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EFFECT OF ETHANOL ON CALCIUM-UPTAKE AND PHOSPHOLIPID TURNOVER BY STIMULATION OF ADRENOCEPTORS AND MUSCARINIC RECEPTORS IN MOUSE BRAIN AND HEART SYNPATOSOMES

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Abstract—The effect of ethanol treatment on mouse brain and heart synaptosomal ^{45}Ca uptake and the incorporation of [^{3}H]inositol and [^{14}C]choline into phosphatidylinositol (PI) and phosphatidylcholine (PC) were investigated. Ethanol in drinking water (15%) was given to mice for 3 weeks. The consumption of ethanol increased gradually during treatment but food intake was almost the same as control. The body weight of ethanol-treated mice was slightly less than that of control. The synaptosomal lipid peroxidation level of ethanol-treated mice was almost the same as control in the brain and heart. On the other hand, the synaptosomal glutathione level of ethanol-treated mice was higher than control in both brain and heart. The ^{45}Ca uptake of brain and heart from ethanol-treated mice was 87% and 216% of control mice, respectively. Not only ethanol but also norepinephrine (NE), carbachol (Carb), or isoproterenol (IsoPro) added *in vitro* increased ^{45}Ca uptake in all cases. The incorporation of [^{3}H]inositol into PI in the brain and heart synaptosomes of ethanol-treated mice was 150% and 113% of control, respectively. The incorporation of [^{14}C]choline into PC in the brain and heart of ethanol-treated mice was 104% and 125% of control, respectively. *In vitro* addition of ethanol, NE, Carb or IsoPro to brain synaptosomes increased the incorporation of [^{3}H]inositol and [^{14}C]choline into PI and PC, respectively, in both control and ethanol-treated mice. In the case of heart synaptosomes, NE and Carb increased the incorporation of [^{3}H]inositol and [^{14}C]choline into phospholipids in control mice but not ethanol-treated mice. However, IsoPro increased the incorporation by both control and ethanol-treated heart synaptosomes. These results suggest that α-adrenoceptors and the cholinergic system of the heart play important roles in modulating the toxic effects of ethanol.

Chronic ethanol administration leads to a resistance to lipid disruption, which is known as "membrane tolerance". Activation of phospholipase A_2 and C by ethanol has been reported in several tissue membranes [1–3]. Thus, ethanol tolerance is clearly a property of the phospholipid portion of the membrane. However, the factors important in long-term functional changes after exposure to ethanol are still unknown. I have studied the direct effects of prenatal ethanol exposure on embryo development, using chick embryo [4, submitted for publication]. I found that ethanol stimulated Ca^{2+} mobilization and/or phospholipid turnover in the differential responses in brain and heart synaptosomal membranes. In the present study, I show that norepinephrine (NE*), isoproterenol (IsoPro) and carbachol (Carb) modify the effect of ethanol on ^{45}Ca uptake, and [^{3}H]inositol and [^{14}C]choline incorporation into phosphatidylinositol (PI) and phosphatidylcholine (PC) in mouse brain and heart synaptosomal membranes.

* Abbreviations: NE, norepinephrine; IsoPro, isoproterenol; Carb, carbachol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol-bis[β-aminoethyl ether]-*N*, *N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl) aminomethane; PI, phosphatidylinositol; PC, phosphatidylcholine.

MATERIALS AND METHODS

Animals. Male ddY mice (Shimizu Lab. Supplies Co., Ltd, Kyoto, Japan), initially weighing 26–28 g, were given either water with 15% ethanol, or water without ethanol, for 3 weeks. Both the ethanol and the control groups were housed with free access to food (MF. Oriental Yeast, Tokyo, Japan).

Synaptosomal preparation. Mice were decapitated and the brain (without the cerebellum) and heart were excised quickly on ice. Synaptosomes were prepared by the modification of Dodd *et al.* [5] of the method of Gray and Whittaker [6]. Briefly, the homogenate with 10 volumes of 0.32 M sucrose was centrifuged (1500 *g*, 10 min, 4°) and the supernatant was laid over 1.2 M sucrose. After high speed centrifugation (50,000 rpm, 10 min; Kontron R. model TGA-65, rotor TST54), the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and laid over 0.8 M sucrose. After centrifugation again at high speed, the synaptosomal pellet was suspended in HEPES (Sigma Chemical Co., MO, U.S.A.) buffer (pH 7.4), and was used in the studies of ^{45}Ca uptake and [^{3}H]inositol and [^{14}C]choline incorporation into phospholipids. The composition of HEPES buffer was (millimolar): NaCl, 125; KCl, 5; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 6; $CaCl_2$, 1; HEPES, 25.

Determination of lipid peroxidation and glutathione levels in synaptosomes. Synaptosomal suspensions in HEPES buffer were brought to a final tissue concentration of 0.5–0.8 mg protein/mL. The synaptosomal suspension was treated with 10% trichloroacetic acid to remove protein. The deproteinized supernatant was used to assay lipid peroxidation and GSH. The mixture of 0.5 mL of the supernatant with 0.5 mL of 1% thiobarbituric acid (TBA) was heated in a boiling water bath for 10 min. Then the absorbance was measured at 530 nm. The data were expressed as malonaldehyde. GSH levels were determined by fluorescence using *o*-phthalaldehyde by modifications of the methods of Cohn and Lyle [7] and Beutler *et al.* [8]. The supernatant (100 μL) was pipetted into 3 mL of 0.4 M Tris–HCl buffer (pH 8.9) containing 10 mM EDTA and then 150 μL of *o*-phthalaldehyde reagent (1 mg/mL in methanol) was added. After 5 min the GSH content was determined fluorometrically (excitation, 343 nm; emission, 428 nm) using a fluorescence spectrophotometer (model F-3010, Hitachi Co. Inc., Tokyo, Japan). The data were expressed as GSH.

Determination of ^{45}Ca *uptake.* Synaptosomal calcium uptake was studied following the method of Blaustein and Weismann [9]. Synaptosomal suspensions in buffered salt solution (BSS) without Ca^{2+} were brought to a final tissue concentration of 0.5–0.8 mg protein/mL. The composition of BSS was (millimolar): NaCl, 120; KCl, 5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.0; glucose, 10; HEPES, 20 (pH 7.5). Aliquots (1.0 mL) of the suspensions were transferred to 12 mL polypropylene centrifuge tubes and preincubated for 3 min at 37°. For the *in vitro* study, ethanol, IsoPro or Carb were added in 20 μL volumes at the beginning of the equilibration period. ^{45}Ca uptake was initiated by adding 1.0 mL of prewarmed (20°) BSS containing 0.5 μCi of ^{45}Ca in to μM $CaCl_2$ ($^{45}CaCl_2$, sp. act. 20.08 mCi/mg, New England Nuclear, MA, U.S.A.). The ^{45}Ca uptake incubations were terminated at specified times 10 min after the addition of the radiotracer by adding 3.0 mL of ice-cold Mg^{2+}, Ca^{2+}-free BSS containing the calcium chelator EGTA at a concentration of 3.0 mM. Incubation mixtures were immediately centrifuged (18,000 *g* for 4 min), and synaptosomal pellets were washed once in ice-cold Ca^{2+}-free BSS. The final pellets were digested in 0.5 mL of 0.5 N NaOH by heating to 50° for 30 min. Following neutralization with 0.5 N HCl, the samples were transferred quantitatively to 10 mL aqueous counting scintillant (ACSII, Amersham, IL, U.S.A.) for determination of the synaptosomal ^{45}Ca content by liquid scintillation spectrometry (LSC-9000, Aloka, Tokyo, Japan).

Assay of [^{3}H]*inositol and* [^{14}C]*choline incorporation into phospholipids.* Synaptosomal suspensions were added to HEPES buffer containing [^{3}H]inositol and [^{14}C]choline. The assay was done as described by Procellati *et al.* [10]. The reaction mixture contained about 600 μg of protein and 0.5 μCi of [^{3}H]inositol in 200 μM inositol (*myo*-[2-^{3}H(N)]inositol, sp. act. 17.10 Ci/mmol, New England Nuclear) and 0.1 μCi of [^{14}C]choline in 200 μM choline ([1,2-^{14}C]choline chloride, sp. act. 7.2 mCi/mmol, New England Nuclear) in 1 mL of the medium. The incubation was continued for 15 min at 37°. It was terminated by adding 6 mL of $CHCl_3$–MeOH (2:1, v/v), and 0.2 mL of 0.17 M KCl including 10 mM inositol and choline was also added. The radiolabeled phospholipids were extracted as described by Eichberg and Dawson [11]. The extracts were evaporated under vacuum and redissolved in small volumes of chloroform, and an aliquot was applied to a 250 μm silica gel G thin layer chromatography plate (TLC). The plate was developed in a solvent system of chloroform, methanol, concentrated acetic acid, and water (25:15:4:2, by vol.). After development, the specific activities of [^{3}H]PI and [^{14}C]PC were determined as described elsewhere [12]. Lipid phosphorus levels were analysed as described by Bartlett [13].

Protein assay. The protein content of the synaptosomal preparation was determined according to Lowry *et al.* [14].

Statistics. Statistical significance was determined using Student's *t*-test.

RESULTS

Ethanol-treated mice

As shown in Fig. 1, ethanol-treated mice consumed gradually increasing amounts of ethanol throughout the experiment. This did not result in any difference between ethanol-treated and control mice in calorie intake or body weight at 2 weeks after the experiment began. However, calorie intake and body weight of ethanol-treated mice were significantly less than those of control mice during the first week.

Synaptosomal lipid peroxidation and GSH levels

Table 1 shows the effect of ethanol on brain and heart synaptosomal lipid peroxidation and glutathione levels. The synaptosomal lipid peroxidation level of ethanol-treated mice was almost the same as control in the brain and heart. On the other hand, the synaptosomal GSH level of ethanol-treated mice was higher than that of control mice in both brain and heart.

^{45}Ca *uptake*

Figure 2 shows the effects of adding EtOH, NE, Carb, and IsoPro *in vitro* on brain and heart synaptosomal ^{45}Ca uptake. The ^{45}Ca uptake of brain and heart from ethanol-treated mice were 87% and 216% of control mice, respectively. The brain ^{45}Ca uptake was lower in the ethanol-treated group, while the uptake in the heart was higher. Not only ethanol but also NE, Carb and IsoPro increased ^{45}Ca uptake.

[^{3}H]*Inositol incorporation into synaptosomal PI*

The synaptosomes of brain and heart were incubated with ethanol, NE, Carb or IsoPro in an incubation medium containing [^{3}H]inositol. The incorporation of [^{3}H]inositol into synaptosomal PI is shown in Fig. 3. The incorporation of [^{3}H]inositol into PI in brain and heart synaptosomes of ethanol-treated mice were 150% and 113% of control, respectively. PI turnover increased significantly in ethanol-treated brain synaptosomes, but not in the heart. *In vitro* addition of ethanol, NE, Carb

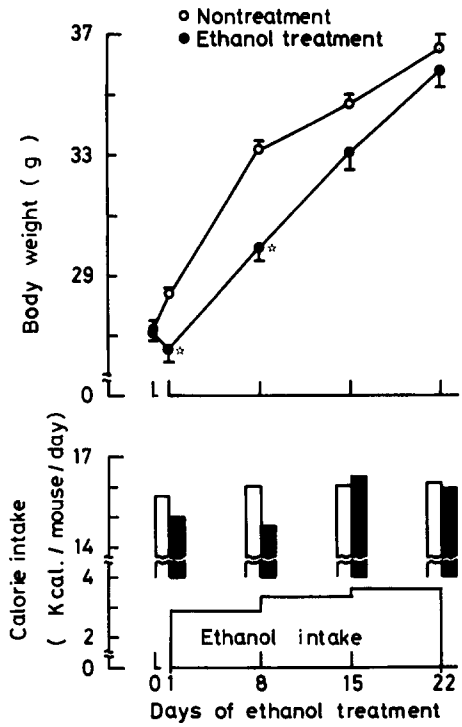

Fig. 1. Body weight and calorie intake of ethanol-treated mice. Calorie intake was calculated from food and ethanol consumption of 9 mice. (□) Nontreatment; (■) ethanol treatment. Body weights are means ± SE (☆) significantly different from nontreatment ($P < 0.01$).

or IsoPro to brain synaptosomes increased the incorporation of [^{3}H]inositol into PI, in both control and ethanol-treated mice. The incorporation was enhanced by NE and Carb in ethanol-treated mice brain but not in the control group. On the other hand, IsoPro increased incorporation markedly in both control and ethanol-treated brains, but the incorporation in ethanol-treated mice was lower than in non-treated mice. In the case of heart synaptosomes, NE and Carb increased the incorporation of [^{3}H]inositol into PI in control mice but not in ethanol-treated mice. However, IsoPro increased the incorporation in both control and ethanol-treated mice.

[^{14}C]*Choline into synaptosomal PC*

The incorporation of [^{14}C]choline into PC in the brains and hearts of ethanol-treated mice were 104% and 126% of control, respectively. *In vitro* addition of ethanol, NE, Carb or IsoPro to brain synaptosomes increased the incorporation of [^{14}C]choline into PC in both control and ethanol-treated mice. However, the increase caused by IsoPro was larger in control than in ethanol-treated brains. In the case of heart synaptosomes, NE and Carb increased the incorporation of [^{14}C]choline into PC in control mice but not in ethanol-treated mice. However, IsoPro increased significantly the incorporation by both control and ethanol-treated mice.

DISCUSSION

Attention has recently been focused on the increased Ca^{2+} sensitivity of neurons previously exposed to ethanol [15]. Ethanol has been shown by various workers using different systems to cause a rise in $[Ca^{2+}]_i$ [16–18]. How ethanol produces this effect, and whether the effect is specific or non-specific for a given regulatory patheway, are open to conjecture. The rise in synaptosomal $[Ca^{2+}]_i$ following exposure to ethanol is probably due to a reduction in cytosolic buffering of Ca^{2+} [18], and to the fact that ethanol causes an increase in synaptosomal $[Ca^{2+}]_i$ [19]. Davidson *et al.* [20] demonstrated that ethanol acts at the level of the endoplasmic reticulum in influencing Ca^{2+} homeostasis in synaptosomes. My work in chick embryo brains indicated that the rise in synaptosomal $[Ca^{2+}]_i$ following chronic exposure to ethanol is probably due to enhanced phospholipid turnover [4, submitted for publication].

On the other hand, in chick embryo hearts the reduction in synaptosomal $[Ca^{2+}]_i$ by ethanol is probably due to a reduction in phospholipid turnover. To assess such specificity, which may be related to characteristics of the lipids surrounding each receptor sytsem, I also compared the acute (*in vitro*) and chronic effects of ethanol on α- and β-adrenergic, and cholinergic receptor characteristics in the brain and heart. The present data show the responses to ethanol in terms of Ca^{2+} uptake, and [^{3}H]inositol and [^{14}C]choline incorporation into PI and PC.

^{45}Ca Uptake of ethanol-treated mouse heart synaptosomes was significantly higher than that of control. *In vivo* addition of EtOH, NE, Carb or

Table 1. Effect of *in vivo* administration of ethanol on brain and heart synaptosomal lipid peroxidation and glutathione

Tissue	Treatment	MDA (malonedialdehyde) (nmol/mg protein × 10^2)	GSH (nmol/mg protein)
Brain	Control	21.0 ± 2.2	134.2 ± 3.5
	Ethanol	22.8 ± 0.6	177.0 ± 4.4*
Heart	Control	13.1 ± 2.2	12.5 ± 1.3
	Ethanol	10.3 ± 1.2	22.4 ± 2.6†

Data are means ± SE from 5 mice. * and † were significantly different from control at $P < 0.01$ and $P < 0.05$, respectively.

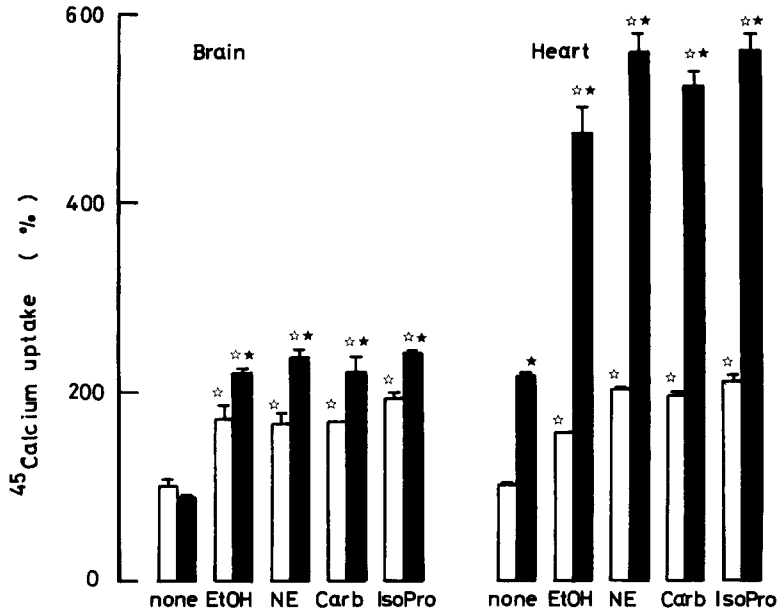

Fig. 2. ^{45}Ca uptake into brain and heart synaptosomes. Brain and heart synaptosomes were incubated with ethanol (100 mM), NE (100 μM), Carb (100 μM) or IsoPro (100 μM) and ^{45}Ca (2 μM) for 10 min at 20°. Data from 5 experiments are expressed at % of the nontreatment group value for each tissue. (□) Nontreatment; (■) ethanol treatment. (☆) Significantly different from the control group with no added drug ($P < 0.01$). (★) Significantly different from the nontreatment group ($P < 0.01$).

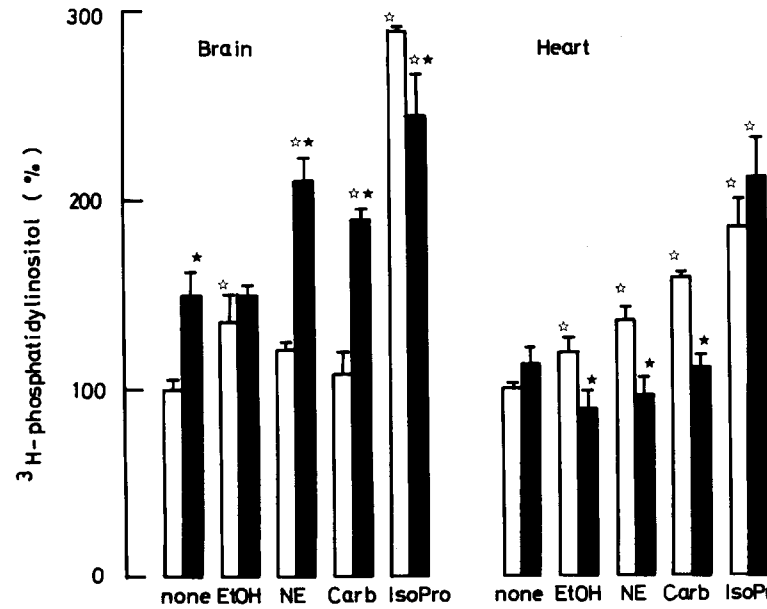

Fig. 3. Incorporation of [^{3}H]inositol into brain and heart synaptosomal PI. Brain and heart synaptosomes were incubated with ethanol (100 mM), NE (100 μM), Carb (100 μM) or IsoPro (100 μM) and [^{3}H]inositol (20 μM) for 20 min at 37°. Data from 5 experiments are expressed at % of the nontreatment group value for each tissue. (□) Nontreatment; (■) Ethanol treatment. (☆) Significantly different from the group with no added drug ($P < 0.01$). (★) Significantly different from the nontreatment group ($P < 0.01$).

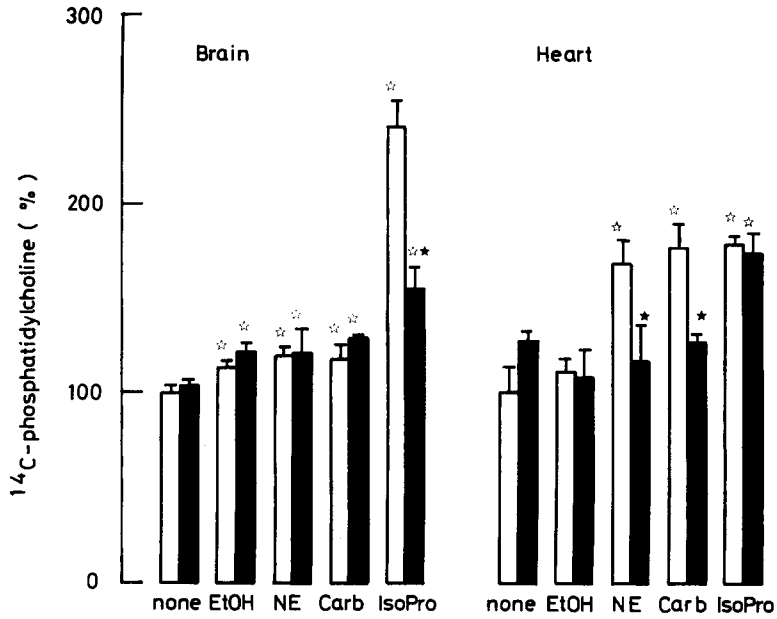

Fig. 4. Incorporation of [^{14}C]choline into brain and heart synaptosomal PC. Brain and heart synaptosomes were incubated with ethanol (100 mM). NE (100 μM), Carb (100 μM) or IsoPro (100 μM) and [^{14}C]choline (20 μM) for 20 min at 37°. Data from 5 experiments are expressed at % of the nontreatment group value for each tissue. (□) Nontreatment group value for each tissue. (□) Nontreatment; (■) ethanol treatment. (☆) Significantly different from the group with no added drug ($P < 0.01$). (★) Significantly different from the nontreatment group ($P < 0.01$).

IsoPro in both brain and heart synaptosomes increased the ^{45}Ca uptake in ethanol-treated mice more than in non-treated mice. This suggests that there were also changes in sensitivities of α- and β-adrenergic, and cholinergic receptors.

In vitro addition of EtOH, NE, Carb or IsoPro in brain synaptosomes increased the incorporation of [^{3}H]inositol and [^{14}C]choline into PI and PC, respectively, in both control and ethanol-treated mice. In the case of heart synaptosomes. NE and Carb increased the incorporation of [^{3}H]inositol and [^{14}C]choline into PI and PC in control mice but not in ethanol-treated mice. However, IsoPro increased the incorporation by both control and ethanol-treated heart synaptosomes.

Hoffman *et al.* [21] reported that ethanol decreased the affinity of the high-affinity site of the brain IsoPro receptor and increased the proportion of low-affinity binding sites. In the present study, the effect of IsoPro on [^{3}H]inositol and [^{14}C]choline incorporation into PI and PC was reduced in ethanol-treated brains, but not in hearts. These results suggest that changes in sensitivity of synaptosomal membrane in brain and heart of ethanol-treated mice may be adaptive responses to the initial effects of ethanol. The differences in response between heart and brain membranes may be in part related to differences in the ratio of those receptors in these tissues. Further work is necessary to resolve this question.

On the other hand, Videla and Valenzuela [22] have reported that the hepatic GSH concentration was decreased and lipid peroxidation was increased by chronic consumption of ethanol. I have also observed the same results (data not shown). However, I observed that ethanol increased GSH-levels in the brain and heart. The mechanism and effects of ethanol-induced increases in GSH is also not known.

Finally, these results suggest that α-adrenoceptors and the cholinergic system in the heart play important roles in modulating the toxic effects of ethanol. It is concluded that α- and β-adrenergic and cholinergic stimulation of ^{45}Ca uptake and [^{3}H]inositol and [^{14}C]choline incorporation into PI and PC are involved in the development of tolerance to ethanol toxicity.

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