Inhibition of proteolysis in the liver by chronic ethanol feeding

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Effects of chronic ethanol feeding on the volume density of lysosomes, the rate of protein degradation and the amount of protein were studied in livers perfused in situ at 07:00, 11:00, 17:00 and 23:00 h. Ethanol was given to the rats in drinking water for either 3 or 8–10 weeks. During week 3 of treatment and onwards, the average daily consumption of ethanol was 12.3 ± 0.3 g/kg body wt. Morphometric analysis revealed that the volume density of autophagosomes and autolysosomes was lower in the ethanol-fed rats than in the controls. When compared with age-matched controls, the rate of proteolysis, measured as release of valine, was 33 % and 26 % less in the ethanol-fed rats after treatment for 3 and 8–10 weeks, respectively. The difference between the two groups was most pronounced at 07:00 and 11:00 h. Protein content of the liver increased significantly after the longer ethanol treatment. According to these results, chronic ethanol feeding inhibits proteolysis in the liver by preventing the sequestration of protein into lysosomes.

INTRODUCTION

Chronic ethanol ingestion disturbs the finely adjusted balance of protein turnover in liver and leads to accumulation of hepatocellular protein (for review, see [1]). Part of this accumulation can be explained by an increase in the amount of fatty-acid-binding protein [2] and by plasma proteins which in the presence of ethanol are retained in the liver [3]. It can, however, be calculated that the amount of protein in the liver together with the fatty-acid-binding protein account for less than 30 % of the total protein accumulation during chronic ethanol feeding [2,3], which leaves the nature of the major contributor(s) unknown.

The ethanol-induced increase in hepatic protein mass can be caused by a change in the rate of either protein synthesis or degradation. Despite extensive studies, effects of chronic ethanol feeding on protein synthesis in situ are far from clear, and the reported results range from stimulation to inhibition (for review, see [1]). Chronic ethanol has been shown to inhibit the rate of intracellular protein degradation in perfused rat liver [4], whereas the chronic effects of ethanol have only been calculated from the changes in the amount of protein and the rate of protein synthesis [5,6].

The bulk of long-lived protein in the liver is degraded intralysosomally [7]. The process has been shown to be more sensitive to nutritional factors than is protein synthesis, and 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 hepatocyte [7,8]. It was shown previously that in perfused rat liver ethanol inhibits the formation of autophagosomes [4], the first step of lysosomal proteolysis, whereas the effect of chronic ethanol feeding on autophagocytosis is not known. The purpose of the present study was to measure the rate of protein degradation and volume densities of lysosomal components in livers of rats that were chronically fed on an ethanol-containing diet. Because protein degradation is sensitive to nutritional factors [7], the circadian variation of proteolysis was also investigated.

EXPERIMENTAL

Animals and diets

Male rats from the mixed strain [9] bred at the Laboratories of Alko (Alko Ltd., Helsinki, Finland) were housed individually in an environmentally controlled room (lights on 06:00–18:00 h) and fed ad libitum on pelleted chow (R3; Ewos, Södertälje, Sweden). The macronutrient content of the diet was (by wt.) 20.5 % protein, 50.5 % fat and 31.5 % carbohydrate. The energy content was 13 MJ/kg. At the beginning of the experiment the rats were 5 weeks old, and their weight averaged 120 g. Ethanol was given to the rats for 3 or 8–10 weeks in drinking water supplemented with sucrose (3 %, w/v). The concentration of ethanol was gradually increased from 5 to 15 % (v/v). Control rats had free access to tap water.

Protein degradation

The portal and hepatic veins of anaesthetized rats (pentobarbital, 50 mg/kg, intraperitoneally) were cannulated and the livers were perfused cyclically in situ for 15 min as described in detail by Hutson et al. [10]. The rate of protein degradation was measured as accumulation of valine into the perfusate, which contained 18 μM-cycloheximide to prevent the re-incorporation of valine. It has been shown previously [11] that during the 15 min period the release of valine into the perfusate is linear and the valine originates from the lysosomes formed in the liver before the perfusion was started. Because valine is not appreciably metabolized in liver [11], its accumulation into the perfusate gives a valid estimate of the rate of protein degradation prevailing in vivo at the moment the perfusion was started. The rate of protein degradation was measured at four time points (07:00, 11:00, 17:00 and 23:00 h).

The perfusate consisted of freshly washed bovine red cells suspended in Krebs–Henseleit bicarbonate buffer and supplemented with 10 mM-glucose and 0.3 mM-valine [12]. Four samples of the perfusate for the determination of valine concentration were taken during the perfusion. Before the perfusion a blood sample was taken from the tip of the tail for measurement of ethanol concentration, and after the perfusion a piece of the liver was frozen in liquid nitrogen for determination of total protein.

Analytical

Concentration of valine in the perfusate was analysed by the competitive tRNA-binding method [13] and the rate of protein degradation was calculated as described previously [12]. Protein [10] was analysed by a previously described method. The con-
centration of ethanol was measured by gas chromatography by a head-space technique [14].

**Electron microscopy**

Livers were fixed in situ for electron microscopy with diluted Karnovsky's fixative [15] after ethanol treatment for 10 weeks. The perfusions for fixation were started at 08:00 h. Thin sections were stained on grids with uranyl acetate and lead citrate and examined with a JEM 100 V (JEOL) electron microscope. Morphometric analysis was performed with point counting at 5300 x primary magnification by using a 1:9/121:1089 double-square lattice [16,17]. The criteria for the classification of the lysosomal components were as detailed by Hirsimäki & Pilström [18].

**Statistical analysis**

Statistical differences were calculated by analysis of variance and Student's t test (Statgraphics; Statistical Graphics Corp.). The results are shown as means ± S.E.M.

**RESULTS**

There was no difference in the intake of energy between the control and the ethanol-fed rats. During week 3 of the experiment, the controls consumed 127 ± 3 kJ/100 g rat per day, and the rats in the ethanol group 124 ± 3 kJ/100 g rat per day. In the ethanol-fed group 23 ± 0.5% of the energy came from ethanol. However, the control rats gained weight faster than the ethanol-fed rats. The final body weights in the 3-week experiment were 245 ± 6 g for the control rats (weight gain 5.5 g/day) and 221 ± 5 g (P < 0.01) for the ethanol-fed rats (weight gain 4.3 g/day). The respective values in the longer experiment were 431 ± 12 g and 3.0 g/day for the controls and 362 ± 6 g and 2.4 g/day for the ethanol-fed rats (P < 0.001). The liver-to-body-weight ratio was significantly (P < 0.001) lower in the 8–10-week (3.18 ± 0.05 %) than in the 3-week (4.52 ± 0.08 %) experiment, which is in keeping with the report by Britton et al. [19] and is due to the difference in the age of the rats at the end of the experiment. Neither the short (3 weeks) nor the long (8–10 weeks) treatment with ethanol increased the liver-to-body-weight ratio significantly.

During the first 2 weeks, the daily ethanol consumption increased from the initial value of 9.1 ± 0.3 to 12.3 ± 0.3 g/kg body wt. Thereafter the ethanol consumption levelled off and remained fairly constant throughout the remaining period of the treatment. The mean ethanol concentrations in the tail blood at the time of the perforusions are shown in Table 1. After ethanol feeding for 3 weeks, concentrations of ethanol higher than 0.5 mM were present at the beginning of the perfusion in all rats at 07:00 and 23:00 h. At 11:00 h 4 out of 7 rats had ethanol in their blood, and the respective value at 17:00 h was 5 out of 9. In the longer (8–10 weeks) experiment ethanol was present in the blood of all rats at 07:00, 11:00 and 17:00 h. At 23:00 h the blood ethanol concentration was analysed only for 2 rats, both of which had ethanol in their blood.

The release of valine into the perfusate that was taken as a measure of the rate of protein degradation is shown in Fig. 1. Taking 465 μmol as the valine content per mg of hepatic protein [20], the measured mean degradation rates for the control rats in the 3-week and 8–10-week experiments are equivalent to fractional rates of degradation of 0.419 day⁻¹ and 0.264 day⁻¹ respectively. The largest differences between the two groups were measured in the morning (07:00 and 11:00 h). The rate of valine release in the ethanol-fed rats was 33% slower than in the controls in the 3-week experiment (Fig. 1). Statistical analysis of the data (ANOVA) revealed that the effect of diet was highly significant (P < 0.001), whereas the time of perfusion had no significant effect in either group. After ethanol feeding for 8–10 weeks, the decrease in the rate of valine release was attenuated, and the inhibition was 26%. The effect of diet was significant (ANOVA) at P < 0.01, and in the ethanol-fed group also the time of perfusion had a significant effect (P < 0.01). The rate of valine release into the perfusate was significantly (P < 0.001) less in the older rats than in the rats in the 3-week experiment in both diet groups. The statistical differences in the rate of valine release did not change when the values were expressed per g liver weight.

Ethanol feeding for 3 weeks had no significant effect on the amount of protein, which was 908 ± 14 mg/100 g body wt. in the control rats and 928 ± 16 mg/100 g body wt. in the ethanol-fed rats. When the treatment lasted for 8–10 weeks, a small

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**Table 1. Concentration of ethanol in the tail blood at the beginning of perfusion**

A blood sample was taken, immediately before perfusion, from the tip of the tail. The results, which include also those animals which did not have detectable concentrations of ethanol in their blood, are means ± S.E.M. for 5–9 rats.

<table>
<thead>
<tr>
<th>Time of perfusion (h)</th>
<th>Duration of treatment...</th>
<th>Ethanol (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 weeks</td>
<td>16 weeks</td>
</tr>
<tr>
<td>07:00</td>
<td></td>
<td>9.1 ± 3.7</td>
</tr>
<tr>
<td>11:00</td>
<td></td>
<td>7.0 ± 2.5</td>
</tr>
<tr>
<td>17:00</td>
<td></td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>23:00</td>
<td></td>
<td>18.2 ± 6.9</td>
</tr>
</tbody>
</table>
Inhibition of proteolysis by ethanol

![Graph showing diurnal changes in hepatic protein content after chronic ethanol treatment](image)

**Fig. 2.** Diurnal changes in hepatic protein content after chronic ethanol treatment

Experimental conditions, numbers of rats and symbols were as in Fig. 1.

<table>
<thead>
<tr>
<th>Vacuole</th>
<th>Volume density (% of the cell volume)</th>
<th>Control</th>
<th>Ethanol-treated</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagosome</td>
<td></td>
<td>0.030 ± 0.006</td>
<td>0.005 ± 0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Autolysosome</td>
<td></td>
<td>0.020 ± 0.005</td>
<td>0.004 ± 0.002</td>
<td>0.05</td>
</tr>
<tr>
<td>Residual body</td>
<td></td>
<td>0.086 ± 0.017</td>
<td>0.079 ± 0.016</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 2. Morphometric analysis of lysosomal components in livers of control and ethanol-fed rats

Livers of fed rats were fixed *in situ* with diluted Karnovsky's fixative for electron microscopy at 08:00 h. The duration of the ethanol treatment was 10 weeks. The classification of the lysosomal elements is described in detail in ref. [17]. Abbreviation: ns, not significant.

but statistically significant increase (*P* < 0.05; ANOVA), was found. The mean values in these groups were 681 ± 13 and 726 ± 13 mg/100 g body wt. for the control and ethanol-fed rats respectively. In the 3-week experiment the total protein content in the control and ethanol-fed rats showed no circadian changes, but after the longer treatment the amount of protein was found to decrease linearly both in the control rats (*r* = −0.972; *P* < 0.05) and in the ethanol-fed rats (*r* = −0.979; *P* < 0.05) during the period when the lights were on in the animal room (from 07:00 to 17:00 h; Fig. 2).

The volume density of autophagosomes after ethanol feeding for 10 weeks was significantly lower than in the controls (Table 2). Also the volume density of autolysosomes was significantly smaller in the ethanol-fed rats than in the controls. No differences in the volume density of residual bodies were observed.

**DISCUSSION**

The results of the present study show that chronic ethanol feeding inhibits the rate of protein degradation. The method used in this study has been shown to give a valid estimate of the rate of protein degradation prevailing in the liver at the time when the perfusion is started [11]. Also in this study, the fractional degradation rates in the livers of the control rats were well in accord with previously reported values [21]. Accordingly, this direct measurement of the effect of ethanol on the rate of protein degradation confirms the suggestions which in previous studies were reached indirectly by measuring the rate of synthesis and the total amount of protein [5,6,22].

Ethanol has been shown to disturb the eating pattern of the rat [23], and, because the rate of protein degradation is sensitive to nutritional factors [7], it can be argued that some of the differences observed between the control and the ethanol-fed rats may be due to the differences in food consumption. However, several lines of evidence indicate that ethanol itself has an inhibitory effect on protein degradation. Firstly only in the ethanol-fed rats was the effect of perfusion time on the release of valine statistically significant. Secondly, at 07:00 and 11:00 h, when the rats in the control group can be considered to be well fed, as indicated, e.g., by the high glycogen content of the livers (results not shown), the difference between the two groups in the rate of protein degradation was the greatest. Thirdly, the volume density of autophagosomes and autolysosomes, also measured in the morning, was significantly smaller in the ethanol-fed rats than in the controls. Furthermore, these results are in accord with an earlier study where it was shown that in perfused liver ethanol decreases the volume density of autophagosomes and the rate of valine release [4].

Another nutritional factor that can be considered to have an influence on the rate of protein degradation is the smaller amount of solid food consumed by the ethanol-fed rats than by the controls. Accordingly, their intake of protein was smaller than that of the controls (20.5 % versus 16 %). An extremely severe restriction of protein is known to suppress the rate of protein turnover [24,25], but in the present study the amount of protein that was consumed by the rats in the ethanol group was well above the recommended minimal level of 12 % [26]. Furthermore, even a 10 % protein diet has been shown to yield growth rates similar to those of the control rats in this study [27].

Most of the constituent proteins in a hepatocyte are degraded intralysosomally [7], and the sequestration of protein into lysosomes is an important regulation site for proteolysis. The volume density of autophagosomes, the early forms of lysosomal vacuoles that contain clearly recognizable material but no acid hydrolases [7], was decreased in ethanol-fed rats, suggesting that ethanol inhibited this first step of lysosomal protein degradation. Thus the site of inhibition by chronic ethanol treatment is the same as that previously found in liver perfusion [4]. The aggregate volume of autolysosomes in the ethanol-fed rats was decreased to the same degree as for autophagosomes. Thus the ratio of these two lysosomal elements was similar in the two groups. This can be taken as indirect evidence that ethanol did not interfere with the degradation of the sequestered proteins [4,28]. This suggestion is supported by reports showing that chronic ethanol feeding does not change the activities of lysosomal enzymes [29,30]; A. R. Pöösö, K. Penttilä & K. O. Lindros, unpublished work.

In conclusion, this study shows that the rate of protein degradation is slower in the livers of ethanol-fed rats than in the controls fed *ad libitum*. The decreased volume density of autophagosomes and autolysosomes suggests that the site of inhibition by ethanol is the sequestration of proteins into the lysosomes.

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REFERENCES


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