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Role of nitric oxide in the induction and expression of morphine tolerance and dependence in mice

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1 The possible involvement of nitric oxide (NO) in the induction and expression of morphine tolerance and dependence was studied in mice. A two-day repeated injection regimen was used to induce morphine tolerance and dependence. Tolerance was assessed by the tail flick test and physical dependence by naloxone challenge, on the third day.

2 Two days pretreatment with L-arginine (20 mg kg^{-1}, twice daily) or D-N^G-nitro arginine methyl ester (D-NAME, 20 mg kg^{-1}, twice daily) alone had no effect on subsequent morphine antinociception. L-N^G-monomethyl arginine (L-NMMA, 10 mg kg^{-1}, twice daily) for two days led to a slight increase (not statistically significant) in morphine antinociception; while L-N^G-nitro arginine methyl ester (L-NAME, 10 mg kg^{-1}, twice daily) for two days led to attenuation of morphine analgesia. None of the animals treated with these drugs alone showed signs characteristic of the opioid withdrawal syndrome upon naloxone challenge.

3 Induction phase L-arginine slowed the development of opioid tolerance and physical dependence, while L-NAME and L-NMMA led to a higher degree of tolerance but had no effect on the development of physical dependence.

4 L-Arginine and D-NAME had no effect on the expression of morphine tolerance and physical dependence. Expression phase L-NAME and L-NMMA, on the other hand, attenuated morphine tolerance and reduced the incidence of withdrawal signs.

5 NO may, therefore, play a role in both phases of morphine tolerance and dependence: elevation of NO levels during the induction phase delays the development of opioid tolerance/dependence, while inhibition of NO synthase accelerates the development of tolerance. Inhibition of NO attenuates the expression of both tolerance and physical dependence.

Keywords: L-Arginine; L-NAME; L-NMMA; nitric oxide; nitric oxide synthase inhibitors; opioid dependence-tolerance; naloxone-precipitated withdrawal

Introduction

The mechanisms involved in the development and expression of opioid tolerance and dependence remain unclear despite a great deal of research. The characteristics of the opioid withdrawal syndrome suggest an involvement of excitatory neurotransmitters in drug-dependence phenomena. This is borne out by reports that show that antagonists of excitatory amino acid receptors suppress opioid withdrawal signs (Rasmussen *et al.*, 1991; Trujillo & Akil, 1991; Koyuncuoglu *et al.*, 1991). The finding that nitric oxide (NO) is produced postsynaptically in response to activation of central excitatory amino acids (Knowles *et al.*, 1989; Garthwaite, 1991) raises the possibility that suppression of the withdrawal signs by NMDA antagonists may be linked to inhibition of NO synthesis. The possible involvement of the NO-guanosine 3′:5′-cyclic monophosphate (cyclic GMP) system (Bredt & Synder, 1989) in these phenomena has been implicated by reports showing that nitric oxide synthase (NOS) inhibitors abolish some aspects of the naloxone-precipitated withdrawal syndrome (e.g. Adams *et al.*, 1993; Cappendijk *et al.*, 1993), and that isosorbide, an NO donor, induced a quasi-morphine abstinence syndrome and exacerbated opioid withdrawal signs in dependent rats (Adams *et al.*, 1993).

It has been postulated that opioid tolerance and dependence may be distinct phenomena, developing independently of each other (Wüster *et al.*, 1985; Johnson & Fleming, 1989). Moreover, both phenomena have two distinct phases, the induction phase during which the processes underlying the observed changes occur, and the expression phase, which refers to the changes noted upon cessation of drug administration of an antagonist. A neurotransmitter or neuromodulator may play different roles in the different phases of tolerance or dependence (Wüster *et al.*, 1985; Bhargava, 1994). 5-Hydroxytryptamine (5-HT) enchances the development of tolerance and dependence (Way *et al.*, 1968; Ho *et al.*, 1972), but has inhibitory effects in the expression phase (Samanin *et al.*, 1980; Neal & Sparber, 1986); noradrenaline, for its part, appears to have little role in the development of opioid tolerance and physical dependence but plays an important role in the abstinence syndrome (Dambisya *et al.*, 1991; Bhargava, 1994); and γ-aminobutyric acid (GABA) enhances the development of tolerance and dependence, with hardly any effect on the expression phase (Ho *et al.*, 1976), to quote but a few. Most of the published reports on the possible involvement of NO in opioid tolerance/dependence have investigated only one of these phenomena or only one of the phases (e.g. Kolesnikov *et al.*, 1992; Adams *et al.*, 1993; Cappendijk *et al.*, 1993). In the present study, the effects of the NOS inhibitors L-N^G-nitro arginine methyl ester (L-NAME) and L-N^G-monomethyl arginine (L-NMMA) and the NO precursor, L-arginine, on the development and expression of both tolerance to and physical dependence on morphine were investigated. A preliminary account of this work was presented at the 11th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology (Dambisya & Lee, 1995a).

Methods

Animals

Male Swiss albino mice (25–30 g) were used in accordance with our institutional guidelines on animal experimentation. The animals were obtained from the Laboratory Animal

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Centre (Singapore) and kept under air-conditioning (21 ± 2°C; 70–80% humidity; 12 h light/darkness cycle) in the Animal Holding Unit (N.U.S.) for at least 72 h before use. Food and water were supplied *ad libitum*. Each animal was used only once. All experiments were conducted in the period between 09 h 30 min and 12 h 00 min.

Induction of opioid tolerance and dependence

Morphine dependence and tolerance was induced in mice by a repeated-injection treatment schedule adapted from that previously described by Dambisya *et al.* (1991). We aimed for at least a 5 fold increase in ED_{50}, which was achieved by a regimen of morphine in graded doses twice daily for two days as follows: Day 1, 30 mg kg^{-1}, a.m. and 45 mg kg^{-1}, p.m.: Day 2, 60 mg kg^{-1}, a.m. and 90 mg kg^{-1}, p.m. On the third day, the animals were assessed for both tolerance and dependence as described by Way *et al.* (1969). Test drugs were given shortly before morphine throughout the induction period for their effects on the development of morphine tolerance and dependence. The controls received saline at the corresponding times.

Assessment of morphine tolerance and dependence

The loss of the antinociceptive effects of morphine in the tail-flick assay (D'Amour & Smith, 1941) was used, with modifications as described in detail elsewhere (Dambisya *et al.*, 1990), to assess the degree of tolerance. A pilot study done showed that after the two-day induction period, the ED_{50} value for morphine shifted from about 1.5 mg kg^{-1} in naive mice (Dambisya & Lee, 1994; 1995b) to 9.6 mg kg^{-1} (9.1–10.4, 95% confidence limits); 3 dose levels with 14 animals per dose were used to calculate the ED_{50}. We subsequently used 10 mg kg^{-1} in the assessment of tolerance. Physical dependence was assessed by the occurrence of jumping and wet dog shakes following administration of naloxone (4 mg kg^{-1}, i.p.). After the naloxone injection, the animals were observed on a platform (40 cm long, 25 cm wide and 45 cm high) in groups of 6–7 for 15 min. An animal was considered positive for jumping behaviour if it jumped off the platform. Only the occurrence or absence of a particular sign was noted for each animal. For the assesssment of the effects of various agents on the induction of morphine tolerance and dependence, the test drugs were co-administered with morphine throughout the induction, with none given on the test day. For the assessment of the effects of the various drugs on the expression of tolerance and dependence, animals that had received only morphine in the induction phase were used, the test drugs were administered only on the test day, 10 min prior to acute morphine.

Drugs

Morphine sulphate (Delta West, U.S.A.); and L-N^G-nitro arginine methyl ester (L-NAME), L-N^G-monomethyl arginine (L-NMMA), D-N^G-nitro arginine methyl ester (D-NAME) and L-arginine (Sigma, U.S.A.) were dissolved in physiological saline solution to such concentrations that requisite doses were administered in a volume of 10 ml kg^{-1}. Saline controls were used in all cases. The doses of the drugs used were 10 mg kg^{-1} for L-NAME and L-NMMA, and 20 mg kg^{-1} for D-NAME and L-arginine, similar to those used in acute interaction studies (Dambisya & Lee, 1995b).

Statistical analysis

The results of the tail flick test are presented as mean ± s.e.mean. The data were analyzed and compared by one-way analysis of variance followed by Bonferroni's *t* tests where indicated. Results for the physical dependence tests are presented as percentage of animals showing a particular sign, and were compared by the Chi-squared test. $P<0.05$ was considered the limit of significance in both tests.

Results

Two days pretreatment with L-arginine (20 mg kg^{-1}) and D-NAME (20 mg kg^{-1}) had no effect on morphine antinociception; L-NMMA (10 mg kg^{-1}) for two days led to a slight increase (not statistically significant) in morphine antinociception; while L-NAME (10 mg kg^{-1}) two-day treatment led to attenuation of morphine analgesia (Table 1). None of the animals treated with these agents alone exhibited any signs characteristic of the opioid withdrawal syndrome after the administration of naloxone; nor did naloxone elicit any jumps or wet dog shakes in other morphine-naive animals. Table 2 shows the effects of the various drugs on the induction of morphine tolerance. Given together with morphine in the induction phase, L-arginine slowed the development of opioid tolerance, while L-NAME and L-NMMA led to a higher degree of tolerance compared to the control group that received saline and morphine at the corresponding times. D-NAME given in the induction phase had no effect on opioid tolerance (Table 2). The effects of these drugs on the induction of morphine-dependence are shown in Figure 1. Concurrent administration of L-arginine and morphine in the induction phase led to less marked dependence, evident from a significant reduction in the withdrawal signs compared to the saline-morphine group; while co-administration of D-NAME, L-NAME or L-NMMA with morphine had no effect on the induction of opioid dependence (Figure 1).

Table 3 shows the effects of these agents on the expression of morphine tolerance in mice. The expression of morphine tolerance was not effected by (acute) pretreatment with L-arginine and D-NAME; while pretreatment with L-NAME and L-NMMA attenuated morphine tolerance (Table 3). The effects of the various drugs on the expression of physical dependence are shown in Figure 2. Pretreatment with L-NAME

Table 1 Modification of morphine antinociception by L-arginine, D-NAME, L-NAME and L-NMMA

Chronic treatment (twice daily × 2 days)	*Treatment*	n	*Tail flick time* (Mean ± s.e.mean)	*% analgesia*
Saline (10 ml kg^{-1})	Saline (10 ml kg^{-1})	12	2.89 ± 0.26	Control
Saline (10 ml kg^{-1})	Morphine (1.5 mg kg^{-1})	13	6.52 ± 0.44	51.1
L-Arginine (20 mg kg^{-1})	Morphine (1.5 mg kg^{-1})	12	6.78 ± 0.61	54.7
D-NAME (20 mg kg^{-1})	Morphine (1.5 mg kg^{-1})	12	6.42 ± 0.57	49.6
L-NAME (10 mg kg^{-1})	Morphine (1.5 mg kg^{-1})	13	5.10 ± 0.52[a]	31.1
L-NMMA (10 mg kg^{-1})	Morphine (1.5 mg kg^{-1})	12	7.29 ± 0.49	61.9

[a]Significantly lower than the corresponding value for saline-morphine group.

Table 2 Effects of L-arginine, L-NAME, D-NAME and L-NMMA on the induction of morphine tolerance

Induction regimen	*Acute treatment*	n	*Tail flick latency* (s) (Mean ± s.e.mean)	*% analgesia*
Saline-saline	Saline (10 ml kg^{-1})	32	3.40 ± 0.22	Control
Saline-morphine	Morphine (10 mg kg^{-1})	23	6.90 ± 0.53	53.0
L-Arginine-morphine	Morphine (10 mg kg^{-1})	21	8.46 ± 0.43[b]	76.7
D-NAME-morphine	Morphine (10 mg kg^{-1})	21	7.01 ± 0.59	54.7
L-NAME-morphine	Morphine (10 mg kg^{-1})	22	4.59 ± 0.47[a]	18.0
L-NMMA-morphine	Morphine (10 mg kg^{-1})	23	5.21 ± 0.45[a]	27.4

[a]Significantly lower than corresponding value for the saline-morphine group; [b]significantly higher than corresponding saline-morphine group.

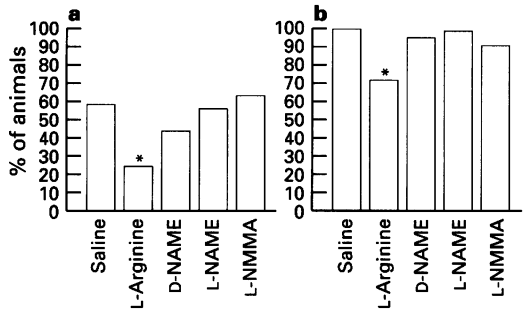

Figure 1 Effects of the various drugs on the induction of physical dependence on morphine. The treatment regimen refers to the drugs administered during the induction period, the test drugs were not administered on the assessment day. (a) Withdrawal jumping: The incidence of jumping in animals given D-NAME-morphine ($n=25$, incidence 11/25), L-NAME-morphine ($n=25$, incidence 14/25) or L-NMMA-morphine ($n=25$, incidence 16/25) is similar to that in the saline-morphine group ($n=24$, incidence 14/24), while withdrawal jumping was significantly reduced in the L-arginine-morphine group ($n=25$, incidence 6/25, $^*P<0.05$). (b) Incidence of wet-dog shakes: The L-arginine-morphine group exhibited a significantly lower incidence of wet dog shakes (18/25, $^*P<0.05$) than the saline-morphine group (24/24). The other agents had no significant effect on the incidence of wet dog shakes, being D-NAME (24/25), L-NAME (25/25) and L-NMMA (23/25).

or L-NMMA prior to naloxone reduced the severity of the withdrawal signs, while pretreatment with L-arginine and D-NAME had no demonstrable effect (Figure 2).

Discussion

These data show that elevation of NO levels during the induction phase (using L-arginine) delays the development of opioid tolerance and dependence, while reducing NO levels (with L-NAME and L-NMMA) accelerates the development of tolerance with no effect on that of opioid dependence. In the expression phase, NOS inhibition diminishes the degree of morphine tolerance and also attenuates withdrawal jumping and wet dog shakes. L-Arginine, in the doses used in the present study, had no effect on the expression of morphine tolerance or physical dependence. In acute interaction studies, we found that L-arginine attenuated, while the NOS inhibitors enhanced, morphine antinociception (Dambisya & Lee, 1995b). L-Arginine appears to lose its attenuating effect on morphine antinociception after repeated administration. The attenuation of morphine effects after repeated treatment with L-NAME suggests that prolonged inhibition of NO synthesis reduces antinociceptive responses to morphine. The apparent difference beween the two NOS inhibitors may be due to the fact that L-NAME is more potent than L-NMMA. Since the time course of the drug-effects were not determined, the possibility cannot be ruled out that the tendency for L-NMMA to accentuate morphine effects after two-day treatment could be an artifact due to its fairly long duration of action; the drug given the previous evening may still be effective in enhancing morphine antinociception. The attenuation of morphine tolerance by the NOS inhibitors administered in the expression phase indicates that NOS inhibition at this stage has a similar effect on morphine antinociception to its effect for acute morphine (Dambisya & Lee, 1995b) and offers further support for the possibility that chronic morphine treatment has no effect on NOS activity (Babey *et al.*, 1994).

Our findings that L-NAME and L-NMMA enhanced the development of morphine tolerance (Table 2) are at variance with those reported by several workers (e.g. Kolesnikov *et al.*, 1992; 1993; Thorat *et al.*, 1993; Elliot *et al.*, 1994; Babey *et al.*, 1994) who showed that NOS inhibitors attenuated the development of morphine tolerance. It is difficult to account for the discrepancy between those studies and ours, but it may be related partly to the degree of tolerance engendered. Elliot *et al.* (1994), for instance, used doses of NOS inhibitors that did not affect the tail-flick reaction time or the ED_{50} of morphine, and the level of tolerance achieved was a 2–3 fold shift in ED_{50}. In contrast, the level of tolerance achieved in the present study was a 6–7 fold shift in ED_{50}. Also, the dose and regimen of L-NAME we employed was capable of attenuating morphine antinociception, the acceleration of morphine tolerance noted in combination may be the result of two mechanisms working to reduce antinociceptive responses to morphine. That, however, would not explain enhancement of morphine tolerance by L-NMMA, given that with that dosing regimen (10 mg kg^{-1} twice daily for two days) L-NMMA tended to enhance morphine antinociception (Table 1). It has been postulated that the development of opioid tolerance and dependence is a continuous process, probably beginning with the first exposure (Kosersky *et al.*, 1974; Wong & Bentley, 1978; Yano *et al.*, 1979; Eisenberg, 1982; Bickel *et al.*, 1988; Heishman *et al.*, 1989). The intensity of dependence and tolerance is, however, related to the total exposure to effective concentrations of opioid (Goldstein & Schulz, 1973). The induction regimen used in the present study is justified by the fact that it led to a measurable (6–7 fold) increase in morphine ED_{50}, and all the animals showed wet dog shakes and/or withdrawal jumping; after all there is apparently no qualitative difference between acutely- and chronically-induced opioid tolerance/dependence (Eisenberg, 1982). While we did not exclude changes in blood pressure as a confounding factor in these results, it is unlikely that they were a major factor. Firstly, the dose of NOS inhibitors used (10 mg kg^{-1}) was low compared to that used by others (for example Cappendijk *et al.* (1993) used up to 100 mg kg^{-1} L-NAME and 400 mg kg^{-1} L-NAME) and was chosen for its lack of side effects (Moore *et al.*, 1991; Dambisya & Lee, 1995b). Secondly, it is not clear whether or not blood pressure changes, *per se*, have any effect on opioid tolerance/dependence as exemplified by the finding

Table 3 Effects of L-arginine, L-NAME, D-NAME and L-NMMA on the expression of morphine tolerance[a]

Pretreatment (10 min before morphine)	*Acute treatment*	n	*Tail flick latency* (s) (Mean ± s.e.mean)	*% analgesia*
Saline (10 ml kg^{-1})	Saline (10 ml kg^{-1})	19	3.01 ± 0.19	Control
Saline (10 ml kg^{-1})	Morphine (10 mg kg^{-1})	21	6.48 ± 0.42	49.6
L-Arginine (20 mg kg^{-1})	Morphine (10 mg kg^{-1})	18	5.97 ± 0.56	42.3
D-NAME (20 mg kg^{-1})	Morphine (10 mg kg^{-1})	17	6.23 ± 0.58	46.1
L-NAME (10 mg kg^{-1})	Morphine (10 mg kg^{-1})	18	8.02 ± 0.52[b]	71.7
L-NMMA (10 mg kg^{-1})	Morphine (10 mg kg^{-1})	19	7.78 ± 0.46[b]	68.2

[a]All the animals received morphine alone during the induction period. [b]Significantly higher than corresponding value for the saline-morphine group.

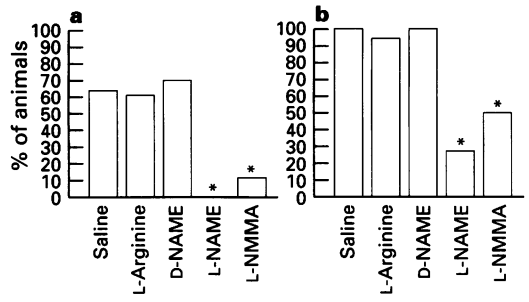

Figure 2 Effects of the various drugs on the expression of physical dependence on morphine. The test drugs were administered only on the assessment day, the induction regimen consisted of morphine alone. (a) Withdrawal jumping: The incidence of jumping in animals pretreated with D-NAME ($n=17$, incidence 12/17) and L-arginine ($n=18$, incidence 11/18) was similar to that in the saline-pretreated group ($n=20$, incidence 13/20), while withdrawal jumping was significantly reduced in the L-NMMA pretreated group ($n=16$, incidence 2/16, $^*P<0.05$), and abolished in the L-NAME pretreated group ($n=18$, incidence 0/18). (b) Incidence of wet-dog shakes: The L-NAME and L-NMMA pretreated groups exhibited significantly lower incidences of wet dog shakes (5/18 and 8/16 respectively, $^*P<0.05$) than the saline pretreated group (20/20). D-NAME or L-arginine pretreatment had no effect on the incidence of wet dog shakes (17/17 and 17/18, respectively).

that clonidine and isosorbide, both of which lower blood pressure, have opposite effects on the abstinence syndrome (Tseng *et al.*, 1975; Adams *et al.*, 1993; Cappendijk *et al.*, 1993).

In the present study L-arginine delayed the development of morphine tolerance (Table 2) and dependence (Figure 1), in sharp contrast to the findings reported by Babey *et al.* (1994) that L-arginine 'induces' tolerance in opioid naive mice through NOS, and accelerates tolerance when co-administered with morphine while NOS inhibitors prevent morphine tolerance. Our findings could be interpreted in terms of the possible role of the L-arginine-nitric oxide pathway in pain and opioid tolerance/dependence. Chronic pain is characterized by a state of abnormal excitability in neurones of the CNS (central sensitization), which may involve the excitatory amino acid receptors and activation of the NO-cyclic GMP pathway (Kitto *et al.*, 1992; Kawabata *et al.*, 1993; Meller & Gebhart, 1993). Chronic administration of L-arginine may lead to such changes in the NO-cyclic GMP pathway as to mimic the situation observed in chronic pain states. It has been shown that morphine fails to produce tolerance when administered in the presence of formalin pain in rats and mice (Vaccarino *et al.*, 1993; Rahman *et al.*, 1993). Furthermore, in clinical practice, opioid tolerance and dependence are rare in patients suffering from chronic pain compared to those who take opioids in the absence of pain (Kanner & Foley, 1981). Indeed, Rahman *et al.* (1994) have sought to account for the escalating analgesic requirements in chronic pain states in terms of the involvement of pain-associated anxiety and not tolerance. Our results suggest that high levels of NO may play a role in delaying the development of opioid tolerance and dependence observed in chronic pain states (Kanner & Foley, 1981; Vaccarino & Couret, 1993; Vaccarino *et al.*, 1993; Rahman *et al.*, 1993; 1994).

The finding that expression-phase NOS inhibitors suppress the naloxone-precipitated withdrawal syndrome (Figure 2) is in line with earlier reports to this effect (Adams *et al.*, 1993; Cappendijk *et al.*, 1993; Kimes *et al.*, 1993; Thorat *et al.*, 1994). The NOS inhibitors may ameliorate the expression of morphine dependence through suppression of the NO-cyclic GMP system, which is thought to mediate the effects of excitatory amino acid receptor activation (Bredt & Synder, 1989; Garthwaite, 1991; Morris *et al.*, 1992). The lack of effect of the NOS inhibitors on the development of opioid dependence is also in line with the conclusions reached by others (e.g. Kimes *et al.*, 1993) that inhibition of NOS affects the expression but not the development of opioid dependence.

The present study provides further support for the possibility that nitric oxide may play a role in the phenomena of opioid tolerance and dependence. Our findings with respect to the effects of L-arginine on the development of tolerance and physical dependence are at variance with some of the published work from elsewhere, but we speculate that the changes associated with L-arginine could be similar to those that occur in chronic pain states, which also show slower rates of development of tolerance and dependence. The present study adds to the growing body of scientific evidence showing that NOS inhibitors may be useful for the amelioration of the opioid withdrawal syndrome.

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