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A role of nitric oxide in WIN 55,212-2 tolerance in mice

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Abstract

The role of nitric oxide (NO) in the development of cannabinoid tolerance was examined by using N^{ω}-nitro-L-arginine methyl ester (L-NAME) as an inhibitor of NO synthase. *R*(+)-[2,3-Dihydro-5-methyl-3 [(morpholinyl)methyl]pyrrolo[1,2,3-de]- 1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2), a cannabinoid receptor agonist, or L-NAME plus WIN 55,212-2 was acutely or chronically injected i.p. to mice and analgesia, body temperature and immobility were measured. A single injection of WIN 55,212-2 induced time- and dose-dependent analgesia, hypothermia and catalepsy. L-NAME (50 mg/kg), which per se was ineffective, administered 20 min before WIN 55,212-2 did not modify the analgesic, hypothermic and cataleptic responses to the cannabinoid. When WIN 55,212-2 was administered once a day, the animals became completely tolerant to the analgesic, hypothermic and cataleptic effects within five, seven and nine days respectively. L-NAME injected once daily 20 min before WIN 55,212-2 inhibited the development of tolerance to the hypothermic and cataleptic actions but not to the analgesic action of WIN 55,212-2. Since L-NAME given chronically by itself did not modify the analgesia, hypothermia and catalepsy induced by acute administration of WIN 55,212-2, our findings suggest L-NAME acts with some selectivity on the mechanisms involved in cannabinoid tolerance. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide (NO); Cannabinoid; Tolerance

1. Introduction

Cannabinoids are a class of psychoactive compounds with many pharmacological properties similar to those of the opioids. These properties include analgesia (Bloom and Dewey, 1978; Bhargava and Matwyshyn, 1980; Bloom and Hillard, 1985), hypothermia (Bhargava, 1980), sedation, hypotension and inhibition of both intestinal motility and locomotor activity (Holtzman et al., 1969). With chronic administration, tolerance develops to the analgesic and hypothermic effects of both classes of drugs (Pertwee, 1988; Bhargava, 1991) and physical dependence of different intensity is induced (Di Chiara and North, 1992; Bhargava, 1994b; Aceto et al., 1996). Moreover opiate antagonists inhibit the analgesia, hypothermia and tolerance induced by Δ^9-tetrahydrocannabinol in rats (Tulunay et al., 1981) and precipitate an opioid-like withdrawal syndrome in rodents chronically treated with crude hashish extract or with purified Δ^9-tetrahydrocannabinol (Kaymakcalan et al., 1977). Further support for an interaction between the cannabinoids and opioids is provided by the synergistic antinociceptive effect of cannabinoids and morphine (Welch and Stevens, 1992) and the cross-tolerance between morphine and Δ^9-tetrahydrocannabinol (Thorat and Bhargava, 1994a). The pharmacological actions shared by cannabinoids and opioids thus could imply a common mechanism. *N*-methyl-D-aspartate (NMDA) receptors are involved in morphine-induced analgesia (Jacquet, 1988; Lipa and Kavaliers, 1990), tolerance and dependence (Marek et al., 1991; Trujillo and Akil, 1991; Elliott et al., 1994). Many effects of NMDA receptor stimulation are mediated through the intracellular formation of the relatively new second messenger, nitric oxide (NO) (Garthwaite, 1991; Snyder and Bredt, 1991). Reflecting the close link between NMDA receptor activation and the production of NO, a role of NO in opiate tolerance and withdrawal has been demonstrated. NO synthase inhibitors block tolerance to the analgesic and hypothermic actions of opiates (Kolesnikov et al., 1993; Thorat et al., 1993; Babey et al., 1994; Bhargava, 1994a, 1995; Elliott et al., 1994) and attenuate the expression of opiate physical dependence in rodents (Adams et al., 1993; Cappendijk et al., 1993; Bhargava, 1995; Vaupel et al., 1995). Moreover it has been reported that, in mice, morphine tolerance is

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associated with increased NO synthase activity in the cerebral cortex, whereas abstinence is associated with increased NO synthase activity in the corpus striatum (Barjavel and Bhargava, 1994). Considering the cross-tolerance and dependence between opiates and cannabinoids, NO might be indirectly involved in cannabinoid tolerance. To date the role of NMDA receptor-NO pathways in cannabinoid tolerance has not been much explored and its involvement in the expression of cannabinoid dependence has not been studied. Concerning cannabinoid tolerance, Thorat and Bhargava (1994b) found that dizocilpine (MK-801), a non-competitive NMDA receptor antagonist and N^G-monomethyl-L-arginine (L-NMMA) did not affect tolerance to the antinociceptive and hypothermic effects of Δ^9-tetrahydrocannabinol. To investigate further the role of NO in cannabinoid tolerance, we examined the effect of the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), on tolerance to the analgesia, hypothermia and catalepsy induced in mice by *R*(+)-[2,3-dihydro-5-methyl-3 [(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate (WIN 55,212-2), a cannabinoid receptor agonist.

2. Materials and methods

2.1. Animals

Male CD-1 (25–35 g; Charles River) mice were used. The animals were housed five per cage in a room with controlled temperature (22 ± 1°C), humidity (60 ± 10%) and light (12 h per day) for at least four days before being used. Food and water were available ad libitum. All experiments were carried out in the same room between 10.00 and 14.00 h to minimize diurnal variations. Separate groups of animals were used for each test.

2.2. Chemicals

L-NAME (Sigma-Aldrich S.r.l., Milan) was dissolved in saline and WIN 55,212-2 (Research Biochemical, Natick, MA) in 10% dimethylsulfoxide (DMSO) (v/v) in saline.

2.3. Treatment

2.3.1. Acute treatment

In preliminary experiments mice were injected i.p. with either vehicle or WIN 55,212-2 to assess the time course and the dose–response curves of the analgesic, hypothermic and cataleptic effects. In a second series of experiments, the effect of L-NAME pretreatment (50 mg/kg i.p.) on analgesia, hypothermia and catalepsy induced by a single injection of WIN 55,212-2 (6 mg/kg for analgesia and 8 mg/kg for hypothermia and catalepsy) was studied. The mice were divided into four groups at random and received: saline + vehicle, L-NAME + vehicle, saline + WIN 55,212-2 and L-NAME + WIN 55,212-2.

Saline or L-NAME was injected i.p. 20 min before vehicle or WIN 55,212-2. Analgesia, hypothermia and catalepsy were measured, respectively, 15, 30 and 60 min after vehicle or WIN 55,212-2.

2.3.2. Chronic treatment

The effect of L-NAME pretreatment on the development of tolerance to the analgesic, hypothermic and cataleptic effects of WIN 55,212-2 was studied by injecting the drug once a day at different time intervals, depending on the effects considered (5 days for analgesia and 9 days for hypothermia and catalepsy). Mice allocated to four groups at random were treated with saline or L-NAME (50 mg/kg i.p.) once a day 20 min before vehicle or cannabinoid (6 mg/kg i.p. when analgesia was to be determined and 8 mg/kg i.p. when hypothermia and catalepsy were to be assessed). On alternate days the analgesia, hypothermia and catalepsy were measured, respectively, 15, 30 and 60 min after vehicle or WIN 55,212-2. In the second series of experiments the effect of chronic L-NAME treatment on the analgesia, hypothermia and catalepsy induced by a single dose of WIN 55,212-2 was examined. Mice allocated to two groups at random were injected daily i.p. with saline or L-NAME (50 mg/kg). On day 5 for analgesia and on day 9 for hypothermia and catalepsy, 20 min after the injection of saline or L-NAME the animals received vehicle or the same i.p. dose of WIN 55,212-2 which had been used to induce tolerance. Thus, the treatment groups then numbered four: saline + vehicle, L-NAME + vehicle, saline + WIN 55,212-2 and L-NAME + WIN 55,212-2. The analgesic, hypothermic and cataleptic effects were determined, respectively, 15, 30 and 60 min after vehicle or WIN 55,212-2.

2.3.3. Analgesia

Antinociceptive activity was measured in a hot-plate test (Eddy and Leimbach, 1953). A plexiglass cylinder (18 cm high, 20 cm diameter) was used to keep the mouse on the plate, which was kept at a temperature of 55 ± 0.5°C. Response latency either to a hind paw lick or to jump was recorded. The cut-off time was set at 30 s. Antinociception was quantified as the percentage of maximal effect (%MPE) using the following formula: $\%MPE = [(\text{test latency} - \text{control latency})/(\text{cut-off time} - \text{control latency})] \times 100$.

2.3.4. Measurement of body temperature

All measurements were made in a quiet room at an ambient temperature of 22 ± 1°C. The mice were allowed to adapt to temperature measurements for three days before the experiment. After 1-h acclimatization in the test room, body temperature was measured with a rectal thermistor probe (PRA-22002A, Ellab, Roedrove, Denmark) inserted to a constant depth of 3.0 cm. After a 30 s equilibration period, the temperature was recorded to the nearest 0.1°C on a CTD-85-M thermometer (Ellab, Roe-

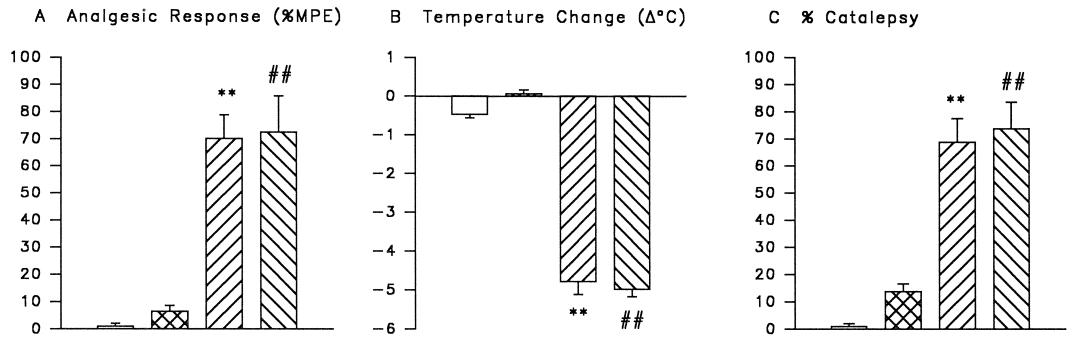

Fig. 1. Effect of L-NAME (50 mg/kg i.p.) on analgesia (A), hypothermia (B) and catalepsy (C) induced by, respectively, 6, 8 and 8 mg/kg i.p. WIN 55,212-2. L-NAME was administered 20 min before WIN 55,212-2 injection. Analgesia, hypothermia and catalepsy (referred to as percent catalepsy) were measured, respectively, 15, 30 and 60 min after WIN 55,212-2 injection (□) saline + vehicle; (hatched area) L-NAME + vehicle; (diagonal stripes from bottom left to top right) saline + WIN 55,212-2; (diagonal stripes from top left to bottom right) L-NAME + WIN 55,212-2 $^{**}P < 0.01$ versus saline + vehicle; $^{\#\#}P < 0.01$ versus L-NAME + vehicle. Tukey's test. Each bar represents the mean ± S.E.M. of measurements from ten mice for analgesia or eight mice for hypothermia and catalepsy.

drove, Denmark). During temperature measurement the mice were unrestrained and were hand-held gently at the base of the tail. Temperature was measured twice before treatment (basal). The data were expressed as the means ± S.E.M. of the °C change from baseline temperature.

2.3.5. Catalepsy

The cataleptic response was measured by the 'ring test' described by Pertwee (1972). The apparatus consisted of a wire ring (5.5 cm in diameter) fixed horizontally to a ring stand at a point 16 cm above the desk. The experimenters placed the mouse across the ring so that it was supported only by its front and rear paws. Its forepaws were placed over the ring and the rear paws were then placed on diametrically opposite points on the ring. The number of seconds the mouse remained motionless on the ring (except for breathing movements) were then recorded. The test was conducted for 5 min and catalepsy was measured as the percentage of the total time for which the mouse stayed immobile on the ring (referred to as percent catalepsy).

2.3.6. Statistical analysis

The results were expressed as the means ± S.E.M. The data were analyzed using a oneway analysis of variance (ANOVA) or a two-way ANOVA (group × time) followed by Tukey's test. Differences were considered significant at $P < 0.05$. Linear regressions were calculated according to Steel and Torrie (1960).

3. Results

3.1. Acute treatment

The time- and dose-dependence of WIN 55,212-2-induced analgesia, hypothermia and catalepsy was initially

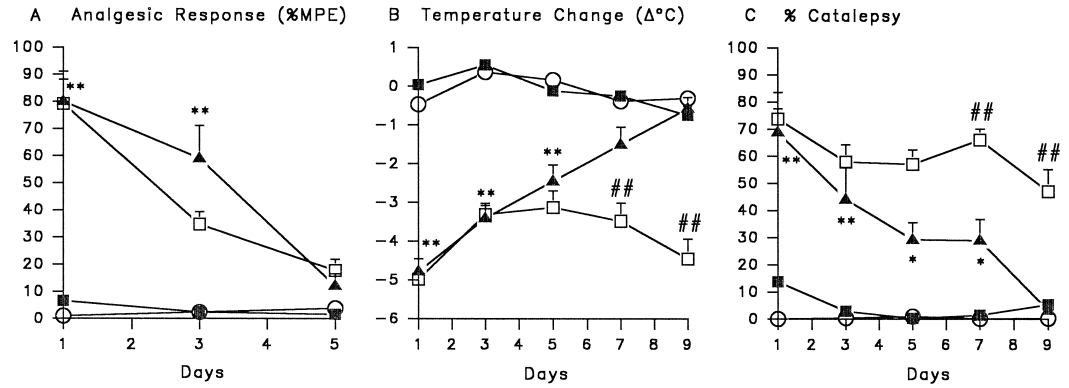

Fig. 2. Effect of L-NAME on the development of tolerance to the analgesic (A), hypothermic (B) and cataleptic (C) effect of WIN 55,212-2. L-NAME (50 mg/kg i.p.) was administered once a day 20 min before WIN 55,212-2 (6 mg/kg i.p. for analgesia and 8 mg/kg i.p. for hypothermia and catalepsy). Analgesia, hypothermia and catalepsy (referred to as percent catalepsy) were measured, respectively, 15, 30 and 60 min after WIN 55,212-2 injection. (○) saline + vehicle; (■) L-NAME + vehicle; (▲) saline + WIN 55,212-2; (□) L-NAME + WIN 55,212-2. $^{*}P < 0.05$, $^{**}P < 0.01$ versus saline + vehicle; $^{\#\#}P < 0.01$ versus saline + WIN 55,212-2. Tukey's test. Each point represents the mean ± S.E.M. for ten mice for analgesia or eight mice for hypothermia and catalepsy.

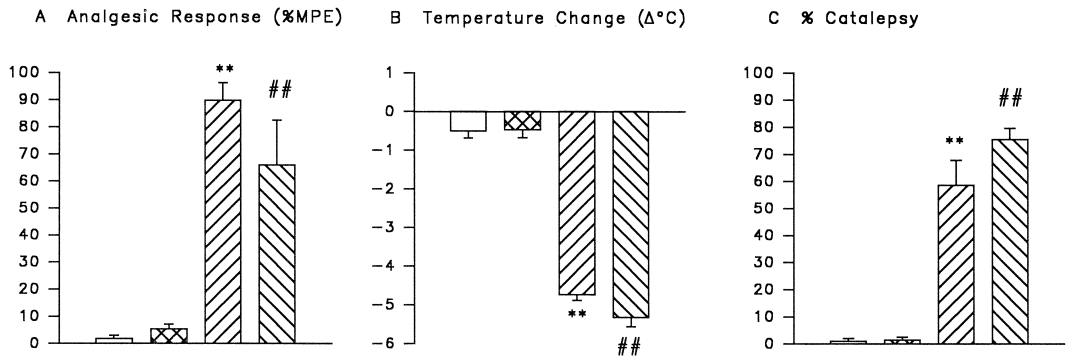

Fig. 3. Effect of repeated injections of L-NAME (50 mg/kg i.p. per day) on analgesia (A), hypothermia (B) and catalepsy (C) induced by a single dose of WIN 55,212-2 administered i.p. 20 min after L-NAME on day 5 (for analgesia) and on day 9 (for hypothermia and catalepsy). The analgesic, hypothermic and cataleptic (referred to as percent catalepsy) responses elicited by, respectively, 6, 8 and 8 mg/kg WIN 55,212-2 were measured 15, 30 and 60 min after cannabinoid injection. (□) saline + vehicle; (hatched area) L-NAME + vehicle; (diagonal stripes from bottom left to top right) saline + WIN 55,212-2; (diagonal stripes from top left to bottom right) L-NAME + WIN 55,212-2. $^{**}P < 0.01$ versus saline + vehicle; $^{\#\#}P < 0.01$ versus L-NAME + vehicle. Tukey's test. Each bar represents the mean ± S.E.M. from six mice for analgesia and hypothermia or eight mice for catalepsy.

examined so as to establish the peak time effect and the doses of WIN 55,212-2 producing an effect of about 70–80% and to be used in the chronic experiment. The analgesic effect peaked 15 min after WIN 55,212-2 (6 mg/kg i.p.) and hypothermia and catalepsy (both induced by 8 mg/kg i.p.) peaked at, respectively, 30 and 60 min post-injection (data not shown). Analgesia was linearly related to the log of the doses administered in the range between 2 and 7 mg/kg i.p., hypothermia between 4 and 12 mg/kg i.p. and catalepsy between 2 and 8 mg/kg i.p. (data not shown). To study the influence of L-NAME (50 mg/kg i.p.) on WIN 55,212-2 analgesia (6 mg/kg i.p.), hypothermia (8 mg/kg i.p.) and catalepsy (8 mg/kg i.p.) the NO synthase inhibitor was injected 20 min before the cannabinoid and the response was measured at the peak time. L-NAME, which per se was ineffective, did not modify the analgesic, hypothermic and cataleptic responses to WIN 55,212-2 (Fig. 1).

3.2. Chronic treatment

The effect of L-NAME on the development of tolerance to the analgesic effect of WIN 55,212-2 is shown in Fig. 2. Continued dosing with WIN 55,212-2 alone elicited a progressively lower response and by five days no mice showed analgesia. L-NAME, 50 mg/kg i.p. per day, coadministered with WIN 55,212-2 did not prevent the development of tolerance.

Tolerance also developed to the hypothermic effect of chronic WIN 55,212-2, and the mice were completely tolerant on days 7 and 9, when the body temperature of mice treated with WIN 55,212-2 did not significantly differ from that of the controls. The concurrent administration of L-NAME significantly blocked the development of WIN 55,212-2 tolerance, so that after nine days of daily WIN 55,212-2 the body temperature change for the L-NAME plus WIN 55,212-2 group was -4.45 ± 0.51°C, compared with -0.57 ± 0.29°C for the saline plus WIN 55,212-2 group (Fig. 2). Repeated administration of WIN 55,212-2 also resulted in the development of tolerance to the cataleptic effect, which was, however, inhibited by coadministration of L-NAME. With WIN 55,212-2 alone, the catalepsy declined from 68.8 to 3.2% over nine days; while with L-NAME, it never fell below 47.1% (Fig. 2).

Chronic administration of L-NAME did not alter either the analgesic response to a single injection of WIN 55,212-2 on day 5, or the hypothermic and cataleptic responses to WIN 55,212-2 given on day 9 of L-NAME treatment (Fig. 3).

4. Discussion

The present study explored the relationship between WIN 55,212-2 tolerance and the NO system. Repeated dosing with L-NAME inhibited the development of tolerance to the hypothermic and cataleptic effects of WIN 55,212-2, but not the tolerance to analgesia. This lack of effect on the development of tolerance to WIN 55,212-2 analgesia is consistent with the findings of Thorat and Bhargava (1994b), who reported that, in mice, repeated administration of L-NMMA, one of the most widely used NO synthase inhibitors, did not affect the development of tolerance to the analgesic action of Δ^9-tetrahydrocannabinol, an active constituent of marijuana.

The failure of L-NAME to alter the tolerance to WIN 55,212-2 analgesia needs particular comment. It has been found that, in mice, L-NAME administered i.p. is antinociceptive in hot plate assay (Moore et al., 1991; Mustafa, 1992; Brignola et al., 1994). Mustafa (1992), who studied the time–dose–response of L-NAME analgesia, reported that the effect peaked at 30 min and was dose-dependent with an ED_{50} value of 65.1 (48.83–80.34) mg/kg i.p. In our hands, however, L-NAME, injected i.p. in a mouse strain different from that used by other authors, at the dose of 50 mg/kg did not significantly affect the nociceptive

threshold either at 30 min or at any time considered within 3 h after drug injection (data not shown). Thus we excluded that a delayed analgesic effect of L-NAME could confuse the interpretation of our findings on the development of tolerance to the analgesic effect of WIN 55,212-2. Moreover L-NAME given chronically does not potentiate the analgesia induced by a single injection of WIN 55,212-2. Thus, together, these findings lead us to suggest that NO is not involved in the mechanisms underlying the tolerance to analgesic effect of WIN 55,212-2. In contrast to the results we present here, Thorat and Bhargava (1994b) observed that chronic treatment with L-NMMA failed to affect the tolerance to the hypothermic action of Δ^9-tetrahydrocannabinol in mice. This might possibly be related to the mouse strain, the type of cannabinoid drugs used, or the NO synthase inhibitor and its dose. Different NO synthase inhibitors have been shown to have different effects on opioid tolerance. Tolerance to the analgesic effect of *trans*-(1*S*,2*S*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide hydrochloride (U-50,488H) is inhibited in a dose-dependent manner by L-NMMA but not affected by N^G-nitro-L-arginine (NO_2Arg) (Kolesnikov et al., 1993; Thorat et al., 1993). L-NAME itself, following either acute or chronic treatment, is not hypothermic or cataleptic. In addition, given acutely or chronically, it does not potentiate the hypothermia and catalepsy induced by a single injection of WIN 55,212-2. The observation that L-NAME blocks the development of tolerance to the hypothermic and cataleptic effect of WIN 55,212-2 without altering its acute actions in mice is particularly intriguing since it implies a direct action of the NO synthase inhibitor on the mechanisms of tolerance itself. The inhibition of tolerance development to the hypothermic and cataleptic effects of WIN 55,212-2 by L-NAME suggests that this tolerance may be associated with an increased formation of NO in the cerebral areas involved in cannabinoid hypothermia and catalepsy. This is not unlikely since NO synthase (Barjavel and Bhargava, 1995) and cannabinoid receptors (CB_1) (Herkenham et al., 1990) were found in the hypothalamus and in the basal ganglia areas which mediate, respectively, the hypothermia (Fitton and Pertwee, 1982; Martin, 1986) and catalepsy (Herkenham, 1995) induced by cannabinoids. Thus it seems interesting to establish whether mice chronically exposed to WIN 55,212-2 show an upregulation of NO synthase and/or an increase of NO synthase activity in these specific brain areas. To date, at least three distinct NO synthase isoforms have been identified: Ca^{2+}-dependent and constitutively expressed NO synthase in neurons (type I, Ca^{2+}-dependent and constitutively expressed NO synthase in endothelial cells (type III) and Ca^{2+}-independent inducible NO synthase, which is found in various cell types stimulated by lipopolysaccharides or cytokines (type II) (Nathan, 1992). L-NAME has a similar in vitro inhibitory potency against type I and type III NO synthase (Salter et al., 1995). Thus, at the moment we cannot establish which NO synthase isoform is involved in the development of tolerance to WIN 55,212-2 hypothermic and cataleptic effects. Recently it has been reported that 7-nitroindazole is more selective against type I NO synthase (Moore et al., 1993a,b) and may therefore be a useful tool for determining the role of type I NO synthase in cannabinoid tolerance.

The mechanism by which NO affects cannabinoid tolerance is not clear. Any event that results in the influx of Ca^{2+} into a cell containing constitutive NO synthase, for example the activation of NMDA receptors, can stimulate constitutive NO synthase and raise the NO levels (Fukuto and Chaudhuri, 1995). Tolerance to the hypothermic effect of WIN 55,212-2, might require mechanisms other than activation of the NMDA receptor. Thorat and Bhargava (1994b) reported that chronic treatment with MK-801, a non-competitive NMDA receptor antagonist, failed to affect the tolerance to the hypothermic action of Δ^9-tetrahydrocannabinol in mice. Concerning the tolerance to catalepsy induced by WIN 55,212-2, it is interesting to note that chronic treatment with MK-801 led to a significant reduction (52%) of Δ^9-tetrahydrocannabinol receptor gene expression in the rat dorsal striatum (Mailleux and Vanderhaeghen, 1994). The authors suggest that the levels of Δ^9-tetrahydrocannabinol receptor mRNA in the rat dorsal striatum are positively modulated by glutamate. In the presence of similar glutamatergic regulation of the Δ^9-tetrahydrocannabinol receptor in mice, L-NAME should favour the development of tolerance to cannabinoid catalepsy. In our hands, however L-NAME inhibits the development of tolerance to the cataleptic effect of WIN 55,212-2, suggesting that the mechanism underlying the NOS inhibitor action is independent of the NMDA receptor.

In summary, the present findings clearly demonstrated that NO is involved in the development of tolerance to the hypothermic and cataleptic effects induced by WIN 55,212-2 but does not mediate tolerance to the cannabinoid's analgesic effect. Recent reports have indicated a role of NO in the development of opiate tolerance and NO synthase inhibitors are known to block the analgesic and hypothermic actions of opiates (Kolesnikov et al., 1992, 1993; Przewlocki et al., 1993, 1994; Thorat et al., 1993; Babey et al., 1994; Bhargava, 1994a, 1995; Elliott et al., 1994).

These findings suggest that the NO system is an important part of the mechanisms mediating tolerance to drugs of abuse.

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