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Studies of the Possible Role of Brain Endorphins in Pentobarbital Anesthesia and Toxicity in Mice

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In an attempt to ascertain whether opiate receptors and brain enkephalins or endorphins are involved in pentobarbital anesthesia and toxicity, the effects of 1) two pure narcotic antagonists, naloxone and naltrexone, 2) morphine sulfate, 3) D-phenylalanine, an inhibitor of carboxypeptidase A, and 4) D-leucine, an inhibitor of leucineaminopeptidase, in combination with D-phenylalanine, were studied in mice. Both naloxone and naltrexone, (1, 5 and 10 mg/kg) administered subcutaneously to mice were unable to modify the duration of anesthesia when they were injected 5 min prior to a challenge dose (75 mg/kg) of pentobarbital (ip). The onset of anesthesia was unaltered by naloxone (1, 5 and 10 mg/kg) and naltrexone (1 mg/kg). Higher doses of naltrexone (5 and 10 mg/kg) delayed the onset of anesthesia slightly. Morphine (1, 2.5 and 5 mg/kg) given 30 min before pentobarbital did not modify the onset or the duration of anesthesia. D-Phenylalanine (250 mg/kg), and D-phenylalanine + D-leucine (250 mg/kg each) injected ip an hour before pentobarbital did not affect either onset or duration of anesthesia. Naltrexone (10 mg/kg, ip) given 5 min before pentobarbital did not alter the LD_{50} of the latter. The studies do not support a role of enkephalins or endorphins in pentobarbital anesthesia or toxicity, and suggest a need for caution in using narcotic antagonists in treating pentobarbital toxicity. (Key words: Analgesics, narcotic: morphine. Anesthetics, intravenous: morphine; pentobarbital. Antagonists, narcotic: naloxone; naltrexone. Brain: endorphins, enkephalins. Receptors: opiate.)

THE RECENT DISCOVERY of endogenous ligands, namely enkephalins and endorphins, for the opiate receptors[1,2] has generated interest in delineating their physiologic function. Furthermore, attempts have been made to implicate these enkephalins and endorphins in pharmacologic actions of drugs. For example, dose-related analgesia produced by nitrous oxide has been shown to be reversed by naloxone in mice,[3] suggesting that the analgesic component of general anesthesia may be related to the release of endogenous opiates. Naloxone has been shown to be a pure antagonist, and can reverse the actions of both morphine and enkephalins.[4] Additional studies indicate that analgesia produced by general anesthetics such as halothane, enflurane and cyclopropane is partially antagonized by naloxone.[5] However, Harper *et al.*,[6] Smith *et al.*,[7] and Bennett[8] found that naloxone in adequately large doses did not modify any variable of anesthesia produced by halothane or nitrous oxide in rats or mice.

Administration of naloxone has been shown to delay the development and decrease the duration of the loss of righting reflex caused by pentobarbital or methohexital.[9] The report also indicated that naloxone antagonized the toxicity of pentobarbital, and the authors suggested the use of an opiate antagonist in the treatment of barbiturate intoxication.

In view of the controversial role of brain enkephalins or endorphins in general anesthesia, the present studies were designed to elucidate the possible role of the endogenous opiates in pentobarbital anesthesia and toxicity in mice. The effects of 1) naloxone and naltrexone, 2) morphine sulfate, 3) D-phenylalanine, an inhibitor of carboxypeptidase A, which presumably increases the concentration of brain enkephalins and endorphins, and 4) a combination of D-leucine, an inhibitor of leucineaminopeptidase, and D-phenylalanine, on the onset and duration of pentobarbital anesthesia have been determined in mice. Furthermore, the effect of naltrexone on pentobarbital toxicity has been determined.

Methods

Male Swiss Webster mice† weighing 25–30 g were housed in an animal room with controlled temperature (23 ± 1 C), humidity (65 ± 2 per cent) and light (L 0600–1800 h) for five days prior to being used. The animals were allowed access to food and water *ad libitum*.

The onset time for sleep and duration of sleep were determined in mice pretreated with saline solution, naloxone 1, 5, and 10 mg/kg, and naltrexone 1, 5, and 10 mg/kg. Sodium pentobarbital, 75 mg/kg, dissolved in saline solution was injected intraperitoneally (ip), 5 min after subcutaneous (sc) administration of saline solution or narcotic antagonists. The drugs were injected in such a way that each mouse received 0.01 ml/g of body weight. The doses of the antagonists used represent the hydrochloride salt form. Sleep times, defined here as loss of the righting reflex, began when the animals could no longer right themselves when placed on their backs and ended when they

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TABLE 1. Effects of Naloxone and Naltrexone on Pentobarbital Anesthesia in Mice

Treatment*	Dose (mg/kg, sc)	Time of Onset of Sleep (Min ± SEM) (n = 15)	Duration of Sleep (Min ± SEM) (n = 15)
Saline solution	—	2.7 ± 0.2	95 ± 11
Naloxone	1	2.7 ± 0.2	103 ± 10
	5	2.8 ± 0.1	97 ± 6
	10	3.1 ± 0.1	100 ± 10
Naltrexone	1	2.5 ± 0.3	96 ± 6
	5	3.3 ± 0.1†	109 ± 10
	10	4.6 ± 0.2‡	112 ± 4

* Pentobarbital sodium (75 mg/kg) was injected intraperitoneally, 5 min after naloxone or naltrexone administration; n indicates the number of mice used for each dose of drug treatment.
† $P < 0.01$ *vs.* saline-treated controls; ‡$P < 0.001$ *vs.* saline-treated controls.

could move from their backs to an upright position within a 30-sec period. The onset time of sleep was determined as the time lapse between the injection of pentobarbital and the loss of the righting reflex. The means of onset time for sleep and duration of sleep in saline- and narcotic antagonist-treated mice were compared using the Student *t* test.

The effects of morphine sulfate, 1, 2.5, and 5.0 mg/kg, on pentobarbital anesthesia were determined. Morphine sulfate was injected subcutaneously 30 min before injection of sodium pentobarbital, 75 mg/kg, since this time interval has been shown to result in maximal analgesia and a peak brain concentration of morphine.[10]

The effects of D-phenylalanine, 250 mg/kg, and the combination of D-phenylalanine and D-leucine, 250 mg/kg each, which presumably increase brain concentrations of enkephalins and endorphins,[11] on pentobarbital anesthesia were also determined. The drugs were dissolved in 0.01 N HCl and injected intraperitoneally 60 min before pentobarbital injection. Control mice were injected with the vehicle (0.01 N HCl). With each drug pretreatment, the onset time and the duration of sleep were measured and the statistical analyses performed as described above.

To determine the effect of naltrexone on pentobarbital toxicity, mice received injections of saline solution or naltrexone, 10 mg/kg, subcutaneously. Five minutes later, the animals received a challenge dose of sodium pentobarbital (ip), and they were then observed for mortality for 24 hours. Four doses of pentobarbital with ten mice for each dose were used to compute LD_{50} values. The LD_{50}, 95 per cent confidence limits, and potency ratio were calculated according to the method of Litchfield and Wilcoxon.[12] A P value of <0.05 was considered significant.

TABLE 2. Effect of an Exogenous Opiate on Pentobarbital Anesthesia in Mice

Treatment*	Dose (mg/kg, sc)	Time of Onset of Sleep (Min ± SEM) (n = 12)	Duration of Sleep (Min ± SEM) (n = 12)
Saline solution	—	2.5 ± 0.2	192 ± 22
Morphine sulfate	1.0	2.8 ± 0.2	159 ± 14
	2.5	2.6 ± 0.1	140 ± 17
	5.0	2.2 ± 0.2	183 ± 17

* Pentobarbital sodium (75 mg/kg) was injected intraperitoneally, 30 min after saline solution or morphine sulfate administration; n represents the number of mice used for each dose of drug treatment.

TABLE 3. Effects of D-Phenylalanine, Alone and in Combination with D-Leucine, on Pentobarbital Anesthesia in Mice

Treatment*	Dose (mg/kg, ip)	Time of Onset of Sleep (Min ± SEM) (n = 11)	Duration of Sleep (Min ± SEM) (n = 11)
Control	—	2.5 ± 0.1	129 ± 15
D-Phenylalanine	250	2.5 ± 0.2	107 ± 11
D-Phenylalanine + D-Leucine	250 250	2.6 ± 0.1	110 ± 13

* Pentobarbital sodium (75 mg/kg) was injected ip 60 min after vehicle or drug administration; n represents the number of mice used for each dose of drug treatment.

Results

Naloxone in the dose range of 1 to 10 mg/kg, administered 5 min prior to the injection of pentobarbital, had no effect on either onset or duration of anesthesia. The onset time for anesthesia was between 2.5 and 3 min, whereas the duration was approximately 100 min in both saline- and naloxone-treated groups (table 1). Naltrexone, on the other hand, had no effect on the duration of pentobarbital anesthesia, but the two higher doses, 5 and 10 mg/kg, increased the onset time significantly.

Administration of morphine, 1, 2.5, and 5 mg/kg, had no effect on onset time or duration of pentobarbital-induced anesthesia (table 2). Although morphine, 2.5 mg/kg, decreased the duration of pentobarbital anesthesia from 192 to 140 min, and 5 mg/kg decreased the onset time for anesthesia from 2.5 to 2.2 min, statistical significance was not found.

D-Phenylalanine, 250 mg/kg, alone or in combination with D-leucine, 250 mg/kg, administered 60 min before pentobarbital injection did not affect either onset time or duration of anesthesia. The onset time remained unchanged at 2.5 min (table 3). Although there was a tendency for the duration of anesthesia to be decreased by D-phenylalanine alone and in

TABLE 4. Effect of Naltrexone on Pentobarbital LD_{50} in Mice

Treatment*	Pentobarbital Sodium LD_{50} (mg/kg)	Potency Ratio†
Saline solution	125 (114–138)	1.0 (0.9–1.2)
Naltrexone (10 mg/kg)	122 (117–126)	—

* Saline solution or naltrexone was injected 5 min prior to the administration of pentobarbital sodium. Four doses of pentobarbital were used with ten mice for each dose.

† The values in parentheses represent the 95 per cent confidence limits.

combination with D-leucine, statistical significance could not be obtained.

Pretreatment with naltrexone had no effect on pentobarbital toxicity in mice. The LD_{50} values of pentobarbital in groups treated with saline solution and with naltrexone, 10 mg/kg, were virtually identical, and were 125 and 122 mg/kg, respectively (table 4).

Discussion

The present studies indicate that brain enkephalins and endorphins do not participate in pentobarbital anesthesia and toxicity. The results were based on both direct and indirect manipulation of brain morphinomimetic substances. Naloxone and naltrexone were unable to antagonize pentobarbital anesthesia. The doses of both pure narcotic antagonists employed in the present investigation were high enough to antagonize motor effects of intraventricularly administered methionine-enkephalin and morphine[4] and also to inhibit the development of dependence on morphine induced by pellet implantation in mice.[13] The duration of anesthesia produced by the dose of pentobarbital used was more than 100 min. Berkowitz *et al.*[14] have reported that naloxone antagonized nitrous oxide analgesia in mice for about 95 min. Thus, if brain endorphins and enkephalins were indeed involved in pentobarbital anesthesia, naloxone should have antagonized it.

The second approach used was to study the effect of morphine on pentobarbital anesthesia. Mouse brain levels of enkephalins have been found to be 56 and 94 ng/brain in the morning and evening hours.[15] Since the molecular weight of enkephalin is about 570, they represent 0.1 and 0.2 nmol/brain in A.M. and P.M., respectively. Our earlier studies[10] have shown that 30 min after morphine 7.5 mg/kg, brain levels of morphine are 250 ng/g or 0.4 nmol/brain. Therefore, doses of morphine (1, 2.5, 5 mg/kg) that would increase brain levels of morphine in the same molar range as that of endogenous enkephalins were used. However, morphine failed to enhance pentobarbital anesthesia. It must also be recognized that morphine is longer-acting and more potent than the newly released enkephalins. We have earlier shown that intracerebral injection of enkephalins as much as to 7 μmol/kg does not produce narcosis,[4] and the amount of endogenous morphinomimetic substances released by drug-induced stimulation will not contribute much to anesthesia.

Enkephalins and endorphins are the naturally occurring opiate-like peptides.[1,2] Although they can produce analgesia[16] and inhibit opiate withdrawal signs[17] when administered intracerebrally, their effect is of short duration. The latter is attributed to rapid degradation, mainly by carboxypeptidase A and leucineaminopeptidase.[18,19] Derivatives resistant to these enzymes have much longer durations of action.[20,21] An approach to prolong and intensify the effects of endogenously produced endorphins was tried by using D-phenylalanine, an inhibitor of carboxypeptidase A.[11] This drug has been shown to produce analgesia in mice.[11] The effect of D-phenylalanine on analgesia was potentiated by combining it with D-leucine, an inhibitor of leucineaminopeptidase.‡ In the present studies, treatment with D-phenylalanine either alone or in combination with D-leucine failed to modify pentobarbital anesthesia.

Fürst *et al.*[9] reported that in the rat, naloxone, 1 mg/kg, significantly antagonized pentobarbital anesthesia and toxicity. In the present studies, naltrexone, 10 mg/kg, was ineffective in altering pentobarbital LD_{50}. The discrepancy between the present data and those of Fürst *et al.*[9] may be explained on the basis of species differences. Furthermore, not only did they inject naloxone concomitantly with pentobarbital, but the injections of naloxone were repeated every 30 min after pentobarbital administration until the return of the righting reflex or death. Our experience indicates tha naltrexone injections need not be repeated so often to antagonize the effects of potent narcotics.[13] More recently, we have found that a single intravenous injection of naltrexone, 10 mg/kg, is able to protect the rat from four lethal doses of pentazocine given every 30 min.§

The authors[9] also suggested the use of naloxone in management of barbiturate intoxication, and cited several references where naloxone was used in treating intoxication produced by a variety of drugs. However, since poly-drug abuse is rather common,[22] the beneficial effects of naloxone in the studies cited may have been due to the presence of narcotics in the drugs consumed.

Many general anesthetics provide slight analgesic

‡ Ehrenpreis S, personal communication.

§ Bhargava HN, Thompson EB, unpublished observations.

effects,[14] which may be related to the release of enkephalins and endorphins in the brain. It is possible that this phase of anesthesia is antagonized by narcotic antagonists. A better approach for measuring anesthesia is to determine minimum alveolar concentrations of the anesthetics. Such a study also failed to show any effect of naloxone even at extremely high doses (250 mg/kg) on general anesthesia produced by halothane.[6]

Drugs that interact with opiate receptors modify the binding of ^{3}H-naloxone to the opiate receptors in brain.[23] Phenobarbital, even at 1 mM, failed to alter the stereospecific opiate receptor binding,[23] indicating that barbiturates do not affect opiate binding sites, and an absence in the commonality in their actions.

In summary, the present studies indicate that the pharmacologic alteration of endogenous opiates or administration of opiates exogenously does not affect pentobarbital anesthesia, and a role of enkephalins and endorphins in barbiturate anesthesia is unlikely. Naltrexone, a pure narcotic antagonist, was also ineffective in modifying barbiturate toxicity. Finally, these studies provide no rationale for the use of narcotic antagonists in treating pentobarbital toxicity.

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