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Disulfiram Causes Sustained Behavioral and Biochemical Effects in Rats

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RAHMAN, M. A., N. E. GRUNBERG, AND G. P. MUELLER. *Disulfiram causes sustained behavioral and biochemical effects in rats.* PHARMACOL BIOCHEM BEHAV **56**(3) 409–415, 1997.—The present experiment examined effects of disulfiram (Antabuse®) administration on behavioral measures of nociception (hot plate and tail flick), peripheral muscular performance (grip strength), motivated performance, balance, and coordination (rotorod) in 24 male Sprague–Dawley rats during and 2 wk after an eight-day administration of disulfiram. In addition, peptidylglycine α-hydroxylating monooxygenase (PHM) activity in several tissues and levels of α-amidated α-melanocyte stimulating hormone (α-MSH) in the neurointermediate lobe of the pituitary were assayed to evaluate biochemical effects of disulfiram. These particular assays were included because it has been reported that disulfiram affects α-amidated peptides via alteration of PHM activity. Decrements in all behavioral measures, except tail flick, occurred after one week of disulfiram administration. Decrements in grip strength continued for the 2 wk after cessation of disulfiram. Dose-related reductions in changes in PHM activity and levels of α-MSH were found 2 wk after cessation of disulfiram administration. The time course of the results suggest that changes in PHM activity may underlie decrements in grip strength. The present experiment provides a paradigm for further investigations of effects of α-amidated peptides on behavior.

Disulfiram Antabuse Hot plate Tail flick Grip strength Rotorod PHM α-MSH

DISULFIRAM (tetraethylthiuram disulfide or Antabuse®) is the most common pharmacological therapy for the treatment of alcoholism and is prescribed to approximately 200,000 patients per year in the United States (18). The effectiveness of disulfiram as an alcohol deterrent results from its ability to irreversibly inhibit the enzyme acetaldehyde dehydrogenase. This action interferes with the metabolism of ethanol, causing the accumulation of acetaldehyde, a noxious intermediate (28). When coupled with psychological and behavioral counseling, disulfiram can be an effective tool in the treatment of alcohol abuse (7). Because of its early use as a therapeutic agent (31), disulfiram has not undergone the rigorous preclinical and clinical evaluations that are required for drug approval today (13). Several case studies have reported peripheral neuropathy after disulfiram administration (1,2,8,20,21) and a rat study (29) reported that disulfiram can decrease body weight gain and damage cholinergic innervation of intestine. The behavioral effects of disulfiram, however, have not been fully investigated and the underlying mechanisms of action on behavior or on the nervous system have not been determined.

In addition to inhibiting acetaldehyde dehydrogenase, disulfiram chelates copper (Cu^{2+}) and thereby inhibits the activity of the Cu^{2+}-dependent enzyme, peptidylglycine-α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) (15). This action is important because PHM catalyzes the rate-limiting step in the α-amidation of many neural and endocrine peptides (4). In fact, more than half of all peptides used in intercellular communication are α-amidated, a modification essential for receptor recognition and biologic activity. Disulfiram has pronounced effects on peptide α-amidation in pituitary and other tissues (3,12,15–17). The responses are greatest in the neurointermediate lobe of the pituitary (NIL) where disulfiram treatment can virtually eliminate α-MSH and alter PHM activity for many days following cessation of one week treatment with a high dose (400 mg/kg). Therefore, disulfiram can be used

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as a tool to study the regulation of α-amidation and the functions of α-amidated peptides (15,22). Disulfiram may affect behavior through the actions on these and other neuroendocrine peptides.

Rats treated with disulfiram exhibit marked decreases in PHM activity in vivo based on decreases in tissue concentrations of α-amidated peptides. Under these conditions, however, PHM appears to undergo a covalent modification that increases its V_{max} (22). Whereas compensatory change is unable to restore levels of α-amidated peptides in vivo, the increased V_{max} is clearly evident when PHM is removed from the animal and placed under optimal conditions in a test tube. This effect may result either from the presence of the disulfiram in vivo or from its lasting effects that persist after the drug is metabolized. Interestingly, the changes in peptide levels and kinetic properties of PHM are sustained for several weeks following the cessation of disulfiram treatment. Therefore, any behavioral effects associated with the actions of disulfiram on α-amidation should show a similar time course of response.

The present experiment was designed to examine behavioral and biochemical effects of disulfiram in rats. The behavioral measures were: hot plate, tail flick, grip strength, and rotorod performance. Hot plate and tail flick were included as indices of nociception. It was hypothesized that disulfiram would decrease levels of α-amidated peptides and pain responses because neurotransmitters involved in pain responses (i.e., neuropeptide-Y [NPY] and substance P [SP]) are α-amidated. Grip strength and performance on rotorod were included because they are reliable and sensitive measures commonly used in behavioral toxicology studies and are, therefore, of value to evaluate the potential behavioral toxicity of disulfiram. It was hypothesized that both measures would be reduced by disulfiram. In addition, body weight was measured because of previous reports of effects of disulfiram (29) and as a gross index of health and possible changes in energy balance. Because α-amidated peptides (e.g., cholecystokinin octapeptide [CCK8] and NPY) are involved in feeding and satiety, it was hypothesized that disulfiram would decrease body weight. To assess disulfiram's biochemical effects, PHM activity was assayed in tissues where the enzyme is most abundant (atrium and pituitary) and where it has clearly defined roles in neural and endocrine communication (pituitary and hypothalamus) (4). Based on previous reports of disulfiram's effect on PHM (22), we hypothesized that disulfiram would increase PHM activity, as measured in vitro, and that this effect would persist following the cessation of administration. Levels of α-amidated α-MSH were assayed to determine whether a product of PHM was similarly affected by disulfiram administration. It was hypothesized that disulfiram administration would produce a sustained decrease in levels of α-MSH.

METHODS

Subjects

Adult male Sprague–Dawley (225–250 g) rats were obtained from Charles River Breeding Laboratories (Wilburn, MA) and housed, two per cage, in standard shoe box cages (44 cm × 23 cm × 20 cm) containing absorbent Pine-Dri wood chip floor covering. Cages were placed on four-shelved metal racks in a room maintained at 22°C and 50% relative humidity under a 12 h light/dark cycle (off at 0700). Standard rat chow pellets (Agway Prolab RMH 3500) and water were available continuously. Rats were assigned to three treatment groups based on baseline body weight and on rotorod training performance.

Drug Administration

Disulfiram was suspended with a Polytron homogenizer (Brinkman Instruments) in 0.9% NaCl containing 0.5% Tween 80. Rats received a single subcutaneous (s.c.) injection between the withers each day (between 0900 and 1000 h) of 1 ml/kg of either: vehicle, 50 mg disulfiram/kg, or 150 mg disulfiram/kg. The doses were based upon reports of the biochemical effects of disulfiram in rats (9,15,22) and on pilot studies.

Behavioral Measures

Behavioral measures included hot plate latency (Omnitech Hot Plate Analgesiometer), tail flick latency (Omnitech Model TF1), grip strength (Chatillon Grip Strength Meter), and rotorod performance (Omnitech). The hot plate and tail flick tests both assess nociception as judged by the latency to react to a thermal stimulus. Changes in tail flick latency are thought to reflect changes in a spinal reflex, whereas changes in hot plate latency are mediated by supraspinal processes (14), including sensory and motor cortical areas. Grip strength assesses muscular strength or weakness (19,27). The rotorod assesses motor coordination, balance, strength, and motivated performance (30).

Procedure

After an initial gentling period of three days, rats were trained on the rotorod task until more than 80% of the rats could perform the task without failure. Total time for each trial and time to maximum speed were maintained at 2 min and 1 minute, respectively, while rotation speed was gradually increased (5–8 rpm) for each training session until a maximum of 40 rpm was reached. After training (approximately one week), there was a 2-day rest period, and then drug administration began.

Behavioral assessments were made on the first day of drug or vehicle administration (day 1); on the last day of administration (day 8); and 3, 7, and 14 days after the cessation of drug administration (days 11, 16, and 23, respectively). These times were chosen to assess acute drug effects (day 1), cumulative drug effects (day 8), and duration of effects after cessation of drug administration (days 11, 16, and 23). All assessments were made during the animals' active (dark) cycle. Body weight was measured daily.

Following sacrifice by decapitation, anterior (AL) and neurointermediate lobes (NIL) of the pituitary, cardiac atrium, and hypothalamus were removed, frozen on dry ice and stored at −70°C until assayed. Specific tissues were selected on the basis of previous reports (22).

Assays

Sample Preparation and Protein Assay: Samples were prepared and assayed as described by Mueller et al. (22). Briefly, tissues were homogenized in 0.05 M 2-[*N*-morpholino] ethanesulfonic acid (MES, pH 6.0), 0.01 M mannitol, and 1 % Triton X 100 containing the protease inhibitors, leupeptin (2.0 μg/ml), benzamidine (16 μg/ml), lima bean trypsin inhibitor (10 μg/ml), and phenylmethylsulfonyl fluoride (15 μg/ml). The homogenates were processed through three freeze-thaw cycles, followed by centrifugation at 15,000 × *g* for 10 min at

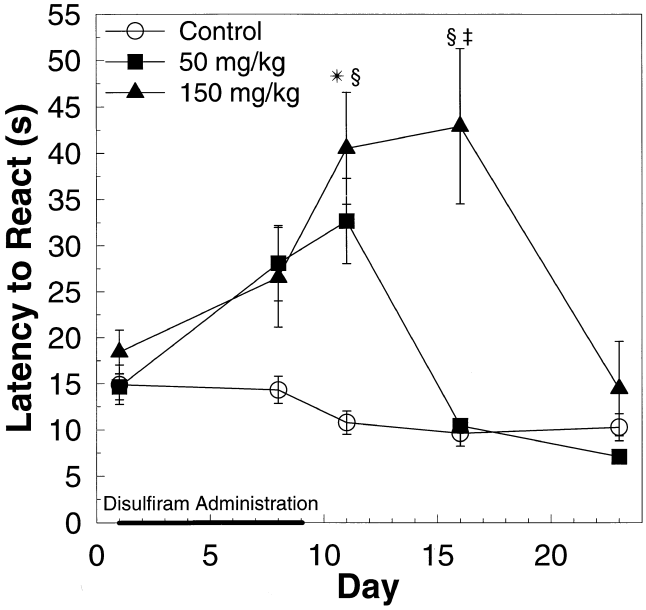

FIG. 1. Effects of disulfiram on hot plate latencies. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days (bold line). Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

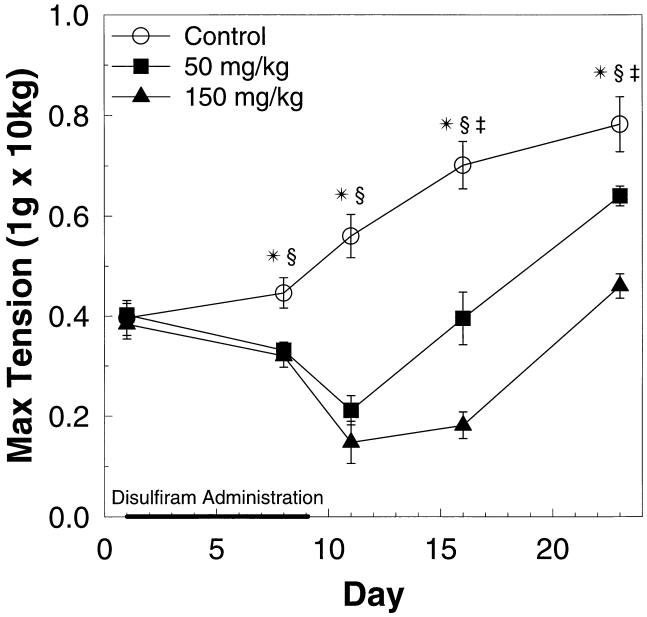

FIG. 2. Effects of disulfiram on hind limb grip strength. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days (bold line). Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

4°C to remove cellular debris. Protein content in supernatants was determined by BCA assay (Pierce Chemical Co., Rockford, IL). Aliquots of NIL supernatants were mixed 1:2 with 3 N acetic acid to prepare samples for determination of α-MSH.

PHM Activity

PHM activity was measured by radioenzymatic assay under optimal conditions of pH, copper, ascorbate, temperature and time (6,24,25). Samples were assayed at 0.5 or 1 μg protein with I^{125}-alpha-N-Ac-Tyr-Val-Gly (20,000–30,000 cpm), 0.5 μM alpha-N-Ac-Tyr-Val-Gly, 1 μM $CuSO_4$, 0.05 μM ascorbate, and 100 μg/ml catalase in 0.15 M MES (pH 5.0) for 45 min at 37°C (final reaction volume of 40 μl). Addition of 15 μl 1 N NaOH terminated the reaction and completed the conversion of hydroxylated intermediate, peptidyl-alpha-hydroxy-glycine, to the α-amidated product (4). Following neutralization of the enzyme reaction mixture (240 μl 1 M Tris, pH 7.0), the amidated peptide product was extracted into hydrated ethyl acetate (640 μl) and measured (320 μl) by gamma counting.

α-MSH Assay

α-MSH was measured by radioimmunoassay using the antiserum, H-50, that has an absolute requirement for a C-terminal α-amide and less than 1% cross-reactivity with α-MSH-free acid, or peptides unrelated to α-MSH. The antiserum is sensitive to less than 3 fmol of synthetic α-MSH at a final dilution of 1:60,000 (26).

Statistical Analysis

A repeated-measures MANOVA was performed on the behavioral dependent measures across all time points in the study. The MANOVA included drug (saline, 50 mg/kg/day, 150 mg/kg/day) as the between-subjects factor and time as the within-subject factor. Subsequent one-way ANOVAs were conducted at each time point to determine the effects of drug on individual dependent measures. Two-way ANOVAs were used to evaluate body weight and one-way ANOVAs were used to evaluate effects of disulfiram on the biochemical results. Post hoc tests (Tukey-HSD procedure) were used to determine which groups differed when significant differences were found by ANOVA. An alpha level of 0.05 was used to determine statistical significance and all tests used two-tailed distributions.

RESULTS

Behavioral Measures

Figure 1 presents hot plate task performance for each of the experimental groups throughout the experiment. Both the high and low doses of disulfiram caused significant increases in hot plate latencies on day 8 [$F(2, 21) = 3.55$, $p < 0.05$] and day 11 [$F(2, 21) = 12.02$, $p < 0.01$]. By one week following the cessation of disulfiram treatment (day 16), latencies in the 50 mg/kg group had returned to control values, whereas latencies in the 150 mg/kg group remained elevated [$F(2,21) = 14.92$, $p < 0.01$]. There were no differences between the groups by the second week following the cessation of treatment. Post-hoc analyses revealed significant differences between drug groups and control on day 11 and between the 150 mg/kg group and both the 50 mg/kg group and control on day 16. Unexpectedly, tail flick nociception latencies were not affected by the disulfiram treatments (data not presented).

Figures 2 and 3 present the effects of disulfiram administra-

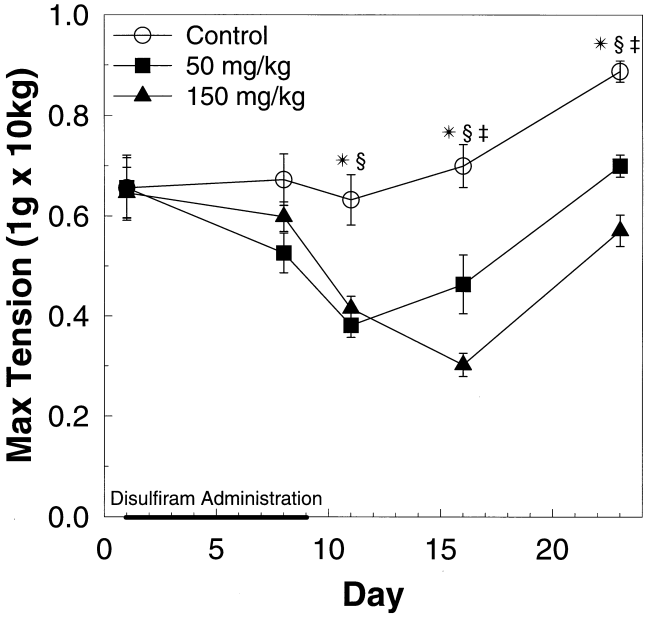

FIG. 3. Effects of disulfiram on fore limb grip strength. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days (bold line). Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

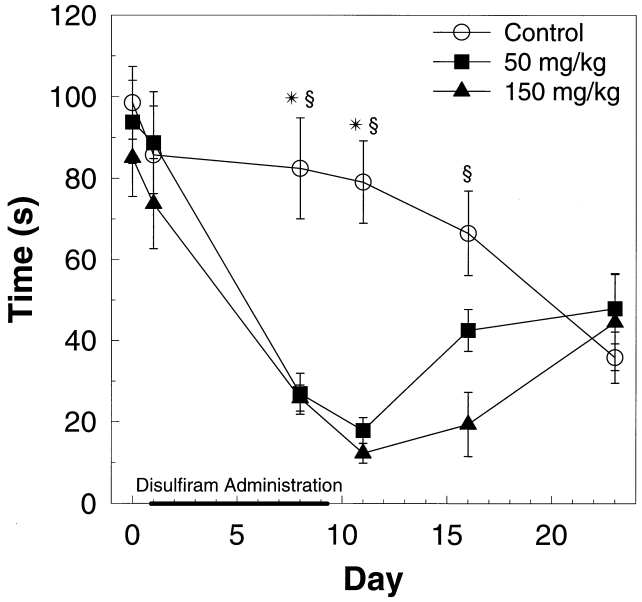

FIG. 4. Effects of disulfiram on moving rotorod task performance. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days (bold line). Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

tion on hind limb and fore limb grip strength, respectively, for each of the treatment groups throughout the experiment. Disulfiram treated animals showed decreased strength for both tasks on all test days after day 1, with a return towards control levels by the last test day. ANOVA revealed significance for hind limb grip strength on: day 8 [$F(2, 21) = 8.55$, $p < 0.0$]), day 11 [$F(2, 21) = 33.05$, $p < 0.01$], day 16 [$F(2, 21) = 56.93$, $p < 0.01$], and day 23 [$F(2, 21) = 19.81$, $p < 0.01$]. Post-hoc analyses revealed significant differences among both dose groups and control on day 8 and day 11, and between all groups on days 16 and 23. ANOVA revealed significance on fore limb grip strength on day 11 [$F(2, 21) = 15.10$, $p < 0.01$], day 16 [$F(2,21) = 20.54$, $p < 0.01$], and day 23 [$F(2, 21) = 39.70$, $p < 0.01$]. Post-hoc analyses revealed significant differences between drug groups and control on day 11, and among all groups on day 16 and day 23.

Figure 4 presents moving rotorod task performance for each of the treatment groups throughout the experiment. Decrements in performance were evident on the day after the termination of disulfiram treatment (day 8) and throughout the two-week recovery period. Maximal decreases were observed at three and seven days following the cessation of treatment. The differences were significant on day 8 [$F(2, 21) = 16.66$, $p < 0.01$] , 11 [$F(2, 21) = 34.70$, $p < 0.01$], and 16 [$F(2, 21) = 8.44$, $p < 0.01$]. Post-hoc analyses revealed significant differences between the control and both drug groups on days 8 and 11, and between the 150 mg/kg group and the control group on day 16. There were no significant differences found on the still rotorod task performance (data not shown).

Body Weight

Figure 5 presents body weight for each experimental group throughout the experiment. Body weights among the groups

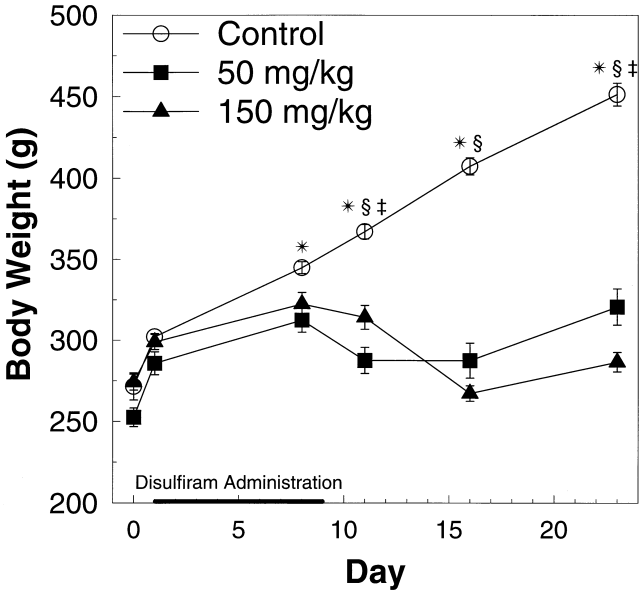

FIG. 5. Effects of disulfiram on body weight. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days (bold line). Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

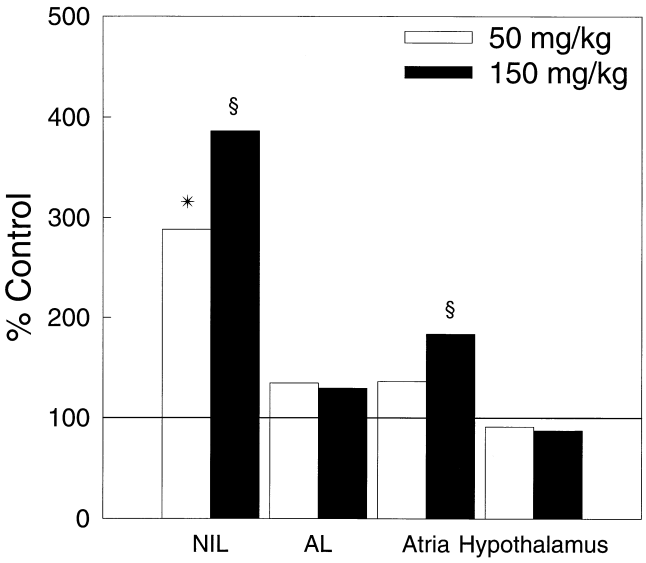

FIG. 6. Effects of disulfiram on PHM activity in NIL, AL, atrium, and hypothalamus 2 wk after cessation of disulfiram administration. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days. Mean data for the tissue samples for each experimental group ($n = 8$ for each group) are expressed as percent of control. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

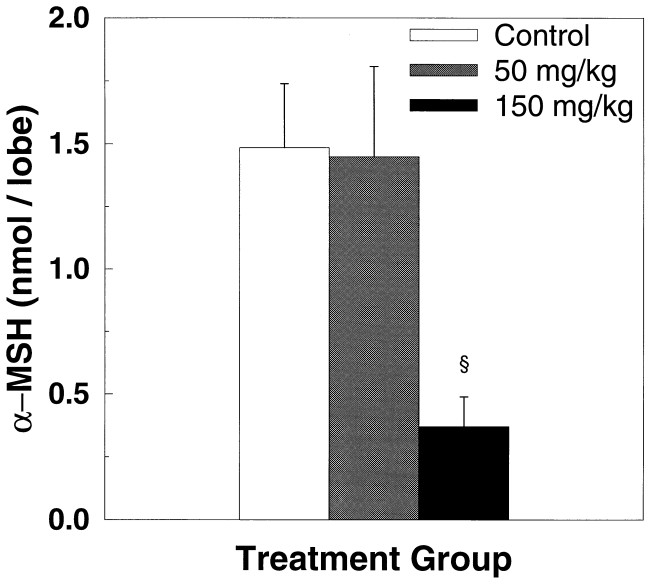

FIG. 7. Effects of disulfiram on α-MSH levels in NIL 2 wk after cessation of disulfiram administration. Disulfiram was administered s.c. daily at 0, 50, or 150 mg/kg in 0.1 ml/kg volume for seven days. Data represent group means ($n = 8$ for each group) ± SEM. * = significant difference between 0 and 50 mg/kg groups ($p < 0.05$); § = significant difference between 0 and 150 mg/kg groups ($p < 0.05$); ‡ = significant difference between 50 and 150 mg/kg groups ($p < 0.05$).

were indistinguishable before and on the first day of drug administration. On the eighth day of administration and throughout the two-week post-administration period, body weights of disulfiram-treated rats were lower than control values. An overall repeated-measures ANOVA revealed a significant main effect for drug [$F(2, 20) = 57.70$, $p < .01$], a significant effect for time [$F(4, 84) = 75.61$, $p < .01$], and an interaction for drug by time [$F(8, 84) = 87.53$, $p < .01$]. Body weights of the three experimental groups were compared for each test day using baseline weight as a covariate and revealed significant differences on day 8 [$F(2, 21) = 30.76$, $p < .01$], day 11 [$F(2, 21) = 34.16$, $p < .01$], day 16 [$F(2, 21) = 98.82$, $p < .01$], and day 23 [$F(2, 21) = 107.29$, $p < .01$].

Biochemical Measures

Figure 6 presents the effects of disulfiram administration on tissue PHM activity 2 wk after cessation of treatment. The sustained effects of previous disulfiram administration on PHM activity were most pronounced in the NIL [$F(2, 21) = 26.20$, $p < .01$]. Activities were increased to roughly 300% and 400% of control values in the 50 mg/kg and the 150 mg/kg groups, respectively. PHM activity in the atrium after the two-week cessation period was significantly affected by disulfiram [$F(2, 21) = 6.78$, $p < .01$]. Post hoc analyses revealed that the control and the 150 mg/kg group differed significantly. In the hypothalamus and AL, mean values for PHM activity were similar across experimental groups. The finding of no difference in PHM activity in some tissues was expected in light of the length of the cessation period (2 wk). The sustained biochemical effects of disulfiram treatment noted in the NIL and atrium are consistent with earlier observations (22,23).

Figure 7 presents levels of α-MSH in NIL for each of the experimental groups 2 wk following the cessation of treatment. ANOVA revealed a significant effect [$F(2, 21) = 14.65$, $p < 0.01$], and post-hoc analyses revealed significant differences between the control group and the 150 mg/kg group with α-MSH levels roughly 30% of controls. In addition, the 150 mg/kg and the 50 mg/kg groups differed significantly.

DISCUSSION

The purpose of this experiment was to examine effects of disulfiram on several behaviors along with the evaluation of PHM activity and peptide α-amidation. Disulfiram impaired supraspinal nociceptive response (hot plate), muscular strength, and motor performance, and decreased body weight. Disulfiram also inhibited α-amidation and evoked compensatory changes in the kinetic properties of the rate limiting enzyme involved, PHM. In atrium and NIL, the biochemical and behavioral responses were sustained for the entire 2 wk following the cessation of administration of disulfiram. The dose and time relations reported here suggest that disturbances in peptide α-amidation may contribute to the effects of disulfiram on behavior. This effect is most clearly evident in the NIL where dose-related changes in α-amidation and PHM activity are most pronounced and prolonged. The PHM activity (see Fig. 6) in NIL is consistent with the behavioral effects, whereas the α-MSH levels (see Fig. 7) are consistent with the behavioral effects in the 150 mg/kg disulfiram group. The present biochemical and behavioral findings also are consistent with the report by Marchand et al. (16) that disulfiram reduces levels of α-amidated Substance P and nociception in mice, and with the report by Mueller et al. (22) regarding sustained effects of disulfiram on PHM. Future studies are needed to determine whether these correlations indicate underlying causality.

Because disulfiram undergoes rapid metabolism in vivo

(see 5), the pharmacologic basis for the sustained effects of disulfiram treatment remains to be determined. Hart and Faiman (10,11) recently reported that disulfiram is metabolized to highly potent inhibitors of aldehyde dehydrogenase (diethyldithiocarbamate [DDC], dieithyldithiomethylcarbamate [Me-DDC], and diethyldithocarbamate-methyl ester [DTC-ME]). Although effects of disulfiram metabolites on peptide α-amidation have been reported (15,23), the effects of these metabolites on behavior have not. Because these metabolites of disulfiram form complexes with copper and because these metabolites also are inhibitors of aldehyde dehydrogenase, it is possible that they play a role in the behavioral and biochemical effects of disulfiram administration. This possibility deserves further research attention.

In the present experiment, PHM activity was affected 2 wk after cessation of both dosages of disulfiram and grip strength also continued to show significant effects of disulfiram at this time point. It is noteworthy that the effects of disulfiram on body weight (see Fig. 5) may contribute to the effects of disulfiram on grip strength because both parameters were affected similarly over time. The prolonged effects of disulfiram on α-amidated peptides is consistent with other reports (15,22,23) . Therefore, it appears likely that effects of disulfiram on PHM activity and α-amidation may underlie effects of disulfiram on peripheral strength or peripheral neuropathy. The fact that some behavioral responses (specifically, hot plate and rotorod) are similar to controls at a time when PHM activity remains altered may indicate a habituation to a related underlying biochemical action or may indicate two separate actions of disulfiram that do not relate to each other. Levels of α-MSH in the NIL, assayed 2 wk after cessation of administration, were significantly lower for the 150 mg/kg group but not for the 50 mg/kg group. These effects of disulfiram on α-MSH may indicate different time courses of recovery for each dose or it may indicate that the higher dosages is necessary to trigger a response. Future investigations should evaluate other α-amidated peptides (e.g. SP, NPY, CCK-8) in conjunction with relevant behaviors (e.g., pain, feeding).

In light of the present findings of marked and prolonged biochemical and behavioral effects of disulfiram, careful, empirical evaluation in normal, nonalcoholic human subjects is warranted. It is important to determine which effects presently attributed to alcoholism and alcohol withdrawal may actually be a result of effects of disulfiram treatment per se. A better understanding of what the beneficial and untoward effects of disulfiram will enable us to better discern what effects help with alcoholism treatment and what effects may be confused with alcoholism.

ACKNOWLEDGEMENTS

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