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Functional tolerance to ethanol in mice: relationship to lipid metabolism

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The development of functional tolerance to ethanol was studied in young adult mice of the TO Swiss and C57BL strains. Ethanol administration was by intermittent inhalation for 7 h and the development of tolerance was assessed by estimating blood ethanol concentrations at successive losses of the righting reflex. The administration of (±)α-tocopherol at a dose previously shown to inhibit the effect of ethanol on peripheral phospholipid composition (750 mg kg^{-1} i.p.) was without effect on functional tolerance. Chronic administration of DL-carnitine, 7% w/w in diet for 10 days, a treatment previously shown to prevent ethanol-induced triglyceride accumulation, was also without effect on ethanol tolerance. Administration of the cholesterol synthesis inhibitor, diazacholesterol (30 mg kg^{-1} i.p.) 3 times per week for 3 weeks from weaning, markedly inhibited the subsequent development of functional tolerance. Administration of the inhibitor of protein synthesis, cycloheximide (300 μg kg^{-1} i.p.) greatly inhibited the development of ethanol tolerance whereas actinomycin D (100 μg kg^{-1} i.p.) was without effect. The results are discussed in relation to the hypothesis that ethanol cellular tolerance results from adaptation in the composition of the lipids of neuronal membranes. It is concluded that synthesis of some protein is involved in the development of cellular tolerance and also that cholesterol plays some part in the development of tolerance.

We have recently investigated the development of functional tolerance to ethanol in mice and have found it to be rapid (Grieve & Littleton 1979a) and strongly influenced by age and genetic complement (Grieve & Littleton 1979b). The genetic influence suggests that tolerance may be under the control of some enzyme system in the brain. The major site of action of ethanol in producing depression of the central nervous system is thought to be the neuronal membrane. Here, ethanol produces a fluidization of mammalian synaptic membranes (Chin & Goldstein 1977a) at concentrations associated in the living organism with intoxication. The mechanism of development of tolerance to ethanol is unknown but it seems logical to seek such a mechanism in some form of adaptation in central synapses to the presence of the drug.

Hill & Bangham (1975) were the first to suggest that tolerance to central depressant drugs, such as ethanol, which increase membrane fluidity, might involve a reduction in the intrinsic fluidity of the neuronal membrane thus reducing the effect of the drug at the cellular level. By analogy with the processes by which lower organisms adapt to increased temperature (which also increases membrane fluidity), it was proposed that tolerance to depressant drugs resulted from a change in the lipid composition of the neuronal cell membrane. There is now some evidence to support this concept. Thus Chin & Goldstein (1977b) have shown that synaptic membranes obtained from ethanol-tolerant mice are relatively resistant to the fluidizing effects of ethanol in vitro. This argues that some change in the membrane composition has occurred which renders the structure less able to be fluidized. That the mechanism may be directly analogous to that associated with adaptation to temperature has been shown by Li & Hahn (1978) using cultured mammalian cells. In these cells cross tolerance between increased growth temperature and addition of ethanol to the growth medium exists.

There are two simple mechanisms, which might underlie adaptation in mammalian cell membrane fluidity and which rely on alteration in membrane lipid composition. To reduce membrane fluidity, and thus to become resistant to fluidizing agents such as ethanol, mammalian cells could either reduce the degree of unsaturation of the fatty acyl groups attached to membrane phospholipids or could increase the cholesterol content of the cell membrane. Evidence that both these changes occur in mammalian synaptosomal membranes in response to the in vivo administration of ethanol has been found (Littleton & John 1977; Chin et al 1978).

Although there is indirect evidence in vitro linking membrane lipid composition with development of

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tolerance to ethanol there is no direct evidence that lipid metabolism is implicated in ethanol tolerance in vivo in the mammal. The purpose of this investigation was to attempt to alter the development of functional ethanol tolerance by pretreatments known to affect lipid metabolism, or the effect of ethanol on lipid metabolism, in the mouse. The treatments used were: (±)-α-tocopherol (Vitamin E), shown by Abu Murad & Littleton (1978) to inhibit changes in peripheral phospholipid composition induced by ethanol, DL-carnitine shown by Hosein & Bexton (1975) to inhibit triglyceride accumulation in liver produced by ethanol, diazacholesterol, which inhibits synthesis of cholesterol and reduces the cholesterol content of brain if given early in development (Ramsey 1978) and the inhibitors of protein synthesis, cycloheximide and actinomycin D. Cycloheximide has recently been shown to prevent the temperature-induced adaptive alteration of membrane phospholipid composition in Tetrahymena (Nozawa & Kasai 1978), actinomycin D was included to make a comparison with morphine tolerance where many inhibitors of protein synthesis effectively block development of tolerance (Cox & Osman 1970).

METHOD AND MATERIALS

The development of functional tolerance to ethanol was investigated using the method described previously (Grieve & Littleton 1979a, b). In this method individual mice are first injected intraperitoneally with a dose of ethanol found to produce severe ataxia, then exposed to ethanol vapour in an inhalation chamber until the righting reflex is lost. They are then removed from the chamber for 30 min while the blood ethanol concentration is estimated, then replaced until the reflex is again lost. The cycle of ethanol inhalation, loss of righting reflex, removal, estimation of blood ethanol, inhalation of ethanol, continues for approximately 7 h. Student's *t*-test was used to compare the means of blood ethanol concentrations at which the righting reflex was lost. Mice used in these experiments were young males of the TO Swiss (A. Tuck, Dagenham) or C57BL (OLAC, Bicester) strains. Investigation of tolerance to ethanol was performed at age 35–40 days except in the case of mice receiving diazacholesterol where the extended period of drug administration (see below) necessitated testing for ethanol tolerance at age 48–55 days. All mice were freely maintained on Spillers modified 41B diet and water before experiments. During investigation of ethanol tolerance no food or water was provided. Mice receiving a 'carnitine supplemented' diet received powdered Spillers 41B diet + 7% w/w (±)-carnitine. Controls for this experiment received powdered Spillers 41B diet alone. All injections were made intraperitoneally in saline except Vitamin E which was injected in soya bean oil (controls for this experiment received an equivalent volume of soya bean oil). Diazacholesterol was administered intraperitoneally 3 times per week from weaning (20 days) for 4 weeks.

Doses of drugs were chosen on the basis of those shown in previous experiments to affect lipid metabolism in rodents or, in the case of inhibitors of protein synthesis, to prevent the development of morphine tolerance (Cox & Osman 1970). All drugs were obtained from Sigma Chemical Co. Ltd., London, except diazacholesterol HCl which was a gift from G. D. Searle, Chicago.

RESULTS

The pre-treatment of mice of the TO Swiss strain with (±)-α-tocopherol (750 mg kg^{-1} i.p.) 16 h before first exposure to ethanol had no effect on the development of tolerance to ethanol (Fig. 1). (±)-α-Tocopherol administration also did not significantly alter the estimated concentration of ethanol in blood (2·92 ± 0·10 mg ml^{-1}, n = 4) at which righting reflex was first lost, when compared with that for soya bean oil injected controls (2·84 ± 0·10 mg ml^{-1} n = 4). The maximum concentration of ethanol in blood at which the righting reflex was subsequently lost (mean ± s.e.m.) was: control, 5·93 ± 0·20 mg ml^{-1}; Vitamin E, 6·06 ± 0·18 mg ml^{-1} ($P > 0·05$).

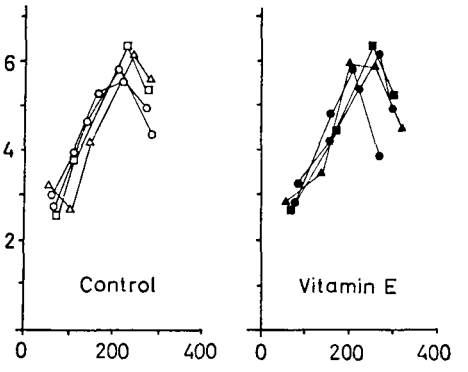

FIG. 1. Lack of effect of pretreatment with (±)-α-tocopherol (Vitamin E, on right) on development of tolerance to ethanol. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1} at loss of righting reflex. Connected symbols represent values obtained for an individual mouse Further details see text.

Chronic administration of (±)-carnitine HCl (7% w/w added to diet for 10 days before testing) did not affect the subsequent ability of mice of the TO Swiss strain to develop ethanol tolerance. The estimated concentration of ethanol in the bloodstream at which righting reflex was first lost was not altered by (±)-carnitine pretreatment (2·77 ± 0·08 mg ml^{-1} ethanol in carnitine-treated mice vs 2·77 ± 0·07 mg ml^{-1} in control mice, n = 4 in each case). The maximum concentration of ethanol in blood at which the righting reflex was subsequently lost (mean ± s.e.m.) was: control; 6·20 ± 0·20 mg ml^{-1}; carnitine, 6·08 ± 0·17 mg ml^{-1} (P >0·05). The results are shown in Fig. 2. An observation of interest was that

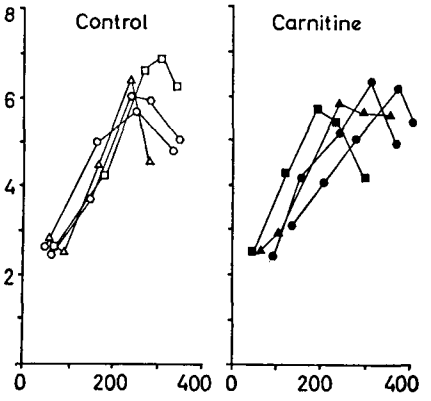

FIG. 2. Lack of effect of (±)-carnitine-supplemented diet on development of tolerance to ethanol. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1} at loss of righting reflex. Connected symbols represent values obtained for an individual mouse. Further details see text.

carnitine-treated mice showed much lower subsequent mortality than controls during the 48 h after exposure to the high tissue concentrations of ethanol achieved in these experiments. Of the 12 animals used in these experiments 5/6 controls subsequently died whereas only 2/6 of those treated with (±)-carnitine died. This confirms previous observations on the protective effect of carnitine in ethanol toxicity (Abu Murad et al 1977).

Pre-treatment of mice of the TO Swiss strain 3 times per week for 4 weeks from weaning with diazacholesterol (30 mg kg^{-1} i.p.) produced a reduction in the ability of the mice to develop ethanol tolerance when compared with saline-injected controls (Fig. 3). There was a small reduction in the concentration of ethanol at which diazacholesterol-treated mice first lost the righting reflex but this was not significant (controls 2·1 ± 0·3 mg ml^{-1}, diazacholesterol 2·0 ± 0·2 mg ml^{-1}, n = 6 in both cases). The maximum concentration of ethanol in blood at which the righting reflex was subsequently lost (mean ± s.e.m.) was: control 4·93 ± 0·15 mg ml^{-1}; diazacholesterol, 2·38 ± 0·21 mg ml^{-1}. These values are significantly different (P <0·01). The development of tolerance and concentrations of ethanol in blood at which righting reflex was lost in these mice differ slightly from the results described previously. This may have been because the mice in this experiment were older than those used before (see Methods). Diazacholesterol-treated mice gained weight normally during development and differed in appearance from control mice only in exhibiting moderate roughness of the coat. There were no gross signs of motor impairment in individuals from either group before ethanol exposure.

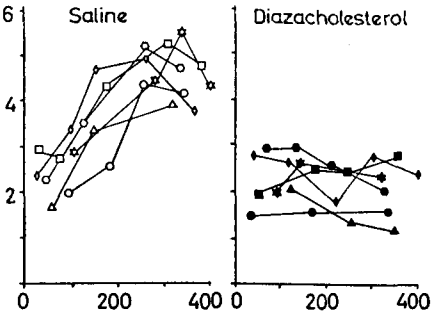

FIG. 3. Effect of chronic treatment with diazacholesterol on development of tolerance to ethanol. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1} at loss of righting reflex. Connected symbols represent values obtained for an individual mouse. Further details see text.

Quantities of cycloheximide and actinomycin D were restricted. For this reason experiments were made only on mice of the highly inbred C57BL strain. We have observed these to show rapid cellular tolerance to ethanol in the same way as mice of the TO Strain, but with less individual variation. Pretreatment with cycloheximide (300 μg kg^{-1} i.p.) 1 h before the first exposure to ethanol greatly inhibited the development of tolerance to ethanol. This inhibition occurred in the absence of any effect on the concentration of ethanol in blood at which the righting reflex was first lost (3·01 ± 0·09 mg ml^{-1} in cycloheximide-treated mice, n = 4, compared with 2·95 ± 0·07 mg ml^{-1} in saline controls, n = 4). The maximum concentration of ethanol in blood at which the righting reflex was subsequently lost (mean ± s.e.m.) was: control; 7·08 ± 0·24 mg ml^{-1};

cycloheximide 4·45 ± 0·15 mg ml^{-1}. These values are significantly different (P <0·01). Cycloheximide pre-treatment produced no gross signs of motor impairment in otherwise untreated animals for the duration of the experiment. The results are shown in Fig. 4.

The administration of actinomycin D (100 μg kg^{-1} i.p.) to C57BL mice one hour before first exposure to ethanol did not appear to affect the development of tolerance in any consistent way, although greater variation in response was noted

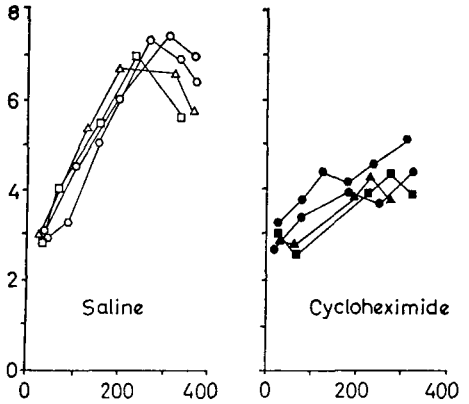

FIG. 4. Effect of pretreatment with cycloheximide on development of tolerance to ethanol. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1} at loss of righting reflex. Connected symbols represent values obtained for an individual mouse. Further details see text.

Similarly, administration of the protein synthesis inhibitor, cycloheximide, which has previously been shown to prevent temperature-induced adaptation in membrane lipids of unicellular organisms (Nozawa & Kasai 1978) prevented the development of ethanol tolerance. The other treatments investigated, intraperitoneal administration of (±)-α-tocopherol and actinomycin D and provision of a diet supplemented with (±)-carnitine were without effect on the development of tolerance to ethanol. In all cases these treatments were without significant effect on

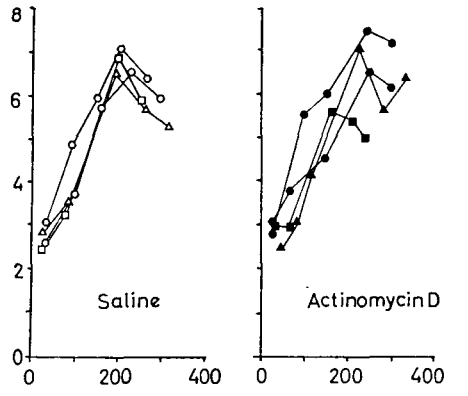

FIG. 5. Effect of pretreatment with actinomycin D on development of tolerance to ethanol. The abscissa shows the time in minutes after first exposure to ethanol; the ordinate the concentration of ethanol in the blood in mg ml^{-1} at loss of righting reflex. Connected symbols represent values obtained for an individual mouse. Further details see text.

(Fig. 5). Actinomycin D did not affect the estimated concentration of ethanol in blood at which the righting reflex was first lost (2·78 ± 0·12 mg ml^{-1}, n = 4) compared to that of saline controls (2·83 ±0·08 mg ml^{-1}, n = 4.) The maximum concentration of ethanol in blood at which the righting reflex was subsequently lost (mean ± s.e.m.) was: control, 6·75 ± 0·24 mg ml^{-1}; actinomycin D, 6·65 ± 0·30 mg ml^{-1} (P >0·05). Actinomycin D pre-treatment had no obvious effect on behaviour of otherwise untreated animals for the duration of the experiment.

DISCUSSION

These experiments provide some evidence to support the concept that alterations in lipid metabolism can affect the development of functional tolerance to ethanol in mice in vivo. Thus, prolonged pretreatment of mice with the inhibitor of cholesterol synthesis, diazacholesterol, inhibited the subsequent ability of mice to develop tolerance to ethanol. the estimated concentration of ethanol in blood at which the righting reflex was first lost. Any alteration in development of tolerance is therefore unlikely to be due solely to a treatment-induced increase or decrease in the central depressant effects of ethanol.

The major lipids of the mammalian membrane are phospholipids and cholesterol. A change in either component could theoretically lead to resistance of the membrane to the physical effects of ethanol. Littleton & John (1977) have shown that reduced proportions of polyunsaturated fatty acids are found in synaptosomal phospholipids from ethanol-tolerant mice whereas Chin et al (1978) have found an increased membrane cholesterol content in the same situation. Both of these changes could alter the intrinsic fluidity of the neuronal membrane and confer resistance to ethanol. In the experiments described here the possibility that ethanol tolerance was related to phospholipid fatty acid composition was investigated by pretreatment with Vitamin E and

carnitine. Vitamin E is capable of preventing (probably by inhibition of peroxidation) the change in fatty acid composition of peripheral tissues associated with administration of ethanol (Abu Murad & Littleton 1978). If it has the same effect centrally it should inhibit the development of ethanol tolerance if this is related to ethanol-induced alteration in membrane phospholipid fatty acid composition. That Vitamin E did not affect tolerance argues either that this is not related to phospholipid fatty acid composition or that peripheral administration of Vitamin E does not affect central phospholipid metabolism.

The rationale for investigating the effect of (±)-carnitine on ethanol tolerance is rather complex. Carnitine can prevent ethanol-induced accumulation of triglycerides in liver (Hosein & Bexton 1975; Abu Murad et al 1977) and in other tissues (unpublished) probably by stimulating fatty acid oxidation. Carnitine acts as the carrier for fatty acyl groups across membranes, including the mitochondrial membrane (hence the stimulatory effect on fatty acid oxidation) and synaptosomal membranes (Abdel Latif et al 1973). It seemed possible that the transfer of fatty acyl groups to membrane phospholipids might depend on the availability of carnitine, and that ethanol tolerance might therefore be influenced by carnitine loading. However, under the conditions of this experiment, carnitine had no effect on the development of tolerance. The experiments utilizing Vitamin E and carnitine therefore provide no evidence for the supposition that a change in central phospholipid metabolism underlies ethanol tolerance. The major problem in such an investigation is to find some treatment which will alter central phospholipid fatty acid composition while leaving the animal in a physical condition conducive to experimentation.

If the hypothesis of Chin et al (1978) is correct and ethanol tolerance can be attributed to an increase in concentration of cholesterol in the neuronal cell membrane, then inhibition of cholesterol synthesis should perhaps prevent the development of tolerance. This was observed to be so, pretreatment with diazacholesterol inhibited the development of tolerance to ethanol. The regime chosen should have significantly reduced the cholesterol content of brain in the treated mice (see Ramsey 1978). In this context it is interesting that no marked increase in ethanol 'sensitivity' (i.e. reduction in blood ethanol concentration at first loss of righting reflex) was seen in the treated mice. The hypothesis of Chin et al (1978) suggests that reduction in cholesterol content of the membrane, and hence increase in intrinsic membrane fluidity, should increase susceptibility to ethanol. This may indicate that membrane cholesterol content does not influence the effect of ethanol in the way proposed, or that membrane cholesterol is replaced, either physically by another sterol (e.g. desmosterol), or functionally by altered phospholipid composition. Biochemical analysis of membranes from these mice should answer these questions.

Lastly, investigation of the effects of inhibitors of protein synthesis has thrown some light on the possible mechanism of development of cellular tolerance to ethanol. Nozawa & Kasai (1978) have shown that the adaptive change in membrane phospholipid composition associated with moving *Tetrahymena pyriformis* to a cold environment is prevented by pre-treatment of the organism with cycloheximide. This argues that synthesis of some protein (Nozawa & Kasai 1978 suggest a desaturase enzyme) is important in adaptive alterations in membrane fluidity. Pre-treatment with cycloheximide inhibited the development of ethanol tolerance, but of course this cannot be adduced as evidence for any specific effect on enzymes involved in lipid metabolism. Cycloheximide is thought to act mainly on the transfer of amino acyl groups to the developing polypeptide chain. Actinomycin D which primarily inhibits RNA synthesis was without effect on ethanol tolerance. Assuming that actinomycin D treatment was sufficient to inhibit protein synthesis, this argues that some step between RNA synthesis and assembly of the polypeptide chain is important in the development of ethanol tolerance. Both actinomycin D and cycloheximide have previously been shown to inhibit the development of morphine tolerance (Cox & Osman 1970) and cycloheximide has been shown to affect the long-term development of ethanol tolerance in rats (Leblanc et al 1976).

In conclusion these experiments provide some evidence that ethanol tolerance in mice may be mediated by changes in lipid metabolism. Membrane cholesterol content probably plays an important part in this change, and synthesis of some protein seems to be necessary.

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