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Daidzin and daidzein suppress free-choice ethanol intake by Syrian Golden hamsters

(*Radix puerariae*/isoflavones/alcohol abuse/alcohol dehydrogenase/aldehyde dehydrogenase)

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ABSTRACT **Syrian Golden hamsters prefer and consume large and remarkably constant amounts of ethanol in a simple two-bottle free-choice regimen. Ethanol intake is significantly suppressed by zimelidine, bromocryptine, buspirone, and lithium carbonate, pharmacological agents that have been shown to be beneficial in controlling ethanol intake in alcohol-dependent humans. These results suggest that this ethanol-drinking animal model has high "predictive validity" and can be used effectively in the search for and identification of new agents for the treatment of alcohol abuse. The model has enabled us to confirm the putative antidipsotropic effect of *Radix puerariae* (RP), an herb long used in traditional Chinese medicine for the treatment of patients who abuse alcohol. A crude extract of RP at a dose of 1.5 g·kg^{-1}·day^{-1} significantly suppresses (>50%) the free-choice ethanol intake of Golden hamsters. Moreover, two major constituents of RP, daidzein (4′,7-dihydroxyisoflavone) and daidzin (the 7-glucoside of daidzein), were also shown to suppress free-choice ethanol intake. Daidzin and daidzein, at doses of 150 and 230 mg·kg^{-1}·day^{-1}, respectively, suppress ethanol intake by >50%. RP, daidzein, and daidzin treatment do not significantly affect the body weight and water or food intake of the hamsters. These findings identify a class of compounds that offer promise as safe and effective therapeutic agents for alcohol abuse.**

Alcohol abuse is a serious human behavioral disorder with major economic, social, medical, and psychological consequences. Recognition of suitable pharmacological agents and their development into effective therapeutic drugs for this disease has been one of the major objectives of alcohol research. However, the lack of a biochemical basis for alcohol abuse has rendered a solely rational approach to the discovery of such agents very difficult if not impossible. Monitoring the search for therapeutic agents for alcohol abuse calls for a laboratory animal that consumes ethanol voluntarily and, preferably, in large quantities. During the past 30 years several lines of ethanol-preferring rats and mice have been developed by selective breeding (1–3). In some of these animal lines—e.g., the P (4) and AA (5) rats—chronic ethanol consumption induces various behavioral patterns, symptoms, and signs that are thought to be equivalent to those found in alcohol-dependent humans. Hence, they are considered to have high "face validity" and match the criteria published for an animal model of "alcoholism" (3, 6). However, these animals are generally not "spontaneous" drinkers and must be bred for 20–30 generations to establish this characteristic and are, therefore, not readily available. Some outbred laboratory animal species studied under a simple two-bottle free-choice regimen have been shown to be useful for determining whether or not a new drug will suppress ethanol consumption in alcohol-dependent humans—i.e., they are thought to have good "predictive validity" (7). However, most of these outbred animals display low preference for and consume only small amounts of ethanol, and hence their usefulness in evaluating the effects of agents on ethanol intake is limited.

We have found the Syrian Golden hamster (*Mesocricetus auratus*) to be exceptionally suitable for the study of voluntary ethanol intake because, by nature, this species prefers and consumes large quantities of ethanol (8). Individual daily consumption of ethanol is remarkably constant. Furthermore, it exhibits excellent predictive validity; agents that have been shown to attenuate ethanol consumption in alcohol-dependent humans also suppress ethanol intake in Golden hamsters. In this study, we have used this animal system to demonstrate that a crude extract of *Radix puerariae* (RP), an herb long used in traditional Chinese medicine for the management of alcohol abuse (9), effectively suppresses ethanol intake. Moreover, we have isolated and shown that two of the major constituents of this extract, daidzin and daidzein, primarily account for this action.

MATERIALS AND METHODS

A crude extract was prepared by heating finely ground RP (Vinh-Kan Ginseng, Boston) with methanol (1:10, wt/vol) in a Soxhlet apparatus equipped with an all-glass thimble (Kontes) for 3 hr. Solvent was evaporated under vacuum and the resultant syrup was dried by lyophilization. Daidzin and daidzein were either isolated from a RP extract according to the procedures described by Keung and Vallee (10) and Keung (11) or synthesized by published procedures (12, 13) and were identified by mass and NMR spectroscopy.

Adult male Syrian Golden hamsters (Sasco, Omaha, NE) weighing 131–135 g were housed (four or five per cage) in a room maintained at 23°C on a 12-hr light/12-hr dark cycle (light on 0600–1800 hr) for a week with ad libitum access to Purina Rodent Laboratory Chow 5001 and a 15% solution of ethanol in water. After this preconditioning period, each hamster was transferred to an individual stainless steel metabolic cage (26 × 18 × 17.5 cm). Two 50-ml drinking bottles fitted with stainless steel sipper tubes were placed in bottle holders attached to the front wall of the cages. The bottle holders were equipped with tilted platforms to collect and direct spillage from the bottles to tubes placed outside of the cages. Two drinking fluids were supplied continuously for each hamster. One was MLQ (Millipore) filtered tap water; the other was a 15% (vol/vol) solution of ethanol (100% United States Pharmacopoeia) in filtered tap water. The positions of the two drinking bottles on each cage were alternated daily to prevent development of positional preference. Fluid intake was measured at 0900 hr each day. Hamsters that drank significant (>8 ml/day) and consistent

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Abbreviations: RP, *Radix puerariae*; ALDH, aldehyde dehydrogenase; ADH, alcohol dehydrogenase; i.p., intraperitoneally.

amounts of ethanol solution were selected for the study of the effects of test compounds. About 70–90% of the hamsters received from Sasco met these criteria. To establish the control ethanol and water intake, sterile saline was administered daily to each hamster at 1500–1600 hr for 6 days (saline control period, day −5 to day 0). After this period, a daily dose of test compound (in sterile saline) was administered for 6 days (treatment period, day 1 to day 6). After the last dose of test compound, all treatments were terminated and ethanol and water intake were monitored for another 6 days (posttreatment period, day 7 to day 12) to determine whether or not the effect(s) of these test compounds on ethanol intake was reversible. Student's t test was used to compare the various fluid consumption measures. Fluid intake measured on the day before drug treatment began (day 0) is compared with fluid intake measured on each day during and after the treatment period.

RESULTS

Free-Choice Ethanol and Water Intake by Golden Hamsters. With free access to food and water, ethanol-naive hamsters (≈130 g) consumed ≈8 ml of fluid per day. When given a free choice between water and a 15% ethanol solution, the water intake declined steadily and ethanol intake increased concomitantly. After about a week, the hamsters' daily water and ethanol intake reached relatively constant levels, and the total intake of fluid increased 2- to 3-fold. The daily urine output also increased from ≈2 ml/day to 5–10 ml/day (data not shown). The free-choice ethanol and water intake of one of the hamsters studied is shown in Fig. 1 and its total daily ethanol intake was ≈14 g per kg of body weight. This amount did not increase significantly when an ethanol solution of higher concentration was provided but it did decrease as the concentration of the ethanol solution was decreased (results not shown). Although ethanol intake by different hamsters varied from 8 to 20 ml/day, the daily ethanol intake by an individual hamster remained remarkably constant.

Effects of Crude RP Extract, Daidzin, and Daidzein on Free-Choice Ethanol Intake in Golden Hamsters. Crude RP extract has been used and claimed to be effective in the management of alcohol abuse in China.* However, to our knowledge, its efficacy has never been examined critically. We have therefore prepared a crude RP extract and studied its effect on free-choice ethanol consumption by the Golden hamster. Six hamsters that consumed similar amounts of ethanol were selected for this study. During the saline control period, this group of hamsters consumed an average of ≈12 ml of 15% ethanol solution per day (Fig. 2 *Upper*, day −5 to day 0) and this was suppressed significantly (≥50%) upon intraperitoneal (i.p.) injection of the RP extract (1.5 $g \cdot kg^{-1} \cdot day^{-1}$ in 1 ml of sterile saline). Ethanol consumption remained low throughout the treatment period (Fig. 2 *Upper*, day 1 to day 6) but was reversed partially after RP injection was terminated (Fig. 2 *Upper*, day 7 to day 12). RP extract had no significant effect on water intake.

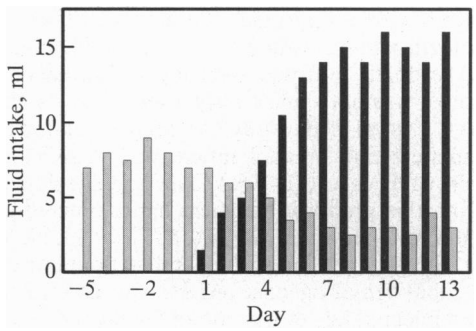

FIG. 1. Free-choice ethanol and water intake by an adult male Golden hamster weighing 130 g. The hamster was preconditioned for 1 week with ad libitum access to Purina Rodent Laboratory Chow and water before it was transferred to a metabolic cage on day −6. Day −5 to day 0, only water was provided; day 1 to day 13, water and a 15% ethanol solution were provided. Shaded bars, water; solid bars, 15% ethanol solution.

Attempts to identify the active principle(s) in RP that suppresses ethanol intake have led to the discovery that daidzin, a glycosylated isoflavone that inhibits human mitochondrial aldehyde dehydrogenase (ALDH) is effective. Thirteen hamsters that consumed similar amounts of ethanol, ≈11 ml of 15% ethanol per day, were selected for this study. Daidzin (150 $mg \cdot kg^{-1} \cdot day^{-1}$) injected i.p. as a suspension in

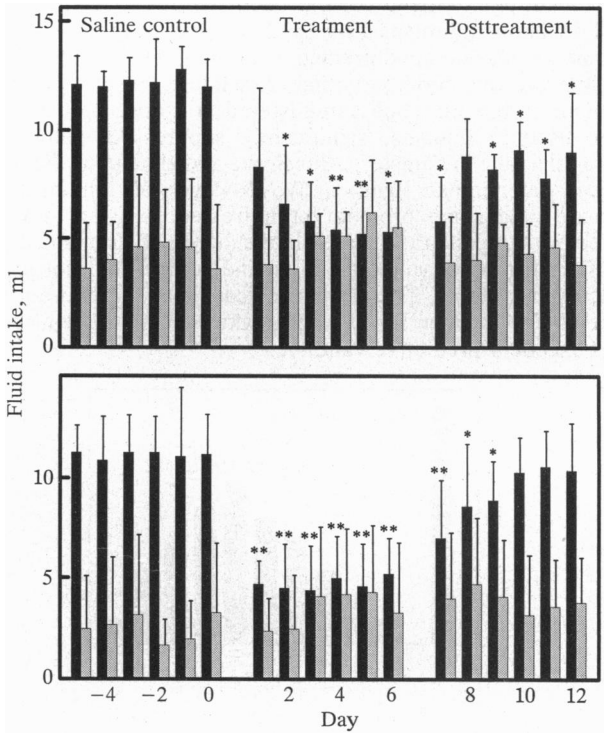

FIG. 2. Effect of RP extract (1.5 $g \cdot kg^{-1} \cdot day^{-1}$; $n = 6$) (*Upper*) and daidzin (150 $mg \cdot kg^{-1} \cdot day^{-1}$; $n = 13$) (*Lower*) on free-choice ethanol intake in Golden hamsters. Values are means ± SD for n hamsters. RP extract and daidzin were suspended in 1 ml of sterile saline and injected i.p. Shaded bars, water; solid bars, 15% ethanol solution; *, $P < 0.05$; **, $P < 0.001$.

*It has not been a general practice of the traditional Chinese physicians to publish or otherwise publicize their mode of therapy. As a consequence, detailed written clinical records on the identities of drugs, their efficacy, dosage, and side effects are not readily available. To collect clinical information on the use of RP in China as remedial agents for alcohol abuse, W.-M.K. visited seven medical colleges and hospitals and three research institutes in China and interviewed 13 traditional physicians and research scientists. Physicians practicing traditional Chinese medicine as well as modern Chinese research scientists generally agree that RP or *F. puerariae* extracts are effective in suppressing the appetite for ethanol and the "deleterious" effects of ethanol on "vital organs." The physicians interviewed had treated a total of ≈300 alcohol abusers with RP- or *F. puerariae*-based medications. In all cases, the medications were considered effective in both controlling and suppressing appetite for alcohol and improving the functions of the alcohol-affected vital organs. Significant improvement is usually said to be observed within a week of treatment. After 2–4 weeks, most patients (≈80%) no longer experienced a craving for alcohol. Alleviation of some of the alcohol-induced damage of some vital organs usually requires longer treatment (4–6 months). No adverse side effects associated with the use of RP or *F. puerariae* have ever been reported.

1 ml of sterile saline suppressed this ethanol intake by >50%. When daidzin administration was terminated, ethanol consumption gradually returned to the level of the saline control. Water intake was not significantly affected by daidzin injection (Fig. 2 *Lower*). This study has been expanded to a total of 71 hamsters and ethanol intake was significantly suppressed in each case (31–79%). The suppression data obtained from this group of hamsters approximated a normal distribution with mean ± SD = 56.5% ± 11.5% (Fig. 3). Daidzein, the aglycone of daidzin and an inhibitor of human class I alcohol dehydrogenase (ADH) was also isolated from RP. When injected i.p. to a group of 5 hamsters at a dose of 230 mg·kg^{-1}·day^{-1}, daidzein suppressed their ethanol intake by ≈50%. Like RP and daidzin, daidzein did not alter water intake by the Golden hamsters to any significant extent (Fig. 4). Hamsters receiving RP extract, daidzin, or daidzein treatment appeared to remain healthy and did not exhibit any significant change in food intake or body weight throughout the experiment (data not shown).

The effects of daidzin and daidzein on ethanol intake by the hamsters are not due to stress caused by the introduction of a crystalline suspension of these isoflavones into the peritoneum of the animals. Two flavones, chrysin (a 4′,5,7-trihydroxyflavone) and 7,8-dihydroxyflavone, which, like daidzin and daidzein, are virtually insoluble in water, were studied for their effect on free-choice ethanol intake. Both were injected as a suspension in 1 ml of sterile saline at the same dose as daidzin (150 mg·kg^{-1}·day^{-1}), but neither of them suppressed ethanol intake. Furthermore, puerarin, the most abundant isoflavone in RP, also failed to suppress ethanol intake (data not shown).

Effects of Some Central Nervous System Agents on Free-Choice Ethanol Intake by Golden Hamsters. The effects on free-choice ethanol intake by Golden hamsters were also examined with four agents thought to be beneficial in the treatment of excessive, uncontrolled ethanol intake in alcohol-dependent humans (14) to determine whether or not hamsters provide good predictive validity. The results (Fig. 5) indicate that bromocryptine, zimelidine, buspirone, and lithium carbonate, each administered at a dose 2–10 times that used in humans, significantly suppressed voluntary ethanol intake in hamsters. Buspirone and zimelidine did not affect water intake significantly. However, all lithium carbonate- and some bromocryptine-treated hamsters developed polyuria and their water intake increased dramatically. Administration of saline alone (saline control) did not produce such effects. The results indicate that the two-bottle free-choice regimen for ethanol intake by Golden hamsters has excellent predictive validity.

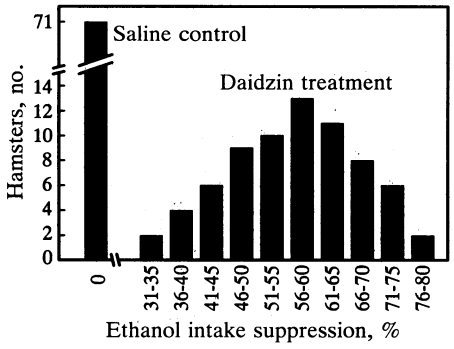

FIG. 3. Suppression of free-choice ethanol intake by daidzin (150 mg·kg^{-1}·day^{-1}) in a population of 71 Golden hamsters. % suppression = $[(V_c - V_t)/V_c] \times 100$, where V_c and V_t are means of ethanol intake during saline control and daidzin treatment period, respectively. Each animal served as its own saline control during the 6 days before treatment.

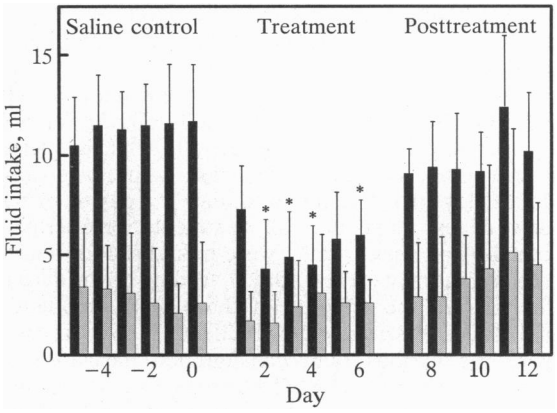

FIG. 4. Effect of daidzein (230 mg·kg^{-1}·day^{-1}) on free-choice ethanol intake in Golden hamsters. Values are means ± SD for five hamsters. Daidzein was suspended in 1 ml of sterile saline and was injected i.p. Shaded bars, water; solid bars, 15% ethanol solution; *, $P < 0.05$.

DISCUSSION

The Syrian Golden hamster, in contrast to most outbred strains of laboratory rodents, displays a high preference for and consumes large quantities of ethanol under a two-bottle free-choice regimen (8), as confirmed in the present study. A typical 130-g male Golden hamster consumes ≈8 ml of water per day. This intake of water is relatively low compared to that of other commonly used laboratory rodents and has been attributed to the fact that Golden hamsters are desert adapted with both renal and respiratory mechanisms for water conservation (15). Their urine output is ≈2 ml/day. Interestingly, when the Golden hamster is provided with free access to both water and a 15% ethanol solution, it acquires most of its total fluid intake from the ethanol solution (Fig. 1). The resultant total ethanol intake reaches a stable value of ≈14 g·kg^{-1}·day^{-1}, ≈10 times more than that consumed by humans considered to be heavy drinkers (16). To consume such large amounts of ethanol, the hamsters must increase their total fluid intake 2- to 3-fold while increasing urine output significantly possibly because the diuretic property of ethanol relaxes the desert-adapted water-conservation mechanisms. Although daily ethanol consumption by different hamsters does vary, ranging from 8 to 20 g·kg^{-1}·day^{-1}, for any individual hamster it is remarkably constant.

Relatively few investigators have used the Golden hamster in experiments for alcohol research largely because it does not meet the criteria published for an animal model of alcoholism (3, 4). After prolonged consumption of large quantities (15–20 g·kg^{-1}·day^{-1}) of ethanol, no signs of ethanol withdrawal or organ disorders have been demonstrated in the Golden hamsters (17). Such failure has frustrated attempts to use this species as a model of alcoholism. The term alcoholism is meant to refer both to the behavioral disorder of excessive, uncontrolled intake of ethanol and to the clinical abnormalities that are the consequences of alcohol abuse. Excessive ethanol intake may or may not necessarily lead to medical or neurologic illnesses that require medical treatment but may nevertheless interfere seriously with normal marital, social, and economic life. Thus, agents that abolish or significantly suppress the uncontrollable desire to drink ethanol without producing undesirable side effects would seem to be the preferable means to treat alcoholism. On this basis, the key feature of an animal model for monitoring the search for such agent(s) would not be its face validity but rather its capacity to predict whether or not an agent will effectively suppress ethanol consumption in alcohol-dependent humans—i.e., its predictive validity (7). Toward this end, we

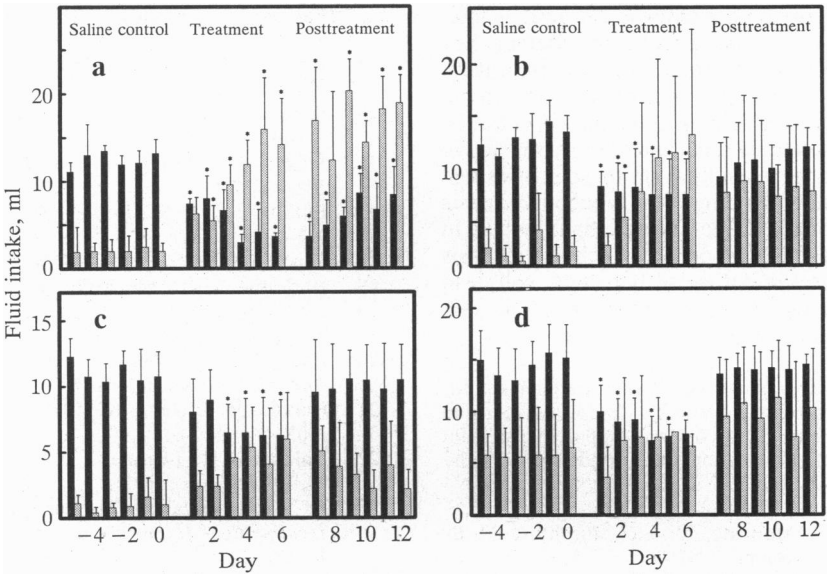

FIG. 5. Effect of lithium carbonate (38 mg·kg^{-1}·day^{-1}) (Sigma) (*a*), bromocryptine (5 mg·kg^{-1}·day^{-1}) (Sigma) (*b*), buspirone (20 mg·kg^{-1}·day^{-1}) (Sigma) (*c*), and zimelidine (38 mg·kg^{-1}·day^{-1}) (Research Biochemicals) (*d*) on free-choice ethanol intake in Golden hamsters. Values are means ± SD for four hamsters. Test compounds were dissolved in 0.5 ml of sterile saline and injected either i.p. (lithium carbonate, bromocryptine, buspirone) or s.c. (zimelidine). Shaded bars, water; solid bars, 15% ethanol solution; *, $P < 0.05$.

have examined the predictive validity of the Golden hamster by testing the effects on free-choice ethanol intake of four pharmacological agents that have been thought to be beneficial to alcohol-dependent humans (14). The results (Fig. 5) clearly indicate that the suppression of free-choice ethanol consumption in the Golden hamster is completely consistent with the beneficial effects of these agents as observed in alcohol-dependent humans. This, together with the fact that the animals consume large and remarkably constant amounts of ethanol each day, identifies them as an excellent laboratory species for monitoring the search for and identification of pharmacological agent(s) for alcohol abuse. We have therefore selected this as a model for the study of the antidipsotropic potential of RP.

RP is an herbal medicine prepared from the root of the Leguminosae *Pueraria lobata* (commonly known as kudzu) and was first described in *Shen-nung Pen-t'sao Ching*, the first Chinese Materia Medica (*ca.* 200 B.C.), as a medication for human consumption with antipyretic, antidiarrheic, diaphoretic, and antiemetic properties. The use of RP, and *Flos puerariae*, the flower of kudzu, in alcohol-related diseases was documented *ca.* 600 A.D. in the Chinese Pharmacopoeia *Beiji-Qianjin-Yaofang* (18), which first described an "anti-drunkenness" effect. About 1000 years later, the use of a *F. puerariae*-based medication for the treatment of alcoholism was described in the book of *Lan-tai Kuei-fan* (9). *F. puerariae*- or RP-based medications are still used in China by traditional Chinese physicians (colloquially referred to as herbalists) for the treatment of patients who abuse alcohol.* The present study confirms the antidipsotropic effect of a crude RP extract on ethanol-drinking Golden hamsters (Fig. 2 *Upper*). Moreover, we have identified daidzin and daidzein, first recognized as inhibitors of enzymes that metabolize ethanol (10, 11), as active principles in this extract that can account for the antidipsotropic activity of RP (Figs. 2 *Lower* and 4).

Daidzein and daidzin are isoflavones, a group of natural products found mostly in leguminous plants (19). The pharmacological effects of isoflavones on animals have been studied since the 1950s when a weak estrogenic activity was detected in most isoflavone aglycones including daidzein (20). Since then, isoflavones have been claimed to have antifebrile, antispasmodic, antihypertensive, and antidysrhythmic activities (21–24). However, an effect of isoflavones on ethanol-drinking behavior has never been demonstrated. Biochemically, isoflavones in general, and genistein in particular, inhibit a number of enzymes including tyrosine-specific protein kinase (25), protein histidine kinase (26), and DNA topoisomerase II (27). Genistein also inhibits endothelial cell proliferation and *in vitro* angiogenesis (28). Daidzein and daidzin affect some of these activities, but only at fairly high (>50 μM) concentrations.

We have shown recently that daidzin is a potent (K_i = 40 nM) and selective inhibitor of human mitochondrial ALDH (10). Since alcohol abuse is rare among the ≈50% of Asians who have inherited an inactive mutant form of this enzyme (29), it is tempting to suggest that daidzin may suppress ethanol consumption by mimicking the effect of this apparently harmless natural mutation of the mitochondrial ALDH gene. Unlike daidzin, however, daidzein does not inhibit human mitochondrial ALDH. Instead, it potently inhibits the class I isozymes of human ADH, especially the γ-type ADH isozymes (11). Studies with 4-methylpyrazole, an inhibitor of human class I ADH, have shown and suggested that it suppresses ethanol intake in P rats by inhibiting the metabolic elimination of ingested ethanol (30). Conceivably, inhibition of ADH or ALDH could suppress ethanol consumption by increasing the bioavailability of ingested ethanol.

In the past decade, the physical, chemical, and enzymatic properties of human ADH and ALDH have been explored thoroughly (29, 31). Both enzymes exist in multiple molecular forms that exhibit different specificities toward a wide variety of alcohol and aldehyde substrates. Among these, many have important physiological roles and some may be involved in the mediation of ethanol-drinking behavior (29, 31). Indeed, it may be that interference with these normal metabolic processes by daidzin or daidzein causes the decrease of ethanol consumption in Golden hamsters. In this context, it should be noted that the structural analogs puerarin, chrysin, and 7,8-dihydroxyflavone, which are not inhibitors of ADH (11) or ALDH (10), do not suppress ethanol intake. This finding does not prove, but it is consistent with the notion, that the γ-type ADH isozymes and mitochondrial ALDH may play an important role(s) in the regulation of ethanol intake.

The metabolic fates of daidzin and daidzein in the Golden hamster are not known. Nor is it known whether daidzin and

daidzein themselves are the pharmacologically active molecules that suppress ethanol intake directly or whether they act as prodrugs that are converted *in vivo* to the pharmacologically active species. Thus, in order to uncover the site(s) and mechanism(s) of action of daidzin and daidzein it will be important to learn the metabolic fates and identify the active species of these isoflavones. Results from these studies will not only allow a better understanding of the biochemical basis for the action of these drugs on free-choice ethanol intake in hamsters and thereby shed light on the mechanistic basis underlying alcohol abuse in humans, but they will also provide a rationale for the design of much needed, safe therapeutic agents for alcoholism/alcohol abuse.

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