Biochemical Pharmacology, Vol. 44, No. 1, pp. 93–98, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

ETHANOL METABOLISM IN RAT BRAIN HOMOGENATES BY A CATALASE–H_2O_2 SYSTEM

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(*Received* 2 *August* 1988; *accepted* 26 *March* 1992)

Abstract—Homogenates of perfused rat brains incubated in the presence of ethanol (50–100 mM) and glucose (10 mM) were found to oxidize ethanol to acetaldehyde. The addition of glucose oxidase, a known hydrogen peroxide generator, to the incubation medium, significantly ($P < 0.05$) increased the generation of acetaldehyde. The presence in the incubation medium of metyrapone, an inhibitor of cytochrome P450, or pyrazole, an alcohol dehydrogenase inhibitor, did not affect the levels of acetaldehyde obtained. Conversely, the presence of 3-amino-1,2,4-triazole, a known catalase inhibitor, induced a concentration-dependent reduction of the amount of acetaldehyde generated after incubation, even in the presence of glucose oxidase. Homogenates of perfused brains of rats treated with 3-amino-1,2,4-triazole or cyanamide (another H_2O_2-dependent catalase blocker) also showed a dose-dependent reduction of the acetaldehyde obtained. These findings support the notion that a catalase-mediated oxidation of ethanol is present in rat brain homogenates. It is suggested that this local oxidation of ethanol may have important biological implications. The data of both studies increase support for the notion that acetaldehyde is produced directly in the brain and that it may be the agent mediating some of the psychopharmacological properties of ethanol and be one of the factors determining the propensity of an animal to voluntarily consume ethanol.

The normal function of the brain can be severely affected by the ingestion of ethanol. It is, therefore, of considerable interest to know whether these central effects are due, in part, to the metabolism of ethanol in brain. Yet, current information on ethanol metabolism in the brain is meager. Previous investigations have shown that there are only trace amounts of alcohol dehydrogenase (the enzyme metabolizing ethanol to acetaldehyde) like activity in rat cerebral tissue [1]. A second proposed pathway could act via cytochrome P450, whose presence and distribution in brain have been established [2, 3]. It has been reported that neuroglial and neuroblastomal cell lines have the capacity to metabolize ethanol *in vitro*, largely, by an alcohol dehydrogenase-independent mechanism that appears to be dependent on one or more isoenzymes of cytochrome P450 [4]. However, to date, there is no direct evidence to support the existence of any of those pathways *in vivo*. Several authors [5, 6] have presented data suggesting that brain catalase in conjunction with endogenous H_2O_2 may oxidize ethanol *in vivo*. They demonstrated that prior administration of ethanol to rats protects brain catalase activity from inhibition by the H_2O_2-dependent catalase inhibitors 3-amino-1,2,4-triazole (AT) [5], cyanamide [6] and 4-hydroxypyrazole [6]. This prevention of the inhibitory effect *in vivo* by these compounds constituted indirect evidence that ethanol had served as substrate for the peroxidatic activity of catalase in brain *in vivo* [5, 6]. The presence and distribution of catalase in brain have been verified by both biochemical [7, 8] and histochemical [9] methods.

While the contribution of this central peroxidative catalase system to the overall metabolism of ethanol in the organism would be insignificant (< 0.06% of total body ethanol oxidation rate) [5], the oxidation of ethanol in brain may nevertheless affect significant central subcellular events. It is important to point out that catalase is not homogenously distributed in rat brain [8, 9]; therefore, acetaldehyde produced in a specific local environment may be of great consequence in that environment. In support of this notion, several studies have presented data suggesting a role for central acetaldehyde in mediating some of the psychopharmacological effects of ethanol [10, 11] which are in turn centrally mediated.

Evidence suggesting a biological significance of this central metabolic process has been reported in several studies. Animals pretreated with AT (a catalase inhibitor) and therefore functionally devoid of brain catalase activity exhibit a significant attenuation or blockade of several known ethanol effects. For example when rats pretreated with AT were compared with untreated controls, the former displayed periods of shorter narcosis [12, 13], less motor depression [14], less lethality [12, 13], less ethanol-induced corticosterone release [15], a complete blockade of ethanol-induced condition taste aversion [16] and finally, a dose-dependent reduction of ethanol intake [17]. These AT effects appear to be specific to ethanol, since AT does not attenuate the behavioral effects induced by other drugs such as morphine or pentobarbital [12, 16]. Moreover, the interaction between AT and ethanol seems to be related to brain catalase since AT fails

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to alter ethanol effects in rats, whose catalase inhibition has been prevented by prior administration of ethanol [12]. In addition to these findings, a direct relationship between brain catalase activity and voluntary ethanol consumption in rats has also been reported [18]. Finally, a significant difference in ethanol-induced locomotor activity between normal and acatalasemic mice has been demonstrated recently [19].

Taken together, these findings suggest that brain catalase may participate in the oxidation of ethanol to acetaldehyde and that this local formation of acetaldehyde may be an important factor in mediating the psychopharmacological effects of ethanol. Therefore, in the present study, we tested the possible capacity of brain homogenates to oxidize ethanol through a catalase–H_2O_2 system. The effects of AT, pyrazole (an alcohol dehydrogenase inhibitor) and metyrapone (a cytochrome P450 inhibitor) [4] on ethanol oxidation by brain homogenates were also studied. In a second experiment, the effects of *in vivo* inhibition of brain catalase by AT and cyanamide (a catalase blocker) [6, 20, 21] on ethanol oxidation were also investigated.

MATERIALS AND METHODS

Materials and subjects. Male rats of the Long-Evans strain were obtained from Charles River (Canada) and maintained on a standard rat chow diet and water *ad lib.* until used. Chemicals were purchased from the Sigma Chemical Co. Glucose oxidase (catalase free) was obtained from Calbiochem.

In vitro *studies.* Animals (weighing 250–300 g) were killed by exsanguination under ether anesthesia. Organs were perfused *in situ* by whole body perfusion using 400–500 mL of heparinized (1000 U/L) isotonic saline [20]. The saline solution was infused into the left ventricle, and a small incision was made in the right ventricle for the effluent. Perfusion was continued until the kidneys were visibly cleared of erythrocytes. Brain was excised and 10% homogenates were prepared with 0.1% Triton X-100 in 0.1 mM potassium phosphate buffer (pH 7.6) at 4°. Triton X-100 was added to solubilized particle-bound (peroxisomal) catalase [7, 22]. All homogenates were stored at 4° and were assayed for catalase activities the same day.

Aliquots of these homogenates equivalent to 50 mg of wet tissue were incubated at 37° in sealed clear 6-mL Pierce hypo-vials (Chromatographic Specialties Inc.) with 90 mM potassium phosphate. buffer (pH 7.6), 10 mM glucose and ethanol (50–100 mM) in the presence or absence of any of the following substances: sodium azide (5 mM), AT (5–10 mM), pyrazole (5–10 mM), metyrapone (2-methyl-1,2-di-3-pyridiyl-propanone) (5–10 mM) and/or glucose oxidase (0.3 U). The effects of AT, pyrazole or metyrapone on ethanol oxidation were studied by adding these inhibitors to the incubation medium 20 min before ethanol. The reaction was stopped at the indicated incubation times by adding 500 μL of 3 M perchloric acid. This treatment prevents further formation of acetaldehyde. Subsequently, the acetaldehyde content of the gaseous phase of each vial was measured by a head-space gas chromatography procedure [23] as follows. The flasks were incubated at 65° for 25 min, and 2 mL of the head-space was injected into a Varian Model 1400 gas chromatograph with flame ionization detectors. A 180 cm × 2 mm column of Chromosorb 101 mesh 80/100 was used with inlet and detector temperatures of 140° and 180°, respectively, and a nitrogen flow rate of about 20 mL/min. Under these conditions, the retention time was 1.8 min for acetaldehyde and 3.4 min for ethanol. Relative peak heights were determined by comparison with standards prepared by the addition of known amounts of acetaldehyde to "zero-time" controls. Blanks with boiled homogenates were employed in each experiment.

Table 1. Ethanol oxidation by brain homogenates, and influence of the addition *in vitro* of sodium azide, pyrazole, metyrapone and aminotriazole (AT) to the incubation medium*

Added drugs	Acetaldehyde obtained (nmol/mg protein)			
	50 mM Ethanol		100 mM Ethanol	
	30 min	60 min	30 min	60 min
Control	5.05 ± 0.28	10.25 ± 0.36	6.22 ± 0.29	12.92 ± 0.31
Sodium azide				
5 mM	0.32 ± 0.02†	0.48 ± 0.05†	0.21 ± 0.07†	0.87 ± 0.03†
Pyrazole				
5 mM	5.38 ± 0.22	10.87 ± 0.54	6.42 ± 0.05	12.31 ± 0.43
10 mM	5.37 ± 0.30	10.91 ± 0.21	6.72 ± 0.24	13.17 ± 0.58
Metyrapone				
5 mM	4.98 ± 0.41	9.92 ± 0.55	5.97 ± 0.33	11.70 ± 0.27
10 mM	4.53 ± 0.17	9.43 ± 0.46	5.68 ± 0.45	11.40 ± 0.66
AT				
5 mM	0.70 ± 0.06†	2.02 ± 0.08†	1.69 ± 0.05†	3.40 ± 0.15†
10 mM	0.46 ± 0.05†	0.75 ± 0.05†	1.46 ± 0.07†	2.59 ± 0.08†

* Each data point is the mean of ± SEM of five different brain tissues.
† $P < 0.01$ vs control.

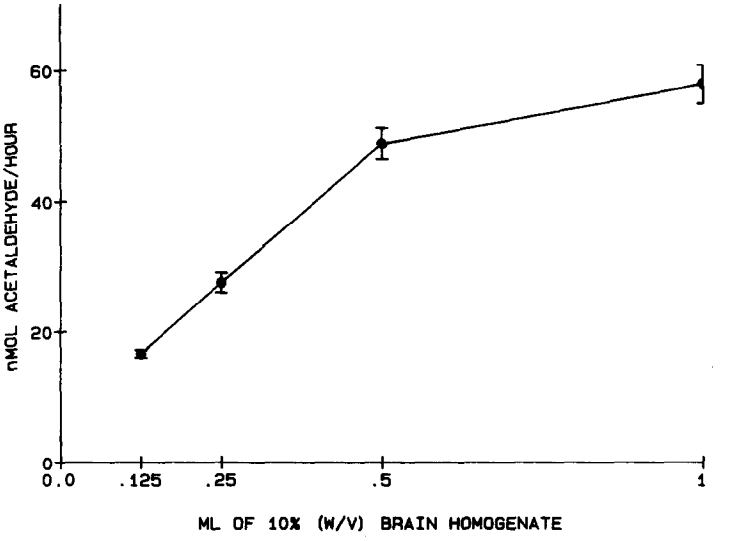

Fig. 1. The effect of different amounts of brain homogenate (10%, w/v) on the generation of acetaldehyde after a 60-min incubation of perfused brain homogenates with ethanol. Reactions were carried out as described in Materials and Methods, and were initiated by the addition of ethanol (50 mM final concentration). Values are means ± SEM, N = 5.

In vivo *studies*. Animals were fasted overnight. AT (0.0625, 0.25 and 1.0 g/kg) and cyanamide (7.15, 14.3 and 28.6 mg/kg) were administered to them intraperitoneally. Control rats were injected with saline. Nine hours later, the rats were killed and brains were assayed as described above.

Catalase activity. All brains were assayed for catalase activity. Brain catalase activity was measured using a Yellow Springs Oxygen Monitor equipped with a Clark style electrode as described by DeMaster *et al.* [20]. The reaction cell was temperature controlled and maintained at 25°. A 0.01 mM potassium phosphate buffer, pH 7.0, (1.7 mL) was deoxygenated with a stream of N_2. Hydrogen peroxide (7.6 μmol in 10 μL) was added to the deoxygenated buffer at zero time and the base line O_2 formation rate was recorded for 2 min. A 25-μL aliquot of brain homogenate was then added to the cell. The difference between the rate of O_2 formation before and after the addition of tissue homogenate was taken as the actual reaction rate. Brain catalase activity is expressed as nanomoles O_2 formed per minute per microgram of protein.

Protein content. Protein was determined in the 10% homogenate by the method of Lowry *et al.* [24].

Statistical analysis. Each data point is the mean ± SEM for a minimum of five animals. Analysis of variance was performed on these means. Post-hoc comparisons using Tukey tests were carried out. P values of < 0.05 were accepted as significant.

RESULTS

Table 1 summarizes the results of acetaldehyde generated after incubation (30 and 60 min) of ethanol (50 and 100 mM) in brain homogenates and in the presence of the enzyme inhibitors: sodium azide, pyrazole, metyrapone and AT. As can be seen, acetaldehyde was detectable after incubation of brain homogenates with ethanol. When AT (5 and 10 mM) or sodium azide (5 mM) was added to the incubation medium, the amount of acetaldehyde generated was significantly lower than in controls. In contrast, no difference from control data was observed in the presence of either pyrazole (5 and 10 mM) or metyrapone (5 and 10 mM). These results support the notion that ethanol could be oxidized by brain homogenates through the catalase peroxidative system at concentrations found *in vivo* to produce behavioral effects [25].

Mean catalatic activity for control brain homogenates was 0.424 ±. 031 nmol O_2/μg protein/min. Addition to the incubation medium of AT to final concentrations of 5 and 10 mM revealed catalase activities of 0.290 ± 0.011 and 0.147 ± 0.010 nmol O_2/μg protein/min, respectively. The results of a Tukey test revealed a significant mean decrease of 31 and 65% catalase activity in these brain homogenates compared with control homogenates ($P < 0.01$). No effect was observed when pyrazole or metyrapone was added to the incubation medium.

The effect of different amounts of brain homogenate on acetaldehyde formation is shown in Fig. 1. Samples were incubated for 60 min in the presence of 50 mM ethanol. A positive relationship between different amounts of brain homogenates and generation of acetaldehyde was observed (see Fig. 1). These observations suggest that the generation of acetaldehyde is related directly to the amount of brain tissue available and that it is not an artifact of the assay method.

The influence of different incubation times on the oxidation of ethanol is displayed in Fig. 2. Maximum

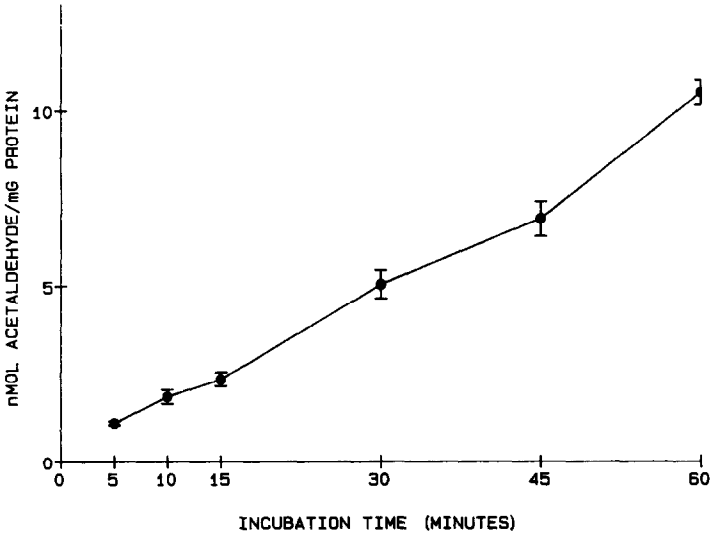

Fig. 2. Time course for the oxidation of ethanol by perfused brain homogenates (≃ 50 mg wet tissue) Reactions were carried out as described in Materials and Methods, and were initiated by the addition of ethanol (50 mM final concentration). Values are means ± SEM, N = 5.

Table 2. Generation of acetaldehyde after incubation (30 or 60 min) of homogenates of perfused rat brains with ethanol in the presence of glucose oxidase (0.3 U)*

Added drugs	Acetaldehyde obtained (nmol/mg protein)			
	50 mM Ethanol		100 mM Ethanol	
	30 min	60 min	30 min	60 min
Control	12.60 ± 0.58	23.19 ± 0.59	18.25 ± 0.86	37.03 ± 0.89
Pyrazole				
5 mM	13.13 ± 0.42	26.30 ± 0.45	19.05 ± 0.60	40.62 ± 1.60
10 mM	11.68 ± 0.60	23.95 ± 0.70	20.11 ± 0.74	38.42 ± 0.99
Metyrapone				
5 mM	12.45 ± 0.38	22.75 ± 0.69	18.75 ± 0.30	36.25 ± 1.40
10 mM	11.52 ± 0.26	24.16 ± 0.42	19.13 ± 0.62	35.78 ± 1.10
AT				
5 mM	1.70 ± 0.09†	3.48 ± 0.20†	5.20 ± 0.14†	10.54 ± 0.32†
10 mM	1.12 ± 0.09†	1.74 ± 0.12†	4.49 ± 0.16†	8.99 ± 0.29†

* Each data point is the mean ± SEM of five different brain determinations.
† $P < 0.01$ vs control.

generation of acetaldehyde was obtained after a 1-hr incubation with a substrate concentration of 50 mM ethanol. However, shorter incubation times yielded detectable levels of acetaldehyde.

The oxidation of glucose by glucose oxidase was used as an H_2O_2-generating system which in the presence of catalase would promote the peroxidatic oxidation of ethanol. The addition of glucose oxidase (0.3 U) to the incubation medium significantly ($P < 0.05$) increased the ethanol oxidation rate. With glucose oxidase, acetaldehyde values were 2–3 times higher at 30 and 60 min of incubation with 50 and 100 mM ethanol (compare Table 1 with Table 2). Pretreatment of this system with pyrazole or metyrapone failed to produce an effect different from controls. However, AT decreased the levels of acetaldehyde obtained at both times and both concentrations of ethanol tested. The highly significant difference between acetaldehyde levels of samples with AT and those without it shows that the stimulation of acetaldehyde production was specifically blocked by AT, allowing the exclusion of other possible ethanol oxidation pathways.

The effects of *in vivo* administration of AT and cyanamide on the generation of acetaldehyde after incubation of brain homogenates with ethanol are shown in Table 3. Brains of rats treated i.p. with these inhibitors, 9 hr before being killed, were

Table 3. Effects of 3-amino-1,2,4-triazole (AT) and cyanamide administration i.p. *in vivo* on ethanol oxidation by brain homogenates and brain catalase activity*

Treatment	(nmol Acetaldehyde/mg protein/hr) 50 mM Ethanol	100 mM Ethanol	O_2 (nmol/μg protein/min)
Saline	9.62 ± 0.69	11.90 ± 0.43	0.514 ± 0.031
AT			
0.0625 g/kg	9.41 ± 0.53	10.08 ± 0.81	0.436 ± 0.025†
0.25 g/kg	3.47 ± 0.20†	5.26 ± 0.36†	0.243 ± 0.008†
1.0 g/kg	1.75 ± 0.12†	3.49 ± 0.21†	0.168 ± 0.013†
Cyanamide			
7.15 mg/kg	8.76 ± 0.60	10.32 ± 0.93	0.496 ± 0.009†
14.3 mg/kg	6.32 ± 0.57†	8.39 ± 0.28†	0.383 ± 0.026†
28.6 mg/kg	3.65 ± 0.19†	5.41 ± 0.38†	0.224 ± 0.015†

* Each data point is the mean ± SEM of five different brain determinations.
† $P < 0.05$ vs saline value.

assayed for ethanol oxidation. Minimum detectable levels of AT are found in brain or liver at this time [26]. A significant dose-dependent decrease in ethanol oxidation was found under these conditions with both ethanol concentrations tested. These findings further support the notion of the oxidation of ethanol in brain homogenates via the peroxidatic activity of catalase. Table 3 summarizes also the catalase activity of these brains.

DISCUSSION

The results reported in this study show that ethanol can be metabolized in brain homogenates of perfused rat brains via the peroxidative activity of catalase. These findings confirm and extend the data base obtained in previous observations by other authors [12, 27, 28] using homogenates of non-perfused UChA rat brains. Acetaldehyde was generated after incubation with low concentrations of ethanol, similar to those found to produce meaningful behavioral effects and well below the lethal one [25]. In fact, ethanol was metabolized at a measurable rate and at concentrations found in rat brain following individual drinking bouts that are sufficient to produce pharmacological effects [25].

The notion that ethanol could be catabolized by a catalase–H_2O_2 system is supported by this study for the following reasons. First, the formation of acetaldehyde during the incubation of rat brain homogenates with ethanol was increased significantly ($P < 0.05$) by the addition of glucose oxidase, a known generator of H_2O_2 in the presence of glucose. Second, the incubation in the presence of different concentrations of AT (catalase blocker) revealed a dose-dependent significant decrease in the generation of acetaldehyde, both in the absence and in the presence of glucose oxidase. The effect of alcohol dehydrogenase and cytochrome P450 in this experimental condition can be ruled out since the presence of pyrazole or metyrapone did not alter the amount of generated acetaldehyde. Finally, the "*in vivo*" inhibition of brain catalase by AT and cyanamide also resulted in a significant decrease in acetaldehyde obtained after incubation of these rat brain homogenates with ethanol.

It has been reported that neuroglial and neuroblastomal cell lines are capable of metabolizing ethanol via an alcohol dehydrogenase-independent pathway [4]. Wickramashinghe reported that this ADH-independent pathway of ethanol metabolism by neural cells appears to be dependent on one or more isozymes of cytochrome P450. However, the apparent discrepancy between these findings and the results obtained in this study may only be meaningful if normal neuroglial or neuroblastomal cells behave with respect to ethanol metabolism like the cell lines derived from their malignant counterparts used in that study.

It is of interest to point out that the rate of ethanol oxidation by the catalase–H_2O_2 system in brain could be limited by the rate of H_2O_2 generation rather than by the amounts of catalase. Production of H_2O_2 in the brain *in vivo* was demonstrated by Sinet *et al.* [22]. In their study the administration of AT, an H_2O_2-dependent inhibitor of catalase, to the rat caused progressive inhibition of brain catalase. These findings were confirmed by other authors [6, 20] using cyanamide, which is also an H_2O_2-dependent inhibitor of catalase. The potential source of H_2O_2 utilized by catalase for ethanol oxidation is at present unknown. A possible source could be mitochondria. Generation of H_2O_2 by mitochondria is a well recognized process, currently considered to be of physiological significance [29]. The brain utilizes large quantities of oxygen, and its mitochondria have all the major redox functions known for these organelles. Moreover, generation of H_2O_2 by brain mitochondria *in vitro* has been reported under different conditions and substrates [22, 30]. In agreement with this notion, Tampier and Mardones [27] have shown catalase-mediated oxidation of ethanol by crude mitochondrial suspension of rat brain homogenates, where enzymatic systems for disappearance of acetaldehyde have been demonstrated. However, other sources of H_2O_2 generation in brain tissue are not discarded (e.g. peroxisomes). The results of the present study show

the same pattern of oxidation at any of the times assayed and/or ethanol concentration used, in the absence of glucose oxidase. This finding seems to support the notion of an endogenous production of H_2O_2 capable of maintaining a measurable rate of ethanol metabolism. *In vivo* the presence of acetaldehyde could be the result of a balance between its rates of formation and oxidation. It is important to note that acetaldehyde could be metabolized by an NAD-dependent aldehyde dehydrogenase [27, 28] or through flavoprotein pathways with formation of H_2O_2 [31], which could be used by catalase for further ethanol oxidation. It seems possible, therefore, that even small amounts of catalase in the neural cells could oxidize ethanol and generate acetaldehyde in significant amounts capable of altering neural function and behavior.

Acknowledgement—This study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (A0991) to C. M. G. Aragon.

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