Pharmacology Biochemistry & Behavior, Vol. 24, pp. 93–98, 1986. © Ankho International Inc. Printed in the U.S.A. 0091-3057/86 $3.00 + .00

Tolerance to and Cross-Tolerance Among Ethanol, Pentobarbital and Chlordiazepoxide

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Received 6 March 1985

LÊ, A. D., J. M. KHANNA, H. KALANT AND F. GROSSI. *Tolerance to and cross-tolerance among ethanol, pentobarbital and chlordiazepoxide.* PHARMACOL BIOCHEM BEHAV **24**(1) 93–98, 1986.—The acute administration of ethanol, pentobarbital and chlordiazepoxide impaired, in a dose-dependent manner, the performance of rats on the moving-belt and two-way shuttle-box avoidance tests. Administration of these drugs for three weeks resulted in tolerance to their motor-impairing effects. Tolerance to ethanol or pentobarbital was characterized by a parallel shift of the dose-response curve to the right. Tolerance to chlordiazepoxide, however, was of greater extent and was accompanied by an apparent flattening of the dose-response curve. Symmetrical cross-tolerance developed between ethanol and pentobarbital. On the other hand, while chlordiazepoxide treatment conferred full cross-tolerance to ethanol and pentobarbital, only partial cross-tolerance to chlordiazepoxide was observed following treatment with ethanol or pentobarbital. These results suggest that at least part of the tolerance to chlordiazepoxide depends on changes in specific benzodiazepine receptors and is independent of the tolerance associated with non-specific changes in the cell membrane.

Tolerance Ethanol Pentobarbital Chlordiazepoxide

THE acute administration of benzodiazepines produces a general depressant effect on the central nervous system [13], and tolerance to this effect has been reported to occur following chronic administration [14,34]. Clinical observations have indicated that the drowsiness occurring during the initiation of diazepam treatment disappears after a few days of chronic treatment, despite a higher blood level of diazepam and its metabolite as a consequence of accumulation in the body [14]. Animal studies have revealed a similar phenomenon. In a conflict behavior paradigm which employs the unpunished bar-pressing response as the dependent measure for the depressant effect, tolerance to a variety of benzodiazepines has been demonstrated following chronic treatment [3, 27, 29, 40]. In addition, tolerance has also been shown to occur to the depression of locomotor activity by these drugs [6, 7, 11] and to their ataxic effect [4, 35, 36]. Despite these demonstrations of tolerance to the depressant effect of benzodiazepines, it is still difficult to draw any general conclusion regarding the nature or extent of the tolerance, since limited doses and test systems were employed in the studies cited.

Besides their use in the treatment of anxiety, benzodiazepines, particularly diazepam and chlordiazepoxide, have been used quite extensively in the management of ethanol withdrawal reactions [39,46] and are known to be co-abused with other drugs [34]. Nevertheless, it is clear that benzodiazepines differ from ethanol and other central depressants with respect to their spectra of effects on human psychomotor performance [45]. While clinical observations have suggested a potential cross-tolerance among the benzodiazepines [46], few experimental studies have been done to assess the development of cross-tolerance between benzodiazepines and other central depressants.Changes in benzodiazepine receptor binding have been observed following chronic ethanol or barbiturate administration, but it was not actually demonstrated that cross-tolerance to benzodazepines existed after such treatment [9, 21, 30]. Recent work by Rosenberg *et al.* [36] has shown that while chronic treatment with flurazepam produced a high degree of tolerance to diazepam-induced ataxia, little or no cross-tolerance to ethanol and pentobarbital was observed. The possibility of cross-tolerance to benzodiazepines following ethanol or pentobarbital treatment was not examined.

In the present study, we report the development of tolerance to ethanol, pentobarbital and chlordiazepoxide, and cross-tolerance between them, as measured by changes in dose-response (D-R) curve for each drug, on two different test systems: shuttle box avoidance [1,44] and the moving belt [25].

METHOD

Male Wistar rats weighing 200–220 g were purchased from Charles River (Montreal, Quebec). They were housed

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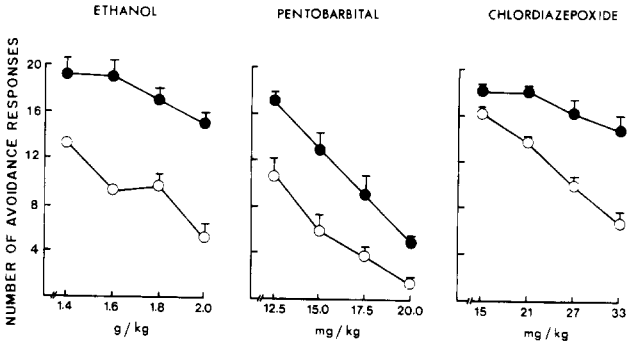

FIG. 1. Two-way shuttle-box avoidance response to various doses of ethanol, pentobarbital and chlordiazepoxide before and after chronic treatment with these drugs. Open symbols are values obtained before, and closed symbols after, chronic treatment. N=12 at each point. Vertical bar represents positive half of S.E.M.

singly and fed a standard rat chow diet. The weight of each animal was held at approximately 320 g by appropriate restriction of the daily ration of chow. Tap water was available ad lib. Ambient temperature was maintained at 21±1°C, and lights were on from 7 a.m. to 7 p.m. daily throughout the whole experiment.

Experiment 1: Shuttle-Box Avoidance Study

The apparatus employed in this study was a Lehigh Valley Electronics Model No. 146-04 toggle floor shuttle-box which was described in detail previously by Stiglick and Kalant [44]. In this test, rats were trained to avoid the unconditional stimulus (US) of an electrical foot shock (0.6 mA), delivered through the grid floor, by crossing to the other compartment. The conditional stimulus (CS) was a compound stimulus consisting of a light and a tone presented together. The light stimulus was the onset of the cue light on the side of the apparatus occupied by the subject at the start of the interval. The tone stimulus was generated from a point directly over the centre of the apparatus by a Mallory Sonalert (standard on the Lehigh Valley shuttle box), adjusted to a sound intensity of 70 dB at 2000 Hz. The CS-UCS interval was 7.0 sec. Both stayed on until the animal crossed to the other side of the apparatus, and then terminated simultaneously. An avoidance response occurred whenever the rat moved to the safe compartment during the CS-UCS interval, i.e., before the footshock was delivered. Each training session consisted of 20 trials with an inter-trial interval of 30 sec. After 10 training sessions, 36 rats which had reached a criterion of 95% correct responses (19 out of 20 trials) were chosen for the study.

Rats were randomly divided into 3 groups of 12 each and were designated for acute testing and subsequent chronic treatment with chlordiazepoxide, ethanol or pentobarbital. A D-R curve for each drug was generated before and after chronic treatment with one of the drugs. Each animal in a group received each of the 4 doses of the drug examined, in a Latin square design, to generate a D-R curve with n=12 per point. The test doses for ethanol were 1.4, 1.6, 1.8 and 2 g/kg; for pentobarbital: 12.5, 15, 17.5 and 20 mg/kg; and for chlordiazepoxide: 15, 21, 27 and 33 mg/kg. All drugs were dissolved in saline and injected intraperitoneally. The volume of injection was maintained constant, and different dosages were attained by varying the drug concentrations. Following the acute testing, animals were then treated once daily by gavage with ethanol (6 g/kg), pentobarbital (50 mg/kg) or chlordiazepoxide (60 mg/kg) for 3 weeks. At the end of this treatment, each animal received a practice session to ensure that it still performed at criterion, then a D-R curve was generated with the same dosages previously employed, to determine the extent of tolerance. Subsequently animals from each treatment group were randomly divided into 2 subgroups (n=6) and were tested for cross-tolerance to one dose of each of the other two drugs. The doses chosen were approximately equieffective in the initial D-R studies (Fig. 1). All chronic treatment doses were given between 10 and 11 a.m.; test doses began at 10 a.m., but in some of the larger experiments with staggered starting times some of the doses could not be given until noon to 1 p.m. On test days, after completion of the test, the difference between the test and treatment doses was given as a supplement by gastric intubation.

Experiment 2: Moving-Belt Study

The moving-belt test has been described in detail previously [10,25]. In summary, rats were trained to remain on a motor-driven belt that moves continuously over a shock grid. If the rat puts one or more paws on the grid it receives a 0.5 mA shock and activates a timer which measures cumulative time off belt during any 2-min trial. Seventy-two rats, trained to a criterion of less than 1% error (i.e., less than 1.2 sec off belt in any 2-min trial), were employed for the study. They were divided randomly into 3 groups of 24 each and were designated for acute testing followed by chronic treatment with ethanol, pentobarbital or chlordiazepoxide. A 3-point (n=8 at each) D-R curve was generated for each drug before and at weekly intervals during chronic treatment. The duration and regimen of chronic treatment were similar to those of Experiment 1. The test doses for ethanol were 1.6, 1.8 and 2 g/kg; for pentobarbital: 15, 17.5 and 20 mg/kg; and for chlordiazepoxide: 35, 45 and 55 mg/kg. To measure the development of cross-tolerance, animals from each treatment group were divided into 4 subgroups (n=6), and were cross-tested with two different doses of the other drugs.

RESULTS

Shuttle-Box Avoidance Experiment

The effects of ethanol, pentobarbital and chlordiazepoxide on the avoidance performance before and after chronic treatment are shown in Fig. 1. Within the dose range employed, these drugs clearly impaired the avoidance performance in a dose-dependent manner. Analysis of variance revealed a significant effect of chronic treatment, $F(1,8)=133$, $p<0.001$ for ethanol; $F(1,8)=48$, $p<0.001$ for pentobarbital; $F(1,8)=114$, $p<0.001$ for chlordiazepoxide. These results clearly demonstrate that chronic treatment gave rise to tolerance to the impairment produced by all three drugs. The same analysis of variance, however, showed no significant interaction between treatment and test doses for ethanol and pentobarbital, $F(3,24)=0.5$, $p>0.05$ for ethanol; $F(3,24)=0.4$, $p>0.05$ for pentobarbital. The lack of significant interaction indicates that there is a parallel shift of the D-R curve to the right following chronic ethanol or pentobarbital treatment. On the other hand, a significant interaction between treatment and test dose was observed for chlordiazepoxide, $F(3,24)=33.7$, $p<0.001$, indicating that the observed extent of tolerance to

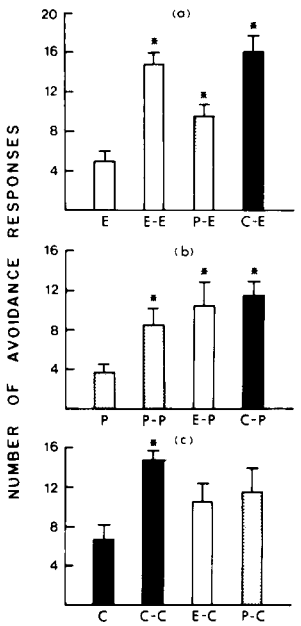

FIG. 2. Number of correct avoidance responses by animals treated with a test dose of (a) 2 g/kg ethanol, (b) 17.5 mg/kg pentobarbital, or (c) 33 mg/kg chlordiazepoxide. The various groups shown are: (a) E—acute treatment with ethanol only; E-E, P-E, C-E—ethanol test after chronic treatment with ethanol, pentobarbital, or chlordiazepoxide respectively, (b) P—acute pentobarbital only; P-P, E-P, C-P—pentobarbital test after chronic pentobarbital, ethanol, or chlordiazepoxide respectively, (c) C—acute chlordiazepoxide only; C-C, E-C, P-C—chlordiazepoxide after chronic chlordiazepoxide, ethanol or pentobarbital respectively. Vertical bar represents the positive half of S.E.M. Asterisk indicates $p<0.05$ for comparison with response of naive animals (E, P and C respectively); the P-E group is also significantly different from E-E and C-E ($p<0.05$ in each case).

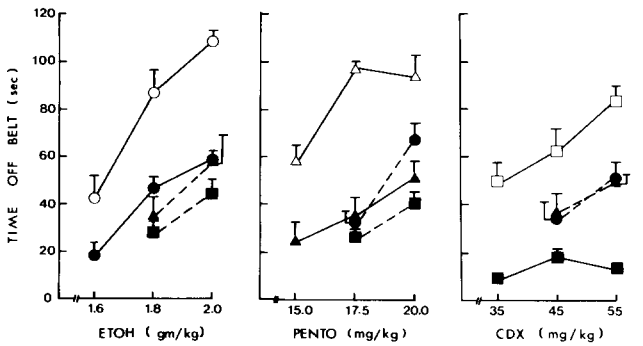

FIG. 3. Motor-impairment on the moving-belt test, induced by the administration of various doses of ethanol (○), pentobarbital (△) and chlordiazepoxide (□). Open symbols indicate results of acute tests with these drugs. Closed symbols indicate tests with the drug named in the abscissa, following 3 weeks of chronic treatment with the drug represented by the symbol. Broken lines represent cross-tolerance testing; N=5–6 animals per point. Solid lines and symbols indicate tolerance test; 7–8 animals per point. Vertical bar represents positive half of the S.E.M.

chlordiazepoxide is dependent on the test dose employed, so that the D-R curve is shifted in a non-parallel fashion.

The development of tolerance to and cross-tolerance among these drugs, as tested by the same dose, are shown in Fig. 2. Chronic treatment with ethanol produced tolerance to ethanol (E-E group, Fig. 2a; $t=5.8$, $p<0.001$ vs. E). In addition, chronic treatment with either pentobarbital or chlordiazepoxide resulted in the development of cross-tolerance to ethanol (Fig. 2a), as both groups showed significantly better performance than that of animals receiving only acute ethanol ($t=2.90$, $p<0.01$ for the pentobarbital-treated group; $t=6.1$, $p<0.001$ for the chlordiazepoxide-treated group; two-tailed Student's t-test for unpaired data). There was no difference between the performances of the E-E and C-E groups, but the P-E rats did not perform as well ($p<0.05$ compared to either E-E or C-E group). Tolerance to pentobarbital was also observed (Fig. 2b, P-P group), as well as cross-tolerance to pentobarbital after ethanol or chlordiazepoxide treatment (E-P and C-P respectively). There were no statistically significant differences among the performances of the P-P, E-P and C-P animals. Chronic treatment with chlordiazepoxide produced significant tolerance to chlordiazepoxide (Fig. 2c, C-C vs. C group). Animals tested with chlordiazepoxide after chronic treatment with ethanol (E-C) or pentobarbital (P-C) were intermediate between the C and C-C groups, but did not differ significantly from them or from each other ($p>0.05$ in all cases). This suggested that cross-tolerance to chlordiazepoxide following ethanol and pentobarbital treatment is of very limited degree.

Moving-Belt Study

The development of tolerance to and cross-tolerance among ethanol, pentobarbital and chlordiazepoxide is shown in Fig. 3. Analysis of variance shows a significant effect of treatment for all three drugs examined, $F(1,40)=31.6$, $p<0.001$ for ethanol; $F(1,40)=57.70$, $p<0.001$ for pentobarbital; $F(1,41)=97.8$, $p<0.02$ for chlordiazepoxide. This indicates that tolerance developed to the motor-impairment effect of all three drugs following chronic treatment. No significant interaction between dose and treatment was observed for either ethanol, $F(2,40)=1.7$, $p>0.2$, or pentobarbital, $F(2,40)=1.8$, $p>0.2$. However, as in Experiment 1, a significant dose × treatment interaction was found for chlordiazepoxide, $F(2,41)=3.6$, $p<0.05$. This again suggests that chronic treatment with chlordiazepoxide produced a non-parallel shift in the D-R curve of this drug.

It is obvious from Fig. 3 that chronic treatment with pentobarbital produced cross-tolerance to ethanol, that was not different in degree from the tolerance to ethanol itself, $F(1,20)=1.4$, $p>0.05$. A slightly greater amount of cross-tolerance to ethanol was observed after chronic chlordiazepoxide treatment than after chronic ethanol treatment, $F(1,21)=9.8$, $p<0.005$. The extent of cross-tolerance to pentobarbital after chronic ethanol or chlordiazepoxide treatment was similar to that produced by chronic treatment with pentobarbital itself, $F(1,20)=0.8$, $p>0.7$ for ethanol vs. pentobarbital, and $F(1,22)=2.5$, $p>0.1$ for chlordiazepoxide vs. pentobarbital. Moreover, there were no significant treatment × dose interaction effects in the tests with ethanol or pentobarbital, so that the cross-tolerance curves were essentially parallel to the primary tolerance curves.

The extent of cross-tolerance to chlordiazepoxide in-

duced by chronic ethanol or pentobarbital treatment, however, was much smaller than that of tolerance to chlordiazepoxide itself, $F(1,22)=30.9$, $p<0.001$ for ethanol; and $F(1,23)=35$, $p<0.01$ for pentobarbital. In addition, the treatment × dose interaction was significant for chlordiazepoxide vs. ethanol groups, $F(1,22)=4.4$, $p<0.05$, and approached significance for pentobarbital vs. chlordiazepoxide groups, $F(1,23)=3.6$, $p=0.06$. This suggests that the pattern of cross-tolerance to chlordiazepoxide following chronic ethanol or pentobarbital treatment was different from that of tolerance to chlordiazepoxide itself.

DISCUSSION

The results of the present study confirm and extend earlier observations that chronic treatment with ethanol, pentobarbital or chlordiazepoxide resulted in the development of tolerance to their respective impairment effects [7, 20, 25]. In addition, the present data also show that chronic treatment with one drug will confer cross-tolerance to the others. In both experiments, since the drug effects were quantified within 10–15 min after administration of the test dose, it is unlikely that changes in the rate of metabolism would play a significant role in the observed tolerance or cross-tolerance.

Although the shuttle-box test and the moving-belt test both contain elements of active avoidance and escape performance, and though all three drugs can exert sedative and hypnotic actions, the patterns of tolerance and cross-tolerance differed according to both test and drug. On both tests, tolerance to ethanol and pentobarbital was characterized by parallel shift of the D-R curves to the right, as previously reported by numerous observers (for references, see [19,20]). In contrast, chronic chlordiazepoxide treatment resulted in chlordiazepoxide D-R curves that were both shifted to the right and significantly less steep in the tolerant group than in the controls, over the dose ranges tested. It is impossible to decide, from these results, whether the curves for the tolerant animals would eventually reach the same maximum as for the controls, or whether their maximum responses are reduced (i.e., "flattening" of the curves). Separate experiments with a much wider dose range are required for this purpose. Ryan and Boisse [37], using the technique of "maximal tolerated dose" administration previously applied to studies of barbiturate tolerance [33], found that chlordiazepoxide-tolerant rats could be brought to the same level of intoxication as naive rats if the dose was raised 5-fold. In contrast, benzodiazepine tolerance in humans appears to be accompanied by very marked flattening of the D-R curve [15,16], such as that observed with morphine tolerance in rats [31].

Although plasma levels of the drugs were not measured, it is well known that the rate of ethanol metabolism [17] in the rat, and the elimination half-lives of pentobarbital [22] and chlordiazepoxide [4,24] are such that no interaction was likely between the chronic treatment doses and the subsequent test doses. Moreover, the possibility of such interaction is even less likely in view of the known increases in rate of biotransformation of all three drugs after chronic administration [5, 17, 18, 22, 24]. However, chlordiazepoxide has active metabolites with longer half-lives than the parent compound [4,5], and the possibility of interaction of these metabolites with the test drugs cannot be ruled out. Nevertheless, this seems unlikely for two reasons. First, the final DR curves for ethanol and pentobarbital were the same in rats treated chronically with chlordiazepoxide as in the chronic ethanol and chronic pentobarbital groups; residual active metabolites should have increased the relative effect of the test doses. Second, accumulated active metabolites should have added to the effects of the chlordiazepoxide test doses as well, yet the tolerance to chlordiazepoxide itself was greater than the cross-tolerance to this drug in the chronic ethanol and pentobarbital groups.

Similar disparities are seen in the cross-tolerance among the three drugs. In the shuttle-box avoidance paradigm, cross-tolerance to ethanol in pentobarbital-treated rats was significantly less than in chlordiazepoxide-treated animals, or than primary tolerance to ethanol itself. This may have been because the pentobarbital treatment dose was not fully equivalent to the treatment doses of the other two drugs. Indeed, primary tolerance to pentobarbital was less than cross-tolerance to pentobarbital in the ethanol- and chlordiazepoxide-treated groups. In contrast, cross-tolerance to chlordiazepoxide in ethanol- and pentobarbital-treated groups was significantly less than primary tolerance to chlordiazepoxide itself. Virtually the same pattern was seen in the moving belt experiment, except that cross-tolerance between ethanol and pentobarbital, as well as primary tolerance to each, were all of approximately the same magnitude. Presumably for the rats in Experiment 2, the chronic treatment doses of the three drugs were more closely equivalent.

These findings are, in general, consistent with the hypothesis that the stimulus to tolerance development is not the drug itself, but the degree of functional disturbance it produces [26]. Nevertheless, this hypothesis must be qualified, since it has already been shown that on other tests equieffective doses of ethanol and pentobarbital do not induce equal degrees of cross-tolerance to each other [12,28].

The findings also suggest a possible difference in the mechanism(s) underlying the development of tolerance to the three drugs. Ethanol and pentobarbital are generally believed to exert their depressant effects by non-specific interactions with lipids and protein inclusions in the cell membrane [19,38]. Benzodiazepines may possibly produce some of their effects in a similar manner, but there is good evidence that at least some of their actions (e.g., anticonvulsant action) result from their combination with stereospecific receptors in the brain [2, 30, 43]. The existence of such receptors might allow additional mechanisms of tolerance to benzodiazepines that do not apply to ethanol and pentobarbital, such as changes in specific receptor density, binding affinity, or in some transduction process between drug-receptor complex and effector mechanisms [19]. Indeed, experiments both in vivo [35] and in neuronal cultures [41,42] indicate that chronic benzodiazepine treatment results in decreased benzodiazepine receptor binding. Thus, chronic treatment with ethanol or pentobarbital may not activate all the potential adaptive responses available to chlordiazepoxide, so that cross-tolerance from ethanol or pentobarbital to chlordiazepoxide is not as great as primary tolerance to chlordiazepoxide itself. Conversely, not all the mechanisms of tolerance activated by chlordiazepoxide can be utilized by ethanol or pentobarbital, so that cross-tolerance from chlordiazepoxide to these drugs is no greater than primary tolerance to these drugs themselves. A similar picture was observed previously in this laboratory concerning the development of cross-tolerance between the hypothermic effects of ethanol and morphine [23].

To test the validity of these speculations, it will be necessary to see whether specific benzodiazepine receptor block-

ers abolish the apparent flattening of the chlordiazepoxide D-R curve, as naloxone did to the flattening of the morphine curve [32], and whether such blockers restore symmetry of cross-tolerance among the three drugs.

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