ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4): 375 - 380
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Correlation between inhibitions of morphine withdrawal and nitric-oxide synthase by agmatine

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KEY WORDS agmatine; drug tolerance; morphine; nitric oxide; nitric-oxide synthase; opioid-related disorders; brain; idazoxan; naloxone; dizocilpine

ABSTRACT

AIM: To study correlation between inhibitions of naloxone-precipitated withdrawal jumps and nitric-oxide synthase (NOS) activity by agmatine. **METHODS**: NOS activities in mouse brain were measured by determination of concentration of [^{3}H]citrulline, the product of [^{3}H]arginine. **RESULTS**: Agmatine inhibited NOS activity in naive and morphine-dependent mouse cerebellum, forebrain, and thalamus in substrate-competitive manner *in vitro*. Naloxone induced withdrawal jumps and an increase in NOS activity in cerebellum, forebrain, and thalamus of abstinent mice. Pretreatment of mice with morphine plus agmatine inhibited the effect of naloxone to precipitate withdrawal jumps and increase in NOS activity. The effect of agmatine was blocked by idazoxan. **CONCLUSION**: The inhibitory effect of agmatine on naloxone-precipitated withdrawal jumps is related to its inhibition of NOS activity by substrate competitive manner and activation of imidazoline receptors.

INTRODUCTION

The diversity of drugs capable of attenuating opiate abstinence suggests that opiate withdrawal syndrome may be modulated at multiple sites involving a complexed variety of neurotransmitter system[1]. Excitatory amino acids were involved in opiate abstinence by activation of NMDA receptors and then increased activity of nitric oxide (NO)[2]. NMDA receptor antagonists and NO synthase (NOS) inhibitors attenuated opiate abstinence by inhibition of NOS[3,4]. The concentration of total nitrogen oxides in brain and cerebrospinal fluid was increased during the period of opiate-withdrawal of rats. Cerebellum NOS activity was increased in opiate withdrawal mice[5]. These results suggested that NO might participate in the formative processes of opiate substance dependence.

Agmatine, an endogenous ligand of imidazoline receptors (I-R), potentiated opiate analgesia, inhibited opiate tolerance in mice[6]. We have also found that agmatine caused weak analgesia and inhibited morphine-induced tolerance and substance dependence in mice and rats *in vivo*, and in guinea pig ileum longitudinal muscle *in vitro*. The aim of this paper was to investigate the relationship between inhibitions of opiate withdrawal and NOS activity by agmatine in naive, morphine-dependent, and abstinent mice.

MATERIALS AND METHODS

Kunming mice ♂, n = 265, 28 g ± s 3 g obtained from Experimental Animal Center of Academy of Military Medical Sciences, Beijing (Certificate № 01-3032) were used. Agmatine, Tris, edetic acid, egtazic acid, *DL*-dithiothreitol, phenylmethylsulphonyl fluoride, leupeptin, soybean trypsin inhibitor, aprotinin, Dowex AG50wx-8, NADPH, N^{ω}-nitro-*L*-arginine, *DL*-citrulline, calmodulin, and other chemicals were obtained from Sigma Co. [^{3}H]arginine (2.0 PBq · mol^{-1}) was purchased from DuPont-New England Nuclear. Idazoxan and dizocilpine were products of Research Biochemicals International, and clonidine was obtained from

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Received 1997-10-10 Accepted 1998-09-22

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Changzhou Pharmaceutical Factory, respectively. Morphine and naloxone were produced by Qinghai Pharmaceutical Factory, Xining and Sihuan Pharmaceutical Factory, Beijing, respectively.

NOS activity assay[5] The naive and morphine-dependent mice were decapitated. Cerebellum, forebrain, and thalamus were homogenized with 20 strokes at 4 ℃ in 4 volumes of a buffer containing Tris 50, egtazic acid 1, *DL*-dithiothreitol 1 mmol·L^{-1}, phenylmethyl-sulphonyl fluoride 100 mg·L^{-1}, leupeptin 10 mg·L^{-1}, soybean trypsin inhibitor 10 mg·L^{-1}, and aprotinin 2 mg·L^{-1} (final concentrations), to pH 7.4 with HCl at room temperature. The crude homogenate was centrifuged at 20 000 × g, at 4 ℃ for 60 min. Protein concentration of the supernatant was determined by Coomassie's brilliant blue method. Activity of NOS in the supernatant was determined by measuring in duplicate the conversion of [^{3}H]arginine to [^{3}H]citrulline. The incubation was initiated by addition of 150 μg protein in the supernatant to Tris buffer 50 mmol·L^{-1} (pH 7.4) containing (final concentration) NADPH 100 μmol·L^{-1}, $CaCl_2$ 2 mmol·L^{-1}, calmodulin 1 μg, [^{3}H]arginine 92.5 MBq in total volume of 100 μL. After 30-min incubation at 37 ℃, the reaction was stopped with 4 mL HEPES 20 mmol·L^{-1} (pH 5.5) containing (final concentration) *L*-citrulline 1 mmol·L^{-1}, egtazic acid 0.2 mmol·L^{-1} and edetic acid 2 mmol·L^{-1}. The incubation mixture was added to Dowex AG50wx-8 (Na^+ form) cation-exchange columns (1.5 mL) to separate [^{3}H]arginine from [^{3}H]citrulline, and then the columns were flushed with 2 mL water to wash out remnant [^{3}H]citrulline. Liquid 100 μL was taken from 4100 μL filtrate and then added into 3 mL absolute ethanol. After amalgamation, xylene 7 mL containing 2,5-diphenyl-oxazole 112.97 mmol·L^{-1} and 1,4-bis-(5-phenyl oxazoyl-2)-benzene 4.12 mmol·L^{-1} was added to the mixture. Concentration of [^{3}H]citrulline was quantified by liquid scintillation spectroscopy (Wallac 1409, LKB Co, Holland). The activity of NOS was expressed as [^{3}H]citrulline pmol·min^{-1}·g^{-1} (protein) or % of control.

Morphine-dependent model Mice were pretreated with morphine (d_1 = 30, d_2 = 40, d_{3-7} = 50 mg·kg^{-1} sc, bid) and normal saline (control). At the appropriate time, withdrawal jumps were precipitated by ip injection of naloxone 5 mg·kg^{-1}. Withdrawal jumps were observed in the first 15 min after ip naloxone and then the mice were killed to determine the activity of NOS at 20 min after ip naloxone.

Administration of agmatine *in vitro* Cerebellum, forebrain, and thalamus were obtained from naive or morphine-dependent mice. The activities of NOS in these tissues were determined as described above in the absence and presence of different concentrations of agmatine, clonidine, dizocilpine, and N^{ω}-nitro-*L*-arginine *in vitro*.

Administration of agmatine *in vivo* To observe the inhibitory effects of drugs on increase in NOS activity during the period of abstinence, agmatine, clonidine, dizocilpine, and N^{ω}-nitro-*L*-arginine were given (sc, bid, for 5 d) 30 min prior to sc morphine. The last doses of the drugs were given 30 min prior to administration of naloxone. Idazoxan was given (ip) 15 min prior to agmatine.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed with t test.

RESULTS

NOS activity, as measured concentration of [^{3}H]citrulline [pmol·min^{-1}·g^{-1} (protein)], was present in cerebellum (230 ± 22), forebrain (46 ± 29), and thalamus (135 ± 53) for naive mice. After direct administration of agmatine to the measure system of NOS activity, the activities of NOS in cerebellum, forebrain, and thalamus of naive mice were inhibited in a dose-dependent manner. The effect of agmatine was not antagonized by selective I-R antagonist idazoxan (Tab 1).

However, dizocilpine and clonidine did not inhibit the cerebellum NOS activity of naive mice (Fig 1). The effects of agmatine, dizocilpine, and clonidine on NOS activity in cerebellum, forebrain, and thalamus of morphine-dependent mice were similar to those of naive mice.

In the kinetic analysis test, Lineweaver-Burk or Dixon plots revealed that the inhibitory effects of agmatine on the two kinds of NOS were competitive, but the K_i values of them were different (Fig 2). The K_i obtained from naive mouse cerebellum [n = 3 samples, (131 ± 61) μmol·L^{-1}] was 4.37-fold greater than that obtained from morphine-dependent mouse cerebellum [n = 3 samples, (30 ± 6) μmol·L^{-1}].

Tab 1. Inhibitory effects of drugs on NOS activities of naive mouse cerebellum, forebrain, and thalamus *in vitro*. $n = 5$ mice. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* NS. $^dP > 0.05$ *vs* agmatine 100 μmol·L^{-1}.

Drug/μmol·L^{-1}	Cerebellum	Forebrain	Thalamus
NS	100 ± 0	100 ± 0	100 ± 0
Agmatine 10	74 ± 23^b	82 ± 19^b	67 ± 21^c
Agmatine 100	44 ± 27^c	26 ± 10^c	32 ± 18^c
Agmatine 1000	15 ± 14^c	10 ± 6^c	14 ± 8^c
Idazoxan 0.01 + agmatine 100	27 ± 28^d	19 ± 11^d	29 ± 12^d
Idazoxan 0.1 + agmatine 100	37 ± 36^d	39 ± 19^d	27 ± 9^d
Idazoxan 1 + agmatine 100	37 ± 38^d	29 ± 16^d	36 ± 12^d

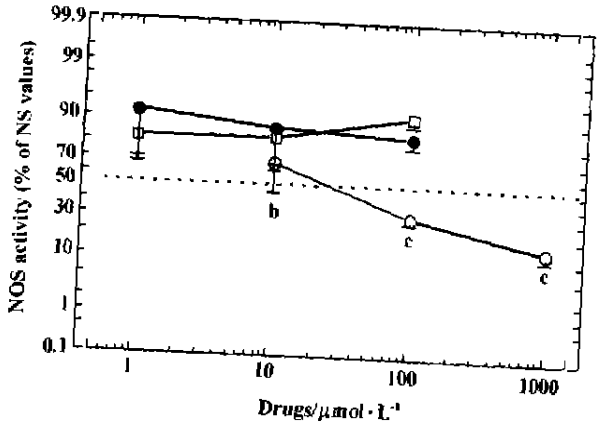

Fig 1. Effects of agmatine (○), dizocilpine (●), clonidine (□), and normal saline (NS) on NOS activity of naive mouse cerebellum *in vitro*. $n = 3$ mice. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* NS.

Although pretreatment of mice with morphine for 3 – 7 d tended to increase the NOS activities of cerebellum, forebrain, and thalamus compared with normal saline, the differences were not significant. When the morphine-dependent mice were in abstinent syndrome induced by naloxone 20 min prior to the beginning of the measurement test of NOS activity, however, NOS activities of cerebellum, forebrain, and thalamus were increased by 2 – 3 times compared with normal saline (Fig 3, Tab 2). Because the increase in NOS activity reached maximal point on d 5 of pretreatment with morphine, the mice in following test were all pretreated for 5 d.

Copretreatment of mice with morphine plus agmatine, clonidine, dizocilpine, or N^{ω}-nitro-*L*-arginine inhibited the effect of naloxone to increase

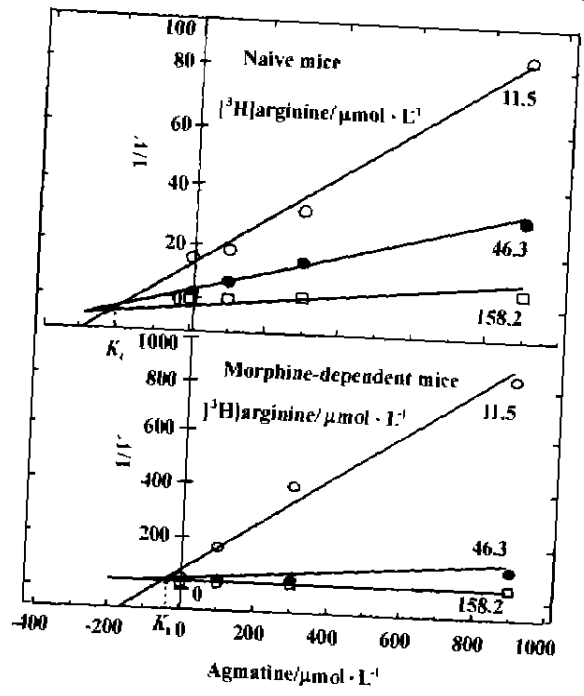

Fig 2. Dixon plots for NOS of cerebellum of naive and morphine-dependent mice. $n = 3$ mice.

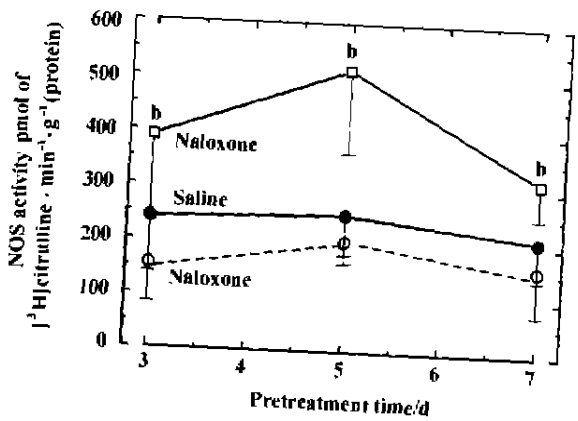

Fig 3. Cerebellum NOS activity [pmol of [^{3}H]citrulline · min^{-1} · g^{-1} (protein)] of mice pretreated with saline (○) or morphine (●, □). Naloxone or saline was given 20 min prior to the beginning of the test. $n = 4$ mice. $\bar{x} \pm s$. $^bP < 0.01$ *vs* saline.

NOS activity of cerebellum, forebrain, and thalamus in morphine-dependent mice in a dose-dependent manner. However, agmatine sc had no effect on NOS activity of naive mouse cerebellum (Tab 2). The inhibitory effect of agmatine was antagonized by selective I-R antagonist idazoxan in a dose-dependent manner (Tab 3).

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Apr; 20 (4)
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Tab 2. Inhibitory effect of pretreatment with drugs *in vivo* on NOS activities [pmol of [^{3}H]citrulline·min^{-1}·g^{-1}(protein)] of cerebellum, forebrain, and thalamus of morphine-dependent and naive mice in the presence of naloxone. $n = 4$ mice. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* NS.

Drugs/mg·kg^{-1}	Morphine-dependent mice			Naive mice
	Cerebellum	Forebrain	Thalamus	Cerebellum
Normal saline	502 ± 146	236 ± 75	473 ± 147	240 ± 36
Agmatine 2.5	303 ± 77^b			238 ± 44^a
Agmatine 10	224 ± 44^c	113 ± 76^c	161 ± 73^c	274 ± 38^a
Agmatine 40	159 ± 95^c			258 ± 21^a
Dizocilpine 0.03	352 ± 55^a			
Dizocilpine 0.1	285 ± 75^b			
Dizocilpine 0.3	266 ± 126^b			
Clonidine 0.3	234 ± 24^c			
N^{ω}-nitro-*L*-arginine 5	408 ± 102^a			
N^{ω}-nitro-*L*-arginine 10	201 ± 63^c			
N^{ω}-nitro-*L*-arginine 20	35 ± 8^c			

Tab 3. Influence of idazoxan in effect of agmatine on NOS activity [pmol [^{3}H]citrulline·min^{-1}·g^{-1}(protein)] of morphine-dependent mouse cerebellum *in vitro*. $n = 4$ mice. $\bar{x} \pm s$. $^cP < 0.01$ *vs* agmatine.

Drug/mg·kg^{-1}	Cerebellum
Normal saline	502 ± 146
Agmatine 10	224 ± 44
Idazoxan 1 + agmatine 10	245 ± 36
Idazoxan 3 + agmatine 10	504 ± 234^c
Idazoxan 9 + agmatine 10	579 ± 142^c

The inhibitory effects of agmatine and other drugs as mentioned above on NOS activity were parallel to their inhibitory effects on mouse withdrawal jumps in a dose-dependent manner except for clonidine, which did not attenuate withdrawal jumps of morphine-dependent mice, although it inhibited NOS activity (Tab 4).

DISCUSSION

Naloxone ip induced a significant increase in NOS activities in cerebellum, forebrain, and thalamus of morphine-dependent mice. These results further support the theory that NO might take part in the pathophysiological processes of tolerance to and substance dependence on opioids[4]. We have found that agmatine prevents and reverses the formation of

Tab 4. Inhibitory effect of pretreatment with drugs on withdrawal jumping % of morphine-dependent mice induced by naloxone. Fisher exact test, $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* NS + morphine.

Drug/mg·kg^{-1}	*n*	Withdrawal jumping %
Normal saline	16	6.3
Normal saline + morphine 750*	16	93.8
Agmatine 2.5 + morphine 750	16	50.0^b
Agmatine 10 + morphine 750	16	37.5^c
Agmatine 40 + morphine 750	16	18.8^c
Idazoxan 1 + agmatine 10 + morphine 750	16	50.0^b
Idazoxan 3 + agmatine 10 + morphine 750	16	87.5^a
Idazoxan 9 + agmatine 10 + morphine 750	12	100.0^a
Clonidine 0.3 + morphine 750	5	100.0^a
dizocilpine 0.03 + morphine 750	12	41.7^b
dizocilpine 0.1 + morphine 750	12	25.0^c
dizocilpine 0.3 + morphine 750	16	6.3^c

*Total dose of morphine for 5 d.

tolerance to and substance dependence on morphine in mice and rats *in vivo*, and in guinea pig ileum longitudinal muscle *in vitro* by activation of I-R. Up to date, it is not known what is the post-receptor mechanism of inhibitory effect of agmatine on morphine-induced substance dependence. In the present test, agmatine sc with morphine not only inhibited naloxone-induced withdrawal jumps, but also at the same time attenuated the increase in NOS activity of abstinent mouse cerebellum, forebrain and thalamus. The inhibitory effect of agmatine was blocked by selective I-R antagonist idazoxan. Agmatine given by the same route and same dose as mentioned above, however, had no significant inhibitory effect on NOS activity of cerebellum in naive mice. These results suggested that agmatine inhibited the increase in NOS activity in abstinent mice by activation of I-R and this effect was related to its inhibition of withdrawal jumps of morphine-dependent mice induced by naloxone.

On the other hand, direct addition of high concentrations of agmatine to the measuring system of NOS activity *in vitro* inhibited the activity of NOS and the inhibitory effect was blocked by idazoxan. In contrast to agmatine, dizocilpine, which inhibited NOS activity *in vivo*[4], and another I-R agonist clonidine had no inhibitory effect on NOS activity in the test. These results indicate that agmatine can directly inhibit NOS activity. The inhibitory effect is related to the

chemical structure of agmatine and has no relation to the activation of I-R. According to the Dixon plot, the direct inhibitory effect might be performed by substrate competitive manner. Although the concentration of agmatine in naive mouse central nervous system under the condition of sc injection might be difficult to achieve its K_i value [(131 ± 61) $\mu mol \cdot L^{-1}$, $n = 3$], agmatine 40 $mg \cdot kg^{-1}$ injected by sc to the abstinent mice might arrive at its K_i value [(30 ± 6) $\mu mol \cdot L^{-1}$, $n = 3$], because the NOS obtained from withdrawal mice was much more sensitive to agmatine than that obtained from naive mice. Because it naturally existed in mammalian including man, agmatine might be an endogenous competitive NOS inhibitor[7].

Multiple NOS inhibitors have been developed to unravel the physiology of NO and to specifically block pathological production of this molecule. Most of the drugs are *L*-arginine analogues with an affinity for NOS similar to or lower than *L*-arginine, whose apparent K_m values range between 1 – 30 $\mu mol \cdot L^{-1}$[8]. Although agmatine is a weaker NOS inhibitor, its relevance lies in the fact that this amine is naturally occurring in mammalian systems. Considering that agmatine binds NOS with significantly lower affinity than *L*-arginine, effective competition is only conceivable if agmatine is more concentrated than *L*-arginine. A comparison of the distribution between the two amines in different organs reveals that *L*-arginine may be 4 – 50 times more abundant in $\mu mol \cdot g^{-1}$ (wet tissue) than agmatine. This observation might imply that agmatine is a weaker endogenous NOS inhibitor, but the values do not necessarily reflect either the amine concentrations at NOS catalytic sites, or their functions in different physiological and pathological stimulations. Recent evidences indeed suggest that conditions may exist in which agmatine may become a more effective competitor of *L*-arginine. In cultured astrocytes, interferon-γ induced 1.5-fold increase in activity of *L*-arginine decarboxylase[9] and agmatine was 20-fold higher in rat aorta after ischemic injury[7]. Moreover, the concentration of *L*-methyl-arginine, which is also an endogenous NOS inhibitor, is normally 1/500 of *L*-arginine, but in the cause of renal failure, its concentration rises enough to inhibit NOS activity, thereby causing immune disfunction and hypertension[10]. In the present research, NOS activity in abstinent mouse cerebellum was much more sensitive to inhibitory effect of agmatine than those in naive mice. It is more important that agmatine inhibits NOS activity by not only substrate competitive manner as mentioned above but also activation of I-R, at least in the period of abstinent syndrome in morphine-dependent mice. So agmatine might be an inhibitory regulator to modulate the pathophysiological process of opiate substance dependence by inhibition of NOS activity.

In conclusion, the inhibitory effect of agmatine on naloxone-induced withdrawal jumps of morphine-dependent mice might be related to its inhibitory effect on NOS activity by activation of I-R and substrate competitive manner.

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胍丁胺抑制小鼠吗啡戒断与其抑制一氧化氮合酶的关系

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关键词 胍丁胺；药物耐受性；吗啡；一氧化氮；一氧化氮合酶；阿片类有关的紊乱；脑；咪唑克生；纳洛酮；地佐环平

目的：观察胍丁胺抑制纳洛酮引起小鼠吗啡戒断跳跃与其抑制一氧化氮合酶（NOS）的关系. **方法**：用测定[^{3}H]胍氨酸浓度的方法确定 NOS 活性. **结果**：在体外胍丁胺底物竞争性抑制正常和吗啡依赖小鼠小脑、端脑和丘脑 NOS 活性. 纳洛酮引起吗啡依赖小鼠戒断跳跃和小脑、端脑、丘脑 NOS 活性升高. 用吗啡和胍丁胺共同处理小鼠显著抑制纳洛酮促使小鼠戒断跳跃和 NOS 活性升高的作用. 咪唑克生抑制胍丁胺的此作用. **结论**：胍丁胺对纳洛酮引起戒断跳跃的抑制作用与其通过激活咪唑啉受体和底物竞争性抑制 NOS 活性相关.

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