Laboratory Investigation

Antinociceptive synergistic interaction between morphine and n^{ω} -nitro l-arginine methyl ester on thermal nociceptive tests in the rats

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Purpose: This study was conducted to demonstrate if subeffective dose of N^{ω} Nitro L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, interacts with morphine when given intrathecally (IT), epidurally (EP), and intravenously (IV) to produce a synergistic antinociceptive effect in normal rats.

Key words

ANALGESIA: acute pain;

ANALGESICS: morphine;

ANAESTHETIC TECHNIQUES: intrathecal, epidural, intravenous;

PHARMACODYNAMICS: dose-response, interaction;

PHARMACOLOGY: L-NAME.

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Methods: Chronically catheterized 72 male Wistar rats were used in this study. We measured the tail flick latency in response to thermal stimulation of the tail on a hot plate (53°C), and determined dose-response functions of IT, EP and IV morphine, L-NAME, and morphine co-administered with subeffective doses of L-NAME. The antinociceptive effect was evaluated using the doses for 50% maximum probable effect (ED_{50}). The interaction between morphine and L-NAME was evaluated using an isobolographic approach. ANOVA was used for the statistical analysis. Significance was taken at $P < 0.05$.

Results: Morphine and L-NAME produced dose-related antinociceptive effects in the IT ($ED_{50} = 1.23 \pm 0.18 \mu\text{g}$ (Mean $\pm$ SEM) and $76.0 \pm 14.5 \mu\text{g}$), EP ($ED_{50} = 32.6 \pm 2.4 \mu\text{g}$ and $560 \pm 97 \mu\text{g}$), and IV ($ED_{50} = 563 \pm 71.8 \mu\text{g}$ and $16.0 \pm 4.0 \text{ mg}$) groups, respectively. Co-administration of small doses of L-NAME and morphine produced reductions in the ED_{50} values for morphine ($0.16 \pm 0.03 \mu\text{g}$ in IT, $1.18 \pm 0.32 \mu\text{g}$ in EP, and $50.5 \pm 11.4 \mu\text{g}$ in IV groups) ($P < 0.01$), suggesting a multiplicative interaction of L-NAME with morphine.

Conclusion: L-NAME has a synergistic antinociceptive interaction with morphine in response to thermal stimulation when given intrathecally, epidurally or intravenously in rats.

Objectif: Rechercher si le N^{ω} nitro L-arginine méthyl ester (L-NAME), un inhibiteur de la synthèse de l'oxyde nitrique administré à doses réduites avec de la morphine par la voie intrathécale (IT), épidurale (EP) ou intraveineuse (IV) agissait en synergie avec celle-ci sur l'effet antinociceptif chez le rat normal.

Méthodes: Cette étude a été réalisée chez 72 rats Wistar porteurs de cathéters à demeure. Nous avons utilisé l'épreuve de

latence de la déviation de la queue en réponse à la stimulation thermique sur plaque chauffante (53%), et déterminé les caractéristiques dose-effet de la morphine IT, EP et IV, du L-NAME, et de la morphine et des doses réduites de L-NAME administrées conjointement. L'effet antinociceptif était évalué avec la moitié des doses de L-NAME susceptibles de produire 50% de l'effet maximal attendu (ED_{50}). L'interaction entre la morphine et la L-NAME était évaluée par isobolographie. L'ANOVA a servi pour l'analyse statistique. La valeur $P < 0,05$ était considérée comme significative.

Résultats: La morphine et L-NAME avaient une activité antinociceptive proportionnelle à la dose dans les groupes IT ($ED_{50} = 1,23 \pm 0,18 \mu\text{g}$ (moyenne $\pm$ SEM) et $76 \pm 14,5 \mu\text{g}$), EP ($ED_{50} = 32,6 \pm 2,4 \mu\text{g}$ et $560 \pm 97 \mu\text{g}$) et IV ($ED_{50} = 563 \pm 71,8 \mu\text{g}$ et $16,0 \pm 4,0 \text{ mg}$). L'administration conjointe de petites doses de L-NAME et de morphine réduisait les valeurs d' ED_{50} pour la morphine ($0,15 \pm 0,03 \mu\text{g}$ en IT, $1,18 \pm 0,32 \mu\text{g}$ en EP et $50 \pm 11,4 \mu\text{g}$ en IV; $P < 0,01$) suggérant une interaction multiplicatrice de L-NAME avec morphine.

Conclusion: Le L-NAME possède une interaction antinociceptive synergique avec la morphine en réponse à la stimulation thermique lorsqu'ils sont administrés conjointement par la voie intrathécale, épidurale ou intraveineuse.

In clinical practice, neuraxial morphine administration is widely used especially for the postoperative pain relief.¹⁻³ They have advantages, compared with local anaesthetics, of potent and long-lasting analgesia accompanied by a reduced incidence of central nervous depression without motor effects or hypotension. However, such use is limited by adverse effects which mainly depend on the dose of morphine.⁴⁻⁷ A key strategy in maintaining analgesic potency and in reducing the incidence of adverse effects with neuraxial morphine is to restrict the morphine dose by potentiating the antinociceptive effect.

Since nitric oxide (NO) was shown to be a central neurotransmitter^{8,9} there have been several reports of the relationship between NO and pain processing in the brain and the spinal cord.^{10,11} Intraperitoneal administration of N^ω-Nitro L-arginine methyl ester (L-NAME), the NO synthase (NOS) inhibitor, has antinociceptive activity in the rat.¹² Recently, a synergistic antinociceptive interaction between morphine and NOS inhibitors was reported when given intrathecally^{13,14} and intraperitoneally,¹⁵ but the L-NAME doses given for the antinociceptive effect were not calibrated, and there are few data on how morphine and NOS inhibitors interact on peripheral nociceptive stimulation when co-administered intrathecally, epidurally and intravenously.

In this study we investigated if intrathecal, epidural,

and intravenous co-administration of morphine and subeffective doses of L-NAME, the NOS inhibitor, interact on the antinociceptive effect using tail flick latency in response to hot plate stimulation in rats.

Methods

Materials and preparations

The following investigations were carried out under a study protocol approved by the Ethical Committee of Animal Research Center, University of Tsukuba, Japan, and the Local Ethical Committee, Department of Anesthesiology, Institute of Clinical Medicine, University of Tsukuba, Japan. Seventy-two male Wistar rats (weighing 400 to 475 g, Animal Breeding Institute, Ibaraki, Japan) were included. They were housed individually in standard cages which provided constant temperature $24 \pm 2^\circ\text{C}$ and light and dark alteration every 12 hr. The rats' food and water was ad libitum.

Polyethylene SP-10[®] catheters (OD = 0.61 mm and 16 cm long with 10 μl dead space, Natsume Company, Tokyo, Japan) were used as intrathecal, epidural, and intravenous catheters. All catheters were placed during pentobarbital anaesthesia, 35 mg $\cdot$ kg⁻¹, *ip*.

Each rat in the intrathecal group was fixed prone and the cervical region was shaved and cleaned with 70% alcohol. The neck fascicular membrane was exposed and the atlanto-occipital membrane was exposed and cut to leak cerebrospinal fluid. A catheter, sterilized by immersion in 70% ethanol and then washed and flushed with sterile saline, was inserted into the intrathecal space 85 mm long caudally so that the tip reached the lumbar enlargement under a microscopic field.¹⁶ The remainder of the catheter was fixed to the skull, tunneled to the skin and the tip was sealed with a dummy cannula. In the epidural group the lumbar fascicular membrane was exposed, and the transverse process of the sixth lumbar spine was exposed and laminectomized. A similarly treated catheter was inserted gently into the peridural space through the laminectomized spine 12 mm cephalad so that the tip reached the lumbar enlargement under a microscope.¹⁷ The remainder of the catheter was fixed to the lumbar spine and skin-tunneled up to the nuchal level. The tip was sealed with a dummy cannula. Chronic intravenous catheters were placed via the left femoral vein. The catheter was skin-tunneled to the nuchal level and the tip was sealed with a dummy cannula.

All the rats were given amikacin sulfate, 15 mg $\cdot$ kg⁻¹, *ip* to control infection. All the rats were kept undisturbed for at least a week before the antinociceptive study. Rats that developed hind limbs paralysis were excluded from the following antinociceptive study.

TABLE Drug and dose protocol, percent maximum possible effect (%MPE), and ED₅₀ values of the thermal tests in the intrathecal, epidural and intravenous groups.

1 Intrathecal groups

ITM (n = 6)		ITL (n = 6)		ITM + L (n = 6)		
M (μg)	%MPE	L-NAME (μg)	%MPE	M (μg)	L-NAME (μg)	%MPE
0	0.5 ± 1.5	0	3.2 ± 0.7	0	38	12.0 ± 2.6
0.58	10.9 ± 3.3	9.75	20.0 ± 3.8	0.066	38	44.0 ± 5.3
2.31	72.8 ± 9.7	39	43.2 ± 3.7	0.28	38	68.8 ± 5.9
9.22	87.1 ± 8.6	156	56.1 ± 10.6	0.11	38	91.6 ± 3.6
1.23 ± 0.18	ED ₅₀	76.0 ± 14.5	ED ₅₀	0.16 ± 0.03*		ED ₅₀

2 Epidural groups

EPM (n = 6)		EPL (n = 6)		EPM + L (n = 6)		
M (μg)	%MPE	L-NAME (μg)	%MPE	M (μg)	L-NAME (μg)	%MPE
0	2.5 ± 0.7	0	4.3 ± 1.9	0	280	17.8 ± 4.4
11.1	7.1 ± 2.9	96	31.7 ± 2.5	0.67	280	48.5 ± 1.9
44.4	57.1 ± 4.1	390	47.5 ± 3.8	2.8	280	62.1 ± 2.5
177	74.1 ± 6.4	1560	57.9 ± 5.1	11.1	280	67.8 ± 3.6
32.6 ± 2.4	ED ₅₀	560 ± 97	ED ₅₀	1.18 ± 0.32*		ED ₅₀

3 Intravenous groups

IVM (n = 6)		IVL (n = 6)		IVM + L (n = 6)		
M (μg)	%MPE	L-NAME (mg)	%MPE	M (μg)	L-NAME (mg)	%MPE
0	1.6 ± 1.0	0	4.4 ± 2.4	0	8.0	16.3 ± 3.1
111	4.9 ± 3.0	2.5	30.2 ± 2.7	13.8	8.0	44.2 ± 2.5
443	47.5 ± 6.1	10	46.5 ± 4.0	55.4	8.0	53.4 ± 3.2
1770	96.7 ± 2.3	40	53.6 ± 5.1	222	8.0	87.0 ± 5.1
563 ± 71.8	ED ₅₀	16.0 ± 4.0	ED ₅₀	50.5 ± 11.4*		ED ₅₀

M = Morphine dose, L-NAME = N^ω Nitro L-arginine methyl ester dose, %MPE = percent maximum probable effect.

Figures are Mean ± SEM.

*P < 0.01 vs ED₅₀ in ITM, EPM and IVM groups in the intrathecal, epidural and intravenous groups, respectively.

Antinociceptive study

Thermal nociceptive test

The terminal half of the tail was exposed to thermal stimulation (53°C hot plate), and the tail flick latency (TFL) was measured with a cutoff time of 30 sec to prevent injury. Tail flick or escape from the hot plate was regarded as positive. The rats were placed beneath a clear plastic cage (6 × 15 × 25 cm) for 5–10 min prior to drug injection to allow them to be accustomed and for TFL determination 15 min after drug injection.

Experimental groups and TFL determination

The agents administered were morphine hydrochloride (Takeda, Osaka, Japan) and N^ω Nitro L-arginine methyl ester (L-NAME) (Sigma, St. Louis, U.S.A.). The doses of L-NAME used were calculated based on our preliminary data.¹⁸ After baseline values of TFL (Predrug laten-

cy) were determined before drug injection, four sets of drug injection and TFL measurement were carried out every hour (the total length of the time was within four hours) in each rat. Drug solutions were injected over 30 sec using a Hamilton syringe, and followed by a 10 μl physiological saline flush. TFL was measured 15 min after each drug injection. The drugs and dose protocols (the first to the fourth injection) are shown in the Table.

1 INTRATHECAL GROUPS

Morphine and L-NAME were dissolved in 10 μl saline, and were given through the intrathecal catheter. Rats in the IT-sham group (n = 6) were given 10 μl saline four times through the intrathecal catheter.

2 EPIDURAL GROUPS

Morphine and L-NAME were dissolved in 30 μl saline, and given through the epidural catheter. Rats in the

EP-sham group ($n = 6$) were given 30 μL saline four times through the epidural catheter.

3 INTRAVENOUS GROUPS

Morphine and L-NAME were dissolved in 100 μL saline, and given through the epidural catheter. IV-sham group ($n = 6$) rats were given 100 μL saline four times through the epidural catheter.

At the conclusion of the study all rats were sacrificed with pentobarbital. Rats in the intrathecal and epidural groups were laminectomized to confirm the proper placement of the catheters.

Data analysis

Data are reported as mean $\pm$ standard error (SEM). In the analysis of an antinociceptive effect, TFL response data were converted to percent maximum probable effect (%MPE)¹⁹ according to the following formula:

$$\%MPE = \frac{\text{Postdrug Latency} - \text{Predrug Latency}}{\text{Cutoff Time} - \text{Predrug Latency}} \times 100\%$$

where, Postdrug Latency was defined as TFL after drug injection. Predrug Latency was defined as TFL before drug injection.

The effectiveness of an agent to produce antinociception is presented as the ED_{50} value which dose produces 50% MPE, calculated from the dose-response relationship. An isobolographic analysis of interaction was used to characterize the functional interaction between morphine and L-NAME on the antinociceptive effect to thermal stimulation.²⁰ The isobologram was constructed by plotting the ED_{50} values of the single agent on the X- and Y-axes. The theoretical additive line was obtained by connecting these points. The experimentally derived interactive point was obtained from ED_{50} values for morphine and L-NAME dose given each time in ITM+L, EPM+L, and IVM+L groups, respectively. The theoretical additive point of morphine and L-NAME on the theoretical additive line was calculated on the basis of an additive interaction in the doses' ratio corresponding to the experimentally derived point, respectively. They were plotted on the same axis (Figure). For statistical comparison of the difference between the theoretical additive combination and the experimentally derived ED_{50} value, one-way ANOVA was used. Post hoc comparisons were made by unpaired t-test where appropriate with Bonferroni's corrections for multiple comparisons. A significantly different potency ratio ($P < 0.05$), showing the dose of the experimental point to be less than the theoretical additive doses, was considered to indicate synergism.

FIGURE 1 Isobolograms for the antinociceptive interaction of morphine and N^o Nitro L-arginine methyl ester (L-NAME) in (A) the intrathecal, (B) the epidural, and (C) the intravenous groups. The $ED_{50} \pm$ SEM values for morphine (from IT-, EP-, or IVM group) and L-NAME (from IT-, EP-, or IVL group) are plotted on the X and Y axes (closed circles), respectively. The dashed line connecting these points is the theoretical additive line, and the point (closed triangle) on this line is the theoretical additive point ($\pm$ SEM) calculated from the ED_{50} values and their variance. In all the injection groups, the experimental ED_{50} points (closed squares) ($\pm$ SEM) (from IT-, EP-, or IVM+L group) were below the theoretical additive point, $P < 0.01$.

Results

No rats in the intrathecal or epidural groups were excluded from the antinociceptive study due to hind limb paralysis or to catheter displacement. The IT-, EP-, and IV-sham groups developed no increases in %MPE after injections compared with the corresponding pre-drug TFL values. Morphine alone and N^ω Nitro L-arginine methyl ester (L-NAME) alone showed dose-related increases in TFL in the intrathecal, epidural and intravenous groups (Table). L-NAME, half doses of ED₅₀ values in the ITL, EPL and IVL groups, produced little increases in %MPE, but reduced ED₅₀ values for morphine when given simultaneously in the intrathecal (ITM+L), epidural (EPM+L) and intravenous (IVM+L) groups ($P < 0.01$, Table).

The isobolographic analysis of the interactions between morphine and L-NAME showed that the experimentally derived ED₅₀ values of the combination groups were below the theoretically additive dose combination points ($P < 0.001$, Figure). The results showed the interaction between morphine and L-NAME is synergistic in the intrathecal, epidural and intravenous groups, respectively.

Discussion

This study demonstrated a synergistic interaction of antinociceptive effect to thermal stimulation between morphine and N^ω Nitro L-arginine methyl ester (L-NAME) when given intrathecally, epidurally or intravenously.

Przewlocki *et al.* reported that intrathecal L-NAME, 400 μ g, produced an increased antinociceptive effect of 0.5 μ g morphine, but the L-NAME dose for synergism was not examined in detail.¹³ Compared with the results in the intrathecal group, the synergistic doses of intrathecal morphine and L-NAME could be less. Thus, it is possible that 400 μ g L-NAME produced the interaction not only through the spinal cord but also through a supraspinal cord action. Xu *et al.* also reported the synergism of intrathecal morphine and nitric oxide synthase (NOS) inhibitor, and their results are identical to ours except that they used N^ω-nitro-L-arginine instead of L-NAME.¹⁴ Brignola *et al.* reported that intraperitoneal L-NAME enhanced subcutaneous morphine-induced antinociceptive effect, supporting our results in the intravenous group.¹⁵ No reports are available to evaluate the epidural morphine and nitric oxide synthase (NOS) inhibitors interaction.

Because the ED₅₀ values for morphine in ITM+L and EPM+L groups are less than in the IVM+L group ($P < 0.001$), the antinociceptive interaction between morphine and L-NAME is at the spinal cord level rather than at the brain. This is supported by Brignola *et al.* who

reported that intracerebroventricle L-arginine did not reduce the antinociceptive effect of subcutaneously administered morphine, while intraperitoneal L-arginine did.¹⁵ However, the possibility of the supraspinal interaction cannot be eliminated and should be evaluated in another study. Morphine given intrathecally or epidurally acts mainly on neurons with opioid receptors at Lamina III in dorsal horns and Laminae V and VII, and produces a segmental antinociceptive effect.²¹ Recent studies showed that NOS containing neurons located in Laminae I through III (dorsal horns)²² probably function as interneurons modulating the sensory processing²³ in the spinal cord. Also, intermediolateral (IML) cell column neurons have projections to dorsal horn neurons, which are said to have connections that regulate sensory neuronal plasticity.²³ These histological investigations may support an antinociceptive interaction between morphine and L-NAME given intrathecally and epidurally.

The observations in this study show that spinal cord or supraspinal cord NO modifies the antinociceptive effect of morphine. Recently, it has been shown that a form of neuropathic pain has sympathetic hyperactivity,²⁴ and that nitric oxide is involved in the mechanism of the hyperaesthetic model^{10,11} and of prolonged chemical nociception.²⁵ The results in this study can be used to titrate hyperaesthetic state by altering L-NAME doses in the intrathecal and the epidural groups.

Nitric oxide is involved in the development of morphine tolerance.²⁶ Morphine tolerance can be treated by NG-nitro-L-arginine²⁷ and prevented by NOS inhibitors.^{28,29} Morphine dependent withdrawal syndrome in animals is suppressed by a NOS inhibitor.³⁰ These observations can be explained by the results obtained in the intravenous groups in the present study and suggest that the addition of L-NAME shifted the dose-response function of morphine to the left. However, it is not known if the long-term use of NOS inhibitors abolishes or enhances morphine tolerance although chronic L-arginine induced morphine tolerance in opioid naive mice.²⁶

Postoperative pain management is another area in which NOS inhibitors can be used with opioids. Recent articles suggest that peripheral tissue injury by trauma or surgery may itself increase dorsal root ganglion and spinal cord neuronal NOS activity³¹⁻³³ resulting in a hyperaesthetic state. The addition of NOS inhibitors, such as L-NAME, to neuraxial morphine may provide improved postoperative pain relief although the adverse effects due to the use of NOS inhibitors should be prevented in advance.

We conclude that subeffective doses of L-NAME, the NOS inhibitor, had synergistic interactions with morphine-induced antinociceptive effects when given

intrathecally, epidurally, and intravenously with morphine in the rats.

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