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FORMATION OF FATTY ACID ETHYL ESTERS DURING CHRONIC ETHANOL TREATMENT IN MICE

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Abstract—Ethyl esters of long-chain fatty acids are formed in the liver and brain of mice after 1–6 days of ethanol intoxication. This observation extends the reports of Lange and co-workers who detected these compounds as unusual metabolites of ethanol in human tissues [E. A. Laposata and L. G. Lange, *Science* **231**, 497 (1986)]. Ethyl esters of oleic and linoleic acids, and, in smaller amounts, ethyl esters of palmitic and stearic acids were found in the livers of mice that had been treated with ethanol by inhalation. In the brain, only the esters of unsaturated fatty acids were found, in lower amounts than in liver. All the fatty acid ethyl esters seemed to have reached steady-state levels in the tissues after 3 or 4 days of alcohol treatment. When incorporated into synaptosomal plasma membranes *in vitro*, in intramembrane concentrations estimated to resemble those observed in the mice, these esters reduced the fluorescence anisotropy, i.e. they disordered the membranes.

Ethanol, a drug that probably acts in a hydrophobic site, affects the composition of lipids throughout the body [1, 2], but it is not clear whether these alterations are related to any pharmacological effects of the drug such as organ toxicity. Recently, a non-oxidative ethanol-metabolizing system that produces fatty acid ethyl esters was discovered in rabbit heart and the esters were detected in various organs of human alcoholics [3, 4]. The formation of these compounds from the free fatty acid pool may affect the composition of cellular phospholipids, and the esters themselves, which are highly lipid-soluble compounds, may be toxic. Organs that do not appreciably oxidize ethanol, such as the brain, are vulnerable to chronic ethanol toxicity, in which the accumulated fatty acid ethyl esters may play a role.

Various tissues can synthesize fatty acid ethyl esters *in vitro* [3, 5–10], and there are a few reports of their formation *in vivo* after administration of a single dose of ethanol. Goodman and Deykin [11] identified esters of palmitic, oleic and linoleic acids in the total body lipids of ethanol-treated rats. Because an ethanolic extraction procedure was used there was some possibility that the esters were formed during the workup [12], although this was considered unlikely at the time [11]. Patton and McCarthy [13], using a chloroform/methanol extraction procedure, reported finding fatty acid ethyl esters in the milk of goats that had been treated with ethanol. We report here the formation of fatty acid ethyl esters in the liver and brain of mice undergoing chronic ethanol treatment (sufficient to produce tolerance and physical dependence) and the membrane-disordering effect of the esters *in vitro*.

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METHODS

Animals. Male Swiss–Webster mice were exposed to ethanol vapor, using daily i.p. injections of pyrazole (68 mg/kg) to stabilize the blood ethanol levels [14]. Control mice received the same daily pyrazole injections. The concentrations of ethanol in the chamber air (10–12 mg/L) and in tail blood (1.9 to 2.3 mg/ml) were determined enzymatically [15]. After 1–6 days of continuous intoxication, the animals were killed, and the brain and liver were removed for lipid analyses. These organs were either extracted immediately or stored for a few days at −80° prior to extraction.

Extraction and isolation of fatty acid ethyl esters. Fatty acid ethyl esters were extracted from the tissue essentially by the procedure of Kinnunen and Lange [12]. To 1 g of tissue (brain or liver) was added 10 μg (33.3 nmol) of heptadecanoic acid ethyl ester (internal standard, Sigma Chemical Co., St. Louis, MO) in 10 ml of cold acetone. The homogenate was centrifuged at 3000 rpm for 10 min. The residue was re-extracted with 10 ml of cold acetone. The acetone extract was chromatographed over a column of 1 g Unisil (silicic acid, Clarkson Chemical Co., Williamsport, PA), and the fatty acid ethyl esters were recovered by elution with 10 ml petroleum ether (40°–60°). The eluate was evaporated to dryness under nitrogen, reconstituted in 50 μl cyclohexane, and analyzed by gas chromatography (GC) with flame ionization detector or by GC–mass spectrometry (GC/MS).

Identification and quantitation of fatty acid ethyl esters. Alternatively, the lipid extracts were subjected to high performance thin-layer chromatography (Silica gel 60, E. M. Science, Cherry Hill, NJ) in a solvent system containing petroleum ether (40°–60°):diethyl ether:acetic acid (75:5:1). Fatty

acid ethyl esters were visualized by exposure of the plates to iodine vapors, and the zone corresponding to standard fatty acid ethyl ester mixtures (Sigma Chemical Co.) was scraped and extracted with 3 ml acetone. The acetone extracts were dried under nitrogen, reconstituted in 50 μl cyclohexane and analyzed by GC or GC/MS.

A Hewlett–Packard 5830 gas chromatograph interfaced with a computing integrator and equipped with a glass column (2 m long and 2 mm i.d.) packed with 10% SP 2330 on Chromosorb 100/120 WAW was employed. Conditions were: oven temperature, 200°; injection temperature, 240°; detector, 275°; and carrier (helium) flow, 30 ml/min. Ethyl esters were identified by comparison of retention times with the standard ethyl esters and were quantitated by comparison of integrated peak area to the peak areas of known amounts of internal standard (heptadecanoic acid ethyl ester). For MS identification of the compounds, a Hewlett–Packard model 5985 GC/MS with electron impact ionization equipped with a column packed with 10% SP2330 on 100/120 Chromosorb was used. Oven temperature was held at 200° and carrier flow was 30 ml/min. The ionization energy was 70 eV.

Fluorescence polarization. Synaptosomal plasma membrane fractions were prepared from whole mouse brains (two to four brains per tube) by the sucrose density centrifugation technique of Jones and Matus [16]. The fraction was pelleted by centrifugation at 27,000 *g* and resuspended in phosphate-buffered saline at pH 7.4 at a concentration of about 20 mg protein/ml. The suspensions were stored in 60-μl aliquots under N_2 at −80°. Protein concentration was determined by the method of Lowry *et al.* [17].

Membrane aliquots were diluted with phosphate-buffered saline. Then 250 μM 1,6-diphenyl-1,3,5-hexatriene (Sigma Chemical Co.) in tetrahydrofuran (Aldrich Chemical Co., Milwaukee, WI) was added to a concentration of about 1 mol of dye per 500 mol of membrane lipid. The dye-labeled membrane suspension (final protein concentration about 30 μg/ml) was dispensed into silanized tubes. Commercially available fatty acid ethyl esters were made up to 0.2 mg/ml in redistilled 95% ethanol and then diluted with phosphate-buffered saline to 0.42 M ethanol. This was done dropwise with stirring, and the resulting solutions were clear. The solutions were added to the membrane suspensions at final concentrations of 1–4 μM fatty acid ethyl ester in 33–100 mM ethanol. The anisotropy values were corrected for the slight disordering produced by these concentrations of ethanol. The samples were incubated in the dark with shaking at 25° for at least 30 min before measurements were taken.

Fluorescence intensity and steady-state anisotropy values were determined at 25° with an SLM polarization fluorometer (SLM Instruments, Urbana, IL) as described [18].

RESULTS

Four fatty acid ethyl esters, namely the ethyl esters of palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids were found in acetone extracts of the livers of the ethanol-treated mice (Fig. 1). Their identity was established by comparison of their retention times with those of the authentic compounds and confirmed by GC/MS analysis. The mass spectra were characterized by the presence of the molecular ion (M^+), parent ion minus ethoxide group (M-45) and minus C_2H_5OH (M-46), as well as successive loss of 14 mass units of methylene groups.

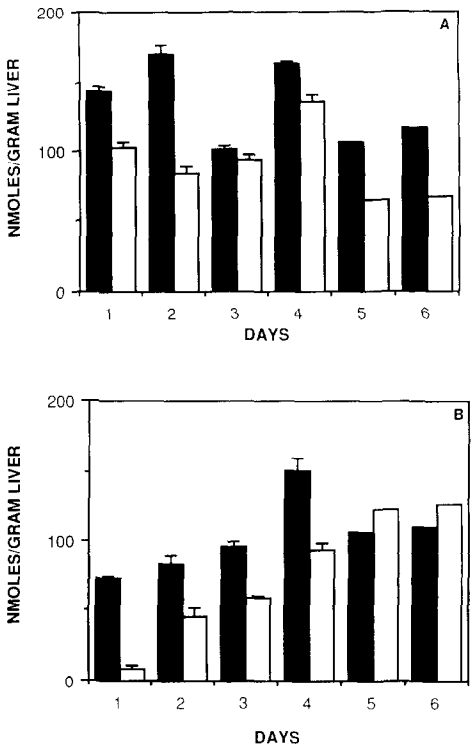

Fig. 1. Effect of chronic ethanol treatment on the formation of fatty acid ethyl esters in mouse liver. Mice were treated with ethanol by inhalation, and their livers were analyzed for fatty acid ethyl esters. The compounds were identified by GC retention times and quantitated by use of an internal standard. Data are means ± SD for N = 3 (days 1–4) and values for N = 1 on days 5 and 6 of ethanol treatment. (A) Esters of saturated fatty acids: filled bars, palmitic acid ethyl ester; open bars, stearic acid ethyl ester. (B) Esters of unsaturated fatty acids: filled bars, oleic acid ethyl ester; open bars, linoleic acid ethyl ester.

No fatty acid ethyl esters could be detected in the control liver or brain, but all four fatty acid ethyl esters were present in the liver after only 1 day of intoxication (Fig. 1). The amount of linoleic acid ethyl ester in liver increased with time during the first few days of treatment, so that the ratio of unsaturated to saturated fatty acid ethyl esters increased up to 72 hr. All the esters seemed to have reached their maximum levels (roughly 50–150 nmol/g) after 72 hr of ethanol exposure.

In brain, appreciable levels of fatty acid ethyl esters were found only after 2 days exposure to ethanol, and only unsaturated fatty acid ethyl esters were detectable (Fig. 2). Ethyl oleate was found at a mean concentration of 20 nmol/g brain and ethyl linoleate at 33 nmol/g after 3 days of ethanol treatment.

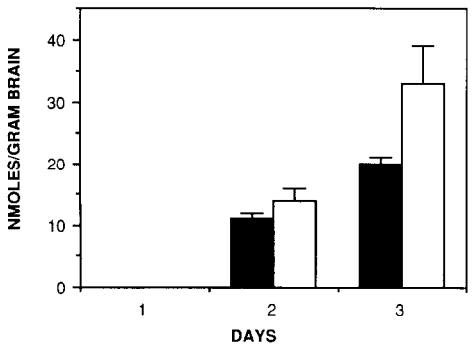

Fig. 2. Effect of chronic ethanol treatment on the formation of fatty acid ethyl esters in mouse brain. Conditions for these experiments were the same as in Fig. 1. Data are means ± SD, N = 3. Filled bars, oleic acid ethyl ester; open bars, linoleic acid ethyl ester.

Brains of mice treated for longer than 3 days were not analyzed.

The effects of three of the fatty acid ethyl esters on membrane fluidity were assessed by measuring the fluorescence anisotropy of diphenylhexatriene in mouse synaptosomal plasma membranes. The ethyl esters of palmitic, oleic, and linoleic acids produced concentration-related decreases in fluorescence anisotropy of 2–3 anisotropy units/mM (nominal concentration in the suspension) (Fig. 3).

DISCUSSION

Fatty acid ethyl esters appeared promptly in the liver of ethanol-treated mice (Fig. 1), and the ratio of unsaturated to saturated fatty acid ethyl esters increased with time. We could detect only unsaturated fatty acid ethyl esters in the brain and they appeared only after 48 hr of ethanol exposure, in smaller amounts than in liver. The tissue concentrations reported here are similar to those reported by Laposata and Lange [4] in autopsy material from intoxicated human subjects.

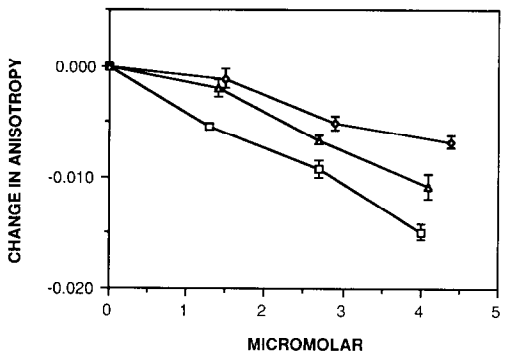

Fig. 3. Disordering of mouse synaptosomal plasma membranes by fatty acid ethyl esters *in vitro*. Points are means and error bars are ranges of duplicate values of changes in fluorescence anisotropy in the presence of the stated concentrations of the esters in the aqueous membrane suspension. Baseline anisotropy values in the absence of added drugs were between 0.236 and 0.238. The slopes of the least-squares lines through these points are −1.7/mM, −3.6/mM, and −2.8/mM for esters of palmitic (diamonds), oleic (squares), and linoleic (triangles) acid respectively.

Could they be artifacts? Probably not, because the ability of tissues to form these esters enzymatically *in vitro* has been well documented [3, 5–10]. The esters can also form non-enzymatically in heat-inactivated homogenates but only at much higher concentrations of alcohol than exist *in vivo* [12].

These metabolites of ethanol should be evaluated to determine whether they contribute to ethanol-induced toxicity. They might do so either by a direct action on cell membranes or by depleting cellular pools of lipid. Although a direct effect is suggested by the strong membrane-disordering action of the compounds at micromolar aqueous concentrations, calculation of their actual potency within the membrane shows them to be approximately equipotent with ethanol. To make this comparison, one must take into account the ratio of membranes (total hydrophobic phase) to aqueous phase, both in tissues and *in vitro*. We calculate the proportion of the total ester fraction that is in the membrane phase as follows: K_p, the membrane/water partition coefficient, is (by definition) $(E_m/V_m)/(E_a/V_a)$, where E_m and E_a are moles of ester in membranes and in the aqueous phase, respectively, V_m and V_a are volumes of membrane and aqueous phase, and E_T is the total amount of ester present. Then,

$$K_p = E_m/V_m \times V_a/(E_T - E_m)$$

or

$$E_m/E_T = K_p/(K_p + V_a/V_m)$$

We estimate K_p to be of the order of 10^4, i.e. about 10-fold higher than the reported partition coefficients of free fatty acids in biomembranes [19]. *In vivo*, assuming that the collective membrane phase is 5% of the wet weight of tissue, V_a/V_m is much smaller than any reasonable value of K_p and $E_m = E_T$; all the ester is in membranes. *In vitro*, however, the membrane concentration must be kept low to avoid turbidity. At the concentration we used, 30 μg protein or 60 μg membrane per ml, V_a/V_m is about 17,000. If $K_p = 10^4$, about 40% of the ester fraction is in the membrane.

Setting aside for the moment the question of where the remaining ester is, we can calculate the intramembrane concentrations *in vivo* and *in vitro*. In tissues, with the observed maximum of 500 nmol total esters per g wet weight and all of it in membranes, we have 500 nmol in 50 mg of membrane lipid or 10 mmolal. *In vitro*, the lowest concentration we used (Fig. 3) was a nominal 1 μM. With 40% of the added 1.0 nmol actually in the 60 μg of membrane per ml of suspension, we have about 7 mmolal ester in the membrane phase. Thus, the concentrations we used *in vitro* were similar to those we observed *in vivo*.

This concentration suffices to reduce the fluorescence anisotropy by about 0.005 (Fig. 3), which is about the same as the effect of 300 mM aqueous ethanol according to our earlier work [20]. The membrane-buffer partition coefficient of ethanol is generally taken to be 0.14 [21], so 300 mM aqueous ethanol corresponds to about 40 mmolal within membranes. Thus, molecule for molecule, the esters

are a few fold more potent than ethanol. Considering the crudeness of our estimates and the unknown effects of the esters that do not partition into membranes *in vitro*, this is a trivial difference. The disordering effect of the observed amounts of esters in brain (which are lower than in liver) would be similar to that of intoxicating concentrations of ethanol. We do not predict serious toxicity on this basis.

Lange, in his study on myocardial ethanol metabolism [22], has identified the donor of the fatty acid moiety to be free fatty acid. The fatty acid composition of the esters was more unsaturated than that in the free fatty acid pool, an observation that is probably explained by the substrate specificity of the cardiac fatty acid ester synthase [10]; esters are formed more rapidly from oleate and linoleate than from saturated fatty acids. The heart has pools of free fatty acids and of cholesterol esters similar in size to the steady-state levels of fatty acid ethyl esters that we observed [22], but we do not know whether these pools can be replenished rapidly enough to prevent their depletion by activity of the synthase. The fatty acid ethyl ester levels seen in our experiments are small compared to the pool size of phospholipids (e.g. 15 μmol/g in heart [22]). Thus, it is difficult to judge whether these synthase activities could account for the changes in membrane lipid species sometimes observed during chronic administration of ethanol [2].

Clearly, the pharmacology of these compounds warrants further detailed investigation. The possibility that the fatty acid ethyl esters may serve as markers for chronic ethanol abusers is of considerable clinical relevance.

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