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The Benzodiazepine Receptor Inverse Agonist RO15-4513 Exacerbates, but Does Not Precipitate, Ethanol Withdrawal in Mice

HOWARD C. BECKER[1] AND RAYMOND F. ANTON

Veterans Administration Medical Center
and Department of Psychiatry and Behavioral Sciences
Medical University of South Carolina, Charleston, SC 29403

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BECKER, H. C. AND R. F. ANTON. *The benzodiazepine receptor inverse agonist RO15-4513 exacerbates, but does not precipitate, ethanol withdrawal in mice.* PHARMACOL BIOCHEM BEHAV 32(1) 163–167, 1989.—RO15-4513, an imidazobenzodiazepine that has been reported to antagonize several behavioral and biochemical actions of ethanol, was given to C3H mice at various times during withdrawal from chronic (72 hours) continuous exposure to ethanol vapor. When administered immediately following chronic ethanol exposure, RO15-4513 (6 or 12 mg/kg) did not influence the withdrawal response. However, when given at subsequent times (3, 5, and 8 hours postethanol withdrawal), RO15-4513 significantly increased the severity of the withdrawal response in ethanol-exposed mice. Moreover, this exacerbation was completely reversed by pretreatment with the benzodiazepine receptor antagonist RO15-1788. Thus, these data indicate that the benzodiazepine inverse agonist, RO15-4513, is capable of exacerbating, but not precipitating, ethanol withdrawal.

RO15-4513 Ethanol withdrawal Benzodiazepine inverse agonist

RO15-4513, a structural analogue of the imidazobenzodiazepine RO15-1788, binds with high affinity to central benzodiazepine (BDZ) receptors (20,27). Recently, RO15-4513 has been reported to antagonize some of the behavioral and biochemical actions of ethanol (EtOH). For example, the compound has been found to attenuate the anxiolytic, anticonvulsant, motor incoordinating, and sedative actions of EtOH (1, 3–5, 12–14, 21, 23, 28). RO15-4513 has been also reported to suppress oral self-administration of EtOH (25) and antagonize the discriminative stimulus properties of EtOH (24), but not influence EtOH-induced hypothermia (12) or the low-dose stimulatory effects of EtOH (1). It has been suggested that the EtOH-antagonistic property of RO15-4513 may be related to its ability to specifically antagonize EtOH's effects on chloride transport at the GABA/BDZ-chloride channel receptor complex (28). However, when administered alone, RO15-4513 presents with a profile characteristic of a BDZ inverse agonist (3, 15, 18, 21). Therefore, it is not clear whether the EtOH-antagonistic action of RO15-4513 is related to this intrinsic activity of the compound.

In a recent report, RO15-4513 was found to exacerbate seizure incidence in mice undergoing EtOH withdrawal (16), an effect attributed to the proconvulsant intrinsic activity of the compound at central BDZ receptors. However, the exacerbation of EtOH withdrawal by RO15-4513 was observed at only one time point (6.5 hr postEtOH withdrawal) and in DBA/2 mice, a strain that is relatively seizure-prone to a variety of pharmacologic treatments (7, 10, 26). Thus, it is not clear whether this effect is strain-specific and whether the exacerbation of EtOH withdrawal by RO15-4513 varies as a function of time following EtOH withdrawal. The present study was designed to address these two questions by using a different mouse strain (C3H/He) and administration of RO15-4513 at various time points following EtOH withdrawal.

METHOD

Ethanol Administration

Adult, male C3H/He mice, initially weighing 27–30 g, were used as subjects. The procedure for chronically administering EtOH by the inhalation route has been described in detail elsewhere (2). Briefly, the animals were housed in triplets or pairs in polypropylene cages with stainless steel wire lids under a 12-hr light/dark cycle (light onset at 0600 hr). One group of mice was continuously exposed to EtOH vapor for 72 hours in an inhalation chamber. Ethanol (95%) was volatilized and delivered to the chamber at a rate of 90.4 μl/min. Air flow to the chambers was maintained at 6 l/min. The remaining control mice were housed in an identical chamber for the same period of time, but in the absence of EtOH.

Ethanol intoxication was initiated by an IP injection of 1.6

[1]Requests for reprints should be addressed to H. C. Becker, Research Service, VA Medical Center, 109 Bee Street, Charleston, SC 29403.

g/kg EtOH and blood alcohol levels (BAL) were stabilized by daily injections of the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg). Controls received an initial loading dose of saline rather than EtOH and daily pyrazole injections. Pyrazole was not administered on the day of EtOH withdrawal.

Mice in the EtOH chamber were given free access to food and water, while controls were maintained on a restricted feeding and water regimen in order to control for body weight loss incurred by EtOH-treated mice. In addition, the chambers were maintained at 26°C to keep the animals close to normal physiologic body temperature during exposure to EtOH vapor. On each day, body weight and body (rectal) temperature were recorded.

At the end of the 72-hour EtOH exposure period, prior to removing the mice from the chambers, an air sample (1 ml) was collected through a port in the EtOH chamber wall for subsequent determination of the air EtOH concentration, as described elsewhere (2). Immediately after removing the mice from the EtOH chamber, blood samples were collected from the orbital sinus for subsequent blood alcohol analysis. Following blood collection, all mice were individually housed and coded.

Withdrawal Testing

In Experiment 1, withdrawal behavior was scored at 1, 3, 5, 7, 8, and 9 hours following EtOH withdrawal. Five minutes prior to withdrawal scoring at hours 5 and 8, half the EtOH-treated mice (N=9) and half the controls (N=7) were given an IP injection of 6 mg/kg RO15-4513. The remaining animals received the vehicle. Withdrawal behavior was observed and recorded at hour 7, but no drugs were administered. At 9 hours postEtOH withdrawal, EtOH-treated mice that received the vehicle at hours 5 and 8 were further divided into two treatment groups. Five minutes prior to withdrawal scoring, five of these mice were given an IP injection of the benzodiazepine receptor antagonist RO15-1788 (6 mg/kg) immediately followed by an IP injection of 6 mg/kg RO15-4513. The remaining four animals were given a single injection of 6 mg/kg RO15-4513 alone.

In Experiment 2, withdrawal scoring was conducted at hours 0, 1, 3, 5, and 8 hours postEtOH exposure. In order to avoid the blood collection procedure from influencing withdrawal behavior observed at hour 0, the mice were scored for withdrawal prior to blood sampling. At each of the time points, one-third of the EtOH-treated mice (N=6–7) and one-third of the controls (N=7) were given an IP injection of either 0, 6, or 12 mg/kg RO15-4513 five minutes prior to withdrawal scoring. At 9 hours following EtOH withdrawal, EtOH-treated mice that received the vehicle were further divided into two drug treatment groups. Half of these animals (N=3) were given an IP injection of RO15-1788 (6 mg/kg) immediately followed by an injection of RO15-4513 (6 mg/kg) while the remaining mice received RO15-4513 (6 mg/kg) treatment alone.

The withdrawal scoring procedure was modified after that described by Goldstein and Pal (11). Subjects were observed for withdrawal by an observer "blind" to the animals' drug history. Briefly, at the specified time points, mice were scored on a scale of 0 to 2 for ambulatory behavior and convulsions (spontaneous as well as handling- or audiogenic-induced seizure). Ambulatory behavior was scored as follows: (0=normal exploratory movements; 1=stationary/pivoting; 2=backing movements). Handling-induced convulsions were assessed by lifting each mouse by the tail and twirling the subject 360°; audiogenic-induced seizures were assessed by jingling keys over the subject's cage top. Seizure susceptibility was scored as follows: (handling-induced convulsions—0=minimal response; 1=seizure after twirl; 2=seizure prior to handling or twirl; audiogenic induced seizures—0=minimal response; 1=run/jump; 2=seizure). Scores for each of these behaviors were summed for a total score, which was then used to compute a group mean for each time point.

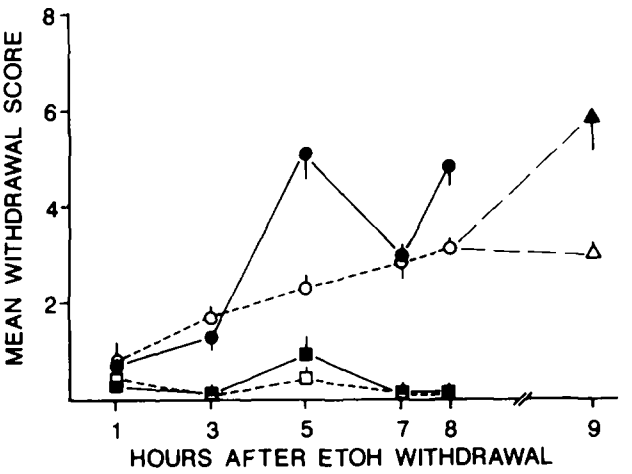

FIG. 1. Mean (±SE) withdrawal scores for EtOH-exposed mice and controls as a function of RO15-4513 treatment (0 vs. 6 mg/kg) and time following EtOH withdrawal. (○) EtOH/Vehicle (N=9); (●) EtOH/RO15-4513 (N=9); (□) Control/Vehicle (N=7); (■) Control/RO15-4513 (N=7). RO15-4513 (6 mg/kg) was injected IP at hours 5 and 8 during EtOH withdrawal. At 9 hours following EtOH withdrawal: (▲) 6 mg/kg RO15-4513; (△) 6 mg/kg RO15-4513 + 6 mg/kg RO15-1788.

Drugs

RO15-4513 and RO15-1788 (donated by Hoffmann-La Roche, Basel, Switzerland) were suspended in distilled water with the addition of a drop of Tween 20 per 10 ml. Pyrazole (Sigma Chemical, St. Louis) was dissolved in saline. All injections were given IP and administered in a volume of 0.02 ml/g.

Statistics

Withdrawal data were analyzed by analysis of variance (ANOVA), with time of withdrawal scoring treated as a repeated measures variable. During the course of withdrawal, some of the animals expired (typically due to audiogenic-induced tonic/clonic seizures). These animals were given a total score of 7 for that time point. However, in order to keep the analyses balanced, these subjects were assigned the group mean at subsequent time points. The two treatment groups tested at hour 9 were analyzed by a *t*-test and nominal data (seizure incidence) were analyzed by Fisher's exact probability test.

RESULTS

Experiment 1

After 72 hours of continuous EtOH exposure through in-

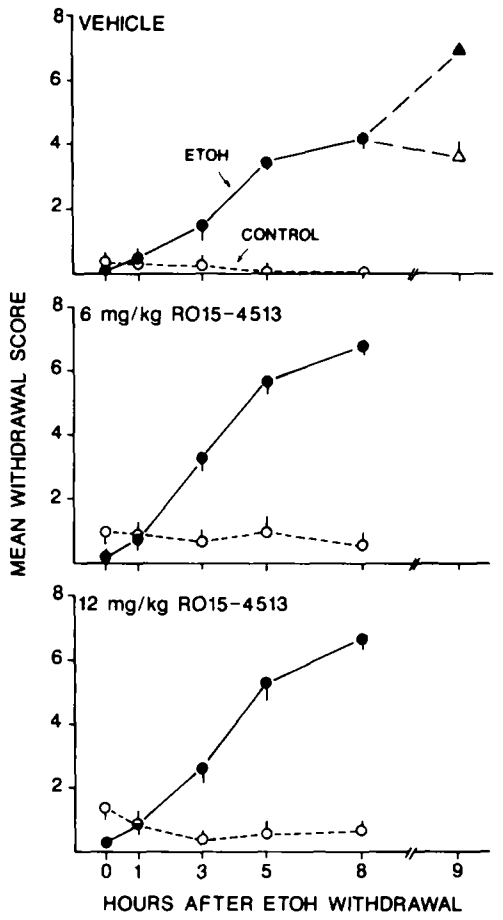

FIG. 2. Mean (±SE) withdrawal scores for EtOH-exposed mice (N=6–7/group) and controls (N=7/group) as a function of RO15-4513 treatment (0 vs. 6 vs. 12 mg/kg) and time following EtOH withdrawal. RO15-4513 was injected IP at 0, 1, 3, 5, and 8 hours following chronic EtOH exposure. At 9 hours following EtOH withdrawal: (▲) 6 mg/kg RO15-4513; (△) 6 mg/kg RO15-4513 + 6 mg/kg RO15-1788.

halation, the mean BAL (±S.E.) was 142.6±11.8 mg% (N=18). Collapsed over four sampling times (at 0, 24, 48, and 72 hours), the mean EtOH concentration in the chamber was 11.25±0.28 mg/l air.

As shown in Fig. 1, physical dependence (denoted by the presence of withdrawal symptomology) was achieved in mice continuously exposed to EtOH vapor for 72 hours, $F(1,28)=162.89$, $p<0.01$. In addition, the EtOH-treated mice exhibited a greater number of withdrawal signs and with greater intensity as time elapsed since their last exposure to EtOH [EtOH × Time interaction: $F(4,112)=27.35$, $p<0.01$]. Moreover, it is clear that treatment with RO15-4513 (6 mg/kg) just prior to hours 5 and 8 exacerbated EtOH withdrawal. This was supported by a significant EtOH × RO15-4513 × Time interaction, $F(4,112)=4.78$, $p<0.01$. Decomposition of this term with Fisher's Least Significant Difference (LSD) test revealed that withdrawal scores for EtOH-exposed mice that received RO15-4513 treatment were significantly greater than that for mice given the vehicle at 5 and 8 hours following EtOH withdrawal ($p<0.01$). In fact, the incidence of audiogenic-induced seizures was significantly greater in mice undergoing EtOH withdrawal if they were treated with RO15-4513 than if they received the vehicle ($p<0.025$). In contrast, none of the controls evidenced audiogenic-induced seizures. Interestingly, while the EtOH-exposed mice could be easily distinguished from controls at hour 7 (with regard to withdrawal-related behavior), the EtOH/RO15-4513 and EtOH/Vehicle groups did not significantly differ from each other (RO15-4513 was not administered at this time point).

Finally, at 9 hours following EtOH withdrawal, increased withdrawal severity in EtOH-exposed mice that received RO15-4513 was markedly attenuated if the animals were pretreated with the benzodiazepine receptor antagonist RO15-1788 (6 mg/kg), $t(7)=3.11$, $p<0.01$. More specifically, two of the four EtOH-exposed mice that received RO15-4513 alone died as a result of a seizure, whereas none of the five EtOH-exposed mice that received the combination of RO15-1788 and RO15-4513 exhibited a seizure. These data may be taken as evidence that the observed exacerbation of EtOH withdrawal by RO15-4513 is due to an interaction of the compound at the benzodiazepine receptor complex.

Experiment 2

Mean (±S.E.) BAL after 72 hours of continuous exposure to EtOH vapor was 179.7±6.0 mg% (N=19). Collapsed over four sampling times, the mean EtOH concentration of the chamber was 15.16±0.25 mg/l air.

Withdrawal data from this experiment are illustrated in Fig. 2. As in the previous experiment, physical dependence was observed in mice following 72 hours of continuous exposure to EtOH vapor, $F(1,35)=119.53$, $p<0.01$, and the severity of the withdrawal syndrome increased with time, $F(4,140)=145.60$, $p<0.01$. In addition, withdrawal severity significantly varied as a function of RO15-4513 treatment, $F(8,140)=2.97$, $p<0.01$. However, decomposition of this three-way interaction term revealed that RO15-4513 did not influence withdrawal behavior when administered immediately following removal from the inhalation chamber (hour 0). Thus, there was *no* statistical indication that RO15-4513 (6 or 12 mg/kg) precipitated EtOH withdrawal. In fact, at hour 0 control mice (not exposed to EtOH) that received 12 mg/kg RO15-4513 evidenced greater withdrawal scores than EtOH-treated mice that received 12 mg/kg RO15-4513 ($p<0.05$).

At subsequent time points, however, RO15-4513 did significantly influence EtOH withdrawal behavior. Similar to the results obtained in Experiment 1, withdrawal severity was exacerbated in mice that received 6 or 12 mg/kg RO15-4513 (in comparison to vehicle-injected mice) at 3, 5, and 8 hours postEtOH exposure ($p<0.01$). The two RO15-4513 dosage groups did not significantly differ from each other. In addition, neither dose of RO15-4513 significantly influenced withdrawal-like behavior in controls.

The incidence of seizures in the different treatment groups over the 8-hour withdrawal test period are presented in Table 1. As in the previous experiment, the frequency of audiogenic-induced seizures was significantly greater in EtOH-exposed mice that received either 6 or 12 mg/kg RO15-4513 than in vehicle-injected mice undergoing EtOH withdrawal ($p<0.005$). The two doses of RO15-4513 were equally efficacious in this regard, and neither dose of RO15-4513 significantly influenced seizure incidence in controls.

At 9 hours following EtOH withdrawal, pretreatment with

TABLE 1

THE NUMBER OF MICE HAVING AT LEAST ONE AUDIOGENIC-INDUCED SEIZURE DURING THE 8-HOUR ETHANOL WITHDRAWAL PERIOD

Group	No. With Seizure/ Group Size	No. Dead
ETOH/ Vehicle	0/6	0
ETOH/ RO15-4513 (6 mg/kg)	6/6*	5
ETOH/ RO15-4513 (12 mg/kg)	7/7*	5
Control/ Vehicle	0/7	0
Control/ RO15-4513 (6 mg/kg)	1/7	0
Control/ RO15-4513 (12 mg/kg)	1/7	0

*Significantly differs from EtOH/Vehicle group ($p<0.005$).

RO15-1788 (6 mg/kg) significantly attenuated the augmented withdrawal response observed in a separate group of mice that received RO15-4513 (6 mg/kg) alone, $t(4)=5.00$, $p<0.01$. These results parallel those obtained in Experiment 1.

DISCUSSION

The results of the present study demonstrated that RO15-4513 (6 and 12 mg/kg) increased the severity of EtOH withdrawal in C3H mice when administered at various time points following chronic exposure to EtOH vapor. As such, these results confirm a recent report (16) in which DBA/2 mice were employed [a strain of mice that are typically seizure-prone (7, 10, 26)], and extend these findings to the C3H mouse strain.

In Experiment 1, at 7 hours postEtOH exposure (when no drug was administered), mice that had received RO15-4513 treatment at hour 5 did not differ from those that received vehicle. This is in agreement with the relatively short half-life of the compound (17).

While RO15-4513 increased seizure incidence in mice undergoing EtOH withdrawal, the drug had only a slight tendency (nonsignificant) to increase the incidence of seizures in control mice (Table 1). This was true for both the 6 and 12 mg/kg doses. Although RO15-4513 has been reported to produce convulsions in primates (19) and limbic seizure activity (without overt behavioral effects) in rats (6), the results of the present study are in general agreement with other reports which indicate RO15-4513 is not convulsant in mice (3,17). Rather, as a partial BDZ inverse agonist, it is a proconvulsant, lowering the seizure threshold to other chemical convulsants (3,17). Data from this study and a previous report (16) indicate this intrinsic proconvulsant activity of RO15-4513 can be also observed in animals that are undergoing EtOH withdrawal.

The results from Experiment 2 indicated that exacerbation of EtOH withdrawal by RO15-4513 depended on when the drug was administered during the abstinence period (Fig. 2). At the doses employed, there was no evidence that RO15-4513 is capable of precipitating EtOH withdrawal. In fact, withdrawal-like behavior was significantly *lower* in EtOH-exposed mice than controls that received the highest dose of RO15-4513 (12 mg/kg). This suggests that at a time point when BAL is at its peak (hour 0), EtOH (an anticonvulsant itself) may be antagonizing any tendency of RO15-4513 to induce seizures (or withdrawal-like behavior).

In contrast, the proconvulsant activity of RO15-4513 is "unmasked" (as indicated by exacerbation of withdrawal behavior) at later time points during EtOH withdrawal, when the BAL is substantially lower. In fact, at 5 hours postEtOH exposure, when exacerbation of withdrawal by RO15-4513 was clearly evident, BAL were approximately 30 mg% (determined from five mice immediately following seizure-induced death). Thus, it appears that exacerbation of EtOH withdrawal by RO15-4513 occurs only later in the abstinence period, when BAL have substantially fallen and seizure threshold is generally lower [e.g., (8, 9, 22)]. This further supports the notion that the effects of RO15-4513 on EtOH withdrawal are related to its BDZ inverse agonist properties.

In both experiments, the exacerbating effect of 6 mg/kg RO15-4513 on EtOH withdrawal behavior at 9 hours post-EtOH exposure was completely reversed by pretreatment with the BDZ receptor antagonist RO15-1788. This suggests that this action of RO15-4513 is due to an interaction of the compound at central BDZ binding sites.

In summary, these data indicate that RO15-4513 does not precipitate withdrawal when administered concurrent with cessation of chronic EtOH treatment. However, when given at times following EtOH withdrawal when BAL are substantially lower, RO15-4513 exacerbates the severity of the withdrawal syndrome in C3H mice. This effect occurred at doses of RO15-4513 that did not produce seizures in controls. Therefore, these results underscore an important undesirable consequence of administering compounds with even partial BDZ inverse agonist properties to animals undergoing EtOH withdrawal.

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