CEREBROVENTRICULAR INJECTION OF CLONIDINE CAUSES ANALGESIA MEDIATED BY A NITROGEN PATHWAY

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SUMMARY

Whereas neuroaxially administered clonidine produces analgesia partially mediated by α_2-adrenoceptor-induced augmented synthesis of nitric oxide (NO), the central mechanisms by which clonidine produces its antinociceptive effects are still speculative. We used the tail-flick model of acute pain in mice to further explore the role of NO in mediating clonidine-induced central analgesia. Cerebroventricular administration of the following agents was studied: clonidine, L-arginine (NO precursor), the NO production inhibitor nitro-L-arginine-methyl ester (L-NAME), the NO antagonist methylene blue (MB), and nitroglycerine (NO-releasing agent). Analgesic response was achieved with clonidine and L-arginine. Simultaneous administration of L-arginine and clonidine produced no additive analgesic effect. Prior administration of L-NAME or MB partially abolished the clonidine-induced analgesic effect, whereas nitroglycerine administration did not

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affect it. NO may be involved in the mediation of the central antinociceptive effects of clonidine. Further investigation is necessary to determine the possible role of NO-promoting agents in analgesia when co-administered with clonidine.

KEY WORDS

analgesia, clonidine, nitric oxide, arginine, L-NAME, cerebroventricular injection, mouse

INTRODUCTION

α_2-Agonists have been used by veterinarians for many years in animal anesthesia and pain relief, but only in the last two decades have these agents begun to be used in humans for their sedative and analgesic properties. Clonidine is an α_2-adrenoceptor agonist currently used in general and regional anesthesia, mainly via the epidural or intrathecal route /1,2/. A link between the α_2-adrenergic and cholinergic systems in the spinal cord has been established, by which acetylcholine in excess is produced in response to α_2-adrenergic stimulation /3/. This in turn stimulates spinal nitric oxide synthase (NOS) to produce nitric oxide (NO), which mediates antinociception /3-6/.

In the central nervous system (CNS), α_2-adrenergic receptors are located within brainstem nuclei involved in antinociception /7/. Although evidence supports a role for α_2-adrenergic receptor activation leading to the attenuation of central sympathetic flow as a possible mechanism of clonidine-mediated analgesia in humans /8/, the exact mechanism by which clonidine induces central analgesia is unclear /9,10/.

The present study was designed to further investigate the involvement of the nitrogen pathway (NO-mediated) in the central antinociceptive effects of clonidine, administered alone or in combination with each of the following: L-arginine, an NO precursor; the NOS inhibitor nitro-L-arginine-methyl ester (L-NAME) /11/; methylene blue (MB), an inhibitor of soluble guanylyl cyclase, a very important enzyme on the NO-cyclic GMP (cGMP) pathway /12/; and

the NO-releasing agent nitroglycerine. We used a noxious thermal stimulus in the mouse tail-flick model.

MATERIALS AND METHODS

Animals and treatment

The study was approved by the Animal Use and Care Committee of the Technion Faculty of Medicine. Experiments were performed on male ICR mice (25 ± 2 g) randomly allocated to different groups and cared for in accordance with national and institutional guidelines. Animals were housed in plastic cages under normal lighting (lights on 07.00-19.00 h) in a temperature- and humidity-controlled animal facility where they had free access to food and water in the home cage.

Under light halothane anesthesia, intracerebroventricular (i.c.v.) injections (1 μl each) were administered as previously described /13/, approximately 2 mm caudal and 2 mm lateral to bregma at a depth of 3 mm, using a 10-μl Hamilton syringe fitted to a 30-gauge needle with V1 tubing. Preliminary studies showed that MB dye injected in this manner reliably produced an i.c.v. injection. Drugs were dissolved in saline.

Drugs

Drugs injected i.c.v. included clonidine (Rafa Laboratories, Jerusalem, Israel), L-arginine, MB, and L-NAME (Sigma Chemical Co., St. Louis, MO, USA), and nitroglycerine (Merck, Darmstadt, Germany).

Tail-flick assay

Analgesia was determined using the tail-flick technique with a 54°C hot-water bath as a heat source /13,14/. The latency from thermal stimulus to withdrawal of the tail was measured using a stopwatch. Before experimental treatments, baseline latencies following saline injection were determined to the nearest 0.1 s for each animal. Post-treatment tail-flick latencies were determined at the indicated times for each experiment, and a maximal latency of 10 s was used to minimize tissue damage. The latency ratio (LR) was defined as the ratio between post-injection latency and baseline

latency. Analgesia was defined quantitatively as at least doubling of the LR as compared with baseline values for each mouse. We chose to study the minimal dose producing analgesia, taking into consideration the planned co-administration set of experiments allowing detection of a response within the non-injurious 10 s time frame. In order to achieve 95% analgesic response (ED_{95}) for a given animal, a 1.1-fold dose of the ED_{50} was used /15/. Each dose was tested on at least six mice (each mouse was checked once).

Study design

We first conducted baseline dose-response analysis for each of the following drugs: clonidine, L-arginine, L-NAME, MB, and nitroglycerine. This provided us with the ED_{50} for the relevant drugs. After obtaining the data for each drug alone, we evaluated the effect of co-administration of clonidine with L-arginine, L-NAME, MB, and nitroglycerine.

Data analysis

Two-tailed *t*-test, one-way analysis of variance, and the Bonferroni post hoc test were used to compare latencies pre- and post-treatment. A p value <0.05 was considered statistically significant. Data are presented as means ± SEM.

RESULTS

Saline

Single as well as multiple i.c.v. injections of 1 μl saline produced no change in mouse behavior, i.e. motor activity, or in the results of the tail-flick assay before as compared with post i.c.v. injection (data not shown). This proved the lack of effect of the saline vehicle or the mode of injection. Therefore each animal served as its own control for the further experiments.

Clonidine (Fig. 1)

In order to determine the dose-response curve (LR vs drug dose), clonidine was injected in increasing dosages (1-4 μg per mouse). The

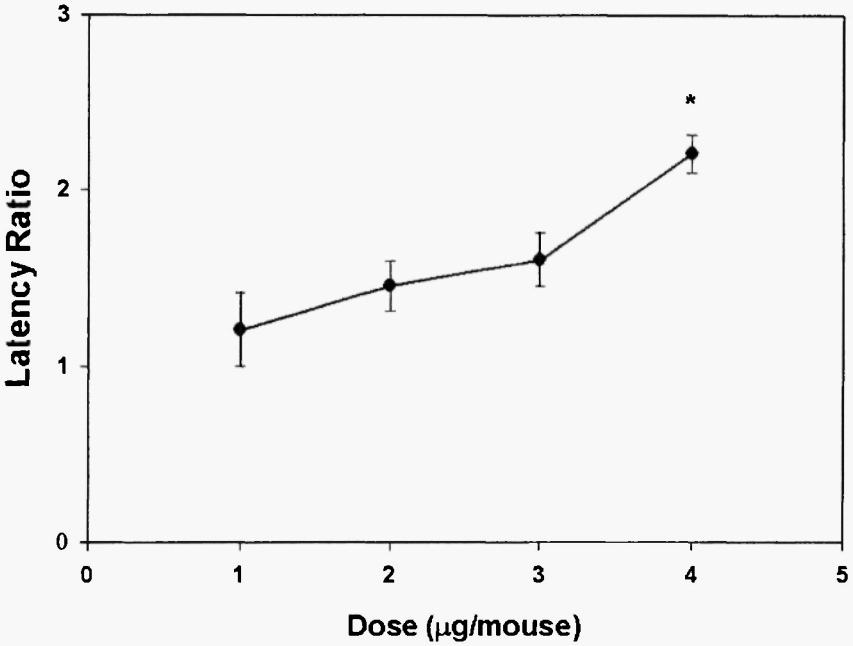

Fig. 1: Dose-response curve for intracerebroventricular injection of clonidine. * Control group received 1.0 μl saline per mouse (for each point, n = 6, $p < 0.001$).

calculated analgesic effective dose (ED_{50}) of clonidine was 3.6 μg per mouse (LR = 2). In order to achieve 95% analgesic response (ED_{95}) for a given animal, a 1.1-fold dose of the ED_{50} (4.0 μg per mouse which produced an LR of 2.43 as compared with control; n = 7, $p < 0.001$) was chosen for the rest of the experiments.

L-Arginine (Fig. 2)

In order to determine the dose-response curve, L-arginine was injected in increasing dosages (10-300 μg per mouse). The calculated analgesic ED_{50} was 272 μg per mouse and the calculated ED_{95} of i.c.v. injections of L-arginine was 300 μg per mouse, which produced an LR of 2.13 as compared with control (n = 6, $p < 0.05$). Interestingly, simultaneous injections of 300 μg per mouse L-arginine with 4 μg per mouse clonidine resulted in no change in the LR. However, i.c.v.

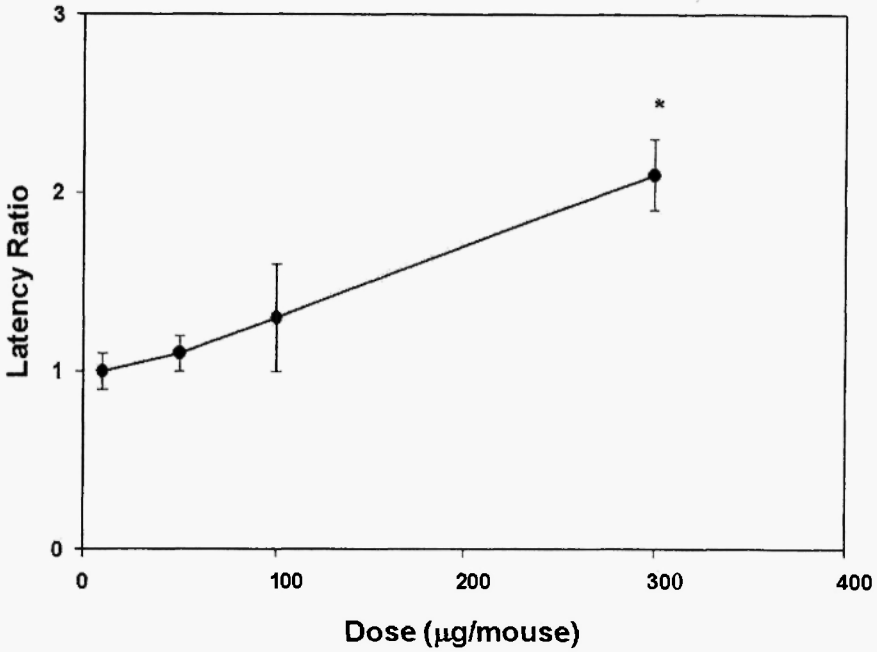

Fig. 2: Dose-response curve for intracerebroventricular injection of L-arginine (for each point, n = 6, p <0.001 compared with control).

injections of L-arginine 15 min before clonidine produced a statistically significant (n = 6, p <0.05) additive analgesic effect of 1.3-fold at a dosage of 100 µg per mouse.

L-NAME (Fig. 3)

I.c.v. injection of L-NAME alone (10-200 µg per mouse) did not produce an analgesic effect (data not shown). In contrast, i.c.v. injection of L-NAME, at doses of 10 and 50 µg per mouse, 15 min before clonidine (4 µg per mouse) caused a reduction of 1.75- and 1.86-fold, respectively, in the LR as compared with i.c.v. injection of clonidine alone (n = 6, p <0.001).

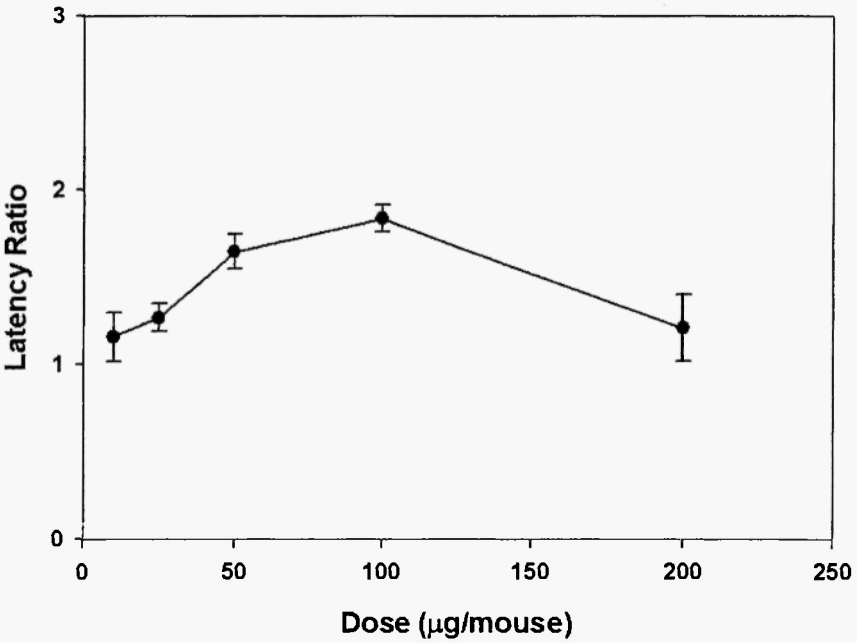

Fig. 3: Dose-response curve for intracerebroventricular injection of L-NAME (for each point, n = 6, p <0.001 compared with control).

Methylene blue (Fig. 4)

I.c.v. injection of MB alone (5-50 μg per mouse) did not produce an analgesic effect (data not shown). Pretreatment with MB (10 and 50 μg/kg) 15 min before clonidine (4 μg per mouse) produced a reduction in LR of 2.15- and 2.52-fold, respectively, as compared with clonidine injections alone (n = 6, p <0.01).

Nitroglycerine

I.c.v. injections of nitroglycerine (2-5 μg per mouse), alone or as a pretreatment 15 min before i.c.v. injection of clonidine (4 μg per mouse), did not affect LR.

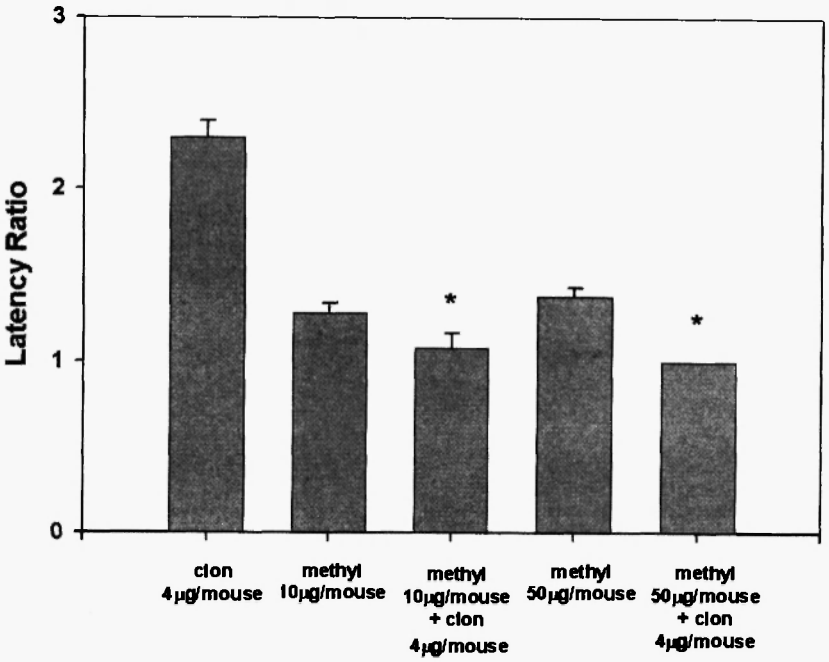

Fig. 4: Effect of methylene blue (methyl) on clonidine (clon) analgesia (for each point, n = 6, $p < 0.01$ compared with control).

DISCUSSION

The tail-flick model enables measurement of the time needed for heat-inflicted pain to be sensed and reacted upon by the animal. Using this model and i.c.v. injections, we found that centrally administered clonidine produced substantial analgesia that was additively affected at some dosages of L-arginine and significantly attenuated when the NO antagonists L-NAME or MB were co-administered.

NO is a small gaseous molecule produced as a free radical with short-term activity in the CNS, where it acts as a non-adrenergic, non-cholinergic neurotransmitter /16/. NO has been identified as an important modulator of neuronal function, responsible in part for communication between neural cells, control of neuronal development, and synaptic plasticity of the CNS /17/. NO may also be

involved in mechanisms of consciousness and anesthesia as well as in nociceptive processes /16,18/.

NO-dependent mechanisms have already been shown to be involved in the sedative effect of clonidine /19/, as well as in its spinal antinociceptive effects /20/. Clonidine is capable of releasing NO by activation of α_2-adrenoceptors on the endothelial surface /21/ and also in the spinal cord /22/. Therefore, we hypothesized that drugs involved in NO metabolism or in its mechanism of action might modulate the analgesic effect of clonidine in the CNS via its NO-related pathways.

Adrenergic mechanisms of analgesia have been explored for nearly 100 years, starting with a report on the analgesic effect of epinephrine when administered intrathecally /23/. This effect is now known to occur secondarily to α_2-adrenoceptor stimulation /9/. The α_2-adrenoceptor, a peptide traversing the cell membrane, can react with extracellular ligands to initiate a cascade of events leading to a physiological outcome. The cytoplasmic portion of the α_2-adrenoceptor interacts with the G-protein system, providing a means for signal transduction and the stimulation of several effector systems /2/. The traditional explanation for clonidine-mediated supraspinal analgesia is its ability to inhibit central sympathetic outflow. However, recent evidence suggests that the central antinociceptive effect produced by α_2-adrenoceptor agonists may be due in part to their ability to release acetylcholine /20/, which stimulates nicotinic and muscarinic receptors /24-26/ to liberate NO /22,27/, known to be involved in central antinociception /28/. A similar mechanism has been reported regarding the spinal effects of clonidine, in which NO in increased concentrations was found in microdialysate samples from the dorsal horn of sheep spinal cord following clonidine administration /15/. Another study showed that spinal NO played an important role in the anti-allodynic effect of intrathecal clonidine in a rat model of neuropathic pain /6/.

L-Arginine and its modulatory effects on chronic pain perception were shown experimentally by Duarte *et al.* /29/. We chose to pre-administer L-arginine and other nitrogen-related drugs following the findings by Kawabata *et al.* /30/, who demonstrated in a tail-flick model in mice that the maximal analgesic effect of intravenously administered L-arginine occurred 10-20 min after injection. It is assumed that the process by which NO is produced from L-arginine is not immediate and therefore needs time and energy. Hence, only

pretreatment with L-arginine produced an additive analgesic effect, whereas co-injection of L-arginine and clonidine did not change the analgesic effect of clonidine in a significant way.

L-NAME suppresses the activity of NOS and prevents the generation of NO. Our results, namely the attenuation of clonidine-induced analgesia by L-NAME, are in accordance with those of Lothe *et al.* /31/, who reported that antinociception from subarachnoid clonidine is blocked by the NOS inhibitor *N*-methyl-L-arginine in sheep. We would have expected a significant analgesic effect produced by injections of nitroglycerine, in view of the anticipated rise of NO concentration, since nitrovasodilators such as nitroglycerine and nitroprusside are NO donors /32/. However, no increased analgesic effect was observed following nitroglycerine treatment. This might be explicable by the low concentrations of nitroglycerine used. We did not use higher doses because, due to safety considerations /11,21/, we were unable to obtain higher concentrations of nitroglycerine. Pretreatment with MB reduced the analgesic effect of clonidine, as it probably acts as an NO scavenger and also competes with NO for the active binding site on soluble guanylyl cyclase (resulting in less production of cGMP) /12,29/.

Future trends in pain management involve multidrug regimens, including α_2-adrenoceptors agonists. The latter, however, possess substantial side effects, e.g. bradycardia, hypotension, and sedation /10/. In this regard, incorporation of NO-promoting agents into the analgesic regimen might contribute to the reduction of the dose of clonidine while maintaining analgesic potency. This subject must be further tested.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Technion Vice-President for Research Fund. We thank Miss Ruth Singer for editing the manuscript.

REFERENCES

1. Tellez S, Colpaert F, Marien M. Acetylcholine release in the rat prefrontal cortex in vivo: modulation by α_2-adrenoceptor agonists and antagonists. J Neurochem 1997; 68: 778-785.

2. Khan ZP, Ferguson CN, Jones RM. Alpha-2 and imidazoline receptor agonists. Their pharmacology and therapeutic role. Anaesthesia 1999; 54: 146-165.
3. Xu Z, Li P, Tong C, Figueroa J, Tobin JR, Eisenach JC. Location and characteristics of nitric oxide synthase in sheep spinal cord and its interaction with α_2-adrenergic and cholinergic antinociception. Anesthesiology 1996; 84: 890-899.
4. Gordh T Jr, Jansson I, Hartvig P, Gillberg PG, Post C. Interactions between noradrenergic and cholinergic mechanisms involved in spinal nociceptive processing. Acta Anaesthesiol Scand 1989; 33: 39-47.
5. Xu Z, Tong C, Pan H-L, Cerda SE, Eisenach JC. Intravenous morphine increases release of nitric oxide from spinal cord by an α-adrenergic and cholinergic mechanism. J Neurophysiol 1997; 78: 2072-2078.
6. Pan H-L, Chen S-R, Eisenach JC. Role of spinal NO in antiallodynic effect of intrathecal clonidine in neuropathic rats. Anesthesiology 1998; 89: 1518-1523.
7. Unnerstall JR, Kopajtic TA, Kuhar MJ. Distribution of alpha 2 agonist binding sites in the rat and human central nervous system: analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. Brain Res 1984; 319: 69-101.
8. Liu N, Bonnet F, Delaunay L, Kermarec N, D'Honneur G. Partial reversal of the effects of extradural clonidine by oral yohimbine in postoperative patients. Br J Anaesth 1993; 70: 515-518.
9. Reddy SVR, Maderdrut JL, Yaksh TL. Spinal cord pharmacology of adrenergic agonist-mediated antinociception. J Pharmacol Exp Ther 1980; 213: 525-533.
10. Eisenach JC, De Kock M, Klimscha W. α_2-Adrenergic agonists for regional anesthesia. A clinical review of clonidine (1984-1995). Anesthesiology 1996; 85: 655-674.
11. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109-142.
12. Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. Annu Rev Pharmacol Toxicol 1985; 25: 171-191.
13. Pick CG. Antinociceptive interaction between alprazolam and opioids. Brain Res Bull 1997; 42: 239-243.
14. D'Amour FE, Smith DL. A method of determining loss of pain sensation. J Pharmacol Exp Ther 1941; 72: 74-79.
15. Trevor AJ, Miller RD. General anesthetics. In: Katzung BG, ed. Basic & Clinical Pharmacology, 6th Ed. East Norwalk, CT: Appleton & Lange, 1995; 381-394.
16. Fürst S. Transmitters involved in antinociception in the spinal cord. Brain Res Bull 1999; 48: 129-141.
17. Garthwaite J, Boulton CL. Nitric oxide signaling in the central nervous system. Annu Rev Physiol 1995; 57: 683-706.
18. Tobin JR, Martin LD, Breslow MJ, Traystman RJ. Selective anesthetic inhibition of brain nitric oxide synthase. Anesthesiology 1994; 81: 1264-1269.

19. Soares de Moura R, Rios AAS, de Oliveira LF, Resende ÂC, de Lemos Neto M, Santos EJA, et al. The effects of nitric oxide synthase inhibitors on the sedative effect of clonidine. Anesth Analg 2001; 93: 1217-1221.
20. Gordh T, Sharma HS, Alm P, Westman J. Spinal nerve lesion induces upregulation of neuronal nitric oxide synthase in the spinal cord. An immunohistochemical investigation in the rat. Amino Acids 1998; 14: 105-112.
21. Vanhoutte PM, Miller VM. Alpha$_2$-adrenoceptors and endothelium-derived relaxing factor. Am J Med 1989; 87 (Suppl 3C): 1S-5S.
22. Xu Z, Chen S-R, Eisenach JC, Pan H-L. Role of spinal muscarinic and nicotinic receptors in clonidine-induced nitric oxide release in a rat model of neuropathic pain. Brain Res 2000; 861: 390-398.
23. Weber HU. Anaesthesia durch Adrenaline. Verh Dtsch Ges Inn Med 1904; 21: 616-619.
24. Bitner RS, Nikkel AL, Curzon P, Donnelly-Roberts DL, Puttfarcken PS, Namovic M, Jacobs IC, Meyer MD, Decker MW. Reduced nicotinic receptor-mediated antinociception following in vivo antisense knock-down in rat. Brain Res 2000; 871: 66-74.
25. Decker MW, Meyer MD, Sullivan JP. The therapeutic potential of nicotinic acetylcholine receptor agonists for pain control. Expert Opin Investig Drugs 2001; 10: 1819-1830
26. Gomeza J, Zhang L, Kostenis E, Felder CC, Bymaster FP, Brodkin J, et al. Generation and pharmacological analysis of M_2 and M_4 muscarinic receptor knockout mice. Life Sci 2001; 68: 2457-2466.
27. Song H-K, Pan H-L, Eisenach JC. Spinal nitric oxide mediates antinociception from intravenous morphine. Anesthesiology 1998; 89: 215-221.
28. Riedel W, Neeck G. Nociception, pain, and antinociception: current concepts. Z Rheumatol 2001; 60: 404-415.
29. Duarte IDG, dos Santos IR, Lorenzetti BB, Ferreira SH. Analgesia by direct antagonism of nociceptor sensitization involves the arginine-nitric oxide-cGMP pathway. Eur J Pharmacol 1992; 217: 225-227.
30. Kawabata A, Nishimura Y, Takagi H. L-Leucyl-L-arginine, naltrindole and D-arginine block antinociception elicited by L-arginine in mice with carrageenin-induced hyperalgesia. Br J Pharmacol 1992; 107: 1096-1101.
31. Lothe A, Li P, Tong C, Yoon Y, Bouaziz H, Detweiler DJ, Eisenach JC. Spinal cholinergic alpha-2 adrenergic interactions in analgesia and hemodynamic control: role of muscarinic receptor subtypes and nitric oxide. J Pharmacol Exp Ther 1994; 270: 1301-1306.
32. Landry DW, Oliver JA. The pathogenesis of vasodilatory shock. N Engl J Med 2001; 345: 588-595.