Inhibition of intracellular protein degradation by ethanol in perfused rat liver

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Ethanol (50 mM) inhibited proteolysis in the perfused rat liver during stringent amino acid deprivation and also in the presence of normal and 10 times normal concentrations of plasma amino acids. The concentration–response curve of ethanol reached a plateau after 5 mM in both the presence and the absence of normal plasma amino acids, suggesting inhibition by oxidation products of ethanol. Intracellular glutamine, tyrosine and proline increased in concentration with ethanol, but the increases were too small to explain the observed inhibition of proteolysis. The uptake of ^{125}I -asialofetuin was slightly decreased and the output of ammonia increased in the presence of ethanol. These, together with a significant suppression of basal proteolysis in the presence of amino acids, suggest that lysosomal function was directly affected. Electron-microscopic examination of lysosomal components showed that the aggregate volume of autophagosomes (initial vacuoles) were significantly smaller in livers perfused with ethanol than in controls. However, the equivalent volume of autolysosomes (degradative vacuoles) was the same in both groups. According to these results, ethanol inhibits protein degradation in the liver by two discrete mechanisms: one decreasing the formation of autophagic vacuoles and the other involving lysosomotropic inhibition, possibly via ammonia.

INTRODUCTION

Several previous reports have documented the observation that the administration of ethanol over prolonged periods in the rat leads to appreciable increases in the content of liver protein (Baraona *et al.*, 1975; Israel *et al.*, 1979; Smith-Kielland & Mørland, 1979; Pösö *et al.*, 1980). No satisfactory explanation for this phenomenon has been found. It is well established that single doses of ethanol do not affect rates of protein synthesis *in vivo* (Lieber, 1980). Although chronic treatment has been claimed to inhibit synthesis, the reports have not been substantiated (Lieber, 1980; Baraona & Lieber, 1982; Mørland *et al.*, 1983). Rates of plasma protein secretion are decreased by ethanol, thereby causing some protein accumulation (Baraona & Lieber, 1982). The effect, however, is too small to account for the measured increase in total hepatic protein (Baraona & Lieber, 1982). Moreover, proteins stored in secretory vesicles are subject to lysosomal degradation (Bienkowski, 1983).

As for the effect of ethanol on intracellular protein degradation, the picture is even less clear. Although ethanol inhibits the turnover of specific proteins such as tyrosine aminotransferase and ornithine decarboxylase (Mørland, 1977; Pösö & Pösö, 1980), it has no acute effect on general protein degradation in isolated hepatocytes (Mørland & Bessesen, 1977), and conflicting results have been reported after chronic treatment (Baraona *et al.*, 1979; Pösö, 1980). Against this background, it is evident that the question of whether ethanol has any effect on hepatic protein degradation is far from settled.

Because there is an increasing body of evidence which supports the view that intracellular protein in the hepatocyte is predominantly regulated through alterations in the rate of protein degradation (Mortimore & Pösö, 1984; Scornik, 1984), a study on the effect of ethanol on intracellular protein degradation seemed warranted. As an experimental model we chose isolated perfused rat liver, which has been shown to be especially well suited for studies on protein metabolism (Mortimore & Surmacz, 1984).

EXPERIMENTAL

Animals

Fed male rats of the Lewis strain (Microbiological Associates, Walkersville, MD, U.S.A.), weighing 120–140 g, served as liver donors. The rats were maintained on standard laboratory chow and water *ad libitum* in an environmentally controlled room with a 12 h-light/12 h-dark cycle (lights off 19:00–07:00 h).

Perfusion medium

The perfusion medium consisted of freshly washed bovine erythrocytes (27%, v/v) suspended in Krebs–Ringer bicarbonate buffer containing 3–4% bovine plasma albumin (Fraction V, Pentex), 10 mM-glucose, and, where indicated, 1 (1×) or 10 (10×) times normal plasma concentrations of 20 amino acids and various concentrations of ethanol (Schworer & Mortimore, 1979). The amino acid mixture (1×) used has been

Abbreviations used: PVP, polyvinylpyrrolidone or povidone; β -acetylglucosaminidase, *N*-acetyl- β -D-glucosaminidase; a number followed by × denotes multiples or fractions of the amino acid concentrations in normal plasma.

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shown (Schworer *et al.*, 1981) to yield perfusate plasma concentrations similar to those of rat plasma (Lunn *et al.*, 1976). A stock solution of ethanol (2 M) in Krebs–Ringer buffer was prepared daily for all perfusate additions. In cyclic perfusions, the initial ethanol concentration was 50 mM; concentrations thereafter were maintained by infusing at 10 μ mol/min per liver (100 g rat). We have shown in separate control experiments that losses of ethanol by evaporation and oxidation decrease the perfusate concentration by about 10% in 30 min.

Perfusions

Livers were perfused *in situ* for 40 min in the single-pass mode as detailed previously (Schworer & Mortimore, 1979; Schworer *et al.*, 1981). Proteolysis was measured as in Schworer *et al.* (1981). When complete amino acid or ammonia analyses were to be performed, the perfused livers were quickly frozen between tongs previously cooled in liquid N₂. For electron microscopy, livers were fixed *in situ* at the end of the 40 min single-pass perfusion. In the ¹²⁵I-asialofetuin experiments, livers were cyclically perfused as described by Mortimore & Mondon (1970) and Woodside *et al.* (1974). Ethanol was added to the perfusate 10 min before asialofetuin. The administration of the ¹²⁵I-asialofetuin and sampling of the perfusate were carried out as described by Surmacz *et al.* (1982). When ammonia production was to be measured, perfusion was started in the antegrade direction, and after 10 min the flow was reversed and the perfusion continued in the retrograde mode for another 10 min. Three to five samples of the perfusate were collected during each perfusion phase.

Proteolysis

Rates of proteolysis were measured from the linear accumulation of valine during the second-stage cyclic perfusion as in Schworer & Mortimore (1979) and Schworer *et al.* (1981). The rates were corrected for short-lived valine release by subtracting a nominal value of 53 nmol/min per 100 g rat from the total (Schworer *et al.*, 1981).

Colloidal-silica/PVP gradients

For measurement of the distribution of lysosomes in colloidal-silica gradients, livers were homogenized after

the 40 min single-pass perfusion as in Neely *et al.* (1974). Mitochondrial and lysosomal fractions, separated from the total homogenate by differential centrifugation, were layered on colloidal silica/PVP; centrifugation and fractionation of the gradients were as in Surmacz *et al.* (1983).

Analytical

Valine in perfusate plasma was analysed chromatographically (Mortimore & Mondon, 1970). Complete amino acid analyses of liver and perfusate plasma were carried out as described in Schworer & Mortimore (1979). Ammonia concentrations in the liver were measured as described by Kun & Kearney (1974). Plasma ammonia was measured enzymically without protein precipitation, with the use of an assay kit from Boehringer (Mannheim, Germany) and a Gilford 3500 analyser. Hexosaminidase in the colloidal-silica/PVP gradient fractions and protein were determined as in Surmacz *et al.* (1983).

Electron microscopy

Livers were fixed and processed for electron microscopy as in Mortimore & Schworer (1977). Thin sections were examined in a Philips 300 electron microscope. Stereological procedures and identification of lysosomal elements were as outlined in Mortimore & Schworer (1977) and Schworer *et al.* (1981).

RESULTS AND DISCUSSION

Protein degradation

The primary aim of this study was to determine whether ethanol has any effect on intracellular protein degradation in liver over the full range of pharmacological concentrations. In our initial experiments, livers were perfused in the single-pass mode with and without 50 mM-ethanol in the presence of 0, 1 or 10 times normal plasma concentrations of amino acids. As shown in Table 1, ethanol elicited significant inhibitory effects on protein degradation both in the absence of added amino acids (0 $\times$), when degradation was maximally induced, and in the presence of maximally suppressive concentrations (10 $\times$). At normal (1 $\times$) plasma concen-

Table 1. Effect of ethanol on protein degradation in perfused rat liver

Livers were perfused for 40 min in the single-pass mode at various concentrations of normal plasma amino acids with and without 50 mM-ethanol. Rates of protein degradation were measured from the release of valine as described in the Experimental section, and were nominally corrected for valine release from short-lived and endocytosed proteins by subtracting 53 nmol/min per liver from the total (Schworer *et al.*, 1981). The results are means $\pm$ S.E.M. for *n* experiments: ***P* < 0.001; **P* < 0.05.

Plasma amino acid concn.	<i>n</i>	Ethanol	Protein degradation (nmol of valine/min per 100 g rat)		Effect of ethanol (nmol/min per 100 g rat)
			(A)	(A – 10 $\times$)	
0 $\times$	17	—	339 $\pm$ 11	214	93**
	15	+	246 $\pm$ 14	121	
1 $\times$	9	—	165 $\pm$ 8	40	12
	6	+	153 $\pm$ 12	28	
10 $\times$	9	—	122 $\pm$ 7	0	35*
	5	+	87 $\pm$ 12	–35

Fig. 1. Effects of different ethanol concentrations on intracellular proteolysis in the presence and absence of normal plasma concentrations of amino acids

Livers were perfused and the rates of proteolysis were measured as in Table 1. The values are means of 4 to 13 determinations. The broken line marks the basal rate of proteolysis ($10\times$). ○, Proteolysis in the absence of perfusate amino acids; ●, proteolysis in the presence of $1\times$ plasma amino acids.

trations, the response to ethanol was small and not statistically significant. These results differ from those of Mørland & Bessesen (1977), who obtained no effect of ethanol in isolated hepatocytes incubated in the absence of amino acids. The reason for this discrepancy is not clear, but it should be pointed out that protein degradation in isolated hepatocytes (Seglen *et al.*, 1980; Ballard & Gunn, 1982) is not as responsive to physiological regulation as it is in the perfused liver (Mortimore & Pösö, 1984; Mortimore & Surmacz, 1984).

In general, the inhibition of proteolysis by ethanol may be caused by ethanol itself, by its products of oxidation, or by metabolic changes induced by its oxidation. We tried to prevent the metabolic effects by the addition of 4-methylpyrazole, a known inhibitor of hepatic alcohol dehydrogenase. Unfortunately, this compound alone decreased protein degradation and could not be used to distinguish between the first and the second mechanisms mentioned above. Instead, we examined the effects of different concentrations of ethanol on proteolysis. As shown in Fig. 1, responses in both the presence and absence of amino acids became constant rather sharply above 5 mM. Since the main pathway of ethanol oxidation, the alcohol dehydrogenase-catalysed reaction, is very likely saturated at this concentration (Wallgren & Barry, 1970), the results support the possibility that the effects are directly linked to the metabolism of ethanol, although the direct effect of ethanol is not ruled out. Thus further experiments are needed to clarify the mechanism of inhibition.

Intracellular amino acid pools

The reduction of the NADH/NAD⁺ couple during ethanol oxidation disturbs amino acid homeostasis in

Fig. 2. Effects of ethanol on intracellular amino acid concentrations

Livers were quickly frozen after 40 min single-pass perfusion without amino acids and with or without 50 mM-ethanol, and intracellular amino acid concentrations were measured. The results are normalized to the respective values without ethanol, marked by the horizontal line (100%). The mean concentrations in control livers were ($\mu\text{mol/ml}$ of intracellular water): Ala, 1.16; Ser, 2.28; Thr, 0.91; Leu, 0.29; Val, 0.32; His, 0.27; Ile, 0.14; Phe, 0.10; Gly, 3.29; Pro, 0.15; Met, 0.11; Tyr, 0.07; Cys, 0.04; Glu, 5.33; Asp, 3.07; Lys, 0.71; Asn, 0.16; Gln, 1.12. Each value is the mean of four to five determinations.

the liver and leads to the accumulation of amino acids (Krebs *et al.*, 1973), which are recognized as the primary inhibitors of hepatic protein degradation (Pösö *et al.*, 1982b). Fig. 2 depicts the effects of 50 mM-ethanol on intracellular amino acid concentrations in the absence of perfusate amino acids. Ethanol significantly increased the hepatic concentration of glutamine, and decreased concentrations of alanine, serine and threonine. However, most of the amino acid concentrations remained about the same. Similar alterations in hepatic amino acids with 50 mM-ethanol were measured after 40 min single-pass perfusions with $1\times$ amino acid concentrations (results not shown). However, in addition to glutamine, proline and tyrosine concentrations were significantly increased, and decreases were observed in aspartate and asparagine as well as in alanine and serine. The most striking response in both conditions was the decrease in alanine concentration. For example, in the presence of $1\times$ plasma amino acids alanine was decreased from $1.92 \pm 0.29 \mu\text{mol/ml}$ of intracellular

water ($n = 4$) to $0.55 \pm 0.16 \mu\text{mol/ml}$ ($n = 5$) with the addition of 50 mM-ethanol.

It is clear from these findings that most of those amino acids that actively regulate hepatic proteolysis did not change in concentration (Pösö *et al.*, 1982b). Glutamine, which was increased in both conditions, has been shown to inhibit protein degradation moderately at plasma concentrations below 3 mM (Pösö *et al.*, 1982a). However, it seems highly doubtful that the relatively small effect of ethanol on intracellular glutamine alone would be sufficient to explain the proteolytic inhibition.

Hepatic ammonia production

As shown in Table 1, ethanol also inhibited basal proteolysis, an effect indicating that intralysosomal proteolysis was directly inhibited. Ethanol has long been known to inhibit urea formation in the liver (Field *et al.*, 1963; Meijer *et al.*, 1975), and we considered the possibility that it might alter hepatic ammonia metabolism as well. We tested this possibility preliminarily by measuring the uptake of ^{125}I -asialofetuin, a protein degraded exclusively by lysosomes (Ashwell & Harford, 1982), which has previously been shown to be inhibited by ammonia (Pösö *et al.*, 1982a). Compared with controls, the fractional rate of uptake appeared to be decreased with the addition of 50 mM-ethanol [0.154 ± 0.010 ($n = 7$) versus 0.130 ± 0.010 ($n = 4$) respectively].

In subsequent experiments, we measured total hepatic ammonia concentration, which after 40 min perfusion was $1.23 \pm 0.049 \mu\text{mol/g}$ of liver tissue ($n = 11$) in control livers and $1.50 \pm 0.124 \mu\text{mol/g}$ ($n = 9$) when 50 mM-ethanol was present in the perfusate. Similar increases in the total hepatic ammonia have been reported by Mohanachari *et al.* (1983). The interpretation of values of ammonia in protein-free extracts of liver is complicated by the release of protein-bound, metabolically inert, ammonia during the precipitation of proteins (Wanders *et al.*, 1980). On the assumption that the total amount of protein-bound ammonia remains unaltered, the increasing effect of ethanol on free intracellular ammonia would be much greater than the measured increase in total ammonia.

To avoid the problem of protein-bound ammonia, we measured ammonia output from the perfused liver. It has been shown (Häussinger, 1983; Pösö *et al.*, 1986) that ammonia is largely produced in the periportal part of the

liver acinus, where most of it is incorporated into urea. Further removal of ammonia occurs in the periveneous region by the reaction catalysed by glutamine synthetase (Häussinger, 1983; Gebhardt & Mecke, 1983). The periportal localization of production, together with its efficient utilization by urea and glutamine synthesis, keeps the concentration of free ammonia low in the effluent when the flow of the perfusate is from portal to caval vein. However, if the direction of perfusion is changed to retrograde (flow from caval to portal vein), ammonia that is formed under these conditions is apparently not utilized, but washed out of the liver and can be taken as a measure of ammonia production (Häussinger, 1983). As indicated in Table 2, the concentration of ammonia in the effluent was greatly affected by the direction of the perfusate flow, a result similar to that obtained by Häussinger (1983). When 50 mM-ethanol was present in the medium during antegrade (flow from portal to caval vein) perfusions, the output of ammonia was raised 3-fold (Table 2). In retrograde perfusions (flow from caval to portal vein) the output of ammonia was the same in control and ethanol-treated livers (Table 2). These findings strongly suggest that the effect of ethanol was one of decreasing ammonia utilization rather than enhancing its production.

In the presence of amino acids at $10 \times$ concentrations, ammonia output from control and ethanol-treated livers perfused in the antegrade mode was greatly elevated, and no further increase was observed with 50 mM-ethanol (Table 2). This general increase may be attributable in part to inhibition of glutamine synthetase by the high concentration of glutamine in the perfusate (Häussinger *et al.*, 1983), and could help to explain the lack of the ethanol effect. Also, in the presence of amino acids the production of ammonia was independent of ethanol (Table 2). Since urea production was significantly inhibited by ethanol (results not shown), the results support the above notion that ethanol decreases the utilization of ammonia. These experiments, although indirect, are consistent with the view that the inhibition of basal proteolysis by ethanol is the result of increased intracellular ammonia concentration.

Lysosomal alterations

As mentioned above, the inhibition by ethanol of basal protein turnover strongly indicates that a lysosomotropic

Table 2. Release of ammonia from livers perfused in the antegrade or the retrograde direction with and without 50 mM-ethanol

Livers were perfused with ($10 \times$) and without ($0 \times$) added amino acids in the single-pass mode for 10 min in the antegrade direction (portal to caval vein); thereafter the flow was switched to the retrograde direction (caval to portal vein) and the perfusion continued for another 10 min. Ammonia concentration was determined in three to five effluent perfusate samples from each direction of flow. The results are means $\pm$ S.E.M. for four to six different perfusion experiments; ns, not significant ($P > 0.05$).

Amino acids	Direction of perfusion	Ammonia production (nmol/min per 100 g rat)		P
		Control	+ Ethanol	
0 $\times$	Antegrade	29 ± 5	80 ± 11	0.005
	Retrograde	152 ± 35	155 ± 27	ns
10 $\times$	Antegrade	266 ± 34	247 ± 44	ns
	Retrograde	747 ± 57	658 ± 77	ns

decrease in intralysosomal proteolysis is one of the mechanisms involved in the overall effect of ethanol. It could also explain the suppression at 0×, but, since most of the increase in protein degradation that occurs in the absence of amino acids is the consequence of induced autophagy (Schworer *et al.*, 1981), one must exclude other possible effects of ethanol on autophagy as well.

Previous studies with colloidal-silica/PVP gradients have shown that decreases in deprivation-induced rates of proteolysis resulting from lysosomotropic inhibition can readily be differentiated from effects on autophagic sequestration by shifts in lysosomal marker away from or into gradient fractions containing dense lysosomes (Surmacz *et al.*, 1987). Because the shifts are measurements of dense-body fusion and, hence, indicate the total quantity of induced autophagy, a suppression of sequestration would be accompanied by a marker shift into the dense fractions. On the other hand, lysosomotropic inhibition is frequently associated with an increase in overt autophagy (Furuno *et al.*, 1982; Kovács *et al.*, 1982; Kominami *et al.*, 1983; Glaumann *et al.*, 1986) and would be expected to produce a shift in the opposite direction (Surmacz *et al.*, 1987). Surprisingly, the results of gradient studies revealed that ethanol did not have any effect on movement of the marker. The percentage of β -hexosaminidase recovered in the dense fractions was $46.0 \pm 0.42\%$ in control and $45.7 \pm 0.95\%$ in ethanol-treated livers. Thus it was immediately apparent that the proteolytic inhibition was possibly the consequence of a combination of more than one lysosomal effect; in this case the lack of any gradient change would have been the result of two opposing effects. Obviously, additional evidence was needed to determine whether or not overt autophagy was indeed affected by ethanol.

Stereologic data in Table 3 showed a highly significant decrease in the fractional cytoplasmic volume of autophagosomes with ethanol treatment. The fact that the aggregate volume of autolysosomes was not similarly altered caused an equally significant decrease in the ratio of autophagosomes to autolysosomes. Taken together,

Table 3. Effect of ethanol on fractional cytoplasmic volumes of lysosomal components

Livers were perfused *in situ* for electron microscopy after 40 min single-pass perfusion with or without 50 mM-ethanol. The following lysosomal components were identified by criteria previously detailed in Schworer & Mortimore (1979) and Schworer *et al.* (1981): initial autophagic vacuoles or autophagosomes (AVi), degradative autophagic vacuoles or autolysosomes (AVd), type A dense bodies (A) and type R dense bodies (R). Results are means $\pm$ S.E.M. for three experiments: ns, not significant ($P > 0.05$).

Lysosomal component	Fractional cytoplasmic volume (%)		P
	Control	+ Ethanol	
AVi	0.478 ± 0.041	0.244 ± 0.040	< 0.05
AVd	0.427 ± 0.051	0.524 ± 0.059	ns
AVi/AVd	1.130 ± 0.050	0.467 ± 0.032	< 0.001
A	0.225 ± 0.048	0.229 ± 0.030	ns
R	0.350 ± 0.127	0.325 ± 0.017	ns

the findings in the gradient experiments and in Table 3 clearly support the conclusion that the effect on protein degradation with ethanol or 0× is mediated by two mechanisms: (i) a decrease in autophagic sequestration and (ii) lysosomotropic inhibition. The deviation in the autophagosome/autolysosome ratio is particularly compelling in this regard, because the proportionality between these two populations has always been extremely close (Schworer *et al.*, 1981; Surmacz *et al.*, 1987).

There are several lines of evidence which indicate that protein degradation rather than synthesis is the dominant site for regulating the cytoplasmic protein content of the hepatic parenchymal cell (Mortimore & Pösö, 1984; Scornik, 1984). Although the present experiments demonstrate only acute effects of ethanol, the continuous inhibition of protein degradation in a chronic situation might explain the measured accumulation of hepatic protein. The observation that the inhibition is attributable to an oxidation product of ethanol or the increased NADH/NAD⁺ redox state is of physiological significance, because only low concentrations of ethanol are needed to evoke maximal effects.

We thank Mr. John J. Wert, Jr., Ms. Ann Bryan-Petersen, Ms. Alice Chow and Ms. Riitta Turtiainen for their excellent technical assistance, and Dr. Bryce L. Munger, of the Department of Anatomy, The Milton S. Hershey Medical Center, for the use of electron-microscope facilities. Ludox HS-40 was a gift from E.I. du Pont de Nemours. A.R.P. was a recipient of a research fellowship from the Research Council for Natural Sciences of the Academy of Finland, and the work was supported by U.S. Public Health Service Grant AM-21624 (G.E.M.) and the Finnish Foundation for Alcohol Studies (A.R.P.).

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Received 3 June 1986/16 October 1986; accepted 6 November 1986