INVOLVEMENT OF NITRIC OXIDE IN CLONIDINE-INDUCED SPINAL ANALGESIA

Stela Aronov[1], Ron Ben-Abraham[2], Dafna Givati-Divshi[3] and Yeshayahu Katz[1,4]*

[1]Laboratory for Research in Anesthesia, Pain, and Neuroscience, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, [2]Department of Anesthesiology, Sourasky Medical Center, Tel Aviv affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, [3]Department of Anesthesiology, Rambam Medical Center, Haifa and [4]Department of Anesthesiology, HaEmek Medical Center, Afula, Israel

SUMMARY

Background: The chronic pain relieving effects following spinal administration of clonidine are probably connected to α_2-adreno-receptor-induced augmented synthesis of nitric oxide (NO) in the spinal cord. In contrast, when acute pain is considered, the possible role of NO is still speculative. The aim of the present study was to explore the role of NO in acute pain relief following intraspinal administration of clonidine.

Methods: We used the mouse tail-flick model of acute pain. Spinal injections of the following agents and their combinations were administered: 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).

* Author for correspondence:
Yeshayahu Katz, M.D., D.Sc.
Department of Anesthesiology
HaEmek Medical Center
18101 Afula, Israel
e-mail: ykatz18@hotmail.com

Results: A 95% analgesic response was achieved with 2.0 μg clonidine. L-Arginine produced analgesia, and L-arginine administration followed by clonidine resulted in a pronounced synergistic analgesic effect. This synergistic effect was attenuated by L-NAME. Pretreatment with MB decreased and nitroglycerine administration did not affect the clonidine-induced analgesia.
Conclusions: NO may be involved in the mediation of the acute pain relieving effects of intraspinally administered clonidine. Further research is warranted to establish the potential benefits and possibility for incorporation of NO promoting agents in therapeutic regional pain regimens.

KEY WORDS

analgesia, clonidine, nitric oxide, spinal injection, tail-flick, mouse

INTRODUCTION

In the last two decades α_2-adrenergic agonists have been increasingly introduced into various regional analgesic regimens in view of their proven potential to cause profound pain control with a non-opioid mechanism /1/. Clonidine is the most popular α_2-adrenergic receptor agonist used clinically in regional anesthesia /2/. Its analgesic effects were shown when used to treat patients with chronic pain epidurally /2-5/. However, the exact mechanism of clonidine's pain alleviating effects is not clear /2/, though evidence supports a role for α_2-adrenergic receptor activation, as shown by the ability to reverse epidural clonidine-mediated analgesia in humans by the α_2-adrenergic antagonist yohimbine /6/. The α_2-adrenergic receptors are located on primary afferent terminals at the peripheral and spinal endings and in superficial laminae of the spinal cord and within brainstem nuclei involved in antinociception /7/. A link between the α_2-adrenergic and cholinergic systems in the spinal cord may exist by which acetylcholine in excess is produced in response to the α_2-adrenergic stimulation, which in turn stimulates nitric oxide synthase (NOS) to produce nitric oxide (NO) in the spinal cord /8-10/.

NO, being an unconventional neurotransmitter in that it has no special storage facility and release apparatus, is generated *de novo*

from its precursor L-arginine /11/. It then diffuses out of the producing cell and acts on neighboring cellular elements to activate soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), which acts as a second messenger to produce the desired effect. NO production has been shown to be involved in mediating antinociception /12/.

The present study was designed to further investigate the antinociceptive effects of intraspinal clonidine administered alone or in combination with: L-arginine - a NO precursor /11/; the NOS inhibitor nitro-L-arginine-methyl ester (L-NAME); methylene blue (MB), an inhibitor of sGC, a very important enzyme on the NO-cGMP pathway /13/; and the NO releasing agent nitroglycerine. We used the thermal noxious stimulus in the mouse tail-flick model.

MATERIALS AND METHODS

Animals and surgery

Male ICR mice (25 ± 2 g) cared for in accordance with national and institutional guidelines were used. Spinal injections (SI) were administered by lumbar puncture /14/ under light halothane anesthesia using a 10-μl Hamilton syringe fitted to a 30-gauge needle with V1 tubing. Preliminary studies showed that the MB dye injected in this manner reliably produced an intrathecal SI. Drugs were dissolved in saline.

The Animal Studies Committee of the Technion Institution approved the study.

Drugs

SI drugs included clonidine (Rafa Laboratories, Jerusalem, Israel), MB, L-arginine, L-NAME (Sigma Chemical Co., St. Louis, MO, USA), and nitroglycerine (Merck, Darmstadt, Germany).

Tail-flick assay

Analgesia was determined using a 54°C hot-water bath as a heat source and the tail-flick technique /15,16/. The latency from the thermal stimulus to the withdrawal of the tail was measured using a stopwatch. Before experimental treatments, baseline latencies follow-

ing saline injection were determined to the nearest 0.1 second for each animal. Post-treatment tail-flick latencies were determined at the indicated times for each experiment, and a maximal latency of 10 seconds 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 to baseline values for each mouse. Each dose was tested on seven mice (each mouse was checked once).

Study design

We first conducted baseline dose-response and time-response analysis for each of the following drugs: clonidine, L-arginine, L-NAME, MB and nitroglycerine. This provided us with the median effective dose and the time to peak response for the relevant drugs. After obtaining the baseline data, we evaluated the effect of co-administration of clonidine with L-arginine, L-NAME, MB and nitro-glycerine.

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 ± standard error (SEM).

RESULTS

Spinal injections

Clonidine (Fig. 1)

In order to determine the dose response curve (LR vs drug dose), clonidine was injected in increasing dosages of 1.0-6.25 μg/mouse ($n = 7$). The calculated analgesic effective dose (ED_{50}) of clonidine was 1.80 μg/mouse. In order to achieve 95% analgesic response for a given animal, a 1.3-fold dose of the ED_{50} (which at 2.0 μg/mouse produced a LR of 2.43 as compared to control, $n = 7$, $p < 0.01$) was chosen for the rest of the experiments /17/.

Fig. 1: Dose response curve for spinal injections of clonidine. * Control group received 2.0 μl/mouse of saline (for each point n = 7).

L-Arginine (Fig. 2)

Spinal injections of L-Arginine at 50 and 100 μg/mouse caused a 1.32- or 2.0-fold increase in LR, respectively ($n = 7$, $p < 0.01$).

L-Arginine and clonidine (Fig. 3)

Spinal injections of L-arginine (50 μg/mouse) followed 20 minutes later by clonidine (2 μg/mouse) produced a latency ratio of 5.0, 2.05-fold greater (5.0/2.43) than that produced by clonidine injection alone ($n = 7$, $p < 0.001$) - a synergistic effect. In contrast, simultaneous injection of clonidine and L-arginine did not produce a significant change in the LR (data not shown).

L-NAME

Spinal injections of L-NAME at a dose of 10 μg/mouse caused a small decrease in LR (0.9). Dosage of L-NAME higher than 10 μg/mouse elicited an immediate response in the tail-flick test.

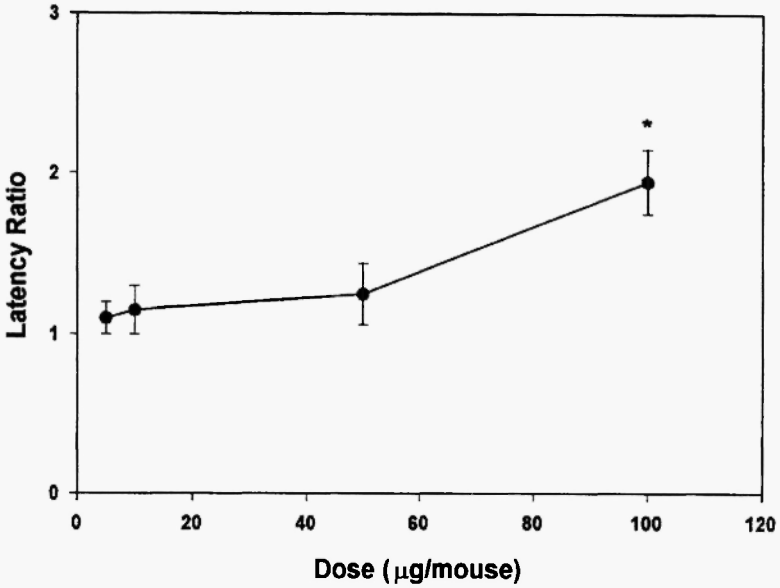

Fig. 2: Dose response curve for spinal injections of L-arginine. Each dose was tested in seven mice. * $p < 0.01$ compared to control.

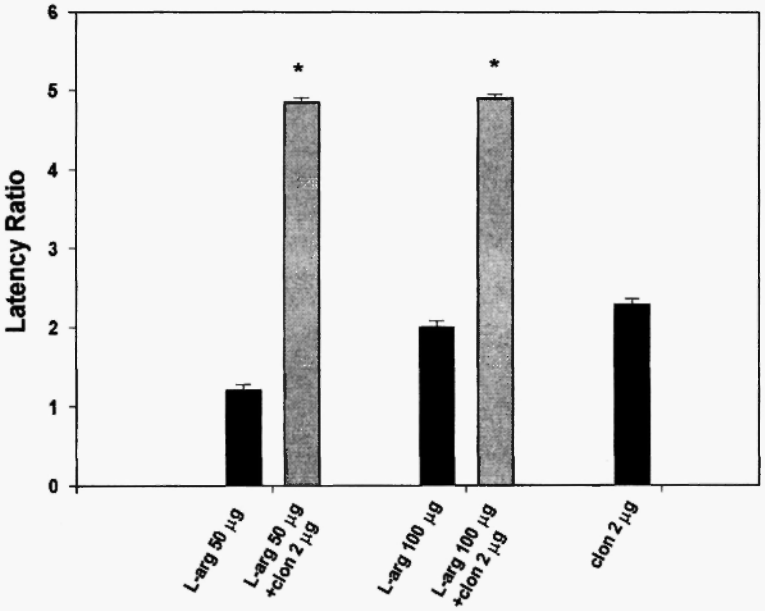

Fig. 3: Effect of L-arginine on clonidine analgesia. Each dose was tested in seven mice. * $p < 0.001$ compared to either clonidine or L-arginine.

L-NAME, L-arginine and clonidine

Spinal injections of L-NAME (10 μg/ mouse) 20 min before L-arginine (50 μg/mouse) and clonidine (2 μg/mouse) caused only a 3.34-fold ($n = 7$, $p < 0.01$) increase in the LR (compared to the 5.0-fold synergistic effect shown in Fig. 3).

Methylene blue (Fig. 4)

Spinal injections of MB alone (5-50 μg/mouse) did not produce an analgesic effect (defined as 2.0-fold increase in the LR). Pretreatment with MB (10-50 μg/mouse) 20 min before clonidine (2 μg/mouse) produced a latency ratio of 1.86 to 1.6, respectively, representing a decrease in the analgesic effect as compared to clonidine injections alone ($n = 7$, $p < 0.05$).

Nitroglycerine

Spinal injections of nitroglycerine up to 2 μg/mouse produced no change in the LR. Pretreatment with nitroglycerine (2 μg/mouse) before clonidine (2 μg/mouse) did not affect the LR.

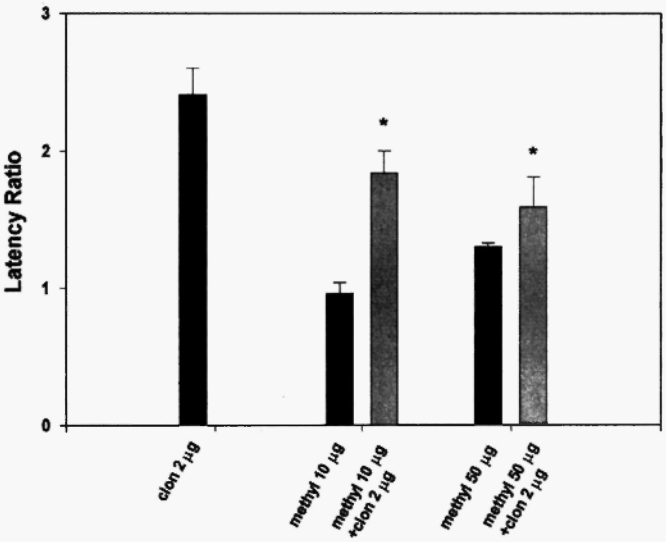

Fig. 4: Effect of methylene blue on clonidine analgesia. Each dose was tested in seven mice. * $p < 0.05$ compared to chlonidine.

DISCUSSION

The aim of this study was to examine the dependence of spinal α_2-adrenoreceptor agonist antinociceptive mechanisms and NO-dependent pathways. We found that the NO precursor L-arginine enhanced whereas NO antagonists reduced antinociception from intrathecally administered clonidine.

NO is a small gaseous molecule produced as a free radical with short-term activity in peripheral nerves, the dorsal horn, and possibly other parts of the central nervous system (CNS) where it acts as a neurotransmitter /18/. NO has high affinity to O_2 and to the heme unit of hemoglobin as well as to other proteins containing heme groups, such as sGC. NO has been identified as an important modulator of neuronal function responsible in part for communication between neural cells, and control of neuronal development and synaptic plasticity of the CNS /19/. NO may also be involved in mechanisms of consciousness and anesthesia as well as in nociceptive processes /20/. Its presence in the CNS may lead to alterations in synaptic transmission, which are expressed as hyperalgesia, facilitation of sensation, and windup /21/.

NO-dependent mechanisms have already been shown to be involved in the sedative effect of clonidine /22,23/ as well as its spinal antinociceptive effects /23/. Clonidine is capable of releasing NO by activation of α_2-adrenoreceptors on the endothelial surface /24/ and also in the spinal cord /25/. Therefore, we hypothesized that drugs involved in NO metabolism or its mechanism of action might modulate the analgesic effect of clonidine in the spinal cord via its NO-related pathways.

In this experimental study we used the tail-flick model for acute pain sensation in mice. The use of this model enables measurement of the time needed for heat-inflicted pain to be sensed and reacted upon by the animal. Our results demonstrated that pretreatment with L-arginine (a precursor of NO) followed 20 minutes later by spinal injection of clonidine produced substantial analgesia, as reflected by the significant increase in the LR compared to baseline. When carefully analyzed, these agents were demonstrated to act synergistically, i.e., an additive effect would have produced a latency ratio of 3.79 (2.43 + 1.32) as compared to the observed 1.36-fold higher analgesic response (5.0). These results, together with the observation of lack of

analgesic effect when L-arginine was injected at the same time as clonidine, support the notion that NO is involved in transmission of pain at the level of the spinal cord. The synergistic effect of L-arginine with that of clonidine probably stems from increased production of NO in the spinal neural tissue given the fact that L-arginine is the substrate for the production of NO. As this L-arginine-related enhanced production of NO needs time and energy to occur, only pretreatment with L-arginine produced an analgesic effect, whereas co-injection of L-arginine and clonidine did not change the analgesic effect of clonidine in a significant way.

Our results are supported by similar findings by Kawabata *et al.* who demonstrated in a tail-flick model in mice that the maximal analgesic effect of 100 μg/kg intravenously administered L-arginine occurred 10-20 min after the injection /26/. Further support for the NO-producing effect of L-arginine as the main mechanism of its analgesic potential comes from the study of Duarte *et al.* /12/ who found that L-arginine caused a significant analgesic effect in the hyperalgesia of carrageenan injected into the hind paws of mice, whereas D-arginine did not. The interpretation is that D-arginine is not a substrate for NOS. Hence, its administration does not cause an augmented production of NO.

L-NAME is an antagonist to the production of NO, suppressing the activity of NOS and preventing the generation of NO. Further support for the hypothesis of NO as a mediator of clonidine's analgesic effect comes from the fact that L-NAME administration alone caused anti-nociception and even hyperalgesia when administered in high concentrations (<10 μg/mouse). This nociceptive effect of L-NAME was attenuated by L-arginine. These observations are in accordance with the report by Lothe *et al.* on the fact that antinociception from subarachnoid clonidine is blocked by the NOS inhibitor *N*-methyl-L-arginine (NMLA) in sheep /27/. Similarly, we would have expected a significant analgesic effect produced by spinal injection of nitroglycerine in view of the anticipated rise of NO concentration in the spinal neural tissue /21/. Nitrovasodilators, such as nitroglycerine and nitroprusside, release NO by a non-enzymatic pathway /28/. However, we did not observe changes in the LR when we used nitroglycerine up to 2 μg/mouse. This might be explained by the low concentration of nitroglycerine used. We did not use higher doses, as we were unable to

obtain higher concentrations of nitroglycerine (due to safety regulations) /11,24/.

Pretreatment with MB reduced the analgesic effect of clonidine due to its NO-antagonizing effects acting either as a competitive inhibitor of NO on the active binding site on GC (resulting in less production of cGMP) or as a NO scavenger /12,13/.

Adrenergic mechanisms of analgesia have been explored for more than 100 years starting with the report on the analgesic effect of epinephrine when administered intrathecally /29/, which is now known to occur secondarily to α_2-adrenoreceptor stimulation /1/. The α_2-adrenoreceptor, a peptide traversing the cell membrane, can react with extracellular ligands to initiate a cascade of events that lead to a physiological effect. The cytoplasmic portion of the α_2-adrenoreceptor interacts with the G-protein system providing a means for signal transduction and stimulator of several effector systems /30/. Recent evidence suggests that the antinociception produced by the α_2-adrenoreceptor agonists may be due in part to acetylcholine release in the spinal cord /31/ which, in turn, stimulates the release of NO /32/. Indeed, clonidine was shown to increase NO production in rat spinal cord *in vitro* and in microdialysate samples from the dorsal horn of the sheep spinal cord /33/. In addition, spinal NO was shown to play an important role in the anti-allodynic effect of intrathecal clonidine in a rat model of neuropathic pain /9/. Nevertheless, the role of NO in spinal mechanisms of nociception is still controversial. Several pieces of evidence suggest that in contrast to our observations, NO possesses a pro-nociceptive action, as demonstrated in animal models of chronic pain in which increased production of NO was measured after nerve constriction or tissue inflammation /34,35/. In addition, intrathecal injection of NOS inhibitors attenuates inflammation-induced hyperalgesia and allodynia in rats /35/, and pretreatment with systemic NOS inhibitors delays the development of thermal hyperesthesia caused by sciatic nerve ligation /36/. NO production in the spinal cord may affect the perception of pain in complex ways. It is possible that sustained noxious stimulation in animals results in hyeralgesia and allodynia accompanied by sensitization of the spinal cord - processes which are NO-dependent and are therefore influenced from the persisting inflammatory process-induced increased synthesis of NO /35/. In contrast, our study was based on a model of acute pain, a fact which may

explain our results, namely that increased NO effects may contribute to antinociception.

Veterinarians have used α_2-agonists (xylazine, medetomidine) for many years for regional anesthesia, but experience with these agents in humans is only from the last 20 years. Clonidine is a non-specific α_2-adrenoreceptor agonist, with an α_2 to α_1 selectivity of 39, proved to be safe for spinal administration /30/. Nevertheless, its use can be accompanied by substantial side effects, including bradycardia, hypotension and sedation /2/. Hence, future advanced regional analgesic regimens should accomplish the goal of maintaining clonidine antinociceptive qualities while minimizing its side effects. In this regard, promotion of the spinal activity of NO with the incorporation of NO-promoting agents in the analgesic regimen might contribute to the reduction of the dose of clonidine while maintaining its analgesic potency.

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