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Mediation of nitric oxide in inhibitory effect of morphine against electroshock-induced convulsions in mice

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Abstract

Nitric oxide (NO) and morphine have been coupled in many physiological as well as pathological processes. The present study examined the involvement of the L-arginine/NO pathway in the anticonvulsant properties of systemic morphine (2–30 mg/kg) against electroshock seizures (ECS) in mice. Morphine decreased the intensity of maximal electroshock seizures (MES) and increased the threshold for ECS. Neither the NOS substrate L-arginine (30, 60, and 100 mg/kg), the reversible nonspecific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 3, 10, and 30 mg/kg), the irreversible specific inducible NOS inhibitor aminoguanidine (20, 50, and 100 mg/kg), nor the opioid receptor antagonist naloxone (0.1, 0.3, and 1 mg/kg) did alter per se the ECS threshold or the intensity of MES at doses used. However, both naloxone and L-NAME, but not aminoguanidine, inhibited the anticonvulsant effects of morphine (30 mg/kg) against ECS, while L-arginine potentiated the anticonvulsant effects of lower doses of morphine (2 or 10 mg/kg). Low doses of naloxone (0.1 or 0.3 mg/kg) or L-NAME (3 mg/kg), which did not alter morphine effect per se, showed additive anticonvulsant effects against MES. Thus, the L-arginine/NO pathway seems to play a role in the anticonvulsant properties of morphine against ECS and this mediation involves the constitutive, but not the inducible, form of nitric oxide synthase.

Keywords: Electroshock seizures; Morphine; Nitric oxide; Naloxone; Nitric oxide synthase inhibitors; Mice

1. Introduction

Electroshock seizure (ECS) is the best-studied animal model of generalized tonic–clonic seizures (Fisher, 1989). Drugs able to inhibit ECS in animals are considered to be candidate therapies for primary and secondary generalized tonic–clonic epilepsies (Löscher and Schmidt, 1988). Meanwhile, the activation of opioid receptors, either endogenously or through opioid receptor agonists, can induce anticonvulsant and/or proconvulsant effects in different seizure paradigms (Frenk, 1983; Lauretti et al., 1994; Przewlocka et al., 1995; Sagratella and Massotti, 1982; Tortella et al., 1985). In this regard, morphine and opioid peptides exert anticonvulsant effects against maximal electroshock seizures (MES) and increase the ECS threshold in experimental animals (Berman and Adler, 1984; Frey, 1988; Karadag et al., 2000; Puglisi-Allegra et al., 1985). This morphine-induced anticonvulsant property is mediated by mu-opioid receptors as evidenced by a blockade by mu-opioid receptor-sensitive low doses of opioid receptor antagonists such as naloxone or naltrexone (Berman and Adler, 1984; Czuczwar and Frey, 1986). Frey (1988) reported that both mu-opioid and kappa-opioid receptors could inhibit the anticonvulsant effect of morphine against electroconvulsive seizure threshold. In a model using graded seizure responses to suprathreshold cerebral electroshock in mice, morphine exerted a proconvulsant effect at a non-mu-opioid receptor plus a simultaneous anticonvulsant effect at a mu-opioid receptor, while delta-opioid receptor blockade increased the seizure severity (Pinsky et al., 1986). Moreover, the anticonvulsant effect of morphine against electrical seizure probably involves increased GABA and histamine release in brain (Karadag et al., 2000; Sagratella and Massotti, 1982).

Nitric oxide (NO) is a small, membrane-diffusing molecule synthesized from L-arginine by nitric oxide synthase (NOS) that acts as a neuronal messenger in the central nervous system (Bredt and Snyder, 1990; Bredt et

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al., 1990). Recently, morphine and NO have been coupled in many physiological as well as pathological processes including morphine-induced antinociception (Brignola et al., 1994; Ferreira et al., 1991), tolerance and physical dependence (Dambisya and Lee, 1996; Kolesnikov et al., 1993), regulation of food intake (Calignano et al., 1993), gastroprotection (Gyires, 1994), constipation (Calignano et al., 1991), and suppression of lymphocyte proliferation (Fecho et al., 1994). Moreover, morphine stimulates NO production in the vasculature of rat median eminence (Prevot et al., 1998; Stefano et al., 1997), implying a NO-mediated mechanism to increase neurotransmitter release. Furthermore, NO has modulatory effects on some seizure models including those induced by excitatory amino acids, while having limited effects on ECS (Baran et al., 1997; Przegalinski et al., 1994, 1996; Urbanska et al., 1996). We recently showed that NO is involved in both anticonvulsant and proconvulsant effects induced by different doses of morphine against seizures induced by the GABA receptor antagonist pentylenetetrazole (Homayoun et al., 2002a). In the present study, we examined the possible involvement of nitricoxidergic pathway in the anticonvulsant effect of morphine against ECS and also assessed the role of inducible versus constitutive NOS in this effect. For this purpose, we used the NOS substrate L-arginine, the reversible nonspecific NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), and the irreversible specific inhibitor of inducible nitric oxide synthase (iNOS) aminoguanidine. The effects of these agents on morphine-induced anticonvulsive properties were separately evaluated using ECS threshold and the intensity of response to MES.

2. Materials and methods

2.1. Animals

Male NMRI mice (Pasteur Institute of Iran) weighing 24–30 g were used. The animals were housed in a temperature-controlled room (24 ± 1 °C) on a 12-h light/dark cycle with free access to food and water. All procedures were carried out in accordance with institutional guidelines for animal care and use and all possible measures were taken to minimize the animals' discomfort including immediate euthanasia after acute experiments. Each animal was used only once.

2.2. Drugs

The drugs used were L-arginine, L-NAME, morphine sulfate (Sigma, Poole, UK), aminoguanidine (Sigma, St. Louis, USA), and naloxone hydrochloride (Tolid-daru, Tehran, Iran). All drugs were dissolved in physiological saline solution to such concentrations that requisite doses were administered in a volume of 10 ml/kg. In all experiments, morphine was administered subcutaneously and all other drugs were administered intraperitoneally.

2.3. Assessment of anticonvulsant activity

The MES test with suprathreshold stimulation was carried out via ear clip electrodes by means of a stimulator that delivered a fixed current of 50 mA with a pulse frequency of 50 s^{-1} for 0.2 s (Löscher and Lehman, 1996). The duration of tonic flexion (TF) and tonic extension (TE) following MES was recorded and the extensor–flexor ratio (TE/TF ratio), known to be a reliable measure of seizure severity (Swinyard, 1972), was calculated. A ratio larger for the test group than for the control group indicates that the seizures in the test group are more severe, and vice versa.

The threshold of ECS was determined using the "staircase" method adapted from Swinyard (1972). The intensity of the stimulus delivered via ear clip electrodes was varied by an up-and-down method in which the current (with a pulse frequency of 50 s^{-1} for 0.2 s) was lowered or raised by 0.1 log intervals, depending on the response of the animal previously tested (starting from an initial baseline current of 10 mA). Each animal was tested once. Thus, the current was increased to the next increment if an animal failed to exhibit a full tonic hindlimb extension, or alternatively decreased if full tonic seizures were induced. The data thus generated in groups of 15 mice were used to calculate the threshold current inducing hindlimb extension in 50% of the mice (CC_{50} with confidence intervals for 95% probability) by the method of Kimball et al. (1957).

2.4. Treatment

Groups of 10 animals were used in MES experiments except for the saline control group (Fig. 1A), which consisted of 16 animals. In ECS experiments, groups of 15 animals were used. In all experiments, morphine or saline was administered 45 min before testing and all other tested substances were administered 60 min before testing.

In the first series of experiments, the effects of different doses of naloxone (0.1, 0.3, and 1 mg/kg), L-NAME (3, 10, and 30 mg/kg), aminoguanidine (20, 50, and 100 mg/kg), and L-arginine (30, 60, and 100 mg/kg)—administered 60 min before testing—on the intensity of MES was determined. In the second series of experiments, the effects of different doses of morphine (2, 5, 10, and 30 mg/kg) administered 45 min before testing on MES were examined. Moreover, the effects of the mentioned doses of naloxone, L-NAME, and aminoguanidine, administered 15 min before 30 mg/kg morphine and 60 min before testing on MES, were determined. Moreover, the effects of L-NAME (10 mg/kg) or aminoguanidine (100 mg/kg) prior to 5 mg/kg morphine were examined using same pretest intervals. The effects of L-arginine (30, 60, and 100 mg/kg),

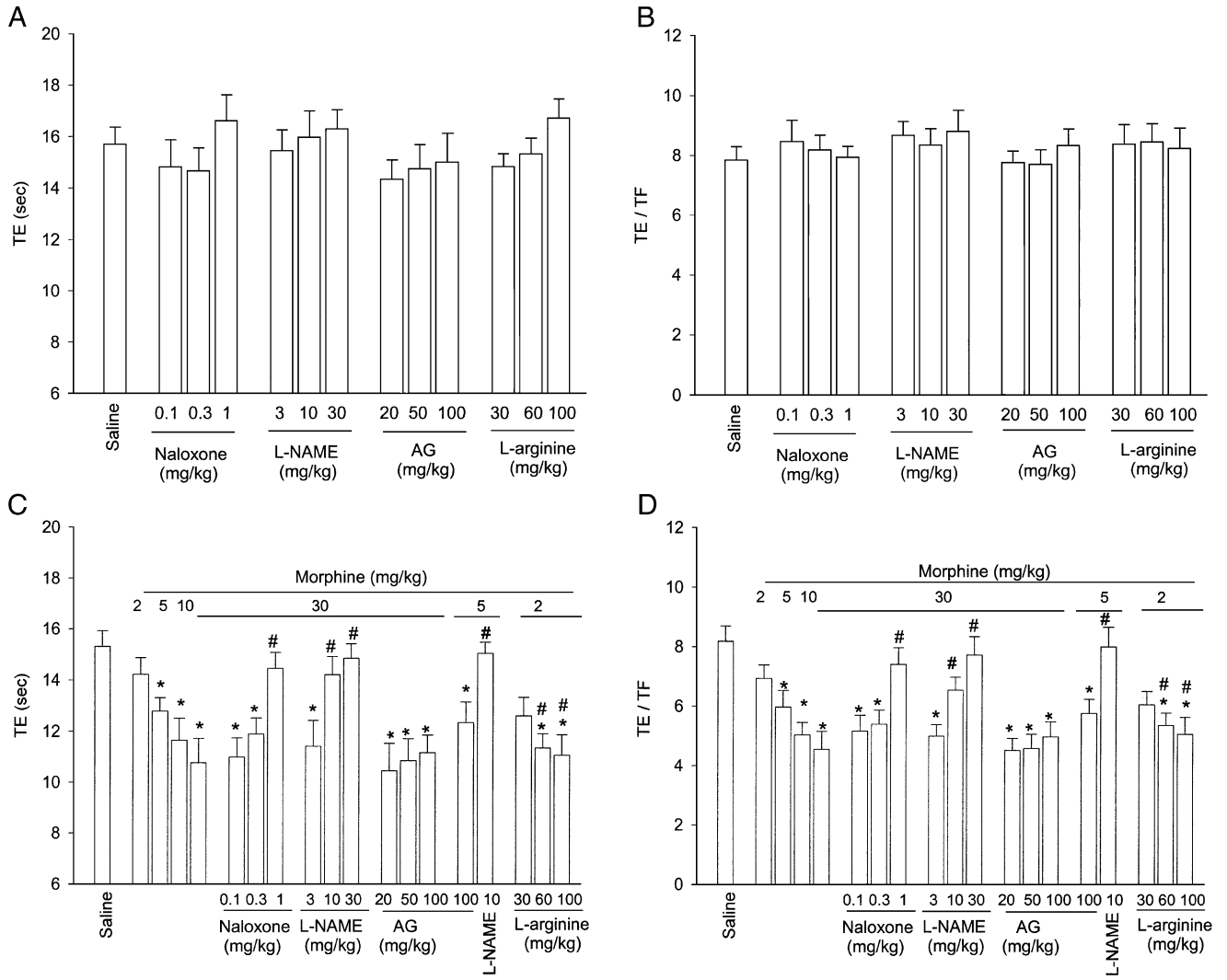

Fig. 1. Effects of naloxone, L-NAME, L-arginine, and aminoguanidine (AG) on MES in the presence (C,D) or absence (A,B) of morphine in mice. (A,C) The duration of TE and (B,D) the ratio of the duration of TE to the duration of TF (TE/TF). Morphine was administered 45 min and other drugs were administered 60 min before testing. Data are expressed as mean ± S.E.M. of 16 (saline control group in A and B) or 10 (other groups) animals. * $P<.05$, ** $P<.01$, *** $P<.001$ compared to saline control group, and $^{\#}P<.05$, $^{\#\#}P<.01$, $^{\#\#\#}P<.001$ compared to the corresponding morphine group.

administered 15 min before 2 mg/kg morphine and 60 min before testing on MES, were also determined. In the third series of experiments, the effects of concomitant administration of lower doses of naloxone (0.1 or 0.3 mg/kg) and L-NAME (3 mg/kg), 15 min before either saline or 30 mg/kg morphine and 60 min before testing, were assessed. In the fourth series of experiments, ECS threshold was determined in groups of mice receiving saline or morphine (10 and 30 mg/kg) 45 min before testing and also in other groups receiving a concomitant administration of L-NAME (3 and 10 mg/kg) or aminoguanidine (100 mg/kg) 60 min before testing, either alone or in combination with 30 mg/kg morphine (45 min before testing). Other groups in this experiment received L-arginine (30 and 60 mg/kg) 60 min before testing, either alone or in combination with 10 mg/kg morphine (45 min before testing). Doses used were chosen based on previously published works (Al-Shabanah et al., 2000; Dehpour et al., 1998; Karadag et al., 2000; Nahavandi et al., 1999).

2.5. Statistics

Data are expressed as mean ± S.E.M. of 10 mice in MES experiments and as CC_{50} with 95% confidence intervals in ECS threshold experiments. In MES experiments, a one-way analysis of variance (ANOVA), followed by post hoc Student–Newman–Keuls test, was used to compare the duration of TE or the TE/TF ratio between different groups. In ECS threshold experiments, the significance of differences between control and drug-treated groups was calculated by the two-sided Student's t test

(Löscher and Lehman, 1996). A probability level of .05 was accepted as significant.

3. Results

3.1. MES

As shown in Fig. 1, different doses of naloxone (0.1–1 mg/kg), L-NAME (3–30 mg/kg), aminoguanidine (20–100 mg/kg), and L-arginine (30–100 mg/kg) did not alter the duration of TE (Fig. 1A) or the TE/TF ratio (Fig. 1B) compared to the control saline group ($P>.05$). Morphine dose-dependently decreased both the duration of TE [Fig. 1C; $F(4,45)=6.22$, $P<.001$] and the TE/TF ratio [Fig. 1D; $F(4,45)=9.32$, $P<.001$]. In mice treated with 30 mg/kg morphine, both naloxone [Fig. 1C: TE, $F(4,45)=8.10$, $P<.001$; Fig. 1D: TE/TF, $F(4,45)=9.66$, $P<.001$] and L-NAME [Fig. 1C: TE, $F(4,45)=6.82$, $P<.001$; Fig. 1D: TE/TF, $F(4,45)=11.06$, $P<.001$], but not aminoguanidine [Fig. 1C: TE, $F(4,45)=5.62$, $P<.001$; Fig. 1D: TE/TF, $F(4,45)=10.48$, $P<.001$; post hoc $P>.05$], inhibited the anticonvulsant effect of morphine. Similarly, in mice treated with 5 mg/kg morphine, L-NAME [10 mg/kg; Fig. 1C: TE, $F(3,36)=4.31$, $P<.01$; Fig. 1D: TE/TF, $F(3,36)=3.79$, $P<.01$; post hoc $P>.05$], but not aminoguanidine [100 mg/kg; Fig. 1C: TE, $F(3,36)=4.31$, $P<.01$; Fig. 1D: TE/TF, $F(3,36)=6.88$, $P<.001$; post hoc $P>.05$] inhibited the anticonvulsant effect of morphine. L-arginine at the dose range used did not, by itself, alter the duration of TE or the TE/TF ratio ($P>.05$), but potentiated the effect of 2 mg/kg morphine against MES [Fig. 1C: TE, $F(4,45)=7.31$, $P<.001$; Fig. 1D: TE/TF, $F(4,45)=6.81$, $P<.001$].

As shown in Fig. 2, the combination of noneffective doses of naloxone (0.1 and 0.3 mg/kg) and L-NAME (3 mg/kg) per se significantly inhibited the anticonvulsant effect of morphine [Fig. 2A: TE, $F(5,54)=5.51$, $P<.001$; Fig. 2B: TE/TF, $F(5,54)=7.45$, $P<.001$]. On the other hand, the combination of aminoguanidine (100 mg/kg) with naloxone did not alter the effect of morphine ($P>.05$).

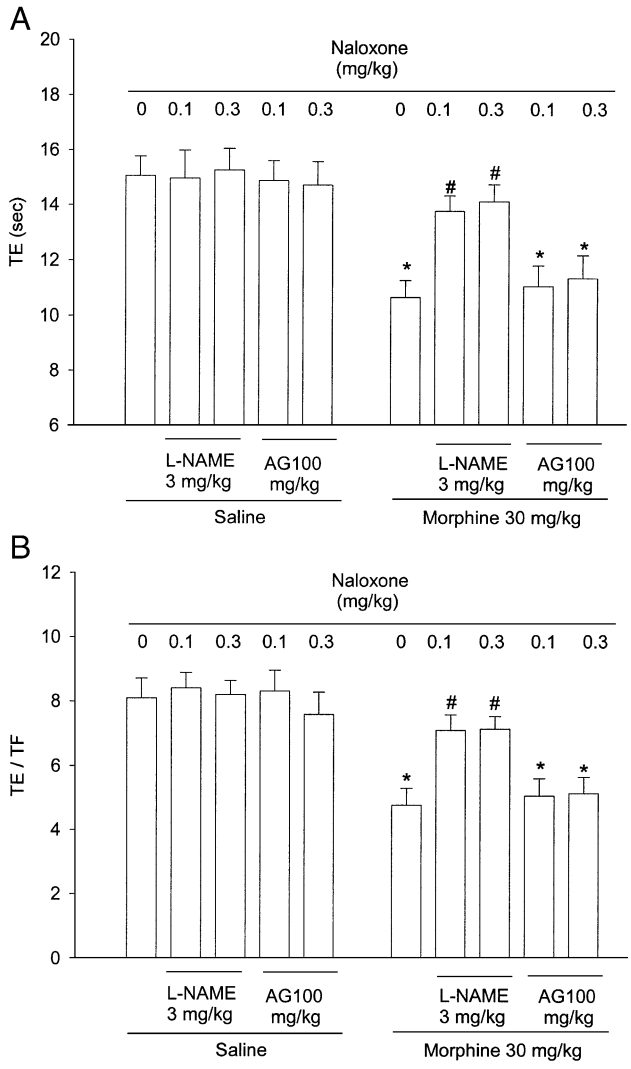

Fig. 2. Effects of concomitant administration of naloxone with L-NAME or aminoguanidine (AG) in the presence of saline or morphine on MES in mice. (A) The duration of TE and (B) the ratio of the duration of TE to the duration of TF (TE/TF). Naloxone concomitant with L-NAME or aminoguanidine was administered 15 min before either saline or morphine (30 mg/kg) and 60 min before testing. Data are expressed as mean ± S.E.M. of 10 animals. $^{*}P<.05$ compared to saline control group, and $^{\#}P<.05$ compared to the group receiving morphine alone.

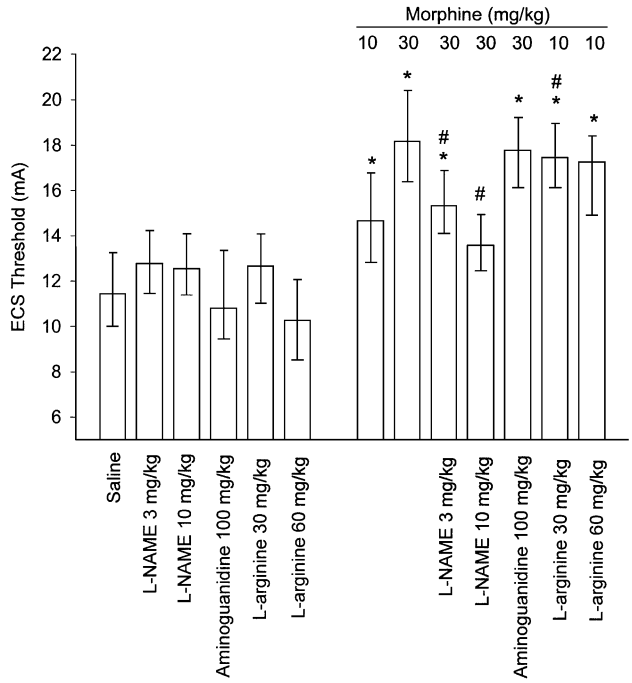

Fig. 3. Effects of different treatments on ECS threshold in mice. Seizure thresholds are shown as the convulsive current (CC_{50}) inducing tonic seizures in 50% of mice per group, with confidence intervals for 95% probability. Each group consisted of 15 mice. Saline or morphine was administered 45 min before and L-NAME, aminoguanidine, or L-arginine was administered 60 min before testing. $^{*}P<.05$ compared to saline control group, and $^{\#}P<.05$ compared to the corresponding morphine group.

3.2. ECS threshold

As shown in Fig. 3, morphine (10 and 30 mg/kg) significantly increased the ECS threshold (two-sided Student's t test, $P < .05$). This effect was inhibited by L-NAME (3 and 10 mg/kg) in a dose-related manner but was not affected by aminoguanidine. Moreover, 30 mg/kg L-arginine, which did not alter the seizure threshold by itself, significantly potentiated the effect of morphine (10 mg/kg) in increasing ECS threshold ($P < .05$).

4. Discussion

Our data show that morphine-induced anticonvulsant effect against ECS is reversible by L-NAME, but not by aminoguanidine, while this effect is restored by a concomitant administration of a noneffective dose of L-arginine with L-NAME. Moreover, L-NAME shows an additive effect with naloxone in inhibiting morphine anticonvulsive property.

Morphine is reported to increase ECS threshold (Czuczwar and Frey, 1986; Frey, 1988; Puglisi-Allegra et al., 1985) and to decrease the intensity of convulsions induced by MES (Berman and Adler, 1984; Karadag et al., 2000). Our data in both models were consistently in favor of the involvement of NO in anticonvulsant effects of morphine. Neither L-arginine nor the NOS inhibitors per se affected ECS susceptibility, which is in accordance with previous reports (Borowicz et al., 1998; Deutsch et al., 1995; Przegalinski et al., 1996; Urbanska et al., 1996). However, it has been reported that L-NAME can impair the anticonvulsant effects of valproate and phenobarbital against maximal electroshock in mice (Borowicz et al., 1998). Both the opioid system (Salzet and Stefano, 1997) and the nitricoxidergic pathway (Stefano et al., 1996) have been well preserved through evolution and show interrelatedness in many central (Calignano et al., 1993; Duarte and Ferreira, 1992; Elliott et al., 1994) as well as peripheral (Fecho et al., 1994; Ferreira et al., 1991) phenomena. NO exerts a dual role as a modulator (Brignola et al., 1994; Przewlocki et al., 1993) or a mediator (Duarte and Ferreira, 1992; Ferreira et al., 1991) of opioid-induced antinociception, while NOS inhibition attenuates the development of morphine tolerance (Elliott et al., 1994; Kolesnikov et al., 1993). Moreover, in pathological conditions like acute cholestasis, which is associated with increased plasma levels and increased activity of endogenous opioids (Bergasa et al., 1994; Dehpour et al., 2000; Swain et al., 1992), the production of NO is also increased (Inan et al., 1997; Nahavandi et al., 1999) and a number of opioid-induced effects including antinociception (Dehpour et al., 1998), naloxone-precipitated withdrawal (Dehpour et al., 1998; Ghafourifar et al., 1997), gastroprotection (Nahavandi et al., 2001; Sadr et al., 1999), and vascular hyporesponsiveness (Namiranian et al., 2001) are reversible by NOS inhibitors. NO is also involved in the rewarding effects of morphine and, in this regard, recently local NO synthesis in hippocampus and nucleus accumbens has been implicated (Gholami et al., 2002; Karami et al., 2002; Kivastik et al., 1996). Both of these areas are also important in seizure susceptibility. Recently, a distinct class of mu-opioid receptors, which are located on the vascular endothelium and are closely related to NO production, has been identified (Cadet et al., 2000; Stefano et al., 2000). These receptors couple NO to morphine-induced neurotransmitter release in some central sites (Prevot et al., 1998; Stefano et al., 1997). Interestingly, the anticonvulsant effect of morphine is completely reversible by mu-receptor-sensitive doses of naloxone (the present data; Berman and Adler, 1984; Czuczwar and Frey, 1986). Our recent study using a pentylenetetrazole model of seizure showed that the inhibition of NOS blocks the anticonvulsant effect of lower doses of morphine completely and decreases the proconvulsant effects of higher doses of morphine partially (Homayoun et al., 2002a). Moreover, the inhibition of NO synthesis blocked the opioid-mediated increased susceptibility of cholestatic mice to pentylenetetrazole-induced seizures (Homayoun et al., 2002b). The molecular mechanisms of morphine interaction with NO may involve the modulation of intracellular calcium transients by morphine (Nieto-Fernandez et al., 1999), leading to the activation of Ca^{2+}/calmodulin-dependent NO synthase. Moreover, opioids can selectively alter the expression of different isoforms of NOS in various brain areas (Cuellar et al., 2000; Lanier et al., 2002; Lysle and Carrigan, 2001; Wong et al., 2000). A further interaction takes place in the mediation of morphine-induced changes in immediate-early gene c-Fos expression in brain areas like the striatum by NO of neuronal origin (Harlan et al., 2001).

In the present study, low and noneffective doses of L-NAME and naloxone per se showed additive effects in blocking the anticonvulsant property of morphine. A similar potentiation between the opioid receptor antagonist and the NOS inhibitor has been recently reported in some other sites (Komjati et al., 2001; Nahavandi et al., 2001), and their parallel inhibitory effects against morphine in this model of seizure warrant further investigation. Although the low doses of naloxone used here are dominantly mu-opioid receptor antagonists, the type of opioid receptor interacting with NO in this effect cannot be concluded here.

Three different forms of NOS have been identified, which include either inducible or constitutive (itself divided to neuronal and endothelial) NOS (Moncada and Higgs, 1993). In the nervous system, neuronal nitric oxide synthase (nNOS) is largely responsible for NO production (Bredt and Snyder, 1990), while iNOS is also reported to be present in normal adult brains and to contribute to the pathophysiology of many neuronal diseases (Licinio et al., 1999). In the present study, the specific irreversible iNOS inhibitor, aminoguanidine, did not alter the ECS threshold or intensity, either per se or concomitant with morphine. This finding implies that the observed involvement of NO in morphine

effect is not mediated by iNOS. L-NAME is a nonspecific inhibitor of all isoforms of NO synthase but the ineffectiveness of the specific inducible NOS inhibitor in this paradigm suggests the involvement of constitutive NOS in the effect of morphine. In this regard, constitutive NOS consists of nNOS that may play the key role in this interaction, plus endothelial nitric oxide synthase (eNOS), which may also contribute to this effect through the modulation of cerebral blood flow (Licinio et al., 1999; Wiesinger, 2001). In addition, drugs that modify the concentration of NO in the central nervous system are reported to alter the uptake and the distribution of morphine in the central and peripheral tissues (Bhargava and Bian, 1997, 1998). However, it should be noted that NO exerts differential effects on various morphine-induced properties and all these effects cannot be consistently explained by NO-induced alterations in central morphine penetration and distribution. In conclusion, NO seems to have a role in the anticonvulsant property of morphine against ECS and this role involves constitutive NOS.

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