
THE EFFECT OF ETHANOL OR HEPATOTOXIN EXPOSURE ON RAT TRANSFERRIN DESIALYLATION

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Abstract — Serum carbohydrate-deficient transferrin (CDT) is being increasingly used as a biological indicator for excessive alcohol consumption. However, the mechanisms behind the changes in the carbohydrate moiety of transferrin are unclear, although they have been suggested to be mediated by acetaldehyde or liver damage. To study this, an animal model involving alterations in serum isotransferrin concentrations would be needed. The present work examined the changes in the carbohydrate moiety of transferrin in rats after different degrees of ethanol exposure, the effects of chronically elevated acetaldehyde levels, and also the changes produced with liver toxins (galactosamine and carbon tetrachloride). Ethanol was administered both in the drinking fluid and by intubation, reaching a dose of 11 g/kg/day over 7 weeks, or 16 g/kg/day over 4 weeks. Serum samples from rats maintained on high ethanol for 10 weeks by intragastric infusion were also analysed. Some rats simultaneously had cyanamide administered to elevate acetaldehyde levels. However, neither ethanol nor acetaldehyde had any effect on transferrin. Intraperitoneal galactosamine, but not carbon tetrachloride, induced transferrin desialylation. Thus, in the rat, neither chronic ethanol consumption nor elevated acetaldehyde induces changes in transferrin microheterogeneity.

INTRODUCTION

Alcohol abuse is often accompanied by increased levels of carbohydrate deficient transferrin (CDT) in the serum, and CDT has been used as a specific biomedical marker of excessive alcohol consumption even in patients with alcohol-induced liver cirrhosis (Stibler and Borg, 1986; Stibler and Hultcrantz, 1987). The carbohydrate deficiency is due to reduced sialic acid and/or N-acetylglucosamine content in transferrin (Stibler and Borg, 1986). The reason for the alcohol-induced increase of serum CDT (disialo-, monosialo- and asialo-transferrin) is not established. The accumulation of CDT in circulation has been suggested to be due to incomplete post-translational protein glycosylation (Malagolini et al., 1989; Stibler and Borg, 1991; Guasch et al., 1992), increased desialylation of completely glycosylated transferrin molecules and impaired hepatic binding of asialoglycoproteins (Casey et al., 1990a, b, 1991).

The purpose of the present study was to investigate if alterations in the microheterogeneity of transferrin could be detected in ethanol-treated rats. An elevation of CDT in rats was not found in an earlier study by Stibler et al. (1989). However, the ethanol treatment may have been too short and the level of acetaldehyde, which has been suggested to be responsible for elevated CDT in human drinkers (Stibler and Borg, 1991), may have been too low. Consequently, we studied the effect of extensive ethanol exposure and the influence of elevated acetaldehyde concentrations. Finally, to evaluate the involvement of liver damage, we examined the effects of liver toxins on CDT in rats.

MATERIALS AND METHODS

Reagents

Galactosamine (G-1639, Sigma Ltd, St Louis,
MO, USA) was dissolved in saline and carbon tetrachloride (Art. 2222, E. Merck, Darmstadt, Germany) in corn oil. Rabbit antiserum to rat transferrin was from Organon Technika corporation (Cappel™55720, West Chester, NY, USA), cyanamide (Dipsan®) was from Lederle, Cyanamid Canada Inc., Montreal, Canada, and neuraminidase (N-5254, type VI-A, from Clostridium perfringens) was from Sigma Ltd (St Louis, MO, USA). The animals were maintained on SDS RM1 (E) SQC pellet rat laboratory diet (Special Diet Food Services Ltd, Witham, Essex, UK) (except in experiment 3). Cyanamide was added to the diet (50 mg/kg) in experiments 1 and 2.

Blood tests

Alanine aminotransferase (ALAT) activity was measured spectrophotometrically using a kit from Boehringer Mannheim, Germany. The microheterogeneity of transferrin was analysed after isoelectric focusing (PhastGel™ IEF 4–6.5) in a pH gradient of 4–6.5 by immunofixation with rabbit anti-rat transferrin, and staining by Coomassie Brilliant Blue R (B-8647, Sigma Ltd, St Louis, MO, USA). Blood samples were taken from the tail vein or from the heart (experiment 4). Serum for ALAT and CDT determination was frozen at -80°C before measurement. The samples for CDT analysis were thawed, diluted with distilled water (20 μl serum + 2 μl Fe³⁺-citrate + 120 μl water) and iron-saturated by Fe³⁺-citrate (10 mM) for 1.5 h at room temperature. In the IEF-technique, 6 samples were applied in a gel by a sample applicator (8/0.5). Sample application took place at cathodal position on the gel. The prefocusation time was 350 Vh. The different isotransferrins were quantified using a dual-wavelength flying spot scanner densitometer (Shimadzu CS-9000, Shimadzu Corporation, Kyoto, Japan), as recently described (Löf et al., 1993). Blood ethanol and acetaldehyde concentrations were analysed from fresh samples taken 90 min after intragastric intubation of alcohol obtained during the last week of ethanol treatment in experiments 1 and 2. The samples were haemolysed with 4 volumes of distilled water before alcohol and acetaldehyde determination by head-space gas chromatography (Eriksson et al., 1977).

Procedures

Experiment 1 (long-term medium ethanol exposure with and without inhibition of aldehyde dehydrogenase). Male Wistar rats were divided into two groups of 4 animals each, with a mean initial weight of 165 g. One group had cyanamide added to their food. Both groups had, as their only drinking fluid, ethanol solution increasing from 5 to 12% (w/v) over 8 days. Thereafter for 7 weeks they had 12% ethanol solution ad libitum in addition to receiving via intragastric intubation 17% (w/v) ethanol solution once a day during the first, second, third, fourth, fifth, sixth and seventh weeks of amount up to 5.5, 9.5, 10.0, 10.5, 11.0, 11.0 and 11.0 g/kg/day respectively. The CDT concentration was measured at the beginning and at the end of the study.

Experiment 2 (medium duration and high exposure with and without inhibition of aldehyde dehydrogenase). The procedure was similar to that in experiment 1, except that the intubation lasted only 4 weeks, employed 30% (w/v) ethanol, and produced ethanol intakes during the first, second, third and fourth weeks of 9.0, 14.0, 16.0 and 16.0 g/kg/day respectively. The groups had 6 rats each, with initial weights averaging 329 g (range: 288–351 g) for the group given cyanamide in their food and 306 g (range: 270–340 g) for the group without cyanamide.

Experiment 3 (total enteral nutrition with or without alcohol consumption). In this experiment the total enteral nutrition (TEN) model was used. Rats were given ethanol as a component of a liquid diet that was infused intragastrically via an inserted cannula as described before (Badger et al., 1993). The mean blood ethanol concentration of the animals during the 10-week treatment was 41 ± 11 mmol/l. The CDT concentration of control (n = 5) and ethanol-treated (n = 5) rats was measured at termination of the study. At that time, the mean blood ethanol levels were 64 mmol/l.

Experiment 4 (hepatotoxin experiment). Rats (mean weight 445 g) received SDS laboratory rat diet and water, but no alcohol throughout the study. The rats received an intraperitoneal injection of the hepatotoxin on the first day of the experiment. Different doses of galactosamine (500, 1000 and 1500 mg/kg) and carbon tetrachloride (0.5, 1.0 and 1.5 ml/kg body weight) were given to subgroups of two rats and blood tests
Table 1. Ethanol and acetaldehyde treatment and CDT concentration

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experiment 1 Ethanol (n = 4)</th>
<th>Experiment 1 Ethanol + cyanamide (n = 4)</th>
<th>Experiment 2 Ethanol (n = 6)</th>
<th>Experiment 2 Ethanol + cyanamide (n = 6)</th>
<th>Experiment 3 (isocaloric model) Control (n = 5)</th>
<th>Experiment 3 (isocaloric model) Ethanol (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>211 ± 18</td>
<td>215 ± 24</td>
<td>306 ± 26</td>
<td>329 ± 28</td>
<td>353 ± 8</td>
<td>347 ± 10</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>350 ± 15</td>
<td>340 ± 25</td>
<td>370 ± 29</td>
<td>373 ± 33</td>
<td>563 ± 8</td>
<td>539 ± 23</td>
</tr>
<tr>
<td>Duration of ethanol</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>consumption (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average ethanol</td>
<td>10 ± 2</td>
<td>10 ± 2</td>
<td>14 ± 3</td>
<td>14 ± 3</td>
<td>0</td>
<td>13 ± 0</td>
</tr>
<tr>
<td>consumption (g/kg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Blood ethanol conc.</td>
<td>29 ± 8*</td>
<td>32 ± 2*</td>
<td>51 ± 16*</td>
<td>52 ± 7*</td>
<td>0*</td>
<td>41 ± 11*</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Blood acetaldehyde conc.</td>
<td>1 ± 0.4*</td>
<td>29 ± 14*</td>
<td>6 ± 5*</td>
<td>56 ± 30*</td>
<td>n.m.</td>
<td>n.m.</td>
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<tr>
<td>(μmol/l)</td>
<td></td>
<td></td>
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<tr>
<td>CDT(%) (initial)</td>
<td>6.3 ± 0.6</td>
<td>6.3 ± 0.8</td>
<td>6.3 ± 0.9</td>
<td>7.8 ± 0.9</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>CDT(%) (final)</td>
<td>6.1 ± 0.8</td>
<td>7.1 ± 0.7</td>
<td>6.6 ± 0.7</td>
<td>8.1 ± 0.7</td>
<td>4.6 ± 0.7</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

*Measured blood concentration (mean ± SD) during last week 1.5 h after intragastric ethanol intubation in experiments 1 and 2.  
*Measured blood concentration (mean ± SD) at end of experiment 3  
CDT = carbohydrate deficient transferrin; n.m. = not measured.

were made 2 and 7 days after toxin administration. In an additional study, 1000 mg/kg of galactosamine was administered to four other rats and 1.5 ml/kg of carbon tetrachloride was administered to four more rats. Blood for CDT and ALAT was taken before toxin administration and after that every day during 1 week. The samples were taken from unanaesthetized rats from the tail vein. At the end of the experiment, the rats were anaesthetized and blood samples were taken by intracardiac puncture before decapitation.

**RESULTS**

In this study attempts were made to maximize the daily ethanol intake and to maintain high blood ethanol levels. The maximal daily ethanol intake was 16.0 g/kg body wt. The mean (±SD) ethanol intake in experiment 1 (7 weeks), 2 (4 weeks) and 3 (10 weeks) was 10 ± 2, 14 ± 3 and 13 ± 0 g/kg/day respectively (Table 1). The maximum blood ethanol concentration was similar in the groups receiving only alcohol or alcohol plus cyanamide. The blood ethanol concentration was the highest in experiment 2, in which higher doses were administered. The acetaldehyde concentration as assayed from peripheral blood 90 min after intubation ranged from 1 to 6 μmol/l among rats drinking alcohol only and from 29 to 56 μmol/l among ethanol plus cyanamide-treated rats (Table 1).

Rat transferrin focused at 8 different bands with two major components at pI 5.6 and 5.8. Rat CDT or desialylated transferrin, in this study, was defined as the second and thereafter situated cathodal bands from the major band (pI 5.8) in the transferrin pattern (Fig. 1). No desialylation
was observed with increasing ethanol administration, regardless of the severity of the exposure or if acetaldehyde levels were elevated by ALDH inhibition (Fig. 1 and Table 1). After intraperitoneal galactosamine injection, there was a difference in transferrin focusing pattern, with cathodal components (high pi) being heavier than in the control sample; one of each is duplicated in Fig. 1, which also shows typical transferrin patterns in control rats and in those treated with ethanol or liver toxins and also after serum neuraminidase addition in vitro. A scan and the relative amounts of desialylated transferrin isoforms after the above treatments can be seen in Fig. 2.

The results in Fig. 3 show the changes in CDT after administration of the hepatotoxins galactosamine (Fig. 3A) and carbon tetrachloride (Fig. 3B). The percentage of CDT during the first 24 h was doubled after galactosamine injection (1000 mg/kg). The CDT was still elevated 48 h after injection, but returned to initial levels after 3
days. The galactosamine dose (500, 1000, 1500 mg/kg body weight) did not correlate with the CDT elevation; the corresponding mean CDT values (two rats for each dose) were 14.1, 14.5, 15.4%, respectively as compared to the CDT value of 6.3% in control rats (data not shown). By contrast, CDT values were not significantly altered after administration of carbon tetrachloride (Fig. 3B), even in a dose as high as 1.5 ml/kg body wt.

Figure 3 also shows changes in serum ALAT activity in the hepatotoxin experiments with galactosamine (Fig. 3A) or carbon tetrachloride (Fig. 3B). After galactosamine treatment, a dramatic but reversible elevation of serum ALAT was observed, which was relatively stronger than after CCl4 (Fig. 3B).

DISCUSSION

In the present study, using three different protocols for long-term ethanol administration, we were unable to observe any changes in transferrin. Also, when acetaldehyde levels were elevated 10-30-fold by partially blocking aldehyde dehydrogenase, no effect on the relative amount of isotransferrin was seen. It is noticeable that in peripheral blood, acetaldehyde levels are considerably much lower than in the hepatic vein, which better reflects the concentration in the liver (Nuutinen et al., 1983). By extrapolation based on a previous study (Eriksson and Sippel, 1977), it can be estimated that a tail blood acetaldehyde level of ~50 μmol/l reflects a hepatic concentration of 150–200 nmol/g, while in the absence of cyanamide this level would be in the range of 50 nmol/g.

Our results are in concordance with those of Stibler et al. (1989), who were unable to observe any significant effect on transferrin, in spite of the ethanol exposure being extensive (4–6 weeks) and blood ethanol reaching moderate to high levels (20–80 mmol/l). We used one protocol with an extended exposure, 10 weeks, but were unable to observe any changes in transferrin pattern. These two studies are at variance with that of Batey and Pattersson (1991), who reported alcohol-induced increase of CDT after 18 weeks of treatment. These effects were observed in spite of the fact that these animals had quite low blood ethanol levels (2–5 mmol/l). These low levels are surprising in comparison with the unusually high daily ethanol intake (22–30 g ethanol/kg) reported. This number exceeds by a factor of 2 the capacity of normal rats to eliminate ethanol. Animals eliminating ethanol at this unusual rate may serve as a model for ethanol-induced transferrin changes, that are not observed in the studies by ourselves or Stibler et al. (1989). Additionally, gender difference in glycoprotein metabolism in rats might not explain the difference because among humans, males as well as females have elevated CDT after alcohol abuse (Stibler and Borg, 1986). Recently Xin et al. (1995) observed an increase in rat serum CDT content after ethanol intake. The use of different rat strain and different ethanol feeding manner may partly explain this discrepancy. It is also possible that nutritional factors have an effect on protein glycosylation. Energy depletion and glucose starvation may disturb protein glycosylation with defects in oligosaccharide chains and a reduced number of oligosaccharide chains (Elbein, 1987).

Both galactosamine and carbon tetrachloride administration caused liver dysfunction, as suggested by the serum ALAT elevation. However, there was no significant alteration in CDT after carbon tetrachloride administration. In contrast, intraperitoneal administration of galactosamine induced clear desialylation of transferrin. This is in agreement with earlier works on galactosamine exposure (Sawamura et al., 1981; Monnet et al., 1985) demonstrating increased amounts of asialglycoproteins. In the present study, CDT elevation existed with ALAT elevation but there does not seem to be a correlation between the two. This may be due to CDT having been maximally elevated earlier, or to the two phenomena being caused by different mechanisms.

Sawamura et al. (1981) found a link between liver damage and increased amount of asialglycoproteins by inducing a reversible hepatocellular injury with galactosamine. They observed accumulation of asialglycoproteins in serum and a concomitant decrease in asialglycoprotein receptors. The increased percentage of asialotransferrin may be due to a decrease in hepatic clearance of desialylated isotransferrins, although the serum survival time of asialotransferrin is not shortened as much as many other asialglycoproteins (Ashwell and Harford, 1982).

The present study did not show an alteration in
the transferrin glycoprotein moiety with ethanol exposure in rats. The reason for the apparent difference between human and rat in these phenomena may be in the ethanol metabolism or it can be related to species differences in transferrin and glycoprotein metabolism. Rat transferrin molecule has a single, mainly tri-sialylated, oligosaccharide side chain (Irie et al., 1988; Spik et al., 1991), but human transferrin molecule has two disialylated glycans. Xin et al. (1995) reported that the glycosylation defect is due to the inhibition of glycosyltransferases by acetaldehyde in the Golgi complex and increase in sialidase activity. According to Xin et al. (1995), this mechanism can explain also the defect in human transferrin glycosylation. However, in human transferrin the abnormal increase is primarily in disialo- and asialoglycoforms rather than continuously in tri-, di-, mono- and asialotransferrins. This indicates perhaps that the defect in glycosylation is earlier in the biosynthesis of oligosaccharide chains, maybe in the synthesis or transfer of a lipid-linked oligosaccharide precursor. The mechanism behind a glycosylation defect induced by heavy alcohol consumption has not yet been completely resolved. Animal models may be helpful, although the species differences can confuse the results.

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REFERENCES


