Title
Regional selectivity in ethanol-induced pro-oxidant events within the brain

Journal
Biochemical Pharmacology, 49(1)

ISSN
0006-2952

Authors
Bondy, Stephen C
Guo, Shirley X

Publication Date
1995

DOI
10.1016/0006-2952(94)00442-o

Pergamon

Biochemical Pharmacology, Vol. 49, No. 1, pp. 69–72, 1995.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/95 $9.50 + 0.00

0006-2952(94)00442-0

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

STEPHEN C. BONDY* and SHIRLEY X. GUO

Department of Community and Environmental Medicine, University of California, Irvine, CA 92715, U.S.A.

(*Received* 23 *May* 1994; *accepted* 22 *August* 1994)

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.

* Corresponding author. Tel. (714) 824-8077; FAX (714) 725-2793.

† Abbreviations: $(Cbz\text{-}Arg\text{-}NH_2)_2$ rhodamine, bis(*N*-benzyloxycarbonyl-L-arginamido)rhodamine; GSH, glutathione; mCBl, monochlorobimane; and ROS, reactive oxygen species.

MATERIALS AND METHODS

Materials

$(Cbz\text{-}Arg\text{-}NH_2)_2$ 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 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.

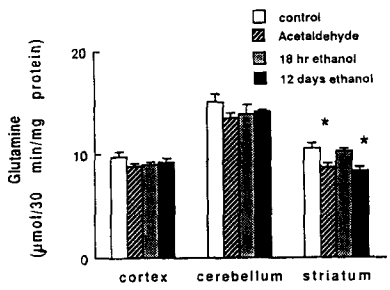

Fig. 1. Levels of glutamine synthetase in brain regions of ethanol-consuming rats. Each data point represents the mean ± SEM of values derived from 6–8 individual animals. Key: (*) level differs significantly from the corresponding control ($P < 0.05$; Student's *t*-test after analysis of variance and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

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]. $(Cbz\text{-}Arg\text{-}NH_2)_2$ 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

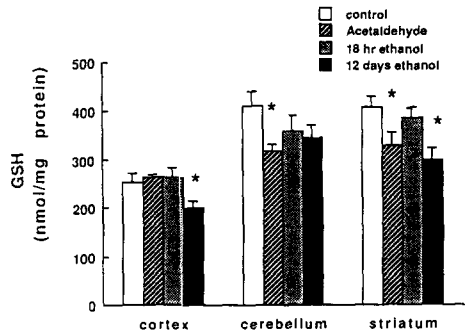

Fig. 2. Cytosolic GSH content in brain regions from ethanol-treated rats. Each data point represents the mean ± SEM of values derived from 6–8 individual animals. Key: (*) level differs significantly from the corresponding control ($P < 0.05$; Student's t-test after analysis of variance and Fisher's Least Significant Difference Test).

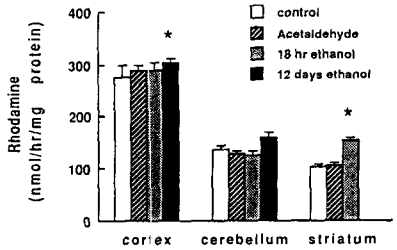

Fig. 3. Proteolysis in post-mitochondrial supernatants from brain regions of ethanol-treated rats. Values represent the rate of hydrolysis of $(Cbz\text{-}Arg\text{-}NH_2)_2$ rhodamine. Each data point is the mean ± SEM of values derived from 6–7 individual animals. Key: (*) level differs significantly 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.

Acknowledgement—This work was supported by a grant from the National Institutes of Health (AA 8281).

REFERENCES

1. Nordmann R, Ribiere C and Rouach H, Ethanol-

induced lipid peroxidation and oxidative stress in extrahepatic tissues. *Alcohol Alcohol* **25**: 231–237, 1990.
2. Bondy SC and Pearson KR, Ethanol-induced oxidative stress and nutritional status. *Alcohol Clin Exp Res* **17**: 651–655, 1993.
3. Anandatheerthavarada HSK, Shankar SK, Bhamre S, Boyd MR, Song BJ and Ravindranath V, Induction of brain cytochrome P-450IIEI by chronic ethanol treatment. *Brain Res* **601**: 279–285, 1993.
4. Phillips SC, Cytoprotective value of lysine, penicillamine and pyridoxal phosphate against the neurotoxicity of acetaldehyde. *Toxicol Appl Pharmacol* **98**: 553–560, 1989.
5. Rouach H, Houze P, Orfanelli MT, Gentil M, Bourdon R and Nordmann R, Effect of acute ethanol administration upon the subcellular distribution of iron in rat liver and cerebellum. *Biochem Pharmacol* **39**: 1095–1100, 1990.
6. Donaldson J, Labella FS and Gesser D, Enhanced autooxidation of dopamine as a possible basis of manganese neurotoxicity. *Neurotoxicology* **2**: 53–64, 1981.
7. Bondy SC and Guo SX, Effect of ethanol treatment on indices of cumulative oxidative stress. *Eur J Pharmacol*, in press.
8. Schor NF, Inactivation of mammalian brain glutamine synthetase by oxygen radicals. *Brain Res* **456**: 17–21, 1988.
9. Stadtman ER, Oxidation of free amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* **62**: 797–821, 1993.
10. Glowinski J and Iversen LL, Regional studies of catecholamines in the rat brain—I. The distribution of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]DOPA in various regions of the brain. *J Neurochem* **13**: 655–659, 1966.
11. Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK and Delaunoy JP, A rapid method for preparing synaptosomes: Comparison with alternative procedures. *Brain Res* **226**: 107–118, 1981.
12. Rowe WB, Ronzio RA, Wellner VP and Meister A, Glutamine synthetase (sheep brain). *Methods Enzymol* **17A**: 900–904, 1970.
13. Shrieve DC, Bump EA and Rice GC, Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J Biol Chem* **263**: 14107–14114, 1988.
14. Rice GC, Bump EA, Shrieve LW, Lee W and Kovacs M, Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: Some applications to radiation and drug resistance *in vitro* and *in vivo*. *Cancer Res* **46**: 6105–6110, 1986.
15. Leytus SP, Melhado LL and Mangel WF, Rhodamine-based compounds as fluorogenic substrates for serine proteases. *Biochem J* **209**: 299–307, 1983.
16. Bucher T and Redetzki H, Eine specifische photometrische Bestimmung von Athylalkohol auf fermantiven Wege. *Klin Wochenschr* **29**: 615–619, 1951.
17. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. *Anal Biochem* **72**: 248–254, 1976.
18. Vince GS and Dean RT, Is enhanced free radical flux associated with increased intracellular proteolysis? *FEBS Lett* **216**: 253–256, 1987.
19. Shaw S and Jayatilleke E, Acetaldehyde-mediated lipid peroxidation: Role of superoxide and ferritin. *Biochem Biophys Res Commun* **143**: 984–990, 1987.
20. Kera Y, Ohbora Y and Komura S, The metabolism of acetaldehyde and not acetaldehyde itself is responsible for *in vivo* ethanol-induced lipid peroxidation in rats. *Biochem Pharmacol* **37**: 3633–3638, 1988.
21. Puntaralo S and Cederbaum AI, Chemiluminescence from acetaldehyde oxidation by xanthine oxidase involves generation of and interaction with hydroxyl radicals. *Alcohol Clin Exp Res* **13**: 84–90, 1989.
22. O'Neill P and Rahwan RG, Protection against acute toxicity of acetaldehyde in mice. *Res Commun Chem Pathol Pharmacol* **13**: 125–131, 1976.
23. Westcott JY, Weiner H, Shultz J and Myers RD, *In vivo* acetaldehyde in the brain of the rat treated with ethanol. *Biochem Pharmacol* **29**: 411–417, 1980.
24. Zuddas A, Corsini GU, Schinelli S, Barker JL, Kopin IJ and Di Porzio U, Acetaldehyde directly enhances MPP^+ neurotoxicity and delays its elimination from the striatum. *Brain Res* **501**: 11–22, 1989.
25. Jenner P, Oxidative stress as a cause of Parkinson's disease. *Acta Neurol Scand* **84** (Suppl 136): 6–15, 1991.
26. Jackson-Lewis V, Prezborski S, Kostic V, Suber F, Fahn S and Cadet JL, Partial attenuation of chronic fluphenazine-induced changes in regional monoamine metabolism by D-α-tocopherol. *Brain Res Bull* **26**: 251–258, 1991.
27. Adams JD Jr and Odunze IN, Biochemical mechanisms of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity. Could oxidative stress be involved in the brain? *Biochem Pharmacol* **41**: 1099–1105, 1991.
28. Liccione JJ and Maines MD, Selective vulnerability of glutathione metabolism and cellular defense mechanisms in rat striatum, to manganese. *J Pharmacol Exp Ther* **247**: 156–161, 1988.
29. Pezzoli G, Ricciardi S, Masotto C, Mariani CB and Cerenzi A, *n*-Hexane induces Parkinsonism in rats. *Brain Res* **531**: 355–357, 1990.
30. Steffen V, Vizuoto ML, Machado A and Cano J, The effect of a vitamin E deficient diet on amino acid levels in the substantia nigra, striatum and hippocampus of rats. *Life Sci* **54**: 375–379, 1994.
31. Donohue DM, Zetterman RK and Tuma DJ, Effect of chronic ethanol administration on protein catabolism in rat liver. *Alcohol Clin Exp Res* **13**: 49–57, 1989.
32. Poso AR and Hirsimaki P, Inhibition of proteolysis in the liver by chronic ethanol feeding. *Biochem J* **273**: 149–152, 1991.