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A Study of the Duration of Acute Tolerance Induced With Hexobarbital in Male Rats

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WAHLSTRÖM, G. *A study of the duration of acute tolerance induced with hexobarbital in male rats.* PHARMACOL BIOCHEM BEHAV **59**(4) 945–948, 1998.—Male rats were infused IV with hexobarbital to obtain a burst suppression of 1 s or more in the EEG (SS). At SS the rats were killed and the concentration of hexobarbital was determined by HPLC in three parts of the brain. Acute tolerance (induced by a 1-h exposure at the SS level) was recorded as an approximately 20% increase in brain concentrations of hexobarbital at the last SS during the exposure when compared with concentrations recorded at the first SS in the controls. Increased brain concentrations (approximately 8%) at SS were recorded 24 h after induction of acute tolerance. After 48 h the increase was uncertain. Thus, acute tolerance to hexobarbital could have cumulative properties if new exposures are imposed after 24 h. © 1998 Elsevier Science Inc.

Acute tolerance Anesthesia Brain concentrations EEG threshold HPLC Hexobarbital Male rats

IN a review where the relation between acute and chronic tolerance was discussed (12) acute tolerance was defined in two ways: "that which occur after one exposure to the drug . . .," or "changes in sensitivity to a drug within the duration of one continuous drug exposure." The other fundamental distinction was between intrasessional adaptation (acute tolerance) and intersessional adaptation (chronic tolerance). Since first investigated (21), interest in acute tolerance has focused on ethanol (5,11,13,18,19,26,29). However, other sedative and anesthetic drugs such as barbiturates (3,5,7,13,28), benzodiazepines (7,10), and propofol (17) have also been investigated. Several neurotransmitter systems such as dopamine (20), noradrenaline (20,22), 5-hydroxytryptamine (5,20), acetylcholine (26), GABA (1), and NMDA (9,14) have been directly or indirectly implicated, which makes it uncertain if acute tolerance to depressants in the central nervous system is an entity with a single common mechanism. Genetic differences (25) have been reported that could support a common mechanism, but such a mechanism is not supported by data pointing at differences between different brain structures (5,8), different results depending on the test system used (11,18), and differences depending on age (13,17).

The possibility of a relation between acute tolerance and the tolerance measured after chronic treatments has been an open question since it was proposed (12). One of the basic issues in this problem complex is the duration of the changes responsible for the acute tolerance. Changes responsible for acute tolerance to ethanol might last for at least a week in P rats (20), but changes lasting for only 20 min has recently been reported after application of ethanol on Purkinje neurons (25). Such differences in the duration of changes in intact animals could be partly due to the difficulties involved in performing repeated tests with the inducing agent. However, such problems can be reduced with a threshold technique, which has been developed in this laboratory (16). This test procedure has no direct effect on the acute tolerance. Because the technique also can be used to accomplish the controlled exposure needed to induce acute tolerance (3,17,28), it was decided to use it to study the duration. Due to our large experience with hexobarbital (16), this anesthetic was used in the present experiments.

METHOD

Fifty-one male outbred rats (Mol:SPRD Han, Möllegaard, Ll Skensved, Denmark), bought at the age of 105 days, were used in the present experiments. They were kept three in each cage in an animal room with a reversed light–dark schedule (lights on 1900–0700 h) and with a temperature of 24°C. The age of the rats was at the start of the first experiment 142 days (Experiment 1) and at the start of the second experiment 289 days (Experiment 2). The corresponding body weights were 515 ± 6 g (n = 30, Experiment 1) and 567± 9 g (n = 11, Experiment 2).

Each experiment consisted of three groups. In all groups

hexobarbital (supplied by Apoteksbolaget, Sweden) was infused with a constant rate of 15 mg/kg/min (volume rate 0.1 ml/min) until the first burst suppression, which was 1 s or longer (the silent second, abbreviated SS) was recorded in the EEG (Mingograf EEG 10, Siemens-Elema AB; Stockholm, Sweden). A dose of 15 mg/kg/min is an optimal dose administration rate defined as the rate where the threshold dose has a minimum (4,16). Subcutaneous stainless steel sutures placed in a bifrontal configuration were used as electrodes. A detailed description of the threshold technique has recently been published (16).

When the first SS were recorded in groups C (Experiments 1 and 2) the infusion was immediately stopped and the rats killed by decapitation. In groups AT (Experiments 1 and 2) the EEG recording was continued after the end of the first infusion and all SSs were indicated. When the EEG record had been without a SS for 1 min, a new infusion of hexobarbital was started. It was again stopped when the next SS appeared. By this technique the rats in groups AT were kept at a level of anesthesia defined by the SS for approximately 1 h. After the last induction of SS the infusion was stopped and the rats in groups AT were also killed by decapitation. In group 24hr (Experiment 1) and group 48hr (Experiment 2) acute tolerance was induced in the same manner as in group AT, but the rats were allowed to recover and the duration of anesthesia measured. At a new induction of SS 24 h (group 24hr) or 48 h (group 48hr) after induction of the acute tolerance the infusion was immediately stopped at the first SS and the rats killed by decapitation in the same manner as group C. At decapitation serum was collected, the brain taken out and dissected into cortex, brainstem, and cerebellum. The tissues were immediately frozen and kept at −70°C until analyzed.

Experiment 1 consisted of three groups. Group C ($n = 9$), which was killed after induction of the first SS, was a control group that estimate the basic sensitivity to hexobarbital. Group AT ($n = 9$), which was killed at SS after 1 h of anesthesia, was a positive control group that estimated the magnitude of the induced acute tolerance. Group 24hr ($n = 12$), which was killed after induction of SS 24 h after an anesthesia of 1 h at the level of the SS, was an experimental group used to evaluate the retention of acute tolerance 24 h after induction. Experiment 2 also consisted of three groups. Group C ($n = 3$) and group AT ($n = 3$) were the same as those in Experiment 1. Group 48hr ($n = 5$) was killed after induction of SS 48 h after an anesthesia of 1 h and corresponded to group 24hr in Experiment 1.

Concentrations of hexobarbital were measured by UV-spectrometry after separation with HPLC essentially according to a method described earlier (15). Briefly, the samples were after extraction put on a reversed-phase HPLC column (Spherisorb ODS II; 100 × 4.0 mm, 5 μm, HPLC Technique, Robertsfors, Sweden). Mobile phase was 40% acetonitril in distilled water. Flow rate was 1 ml/min. The variable UV-spectrometer was set at 210 nm and secobarbital (supplied by Apoteksbolaget, Sweden) was used as internal standard. Acute tolerance to hexobarbital was defined as an increased concentration in the brain at SS after an anesthesia lasting for 1 h when compared with the concentration recorded immediately after induction of the first SS. This method to record acute tolerance has been described in detail earlier (3,17,28).

Parametric statistical methods were used and the probability of differences were estimated by Student's *t*-test. Because the working hypothesis was induction and retention of acute tolerance, one-sided probabilities were used. All doses and concentrations of hexobarbital are given as the sodium salt. The experiment has been approved by the animal ethical committee at Umeå University.

RESULTS

As expected (3,28), the cumulated doses of hexobarbital needed to maintain the approximately 1 h anesthesia in group AT and group 24hr had a linear function. The rates were 2.92 ± 0.11 mg/kg/min and 3.10 ± 0.11 mg/kg/min, respectively. The corresponding average durations of this anesthesia were due to the variability between the rats 61.0 and 57.0 min. The doses of hexobarbital needed to induce the initial SS in earlier unexposed rats was in group C 64.53 ± 3.52 mg/kg, in group AT (starting the 1 h anesthesia) 60.34 ± 1.75 mg/kg, and in group 24hr (starting the 1 h anesthesia) 61.24 ± 2.34 mg/kg. There were no significant differences between these doses. The dose of hexobarbital in group 24hr needed to induce the SS at the test 24 h after the 1 h anesthesia was 58.27 ± 2.82 mg/kg. This dose did not significantly differ from the first dose needed to induce anesthesia 24 h earlier nor from the dose needed in group C.

The results of the analysis of hexobarbital in serum and three parts of the brain are given in Fig. 1. The data clearly indicate that there was a significant increase in brain concentrations in all brain parts [cortex, $t(16) = 4.938$; brainstem, $t(16) = 5.734$; cerebellum, $t(16) = 3.368$] when group AT is compared with group C (Fig. 1B–D). Thus, acute tolerance had been induced by the 1-h anesthesia at the end of which the rats in group AT were killed. This increase was around 20%, but there was no corresponding increase in serum concentration (Fig. 1A). At induction of SS 24 h after a 1-h anesthesia there was a significant decrease in concentration of hexobarbital in all brain parts [cortex, $t(19) = 3.277$; brainstem, $t(19) = 2.937$; cerebellum, $t(19) = 2.525$] in group 24hr when compared with the positive controls in group AT (Fig. 1B–D). However, there was in two brain parts still a significant increase [cortex, $t(19) = 2.479$; brainstem, $t(19) = 1.936$] when group 24hr was compared with group C (Fig. 1B and C). This remaining increase (retention of acute tolerance) amounted to approximately 8%. There was a corresponding slightly larger (approximately 13%), but not significant increase in serum concentration (Fig. 1A). Thus, in the intact animal no behavioral tolerance, seen as an increase in the dose needed to induce SS, was recorded 24 h after induction of acute tolerance, but the analyses of brain concentrations indicated that the changes responsible for the acute tolerance were retained because the concentrations at SS found in group 24hr had not returned to the level found in the controls.

Due to the remaining acute tolerance seen 24 h after induction, a second experiment that consisted of a smaller number of animals was performed approximately 4 months later. Brain concentrations in group C ($n = 3$) and group AT ($n = 3$) were in this experiment similar to the corresponding groups in Experiment 1. The remaining tolerance seen in group 48hr ($n = 5$) was tested 48 instead of 24 h after induction of the acute tolerance. The data (not shown) indicated that there still remained a slight increase in brain concentration (around 6%). This change was not significant when compared to group C, nor was the difference to group AT significant. An indication of a remaining behavioral tolerance was recorded in this experiment as an increase in dose needed to induce SS 48 h after the 1-h anesthesia in group 48hr when compared to the corresponding dose recorded in group C [70.10 ± 2.79 mg/kg vs. 58.58 ± 0.30 mg/kg, $t(6) = 3.089$, $p < 0.05$]. Thus, in Experiment 2 there was a slight significant behavioral tolerance that

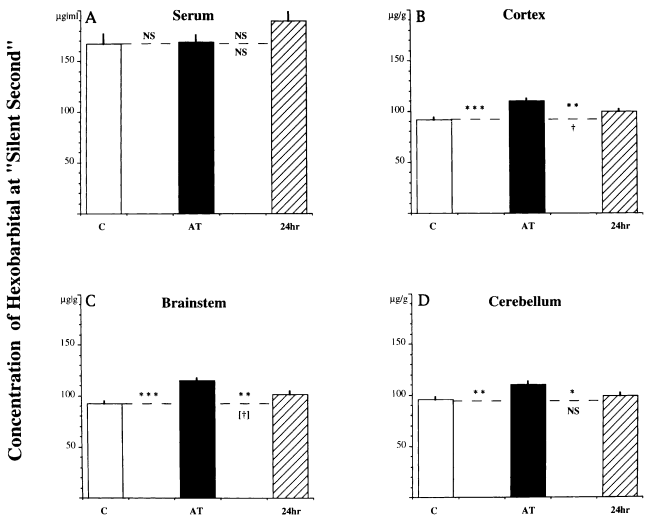

FIG. 1. Tissue concentrations of hexobarbital recorded in Experiment 1. The different groups are given below the abscissa. The broken line gives the value of the group C as a reference. Significance of differences between group C and group AT and between group AT and group 24hr are given with stars above the broken line. Significance between group C and group 24hr are given with crosses below the broken line. Levels of significance given as follows (stars or crosses): (*)$p < 0.10$, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Standard error of the mean is indicated at the top of each bar. A gives concentrations of hexobarbital in serum, B gives concentrations of hexobarbital in the cortex, C gives concentrations of hexobarbital in the brainstem, and D gives concentrations of hexobarbital in the cerebellum.

could not be verified by significant differences in the analysis of brain concentrations.

DISCUSSION

The slight decrease in dose needed to induce SS seen in group C in Experiment 2 (58.58 mg/kg) when compared with group C in Experiment 1 (64.53 mg/kg) was expected because a significant increase in brain sensitivity (decrease in brain concentration) due to age has been recorded earlier (2) when a larger age span was investigated. However, the differences in dose for SS or in brain concentrations between group C in Experiments 1 and 2 in the present experiment were not significant. This means that the difference in body weight between the two experiments (see the Method section) was of minor pharmacokinetic importance with regard to the threshold determinations. Furthermore, the use of the optimal dose administration rate in the threshold test reduces the importance of differences in the pharmacokinetic situation (16). The two experiments were, nevertheless, in the present article reported separately due to results obtained with propofol (17) and ethanol (13), which indicated that age could be a critical variable when inducing acute tolerance. If age is disregarded the brain concentration in cortex, $t(15) = 2.196$, $p < 0.05$, and brainstem, $t(15) = 2.088$, $p < 0.10$, of group 48hr was significantly increased when compared to the combined group C.

In the earlier experiments when the method to study acute tolerance to hexobarbital in rats was developed (3,28), it was clear that there was an increase in acute tolerance that depended on the duration of anesthesia. A maximum seemed to occur after 1 h. This means that when the hexobarbital infusion was stopped, maximal biochemical changes responsible for the acute tolerance must have been present. The time aspect could be further evaluated by analyzing the anesthesia times recorded after the 1-h anesthesia used to induce acute tolerance in group 24hr (Experiment 1) and group 48hr (Experiment 2). This duration of anesthesia was defined as the time from the last SS to the return of righting reflex. These durations were 31.1 ± 2.2 min ($n = 12$) in group 24hr and 26.1 ± 5.3 min ($n = 5$) in group 48hr, respectively, which is approximately twice the anesthesia times seen after induction of the first SS in other experiments (2,4). These data could indicate that the changes involved in acute tolerance had started to be reduced already during the ensuing anesthesia following the induction. If no change in acute tolerance had occurred during the ensuing anesthesia, the anesthesia times would probably have corresponded to the ones seen after induction of SS in the other experiments. This is the case when the hexobarbital thresholds are increased due to the excitation found in the abstinence after prolonged barbital treatments (27). A tentative conclusion could then be that changes recorded as tolerance in the abstinence after chronic treatments with a long-acting barbiturate have a longer half-life than changes involved in the induction of acute tolerance with hexobarbital. Such a difference is not absolute, but could initially separate the changes causing acute tolerance from the corresponding changes seen after chronic treatments. However, caution must always be exercised when dealing with explanations founded on changes in anesthesia times because changes in duration could involve both pharmacodynamic and pharmacokinetic properties of the tested drug, and the possibility of pharmacokinetic differences are obvious despite the use of SS as the end point in both cases.

Rapid tolerance has been introduced as a term to describe a tolerance that was recorded on the second but not on the first day of exposure to ethanol (14). The data on concentrations of hexobarbital in Experiment 1 clearly indicated a retention of changes associated with acute tolerance 24 h after induction (Fig. 1). This also means that the changes recorded as an acute tolerance after the first exposure could accumulate if the exposure to the inducing agent is repeated in the same way on a 24 h time scale. If hexobarbital had been tested with fixed doses against a criterion with larger variability compared to the threshold for SS, the initial change (the acute tolerance) might have been undetected and the tolerance recorded only after the second exposure (rapid tolerance) due to the retention of changes that could be added to a more easily detected cumulative effect. Thus, if hexobarbital had been tested in such a manner, a distinction between acute and rapid tolerance would probably have been very hard to verify experimentally. The carryover also means that there is a distinct possibility that acute tolerance could develop into a chronic tolerance, and the distinction between intrasessional and intersessional (12) as definitions of different kinds of tolerances could not be applied to hexobarbital.

An extrapolation of the present results to the induction of acute tolerance with other drugs assumes that a common mechanism is involved. As stated in the introductory paragraphs, it is, at present, hard to make such an assumption. The CNS could have a number of ways in which it can oppose the depressive effects of drugs with different mechanisms of action. Because ethanol could influence a number of transmitter system (6) differences in duration of acute tolerances recorded with different techniques, as those described in the introductory paragraphs, are not surprising. With regard to hexobarbital, it has been shown that besides an effect on the GABA ionophore (24), there also is an interaction with mus-

carinic receptors in the CNS at the concentrations involved in the SS testing (23) and a reduction of acute tolerance by disulfiram (22). Thus, there is a possibility that different biochemical mechanisms could also be involved in the case of induction of acute tolerance to hexobarbital.

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