

Tolerance to Disulfiram Induced by Chronic Alcohol Intake in the Rat

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Background: Disulfiram, an inhibitor of aldehyde dehydrogenase used in the treatment of alcoholism, is an effective medication when its intake is supervised by a third person. However, its therapeutic efficacy varies widely, in part due to the fact that disulfiram is a pro-drug that requires its transformation into an active form and because it shows a wide range of secondary effects which often prevent the use of doses that ensure full therapeutic effectiveness. In this pre-clinical study in rats we report the development of tolerance to disulfiram induced by the chronic ingestion of ethanol, an additional source of variation for the actions of disulfiram with possible therapeutic significance. We also addresses the likely mechanism of this effect.

Methods: Wistar-derived rats bred for generations as high ethanol drinkers (UChB) were trained for either 3 days (Group A) or 30 days (Group B) to choose between ethanol (10% v/v) or water, which were freely available from 2 bottles on a 24-hour basis. Subsequently, animals in both groups were administered disulfiram or cyanamide (another inhibitor of aldehyde dehydrogenase) and ethanol intake in this free choice paradigm was determined. Animals were also administered a standard dose of 1 g ethanol/kg (i.p) and arterial blood acetaldehyde was measured.

Results: Disulfiram (12.5 and 25 mg/kg) and cyanamide (10 mg/kg) markedly inhibited ethanol intake (up to 60 to 70%) in animals that had ethanol access for only 3 days (Group A). However both drugs were inactive in inhibiting ethanol intake in animals that had consumed ethanol for 30 days (Group B). Following the injection of 1 g ethanol/kg, arterial blood acetaldehyde levels reached levels of 150 and 300 μ M for disulfiram and cyanamide respectively, values which were virtually identical regardless of the length of prior ethanol intake of the animals.

Conclusions: Chronic ethanol intake in high-drinker rats leads to marked tolerance to the aversive effects of disulfiram and cyanamide on ethanol intake despite the presence of consistently high levels of blood acetaldehyde. These findings may have implications for the use of disulfiram for the treatment of alcoholism in humans.

Key Words: Reward, Diversion, Ethanol, Disulfiram, Tolerance.

DISULFIRAM, A DRUG that inhibits aldehyde dehydrogenase, reduces ethanol consumption in alcoholics (Chick et al., 1992; Fuller and Gordis, 2004; Fuller et al., 1986). Its therapeutic effect is most evident when compliance with drug intake is supervised by a third person (Chick et al., 1992; De Sousa and de Sousa, 2005; Fuller and Gordis, 2004; Laaksonen et al., 2007; Weiss and Kueppenbender, 2006). While the use of supervised disulfiram is increasing, only 10% of patients treated by specialist physicians in the U.S. are prescribed disulfiram (Mark et al., 2003).

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In addition to the lack of compliance, other reasons also account for the reduced use of disulfiram. There is large inter-individual variation both in its deterrent action (Beyeler et al., 1985) as in its side effects (Dupuy et al., 1995; Gallant, 1987; Peachey and Annis, 1989;). It has been reported that at the regularly prescribed doses (250 mg to 300 mg/daily) used to avoid major secondary effects, only 50% of alcoholics develop the dysphoric disulfiram-ethanol reaction (Brewer, 1984; Christensen et al., 1991). A study on the use of disulfiram at the National Health Service of the U.K showed that the doses needed to induce the disulfiram-ethanol reaction in 90% of patients ranged from 250 mg to 650 mg daily (Brewer, 1984). One source of inter-individual variation stems from the fact that disulfiram must be metabolized into an active metabolite (Madan and Faiman, 1995; Mays et al., 1995).

In humans, the inhibition of liver ALDH2 by disulfiram and high blood acetaldehyde levels upon alcohol intake are most likely responsible for the dysphoric effects of the disulfiram-ethanol reaction, which include nausea, vomiting, flushing, sweating, hypotension and tachycardia, that curtail ethanol intake. However, there are also anecdotal reports that some individuals enjoy drinking in moderation while on disulfiram (Peachey et al., 1980) Animal studies may contribute to

shed light on the wide range of variability of disulfiram effectiveness. In the rat, brain acetaldehyde has been shown to be a reinforcing metabolite that is self administered intracranially (Amit et al., 1977; Brown et al., 1979; Rodd-Henriks et al., 2002). When ethanol is consumed, acetaldehyde is generated in the brain mainly by the action of catalase (Aragon et al., 1992; Jamal et al., 2007; Zimatkin et al., 2006). In line with a reinforcing effect of brain acetaldehyde, in the rat administration of aminotriazole, an inhibitor of catalase, reduces voluntary ethanol consumption (Aragon and Amit, 1992; Koechling and Amit, 1994; Tampier et al., 1995). Since disulfiram inhibits the low Km aldehyde dehydrogenase (ALDH2) not only in the liver but also in the brain (Hellstrom and Tottmar, 1982; Tampier and Quintanilla, 2003), an elevation of brain acetaldehyde following disulfiram administration may, in some individuals, counteract the aversive effects of acetaldehyde in the periphery.

In the East Asian population, an important proportion of individuals carries a largely inactive form of ALDH2 (ALDH2*2). Upon ethanol drinking, blood acetaldehyde is markedly increased in these subjects. Due to the presence of ALDH2 in the brain, in some individuals who carry the *ALDH2*2* allele a rewarding effect of acetaldehyde at the CNS levels may counteract the dysphoric reaction at the periphery. However, since the *ALDH2*2* allele exerts an overall protection against alcohol abuse and alcoholism in the population (Chen et al., 1999; Harada et al., 1982; Li, 2000; Thomasson et al., 1991; Tu and Israel, 1995) the dysphoric effect of acetaldehyde in the periphery must prevail over any rewarding effects in the CNS in most individuals. Despite this fact, some individuals who carry the *ALDH2*2* allele do become alcoholics.

Two experimental gene therapy drugs which do not cross the blood-brain barrier: (i) anti *aldh2* antisense oligonucleotides (Garver et al., 2001) and (ii) antisense genes (Ocaranza et al., 2008) reduce liver ALDH2 activity and, markedly inhibit ethanol intake, thus indicating that acetaldehyde elevations in the periphery per se lead to aversive effects. On the other hand, for disulfiram the dual inhibitory effect on both brain and liver ALDH2 may result in effects that depend on the balance of brain reward versus peripheral aversion. In this communication we report the loss of aversive effect of disulfiram elicited in rats by prior chronic intake of ethanol. The findings may have implications with regard to initiation of disulfiram treatment of alcoholics. The study may also provide new leads on why some individuals who carry the *ALDH2*2* allele become alcoholics.

METHODS

Animals

The experiments were performed in female UChB (*Aldh2*¹/*Aldh2*¹) rats originally derived from the Wistar strain, presently F 78 generation (see Quintanilla et al., 2006). Two to 3 month old rats were housed in individual cages and offered the choice of a 10% ethanol solution or distilled water from 2 bottles. Rat chow devoid of animal products (to avoid the presence heated fish bone which yields cyana-

mid) was available *ad-libitum*. The room was kept on a 12-hour light/dark cycle at 22 ± 2°C. The study was approved by the Animal Committee of the Faculty of Medicine, University of Chile.

Effect of Disulfiram and Cyanamide on Ethanol Consumption

The aldehyde dehydrogenase inhibitors disulfiram or cyanamide were administered to rats following 3 days of ethanol consumption (group A) and in another group after 1 month of ethanol consumption (group B). Groups of 5 to 9 rats of A or B received either an intraperitoneal dose of disulfiram (0, 12.5, 25 mg/kg suspended in a 0.5% Arabic gum solution) or of cyanamide (10 mg/kg in saline). Disulfiram or cyanamide were injected to rats 2 hours prior the 12-hour dark cycle, and ethanol and water consumption were measured for the subsequent 24 hours.

Acetaldehyde Blood Levels Induced by Disulfiram and Cyanamide After a Standard Dose of Ethanol

Rats treated with disulfiram or cyanamide (3 animals per group A and B) were used to determine arterial blood acetaldehyde levels produced by these inhibitors following the administration (i.p.) of a standard dose of 1 g ethanol/kg (as a 20% v/v solution in saline). Disulfiram (12.5 and 25 mg/kg) was administered 14 hours before the experimental dose of ethanol, while cyanamide (10 mg/kg) was administered 30 minutes before the dose of ethanol. Blood acetaldehyde levels were measured in 0.1 ml of blood samples taken from the carotid artery of anesthetized rat (ketamine hydrochloride, 60 mg/kg plus acetopromazine 2 mg/kg) at 5, 15, 30 and 60 minutes of the administration of ethanol. Acetaldehyde was measured in arterial blood by the head space method using gas chromatography as described in detail in Quintanilla et al. (2007).

Voluntary Ethanol Consumption

The amount of ethanol consumed each day by the rats under the free choice condition was registered and data were expressed as g ethanol consumed/kg body weight/d.

Statistical Analyses

Results are expressed as means ± SEM. Data were analysed by one-way ANOVA and compared using the Newman-Keuls post hoc test. $p < 0.05$ was considered statistically significant.

RESULTS

Tolerance to the Disulfiram and Cyanamide Effects Following Chronic Ethanol Exposure

Animals that had been trained to ingest ethanol for 3 days (Group A) or 30 days (Group B) in the ethanol versus water choice paradigm were administered disulfiram (12.5 or 25 mg/kg) or cyanamide (10 mg/kg) and subsequently 24-hour ethanol intakes were measured on day 4 and 31. Figure 1 shows that a significant reduction in ethanol intake follows the administration of disulfiram [$F(3,24) = 7.33, p < 0.005$] for animals in group A. The Newman-Keuls post hoc comparisons also showed significant reductions in ethanol intake by each dose of disulfiram or cyanamide ($p < 0.05$). When disulfiram or cyanamide were administered to rats that had a previous access to 10% ethanol versus water for 30 days (Group B), the drugs did not inhibit ethanol intake for disulfiram or cyanamide [$F(3,27) = 2.12, p < 0.122$], thus showing a

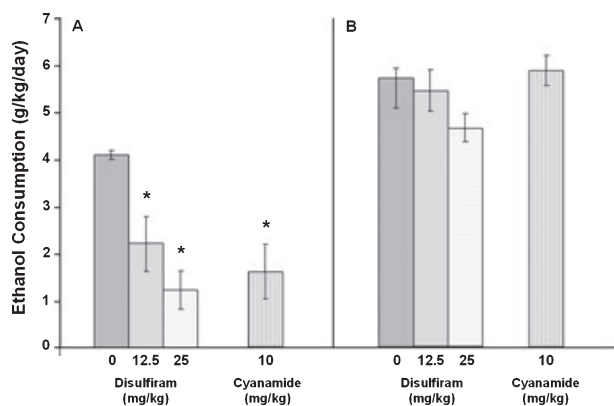


Fig. 1. Effect of disulfiram (12.5 or 25 mg/kg) and cyanamide (10 mg/kg) on ethanol intake by rats trained for 3 days (A) or 30 days (B) on the 2-bottle ethanol versus water choice paradigm $n = 5$ to 9 rats each group. * $p < 0.05$.

marked loss in the aversive effects of these drugs on ethanol consumption following prior chronic ethanol ingestion. Total fluid intake was not altered by disulfiram or cyanamide administration at 3 or 30 days of ethanol access (mean group values ranging from of 92 ± 6 to 99 ± 3 ml water/kg/d). The question posed was whether the tolerance observed to the aversive effects of these drugs on ethanol consumption was due to a reduction in blood acetaldehyde levels in the animals that had the prolonged access to ethanol.

Blood Acetaldehyde Levels Induced by Disulfiram and Cyanamide in Animals Allowed Chronic Access to Ethanol

Disulfiram and cyanamide at the doses used previously led to marked increases in blood acetaldehyde levels in both groups of rats (A and B) (Fig. 2) when administered the standard dose of 1 g ethanol/kg i.p. The levels of blood acetaldehyde achieved were virtually identical in both groups of animals, whether subjected to the brief period of ethanol intake (Group A) or the prolonged access to ethanol (Group B). Thus, tolerance to these ALDH inhibitors induced by chronic prior ethanol intake was not due to the generation of lower blood acetaldehyde levels.

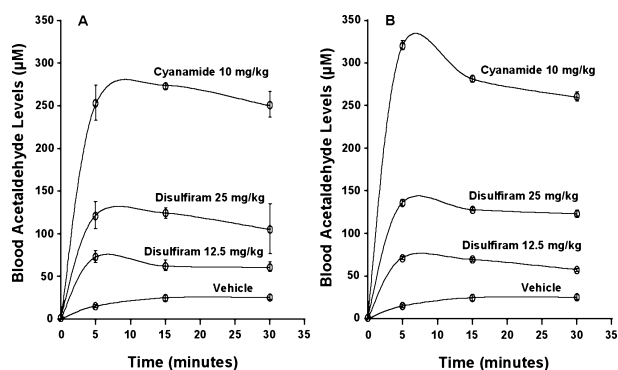


Fig. 2. Blood acetaldehyde levels following the administration of disulfiram (12.5 or 25 mg/kg) or cyanamide (10 mg/kg) after a standard dose of ethanol (1 g/kg, i.p.) to rats trained for 3 days (A) or for 30 days (B) on the 2-bottle ethanol versus water choice paradigm $n = 3$ rats each group.

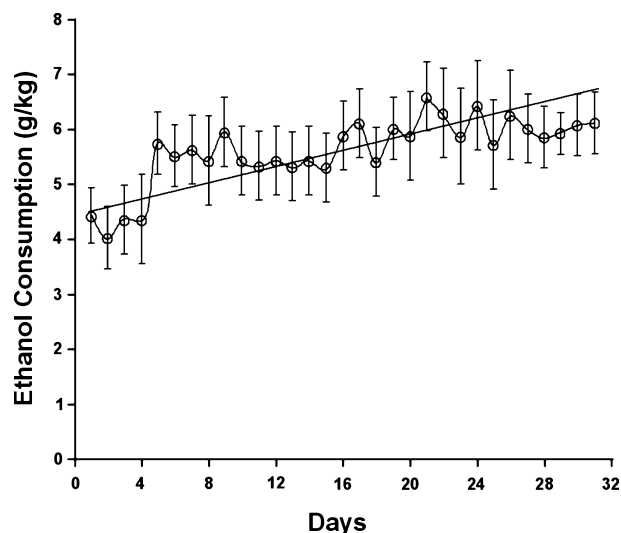


Fig. 3. Increase in voluntary ethanol consumption by UChB rats along time during free access to a 10% (v/v) ethanol solution and water. Ethanol consumption is expressed as g ethanol per kilogram body weight per day. $n = 27$ rats. The slope of the line is significantly different from zero ($r^2 = 0.634$; $p < 0.001$).

Changes in Ethanol Consumption in the Free Choice Paradigm

The change of ethanol consumption during 1 month under the 2 bottles free choice paradigm is shown in Fig. 3. Rats initially consumed an average of 4 g/kg (ethanol/water preference ratio of 50%) while increasing by about 50% reaching nearly 6 g/kg/d by the fourth week of exposure ($p < 0.001$) (preference ratio of 75%), indicating either the development of tolerance to the aversive effects of ethanol or a sensitization to its rewarding effects (see Discussion).

DISCUSSION

Agabio et al. (1996) investigating the drinking behaviour of Sardinian alcohol-preferring rats, put forth the hypothesis that voluntary ethanol intake is sustained in rats by the search for specific pharmacological effects of ethanol that are regulated by a central set-point mechanism which promotes or limits ethanol intake on the basis of the positive (rewarding) and negative (aversive) perception of those effects. Alcohol drinking is initially promoted until specific effects are perceived, and then limited, presumably to avoid possible aversive effects, which would be produced by high doses of alcohol. The possible mechanisms by which chronic exposure may lead to an increased ethanol intake are the development of tolerance to the aversive effects or an increase in its rewarding effects.

Following chronic administration, many drugs of abuse lead to sensitization (see Lessov et al., 2001; Robinson and Berridge, 1993) a condition in which a greater effect is obtained at a constant dose. Thus, it is conceivable that after chronic ethanol intake a time-dependent sensitization to the

rewarding effects of brain acetaldehyde may overcome the dysphoric effects of acetaldehyde in the periphery. Such an effect may account for the increases in ethanol volition seen following chronic alcohol intake. This notion is consistent with the observation that a single large injection of acetaldehyde increases subsequent voluntary alcohol consumption in alcohol-preferring UChB rats (Tampier and Quintanilla, 2002). Further, the administration of a single large dose of ethanol also leads to increases in ethanol consumption, an effect that is abolished by the administration of 3-Amino-1,2,4 triazole, a catalase inhibitor, prior to the dose of ethanol (Tampier and Quintanilla, 2003).

It has been well documented that rats learn to self-administer acetaldehyde into the cerebral ventricles (Amit et al., 1977; Brown et al. 1980) or the ventral tegmental area (Rodd-Henriks et al., 2002). The reinforcing properties of acetaldehyde have also been confirmed by place preference studies; rats exhibit a strong preference for a place that has been associated with intraperitoneal acetaldehyde injections (Quertemont and de Witte, 2001; Quintanilla and Tampier, 2003). Taken together a number of studies suggest that for the rat the overall hedonistic value of acetaldehyde represents the balance between its central reinforcing effects and its peripheral aversive effects, in line with the dual effect proposed for the rat by Quertemont and associates (see Quertemont, 2004).

From the above studies, chronic ethanol ingestion by alcohol preferring rats may result in a state in which the brain rewarding effect of acetaldehyde could exceed its peripheral aversive effect. If such is the case, alcohol drinker rats which are allowed access to ethanol for a prolonged period may lose the aversive effects of drugs such as disulfiram or cyanamide. That was indeed observed in the present study. These drugs not only increase the peripheral levels acetaldehyde but, by inhibiting brain aldehyde dehydrogenase, also the level of acetaldehyde in the CNS (Jamal et al., 2007; Tampier and Quintanilla, 2003). Data obtained show that tolerance to the aversive effects of disulfiram and cyanamide is seen following 30 days of continuous ethanol intake when the maximal intake of ethanol has stabilized. The tolerance observed for disulfiram and cyanamide in the present studies is contrasted with the marked inhibitory effect on ethanol intake generated by the administration of an anti ALDH2 antisense-gene based drug which does not penetrate the blood brain barrier (Ocaranza et al., 2008). The latter greatly reduced ethanol intake in UChB drinker rats animals previously exposed to ethanol for 60 days. These differences suggest that part of the *tolerance* to disulfiram and cyanamide observed in the present study requires the central inhibition of ALDH2 exerted by these drugs.

It is noteworthy that the levels of blood acetaldehyde achieved in animals treated with disulfiram and ethanol was equally high in animals that were allowed voluntary access to ethanol for only 3 days as for 30 days, thus ruling out that the mechanism of tolerance to disulfiram would be due to differences in the metabolism of ethanol or acetaldehyde. Further, the fact that tolerance to the aversive effect was similarly

observed for cyanamide also rules out possible changes in the metabolism of disulfiram as responsible for the effects observed. While we have not ruled out that tolerance to the aversive effects of acetaldehyde may in part result from tolerance to the peripheral effects of acetaldehyde, the studies presented, independently of the mechanism, may have implications on the time and dosing of disulfiram administration as an adjunct in the treatment of alcoholics.

An additional observation supporting the notion of a rewarding effect of brain acetaldehyde is the marked concordance between nicotine dependence and alcohol dependence. Some 70% of alcoholics are heavy smokers versus only 10% of individuals in the general population (Collins and Marks, 1995). Further, smokers are 10 times more likely to develop alcoholism than are nonsmokers (Hurt et al., 1994). Recent studies (Joshi and Tyndale, 2006) have shown that nicotine increases by the levels of brain CYP2E1, an enzyme that can also generate acetaldehyde in the brain (Vasiliou et al., 2006; Zimatkin et al., 2006).

We wish to point out that we have used 2 conditions that could have magnified the tolerance observed; namely the use of low doses of disulfiram (however a dose equivalent to 1.75 g per 70 Kg). While it might be argued that tolerance is observed because the dose-response curve for disulfiram has moved to the right, the qualitative conclusion remains unaltered. Also, it is noted that we used a line of rats bred for generations as alcohol-preferring rats which may have selected genes for acetaldehyde reward. Future studies should address the off-kinetics of disulfiram tolerance following withdrawal from ethanol, and whether the duration of this effect is influenced by other drugs currently tested for treatment of alcoholism, such as naltrexone, acamprosate and topiramate.

Overall, studies presented show that chronic ethanol intake in an alcohol-preferring rat line results in tolerance to the deterrent effect of disulfiram on ethanol volition, an effect that is seen in despite an unchanged elevation in systemic acetaldehyde after the administration of ethanol. Data might also explain why some individuals who carry the *ALDH2**2 allele who continue to drink chronically become alcoholics.

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