state levels of precursor and mature forms of cathepsin L in hepatic post-nuclear fractions. Ethanol administration caused a significant elevation in the steady-state level of the 39 kDa cathepsin L precursor relative to its 30 kDa intermediate and 25 kDa mature product. These results were confirmed by pulse-chase experiments using isolated hepatocytes exposed to [^{35}S]methionine. Hepatocytes from both control and ethanol-fed rats incorporated equal levels of radioactivity into procathepsin L. However, during the chase period, the ratios of the 39 kDa procathepsin L to its 30 kDa intermediate and 25 kDa mature product in cells from ethanol-fed rats were 1.5–3-fold higher than those in controls. These results demonstrate that ethanol consumption caused a marked impairment in the processing of procathepsin L to mature enzyme, without affecting its synthesis. Taken together, our findings suggest that chronic ethanol consumption caused a deficiency in intralysosomal enzyme content by altering the trafficking and processing of these hydrolases into lysosomes.

Keywords: Liver; Ethanol; Lysosome; Proteinase; Hydrolase; Trafficking; Cathepsin L; Intracellular processing

1. Introduction

In earlier studies, we showed that the intralysosomal content of cathepsins B and L in livers of ethanol-fed rats was lower than that in pair-fed control rats. However, while ethanol consumption also lowered the total cellular amount of cathepsin B, it did not affect the cellular quantity of cathepsin L [1]. These findings suggest that ethanol administration alters the biosynthesis and/or trafficking of these proteases to lysosomes. In the present study we sought to ascertain whether ethanol administration altered the intracellular trafficking of these and other lysosomal enzymes. In this study, we determined the activities of lysosomal acid phosphatase and β-galactosidase in hepatic extracts as well as in isolated lysosomes from both control and ethanol-fed rats. We then examined the distribution of these enzymes and those of cathepsins B, L and H, in Percoll density gradients to determine whether ethanol administration affected their sedimentation properties. We then focussed our attention on cathepsin L and conducted immunoblot analyses to determine the steady state levels of precursor and mature forms of this enzyme. In addition, we measured the synthesis and processing of cathepsin L

Abbreviations: IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; N-Cbz, *N*-carbobenzoxy derivatives; BSA, bovine serum albumin; Hepes, *N*-[2-hydroxyethyl]piperazine *N'*-[2-ethanesulfonic acid]; KRH, Krebs-Ringers Hepes buffer; MEM, minimum essential medium; V.I.U., volume integration units.

* Corresponding author. Fax: +1 (402) 4490604.

PII S0304-4165(96)00043-8

by pulse-chase analysis to directly examine whether ethanol administration affected its synthesis and proteolytic processing. Preliminary versions of this work have been published as abstracts [2,3].

2. Materials and methods

Male Sprague-Dawley-derived rats were purchased from Small Animal Supply Company (Omaha, NE). Control and ethanol liquid diets, formulated by Lieber and DeCarli [4], were from Dyets (Bethlehem, PA). Immunoglobulins conjugated to alkaline phosphatase and reagents for electrophoresis and electroblotting were obtained from BioRad (Richmond, CA). Percoll, density marker beads, the respective substrates for cathepsins H, B, L, β-galactosidase and acid phosphatase, namely L-arginine-7-amido-4-methylcoumarin hydrochloride, *N*-Cbz-L-arginyl-L-arginine-7-amido-4-methylcoumarin hydrochloride, *N*-Cbz-phenylalanyl-arginine-7-amido-4-methylcoumarin hydrochloride, 4-methylumbelliferyl-1-β-D-galactoside, Sigma-104 phosphatase substrate, methionine-free MEM, Hepes, BSA, collagenase (Type IV), Trypan blue, L-methionine, leupeptin, chymostatin, pepstatin A, antipain, Protein A-Sepharose 4B and ^{14}C-labeled standard marker proteins were from Sigma (St. Louis, MO). Prestained molecular weight standards for electrophoresis and L-[^{35}S]methionine (> 1000 Ci/mmol) were from Amersham (Arlington Heights, IL). Immobilon-P membranes were from Millipore (Bedford, PA). Spectramesh polypropylene filter mesh (100 μm pore size) was purchased from Spectrum, (Houston, TX). Ultima Gold scintillation fluid was purchased from Packard Instrument (Meriden, CT). All other chemicals were reagent-grade quality. Antiserum to cathepsin L was a gift from Dr Michael Gottesman, National Cancer Institute, Bethesda, MD, USA and was raised in rabbits against the major excreted protein (MEP) derived from the culture medium of Kirsten-virus transformed 3T3 mouse fibroblasts.

2.1. Animal procedures

These were performed exactly as described [1,5] in accordance with Public Health Service Guidelines for care and use of laboratory animals. Animals were maintained in an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). When rats reached a weight of 150 to 170 g, they were weight-matched and pair-fed with ethanol or isocaloric control diet for one to five weeks, as detailed [1,5].

2.1.1. Preparation of liver homogenates and isolated lysosomes

Each rat was anesthetized with sodium pentobarbital (50 mg/kg body wt), the portal vein cannulated and each liver perfused with ice-cold 0.25 M sucrose (pH 7.4). Following perfusion, the liver was excised, blotted and weighed. Twenty percent homogenates (w/v) were then prepared in 0.25 M sucrose (pH 7.4) using 4 strokes of a Teflon pestle spinning at 200 rpm in a glass homogenizing vessel. Lysosomes were isolated by the procedure of Yamada et al. [6] as previously described [1].

2.2. Density gradient fractionation

Percoll gradient fractionation of the hepatic post-nuclear fraction was performed by the procedure of Yamada et al. [6]. Briefly, calcium chloride-treated post-nuclear supernatants from control and ethanol-fed rats were layered onto 27 ml of iso-osmotic Percoll (density 1.08 g/ml, pH 7.4). The tubes were centrifuged at $80\,000 \times g$ for 15 min, using a 50.2 Ti rotor, generating a density gradient to achieve separation of dense lysosomes from other subcellular organelles. The gradient was collected in 2 ml fractions by pumping from the bottom of each tube. The density profile of the Percoll gradient was determined by using colored latex beads.

Each fraction was diluted two-fold with 0.25 M sucrose (pH 7.4) containing 0.2% Triton X-100 and analyzed for the activities of lysosomal enzymes [7–10]. In addition, the distribution of other subcellular fractions were determined by assays of their respective marker enzymes, glucose-6-phosphatase (microsomes), succinic dehydrogenase (mitochondria), 5′-nucleotidase (plasma membrane) and lactic dehydrogenase (cytosol) [11–14].

2.3. Enzyme assays and analytical procedures

The activities of lysosomal marker enzymes, acid phosphatase and β-galactosidase were determined in post-nuclear fractions and in isolated lysosomes obtained from both control and ethanol-fed rats [7–10]. One milliunit of cathepsin B, L or H activity catalyzed the formation of one nmol aminomethylcoumarin per min from its respective substrate. One milliunit of acid phosphatase and one milliunit of β-galactosidase each catalyzed the release of one nmol of *p*-nitrophenol and 7-hydroxy-4-methylumbelliferone per min, respectively. Protein concentrations were assayed by the BioRad assay using BSA as the standard [15].

2.4. Immunoblot analyses

Thirty micrograms of protein from hepatic post-nuclear supernatant fractions were subjected to SDS-PAGE [16] in 12% polyacrylamide gels. Proteins were electrophoretically transferred from the gels to Immobilon-P membranes using a modification of the method of Towbin and Gordon [17]. Membranes were incubated with 1000-fold diluted rabbit anti-cathepsin L. Membranes were then washed and incubated with a 1000-fold diluted appropriate second

antibody conjugated to alkaline phosphatase as detailed before [1]. Immunoreactive protein bands were developed enzymatically with BCIP and NBT as the substrate and developer respectively. The color intensity of each reactive protein band was quantitated as arbitrary volume integration units (V.I.U.) by reflectance densitometry on a Molecular Dynamics densitometer using the Molecular Dynamics ImageQuant software.

2.5. Pulse-chase analyses

2.5.1. Preparation of hepatocytes

Hepatocytes were prepared by the collagenase perfusion method of Seglen [18] as modified by Casey et al. [19]. Briefly, rats were anesthetized and livers perfused in situ via the portal vein at a rate of 30 ml/min with Ca^{2+}-free Krebs-Ringer Hepes (KRH) buffer (pH 7.4). This was followed by perfusing in a recirculating mode with 0.03% collagenase (Type IV) in KRH containing 0.5% BSA and 2.5 mM calcium chloride. Livers were excised and gently swirled in Ca^{2+}- and 2% BSA-containing KRH to disperse the cells which were filtered through polypropylene mesh (100 μm opening) and pelleted by centrifugation. Cells were further purified using continuous Percoll gradients [19]. After washing, the cell pellets were resuspended in methionine-free MEM containing 0.5% BSA. Viability, as assessed by trypan blue exclusion, was routinely greater than 95%.

2.5.2. Methionine labeling

Hepatocytes from control and ethanol-fed rats were preincubated at 37°C for 30 min in methionine-free MEM supplemented with 0.5% BSA at densities of 3 million cells /ml in a total of five ml. Hepatocellular proteins were pulse-labeled by incubating cells for 30 min in the same medium containing 100 μCi of [^{35}S]methionine/ml in rotary shaker incubator. Hepatocytes were pelleted, washed twice, resuspended and incubated for up to 4 h in chase medium (MEM containing 10 mM unlabeled methionine). After harvesting at 0, 2 and 4 h of chase, the cell pellets and extracellular media were prepared for immunoprecipitation of cathepsin L as described by Nishimura et al. [20].

2.5.3. Immunoprecipitation

Solubilized hepatocyte lysates and extracellular media were preadsorbed for 3 h at 4°C with Protein A-Sepharose, centrifuged and each supernatant was incubated with a previously-optimized quantity of anti-cathepsin L at 4°C for 16 h. The immune complex was adsorbed to 10 μl of Protein A-Sepharose with continuous gentle mixing for 3 h at room temperature. The beads were sedimented and washed as described by Nishimura et al. [20]. The sedimented beads were boiled for 2 min at 100°C in 25 μl of SDS-PAGE sample buffer [16] and subsequently centrifuged. Radioactivity in the immunoprecipitate was quantified by counting an aliquot (2 μl) of resolubilized immunoprecipitate and the remainder was subjected to SDS-PAGE. The incorporation of label into total proteins was determined in an aliquot of cell suspension by the filter disc method of Mans and Novelli [21].

2.5.4. SDS-PAGE and autoradiography

20 μl of each resolubilized immunoprecipitate was subjected to SDS-PAGE [16] in 12% polyacrylamide gels. Immunoprecipitates from each pair of control and ethanol-fed rats were analyzed on the same gel. Radioactive bands were detected by exposing the dried gel to a Phosphorscreen (Molecular Dynamics) and quantitated as arbitrary V.I.U. by reflectance densitometry on a Molecular Dynamics phosphorimager using the Molecular Dynamics ImageQuant software. Apparent molecular masses were determined by using ^{14}C-methylated protein standards.

2.6. Data analyses

All results are expressed as mean values ± S.E. The data were analyzed by Student's *t*-test. A probability value of ≤ 0.05 was considered significant.

3. Results

3.1. Hydrolase activities in isolated lysosomes and hepatic post-nuclear fractions

Purified lysosomes from rats fed ethanol for one and five wk consistently exhibited 20–50% lower specific activities ($P < 0.05$) of acid phosphatase and β-galactosidase compared with those from pair-fed controls (Table 1). We previously reported that the specific activities of cathepsins B, L and H in hepatic post-nuclear fractions from ethanol-fed rats did not decrease in a manner consistent with the decrease seen in lysosomes [1]. Here, we

Table 1
Specific activities of non-proteolytic hydrolases in isolated hepatic lysosomes from control and ethanol-fed rats

Enzyme	1 wk		5 wk	
	control	ethanol	control	ethanol
Acid phosphatase	2.2 ± 0.3	1.8 ± 0.3 *	1.5 ± 0.2	0.7 ± 0.1 *
β-Galactosidase	704 ± 80	497 ± 94 *	265 ± 60	171 ± 38 *

Results expressed as units (acid phosphatase, β-galactosidase) per mg protein are mean values (±S.E.) from 11–13 pairs of rats fed for 1 or 5 wk.
* Indicates statistical significance ($P \leq 0.05$).

Table 2
Total activities of non-proteolytic hydrolases in livers of control and ethanol-fed rats

Enzyme	1 wk		5 wk	
	control	ethanol	control	ethanol
Acid phosphatase	43 ± 6.5	49 ± 6.2	29 ± 1.9	34 ± 3.5
β-Galactosidase	3883 ± 227	3470 ± 227	4682 ± 876	3898 ± 266

Results expressed as units per liver are mean values (±S.E.) from 11–13 pairs of rats fed for 1 or 5 wk.

made similar observations with acid phosphatase and β-galactosidase, the specific activities of which were essentially the same in post-nuclear fractions from both groups of rats (data not shown). In addition, the total activities of these two enzymes were equal in livers of both control and ethanol-fed rats (Table 2).

3.2. Percoll gradient analysis

Chronic ethanol consumption altered the distribution on Percoll gradients of nearly all lysosomal marker enzymes studied. Since the results obtained after one week of pair-feeding were similar to those after five weeks, the data from both were combined. Compared with those from controls, extracts from ethanol-fed rats had significantly lower percentages of cathepsins B and L, acid phosphatase and β-galactosidase activity sedimenting in heavy fractions (fractions 1–4) of these gradients which correspond to dense lysosomes. A significant amount of these enzyme activities had shifted to fractions of lower density, corresponding to microsomes, plasma membrane, cytosol and prelysosomal compartments (data not shown). We separately pooled fractions 1–4 and 10–17 from these gradients and determined the percent of each enzyme activity in the pooled samples relative to the total activity applied to each gradient. In heavy lysosomes (fractions 1–4) derived from ethanol-fed rats, the percent of total enzyme activity of cathepsins B and L, acid phosphatase and β-galactosidase was lower than the percent activity in corresponding fractions from control animals. Conversely, in lower density fractions (10–17) from ethanol-fed rats, there was a significantly higher proportion of lysosomal enzyme activity compared with controls (Table 3). While cathepsin H from ethanol-fed rats showed a similar tendency for redistribution in Percoll gradients, its pattern was not significantly different from that in controls (Table 3). The enzyme markers for mitochondria (succinic dehydrogenase), microsomes (glucose-6-phosphatase), plasma membrane (5′-nucleotidase) and cytosol (lactic dehydrogenase) cosedimented in buoyant fractions 10–17 and showed comparable distributions in gradients from both control and ethanol-fed rats (data not shown).

3.3. Western blot analyses

In rat hepatocytes, cathepsin L is synthesized as a 39 kDa precursor which is processed into a 30 kDa intermediate, and finally into a 25 kDa mature form [20]. We immunochemically quantified all three forms of the enzyme in hepatic post-nuclear fractions on immunoblots to determine whether their steady-state levels were altered by ethanol administration. We then calculated the densitometric ratios of the different molecular forms in each group of animals to determine whether any quantitative changes occurred in the conversion of procathepsin L to its intermediate and mature forms. The absolute quantity of the 39 kDa cathepsin L proform in ethanol-fed rats (173 ± 38 V.I.U.) was numerically but not significantly higher than that in controls (127 ± 29 V.I.U.). However, the densitometric ratios of the 39 kDa precursor to the 30 kDa intermediate and to the 25 kDa final product in ethanol-fed rats were both significantly higher than those in pair-fed controls after one to five wk of pair-feeding (Table 4). At the same time, we found no difference between the two

Table 3
Percent of lysosomal enzyme activity in pooled fractions from Percoll gradients relative to the total activity in the post-nuclear fractions of control and ethanol-fed rats

Enzymes	Dense lysosomes fractions 1–4		Lower density fractions 10–17	
Enzymes	control	ethanol	control	ethanol
Cathepsin L	49 ± 1.8	44 ± 2.1 *	34 ± 2.0	39 ± 2.7 *
Cathepsin B	40 ± 2.2	32 ± 2.1 *	46 ± 2.6	54 ± 2.6 *
Cathepsin H	39 ± 2.7	35 ± 1.8	49 ± 3.3	54 ± 2.3
Acid phosphatase	20 ± 1.2	15 ± 1.3 *	70 ± 1.7	76 ± 1.8 *
β-Galactosidase	43 ± 1.7	37 ± 2.5 *	44 ± 1.8	51 ± 2.6 *

Results expressed as percent activity to total activity are mean (±S.E.) from 15 pairs of rats fed for 1 and 5 wk.
* Indicates statistical significance ($P \leq 0.05$).

Table 4
Densitometric ratios of the molecular forms of cathepsin L on immunoblots of hepatic post-nuclear fractions from control and ethanol-fed rats

Ratio	Control	Ethanol
39 kDa/30 kDa	2.04±0.76	3.89±0.57 *
39 kDa/25 kDa	0.53±0.12	0.86±0.19 *
30 kDa/25 kDa	0.16±0.08	0.20±0.05

Results are mean values (± S.E.) from 3–9 pairs of rats fed for 1 and 5 wk.
* Indicates statistical significance ($P \leq 0.05$).

groups in the ratio of the 30 kDa intermediate molecular form to the 25 kDa mature form of cathepsin L (Table 4).

3.4. Pulse chase analyses

3.4.1. Methionine incorporation

Immediately following pulse labeling of hepatocytes, the incorporation of [^{35}S]methionine into total hepatocellular proteins of ethanol-fed rats was lower than that from pair-fed controls. However the radioactivity in immunoprecipitated cathepsin L was the same in cells from both groups (Table 5).

3.4.2. Autoradiographic analyses

These analyses revealed the presence of a predominant 39 kDa protein, corresponding to the procathepsin L, in hepatocytes immediately after pulse-labeling (zero time). After 2 and 4 h of chase, the intensity of the precursor diminished with a concomitant increase in the intensities of the 30 kDa intermediate and mature 25 kDa subunit proteins (Fig. 1). We densitometrically quantified the different molecular forms of cathepsin L in each lane and determined the ratio of the 39 kDa procathepsin L to its 30 kDa intermediate and 25 kDa product. The ratios of both the 39 to 30 kDa and the 39 to 25 kDa cathepsin L forms in hepatocytes from ethanol-fed rats at both 2 and 4 h of chase were 1.5- to 3-fold higher than those in pair-fed controls (Table 6). However, the ratio of the 30 kDa intermediate to the 25 kDa product was not significantly different in the two groups of animals. We could not attribute these results to differential viabilities of cells

Table 5
Methionine incorporation into total cellular protein and immunoprecipitated cathepsin L in hepatocytes from control and ethanol-fed rats

	Control	Ethanol	P value
Total cellular proteins (cpm·10^{-6}/million cells	3.64±0.68	2.49±0.48	0.004
Cathepsin L (cpm·10^{-3}/million cells	3.33±0.65	2.90±0.80	0.43

Data are mean values (±S.E.) from 18–20 pairs of rats fed for 1 and 5 wk.

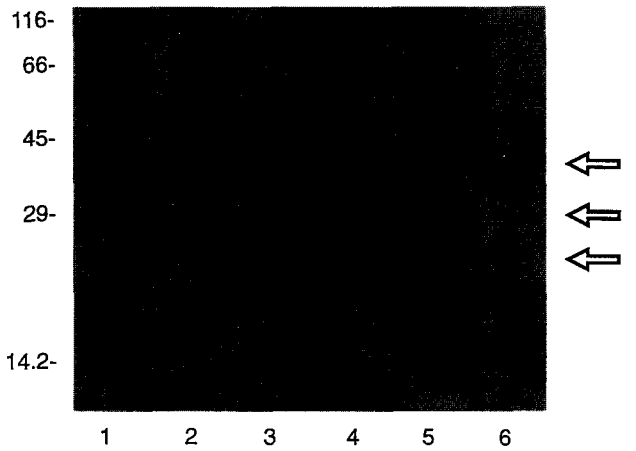

Fig. 1. Autoradiogram of [^{35}S]methionine-labeled cathepsin L from hepatocytes of control (lanes 1, 3, and 5) and ethanol-fed rats (lanes 2, 4 and 6). Cells were labeled and incubated in chase medium containing unlabeled methionine for 0, (lanes 1 and 2), 2 h (lanes 3 and 4) and 4 h (lanes 5 and 6). Numbers on the left indicate the apparent molecular mass in kilodaltons. The positions of the three cellular forms of the enzyme are indicated by arrows on the right.

from the control and ethanol-fed rats. Hepatocytes from both groups were always greater than 95% viable after both 2 and 4 h of chase.

We detected only the 39 kDa precursor form of cathepsin L in immunoprecipitates obtained from the extracellular medium of hepatocytes from both control and ethanol-fed rats over the chase period (Fig. 2). However, we found no significant difference in the amount of procathepsin L

Table 6
Densitometric ratios of the molecular forms of radiolabeled cathepsin L immunoprecipitated from hepatocytes from control and ethanol-fed rats

Chase time	120 min		240 min	
Ratios	control	ethanol	control	ethanol
39/30 kDa	3.5±0.5	10.3±1.8 *	2.5±0.6	5.6±1.2 *
39/25 kDa	9.8±2.5	14.8±3.5 *	5.8±1.7	14.7±6.4
30/25 kDa	1.9±0.4	2.1±0.5	2.3±0.6	1.5±0.3

Results expressed are mean values (±S.E.) from 10–11 pairs of rats fed for 1 and 5 wk. Ratios of the V.I.U. were calculated after quantitative densitometry of bands from the same lane following SDS-PAGE of immunoprecipitated cathepsin L.
* Indicates statistical significance ($P \leq 0.05$).

Table 7
Amount of 39 kDa cathepsin L in the extracellular medium at 2 and 4 h of chase of hepatocytes from control and ethanol-fed rats

Chase time (min)	Control (·10^{-3})	Ethanol (·10^{-3})	P value
120	637±157	561±186	0.8
240	787±244	670±181	0.7

The labeling in each band was measured as V.I.U. and results are mean values (±S.E.) from 6–11 pairs of rats fed for 1 and 5 wk.

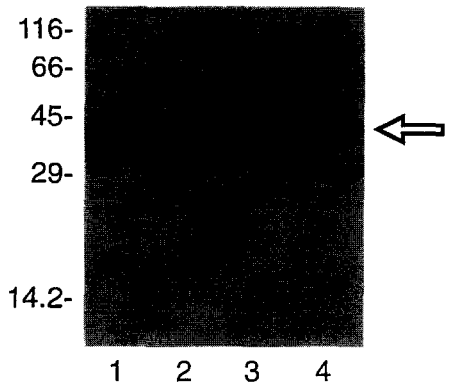

Fig. 2. Autoradiogram of [^{35}S]methionine-labeled procathepsin L immunoprecipitated from the extracellular medium of hepatocytes of control (lanes 1 and 3) and ethanol-fed rats (lanes 2 and 4) at 2 (lanes 1 and 2) and 4 h (lanes 3 and 4) of chase. Numbers on the left indicate the apparent molecular mass in kilodaltons. The position of the 39 kDa precursor is indicated by the arrow on the right.

secreted by hepatocytes from the two groups of rats at either 2 or 4 h of chase (Table 7).

4. Discussion

We previously reported that ethanol consumption for one or five wk results in decreased hepatic intralysosomal activities and/or contents of cathepsins B, L and H [1]. Here, we extended those studies to two non-proteolytic hydrolases, acid phosphatase and β-galactosidase, and observed an ethanol-induced decline in the specific activity of both enzymes in isolated hepatic lysosomes (Table 1). These findings imply that ethanol administration may exert a generalized effect on all lysosomal hydrolases, lowering their content and/or activities inside lysosomes. Similar to our previous studies [1], ethanol consumption affected neither the specific activity nor the total activity of lysosomal acid phosphatase and β-galactosidase in hepatic post-nuclear fractions (Table 2). The latter findings indicate that ethanol consumption reduces hydrolase activity by specifically affecting the lysosomal fraction and not necessarily the total cellular activities of these enzymes.

The sedimentation properties of lysosomal enzymes (Table 3) further showed that ethanol administration produced a generalized deficiency in the enzyme content of hepatic lysosomes which sedimented in fractions 1–4 in Percoll gradients. The ethanol-elicited redistribution of lysosomal enzymes toward lower density fractions (fractions 10–17) suggests an alteration in the ability of liver cells to target these enzymes to lysosomes. We recognize that these experiments did not completely rule out the possibility that some of this enzyme redistribution may have represented the release of hydrolases into the cytosol fraction due to rupture of more fragile hepatic lysosomes in ethanol-fed rats. Hagen et al. reported results with lysosomal acid phosphatase that were similar to ours and concluded that altered sedimentation of this enzyme is a consequence of enhanced lysosomal fragility due to chronic ethanol administration [22]. However, data from our laboratory have demonstrated that the lysosomal fraction from ethanol-fed rats is not as fragile as previously thought. First, we have observed that, following subcellular fractionation of liver extracts from control and ethanol-fed rats, there is no change in the acid phosphatase activity in liver cytosols, relative to the total hepatic activity (control: 21.1 ± 2.7 percent; ethanol-fed: 25.7 ± 5.9 percent). We have also obtained similar results with β-galactosidase and cathepsin L (unpublished observations). Second, we previously reported that, at neutral pH, the in vitro stability of lysosomes obtained from ethanol-fed rats is identical to that from controls, even after 2 hr of agitation at 37°C [1,5]. Therefore, we attribute the shift in the density gradient distribution of hydrolases not to ethanol-induced lability of liver lysosomes, but to an ethanol-elicited alteration in the routing of these enzymes to their target organelles.

Our conclusions are based on well-documented evidence that ethanol and/or its metabolite(s) impairs major protein trafficking processes in the liver and pancreas [23–29]. In corroboration with our results, Hirano and Manabe demonstrated that ethanol consumption causes a significant decrease of cathepsin B activity in the pancreatic lysosomal fraction. This was accompanied by an increase in enzyme activity in the zymogen fraction, indicating an ethanol-elicited missorting of lysosomal enzymes in pancreatic acinar cells [29]. Nishimura et al. [30], recently reported that acute ethanol administration inhibited the intracellular processing of β-glucuronidase in rat liver. In addition, there are numerous reports indicating that the glycosylation and transport of plasma proteins are both impaired by ethanol consumption [23–28,31–35]. Thus, it is possible that glycosylation and subsequent trafficking of lysosomal hydrolases, are similarly altered. This is further supported by data showing increased activity of lysosomal enzymes in the sera of alcohol abusers, suggesting that these proteins are secreted rather than routed to lysosomes [36–38].

Our immunoblot analyses of post-nuclear fractions of ethanol-fed rats revealed higher ratios of the 39 kDa cathepsin L precursor to its 30 kDa intermediate and 25 kDa product than those of controls (Table 4). This suggests that the conversion of the 39 kDa proform to its lower molecular mass forms was impaired. Proteolytic processing of the 39 kDa cathepsin L proform to its final product is initiated after the proform is delivered to the lysosomes [39]. Therefore, in ethanol-fed rats the impairment in the processing of the 39 kDa proform to its intermediate and final products may have resulted either from altered trafficking of the proform to the lysosomes or incomplete processing within the lysosomes. It is noteworthy that although these western blot analyses were static determinations of steady state levels of the enzyme, they still

revealed significant elevations of the precursor/intermediate and precursor/product ratios in ethanol-fed rats over those in pair-fed controls (Table 4). These results were confirmed by our pulse chase experiments which showed that prior ethanol consumption caused a significant delay in the intracellular processing of the isotopically labeled 39 kDa precursor to its intermediate and mature forms (Fig. 1 and Table 6). In addition, while ethanol consumption decreased overall protein synthesis in hepatocytes, it had no effect on cathepsin L synthesis (Table 5). This effectively resulted in an apparent higher relative synthesis rate of cathepsin L in hepatocytes from these animals compared with those from pair-fed controls.

Ethanol administration appeared to disrupt cathepsin L processing at an early step, since only the ratios of the 39 kDa cathepsin L proform to its intermediate (30 kDa) and final (25 kDa) products were significantly elevated in ethanol-fed rats (Tables 4 and 6). However, the ratio of the 30 kDa to the 25 kDa forms of cathepsin L was the same in the two groups of animals (Tables 4 and 6). These results suggest that while ethanol ingestion significantly impaired the processing of the cathepsin L proform, it did not affect the step required for conversion of its intermediate form to the mature 25 kDa enzyme. The proteolytic conversion of procathepsin L to its intermediate single chain 30 kDa mature form is thought to be catalyzed by an aspartic proteinase, probably cathepsin D [40–44]. This enzyme has a narrow pH optimum in the acidic range [45,46]. This initial proteolytic step is thought to occur as an early event in the lysosomes, although some reports indicate that this process may be initiated in pre-lysosomal compartment(s) [42,44]. Subsequent processing of cathepsin L to the final 25 kDa and 5 kDa double chain forms appears to be an autolytic process that is mostly cathepsin L-dependent [42,43,47,48]. We have reported a deficiency in the intralysosomal activities of cathepsins B, L and H [1] and two non proteolytic hydrolases, acid phosphatase and β-galactosidase in hepatic lysosomes from ethanol-fed rats. It is therefore possible that the level of intralysosomal cathepsin D was similarly reduced by ethanol consumption. Alternatively, ethanol administration may have elevated the intralysosomal pH, thus reducing the catalytic activity of cathepsin D. In either case, the maturation of cathepsin L would be slower than that in controls.

Conditions that impair the processing of cathepsins are, in many cases, accompanied by increased secretion of the procathepsin [20,47,49–52]. Here, we found no evidence of a concomitant increase in the amount of cathepsin L precursor in the culture medium of hepatocytes from ethanol-fed rats (Table 7). This finding may reflect the anti-secretory effects of ethanol which we and others have previously demonstrated [23,24,26,31,32,53–55]. Hara et al. [47] using 1,10-phenanthroline and Scheel et al. [52] using colchicine, also observed a dissociation between altered processing of procathepsins and increased secretion of the proforms by cultured cells. Similar to ethanol, both 1,10-phenanthroline and colchicine are known to be anti-secretory agents.

In summary, our data suggest that ethanol consumption impairs the trafficking of hydrolases to lysosomes and also disrupts the processing of procathepsin L without affecting its biosynthesis. This is supported by data presented herein showing (i) an ethanol-elicited alteration in the distribution of hydrolases on Percoll gradients and (ii) a significantly higher ratio of the precursor to the mature molecular form of cathepsin L in ethanol-fed rats. An ethanol-elicited impairment of intracellular trafficking resulting in reduced intralysosomal cathepsin content may be responsible for the decreased proteolytic capacity of lysosomes from ethanol-fed rats [1]. The long-term consequence of these changes is that hepatocellular damage could eventually result from the accumulation of undegraded, potentially cytotoxic macromolecules due to deficiencies in the lysosomal degradative pathway. Such damage could contribute to the pathogenesis of alcoholic liver disease.

Acknowledgements

This investigation was supported by Grant No. R01 AA09384 from the National Institute on Alcohol Abuse and Alcoholism and by Medical Research Funds from the Department of Veterans affairs. Rabbit-anti-mouse cathepsin L was a generous gift of Dr Michael Gottesman, National Cancer Institute, Bethesda, MD, USA.

References

[1] Kharbanda, K.K., McVicker, D.L., Zetterman, R.K. and Donohue, T.M., Jr. (1995) Biochim. Biophys. Acta 1245, 421–429.
[2] Kharbanda, K.K., McVicker, D.L., Zetterman, R.K. and Donohue, T.M., Jr. (1995) Alcoholism: Clin. Exp. Res. 19, 97A.
[3] Kharbanda, K.K., McVicker, D.L., Zetterman, R.K. and Donohue, T.M., Jr. (1995) Hepatology 22, 242A.
[4] Lieber, C.S. and DeCarli, L.M. (1986) Alcoholism: Clin. Exp. Res. 10, 550–553.
[5] Donohue, T.M., Jr., McVicker, D.L., Kharbanda, K.K., Chaisson, M.L. and Zetterman, R.K. (1994) Alcoholism: Clin. Exp.. Res. 18, 536–541.
[6] Yamada, H., Hayashi, H. and Natori, Y. (1984) J. Biochem. 95, 1155–1160.
[7] Gomori, G. (1952) in Microscopic Histochemistry, Principals and Practice, University of Chicago Press, Chicago.
[8] Walter, L. and Schutt, C. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.V., ed.), Vol. 2, pp. 856–860, Verlag Chemie/Academic Press, New York.
[9] Barrett, A.J. (1972) in Lysosomes (Dingle, T.T., ed.), pp. 83–84, Elsevier/North Holland, New York.
[10] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[11] Baginski, E.S., Foa, P.P. and Zak, B. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.V., ed.), Vol. 2, pp. 876–878, Verlag Chemie Academic Press, New York.
[12] Ackrell, B.A.C., Kearney, E.B. and Singer, T.P. (1978) Methods Enzymol. 53, 406–483.

[13] Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31A, 20–102.
[14] Bergmeyer, H.U. and Bernt, E. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.V., ed.), Vol 2, pp. 574–579, Verlag Chemie/Academic Press, New York.
[15] Spector, T. (1978) Anal. Biochem. 86, 142–146.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Towbin, H. and Gordon, J. (1984) J. Immunol. Methods 72, 313–340.
[18] Seglen, P.O. (1976) Methods Cell Biol. 13, 29–83.
[19] Casey, C.A., Kragskow, S.L., Sorrell, M.F. and Tuma, D.J. (1987) J. Biol. Chem. 262, 2704–2710.
[20] Nishimura, Y., Furuno, K. and Kato, K. (1988) Arch. Biochem. Biophys. 263, 107–116.
[21] Mans, R.J. and Novelli, G.D. (1961) Arch. Biochem. Biophys. 94, 48–53.
[22] Hagen, B.F., Bjørnboe, A., Bjørnboe G.A. and Drevon, C.A. (1989) Alcoholism: Clin. Exp. Res. 13, 246–251.
[23] Sorrell, M.F., Nauss, J., Donohue, T.M., Jr. and Tuma, D.J. (1983) Gastroenterology 84, 580–586.
[24] Volentine, G.D., Tuma, D.J. and Sorrell, M.F. (1984) Gastroenterology 86, 225–299.
[25] Tuma, D.J., Mailliard, M.E., Casey, C.A., Volentine, G.D. and Sorrell, M.F. (1986) Biochim. Biophys. Acta 856, 571–577.
[26] Volentine, G.D., Tuma, D.J. and Sorrell, M.F. (1986) Gastroenterology 90, 158–165.
[27] Casey, C.A., Kragskow, S.L., Sorrell, M.F. and Tuma, D.J. (1989) Alcoholism: Clin. Exp. Res. 13, 258–263.
[28] Piqueras, J.R., Sancho-Tello, M., Cervellera, R.B. and Guerri, C. (1989) Alcoholism: Clin. Exp. Res. 13, 817–823.
[29] Hirano, T. and Manabe T. (1993) Arch. Jpn. Chir. 1, 16–23.
[30] Nishimura, Y., Kato, K., Oda, K. and Himeno, M. (1995) Biol. Pharm. Bull. 18, 938–944.
[31] Tuma, D.J. and Sorrell, M.F. (1988) Sem. Liver Dis. 8, 69–80.
[32] Guash, R., Piqueras, J.R. and Geurri, C. (1992) Alcoholism: Clin. Exp. Res. 16, 942–948.
[33] Stilber, H. (1991) Clin. Chem. 37, 2029–2037.
[34] Anton, R. and Bean, P. (1994) Clin. Chem. 40, 364–368.
[35] Allen, J.P., Litten, R.Z., Anton, R.F. and Cross, G.M. (1994) Alcoholism: Clin. Exp. Res. 18, 799–812.
[36] Geokas, M.C. and Rinderknecht, H. (1973) Clin. Chim. Acta 46, 27–33.
[37] Hultberg, B., Isaksson, A. and Tiderstrom, G. (1980) Clin. Chim. Acta 105, 317–323.
[38] Wehr, H., Czartoyska, B., Gorska, D. and Matsumoto, H. (1991) Alcoholism: Clin. Exp. Res. 15, 13–15.
[39] Nishimura, Y., Kato, K., Furono, K. and Himeno, M. (1995) Biol. Pharm. Bull. 18, 945–950.
[40] Nishimura, Y. Kawabata, T. and Kato, K. (1988) Arch. Biochem. Biophys. 261, 64–71.
[41] Weideranders, B. and Kirschke, H. (1989) Arch. Biochem. Biophy. 272, 516–521.
[42] Nishimura, Y. Kawabata, T., Yano, S. and Kato, K. (1990) Acta Histochem. Cytochem. 23, 53–64.
[43] Salminen, A. and Gottesman, M.M. (1990) Biochem. J. 272, 39–44.
[44] Nishimura, Y., Kawabata, T., Furuno, K. and Kato, K. (1989) Arch. Biochem. Biophys. 271, 400–406.
[45] Kirshke, H. and Barrett, A.J. (1985) in Intracellular Protein Catabolism (Khairallah, E.A., Bond, J.S. and Bird, W.C., eds.), pp. 61–69, Alan R. Liss, New York.
[46] Kirshke, H. and Barrett, A.J. (1987) in Lysosomes: Their Role in Protein Breakdown (Glaumann, H. and Ballard, F.J., eds.), pp. 193–238, Academic Press, London.
[47] Hara, K., Kominami, E. and Katanuma, N. (1988) FEBS Lett. 231, 229–23.
[48] Nishimura, Y., Kato, K., Furono, K. and Himeno, M. (1995) Biol. Pharm. Bull. 18, 829–836.
[49] Nishimura, Y., Amano, J., Sato, H., Tsuji, H. and Kato, K. (1988) Arch. Biochem. Biophys. 262, 159–170.
[50] Mach, L., Stüwe, K., Hagen, A., Ballaun, C. and Glössl, J. (1992) Biochem. J. 282, 577–582.
[51] Lindmark, A., Gullberg, U. and Olsson, I. (1994) J. Leukocyte Biol. 55, 50–57.
[52] Scheel, J., Matteoni, R., Ludwig, T., Hoflak, B. and Kreis, T. E. (1990) J. Cell Sci. 96, 711–720.
[53] Tuma, D.J. and Sorrell, M.F. (1982) Toxicol. Appl. Pharmacol. 63, 303–311.
[54] Ghosh, P., Chirtel, S.J. and Lakshman, M.R. (1991) Alcoholism: Clin. Exp. Res. 15, 725–729.
[55] Donohue, T.M., Jr., Chaisson, M.L. and Zetterman, P.K. (1991) Alcoholism: Clin. Exp. Res. 15, 7–12.