RAPID COMMUNICATION

PREVENTION BY CYCLOHEXIMIDE OF THE AUDIOGENIC SEIZURES AND TRYPHTHAN METABOLIC DISTURBANCES OF ETHANOL WITHDRAWAL IN RATS

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Abstract — Cycloheximide (20 mg/kg body wt, given intraperitoneally at —1 and 3 h after withdrawal of an ethanol-containing liquid diet) prevents the activation of liver tryptophan pyrrolase, the consequent inhibition of synthesis of brain 5-hydroxytryptamine, and the audiogenic seizures observed at 7 h after alcohol withdrawal. We suggest that a rapidly-turning-over protein mediates the alcohol withdrawal syndrome and discuss the possible role of liver tryptophan pyrrolase.

INTRODUCTION

We have previously shown (Badawy et al., 1980) that chronic ethanol administration in drinking water causes an enhancement of rat brain 5-hydroxytryptamine (5-HT, serotonin) synthesis and turnover as a result of inhibition of activity of the major tryptophan (Trp)-degrading enzyme, hepatic Trp pyrrolase (tryptophan 2,3-dioxygenase, EC 1.13.11.11), which, in turn, increases circulating Trp and its availability to the brain. We also showed (Badawy et al., 1980) that subsequent withdrawal of ethanol led to inhibition of brain 5-HT synthesis and turnover secondarily to a decrease in circulating Trp availability to the brain, because of induction of liver Trp pyrrolase activity. Under these conditions, the withdrawal changes developed slowly, reaching their maxima after 7 days, and were not accompanied by any gross behavioural disturbances. To study further the possible role of Trp-metabolic disturbances in alcohol dependence, a robust withdrawal syndrome was needed, which necessitated administration of ethanol by another procedure. We have recently confirmed that the above Trp-metabolic disturbances also occur in rats to which ethanol is administered chronically by the liquid diet procedure (Bano et al., 1996). Here, removal of the liquid diet was followed by rapid appearance of the behavioural manifestations of the alcohol-withdrawal syndrome (AWS) (R. G. Oretti et al., unpublished observations) and rapid increases in both Trp pyrrolase activity and mRNA expression, with the consequent changes in Trp metabolism and disposition (Bano et al., 1996).

Liver Trp pyrrolase has a rapid turnover rate of 2 h for the apoenzyme, and increases in its activity resulting from enhanced synthesis can therefore be rapidly prevented by the translational inhibitor cycloheximide (CHX) (see, e.g. Badawy and Evans, 1975). The initial purpose of the present work was therefore to find out if prevention by CHX of liver Trp pyrrolase activation during alcohol withdrawal can also lead to blockade of the consequent changes in Trp metabolism and disposition in rats. The results of the present paper show that this is so, and additionally demonstrate...
Table 1. Prevention by cycloheximide of alcohol withdrawal seizures and the associated tryptophan metabolic disturbances in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control rats</th>
<th>Alcohol-withdrawn rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>Seizures</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Liver TP:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>1.9 ± 0.15</td>
<td>1.5 ± 0.18</td>
</tr>
<tr>
<td>Total enzyme</td>
<td>4.0 ± 0.31</td>
<td>1.6 ± 0.17***</td>
</tr>
<tr>
<td>Apoenzyme</td>
<td>2.1 ± 0.16</td>
<td>0.1 ± 0.06***</td>
</tr>
<tr>
<td>% Saturation</td>
<td>47 ± 0</td>
<td>-</td>
</tr>
<tr>
<td>Serum [Trp]:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free</td>
<td>2.15 ± 0.04</td>
<td>4.02 ± 0.15***</td>
</tr>
<tr>
<td>Total</td>
<td>14.98 ± 0.78</td>
<td>28.49 ± 1.11***</td>
</tr>
<tr>
<td>Liver [Trp]</td>
<td>6.43 ± 0.20</td>
<td>7.47 ± 0.20**</td>
</tr>
<tr>
<td>Brain indoles:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Trp]</td>
<td>3.17 ± 0.08</td>
<td>4.29 ± 0.06***</td>
</tr>
<tr>
<td>[5-HT]</td>
<td>0.81 ± 0.020</td>
<td>0.88 ± 0.022*</td>
</tr>
<tr>
<td>[5-HIAA]</td>
<td>0.39 ± 0.005</td>
<td>0.46 ± 0.007***</td>
</tr>
</tbody>
</table>

Experiments were performed 7 h after withdrawal of the liquid diets. In seizure assessment, the numbers of rats fitting/the total numbers of rats tested are given. All other values are means ± SEM of 5 rats per group. Liver tryptophan pyrrolase activity was determined in either the absence (holoenzyme) or the presence (total enzyme) of added (2 μM) haematin. The apoenzyme activity was calculated by difference and the haem saturation of the enzyme was expressed as a percentage (100 × holoenzyme activity/total enzyme activity). Where the apoenzyme or the % saturation are not given (−) is because in the former case there is no free apoenzyme because of increased haem saturation, whereas in the latter case, there is no increased saturation because of inactivation of the free apoenzyme. Liver tryptophan pyrrolase activities are expressed in μmol of kynurenine formed/h per g wet wt, whereas all other expressions are in ng/ml of serum or per g wet wt of tissue. The values in columns 2 and 3 have been compared with those in column 1, whereas those in column 4 were compared with those in column 2, and the significance of the differences is indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations used: TP, tryptophan pyrrolase; Trp, tryptophan; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindol-3-ylacetic acid.

The ability of CHX to block the audiogenic seizures of the AWS. A summary of this work has appeared in Abstract form (Oretti et al., 1995).

MATERIALS AND METHODS

Animals and treatments

Locally bred male Wistar rats (150–200 g at the start of experiments) were housed in groups of five per cage under standard conditions and were maintained on lab chow and water. Some groups were then given ad libitum the liquid diet (Dyets Inc., 2508 Easton Avenue, Bethlehem, PA 18017, USA) of Lieber and DeCarli (1989) containing 8% (v/v) ethanol for 4 weeks. Matched control groups were given equal volumes of an ethanol-free control liquid diet in which ethanol was isocalorically substituted for by maltose-dextrin. Other relevant experimental details have already been described (Bano et al., 1996). For withdrawal, the ethanol and control liquid diets were removed at 07:00 and both control and test animals were treated i.p. at −1 and 3 h after withdrawal with either CHX (20 mg/kg) or an equal volume (2 ml/kg) of saline, and were killed (by a rising concentration of CO₂ followed by decapitation) 4 h after the last injection, i.e. 7 h after withdrawal. The above CHX dosage regimen was designed to ensure the maximum possible sustained inhibition of liver Trp pyrrolase activity (in the light of previous work by Badawy and Evans, 1975) over the 7 h ethanol withdrawal period of study. The CHX solution for injection was prepared as described by Badawy and Evans (1975). Rats used for the biochemical studies were different from those used for assessment of the withdrawal seizures.
**Behavioural, enzymic and other determinations**

Alcohol withdrawal was assessed by measuring audiogenically-induced seizures as described by Hunter et al. (1975). Although the other less severe behavioural manifestations of the AWS were also observed, they were not assessed systematically in the present work and could not therefore be described here. Also, audiogenic seizures were studied in the present work only at the 7 h time point, because this is the time interval after withdrawal of ethanol-containing liquid diets at which seizure intensity as well as the Trp metabolic changes are both maximal under our experimental conditions (unpublished observations).

Liver Trp pyrrolase activity was determined either in the absence (holoenzyme) or the presence (total enzyme) of added (2 µM) haematin as described by Badawy and Evans (1975). The apoenzyme activity was obtained by difference and the saturation of the pyrrolase with its cofactor haem was expressed as a percentage (100 x holoenzyme activity/total enzyme activity). Other parameters of Trp metabolism and disposition were determined by established procedures (for references, see Badawy et al., 1980). Statistical analysis of the biochemical results was performed by Student’s t-test.

**RESULTS**

**Prevention of alcohol-withdrawal seizures by cycloheximide**

As shown in Table 1, whereas most saline-treated ethanol-withdrawn rats exhibited audiogenic seizures, none of those given cycloheximide did. This is the first demonstration of blockade of ethanol withdrawal seizures by a protein synthesis (translational) inhibitor.

**Effects of alcohol withdrawal on tryptophan metabolism and disposition and their prevention by cycloheximide**

The biochemical changes observed during ethanol withdrawal are also shown in Table 1. The holoenzyme activity of liver Trp pyrrolase was enhanced at 7 h after withdrawal by 342%, whereas that of the total enzyme was elevated by 118%. As a result, the haem saturation of the enzyme was increased from a basal value of 47% in saline-treated controls to near-maximum saturation (97%). When ethanol-withdrawn rats were treated with CHX, the activation of Trp pyrrolase was totally abolished and the values for the three forms of the enzyme were not dissimilar to those observed in control rats treated with CHX alone; the latter caused inhibition of the basal activities of the holoenzyme, total enzyme and apoenzyme of 21%, 60% and 95% respectively.

Free (ultrafiltrable) serum, total (free + albumin-bound) serum, liver and brain Trp concentrations and those of brain 5-HT and its major metabolite 5-HIAA were decreased at 7 h after ethanol withdrawal by 26%, 30%, 23%, 31%, 17% and 28% respectively (Table 1). Because of the proportionate decreases in free and total serum [Trp], Trp binding to albumin was not significantly altered (data not shown). By contrast with the above effects of Trp pyrrolase activation during ethanol withdrawal on Trp metabolism and disposition, the effects of CHX administration to control rats demonstrated all the consequences of Trp pyrrolase inhibition, namely significant increases in concentrations of free serum, total serum, liver and brain Trp and brain 5-HT and 5-HIAA, of 87%, 90%, 16%, 35%, 9% and 18% respectively (Table 1). Furthermore, CHX administration to ethanol-withdrawn rats prevented all the above decreases in Trp and 5-hydroxyindole concentrations caused by withdrawal and even maintained the elevations of serum total Trp and brain indoles which it produced by itself in control rats (Table 1). It should be pointed out here that the CHX elevation of free and total serum [Trp] in control rats (Table 1) is far greater than would be expected from liver Trp pyrrolase inhibition alone; it could therefore also be caused by inhibition of protein synthesis leading to a further accumulation of Trp.

**DISCUSSION**

We have recently demonstrated (Bano et al., 1996) that withdrawal of ethanol-containing liquid diets induces a rapid activation of rat liver Trp pyrrolase, secondarily to a possible increase in its synthesis, leading to a decrease in circulating Trp availability to the brain and a consequent inhibition of cerebral 5-HT synthesis and turnover. The present results (Table 1) in control rats confirm these observations, whereas those demonstrating
the CHX prevention of the ethanol withdrawal effects provide further support to the conclusion that inhibition of brain 5-HT synthesis during alcohol withdrawal is due to activation of liver Trp pyrrolase (see also Badawy et al., 1980). It could be argued that CHX masks, rather than prevents, the effects of alcohol withdrawal on the various parameters of Trp disposition (i.e. Trp and 5-hydroxyindole concentrations in serum, liver and/or brain) because it exerts opposite effects by itself in control rats. However, because these latter effects are exerted in control rats by virtue of inhibition of liver Trp pyrrolase activity, the conclusion must be reached that CHX also prevents the pyrrolase-dependent decreases in the various parameters of Trp disposition in withdrawn rats.

Because the increase in liver Trp pyrrolase activity during ethanol withdrawal is associated with an increased haem saturation of the enzyme (Table 1; see also Bano et al., 1996), the mechanism of activation could either be substrate (Trp) or cofactor (haem) dependent, rather than hormonally (corticosterone) mediated (for differences between these mechanisms, see Badawy and Evans, 1975). Further work is, however, required to establish which of the above two mechanisms is responsible.

The present experiments were not, however, designed to examine the possibility that the Trp disposition disturbances described here play a role in the behavioural manifestations of the AWS. It is unlikely that they do, although the possibility could not be ruled out that the observed decreases in 5-HT synthesis and turnover may.

One of the severest features of the AWS is seizure and the present results also demonstrate the ability of the translational inhibitor CHX to block audiogenically-induced seizures during alcohol withdrawal in rats (Table 1). This is the first demonstration of the ability of a translational inhibitor to block alcohol-withdrawal seizures. Although, as stated earlier, the 7 h time point was chosen in the present work as the peak seizure response time, a more detailed time-course examination of the CHX effect is desirable. Also, whether CHX can block other types of seizures remains to be investigated, to establish the specificity of this effect in relation to alcohol withdrawal.

The present results with CHX, however, suggest that the AWS may be mediated by a rapidly-turning-over protein(s). Current opinion has implicated two proteins in the AWS, namely the GABA_A receptor and the NMDA receptor. The possible effects of CHX on the functions of these two receptors during alcohol withdrawal have not as yet been investigated. Also, the turnover rate of the NMDA receptor is so far unknown and thus requires assessment. An estimate of that of the GABA_A receptor by one group initially suggested two phases of 4 h and 32 h respectively (Borden and Farb, 1988), but was later revised to 18 h (Roca et al., 1990). Evidence from electrophysiological and other studies, however, suggests that the hyperexcitability of the AWS may not be mediated by primary decreases in inhibition mediated by the GABA_A receptor (Whittington et al., 1992; see also the recent comment by Tabakoff, 1995).

A third candidate is liver Trp pyrrolase, whose activity controls the rate of Trp degradation along the hepatic kynurenine pathway, a product of which is the excitotoxin and NMDA receptor agonist quinolinic acid. Morgan (1991) suggested that quinolinate may be involved in the AWS, and our preliminary results suggest that quinolinate concentration is elevated by twofold in rat liver and brain at 7 h after ethanol withdrawal. Further work to establish the possible role of this excitotoxic Trp metabolite in the AWS in relation to NMDA receptor functional disturbances is being planned. The present results also suggest that cycloheximide and possibly also other (protein-synthetic as well as Trp-metabolic) inhibitors may be useful in further studies of the molecular and other events associated with alcohol withdrawal.

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REFERENCES


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