TOLERANCE TO AND DEPENDENCE ON BARBITURATES IN MICE WITH REFERENCE TO THE DATA IN RATS

Eijiro TAGASHIRA, Tomoko URANO, Ken-ichi YASUKOUCHI, Tameo HIRAMORI and Saizo YANAURA
Department of Pharmacology, Hoshi College of Pharmacy, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142, Japan
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Abstract—The experimental results on tolerance to and dependence on phenobarbital (PhB) in male, ICR mice were compared with results previously reported in the case of rats. When food admixed with 1 and 2 mg PhB/g food was administered daily for 13 consecutive days, the mice began to acquire tolerance to PhB from the 4th day or so (rotarod performance test), with little suppression being observed on days 6 or 7. These results indicate that tolerance to PhB is acquired earlier in mice than in rats. The blood and brain concentrations of PhB during the dosing period were reduced abruptly on the 3rd or 4th day, corresponding well with the time course changes in the development of tolerance shown by rotarod performance. The mice were given daily doses of PhB increasing stepwise from 0.5 and 1 mg PhB/g food to 4 mg PhB/g food, over 39 consecutive days. With this gradually increasing dose regimen, the animals maintained moderate to severe depression of CNS throughout the dosing period. The blood and brain half-life of PhB after withdrawal were 16 and 8 hr respectively. From 17 to 24 hours after withdrawal of PhB, the animals showed signs of systemic tremors, Straub-tail, hyperkinesia, wild running "rum fits" and clonic-tonic convulsions. Contrary to the findings in rats, in which there was a frequent incidence of convulsion from 17 to 48 hours after withdrawal, the duration of the characteristic signs after barbiturate withdrawal was obviously short-term. These results suggest that it may be more reasonable to use rats in preference to mice as a preclinical model of dependence, especially in cross-physical dependence tests for sedative-hypnotic drugs.

In our previous reports (1, 2) of a model designed to form physical dependence on phenobarbital (PhB) or barbital in rats, we stressed the necessity of maintaining continuous CNS depression day and night for about one month in order to precipitate acquisition of a sedative-hypnotics dependence comparable to that in humans, in terms of the time course of the blood and brain PhB concentrations during the dependence acquisition process. Studies of physical dependence on barbiturates in mice have been reported by Goldstein (alcohol: 3, 4), Kaneto et al. (5) and Belknap et al. (6, 7). Belknap et al. used a rapid induction method in which high (and rather toxic) doses of PhB mixed with food (2.5 or 3.2 mg PhB/g food) were administered for a short period (6–9 days) to mice of the G57 BL/6J and DBA/2J strains, these strains being...
susceptible to convulsion. After withdrawal, tonic-clonic convulsions occurred when the mice were handled. Given our previous experience with rats, it was surprising that such a severe dependence could be successfully developed within only a week or so, but this discrepancy is probably due to the difference in animal strain or species. In addition, a technical discrepancy between Goldstein and Belknap et al. on the one hand and our study on the other may have strong bearing: whereas the former two studies used the occurrence of seizures on handling as the indicator, we used spontaneously occurring convulsion upon withdrawal of barbiturate as the indicator in our rat model.

In the present study, we used the ICR strain to observe tolerance to and dependence on PhB. The same dosage schedule as in our previous report (1), in which severe PhB-withdrawal signs were induced in rats, was applied to the mice in order to allow for a comparison of the progress of tolerance acquisition and dependence formation as well as the degree and duration of withdrawal signs.

**MATERIALS AND METHODS**

Five-week-old male ICR mice were fed food pellets and tap water ad libitum. After 1 week of preliminary caging for acclimatization mice weighing 28–30 g were assigned to groups of 6–7 mice each. PhB was added to and mixed with powdered food, solidified by compression, and made available to the mice ad libitum the whole day (DAF method).

**Experiment I: Test for tolerance development**

The mice were fed on a fixed dosage of 1 and 2 mg PhB/g food for 13 consecutive days. The rotarod performance test (φ 30 mm, 10 rounds/min for 5 minutes) was carried out daily. Time course changes were observed as to whether the mice had acquired tolerance to the drug, using motor incoordination as an indicator. In other mice, time course changes in serum and brain concentrations of PhB were measured on the 1–5th days and 7th, 9th, 11th and 13th days after the initiation of dosing.

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**Fig. 1.** Dosage schedule for the formation of dependence on phenobarbital in ICR strain mice. The absolute amount of phenobarbital intake (mg/kg/day) was increased stepwise by the DAF (Drug-Admixed Food) method.
Experiment II: Formation of physical dependence on PhB

The mice were fed PhB-admixed food according to the dosage schedule shown in Fig. 1 for 39 consecutive days. The rotarod test was carried out twice a day at 10:00 a.m. and 5:00 p.m. Serum and brain concentrations of PhB were determined on the initial day and the last day at every dose level, and also at 8, 17, 24, 41 and 48 hr after withdrawal of the drug. The blood and brain half-life of PhB was also calculated in cases of continuous dosing and application of the DAF method.

In both experiments, general behaviors were observed, and body weight and food intake were measured at fixed times every day. The net intake of PhB was calculated from the daily total intake of the drug-admixed food.

RESULTS

Experiment I: Test for tolerance development

After initiation of the PhB-admixed diet food, the mice showed the following signs: on the 1st day: staggering gait, increased spontaneous motor activity (exaltation), ptosis, relaxation of the hind legs; on the 3rd–4th days: enhancement of the above-mentioned signs, general relaxation of the skeletal muscles, straddling of the hind legs, suppression of the righting reflex; on the 6th day: toxic signs such as piloerection, roughening of the hair, reduction of food intake and body weight; on the 6th–7th days: a tendency toward restoration due to a reduction of the CNC depressive signs. The animals eventually acquired complete tolerance to PhB on the 6th or 7th day of dosing. The extent of motor incoordination corresponded well to the severity of the general signs of CNS depression. On the 1st day of administration, the mice could scarcely remain on the rotarod due to severe CNS depression, up to 90–95% inhibition of rotarod performance day and night (Fig. 2). But the rate of depression gradually decreased from the 2nd or 3rd day on, with complete recovery to the initial level by day 6 or 7. After that, with the initiation

Fig. 2. Development of tolerance to phenobarbital-induced motor incoordination, and changes in body weight and eating pattern of drug-admixed food. Motor incoordination was assessed in the mornings and evenings by the rotarod performance test.
of administration of the higher dose (2 mg PhB/g food), the mice again showed a inhibition of rotarod performance, staggering gait, increased spontaneous motor activity, and ptosis. However, there was also a tendency toward recovery at this dose level, after the 3rd day. Both the blood and brain concentrations of PhB were highest on the initial day of dosing. The levels suddenly decreased on the 3rd day in the case of serum, and on the 4th day in the brain tissue. Thereafter, almost constant levels of 65 μg/ml for serum and 25 μg/g for brain were maintained (Fig. 3).

Experiment II: Formation of physical dependence on PhB

From the initiation of dosing at 0.5 and 1 mg PhB/g food, the mice sustained signs of exaltation with slight CNS depression, including inhibition of rotarod performance, ptosis, general relaxation of skeletal muscles, and staggering gait. But from the 8th day on, there was a tendency to recover from motor incoordination and the CNS signs (Fig. 4). When the dose level of PhB increased from 0.5 and 1 mg PhB/g food to 1 and 2 mg PhB/g food, there was an increase in the inhibition of motor coordination as well as general CNS depression. However, there was also a tendency toward recovery at these dose levels, after the 4th day. The animals consumed apparently lesser amounts of food, at this dose level, as compared with the control animals. The body weight increase was also inhibited. When the dose was increased from 1 and 2 mg PhB/g food to 2 and 4 mg PhB/g food, motor coordination sustained a 50–60% suppression up to the 7th day, with gradual recovery thereafter. In general, the mice showed moderate or severe depression of CNS, both day and night. Body weight increase was retarded about 15–25% at this stage. When the dose was increased from 2 and 4 mg PhB/g food to 4 mg PhB/g food, there was a remarkable inhibition of rotarod performance (70–100%)

Fig. 3. Correlation of tolerance development to motor incoordination and phenobarbital concentrations in the serum and brain. The method of determination of phenobarbital concentration in the serum and brain was as described previously (1).
and severe CNS depression in all mice. With increase in the dose of PhB the mice showed toxic signs and there was 25–30% decrease in weight as compared with the controls. No mouse died throughout the experiment.

The PhB blood and brain concentrations increased with graded increases in the dose. There was a good correlation between the increase in the blood and brain levels of PhB and time course change in the suppression of motor coordination (Fig. 5). At each dose level, the blood and brain concentrations of PhB increased abruptly on the initial day of dosing at the levels and decreased gradually thereafter. The brain concentration of PhB fluctuated within a more limited range than did the blood concentration at all dose levels. There were some cases of food retention in the stomach (autopsy at approximately 1:00 p.m.).

After PhB-admixed food (4 mg PhB/g food) was replaced by regular PhB-free food (PhB withdrawal), retention of food in the stomach was observed until 8 hr after withdrawal, but none was seen from 17th hr to 41th hr. Body weight decreased until 41th hr after withdrawal, with the maximum rate being 20%. The serum and brain concentrations of PhB at the time of withdrawal were 125.5 µg/ml and 50.8 µg/g respectively. These levels were reduced to 91.8 µg/ml and 28.8 µg/g, respectively, after 8 hr. PhB could hardly be detected in the serum and brain after 41–48 hr, and the half-life after elimination was 16 and 8 hr, respectively (Fig. 6). At 17–24 hr after withdrawal, moderate to severe withdrawal signs such as hyperirritability, tremor, ataxia, Straub-tail, wild running, and hyperkinesia were observed, and in some cases, such

![Graph](image-url)
typical withdrawal signs as spontaneous clonic convulsions and clonic-tonic convulsions (2–3 times per mouse) were seen. These signs all but disappeared by 41 hr after withdrawal. In contrast with rats, the moderate withdrawal signs including hyperirritability, termor, and Straub-tail (convulsion excepted; intermediate degree of withdrawal signs) lasted continuously with the mice, although the rats continued to manifest severe withdrawal signs including frequent convulsions.

**DISCUSSION**

From observation of the time course changes in suppression of motor coordination during dosing at 1 and 2 mg PhB/g food in the mice, this species acquired tolerance to PhB earlier than did rats (1, 2). As the simultaneously determined serum and brain concentrations of PhB decreased abruptly on the third or fourth day of dosing, the earlier tolerance acquisition may be attributed to enzyme induction with the PhB. On the other hand, the suppression of motor coordination was reduced despite maintenance of steady levels of PhB at the early dosing stage, in both serum and the brain. It is conceivable that this may have originated from a reduced susceptibility in the brain rather than to any change in the PhB level. There was a tendency for the inhibition rate of motor coordination to be lower in the evening than the morning, while duration of CNS suppression was found to be shorter than that in rats, at each dose level.

In a previous work (2), we noted that the serum and brain concentrations of PhB were maintained at a constant level in rats, when determined at 4-hr intervals within 24 hr after feeding the PhB-admixed food for 7 consecutive days. From the simultaneously
measured amounts of food intake and fresh contents remaining in the stomach it was found that the bulk of the food ingested remained in the rat stomach, even though little food had actually been eaten. In contrast to the rats, the stomachs of mice contained little food. This may well explain the reduced PhB activity in the evening in the mice, because concentrations of PhB sufficient for CNS suppression would not be sustainable due to the short-term retention of the drug-admixed food in the stomach. In addition, it was confirmed that mice absorb PhB-admixed food from the gastrointestinal tract as rapidly as in the case of oral administration via gavage. It may be concluded that the mouse can acquire tolerance to PhB more quickly than the rat owing to more rapid increases in the blood concentration and, in turn, at the reactive sites.

The biphasic changes in motor incoordination shown in Fig. 2 may be regarded as being due to the difference between the eating patterns during the initial week (average dose: 120 mg/kg/day) and the later week (200 mg/kg/day). Nevertheless, progress in the acquisition of tolerance in mice can be readily understood. In both phases,
the reduced suppression of motor coordination commenced from the 3rd to 4th days. The PhB withdrawal signs were clearly of a lesser extent and were shorter in duration in mice than in rats, although suppression of motor coordination was much the same in both species during dosing, under equivalent schedules. The mice showed severe withdrawal signs accompanied by clonic-tonic convulsions, but such were of a short duration and there was no relapse of convulsion in the same subject. In the mouse model by Belknap et al. (6), severe withdrawal signs of PhB appeared at 22 to 36 hr, or from 18 to 24 hr after withdrawal, while thereafter there was an abrupt recovery, with the signs becoming mild after 49 hr and completely disappearing after 61 hr. Our results coincided well with those of Belknap et al. (6) with regard to the intensity and duration of the withdrawal signs, but due to the significant differences in nature between our ICR strain, and the DBA/2J and C57 BL/6J strains of Belknap et al., we found that the mice were unable to obtain such a severe dependence on PhB within one week or so.

Our experiment is characterized by a short duration of withdrawal signs, which thus differs from the models in man (9) and large animals (10, 11). In drug dependence screening, the mouse is a good species to use for testing for single dose suppression of barbiturate withdrawal signs (5, 8). However, it is difficult with this test to assess the capability of maintaining a barbiturate-dependence, which is a useful for discriminating between merely apparent suppressive action (e.g., general CNS depression with chlorpromazine, anticonvulsive action with phenytoin, etc.) and substantial suppression of withdrawal signs. When the mouse is compared with the rat with regard to tolerance to and dependence on PhB and withdrawal signs in general, the dosage schedule for dependence formation with continuous dosing of sedative-hypnotics is more difficult in mice than in rats owing to the smaller safety margin and to the rapid acquisition of tolerance to CNS depression.

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