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Daidzin suppresses ethanol consumption by Syrian golden hamsters without blocking acetaldehyde metabolism

(mitochondrial aldehyde dehydrogenase/inhibitor/disulfiram)

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ABSTRACT **Daidzin is a potent, selective, and reversible inhibitor of human mitochondrial aldehyde dehydrogenase (ALDH) that suppresses free-choice ethanol intake by Syrian golden hamsters. Other ALDH inhibitors, such as disulfiram (Antabuse) and calcium citrate carbimide (Temposil), have also been shown to suppress ethanol intake of laboratory animals and are thought to act by inhibiting the metabolism of acetaldehyde produced from ingested ethanol. To determine whether or not daidzin inhibits acetaldehyde metabolism *in vivo*, plasma acetaldehyde in daidzin-treated hamsters was measured after the administration of a test dose of ethanol. Daidzin treatment (150 mg/kg per day i.p. for 6 days) significantly suppresses (>70%) hamster ethanol intake but does not affect overall acetaldehyde metabolism. In contrast, after administration of the same ethanol dose, plasma acetaldehyde concentration in disulfiram-treated hamsters reaches 0.9 mM, 70 times higher than that of the control. *In vitro*, daidzin suppresses hamster liver mitochondria-catalyzed acetaldehyde oxidation very potently with an IC_{50} value of 0.4 μM, which is substantially lower than the daidzin concentration (70 μM) found in the liver mitochondria of daidzin-treated hamsters. These results indicate that (*i*) the action of daidzin differs from that proposed for the classic, broad-acting ALDH inhibitors (e.g., disulfiram), and (*ii*) the daidzin-sensitive mitochondrial ALDH is not the one and only enzyme that is essential for acetaldehyde metabolism in golden hamsters.**

Daidzin, a glucosylated isoflavone isolated from *Radix puerariae*, suppresses free-choice ethanol intake by Syrian golden hamsters (1). It also potently and selectively inhibits human mitochondrial aldehyde dehydrogenase (ALDH) (2). Almost half of all Asians have inherited an inactive variant form of mitochondrial ALDH, and in this population group alcohol abuse/alcoholism is rare (3–5). Hence, daidzin might be assumed to act by mimicking the consequences of this mutation of the mitochondrial ALDH gene. Individuals carrying the null mitochondrial ALDH gene are not known to suffer from any physiologic problems, except for a significantly compromised capacity to metabolize acetaldehyde. When they consume even small amounts of ethanol (6) their blood acetaldehyde concentration increases markedly.

The premise that acetaldehyde accumulation after ethanol consumption may deter further drinking has attracted considerable attention ever since disulfiram was introduced as a therapeutic agent for human alcohol addiction. It was first proposed as an aversive drug based on the observation that workers in the rubber industry who had been exposed to thiuram compounds experienced unpleasant effects after drinking (7). Since then, it has been established that disulfiram—as well as other ALDH inhibitors—suppresses the metabolism of acetaldehyde and causes it to accumulate in peripheral tissues when ethanol is consumed. As a consequence, it induces a broad spectrum of disagreeable effects (8). Association of these noxious, unpleasant experiences with drinking ethanol has been thought to be part of the widely accepted, although unproven, mechanism of action of disulfiram. To determine whether or not daidzin suppresses hamster ethanol intake by inhibiting acetaldehyde metabolism, plasma acetaldehyde concentrations of daidzin-treated hamsters were determined after ethanol administration. Acetaldehyde clearance in disulfiram-treated hamsters was also studied for comparison.

MATERIALS AND METHODS

Animal Drinking Experiments. Animal drinking experiments were done in metabolic cages as described (1). Thirty-nine ethanol-preferring hamsters (Sasco, Omaha, NE) were used for this study. During the saline control period (day −6 to day −1) each hamster received (i.p.) 1 ml of sterile saline per day. On day 0, nine hamsters were used for the ethanol challenge experiment (see below). The remaining hamsters entered the drug treatment period (day 0 to day 5) with 18 of them receiving daidzin (150 mg/kg per day i.p.) and 12 receiving disulfiram (Sigma; 150 mg/kg per day i.p.). On day 6, an ethanol challenge experiment was done with 9 of the daidzin- and 6 of the disulfiram-treated hamsters. The remaining 15 hamsters entered the posttreatment phase of the experiment and received no further treatment until their ethanol intake returned to that of the saline control (day 13). Ethanol challenge experiments of the posttreatment hamsters were done on day 14. Ethanol intake measurements obtained throughout the experiment were compared by Student's *t* test. Intake measured on the day before treatment began (day 0) was compared with that measured on each day during and after the treatment phase. Daidzin was synthesized by using published procedures (9, 10) and identified by mass and NMR spectroscopy.

Ethanol Challenge Experiments. Hamsters were deprived of ethanol for 24 hr before the challenge experiments to allow ethanol and acetaldehyde to clear from their circulations. Hamsters were given their last doses of drugs 30 min before being anaesthetized with urethane (Sigma; 1.2 g/kg i.p.). Anesthetized animals were given a test dose of ethanol (1.3 g/kg i.p.), and blood samples (100 μl) were drawn at $t = 0$ (≈10 sec before ethanol was given), 20, 40, 90, 160, and 220 min from the jugular vein into tubes containing 5 μl of 9% K_3EDTA. Plasma was obtained by centrifugation.

Plasma acetaldehyde was analyzed by a described fluorescent method (11–14) with slight modifications. Briefly, 10 μl of plasma was treated with 190 μl of 1,3-cyclohexanedione solution (0.2 M/2 M NH_4Cl) containing 1-propanal as internal standard, at 70°C for 60 min. The reaction was stopped by chilling on ice. Samples were then centrifuged at 4°C, 13,000 × *g* for 10 min, and 10 μl of supernatant was injected onto a Beckman Ultrasphere C_{18} column (4.6 × 250 mm, 5 μm). The

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Abbreviation: ALDH, aldehyde dehydrogenase.

column was eluted isocratically at 27°C with a 20/80 (vol/vol) acetonitrile/water mobile phase at a flow rate of 1 ml/min. The product was detected by a fluorimeter with excitation and emission wavelengths set at 365 and 455 nm, respectively. Data were collected and analyzed with a Nelsen data collection system (Perkin–Elmer). Plasma ethanol was determined enzymatically with a Sigma assay kit (procedure no. 332-UV).

Acetaldehyde Oxidation by Isolated Liver Mitochondria. Mitochondrial preparations were obtained by the procedure of Johnson and Lardy (15), and organelle integrity was evaluated by latent glutamate dehydrogenase activity (16). Mitochondria-catalyzed acetaldehyde oxidation was assayed by monitoring the disappearance of acetaldehyde in 1 ml of assay medium containing intact mitochondria (0.3 mg of protein), 10 mM Tris·HCl (pH 7.4), 0.3 M mannitol, 2.5 mM $MgCl_2$, 10 mM K_2HPO_4, 10 mM KCl, 100 μM acetaldehyde, and a specified concentration of daidzin (17). Reactions were initiated by the addition of mitochondria and allowed to proceed at 37°C for 10 min. Acetaldehyde in the reaction mixture was assayed as described above. The integrity of the mitochondrial preparations measured before and after the reaction was >97% and >93%, respectively.

RESULTS

Effect of Daidzin and Disulfiram on Free-Choice Ethanol Intake. Only hamsters that consume large (10–15 ml/day) and consistent (<±2 ml) amounts of ethanol during the saline control period (Fig. 1, day −5 to day 0) were used for these studies. Treatment with either daidzin or disulfiram reduces their ethanol consumption significantly ($P < 0.001$). At a daily dose of 150 mg/kg, daidzin (0.36 milliequivalent per day) and disulfiram (0.5 milliequivalent per day) suppress ethanol intake by ≈70 and ≈80%, respectively (Fig. 1, day 1 to day 6). On termination of treatment, ethanol intake gradually returns to that of the saline controls (Fig. 1, day 7 to day 13).

Acetaldehyde Metabolism in Daidzin-Treated Hamsters. Fig. 2*A* shows the plasma acetaldehyde concentration vs. time profiles obtained for the hamsters that are under saline treatment (control), daidzin treatment (daidzin), and those that have recovered from daidzin treatment (postdaidzin). Before ethanol challenge, the residual acetaldehyde concentrations found in the plasma of these hamsters were 1.2, 2.5, and 3 μM, respectively (Fig. 2*A*, $t = 0$)—i.e., in the range of background values normally found in untreated hamsters kept in our animal facility. After injection of a test ethanol dose (1.3 g/kg i.p.), there is a 4-fold increase in plasma acetaldehyde concentration in all hamsters. It reaches a plateau of ≈12 μM at ≈50 min and begins to decline slowly when the ethanol concentration falls below 10 mM (Fig. 2*B*). Analysis by paired *t* test shows that the acetaldehyde concentrations in daidzin and postdaidzin hamsters do not differ significantly ($P > 0.05$) from those of the saline controls throughout the time course of the experiment.

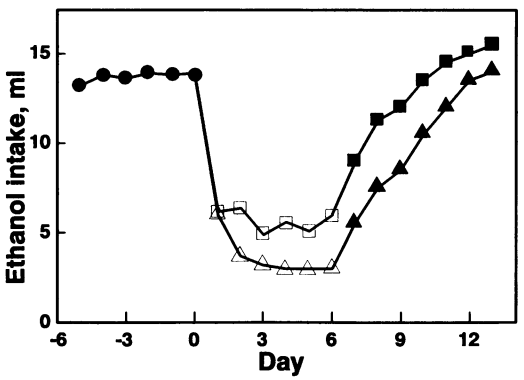

FIG. 1. Free-choice ethanol intake by golden hamsters under saline (●, 1 ml per day i.p., $n = 39$), daidzin (□, 150 mg/kg per day i.p., $n = 18$), or disulfiram (△, 150 mg/kg per day i.p., $n = 12$) treatment, and by those that were recovering from daidzin (■, $n = 9$) or disulfiram (▲, $n = 6$) treatment. Values are means for n hamsters.

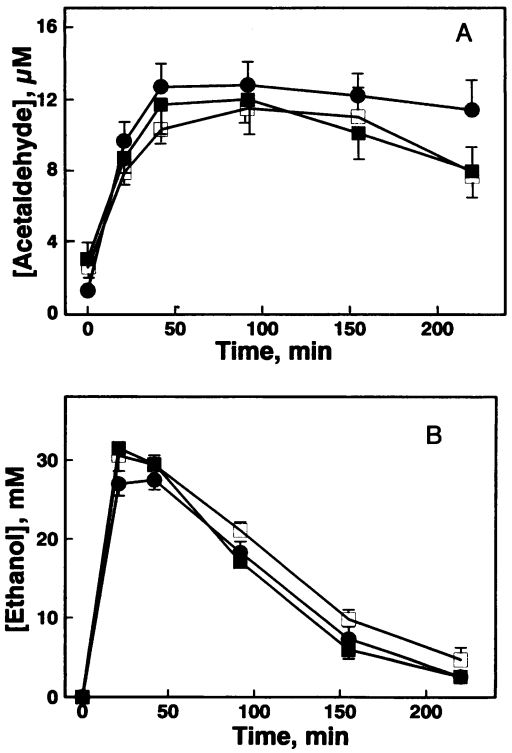

FIG. 2. Time course of plasma acetaldehyde (*A*) and ethanol (*B*) concentrations in the control (●), daidzin (□), and postdaidzin (■) hamsters after receiving an acute dose (1.3 g/kg i.p.) of ethanol at time = 0. Values are means ± SEMs for nine hamsters.

No residual ethanol was detected in the control, daidzin, and postdaidzin hamsters before ethanol administration (Fig. 2*B*, $t = 0$). After administration, plasma ethanol rises rapidly in all hamsters, reaches a maximal level of ≈30 mM, and gradually decreases to approach the base line over 4 hr. There is no significant difference ($P > 0.05$) in either the peak ethanol concentration (≈30 mM) or the elimination rate (0.18 mM/min) among the three different groups of animals.

Ethanol and Acetaldehyde Metabolism in Disulfiram-Treated Hamsters. Fig. 3*A* shows the plasma acetaldehyde concentration vs. time profiles obtained on ethanol challenge of control, disulfiram-treated, and postdisulfiram-treated hamsters. Before ethanol challenge, the residual acetaldehyde in the plasma of disulfiram and postdisulfiram hamsters is 3.7 and 4 μM, respectively, not significantly different ($P > 0.05$) from that in saline controls (Fig. 3*A*, $t = 0$). Unlike the daidzin-treated hamsters, those treated with disulfiram register extremely high plasma acetaldehyde concentrations when challenged with the same dose of ethanol. Plasma acetaldehyde rises rapidly, reaches 0.6 mM within 50 min, and continues to rise and approaches 0.9 mM (a 225-fold increase over the basal value) 4 hr after ethanol administration (Fig. 3*A*).

No residual ethanol is detected in the plasma of the disulfiram and postdisulfiram hamsters before ethanol injection (Fig. 3*B*, $t = 0$). After the injection of a test ethanol dose, plasma ethanol rises rapidly, reaches a maximal level of ≈30 mM in 40 min, and starts to decline thereafter. No significant difference is found among the control, disulfiram, and postdisulfiram hamsters in the peak ethanol concentration attained ($P > 0.05$). However, the ethanol elimination rate observed in the disulfiram hamsters is significantly slower than that ob-

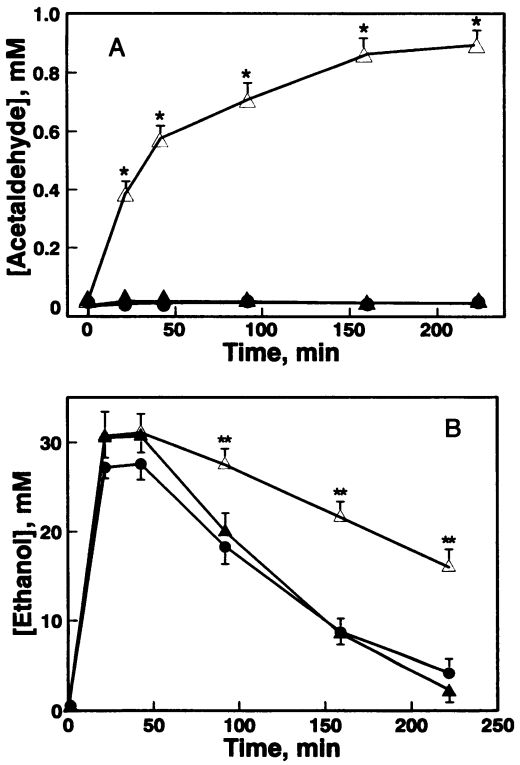

FIG. 3. Time course of plasma acetaldehyde (*A*) and ethanol (*B*) concentrations in the control (●), disulfiram (△), and postdisulfiram (▲) hamsters after ethanol administration (1.3 g/kg i.p.). Values are means ± SEMs for six to nine hamsters. ⋆, $P < 0.001$; ⋆⋆, $P < 0.01$.

served in the control and the postdisulfiram hamsters. The plasma ethanol elimination rate estimated for disulfiram hamsters is 0.08 mM/min, ≈50% of that observed in saline controls. Four hours after ethanol administration substantially higher levels (≈16 mM) of ethanol still remain in the plasma of the disulfiram hamsters (Fig. 3*B*).

Effect of Daidzin on Mitochondria-Catalyzed Acetaldehyde Oxidation. Freshly prepared hamster liver mitochondria catalyze acetaldehyde oxidation very efficiently. The rate of oxidation measured with 100 μM of acetaldehyde is 16 nmol/min per mg of protein, approximately two times that reported for rat liver mitochondria preparations (17). Daidzin, added to the incubation medium, markedly inhibits mitochondria-catalyzed acetaldehyde oxidation with an IC_{50} value of 0.4 μM (Fig. 4).

Daidzin Concentration in Liver Mitochondria of Daidzin-Treated Golden Hamsters. To determine whether or not injected daidzin can be absorbed and delivered to its putative site of action, mitochondria from livers of the saline control and daidzin-treated hamsters were isolated, and their daidzin contents were analyzed by HPLC. No daidzin can be detected in the mitochondria of the control hamsters (Fig. 5*A*), but significant amounts (70 μM) are found in that of the daidzin-treated hamsters (Fig. 5*B*).

DISCUSSION

The data demonstrate that daidzin, at a dose that suppresses voluntary ethanol intake by ≈70% in golden hamsters (Fig. 1), does not affect their ability to metabolize ethanol-derived acetaldehyde (Fig. 2*A*). Further ethanol elimination in these animals is not affected (Fig. 2*B*). In contrast, a similar dose of disulfiram both greatly suppresses acetaldehyde metabolism (Fig. 3*A*) and inhibits the rate of ethanol elimination (Fig. 3*B*). Clearly, the mechanism by which daidzin suppresses ethanol

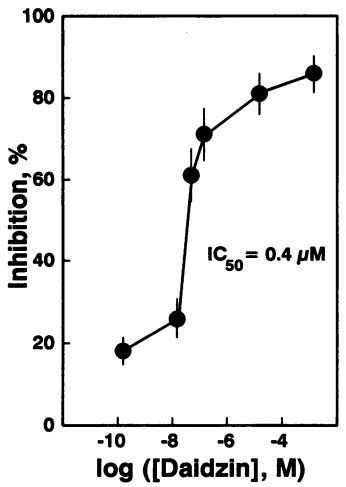

FIG. 4. Effect of daidzin on mitochondria-catalyzed acetaldehyde oxidation. Values are means ± SEMs for four separate determinations.

intake in these animals must differ from that proposed for the classic, broad-acting ALDH inhibitor disulfiram.

It is widely accepted that in humans, acetaldehyde produced from consumed ethanol is oxidized primarily by the low K_m mitochondrial ALDH isozyme (18). Surveys showing that individuals who have inherited the null mitochondrial ALDH gene are unable to metabolize acetaldehyde efficiently (6) support this view. Mitochondrial ALDH activity has also been shown to be the rate-limiting factor for acetaldehyde metabolism in species other than the human—e.g., pigs, cows (19), and rats (20). Hence, it was unexpected to find that daidzin, a potent inhibitor of human mitochondrial ALDH, does not affect acetaldehyde metabolism in golden hamsters. Three possibilities could account for this observation: (*i*) unlike the human enzyme, hamster mitochondrial ALDH is not sensitive to daidzin inhibition; (*ii*) daidzin administered i.p. is not absorbed and/or transported into hamster liver mitochondria in quantities sufficient to inhibit acetaldehyde oxidation; and (*iii*) the liver mitochondrial ALDH of golden hamsters, although sensitive to daidzin, is not critical for acetaldehyde oxidation.

To evaluate these various propositions, we isolated intact mitochondria from hamster livers and studied the effect of daidzin on their acetaldehyde-oxidizing activities. Daidzin markedly suppresses mitochondria-catalyzed acetaldehyde oxidation *in vitro* (Fig. 4), indicating not only that the hamster's mitochondrial acetaldehyde-oxidizing capacity is very sensitive to daidzin inhibition (IC_{50} = 0.4 μM) but, further, that the mitochondrial membrane is permeable to daidzin. Moreover,

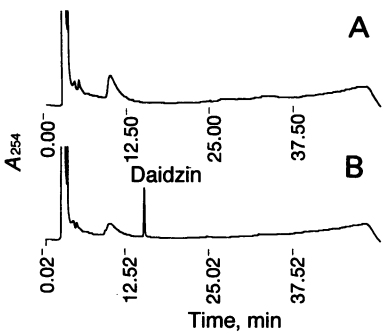

FIG. 5. HPLC chromatograms of extracts of liver mitochondria from control (*A*) and daidzin-treated (*B*) golden hamsters. Daidzin was extracted from mitochondria preparations with methanol and analyzed by HPLC as described (2).

liver mitochondria isolated from daidzin-treated hamsters contain ≈70 μM daidzin. Hence, daidzin administered i.p. is absorbed and delivered to the mitochondria in concentrations that are sufficiently high to suppress ≈80% of the acetaldehyde-oxidizing capacity of this organelle. Because daidzin does not affect overall acetaldehyde metabolism in golden hamsters, one or more extramitochondrial, daidzin-insensitive acetaldehyde-metabolizing enzyme(s) might exist in this species.

In a separate study, we have isolated and characterized three liver ALDH isozymes from golden hamsters, one mitochondrial (ALDH-M) and two cytosolic (ALDH-C1 and ALDH-C2). The high K_m of ALDH-C2 toward acetaldehyde (3.6 mM) indicates that it is not involved in its metabolism. ALDH-M and ALDH-C1 have K_m values (0.2 and 12 μM, respectively) that are lower than or similar to the concentration of acetaldehyde found in hamsters during ethanol metabolism; hence, both should participate in its metabolism. Daidzin inhibits only the mitochondrial isozyme ALDH-M (unpublished data) and not ALDH-C1, which can therefore metabolize acetaldehyde unimpeded. These results further support the proposition that the daidzin-sensitive liver mitochondrial ALDH is not critical for acetaldehyde metabolism in this species.

An inverse relationship between ethanol consumption and blood acetaldehyde concentration after ethanol administration has been observed in laboratory animals in which acetaldehyde metabolism was artificially suppressed with ALDH inhibitors (21, 22) and in animal strains that were bred selectively to prefer (AA rats) or avoid (ANA rats) ethanol (23). However, there has never been any direct evidence presented to show that high acetaldehyde concentrations suppress ethanol intake. In fact, studies with rats in which a low dose of alcohol dehydrogenase inhibitor was used to diminish acetaldehyde accumulation suggest that accumulated acetaldehyde does not mediate the ethanol intake effect of calcium carbimide, an ALDH inhibitor. 4-Methylpyrazole, at a dose that prevents the accumulation of blood acetaldehyde in carbimide-treated rats that were given ethanol, did not abolish the capacity of carbimide to suppress ethanol intake (24). This, together with our present findings, suggests that a physiological substrate of the mitochondrial ALDH, not acetaldehyde, may play a critical role in the regulation of ethanol consumption in laboratory animals. However, acetaldehyde derived from ethanol metabolism or other sources may compete with that substrate for the mitochondrial ALDH and thereby alter ethanol drinking behavior.

ALDH inhibitors are anti-dipsotropic in most animal models. However, they have not proved to be efficacious in the treatment of humans addicted to alcohol. Disulfiram inactivates both the mitochondrial and cytosolic ALDH isozymes *in vivo*, and for almost 50 yr it has been the only agent approved in the U.S. for the treatment of alcoholism. Disulfiram plus even small amounts of ethanol, produces a wide spectrum of unpleasant, and in some cases severe reactions that may result in respiratory depression, cardiovascular collapse, arrhythmias, myocardial infarction, acute congestive heart failure, unconsciousness, convulsion, and death (25). These effects have been attributed to inhibition of ALDHs and other enzymes critical in neurotransmitter metabolism, drug metabolism and detoxification, and multiple pathways of intermediary metabolism (26). The rationale for treatment with antabuse is that fear of these reactions will deter further drinking. In fact, results of double-blind, placebo-controlled clinical trials of disulfiram have proven just that: disulfiram is no more effective than the placebo control in reducing alcohol consumption (27). Low efficacy and high risk have greatly limited the use of disulfiram in the treatment of alcoholism.

The effect of daidzin on human ethanol consumption is not known. However, extracts of *R. puerariae*, which contain daidzin, have been used effectively in humans in China for >400 yr (2). No adverse side effects, including those associated with the use of ALDH inhibitors, have ever been reported. This anecdotal information suggests that these extracts, unlike disulfiram or carbimide, do not contain an ethanol-sensitizing agent and should not act via the ALDH enzyme system. Hence, it is likely that daidzin, the putative active principle of *R. puerariae*, may exert its anti-dipsotropic effects via a pathway other than that catalyzed by mitochondrial ALDH.

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