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TOLERANCE TO CENTRALLY ADMINISTERED PHENOBARBITAL

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Abstract—Rats with permanent indwelling cerebral ventricular cannulae were shown to lose their righting reflex in response to centrally injected phenobarbital in a dose-related manner. Administration of four daily injections of 800 μg each over a 4- to 5-day period produced a gradual tolerance to the hypnotic effect, which was reversible after suspension of the drug regimen. This tolerance was associated with an increase in hepatic drug-metabolizing activity, measured as *p*-nitroanisole demethylase. The microsomal enzymes have been excluded as mediators of tolerance, however, on the basis of temporal and quantitative comparisons between the level of hepatic enzymatic activity and the degree of tolerance. The data are consistent with the concept that tolerance to the hypnotic action of phenobarbital is mediated through an adaptation of the central nervous system.

Accelerated hepatic metabolism has been implicated in the development of tolerance to the short-acting barbiturates, e.g. pentobarbital and hexobarbital [1–3]. However, a number of reports [4–9] suggest that the increased hepatic disposition resulting from induction of the hepatic microsomal mixed-function oxygenases by chronic administration of the barbiturates cannot account for the tolerance observed with the long-acting barbiturates, such as phenobarbital and barbital, which are biotransformed minimally or not at all [5]. For example, blood levels of the barbiturates in tolerant animals or patients continue to rise at a time when tolerance to the action of the drug is evident. Consequently, these observations led to the proposal that the attenuated hypnotic response reflects an altered sensitivity of the central nervous system (CNS) to the drugs.

It was the purpose of this investigation to evaluate directly the relative contributions of the liver and CNS to tolerance development to phenobarbital by administering the drug directly into the lateral cerebral ventricles of conscious rats. Evidence has been obtained which supports the concept of a centrally mediated process being involved in the production of tolerance to centrally injected phenobarbital, as determined by the progressive shortening of the loss of righting reflex to a given dose of drug.

EXPERIMENTAL

Preparation of animals. Male Sprague Dawley rats (150–170 g) were placed under ketamine anesthesia (150 mg/kg intraperitoneally) and a burr hole was drilled through the skull 1·5 mm lateral to bregma. A 23 gauge stainless steel guide for intracerebroventricular (i.c.v) injections was then inserted through the burr hole and directed toward the lateral ventricle. The guides were permanently fixed to the skull with dental cement. Each animal received 6000 units of benzathine pencillin intramuscularly to minimize infection. The animals were allowed to recover from surgery for at least 1 week before they were subjected to the experimental protocols.

Drug administration. Phenobarbital sodium was dissolved in 0·9% NaCl and injected into conscious animals in a 10-μl volume over a 20-sec interval via a 30 gauge cannula inserted through the guide. Control rats were injected with the saline vehicle adjusted with NaOH to the same pH as that of the phenobarbital solution, i.e. pH 9. (Phenobarbital has a p*K*a of 7·2 and below pH 9 precipitates from solution.) At the end of the experiment, methylene blue was injected through the guide and the brain was examined microscopically in order to confirm the i.c.v injection site.

The hypnotic effect of the barbiturates was recorded as the interval between the time the animal lost and subsequently regained its righting reflex.

O-demethylase activity. A 20% homogenate of liver was prepared in 0·25 M sucrose containing 0·02 M Tris buffer and 0·006 M disodium EDTA. After an initial centrifugation at 15,000 *g* for 30 min, the supernatant fluid was re-centrifuged for 20 min at the same force. The postmitochondrial supernatant thus prepared was analyzed for *O*-demethylase activity with *p*-nitroanisole as substrate according to the method of Netter and Seidel [10]. Results are expressed as μmoles *p*-nitrophenol formed/g wet weight of liver/hr.

RESULTS

Dose response to i.c.v phenobarbital. The duration of loss of righting reflex for a dosage range of 400–1000 μg is presented in Fig. 1. The hypnotic effect occurred 2–3 min after the start of the injection and lasted for 24 ± 3 min (mean ± S. E. M.) at the highest dose. No observable effects were produced by i.c.v-administered saline.

Since the rats weighed an average of 218 g at the time of barbiturate injection, the 400 and 1000 μg doses are equivalent to a total body dose of 1·8 to 4·5 mg/kg. Intraperitoneal (i.p.) or intravenous administration of these doses did not cause a loss of righting reflex. In order to produce a loss of righting

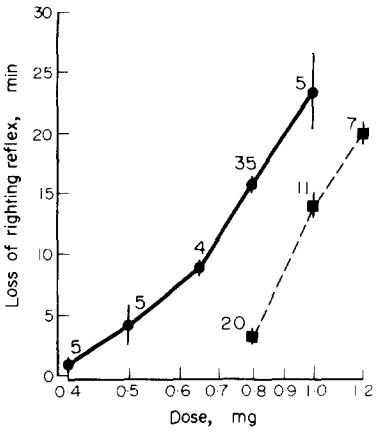

Fig. 1. Duration of loss of righting reflex after injection of various doses of phenobarbital sodium into the lateral ventricle of the rat. Each point represents the mean ± S.E.M. The numbers along the graph lines indicate the number of animals examined at each dose. The solid line represents control animals; the broken line represents tolerant animals.

reflex by the intraperitoneal route of drug administration, a dose of 90 mg/kg was required. This constitutes a dose which is 50 times larger than the centrally injected threshold dose.

Tolerance development. Since an acute dose of 1000 μg phenobarbital sodium was occasionally lethal, 800 μg was chosen as our standard i.c.v dose to study tolerance development.

Administration of 800 μg phenobarbital four times daily (i.e. a total daily dose of 13 mg/kg) for 4 consecutive days resulted in the attenuation of the hypnotic response (Fig. 2). This regimen consisted of two injections 30 min apart in the morning and repeated in the afternoon. To minimize possible variability in response due to diurnal factors, the animals were injected during the same time periods each day. Tolerance developed progressively as indicated by a shortening in the duration of loss of righting reflex which ultimately attained a stage in which the animals were virtually unaffected by the test dose of phenobarbital. When control rats that had received i.c.v saline for 4 days were challenged with 800 μg phenobarbital on day 5, their response was indistinguishable from that found with naïve animals.

Suspension of drug treatment after the 4 consecutive days of barbiturate administration resulted in a gradual return of the duration of the righting reflex toward normal. After 1 week, the duration of the response was not significantly different from that of saline-treated controls that had been challenged with the test dose (Fig. 2).

One of the characteristics of drug tolerance is the restoration of the original response by increasing the administered dose. The duration of the loss of righting reflex in the tolerant rats could be restored to the initial value, i.e. about 15 min, by administration of 1000 μg centrally. Indeed, the entire dose response curve shifts to the right as depicted in Fig. 1.

Relationship of tolerance to the hepatic drug metabolism activity. To ascertain the extent to which increased hepatic drug oxidation might contribute to the tolerance produced by central administration of phenobarbital, the oxidative drug-metabolizing activity, i.e. the *O*-demethylation of *p*-nitroanisole, was measured in the postmitochondrial fraction of liver homogenates prepared from animals at various points in the treatment schedule (Fig. 2).

On day 5 of successive i.c.v injections, the hepatic *O*-demethylase activity was 3·4-fold greater than that of saline-treated controls, in which the formation of *p*-nitrophenol averaged 1·0 ± 0·1 μmole/g/hr. Although the stimulation of demethylase activity varied inversely with the development of tolerance, important temporal differences exist between the two parameters. Thus, in spite of the fact that both were maximal at day 5, enzyme activity appeared more rapidly than did tolerance during day 1 of development. Of even greater significance, however, is that, when drug treatment was discontinued after day 4, the enzyme activity dropped to control values by day 8, at a time when maximal tolerance to the hypnotic effect was still maintained. Indeed, the hypnotic response did not return to that of identically handled saline-treated controls until 1 week (day 11) after cessation of the drug regimen.

A similar increase in enzyme activity was found on day 5 after daily intraperitoneal administration of phenobarbital at a dose equivalent to that injected centrally, i.e. 13 mg/kg per day. This latter observation suggests that the centrally injected drug enters the peripheral circulation and exerts an inductive effect on the hepatic mixed-function to oxidase system.

Effect of hepatic enzyme induction on response to i.c.v phenobarbital and i.p. hexobarbital. In order to test whether the tolerance to centrally administered phenobarbital could also be observed in rats made tolerant to systemically administered barbiturate, rats were treated intraperitoneally with phenobarbital to induce the hepatic mixed-function oxygenases and were compared to rats that were centrally treated. Two doses of phenobarbital were used, 80 mg/kg per day to maximally induce the hepatic enzymes and 13 mg/kg per day, which is equivalent to the daily

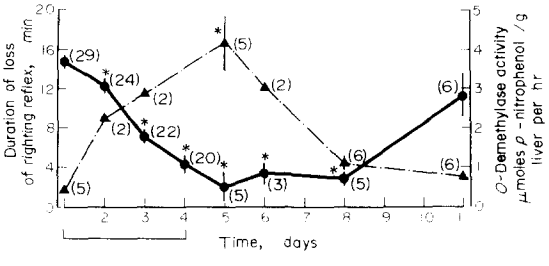

Fig. 2. The duration of hypnotic response to centrally administered phenobarbital (solid line) compared to hepatic *p*-nitroanisole *O*-demethylase activity (broken line) as a function of day of treatment. During days 1–4 (horizontal bar), all rats received four daily i.c.v injections of phenobarbital (800 μg/injection) as described under Experimental and the hypnotic response to the first of these daily injections is presented in the graph. Subsequent values for days 5, 6, 8 and 11 represent hepatic *O*-demethylase activity and hyponotic response to a single 800 μg i.c.v dose of phenobarbital in the rats which had been made tolerant. All points are expressed as the mean ± S. E. M.; numbers in parentheses denote the number of animals. The asterisk indicates $P < 0{\cdot}005$.

dose injected i.c.v. The results of this experiment are listed in Table 1.

Pretreatment centrally and intraperitoneally with daily doses of phenobarbital resulted in enhanced *p*-nitroanisole demethylase activity and tolerance to i.p. hexobarbital. The hypnotic response to hexobarbital administration in the tolerant animals ranged from 34 to 54 per cent of that found in control animals. The actual sleep time values, however, were lower in the case of the control animals in the group receiving 80 mg/kg of phenobarbital i.p. as well as in all subsequent animals. This change coincided with the use of a new batch of hexobarbital.

Tolerance to centrally administered phenobarbital also was observed in rats that had received either phenobarbital centrally or the high (80 mg/kg) dose of the drug intraperitoneally daily for 4 days. The degree of tolerance was considerably less in the latter group as compared to the former, however, despite the fact that the demethylase activity was greater in the rats treated i.p. Indeed, animals treated daily with 13 mg/kg were not tolerant to i.c.v PB, although the hepatic enzyme activity was equivalent to that in the i.c.v-treated rats.

DISCUSSION

Tolerance to barbiturates might be a consequence of CNS adaptation, increased metabolic disposition, or a combination of these factors [3]. By administering phenobarbital directly into the cerebral ventricles, it was hoped to resolve the relative contribution of hepatic enzyme induction and brain adaptation in the development of barbiturate tolerance. This technique offers the advantages of bypassing the blood-brain barrier and of injecting a precise amount of drug into a circumscribed site. The initial work showed that acute injection of phenobarbital by this route produced a dose-related loss of righting reflex.

Repeated central administration of phenobarbital for 4 consecutive days resulted in tolerance to the hypnotic response. That the shortened duration of the loss of righting reflex did represent true tolerance rather than nonspecific cell damage was substantiated by the following evidence: (1) the duration of the loss of righting reflex in tolerant rats could be restored to the initial value by increasing the dose; (2) control rats that had received i.c.v saline for 4 days responded as if they were naïve animals when challenged with phenobarbital on day 5; and (3) upon suspension of drug treatment after 4 consecutive days of administration, the response gradually returned toward normal and after 1 week was indistinguishable from that obtained with saline-treated controls. Furthermore, histological examination revealed minimal cellular injury confined to the immediate area along the sides of the cannula guide.

Oxidative drug-metabolizing activity, as reflected by *p*-nitroanisole demethylase activity, was increased in the livers of the barbiturate-treated rats. Thus it was important to establish what role, if any, this enzymatic system played in shortening the duration of hypnotic response.

The observed induction of the oxidative system is probably not due to any central process, inasmuch as enzymatic activity could be induced to a similar degree by intraperitoneal administration of doses comparable to those administered by the central route. Thus it appears that the barbiturate is cleared completely from the brain. Recent preliminary experiments measuring the rate of phenobarbital appearance in peripheral blood bear this out.

However, the possibility that increased hepatic metabolism of the drug and subsequent elimination of the drug could account for the tolerance observed under the conditions of these experiments seems unlikely. The central dose is only 1/50 that required to cause a loss of righting reflex upon intraperitoneal administration. Thus, upon clearing the CNS, it would be diluted by the peripheral blood and tissues to result in concentrations which would be profoundly lower than those capable of producing CNS

Table 1. Effect of chronic phenobarbital pretreatment on hepatic demethylase activity and the hypnotic response to phenobarbital (i.c.v) and hexobarbital (i.p.)*

Days 1–4	Day 5		
	Sleep time (min)		p-NO_2-anisole demethylase activity
Pretreatment	i.c.v PB (800 μg)	i.p. HX (100 mg/kg)	(μmoles p-NO_2-phenol/g liver/hr)
Intraventricular			
Saline	15·7 ± 0·7 (12)	38·4 ± 2·4 (8)	1·1 ± 0·2 (13)
Phenobarbital†	1·7 ± 0·5‡ (14)	13·0 ± 3·2‡ (8)	4·4 ± 0·5‡ (14)
Intraperitoneal			
Saline	15·5 ± 0·8 (8)	42·6 ± 9·0 (8)	1·0 ± 0·1 (8)
Phenobarbital§	15·5 ± 1·2 (14)	17·8 ± 1·2‡ (8)	3·3 ± 0·3‡ (14)
Saline	16·2 ± 0·6 (10)	22·1 ± 1·3 (6)¶	0·8 ± 0·06 (4)
Phenobarbital‖	10·4 ± 0·5‡ (10)	11·9 ± 0·7‡ (6)¶	7·2 ± 0·6‡ (4)

* Figures in parentheses are numbers of animals; values represent means ± S. E. M. PB = phenobarbital; HX = hexobarbital.

† Four daily doses of 800 μg each = 13 mg/kg per day.

‡ $P < 0·002$ compared to saline control values.

§ One daily dose = 13 mg/kg per day.

‖ One daily dose = 80 mg/kg per day.

¶ Hypnotic response was tested using a new batch of HX.

depression. In this case, the contribution of hepatic metabolism would be expected to have a negligible role in terminating barbiturate activity.

On the other hand, it was possible that a barbiturate metabolite, formed as a consequence of accelerated hepatic biotransformation, might penetrate into the brain and block a locus involved in barbiturate action, thereby producing tolerance to subsequent injections. Such a metabolite–receptor complex might have a longer half-life than that of the drug-metabolizing enzyme system and could explain why tolerance persists at a time when the demethylase activity has returned to normal. If this were the case, then induction of the drug-metabolizing enzymes by i.p. phenobarbital received chronically should protect the rat from the hypnotic action of centrally injected phenobarbital. Such protection (tolerance) to icv phenobarbital was observed in rats injected with a dose of 80 mg/kg intraperitoneally for 4 days but not in those receiving the lower dose, which corresponds to that administered centrally. Although the possibility of a metabolically derived substance acting to protect the animal against the hypnotic effect of the barbiturate is not completely excluded, it seems unlikely as an explanation for the tolerance observed at the doses employed in the central administration experiments.

Even though i.p. phenobarbital administration resulted in an attenuation of the hypnotic response to centrally injected drug, the degree of tolerance was not as great as with the chronic central injection. Since these data might be explained by relative differences in drug levels present in the brain as a consequence of the two routes of administration, it would be important to measure these levels after each treatment. The high local concentration of phenobarbital following icv injection could overcome any adaptive change in the CNS which may have occurred during 4 days of systemic administration. It is also possible that exposure to the drug for longer periods (greater than 4 days) might protect the animals from the hypnotic effect of centrally administered drug. For example, Stevenson and Turnbull [11] pretreated young rats with various drugs for several weeks and found that the response to i.c.v pentobarbital could be influenced by the type and degree of tolerance.

The results reported herein provide the first demonstration that tolerance to phenobarbital can be produced by chronic central administration of this barbiturate. For the reasons cited above, it is unlikely that the liver and its ability to metabolize barbiturates play a role in this phenomenon. Oxidative biotransformation of the drug by the brain tissue is also unlikely, since cytochrome P-450 associated with this activity is absent [12]. We have also been unable to demonstrate oxidative activity in some preliminary work. Thus, we conclude that tolerance to centrally administered phenobarbital is a phenomenon separate from that of accelerated breakdown due to enhanced metabolism.

Consequently, the nature of the tolerance appears to be a function of adaptation of one or more processes in the CNS. Whether such an adaptation depends on alterations in neurotransmitter concentrations or turnover [13–15], or by some other process, e.g. more rapid clearance of the drug from the ventricles, is under investigation.

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