Br. J. Pharmacol. (1988), **95**, 77–82

Effects of ethanol and pantothenic acid on brain acetylcholine synthesis

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1 Measurements of brain acetylcholine (ACh) synthesis from precursor [^{14}C]-pyruvate, pantothenic acid (PA) concentration in the brain, and blood ethanol (EtOH) concentration were made in rats treated with either ethanol (5–6 g kg^{-1} body wt daily) alone or ethanol with PA supplementation (100–200 mg kg^{-1} body wt daily). EtOH with or without PA was administered orally by either Lieber-Decarli liquid diet for 4 weeks and 4 months or by oral intubation for 1 and 4 days. Matched controls were given either ethanol-free liquid diet or saline.

2 ACh synthesis in the brain of rats treated with ethanol alone for 4 months was significantly ($P < 0.01$) inhibited. PA concentration of the brain was diminished to 7.0% of the control value.

3 PA concentration in the brain of rats treated with ethanol plus PA for 4 months was three times that of rats treated with ethanol alone. ACh synthesis in rats with ethanol and PA supplementation was also significantly ($P < 0.01$) higher.

4 There was no difference in blood EtOH concentration between rats treated with ethanol with or without PA supplement.

5 The EtOH effect on ACh synthesis and PA concentration in the brain was observed in the chronic treatments but not in the acute treatments.

6 Data suggest that chronic ethanol exposure may decrease ACh synthesis by depleting PA, a precursor for the synthesis of acetyl CoA. Acetyl CoA is an essential substrate for ACh synthesis.

Introduction

Alterations in cholinergic transmission occur in the brain of ethanol-exposed animals. Kalant & Grose (1967) and Kalant *et al.* (1967) showed that alcohol decreased acetylcholine (ACh) release by rat brain slices *in vitro*. Erickson & Graham (1973) reported depressed spontaneous release of ACh in rabbits *in vivo*, with no diminution in ACh content in rat brain. Rawat (1974) showed decreased brain ACh, acetyl CoA and coenzyme A in mice chronically treated with ethanol. A possible association of diminished brain acetylcholine, due to ethanol, with the cognitive and memory impairment observed in chronic alcoholics may be of clinical significance. An understanding of how ethanol decreases acetylcholine in the brain is important for prevention and treatment of ethanol neurotoxicity.

Acetylcholine is synthesized from substrates acetyl CoA and choline by the enzymatic activity of choline acetyltransferase. Studies have indicated that chronic ethanol treatment diminished the availability of acetyl CoA, a precursor for acetylcholine synthesis, by inhibiting the incorporation of pantothenic acid (PA) into coenzyme A (Iannucci *et al.*, 1982; Israel & Smith, 1987; Smith *et al.* 1987). This effect may be compounded further by ethanol-induced vitamin deficiencies and malnutrition with consequent defects in carbohydrate and pyruvate metabolism (Frank & Baker, 1980; Eisenstein, 1982). We have

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studied the effect of ethanol on brain PA content and synthesis of ACh from precursor [^{14}C]-pyruvate in rats fed with normal diet and ethanol diet with and without PA supplementation. The present study showed that rats on chronic ethanol treatment had significantly lower brain PA concentration, associated with diminished brain acetylcholine synthesis from precursor [^{14}C]-pyruvate. Studies *in vitro* and *in vivo* suggested that PA can prevent or reverse the inhibitory effect of ethanol on brain acetylcholine synthesis from [^{14}C]-pyruvate.

Methods

Animals

Male Sprague-Dawley rats, age 3–6 months, were purchased from Charles River Breeding Facilities. Rats were housed one to a cage with food and water *ad libitum*, unless otherwise stated. Animal rooms were maintained at 70°F with 12 h light/dark cycles.

Analytical methods

ACh synthesis was measured by a modification of the radiochemical assay of Fonnum (1975). Rats were killed by decapitation and the brains were weighed and dissected. The cerebri were homogenized in 10 mM EDTA buffer, pH 7.4, (10% w/v) in a Potter Elvehjem homogenizer. Samples were treated with 0.05% Triton X-100 to release full enzyme activity. The brain homogenate (20 mg tissue per 200 μl) was incubated with [^{14}C]-pyruvate (5 mM), choline bromide (16 mM), physostigmine (a cholinesterase inhibitor, 0.1 mM), ATP (10 mM), EDTA (20 mM pH 7.4), and oxygenated Krebs Ringer buffer in a total incubation volume of 0.7 ml. Blanks consisted of the whole incubation mixture without the homogenate. The composition of the Krebs-Ringer buffer (pH 7.4) was (mM): NaCl 140, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 0.8 and Tris-HCl 20. The solution was oxygenated with 95% O_2 and 5% CO_2, for 5 min before use. The incubation was carried out at pH 7.4 at 37°C for 30 min in a Dubnoff metabolic shaker. After the incubation, 5 ml of 10 mM sodium phosphate, pH 7.4, was added and the synthesized [^{14}C]-ACh was reacted with 10 mg sodium tetraphenyl boron in 2 ml acetonitrile for extraction. The mixture was swirled to mix, transferred to a scintillation vial containing 10 ml of toluene scintillation liquid and shaken for 1 min. The toluene and aqueous layers were allowed to separate for 10 min in the scintillation counter before counting. The radioactivity was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. Counting efficiency for ^{14}C was 90%, and efficiency for the bilayer assay was 63%. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

Standardization of the ACh synthesis assay from various substrates was performed with dose-response and time-response experiments to establish the validity of the procedure and to optimize concentrations of substrates and tissues, incubation time and incubation medium for the assay (optimum is defined as the greatest response achievable under physiological conditions). We tested the specificity and efficiency of the ACh extraction by mixing radiolabelled standards of [^{14}C]-ACh, [^{14}C]-acetyl CoA, [^{14}C]-pyruvate and [^{14}C]-acetate with 20 mM EDTA and 0.2 mM physostigmine in 500 μl of Krebs Ringer buffer, incubating for 30 min and extracting with sodium tetraphenyl boron (10 mg in 2 ml acetonitrile) and the toluene scintillation fluid (10 ml) by gentle shaking for 1 min. Results showed that [^{14}C]-ACh was 99% extracted into the toluene layer while negligible amounts of [^{14}C]-acetyl CoA, [^{14}C]-acetate and [^{14}C]-pyruvate (0.5–1.0%) were extracted into the toluene layer.

Radioimmunoassay of pantothenic acid (PA) was carried out by the method of Wyse *et al.* (1979) as follows:

Preparation of tissue extracts brain tissues were homogenized in two volumes of distilled water and centrifuged. The pH of the supernatant was adjusted to pH 8 with tris (hydroxymethyl) methylamine. The supernatant (0.5 ml) was incubated with intestinal alkaline phosphatase (2 units) and pigeon liver enzyme (12 units) at 37°C for 22–24 h to liberate pantothenate. After incubation, the tissue extracts were assayed for PA.

Assay procedure The antibody (50–75 μl) was mixed with 1% rabbit serum albumin (Sigma) in a 1:200 dilution at room temperature and stirred for 5 min. [^{14}C]-pantothenic acid (~50,000 c.p.m. per sample) was added and stirred for 5 min; 0.5 ml of the antibody/albumin/[^{14}C]-PA mixture was incubated with the standard PA (5 ng to 100 ng in 150 μl solution), 150 μl unknown and 150 μl water (blank). Another tube contained 1% rabbit serum albumin and the [^{14}C]-PA alone. The mixtures were incubated for 15 min at room temperature in a Dubnoff metabolic shaker. After incubation, 100% saturated ammonium sulphate (0.7 ml) was added, and the mixture was vortexed and centrifuged at 10,000 *g* for 20 min at 4°C in a Sorvall centrifuge. The supernatant was discarded. Pellets were washed with 0.5 ml of 50% ammonium sulphate, centrifuged at 10,000 *g* for 20 min at 4°C and the supernatant was discarded. NCS tissue solubilizer (0.5 ml) was added to each tube to dissolve the pellet. Dissolved pellets

were transferred to the scintillation vial, with 10 ml triton-X scintillation liquid and the radioactivity was counted in a Packard Scintillation counter.

Calculation From the antiserum dilution curves, the mean c.p.m. value of duplicate samples (after subtraction of the c.p.m. for mean nonspecific precipitation in the presence of normal rabbit serum) was expressed as a percentage of the mean c.p.m. added to each dilution. The calculations for the dose-response curve and unknown samples were similar except that they were expressed as a percentage of the counts bound when no PA was present in the standard tubes, i.e.,

$$\% \text{ binding} = B/B_0 \times 100$$

where B = c.p.m. in sample tube minus nonspecific binding c.p.m.; B_0 = c.p.m. in blank tube where phosphate buffer replaced sample aliquot minus nonspecific binding c.p.m.

The antibody-bound fraction is expressed as a percentage of B/B_0 vs. log of the concentration of added nonradioactive pantothenic acid and plotted on logit-semilog paper. The linear fit (logit B/B_0) vs. log of added nonradioactive PA of preselected standards was computed and the amount of PA in each unknown was determined.

Data analyses were performed using ANOVA tests (one way and the randomized block test).

Experimental procedure

Utilizing the methods and assay procedures, experiments were designed to test the effect of acute and chronic ethanol on rat brain ACh synthesis from [^{14}C]-pyruvate, and on the PA concentration in the brain.

The effect of ethanol treatment of rats, with and without pantothenic acid supplementation, on brain ACh synthesis from [^{14}C]-pyruvate and brain concentration of pantothenic acid Twelve Sprague-Dawley rats weighing 160–200 g were divided into three groups: Group A, matched control rats were maintained on Lieber-Decarli control rat liquid-test diet without ethanol (BioServe Inc., Frenchtown, NJ), which was tested as nutritionally adequate as to its protein, carbohydrate, fat, mineral and vitamin content by the manufacturer. Group B, 'ethanol' rats were maintained on the same liquid diet but the carbohydrate was replaced by ethanol at a concentration of 50 g l^{-1}. Group C, 'pantothenic acid' rats were maintained on the ethanol liquid diet with PA supplementation of 100 mg kg^{-1} body weight daily.

The three groups were maintained on their respective diets for 4 months. Daily consumption of the diet and weekly weights of the rats were recorded. The rats were given the diet up to the day before they were killed. From the data of the diet consumed, daily ethanol consumption was 5–6 g kg^{-1} daily.

On the day of the experiment, the rats were killed by decapitation. Blood was collected in heparinized vacutainers with fluoride for blood ethanol determination. The brain was removed, washed three times with Krebs-Ringer solution, weighed and placed on ice. ACh synthesis from [^{14}C]-pyruvate was assayed in brain tissues as described. The PA concentration of the brain tissue was analysed by radioimmunoassay. Blood ethanol concentration was assayed with gas-liquid chromatography by direct injection using a gas chromo-flame ionization detector, 5′ column, 2 mm in diameter packed with 0.2% carbopack C on carbowax 1500. We also studied the *in vitro* addition of 12 mM PA, 10 mM thiamine HCl and combined PA and thiamine on ACh synthesis in brain homogenates obtained from the control, ethanol and ethanol with PA supplement chronically-treated rats.

The effect of duration of ethanol treatment on brain ACh synthesis Groups of rats were maintained on the specified diets, as described, for four weeks instead of four months. Similarly, the effect of acute oral ethanol administration for one and four days on brain ACh synthesis from [^{14}C]-pyruvate was also tested. Groups of 4 male Sprague Dawley rats were incubated with ethanol (6 g kg^{-1} body weight) alone or combined with PA, 200 mg kg^{-1} body weight, for 1 day and 4 days duration. After the treatment period, the rats were killed and the brain ACh synthesis from [^{14}C]-pyruvate was measured as described and compared to matched controls treated with saline solution.

Materials and reagents

Acetyl-[1-^{14}C]-coenzyme A (sp. act. 51.9 mCi mmol^{-1}), [1-^{14}C]-acetylcholine (sp. act. 55.3 mCi mmol^{-1}), [2-^{14}C]-acetic acid (sp. act. 2.1 mCi mmol^{-1}), [2-^{14}C]-pyruvate (sp. act. 18.5 mCi mmol^{-1}) and D-[1-^{14}C]-pantothenic acid (sp. act. 57.6 mCi mmol^{-1}) were purchased from New England Nuclear Corp. All radioisotopes were of 98.5–99% purity as assayed by paper chromatography and/or h.p.l.c. Choline bromide, physostigmine, sodium tetraphenyl boron, D-pantothenic acid, pyruvate, adenosine 5′-triphosphate, triton X-100 and acetonitrile were purchased from Sigma Chemical Co. Scintillation grade PPO and POPOP were purchased from the Packard Instrument Company (LeGrange, IL, U.S.A.). Scintillation grade toluene was purchased from Baker Chemical Co. All other chemicals used were of analytical grade, and purchased from the Fisher Scientific Co. Distilled,

deionized water was used in all solutions, and prepared by use of a Crystalab filtration system.

Materials for the radioimmunoassay of pantothenic acid The pantothenic antibody was a gift from Dr Wyse's laboratory, (Department of Nutrition/Food Sciences and Chemistry Biochemistry, Utah State University, Logan, UT 84322, U.S.A.) (antibody potency: 1:100 dilution in 1% albumin gave 43% binding of [^{14}C]-PA). The PA standards were prepared fresh from Ca-pantothenate (Sigma) and made up in 1 mM ethylene glycol tetraacetic acid. D-[^{14}C]-PA was purchased from New England Nuclear Co. (sp. act. 57.6 mCi mmol^{-1}).

Results

Figure 1 shows the ACh synthesis from [^{14}C]-pyruvate in the brains obtained from rats treated with ethanol alone and ethanol with PA supplementation compared to controls. Durations of treatment were 1 day, 4 days, 4 weeks and 4 months.

Significant differences in ACh synthesis ($P < 0.01$) were observed with the 4 months treated rats. There was 50% inhibition of ACh synthesis with ethanol alone, but only 26% inhibition when ethanol was administered with PA supplementation. In the 4 weeks treated rats, the differences among the groups did not reach significance by one way analysis of variance (ANOVA), but comparison of ethanol rats vs. control showed a significant difference ($P < 0.05$)

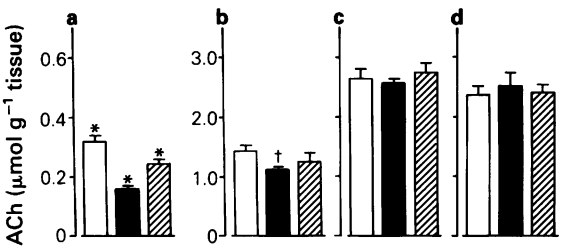

Figure 1 Acetylcholine (ACh) synthesis (μmol ACh synthesized g^{-1} tissue) in the rat brain of control (saline-treated) (open columns), ethanol (closed columns) and ethanol plus pantothenic acid supplement (hatched columns) treatments for the duration of four months (a), four weeks (b), four days (c), and 1 day (d). The dose of ethanol was 5-6 g kg^{-1} body wt daily given as Lieber-Decarli ethanol liquid diet in (a) and (b), and as ethanol solution administered by oral intubation in (c) and (d). The dose of pantothenic acid was 100 mg kg^{-1} daily in (a) and (b) and 200 mg kg^{-1} daily in (c) and (d). The values represent the mean ($n = 4$) with s.e.mean indicated by vertical bars. * $P < 0.01$ by ANOVA; † $P < 0.05$ from control when tested with Student's t test.

Table 1 Blood ethanol and brain pantothenic acid (PA) concentration in rats after 4 months oral treatment with ethanol and pantothenic acid

Experimental group	*Blood ethanol concentration*	*Brain PA* (ng mg^{-1} tissue)
Control	0%	7.00 ± 1.86
Ethanol	0.23% (0.18–0.28)	0.49 ± 0.08*
Ethanol + PA	0.24% (0.17–0.29)	1.41 ± 0.28*

Blood ethanol concentration was measured by gas chromatography and PA by radioimmunoassay. Values represent the mean concentration and range for blood ethanol and the mean ± s.e.mean for PA concentration ($n = 4$). Control rats were fed Lieber-Dicarli control liquid rat diet (BioServe Inc., Frenchtown, NJ). The ethanol rats were fed the same liquid diet, but the carbohydrate was replaced by ethanol at a concentration of 50 g l^{-1}. The ethanol + PA rats were fed the ethanol liquid diet plus 30 mg% PA. Calculated daily doses were 5–6 g kg^{-1} body wt ethanol and 100 mg kg^{-1} body wt PA. * Significance of $P < 0.025$ from control using unpaired Student's t test.

by Student's t test. Of interest is the concomitant ethanol depressant effect on the PA concentration in the brain tissues of the 4 months treated rats as compared to controls (Table 1). The brain PA concentration of the ethanol rats was significantly diminished to 7% of controls. Brain PA concentrations of the rats that received daily PA supplementation with ethanol ingestion were three times greater than those of the rats treated with ethanol alone. There was, however, no significant difference in blood concentrations of ethanol between the two groups.

Figure 2 shows the effect of the *in vitro* addition of PA (12 mM) and thiamine (10 mM) and their combination on the ACh synthesis of brain homogenates obtained from the 4 months ethanol-treated rats. ACh synthesis of the ethanol brain was increased significantly by PA and thiamine, alone and in combination and the results suggest synergism between PA and thiamine. Data were analysed by the randomized block analysis of variance (ANOVA) and the differences between the treatment data and baseline (4 months ethanol), as well as the single treatments of PA and thiamine alone from their combined treatment were significant at $P < 0.01$.

Discussion

The association of the marked decline in PA concentration in the brain of rats and the significant inhibition of brain ACh synthesis from precursor [^{14}C]-pyruvate was observed in rats after 4 months ethanol

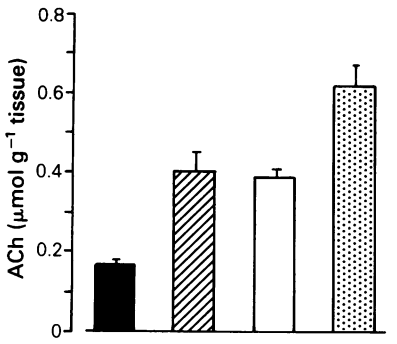

Figure 2 Acetylcholine (ACh) synthesis (μmol ACh synthesized g^{-1} tissue) in the rat brain obtained from 4 months ethanol-treated rats (5–6 g kg^{-1} body wt daily). Brain was treated *in vitro* as control (closed column), with addition of 12 mM pantothenic acid (hatched column), with addition of 10 mM thiamine (open column), or with addition of both 12 mM pantothenic acid and 10 mM thiamine (stippled column). The values represent mean ($n = 4$) with s.e.mean indicated by vertical bars. Statistical analysis by ANOVA. $P < 0.01$ for all pairs except pantothenic acid alone to thiamine alone.

treatment. Interestingly, ACh synthesis in rats that received PA supplement during the ethanol treatment was significantly higher compared to that of ethanol treatment without PA supplementation. These observations suggest that ethanol inhibition of ACh synthesis could have resulted from depletion of PA by ethanol. Previous studies have shown that PA, the precursor of CoA, increased brain ACh synthesis from [^{14}C]-pyruvate in both *in vitro* and *in vivo* experiments in aged rats (Rivera-Calimlim, 1981). Likewise, it was shown that brain ACh synthesis of diet-induced PA-deficient rats was significantly diminished (Rivera-Calimlim, 1981).

The influence of PA on ACh synthesis from [^{14}C]-pyruvate could be explained by the crucial role of PA in the synthesis of coenzyme A, which is essential to the production of acetyl CoA. Ethanol was shown to inhibit the incorporation of PA into CoA in liver cells (Ianucci *et al.*, 1982). Likewise, ethanol inhibited the synaptosomal uptake of [^{14}C]-PA (Rivera-Calimlim, unpublished data). Spector (1986) showed that PA is transported into the brain by active transport. Conceivably ethanol, a universal solvent and tissue poison, could inactivate the enzymes and carriers for the active transport of PA.

If indeed the ethanol inhibition of ACh synthesis was due to depletion of PA in the brain, then PA supplementation might prevent or counteract the ACh synthesis inhibition by ethanol. Our data showed that PA supplement at a dose of 100 mg kg^{-1} body weight daily counteracted the ethanol inhibition of ACh synthesis by only 50%. These results could mean that the ethanol inhibition of ACh synthesis is associated with some other enzymatic changes (i.e., decline of pyruvate dehydrogenase enzymes) in addition to ethanol-induced PA deficiency. Another possibility is that the daily dose of 100 mg kg^{-1} PA was insufficient to replace the ethanol-induced PA deficiency in the brain. This latter possibility was supported by the fact that when PA (12 mM) was added *in vitro* to the brain homogenates prepared from the chronically ethanol-treated rats, ACh synthesis increased by 122% (Figure 2). Interestingly, thiamine had similar effects, and appeared to potentiate the PA effect.

Several reasons can account for lower brain PA concentrations achieved by the rats fed with a daily dose of PA supplement in the ethanol diet in our study compared to controls: (1) possible degradation of PA in the ethanol-liquid diet (PA is known to be unstable in solution); (2) increased metabolism of PA in the presence of ethanol; (3) the brain uptake of PA has been shown to be 0.03% of administered dose (Rivera-Calimlim, 1981) and, therefore, could require a much higher daily dose to achieve plasma concentrations adequate for brain uptake.

The ethanol-induced inhibition on ACh synthesis in the brain of rats was not observed in rats treated for one and four days. In the four weeks ethanol-treated rats, ACh synthesis was inhibited, but did not reach significance using the analysis of variance. However, inhibition of ACh synthesis in the ethanol treated rats compared with control showed significance ($P < 0.05$) using Student's t-test. The mechanisms of the ethanol-PA interaction have not been studied. The interaction has been observed in behavioral studies in rats and monkeys, where intravenous PA (100–200 mg kg^{-1}), preceding ethanol administration, significantly counteracted acute ethanol-induced behavioural toxicity (Newland *et al.*, 1987a,b).

It is apparent, therefore, that the ethanol-PA interaction on ACh synthesis is time-dependent (being observed only after chronic treatment), whereas the interaction on behaviour was observed even after a single dose. This suggests that chronic and acute ethanol toxicities may have two separate underlying mechanisms. Further study at the cellular and molecular level may explain the possible differences in the mechanism. The significance of the stimulant effect of PA on brain ACh synthesis can be extended to conditions where ACh deficiency has been implicated, like aging, dementia and Alzheimer's disease.

This research was supported by a grant from the Foundation for Nutritional Advancement.

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(Received November 24, 1987
Revised April 21, 1988
Accepted May 3, 1988)