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Research report

Effect of an NMDA receptor antagonist and a ganglioside GM₁ derivative upon ethanol-induced modification of parameters of oxidative stress in several brain regions

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Abstract

Dietary administration of ethanol to rats for 2 weeks was able to depress levels of glutathione (GSH) and Cu/Zn superoxide dismutase (SOD) in several brain regions. This was indicative of the generation of excess levels of reactive oxygen in treated animals. The potentially protective effect of both an NMDA receptor blocker (MK-801) and an internally esterified derivative of ganglioside GM₁ (AGF₂) upon ethanol-induced changes in these indices of oxidative stress, was studied. Both of these agents are reported to have neuroprotective properties, but neither was able to prevent ethanol-induced reduction of GSH and SOD levels in any brain area studied. In fact, both agents depressed SOD and GSH levels in midbrain independently of ethanol. MK-801 had a pronounced pro-oxidant potential, and when administered in combination with ethanol, GSH and SOD were reduced in midbrain and striatum to levels below those obtained with either agent alone. The pro-oxidant properties of ethanol may thus act independently of its actions upon the NMDA receptor. The protective properties of NMDA receptor inhibitors or gangliosides cannot be attributed to any antioxidant effect.

Keywords: Ethanol; Ganglioside GM₁; Free radical; Oxidative stress; Glutamate antagonist

1. Introduction

The toxicity of ethanol has been in part attributed to its ability to promote lipid peroxidation and other indices of oxidative stress [10,29,31]. While this has been most unequivocally demonstrated in the liver, parallel findings have been reported for the central nervous system of ethanol-treated animals [2,28]. The pro-oxidant effects of ethanol upon nervous tissue have been found to be attenuated following pretreatment with various antioxidants including α -tocopherol ([27]; Bondy et al., unpublished data).

Other targets of ethanol effects in the CNS include inhibition of, followed by upregulation of, the NMDA receptor for glutamate [11,19,38]. This has led to investigations of the potential of calcium gating glutamate receptors to protect against the adverse excitatory effects of ethanol withdrawal [23]. In addition, the protective potential of

other materials which may protect intracellularly against glutamate toxicity, such as ganglioside GM₁, has also been reported [23]. Specifically, ganglioside GM₁ may antagonize the behavioral effects of ethanol [18,25] and the developmental deficits incurred by ethanol [24].

The intent of the current study was to link the above-described aspects of ethanol toxicity by examining the effect of two potentially protective agents upon ethanol-induced oxidant events in several brain regions. To this end, an NMDA channel blocker, MK-801 (dizocilpine), was utilized as well as a synthetic ganglioside (AGF₂), the internal ester of ganglioside GM₁ which has both neurochemical and behavioral neuroprotective effects similar to the parent ganglioside [15,30]. A further rationale for the selection of these two agents is the frequently described potentially protective properties of both MK-801 and ganglioside GM₁ against a wide variety of excitatory and ischemic events [13,36,41]. More specifically, both NMDA antagonists and ganglioside GM₁ have been reported to inhibit the increased susceptibility to excitotoxic agents in isolated cell explants treated with ethanol [22].

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2. Materials and methods

2.1. Animals and dosing schedule

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 120–130 g and aged 6–7 weeks were utilized. Rats were housed three per cage with wood chip bedding and maintained on a 12:12 h light/dark cycle in a temperature controlled ($20 \pm 1^\circ\text{C}$) room. Food (Purina Laboratory Chow, St. Louis, MO) and water were provided ad lib. until the start of the feeding protocol.

An all-liquid nutrient diet (Ensure, Abbott, Columbus, OH) was supplied to all rats [6]. All rats received 88% Ensure, 12% water (v/v) for 3 days. Then half of the rats received 88% Ensure/7% water/5% ethanol (v/v) for 3 further days. Fresh solutions were made up daily. At this time the ethanol proportion was raised to 7% while water was reduced to 5%. Animals consumed ethanol for a total of 14 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 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. During the 2-week weight-matched feeding period, groups of control and ethanol-consuming rats also received a daily administration by i.p. injection of either MK-801 (0.025 mg/kg body wt.) or AGF₂ (30 mg/kg) dissolved in distilled water. The dose of MK-801 used, caused no overt behavioral changes. Each study group consisted of six animals.

2.2. Tissue and membrane preparation

Rats were decapitated, the brains were excised quickly on ice and the cerebrocortex, striatum, cerebellum and midbrain were dissected out. Blood was collected, allowed to coagulate and serum centrifuged down for determination of ethanol content. All tissues were placed into screw-capped vials, and stored at -70°C until preparation. Each tissue was weighed and homogenized in 10 vols. of 0.32 M sucrose and centrifuged at $1800 \times g$ for 10 min. The resulting supernatant fraction was then centrifuged at $31,500 \times g$ for 10 min to yield the final supernatant (S2). The final protein content of the S2 fraction was 3.8–10.2 mg/ml. The precipitate was resuspended in HEPES buffer and recentrifuged in order to obtain the crude synaptosomal (P2) fraction. The composition of the HEPES buffer was (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. Crude synaptic membranes were prepared from the P2 precipitate by dispersal in distilled water followed by centrifugation at $48,000 \times g$ for 20 min. This procedure was repeated after further lysis of membranes by freezing at -80°C . In order to facilitate the removal of endogenous glutamic acid, membranes were incubated with

0.04% (w/v) Triton X-100 before being recentrifuged at $48,000 \times g$ for 20 min. Detergent was then removed by two more cycles of membrane resuspension in 50 mM Tris, pH 7.4, and centrifugation.

2.3. Glutathione content

Glutathione levels were determined using a modification of the method of Shrieve et al. [35]. The principle behind the assay is that monochlorobimane (mBCI), a nonfluorescent compound, reacts with glutathione to form a fluorescent adduct. It has been shown that there is very little reaction between mBCI and protein sulfhydryl groups [33]. mBCI was dissolved in ethanol to a concentration of 5 mM and stored at -10°C in the dark. mBCI was added to 0.1 ml of a S2 suspension and 1.9 ml HEPES buffer to a final concentration of 10 μM , after which the suspension was incubated for 15 min at 37°C . The fluorescence of the supernatant was read on a Perkin-Elmer Spectrophotofluorometer at excitation wavelength 395 nm and emission wavelength 470 nm. The tissue GSH concentration was determined using a GSH standard curve. This determination was performed in the presence of 0.1 unit/ml of liver glutathione transferase in order to accelerate the formation of the fluorescent adduct derivative.

2.4. Superoxide dismutase

Superoxide dismutase was assayed in the S2 fraction using the method of Bose et al. [7]. 0.2 ml S2 was added to 0.6 ml 50 mM Tris-HCl, pH 7.4, containing 1.25 mM cacodylic acid and 1.25 mM diethylene triamine pentaacetic acid (DTPA), 0.1 ml 16% Triton X-100, 0.2 ml 1 mM nitroblue tetrazolium (NBZ) and 0.05 ml 1 mM freshly prepared pyrogallol. After incubation for 60 min. at 37°C , the superoxide dismutase-effected retardation of the reduction of NBZ to dihydroformazan was followed at 540 nm. Bovine erythrocyte superoxide dismutase was used as a standard. One unit activity is defined as that which will inhibit the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 and 25°C . Enzyme activity was totally inhibited by 2 mM KCN. Since the mitochondrial Mn enzyme is not inhibited by cyanide [28], this indicated that solely the cytosolic Cu/Zn form of the enzyme was assayed.

2.5. [^3H]MK-801 binding assay

100 μl of the washed cortical membrane fraction containing 0.2–0.3 mg protein was incubated for 60 min at 22°C in 1 ml Tris buffer, pH 7.4, together with 1 μM glycine, 10 μM glutamate, 50 nM 5,7-dichlorokynurenine acid and 0.1 μCi [^3H]MK-801 (22.0 Ci/mmol). Samples were then diluted with 5 ml cold distilled water and filtered through Whatman GF/C glass fiber filters (Whatman, Clifton, NJ) using a Brandel cell harvester.

Filters were washed once with 5 ml water and twice with 5 ml Tris buffer and their radioactive content was then determined in a liquid scintillation spectrometer. Non-specific binding was defined as that determined in the absence of glutamate and glycine, and was less than 20% of the total.

2.6. Plasma levels of ethanol

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 [8].

2.7. Statistical analyses

Differences between groups were assessed by one-way Analysis of Variance 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 six individual rats.

2.8. Materials

The internally cyclized ester of ganglioside GM₁, AGF₂, was a gift from Fidia Research, Abbano Terme, Italy.

Fig. 1. Levels of glutathione (GSH) and superoxide dismutase (SOD) in cerebral cortex of control rats and animals treated with ethanol (E), AGF₂ (AG), MK-801 (MK), singly or in combination. Values $\pm$ S.E.M. are derived from 6 animals/group. * Differs from level in untreated control (C) group. † Differs from control group and from corresponding levels in rats receiving only a single agent ($P < 0.05$).

Fig. 2. Levels of glutathione (GSH) and superoxide dismutase (SOD) in cerebellum of control rats and animals treated with ethanol (E), AGF₂ (AG), MK-801 (MK), singly or in combination. Values $\pm$ S.E.M. are derived from 6 animals/group. * Differs from level in untreated control (C) group ($P < 0.05$).

Fig. 3. Levels of glutathione (GSH) and superoxide dismutase (SOD) in midbrain of control rats and animals treated with ethanol (E), AGF₂ (AG), MK-801 (MK), singly or in combination. Values $\pm$ S.E.M. are derived from 6 animals/group. * Differs from level in untreated control (C) group. † Differs from control group and from corresponding levels in rats receiving only a single agent ($P < 0.05$).

Fig. 4. Levels of glutathione (GSH) and superoxide dismutase (SOD) in striatum of control rats and animals treated with ethanol (E), AGF₂ (AG), MK-801 (MK), singly or in combination. Values $\pm$ S.E.M. are derived from 6 animals/group. * Differs from level in untreated control (C) group. † Differs from control group and from corresponding levels in rats receiving only a single agent ($P < 0.05$).

Monochlorobimane was from Molecular Probes, Eugene, OR. Isotope was from New England Nuclear, Bedford, MA and 5,7-dichlorokynurenine acid from Research Biochemicals International, Natick, MA. All other chemicals were from Sigma Corp., St. Louis, MO.

3. Results

Levels of ethanol circulating in blood did not differ significantly between groups receiving ethanol alone or ethanol in conjunction with either MK-801 or AGF₂, and were 126 ± 23 mg/dl.

Glutathione levels were depressed by ethanol treatment in all brain regions examined, except the cerebral cortex (Figs. 1–4). Both MK-801 and ganglioside AGF₂ dosing also lowered levels of glutathione in midbrain and striatum (Figs. 3 and 4). Animals receiving ethanol together with one of the above chemicals, had glutathione levels in the midbrain and cerebellum that did not differ from values obtained with ethanol alone. However, a combination of ethanol with MK-801, profoundly depressed striatal glutathione to values significantly below those obtained with either agent alone (Fig. 4).

Cytosolic superoxide dismutase levels were broadly reduced in rats consuming ethanol, in all brain areas studied except striatum where this did not reach significance. This may have been due to the relatively large

variance resulting from the small amount of striatal tissue available. A parallel depression of SOD activity was apparent in the cortex, cerebellum and midbrain of rats receiving MK-801 alone (Figs. 1–3). The co-administration of ethanol with either pharmacological agent under test, also led to depressed SOD levels. In the case of cortex and striatum, simultaneous treatment with ethanol and MK-801 led to SOD values which were significantly below those obtained using either agent singly (Figs. 1 and 4).

The overall pro-oxidant tendency of MK-801 was generally greater than in the case of AGF₂, as judged both by the magnitude of its effects alone and by its potential to exacerbate the effects of ethanol. Neither MK-801 nor AGF₂ was able to modulate SOD levels or promote generation of reactive oxygen species in vitro when added to an isolated synaptosomal preparation (data not shown), suggesting that their mode of altering GSH or SOD levels was by way of an indirect influence on cerebral metabolism.

The extent of binding of [³H]MK-801 was increased in cortical membranes derived from MK-801-treated rats, whether or not they were also receiving ethanol. Ethanol treatment had no effect on binding of [³H]MK-801 and neither ethanol treatment alone nor AGF₂ altered such binding (Fig. 5).

4. Discussion

The depression of cerebral SOD and glutathione levels consequent to ethanol treatment has been described previously by several laboratories. Both of these reductions have been used as parameters of oxidative stress and have been attributed to enhanced pro-oxidant activity within the brain [4,5,20,26,32,34]. The especial vulnerability of the striatum to ethanol-effected pro-oxidant and metabolic changes has been noted before [5,41], and may relate to the high dopamine content of this region which makes it prone to oxidative and excitotoxic damage [12]. The reasons for especial susceptibility of the thalamus is less clear but this area selectively expresses depressed levels of glutathione in thiamine-deficient animals (P.J. Langlais and S.C.

Fig. 5. Extent of binding of [³H]MK-801 in washed cortical membranes of control rats and animals treated with ethanol (E), AGF₂ (AG), MK-801 (MK), singly or in combination. Values $\pm$ S.E.M. are derived from 6 animals/group. * Differs from level in untreated control (C) group.

Bondy, unpublished results). Interestingly, ethanol treatment may depress levels of ganglioside GM₁ specifically in the thalamus [39].

Any direct effects of low molecular weight compounds upon the NMDA complex would have not been detectable in the extensively washed membrane preparation used in the binding study. Therefore the elevation of cortical binding of [³H]MK-801 observed following MK-801 dosing must reflect increased receptor site number or affinity. The hippocampal region where ethanol-related upregulation of NMDA receptors may take place predominantly [40], was not assayed individually, but constituted part of the cortical preparation. This may account for our failure to detect any binding changes attributable to ethanol. Protective effects of both ganglioside GM₁ and several NMDA receptor antagonists have been reported for glutamate-induced neurotoxicity in cerebellar granule cells where NMDA receptors have been upregulated by extended exposure to ethanol [23]. Such protective effects may have been related to the attenuation of glutamate-induced elevations of intracellular calcium. While excitotoxicity and oxidative stress are separate phenomena, there is a suspected but not clearly defined relation between these potentially harmful expressions of cell abnormality [3]. Both are undoubtedly relevant to the toxicity of ethanol and the report of Hoffman et al. [23] shows that these features are closely related. However, our findings suggest that some of the toxicity of ethanol may also be attributable to pro-oxidant events occurring independently of the ability of extended ethanol treatment to cause up-regulation of NMDA receptor content. Thus agents providing protection against excitotoxicity may actually enlist events that increase the potential for adverse changes in cell redox. This may account for the inability of ganglioside GM₁ to protect against neuronal degeneration occurring as a result of ethanol withdrawal [9]. Our findings of parallel effects of ethanol and AGF₂ upon oxidant status, are concordant with the reported synergistic relationship between gangliosides and ethanol upon membrane order [21,37].

In addition to the data reported here, another situation where ethanol and MK-801 have similar effects is that both cause dopaminergic activation in the mesolimbic system [14]. In view of the heterogeneity of sites of action of NMDA antagonists, our findings concerning initiation of pro-oxidant events by MK-801 may not be generalizable to all NMDA channel blockers.

The overall conclusion from this work is that some of the adverse effects of ethanol upon the brain may be attributable to enhanced pro-oxidant events. However, the mechanism of action of reported protective effects of MK-801 or gangliosides on ethanol neurotoxicity are not likely to be by way of reduction of any induced oxidative stress. In fact these agents may promote rather than attenuate rates of generation of reactive oxygen species. Finally, the ability of MK-801 and AGF₂ to promote oxidant events in some brain regions may relate to the toxicity that

has been reported for gangliosides and also for NMDA receptor antagonists [1,16,17].

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