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Attenuation of morphine tolerance and dependence by aminoguanidine in mice

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Abstract

The effect of aminoguanidine, an inducible nitric oxide synthase (iNOS) inhibitor, on morphine-induced tolerance and dependence in mice was investigated in this study. Acute administration of aminoguanidine (20 mg/kg, p.o.) did not affect the antinociceptive effect of morphine (10 mg/kg, s.c.) as measured by the hot plate test. Repeated administration of aminoguanidine along with morphine attenuated the development of tolerance to the antinociceptive effect of morphine. Also, the development of morphine dependence as assessed by naloxone-precipitated withdrawal manifestations was reduced by co-administration of aminoguanidine. The effect of aminoguanidine on naloxone-precipitated withdrawal was enhanced by concurrent administration of the non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, dizocilpine (0.25 mg/kg, i.p.) or the non-specific nitric oxide synthase (NOS) inhibitor, L-*N*(G)-nitroarginine methyl ester (L-NAME; 5 mg/kg, i.p.) and antagonized by concurrent administration of the nitric oxide (NO) precursor, L-arginine (50 mg/kg, p.o.). Concomitantly, the progressive increase in NO production, but not in brain glutamate level, induced by morphine was inhibited by repeated administration of aminoguanidine along with morphine. Similarly, co-administration of aminoguanidine inhibited naloxone-induced NO overproduction, but it did not inhibit naloxone-induced elevation of brain glutamate level in morphine-dependent mice. The effect of aminoguanidine on naloxone-induced NO overproduction was potentiated by concurrent administration of dizocilpine or L-NAME and antagonized by concurrent administration of L-arginine. These results provide evidence that blockade of NO overproduction, the consequence of NMDA receptor activation, by aminoguanidine, via inhibition of iNOS, can attenuate the development of morphine tolerance and dependence.

Keywords: Aminoguanidine; NO (Nitric oxide); Tolerance; Dependence

1. Introduction

Repeated use of opiate drugs, such as morphine, for pain relief leads to the development of tolerance and dependence. Activation of the central *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors and elevations of nitric oxide (NO) have been implicated in the development of morphine tolerance and dependence (Elliott et al., 1994; Majeed et al., 1994; Kolesnikov et al., 1998; Heinzen and Pollack, 2004; Wang et al., 2004).

The NMDA receptor, an ionotropic glutamate receptor, is widely distributed in the mammalian central nervous system (Zhu et al., 1998). Glutamate activation of these receptors stimulates Ca^{++} influx into cells. Calcium then binds to calmodulin and activates nitric oxide synthase (NOS) resulting in stimulating NO formation (Bredt and Snyder, 1992; Mao et al., 1995). Thus, the neuronal NOS (nNOS), a Ca^{++}-dependent low-output NOS isoform, has a pivotal role in development of morphine tolerance and dependence (Babey et al., 1994; Lue et al., 1999; Cuellar et al., 2000; Homayoun et al., 2003; Heinzen and Pollack, 2004).

However, it has been found that the activity of inducible NOS (iNOS), a Ca^{++}-independent high-output NOS isoform (Ogden and Moor, 1995), is induced as a consequence of glutamate release and NMDA receptors activation in rat brain cortex during restraint stress (Madrigal et al., 2001) and in rat

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forebrain slices during ischemia (Cardenase et al., 2000). Similarly, activation of microglia by interferon-gamma and lipopolysaccharide was found to cause neuronal release of glutamate, NMDA receptors activation and iNOS stimulation (Golde et al., 2002).

In light of these observations and since the glutamate release in the brain is augmented in morphine tolerance, dependence and withdrawal (Zhang et al., 1994; Sepulveda et al., 1998; Vandergriff and Rasmussen, 1999), the possibility of iNOS induction during these phenomena exists. Therefore, the objective of this work is to investigate the potential role of aminoguanidine, a selective iNOS inhibitor (Misko et al., 1993), in attenuation of morphine tolerance and dependence.

2. Materials and methods

2.1. Animals and treatments

Male adult Swiss–Webster mice weighing 20–30 g from the animal house of Assiut University were used in all experiments. The animals were housed in a temperature-controlled room in groups, 6 mice each, maintained on a 12 h light/dark cycle. Mice were allowed water and food (laboratory chow) ad libitum. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines. The experiments reported here were approved by our institutional ethics committee.

Animals of Group-I were treated s.c. with 10 mg/kg morphine (0.1% solution in saline) twice daily at 12 h intervals for 5 days. Group-II mice received 20 mg/kg aminoguanidine (0.4% solution in saline) orally (p.o.) by means of a stomach tube 30 min before every morphine treatment for 5 days. Animals of groups-III, IV and V were treated, in addition to 20 mg/kg aminoguanidine, with 0.25 mg/kg dizocilpine (MK-801, 0.005% solution in saline) i.p.; 5 mg/kg L-*N*(G)-nitroarginine methyl ester (L-NAME, 0.125% solution in saline) i.p. and 50 mg/kg L-arginine (1% solution in saline) p.o., respectively 30 min before every morphine treatment for 5 days. Control groups of animals were treated likewise with the vehicle (isotonic saline).

2.2. Hot plate test

In this method, the time taken by the mouse to lick its hind paws or to jump with all four feet within a plexiglass cylinder placed on a hot plate surface (55 °C) was determined. This reaction time was taken as the end point and the increase in hot plate latency was taken as a measure of the analgesic activity. Prior to administration of drugs, mice were tested on the hot plate for 4 days in order to obtain a stable control response level. The animals were removed from the hot plate if they did not respond within 30 s in order to avoid tissue damage. Any animal failed to respond within 30 s was excluded immediately and retested again after 30 min. The antinociceptive effect of morphine was determined 60 min after the first injection on the first, third, and fifth day.

2.3. Induction of withdrawal syndrome

On the sixth day, animals of the different groups were treated once as previously mentioned. Two hours later, all mice received 5 mg/kg naloxone (0.1% solution in saline) i.p. Immediately after naloxone injection each animal was placed in a transparent acryl cylinder (20 cm in diameter, 35 cm in height) to observe withdrawal manifestations (jumping, rearing, paw tremor and teeth chatter) for 30 min. The withdrawal manifestations were manually evaluated by co-works blind to the treatment protocol.

2.4. Biochemical measurements

After recording of the withdrawal manifestations, animals were sacrificed by decapitation. Brain and blood tissues were obtained from each animal for estimation of glutamate and nitric oxide levels. Brain and blood tissues were also obtained on the first, third and fifth day of treatment of other different groups of mice with 10 mg/kg morphine s.c. and 10 mg/kg morphine in combination with 20 mg/kg aminoguanidine p.o. or saline twice daily at 12 h intervals.

The brain was rinsed in ice-cold saline, blotted carefully and placed in a previously weighed ice-cold glass homogenizer containing ice-cold perchloric acid (1 mol/l). The wet weight of the added tissue was then determined. After homogenization and centrifugation for 10 min at 3000 ×*g*, the pH of the supernatant was adjusted with the tripotassium phosphate solution (1.93 mol/1). After mixing by vortexing and centrifugation, the supernatant was collected carefully without disturbing the precipitate. The glutamate content in the supernatant was measured spectrophotometrically via its enzymatic dehydrogenation with conversion of NAD^+ to NADH according to the method of Lund (1986). A standard reference curve must be prepared for each assay.

Nitric oxide formation was measured in serum samples by assaying nitrite, one of the stable end-products of NO oxidation. Serum nitrite concentration was measured spectrophotometrically using Griess reagents [1% sulfanilamide in 5% phosphoric acid (sulfanilamide solution) and 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in bidistilled water (NED solution)] as described by Green et al. (1982). A standard curve must be run simultaneously with each set of samples.

2.5. Drugs

The following drugs were used: morphine sulphate (Misr Co. Pharma, Egypt), aminoguanidine (Merk, Germany), Dizocilpine hydrogen maleate (MK-801) (ICN Biomedicals Inc., USA), L-*N*(G)-nitroarginine methyl ester (Sigma Chemical Co, USA) and L-arginine (Sigma Chemical Co., USA). All other chemicals were of analytical grade.

2.6. Statistical analysis

The variability of results was expressed as the mean ± S.E.M. Statistical analysis of the difference between groups was done

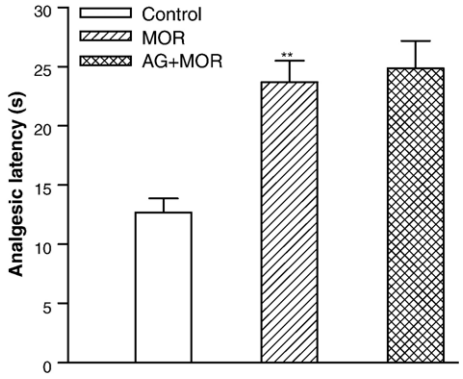

Fig. 1. Effect of 30 min pretreatment with 20 mg/kg aminoguanidine (AG) p.o. on analgesia induced by s.c. administration of 10 mg/kg morphine (MOR) in mice as measured by the hot plate test. Values are means±S.E.M. (n=6). **P<0.01 vs. control values.

with the one-way analysis of variance (ANOVA) and Student's t-test as a post hoc analysis.

3. Results

3.1. Effect of aminoguanidine on morphine-induced analgesia

The results presented in Fig. 1 illustrate that treatment of mice with 10 mg/kg morphine s.c. produced an antinociceptive effect in the hot plate test. The analgesic latency in morphine-treated mice was increased significantly compared to that in saline-treated mice. Pretreatment with 20 mg/kg aminoguanidine 30 min before morphine administration produced no effect on the morphine-induced analgesia.

3.2. Effect of aminoguanidine on the development of morphine tolerance to analgesia

Repeated administration of 10 mg/kg morphine s.c. to mice twice daily resulted in a decrease in the analgesic latency in hot plate test. After 5 days of morphine administration the analgesic

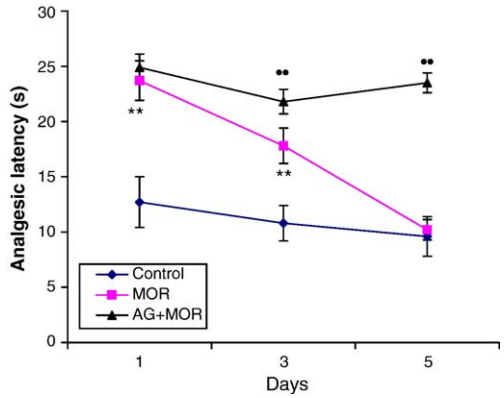

Fig. 2. Effect of aminoguanidine (AG) on the development of morphine (MOR) tolerance to analgesia in mice. Animals were treated with 20 mg/kg AG p.o. 30 min before s.c. administration of 10 mg/kg MOR twice daily for 5 days. Values are means±S.E.M. (n=6). **P<0.01 vs. control values; ●●P<0.01 vs. MOR values.

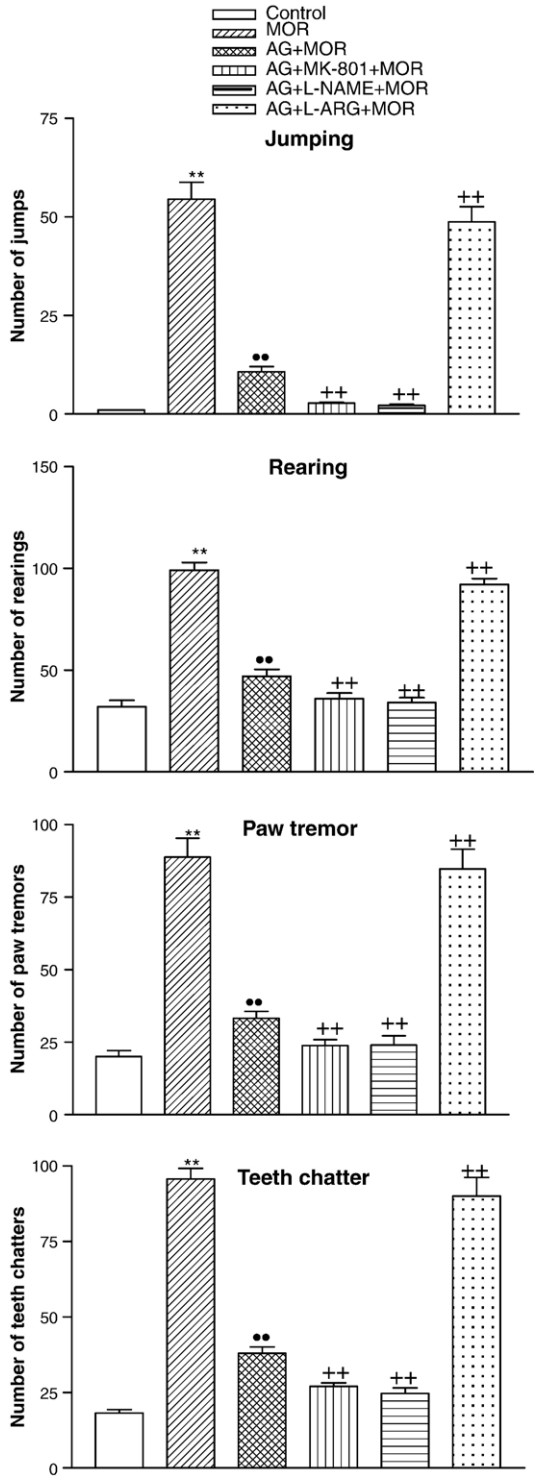

Fig. 3. Effect of repeated administration of 20 mg/kg aminoguanidine (AG) p.o. and 20 mg/kg AG in combination with 0.25 mg/kg MK-801 i.p., 0.5 mg/kg L-NAME i.p. or 50 mg/kg L-arginine (L-ARG) p.o. on the naloxone-induced withdrawal manifestations in morphine (MOR)-dependent mice. Animals received AG and AG in combination with MK-801, L-NAME or L-ARG 30 min before s.c. administration of 10 mg/kg MOR twice daily for 5 days. Naloxone was injected into mice on the sixth day after the first treatment. Values are means±S.E.M. (n=6); **P<0.01 vs. control values; ●●P<0.01 vs. MOR values; ++P<0.01 vs. AG+MOR values.

latency was not significantly different from that in mice treated likewise with saline. Pretreatment of mice with 20 mg/kg aminoguanidine 30 min before each morphine injection inhibited the development of tolerance to morphine analgesia (Fig. 2).

3.3. Effect of aminoguanidine on naloxone-precipitated withdrawal manifestations in morphine-dependent mice

In morphine-treated mice administration of 5 mg/kg naloxone i.p. on the sixth day of treatment, 2 h after morphine injection induced withdrawal manifestations such as jumping, rearing, paw tremor and teeth chatter. Pretreatment of mice with 20 mg/kg aminoguanidine 30 min before each morphine injection attenuated the development of these withdrawal manifestations (Fig. 3). It is evident from the same figure that concurrent administration of 0.25 mg/kg MK-801 i.p. or 5 mg/kg L-NAME i.p. with aminoguanidine potentiated its inhibitory effect on the development of morphine withdrawal manifestations following the naloxone challenge. Concurrent administration of 50 mg/kg L-arginine with aminoguanidine antagonized its inhibitory effect on the development of withdrawal manifestations.

3.4. Brain glutamate and serum nitrite levels

Administration of 10 mg/kg morphine s.c. to mice twice daily produced, 2 h after the second injection, a significant decrease in brain glutamate and serum nitrite levels. Treatment of mice with this dose level of morphine twice daily for 3 and 5 days resulted in a progressive increase in brain glutamate and serum nitrite levels. Pretreatment of mice with 20 mg/kg aminoguanidine 30 min before each morphine injection inhibited the progressive increase in serum nitrite level induced by morphine. The progressive increase in brain glutamate level induced by morphine was not changed by co-administration of aminoguanidine (Fig. 4).

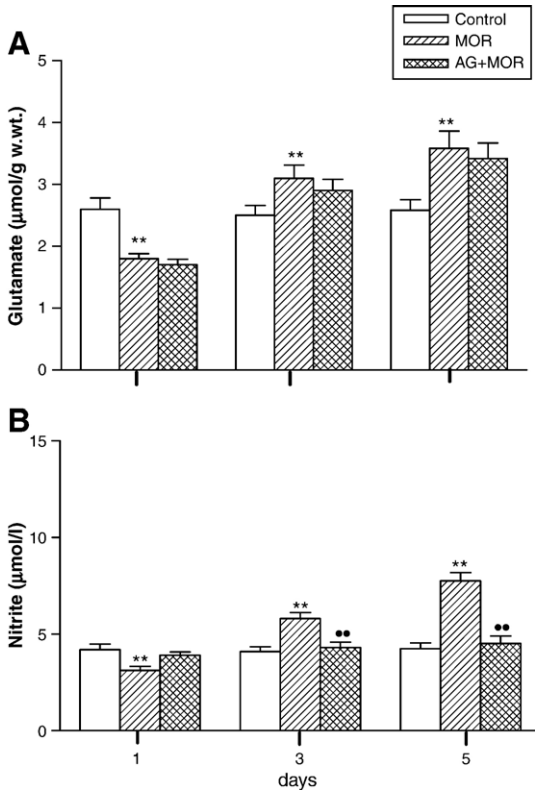

Fig. 4. Time course of changes in the brain glutamate (A) and serum nitrite (B) levels of mice receiving 10 mg/kg morphine (MOR) s.c. or 10 mg/kg MOR in combination with 20 mg/kg aminoguanidine (AG) p.o. twice daily. Values are means±S.E.M. ($n=6$); **$P<0.01$ vs. control values; ●●$P<0.01$ vs. MOR values.

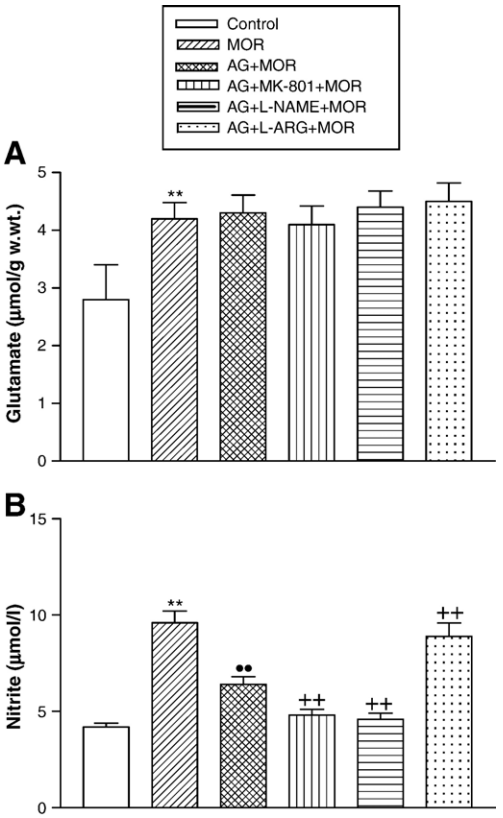

Fig. 5. Effect of repeated administration of 20 mg/kg aminoguanidine (AG) p.o. and 20 mg/kg AG in combination with 0.25 mg/kg MK-801 i.p., 0.5 mg/kg L-NAME i.p. or 50 mg/kg L-arginine (L-ARG) p.o. on the naloxone-induced elevations of brain glutamate (A) and serum nitrite (B) levels in morphine (MOR)-dependent mice. Animals received AG and AG in combination with MK-801, L-NAME or L-ARG 30 min before s.c. administration of 10 mg/kg MOR twice daily for 5 days. Naloxone was injected into mice on the sixth day after the first treatment. Values are means±S.E.M. ($n=6$); **$P<0.01$ vs. control values; ●●$P<0.01$ vs. MOR values; ++$P<0.01$ vs. AG+MOR values.

In morphine-treated mice administration of 5 mg/kg naloxone i.p. on the sixth day of treatment 2 h after morphine injection produced a significant increase in brain glutamate and serum nitrite levels. Pretreatment of mice with 20 mg/kg aminoguanidine 30 min before each morphine injection inhibited naloxone-induced elevation of serum nitrite level but it did not inhibit naloxone-induced elevation of brain glutamate level. The effect of aminoguanidine on the serum nitrite level elevated by naloxone challenge was potentiated by concurrent administration of 0.25 mg/kg MK-801 i.p. or 5 mg/kg L-NAME i.p. and antagonized by concurrent administration of 50 mg/kg L-arginine. (Fig. 5).

4. Discussion

It has been shown that acute treatment with morphine prevented the release of endogenous glutamate in the motor cortex of rats (Coutinho-Netto et al., 1980) and in both brain slices and synaptosomes from the cerebral cortex (Crowder et al., 1986). Also, inhibition of glutamate release by opioids was observed in primary cultures of rat cerebral cortex (Vlaskovska et al., 1997) and in rat cerebral cortex nerve terminals (Yang et al., 2004).

However, Sepulveda et al. (1998) found that injection of morphine into rats decreased glutamate release in the brain. After repeated morphine injections, this effect disappeared, suggesting tolerance. Naloxone injections to morphine-dependent rats increased glutamate release. Direct neurochemical evidence obtained from in vivo microdialysis studies has shown an elevation in glutamate levels in the locus coeruleus and nucleus accumbens during naltrexone or naloxone-precipitated withdrawal in morphine-dependent rats (Zhang et al., 1994; Sepulveda et al., 1998; Vandergriff and Rasmussen, 1999). To investigate further the role of the augmented glutamate release in morphine dependence and withdrawal, glutamate was injected intracerebroventricularly into morphine-dependent animals (Tokuyama et al., 1996). It is interesting to note that such glutamate injections can dose-dependently precipitate withdrawal signs in morphine-dependent rats. The behavioral signs precipitated by glutamate are generally comparable to those precipitated by the morphine receptor antagonist, naloxone.

Our results are consistent with these previous observations. Administration of morphine to mice in this study, resulted in a marked decrease in brain glutamate level. Repeated administration of morphine for several consecutive days produced a progressive increase in glutamate level. Treatment of morphine-dependent mice with naloxone resulted in a profound increase in brain glutamate level.

There is a body of evidence implying the involvement of the central glutamatergic system in morphine tolerance and dependence. Glutamate, the major excitatory neurotransmitter in the brain, was found to stimulate both ionotropic and metabotropic receptors (Conn and Pin, 1997). Activation of the ionotropic NMDA subtype of glutamate receptors, has been implicated in the development of morphine analgesic tolerance and dependence (Elliott et al., 1994; Trujillo, 1995; Popik et al., 2000; Wang et al., 2004).

It has been established that many glutamate actions mediated through NMDA receptors result from the subsequent activation of NOS and the formation of NO (Bredt and Snyder, 1992; Kolesinikov et al., 1993). Thus, NO, the intracellular messenger linked to NMDA receptor activation, plays a role in morphine tolerance (Mao et al., 1995) and dependence (Buccafusco et al., 1995).

In the present work, administration of morphine to mice decreased the production of NO. Repeated administration of morphine for several consecutive days produced a progressive increase in NO production. Treatment of morphine-dependent mice with naloxone was associated with NO overproduction. These results are in favor of the possibility that the increase in NO production plays an important role in the development of tolerance to and dependence on morphine (Elliott et al., 1994; Majeed et al., 1994; Homayoun et al., 2003; Heinzen and Pollack, 2004).

It has been demonstrated that nNOS, via NMDA receptor activation, may involved in the development of morphine tolerance (Babey et al., 1994). In rat hippocampal slices, NO production, via nNOS activation, may play a key role in the development of morphine tolerance (Lue et al., 1999). Furthermore, Heinzen and Pollack (2004) using genetically modified mice found that NO overproduction, via nNOS, is implicated in the development of morphine antinociceptive tolerance. Similarly, Cuellar et al. (2000) found that morphine dependence up-regulates nNOS in several brain regions in mice. Administration of naloxone to morphine-dependent mice up-regulates nNOS in the hypothalamus and locus coeruleus. Moreover, the selective inhibitors of nNOS, such as 7 nitroindazole, were found to be able to prevent opiate withdrawal (Vaupel et al., 1997).

However, it has been found that the release of glutamate and subsequent NMDA receptors activation in acute restraint stress increase the activity of iNOS and induce its expression in brain cortex of rats. Pretreatment with the NMDA receptors antagonist, MK-801, inhibits the stress-induced increases in iNOS expression (Madrigal et al., 2001). Similarly Olivenza et al. (2000) found that chronic stress increases the activity and expression of iNOS in cortical neurons. Also, Golde et al. (2002) reported that activation of microglia by interferon-gamma and lipopolysaccharide causes neuronal release of glutamate and iNOS stimulation. These effects were inhibited by MK-801. Furthermore, in rat forebrain slices, Cardenase et al. (2000) found that activation of NMDA receptors by glutamate released in ischemia is involved in the expression of iNOS. In addition, Perez-Asensio et al. (2005) demonstrated that transient focal cerebral ischemia in rats resulted in increase in brain glutamate level and expression of iNOS. Moreover, Iravani et al. (2004) found that unilateral intrastriatal administration of *N*-methyl-D-aspartic acid to rats results in marked iNOS expression within both astroglial and microglial cells.

In light of these considerations, the effect of aminoguanidine, a selective iNOS inhibitor (Misko et al., 1993) on morphine-induced tolerance and dependence was investigated in this study. Our results indicate that administration of aminoguanidine produced no change in the acute morphine analgesia in mice as measured by the hot plate test. Repeated administration of aminoguanidine along with morphine attenuated the development of tolerance to the antinociceptive effect of morphine. Also, the development of morphine dependence as assessed by naloxone-precipitated withdrawal manifestations was attenuated by repeated administration of aminoguanidine along with morphine. It is of interest that the progressive increase in NO production but not in brain glutamate level induced by morphine was inhibited by repeated administration of aminoguanidine along with morphine to mice. Also, the NO overproduction but not the elevation of brain glutamate level associated naloxone-induced withdrawal in morphine-

dependent mice was inhibited by co-administrations of aminoguanidine.

It is well established that the non-competitive NMDA receptor antagonist, MK-801 inhibited the development of morphine tolerance (Trujillo and Akil, 1991; Elliott et al., 1994; Popik et al., 2000) and attenuated the development and expression of morphine dependence (Trujillo and Akil, 1991; Elliott et al., 1994; Wang et al., 2004). Similarly, the non-selective NOS inhibitor, L-NAME has been found to attenuate the development of tolerance to the analgesic action of morphine (Majeed et al., 1994; Homayoun et al., 2003) and to inhibit the expression of morphine dependence as assessed by naloxone-precipitated withdrawal syndrome(Majeed et al., 1994; Dehpour et al., 2000; Homayoun et al., 2003).

In this study the attenuating effect of aminoguanidine on the development of morphine dependence in mice was potentiated by concurrent administration of MK-801 and L-NAME. Also, the inhibitory effect of aminoguanidine on naloxone-induced NO overproduction in morphine-dependent mice was potentiated by concurrent administration of MK-801 and L-NAME.

In light of these observations, the most likely explanation for our results is that the elevation of the brain glutamate level by repeated administration of morphine to mice and by injection of naloxone into morphine-dependent mice activated NMDA receptors. Activation of these receptors stimulated inducible and constitutive isoforms of NOS resulting in NO overproduction. Inhibition of iNOS by aminoguanidine, suppresses NO production and thereby attenuated the development of morphine tolerance and dependence. Additional blockade of NO production by using a NMDA receptor antagonist or a non-selective NOS inhibitor potentiated the effect of aminoguanidine.

In support for our findings, pretreatment of rats with aminoguanidine was found to decrease the extent of *N*-methyl-D-aspartic acid-induced marked expression of iNOS activity within both astroglial and microglial cells of rats (Iravani et al., 2004). Also, aminoguanidine was found to prevent the increase in activity and expression of iNOS in rat cortical neurons during chronic stress (Olivenza et al., 2000).

L-arginine is the only natural substrate for all isoforms of NOS and the sole metabolic precursor for NO biosynthesis (Brosnan et al., 1994;). Sustaining high levels of NO synthesis is a poignant issue in the brain because this organ lacks the urea cycle enzymes which are required to maintain adequate intracellular synthesis of L-arginine (Masters, 1994). Induced NO biosynthesis occurs in brain cells only if the extracellular cerebrospinal fluid contains L-arginine (Brosnan et al., 1994). Furthermore, there is a coordinated regulation between intracellular iNOS activity and membrane L-arginine transport in brain (Stevens et al., 1996). In activated microglial cells, both arginine transport by cationic amino acid transporter-2 and citrulline-arginine recycling were found to be important for high-output production of NO, via iONS (Kawahara et al., 2001).

Data obtained in the present study demonstrate that concurrent administration of L-arginine with aminoguanidine antagonized the inhibitory effects of aminoguanidine on the development of morphine dependence in mice. At the same time, the inhibitory effect of aminoguandine on naloxone-induced NO overproduction in morphine-dependent mice was antagonized by concurrent administration of L-arginine. It is noteworthy that in preliminary experiments in this study, repeated administration of L-arginine along with morphine enhanced the development of morphine tolerance in mice. The withdrawal manifestations and NO overproduction induced by injection of naloxone into morphine-dependent mice were potentiated by co-administration of L-arginine. These results provide further evidence implicating the role of inhibition of iNOS by aminoguanidine in the prevention of the development of morphine tolerance and dependence.

In studies of a similar nature; it has been found that L-arginine accelerated the development of tolerance when co-administered with morphine in mice (Babey et al., 1994; Pataki and Telegdy, 1998). The effect of L-arginine on morphine-induced analgesia can be reversed by MK-801 and L-NAME (Brignola et al., 1994; Bhargava et al., 1998). Chronic administration of L-arginine was found to induce NO synthase activity in brain (Bhargava et al., 1997). L-arginine was also found to increase the withdrawal signs in morphine-dependent mice (Dehpour et al., 2000).

Taken together, the results of this study suggest that aminoguanidine can attenuate the development of morphine tolerance and dependence by inhibiting the consequences of NMDA receptor activation; iNOS induction and NO overproduction. Thus, iNOS may play a pivotal role in the development of morphine tolerance and dependence.

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