REGIONAL SELECTIVITY IN ETHANOL-INDUCED PRO-OXIDANT EVENTS WITHIN THE BRAIN

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Abstract—Several biochemical parameters that reflect the presence of excess levels of reactive oxygen species were modulated in the brains of rats exposed acutely or subchronically to ethanol. These parameters included depression of cytosolic glutathione (GSH) concentration and of glutamine synthetase levels. However, using these indices, there was a significant difference in susceptibility to ethanol in different brain regions. After dietary exposure to ethanol for 12 days, these indices were selectively depressed in the striatum but not in the cerebral cortex or cerebellum. Eighteen hours after a single acute dose of ethanol (4.5 g/kg body wt), the striatum was also the only one of these areas in which proteolytic activity was elevated by ethanol treatment. Two injections of acetaldehyde (300 mg/kg), given 18 and 2 hr prior to tissue preparation, caused a specific reduction of glutamine synthetase in the striatum and a decrease of GSH levels in both striatum and cerebellum. Taken together, the results suggest a distinctive vulnerability of the striatum to ethanol-promoted oxidative events. Rather than ethanol exerting effects directly, the metabolite acetaldehyde may be the primary agent responsible for these changes.

Key words: ethanol; free radicals; oxidative stress; brain regions; striatum

The evidence for ethanol inducing pro-oxidant events in the liver is unequivocal. Smaller but significant oxidative events have been described frequently in the central nervous system after ethanol treatment [1–3]. The difficulty of obtaining more definitive data in this case may be due to the considerable regional heterogeneity of the central nervous system. For this reason, we have studied the response of several brain regions to ethanol exposure. The regions selected were the cortex, often described as a target of ethanol toxicity [4]; the cerebellum, where distinctive, localized morphological changes have been reported [5]; and the striatum, postulated as especially susceptible to oxidative stress because of its high dopamine content [6]. The selection of these parameters for assay was based on previous work indicating that they might be useful in detecting ethanol-induced oxidative events [7]. Glutamine synthetase is a particularly sensitive index, since its degradation can represent the temporal integration of chronic low level alterations in free radical production [8], and this is apparently due to a site-specific, metal-catalyzed oxidative attack on an amino acid residue [9]. GSH† levels may represent changes occurring over a shorter time period. The direct measurement of free radical production may be limited in that it represents a single instant in time. Furthermore, ethanol is a direct quencher of free radicals and thus can prevent their appearance.

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† Abbreviations: (Cbz-Arg-NH₂)₂ rhodamine, bis(*N*-benzyloxycarbonyl-L-arginamido)rhodamine; GSH, glutathione; mCBl, monochlorobimane; and ROS, reactive oxygen species.

MATERIALS AND METHODS

Materials

(Cbz-Arg-NH$_2$)$_2$ rhodamine and mBCl were purchased from Molecular Probes, Inc. (Eugene, OR). All other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 150–175 g, were utilized. Rats were housed four per cage with wood chip bedding and maintained on a 12-hr light/dark cycle in a temperature-controlled (20 ± 1°) room. Food (Purina Laboratory Chow, St. Louis, MO) and water were provided *ad lib.* until the start of the feeding protocol.

Ethanol and acetaldehyde treatment

Acute dosing. Rats were injected intraperitoneally with 25% (v/v) ethanol at a dose of 4.5 g/kg body weight, and were killed 18 hr after dosing. No circulating ethanol was detectable at the 18 hr time point, and this interval has been found appropriate for the parameters under study [7]. Acetaldehyde dosing was performed with a 7.5% (v/v) solution at 300 mg/kg injected twice, 2 and 18 hr prior to decapitation.

Subchronic dosing. An all-liquid nutrient (Sustercal, Mead Johnson, Evansville, IN) was employed [2]. All rats received 88% Sustercal, 12% water (v/v) for 2 days. Then half of the rats received 88% Sustercal, 7% water, 5% ethanol (by vol.) for another 2 days. Fresh solutions were made up daily. At this time, the ethanol proportion was raised to 7% while water was reduced to 5%. Animals were

decapitated after a total of 12 ethanol drinking days. The fluid intake of the control group drinking the liquid nutrient alone was limited to 120% of the volume drunk on the previous day by the group receiving the ethanol-containing liquid diet. This was isocaloric with the original ethanol-contained nutrient. Rat weights were monitored and recorded every second day, together with fluid consumption of each group.

Tissue preparation

Rats were decapitated, the brains were excised quickly on ice, and the cerebrocortex, cerebellum and striatum were dissected out [10]. All tissues were placed into screw-capped microcentrifuge tubes, stored at $-20°$ for 24 hr, and subsequently stored at $-70°$ until used. A relatively slow freezing rate was intended to maintain the integrity of subcellular structures [11]. Each tissue was weighed and homogenized in 10 vol. of 0.32 M sucrose and centrifuged at $1800\,g$ for 10 min. The resulting supernatant fraction was then centrifuged at $31,500\,g$ for 10 min to yield the supernatant (S2) fraction. This was suspended in HEPES buffer to a concentration of 0.2 g-Eq/mL. The final protein content of S2 suspensions was 1.6 to 3.1 mg/mL. The composition of the HEPES buffer was (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10; pH 7.4.

Glutamine synthetase

This enzyme was assayed as γ-glutamyl transferase activity by incubation (30 min, 37°) of 0.1 mL of S2 preparation together with (mM) l-glutamine (50), hydroxylamine (75), sodium ADP (0.5), $MnCl_2$ (0.2), imidazole–HCl, (50), and sodium arsenate (25), in a final volume of 1 mL. The γ-glutamyl hydroxylamate formed could then be quantitated after centrifugation ($5000\,g$, 5 min) by spectrophotometric assay of the colored product formed with acidified $FeCl_3$ [12]. A standard curve was concurrently generated with γ-glutamyl hydroxylamate. The iron complex of 1 mmol/mL of this compound gave an absorbance of 0.340 at 535 nm. When the effects of various agents on enzyme activity were studied, a 30-min preincubation of the S2 fraction with these chemicals preceded enzyme assay.

Determination of reduced GSH

GSH levels were determined using a modification of the method of Shrieve et al. [13]. The principle behind the assay is that mBCl, a nonfluorescent compound, reacts with GSH to form a fluorescent adduct. It has been shown that there is very little reaction between mBCl and protein sulfhydryl groups [14]. mBCl was dissolved in ethanol to a concentration of 5 mM and stored at $-10°$ in the dark. mBCl was added to 2 mL of an S2 suspension to a final concentration of 20 μM, after which the suspension was incubated for 15 min at 37° in 1% (w/v) Triton and then centrifuged for 10 min at $31,500\,g$. The fluorescence of the supernatant was read on a Perkin–Elmer spectrofluorometer at an excitation wavelength of 395 nm and an emission wavelength of 470 nm. The tissue GSH concentration
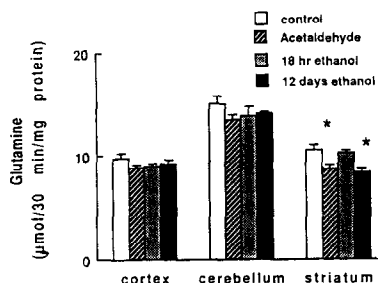

Fig. 1. Levels of glutamine synthetase in brain regions of ethanol-consuming rats. Each data point represents the mean ± SEM of values derived from 6–8 individual animals. Key: (*) level differs significantly from the corresponding control ($P < 0.05$; Student's t-test after analysis of variance and Fisher's Least Significant Difference Test).

was determined using a GSH standard curve. This determination was performed in the presence of 0.1 U/mL of liver GSH transferase in order to accelerate the formation of the fluorescent adduct derivative.

Assay for proteolytic activity

Serine protease activity was measured by a method utilizing the cleavage of a synthetic peptide, whose hydrolytic cleavage leads to the appearance of rhodamine, which is quantitated by fluorescent assay [15]. $(Cbz-Arg-NH_2)_2$ rhodamine, dissolved in ethanol, was added at a final concentration of 10 μM, to 2 mL of an S2 suspension in Tris–HCl buffer. The appearance of rhodamine over a 1 hr incubation at 37° was determined fluorometrically (excitation: 492 nm; emission: 523 nm) and calibrated using a rhodamine standard.

Ethanol determination

Ethanol was assayed in plasma by enzymic conversion to acetaldehyde in the presence of NAD. The consequent rise in NADH was then monitored at 340 nm [16].

Protein determination

Protein concentration was assayed using the method of Bradford [17].

Statistical analyses

Differences between groups were assessed by ANOVA followed by Fisher's Least Significant Difference Test. The acceptance level of significance was $P < 0.05$, using a two-tailed distribution. Each value presented was obtained from 6–8 individual rats.

RESULTS

The subchronic ethanol dosing regimen resulted in blood ethanol levels of 164 ± 22 mg/100 mL. Levels of glutamine synthetase were depressed significantly in the striatum of exposed animals, but no parallel changes were observed in the
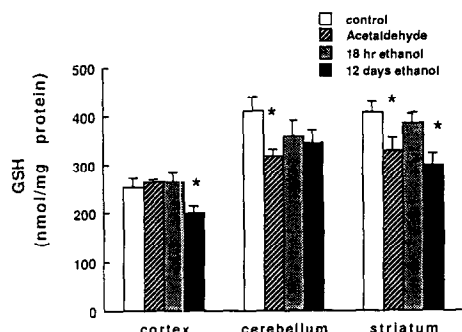
Fig. 2. Cytosolic GSH content in brain regions from ethanol-treated rats. Each data point represents the mean ± SEM of values derived from 6–8 individual animals. Key: (*) level differs significantly from the corresponding control (P < 0.05; Student's t-test after analysis of variance and Fisher's Least Significant Difference Test).
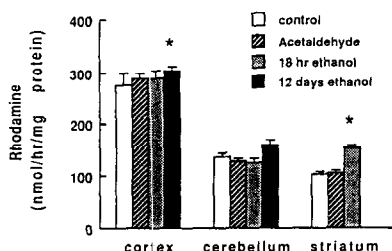

Fig. 3. Proteolysis in post-mitochondrial supernatants from brain regions of ethanol-treated rats. Values represent the rate of hydrolysis of (Cbz-Arg-NH$_2$)$_2$ rhodamine. Each data point is the mean ± SEM of values derived from 6–7 individual animals. Key: (*) level differs significantly from the corresponding control (P < 0.05; Student's t-test after analysis of variance and Fisher's Least Significant Difference Test).

cerebrocortex or the cerebellum (Fig. 1). GSH levels were depressed in both the striatum and the cortex (Fig. 2). In rats receiving a single intraperitoneal injection of ethanol, these parameters were unchanged after 18 hr, in all brain regions studied. However, following two injections of acetaldehyde (300 mg/kg) at 18 and 2 hr prior to tissue preparation, the glutamine synthetase content of the striatum was selectively depressed, while GSH concentration was reduced in both striatum and cerebellum (Figs. 1 and 2).

Cytosolic proteolytic activity may be induced by oxidative events consequent to an elevated level of damage to proteins [18]. For this reason, levels of proteolysis were examined in the regions under study. Basal proteolytic rates were lowest in the striatum. Acute treatment with ethanol significantly increased striatal levels of proteolytic activity, but this was not the case for cerebellum or cortex. In the subchronically treated rats, proteolysis was increased significantly in cortex, but there was

insufficient tissue to allow a parallel determination within the striatum (Fig. 3). Acetaldehyde treatment did not affect proteolysis levels in any region studied.

DISCUSSION

The induction of excess ROS by ethanol may be primarily by way of its metabolite, acetaldehyde. This conclusion has been reached by other groups [19–21]. The report that behavioral changes caused by acetaldehyde can be prevented by pretreatment with anti-oxidants [22] implies that this concept may also apply to CNS metabolism of ethanol. The dose of ethanol used in this study has been reported to lead to intracerebral acetaldehyde concentrations of around 5 μM [23]. Acetaldehyde appears to be able to penetrate into the brain following a single intraperitoneal injection [4], but the concentrations attained within nerve tissue are not known. The enhancement of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity by ethanol [24] suggests that acetaldehyde may also underlie the effects upon the striatum described here. The failure of acetaldehyde to modulate proteolytic activity after 18 hr of treatment may be because the residence period of acetaldehyde within the brain was insufficient to allow major induction of proteolytic enzymes.

The general conclusion from these data is that the striatum shows a distinctive vulnerability to elevation of features suggestive of increased pro-oxidant status, and that this feature is not characteristic of all brain regions that are considered to be susceptible to ethanol. Excess ROS generation in the striatum is suspected to play a role in Parkinson's disease [25] and in tardive dyskinesia [26]. Increased striatal rates of ROS generation have been reported for dopamine-selective neurotoxic agents such as MPTP [27]. However, there are also reports of selective damage to the striatum being initiated by neurotoxic agents that are not clearly related to dopamine metabolism but which may be general effectors of oxidative stress [28, 29]. Thus, the striatum may be intrinsically more susceptible to oxidative damage than other brain regions [30]. However, Parkinsonian symptoms are not a predominant feature of alcoholism. The reason for this discrepancy may be due to the fact that the striatum has considerable redundancy and that behavioral changes reflecting dopaminergic insufficiency do not appear before the loss of around 80% of dopaminergic neurons.

Ethanol has been found to inhibit proteolysis in the liver and to lead to an accumulation of excess protein in this organ [31, 32]. In the present work, the cytosolic fraction of liver from subchronically treated rats also showed a significant (18%) reduction of proteolytic activity (data not shown), similar in magnitude to those of earlier reports. It may be that events unrelated to increased ROS production predominate in inhibiting proteases in the liver after ethanol treatment.

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