Is nitric oxide involved in the antinociceptive activity of tramadol? Findings in a rat model of neuropathic pain

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ÖZET

Nitrik oksit, tramadolun antinosiseptif aktivitesinde yer almakta mıdır?: Sıçanlarda nöropatik ağrı modelindeki bulgular

Çalışmanın amacı, sıçanlarda deneysel olarak oluşturulan nöropatik ağrıdaki tramadolun antinosiseptif etkisinde NO'in rolünü ortaya koymaktır. Çalışmada Wistar türü, 200 - 250 gr ağırlığında, kronik konstriktif yaralanma (CCI) modeli ile nöropatik ağrı oluşturulan erkek sıçanlar beş eşit gruba ayrılarak kullanıldı. Cerrahi işlemden üç hafta sonra her deneğin mekanik ağrı eşik değerleri, elektronik algometre kullanılarak gram cinsinden ölçüldü. CCI uygulandıktan sonra intraperitoneal (i.p.) tramadol tüm gruplarda verildi. Ayrıca, N(omega)-nitro-L-arjinin (L-NA) ve L-arjinin, i.p. veya intratekal (i.t.) olarak değişik gruplarda uygulandı. Tramadol 10 mg/kg i.p. uygulanarak etki başlama zamanı belirlendi. L-NA 10 mg/kg i.p., 30 μg/kg i.t. olmak üzere ve L-Arjinin 10 mg/kg i.p. ve 50 μg/kg i.t. olarak verildi. Çoklu ajanlar 30 dk. aralıkla uygulandı. Grup 1'de sadece i.p. tramadol verildi ve sıçanların sol arka ayaklarında mekanik ağrı eşiğini yükseldi. Grup 2'de i.p. L-NA (10 mg/kg) ve Grup 4'de de i.t. L-NA (30 μg/kg) ortalama mekanik antinosiseptif eşik değerinde anlamlı azalma oluşturdu ($p<0.05$). Grup 2 (tramadol i.p. + L-NA i.p.) ve Grup 4 (tramadol i.p. + L-NA i.t.) birbirleriyle karşılaştırıldıklarında ortalama mekanik antinosiseptif eşik değerlerinde fark yoktu. Ortalama mekanik antinosiseptif eşik değerlere bakıldığında Grup 3 (tramadol i.p. + L-NA i.p.+ L-arjinin i.p.) ile Grup 5 (tramadol i.p. + L-NA i.t.+ L-arjinin i.t.) arasında da anlamlı fark gözlenmedi. Grup 3 ve Grup 5'te, Grup 1'e göre ortalama mekanik antinosiseptif eşik değerlerinde anlamlı derecede yükselme oldu (her iki grupta $p<0.05$). Bu bulgular, sıçanda geliştirilmiş nöropatik ağrı modelinde tramadolun antinosiseptif etkisinde L-arjinin/nitrik oksit rolünün olduğunu desteklemektedir.

Anahtar kelimeler: Kronik konstriksiyon, tramadol, nitrik oksit, N (omega)-nitro-L-arjinin, L-arjinin, antinosisepsiyon

SUMMARY

The aim of this investigation was to assess the role that NO plays in the antinociceptive activity of tramadol using a rat model of neuropathic pain. Thirty male Wistar rats weighing 200-250 g were randomly divided into five equal groups. The neuropathic pain model used for the study was chronic constrictive injury (CCI) model. Three weeks after the surgical procedure, each rat was tested to assess mechanical threshold in grams using an electronic algometer. After CCI was induced, tramadol hydrochloride was administered by intraperitoneal (i.p.) injection in all groups, and N(omega) - nitro - L - arginine (L-NA) and L-arginine were administered i.p. or intrathecally (i.t.) depending on the group. Tramadol was administered in 10 mg/kg doses i.p., L-NA was given in 10 mg/kg doses i.p. and in 30 μg/kg doses i.t.. L-arginine was given in 10 mg/kg doses i. p. and in 50 μg/kg doses i.t.. The multiple agents were given 30 minutes apart from each administration. Intraperitoneal administration of tramadol (Group 1) only increased mechanical threshold in the rats' left hind paw, whereas in i.p. L-NA group (10 mg/kg) (Group 2) produced a significant reduction of the mean mechanical antinociceptive threshold ($p<0.05$). Like this, in i.t. L-NA group (30 μg/kg) (Group 4) a significant reduction of the mean mechanical antinociceptive threshold ($p<0.05$) was also observed. The mean threshold values in Group 2 (i.p. tramadol + i.p. L-NA) and Group 4 (i.p. tramadol + i.t. L-NA) were not significantly different. The mean threshold values in Groups 3 (i.p. tramadol + i.p. L-NA + i.p. L-arginine) and 5 (i.p. tramadol + i.t. L-NA + i.t. L-arginine) were also similar. The mean mechanical antinociceptive threshold was significantly increased in Group 3 (i.p. L-NA + L-arginine) and Group 5 (i.t. L-NA + L-arginine) when compared to Group 1 (i.p. tramadol only) ($p<0.05$ for both). The results of this study support the involvement of the L-arginine/nitric oxide pathway in the antinociceptive effect of tramadol in a rat model of neuropathic pain.

Key words: Chronic constriction nerve injury, tramadol, nitric oxide, N(omega) - nitro - L - arginine, L-arginine, antinociception

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Introduction

Pain has traditionally been classified as either nociceptive or neuropathic. Neuropathic pain is that linked to or characterized by neuropathy. Neuropathic pain syndromes involve injury to the nervous system and involve different mechanisms of pain associated with chronic tissue inflammation. In these conditions, the nerve fibers are intact and the peripheral nerve endings react to inflammatory mediators and cell breakdown products. This results in altered afferent and efferent function of peripheral and central sensory nerve fibers. When a nerve is damaged, connections with the periphery are disrupted and the fiber injury results in axonal damage, local neuritis, atrophy, altered Schwann cell activity, and altered signaling. Neuropathic pain is caused by a heterogeneous group of disorders with varying etiologies and presentations. This type of pain tends to be difficult to treat because the underlying pathophysiology is usually complex. The clinical features of neuropathic pain include the paradoxical combination of sensory loss and hypersensitivity phenomena, such as allodynia, in the same region (Jensen 1996). The allodynia is assumed to reflect neuronal hyperexcitability in the central nervous system (Koltzenburg et al. 1992).

To date, the animal models for neuropathic pain that have been most intensively studied are those in which a peripheral nerve is subjected to mechanical trauma. The most straightforward of these is transection and ligation of the sciatic nerve. Four main animal models for pain associated with nerve injury are now in use: a) total nerve transection and ligation (Bennett 1994); b) partial nerve lesion with a tight ligature compressing approximately 50 % of the nerve fascicles (Seltzer et al. 1990); c) chronic constriction injury (CCI), in which several loose ligatures are placed on the nerve such that it is compressed to a smaller diameter than the original nerve (Bennett and Xie 1988); and d) tight ligation of a spinal nerve (Kim and Chung 1992) or transection of one or several dorsal roots (Brinkhus and Zimmermann 1983).

Tramadol hydrochloride is a centrally acting analgesic that binds opioid receptors and also appears to modify transmission of pain impulses by inhibiting monoamine reuptake. The chemical name of this agent is (1RS;2RS) - 2 - ([dimethylamino] - methyl) - 1 - (3 methoxyphenyl) - cyclohexanol hydrochloride. Research has shown that this drug has analgesic activity in a number of animal models. Dose-dependent analgesic action of tramadol has been demonstrated in mice and rats using various tests of analgesia, including tail-flick response, and vocalization thresholds for paw pressure, hot plate application, and abdominal constriction (Bernatzky and Jurna 1986, Carlsson and Jurna 1987). Early preclinical studies revealed that tramadol has an affinity for μ-opioid receptors, albeit with orders of magnitude less than morphine and codeine (Hennies et al.1988).

However, the binding affinity of tramadol for opioid receptors in the brain appears to be too low to account for the antinociceptive efficacy of this drug in animal models, or for its analgesic effects in humans. More recently, another mode of tramadol action has been identified, namely, inhibition of reuptake of norepinephrine (NE) and serotonin (5-HT) by neurons (Codd et al.1995, Giusti et al. 1997). However, the mechanisms involved in the potent analgesic action of this agent are still not completely understood (Rafa and Friderichs 1996).

Nitric oxide (NO) is suggested to play a role in synaptic transmission in both the central and peripheral nervous systems (Meller and Gebhart 1993). In the brain, NO is thought to be involved in synaptic plasticity or to act as a neurotoxin when produced in excess (Vincent 1994). In the peripheral nervous system, NO is now known to be the mediator released by a widespread network of nerves, previously recognized as nonadrenergic and noncholinergic nerves. In body tissues, NO is produced from L-arginine by calcium-dependent constitutive NO synthase (NOS) isoforms, specifically neuronal NOS (nNOS), endothelial (eNOS), and calcium-independent inducible NOS (iNOS). The latter requires activation by endotoxin or cytokines (Moncada 1997). The NO that is formed activates soluble guanylate cyclase, which results in increased cyclic guanosine 3',5'monophosphate (cGMP) levels (Meller and Gebhart 1993). Generation of NO from L-arginine proceeds via the formation of N(omega)-hydroxy-L-arginine (Pufahl et al.1992). This L-arginine/NO pathway can be inhibited by several analogues of L-arginine, one of which is N(omega)-nitro-L-arginine (L-NA).

Since NO has been implicated in nociceptive processing, the present study examined whether NO synthase inhibition with N(omega)-nitro-L-arginine would alter antinociception elicited by tra-

madol on the mechanical nociceptive threshold test using a rat model of neuropathic pain.

Material and Method

Animals

Thirty male Wistar rats weighing 200-250 g were supplied by the Experimental Research Center affiliated with the Firat University Faculty of Medicine in Elazig, Turkey. The local ethics committee approved the study protocol. The animals were randomly divided into five equal groups (group details explained below). Each group was housed in a plastic cage, and maintained in a temperature-controlled environment ($22 \pm 2°C$) under a 12/12-h light/dark cycle, with the dark cycle beginning at 09:00. After the surgical procedure (details below) was done to induce neuropathic pain, each rat was isolated individually in a separate cage. The floors of these cages were covered in sawdust to minimize the possibility of painful mechanical stimulation. Each animal was allowed to recover for 3 weeks before being subjected to pain testing. Food and water were available ad libitum and the operated animals with neuropathic pain were able to eat and drink unaided.

Surgical Procedure

The neuropathic pain model used for the study was a modified version of the CCI model described by Bennet and Xie (1988). Each of the 30 rats was anesthetized with intramuscular injections of ketamine (60 mg/kg) and xylazine (5 mg/kg) (Hall et al. 2001). A 2 cm-long skin incision was made on the inside of the left thigh, and the left sciatic nerve was exposed at mid-thigh level and dissected free from the tissues proximal to its trifurcation. Four ligatures of 4/0 chromic gut suture were then placed in this section of the nerve, with 1 mm spacing between them. Each was tied such that it very slightly constricted the nerve and a twitch was observed in the corresponding hind limb. After the ligatures were in place, the wound was closed in layers and the skin was closed in standard fashion with 2/0 silk. None suffered autotomy or showed complete anesthesia in the distribution of the sciatic nerve.

Each rat also underwent sham surgery on the contralateral limb, and these limbs were used as controls. In this procedure, the sciatic nerve was exposed exactly as detailed above, but the tissues were then closed without any damage induced or ligatures applied.

Testing of Mechanical Antinociceptive Threshold in Operated Rats

Three weeks after the surgical procedure, each rat was tested to see whether the induced neuropathy had progressed or not by assessing mechanical threshold in grams using an electronic algometer (Khall and Khodr 2001). All animals who exhibited progression of neuropathy with pain-like behavior and facilitated withdrawal reflexes were subjected to intraperitoneal (i.p.) or intrathecal (i.t.) administration of the drugs tested in the study (see group details below). All 30 animals exhibited these features on the operated side, so all underwent the drug testing. After CCI was induced but before these drug experiments were performed, we initially investigated the onset and duration of the analgesic effect of tramadol. This was done by randomly dividing the rats into two groups, one that received i.p. tramadol (n=15) and one that received i.p. saline (n=15). Testing was started few minutes after the agent was administered, and each animal's mechanical thresholds were determined by applying von Frey filaments at 10-minute intervals for a period of 230 minutes.

Drugs and Injection Procedures

The drugs used were tramadol hydrochloride [Fluka Chemie (Sigma Chemical Co. St. Louis, MO, USA)], NΩ-nitro-L-arginine (Sigma Chemical Co. St. Louis, MO, USA), and L-arginine (Sigma Chemical Co. St. Louis, MO, USA). Each agent was dissolved in 0.9% NaCl. As explained above, N (omega)-nitro-L-arginine (L-NA) inhibits all three forms of NOS, and L-arginine acts as a substrate for NO formation.

As detailed, the rats were divided into five equal groups by random assignment. Tramadol hydrochloride was administered by i.p. injection in all groups, and L-NA and L-arginine were administered i.p. or i.t. depending on the group.

The study groups were as follows:

Group 1: i.p. tramadol only (n=6);

Group 2: i.p. tramadol + i.p. L-NA (n=6);

Group 3: i.p. tramadol + i.p. L-NA + i.p. L-arginine (n=6);

Group 4: i.p. tramadol + i.t. L-NA (n=6);

Group 5: i.p. tramadol + i.t. L-NA + i.t. L-arginine (n=6).

Tramadol was administered in 10 mg/kg doses i.p.; L-NA was given in 10 mg/kg doses i.p. and in 30 µg/kg doses i.t.; and L-arginine was given in 10 mg/kg doses i.p. and in 50 µg/kg doses i.t. In Groups 2 through 5, the multiple agents were given 30 minutes apart from each administration.

For i.t. drug administration, each rat was lightly anesthetized by placing it in a glass enclosure and allowing it to breathe 2% halothane for an average of 2 minutes. Once the animal was anesthetized, a 25 G needle was carefully inserted into the subarachnoid space at the L_5-L_6 intervertebral space. Entry into the spinal canal was confirmed by trembling movements of the tail (Li and Clark 2001). The total volume administered to each animal via the i.t. route was 10 µl.

Measurement of Antinociceptive Effects

Mechanical nociceptive testing of the left and right paws of the 30 rats was initially done prior to surgery (before sciatic nerve ligation). A second round of testing was done 3 weeks after CCI but prior to administration of the drugs that were studied. A third round was done to compare findings in the operated rats after they received either tramadol (n=15) or saline (n=15). Finally, a fourth round of testing was completed 30 minutes after tramadol administration in Group 1 (tramadol only), and a few minutes after L-NA administration or L-NA + L-arginine administration in the other groups.

All the nociception assessments were done during the day portion of the circadian cycle (09:00-17:00). For testing, each rat was placed in a cage with a wire mesh bottom, which allowed easy access to the plantar surfaces of the paws. Initially, the animal was left undisturbed in the cage for approximately 15 minutes to allow behavioral accommodation. Mechanical pain threshold measurements were then performed using an electronic algometer (Electro von Frey, model 1601 CE, IITC INC., Life Science Instruments, Los Angeles, CA, USA). We tested only the mid-plantar region of the right and left hind paws (an area innervated by the sciatic nerve), thus avoiding the less sensitive foot pads. Each von Frey filament was applied from beneath the grid floor. The filament was pressed into contact with the foot perpendicular to the plantar surface until it buckled slightly, and then held in place for approximately 3-4 seconds. Testing was initiated with the 2.04 g

filament in the middle of the series. Withdrawal of the paw signified a positive response. In the absence of a response to a particular filament, the next stronger filament was applied; in the case of a response, the next weaker filament was presented. Ambulation was considered an ambiguous response, and the stimulus was repeated if this occurred.

In each test session, three different measurements were taken and the mean was recorded as the animal's mechanical antinociceptive threshold (in grams).

Statistics

All results are expressed as mean ± standard deviation. The mechanical antinociceptive threshold results for the left and right hind paws of the 30 rats before sciatic nerve ligation were compared using the Student's t-test. The same test was used to compare the left-paw and right-paw data 3 weeks after CCI (before drug administration).

The initial data related to onset and duration of tramadol (i.p.tramadol versus i.p.saline administration) were assessed by analysis of variance (ANOVA) followed by Tukey's test.
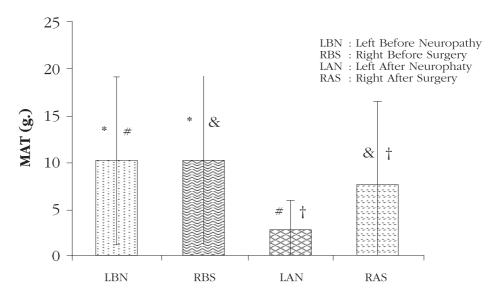
For the drug testing results, the Mann-Whitney U test was used to make intergroup comparisons of the mean threshold findings for the left paws. The Wilcoxon ranks test was used to compare the median threshold findings for the left and right paws within each group.

All analyses were performed using the statistical software package SPSS for Windows, version 10.0, and p values less than 0.05 were considered as significant.

Results

Mean Threshold Findings 3 Weeks After CCI Before Drug Testing

The mechanical antinociceptive threshold results for the left and right paws of the 30 rats before and after sciatic nerve ligation on the left are illustrated in Figure 1. There was no statistically significant difference between the mean mechanical antinociceptive thresholds for the rats' left and right paws before the nerve ligation surgery was performed (Student's t-test; $p>0.05$). However, the mean threshold value for the left paws (the side affected by nerve ligation) 3 weeks after surgery was significantly lower than the mean value for the left paws before CCI ($p<0.001$). The left-paw mean threshold value 3

Figure 1: Changes produced by CCI or sham surgery on the paw mechanical antinociceptive thresholds (MAT) to tactile stimuli.

* Comparison of mean left -and mean right- paw values before neuropathy; $p > 0.05$
\# Comparison of mean left-paw values before and after nerve injury; $p > 0.001$
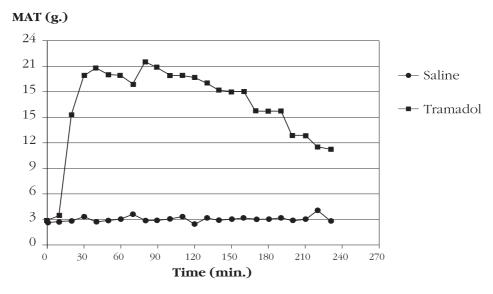& Comparison of mean right-paw values before and after sham surgery; $p > 0.001$
† Comparison of mean left -and mean right- paw values after intervention (nerve injury on left, sham surgery on right); $p > 0.001$

weeks after surgery was also significantly lower than the right-paw (sham surgery) mean threshold value at this stage ($p < 0.001$). The right-paw mean threshold value 3 weeks after surgery was also significantly lower than the right-paw mean threshold value before sham surgery ($p < 0.001$).
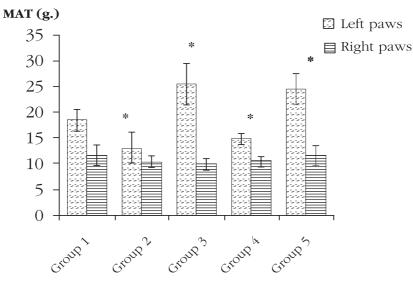
Effect of i.p. Tramadol After CCI

As described above, all 30 rats exhibited neuropathy on the left side 3 weeks after sciatic nerve surgery, so we divided them into two equal groups and administered tramadol 10 mg/kg i.p. or sterile saline i.p. Twenty minutes later, testing showed that the mean mechanical threshold for the left hind paws in the tramadol group was significantly higher than the corresponding mean in the saline group (ANOVA followed by Tukey's test, $p < 0.05$). The difference between the group thresholds declined as the testing continued over the 230 minutes, and the differences remained statistically significant throughout the test period (Figure 2).

Figure 2: Effect of intraperitoneal tramadol (10 mg/kg) versus intraperitoneal saline after CCI on the paw mechanical antinociceptive threshold (MAT) to tactile stimuli.

$p > 0.05$ at minutes (one-way ANOVA and Tukey's test)

MAT (g.)

Figure 3: Intergroup comparisons of mean left-paw values

MAT: Mechanical Antinociceptive Threshold, *p<0.05 (compared to Group 1)

Effects of Drug Combinations After CCI

Figure 3 shows the left hind paw (neuropathy) and the right hind paw (sham surgery) mechanical threshold findings for each of the drug combinations tested 3 weeks after CCI. In all groups except Group 2, there was a significant difference between the median thresholds for the rats' left and right paws (Wilcoxon ranks test, $p<0.05$ for each).

Compared to the mean left hind paw threshold in Group 1 (i.p. tramadol alone), the corresponding values in Group 2 (i.p. tramadol + i.p. L-NA) and Group 4 (i.p. tramadol + i.t. L-NA) were significantly lower (Mann-Whitney U test, $p<0.05$ for both). The mean left-paw values in Groups 2 and 4 were statistically similar. Compared to the mean left hind paw threshold in Group 1, the corresponding means thresholds in Group 3 (i.p. tramadol + i.p. L-NA + i.p. L-arginine) and Group 5 (i.p. tramadol + i.t. L-NA + i.t. L-arginine) were significantly higher ($p<0.05$ for both). The mean left hind paw values in Group 3 and 5 were similar.

Discussion

The results of this study clearly show that tramadol reduces the neuropathic pain associated with CCI of the sciatic nerve in rats. The findings also reveal that the effect of the NOS inhibitor L-NA is reversed when L-arginine (NOS) is coadministered. The mechanical threshold data from the different groups prove that NO alters the analgesic effects of tramadol in rats suffering neuropathic pain.

Attal and coworkers (1990) found that hyperalgesia in rats subjected to CCI of the sciatic nerve peaked 2 weeks after the intervention. In all the behavioral tests they used, they found that the time course of pain-related disorders was comparable, with recovery 2 months after surgery. Work by Goff and colleagues (1998) on CCI of the sciatic nerve in rats determined that changes in the animal's threshold to stimulus were remained significant for mechanical stimuli at day 14, and the difference in withdrawal threshold was non-significant at 28 days. In our study, neuropatic model was at the third week after CCI, and we terminated the investigation in 5 days.

Substance P and calcitonin gene-related peptide (CGRP) decrease, whereas galanin and NOS increase dramatically in the weeks and months after axotomy (Zimmermann 2001). Levy and Zochodne (1998) suggested that NO is locally elaborated within the injured sciatic nerve after CCI. Local NO may contribute to the development of hyperalgesia and allodynia directly or indirectly by influencing local inflammatory and repair processes in a partially injured peripheral nerve. This substance may activate and sensitize primary afferents. The results of this study support evidence for the change produced by CCI on the paw mechanical antinociceptive thresholds to tactile stimuli in an animal model.

Many researchers have used the CCI model to investigate the possible role of NO in mechanisms of neuropathic pain, and these studies have yielded differing results. Yamamoto and Shimoyama (1995) found that i.t. administration of an NOS in-

hibitor before induction of sciatic nerve damage in the rat led to delayed hyperalgesia, whereas there was no such delay with i.t. administration of these agents after nerve injury. This suggests that NO is an important contributor to the initial stages of hyperalgesia. In contrast to this data, Luo et al. (1999) found that specific pre- and post-nerve injury administration of an NOS inhibitor in the rat had no effect on the development of allodynia associated with pain from nerve injury. Other work by Aley and Levine (2002) in a rat CCI model showed that second messengers such as protein kinase A, protein kinase C and NO had no effect on the development of neuropathy. Our results indicate that central or peripheral administration of NOS inhibitor after CCI yields a decrease in mechanical nociceptive threshold.

Tsai and associates (2000) demonstrated that both acute and semi-chronic tramadol treatment relieves thermal hyperalgesia effectively in rats with CCI of the sciatic nerve. Bianchi and Panerai (1998) revealed that i.p. administration of higher doses of tramadol (5-10 mg/kg) in rats had effects on central hyperalgesia. Apaydın et al. (2000) compared antinociceptive effect on both lesioned and non-lesioned hind paws to different doses (2.5, 5 and 10 mg/kg) of i.p. tramadol in rats with CCI neuropathy. They found that 10 mg/kg tramadol provided more potent antinociceptive effect at 50 minutes on the lesioned hind paw, and at 40 minutes on the non-lesioned hind paw. In our study, administration of 10 mg/kg i.p. tramadol alone significantly increased the mean mechanical threshold at 20 minutes in rats with CCI.

The ways in which NO affects nociception and antinociception are still unclear. Research has shown that the superficial dorsal horn and intermediolateral cell column of sheep contain NOS (Xu et al. 1996). In the spinal cord, NO is an important second messenger with neurotransmitter-like function. However, this molecule differs from classical neurotransmitters/modulators in that it diffuses through biological membranes and lacks specific release or uptake mechanisms. After it is synthesized in the soma or processes of a neuron, NO diffuses out of the cell and causes effects in local neural tissue. Research has demonstrated that as a result of nerve compression and nervous tissue inflammation, there is rapid upregulation of NOS and NO formation in the spinal cord (Callsen-Cencic et al. 1999). It has also been shown that, in the CCI model of neuropathy, the thermal hyperalgesia that develops can be alleviated by i.t. administration of NG-nitro-L-arginine

methyl ester (L-NAME) at the lumbar and cerebral levels (Salter et al. 1996). Choi and colleagues (1996) studied neuropathic pain in rats. They found that, in dorsal root ganglion neurons, nNOS activity was increased, whereas in the dorsal horn nNOS activity was decreased. The changes developed within 3-4 days and persisted for 2-4 weeks or longer. Work by Luo and coworkers (1999) revealed that interruption of retrograde axonal transport in a CCI model in rats, led to upregulation of nNOS. However, they concluded that regulation of nNOS expression did not explain the development of neuropathic allodynia. It is likely that, like N-metyl-D-aspartate (NMDA), NO produced in spinal cord neurons containing NOS plays a pivotal role in multisynaptic local circuit nociceptive processing in the cord (Meller and Gebhart 1993). Mense and Hoheisel (2001) reported that NO is normally released in tonic fashion in the spinal cord, and that this inhibits discharges from nociceptive dorsal horn neurons. Accordingly, diminished local NO synthesis leads to an increase in the electrical activity of these neurons.

Studies of different experimental pain models in rats in which L-arginine and different NOS inhibitors have been administered alone intrathecally have demonstrated that these agents have no antiallodynic effects in the setting of diabetic neuropathy (Aley and Levine 2002, Chen et al. 2001). In line with this, Pan et al. (1998) observed that i.t. injection of 20-200 μg L-arginine did not alter paw withdrawal thresholds in rats with neuropathic pain. The same study also revealed no significant differences among mechanical pain thresholds recorded after i.t. injections of different types of NOS inhibitors. In summary, the above-mentioned investigations showed that i.t. injections of NO donors, and NOS inhibitors had no effect on allodynia in rats with neuropathic pain. Secondly, the above results suggest that spinal NO is unlikely to be an important factor in the maintenance of allodynia in this animal model. In contrast to these data, work by Sousa and Prado (2001) has shown that the NO-donor 3-morpholinosydnonimine (SIN-1) and NOS inhibitors reduce pain through a spinal mechanism that involves activation of guanylate cyclase. It has also been demonstrated that NO in rat spinal cord can directly excite or inhibit electrical activity of spinal neurons (Pehl and Schmid 1997). Further, Inoue and coworkers (1998) documented rapid development of thermal hyperalgesia after i.t. injection of NOC-18 (an NO-releasing compound)

in a rat CCI model. Currently, there are no clear explanations for the effects that spinal NO has on pain caused by nerve injury.

Separate studies have shown that spinal NO levels influence the antiallodynic effects of i.t. clonidine (Pan et al. 1998), and i.t. neostigmine (Chen et al. 2001) in rats with neuropathic pain. A report by Song and colleagues (1998) concluded that spinal NO mediates the antinociceptive effects of intravenous morphine. These authors found that the antinociceptive effects of intravenous morphine were reduced by i.t. injection of α2-adrenergic inhibitors or NOS inhibitors. Work by Li and Clark (2001) supported this. They found that morphine stimulates cGMP production in the spinal cord by activating nNOS and heme-oxygenase (HO-2), thus reducing the overall level of analgesia obtained. Bulutcu and coworkers (2002) observed that i.p. administration of the NOS inhibitor L-NAME to investigate the contribution of the NO-cGMP pathway in the antinociceptive effects of ketamine in mice produced no antinociceptive effects on its own, and inhibited the antinociceptive effects of i.p. ketamine. However, intraspinal L-NAME neither altered the antinociceptive effect of i.t. ketamine nor did it produce an antinociceptive effect alone.

The results of these studies are in line with our findings in rats with CCI. We observed that both i.p. and i.t. L-NA decreased the antinociceptive effects of i.p. tramadol. In contrast, the groups that received L-arginine in addition to tramadol and L-NA showed even higher pain thresholds than the group that received tramadol alone. In other words, when L-arginine (an NO donor) was coadministered intraperitoneally or intrathecally with L-NA (a NOS inhibitor), it reversed the effect of L-NA on the animals' mechanical thresholds.

No previous study has compared the i.p. and i.t. effects of both NOS inhibitors and NO donors in neuropathic pain. Our findings indicate that NOS inhibitors and NO donors administered intraperitoneally or intrathecally can alter the effects of tramadol.

Our data revealed no significant difference in effects on mechanical threshold when these substances were administered i.p. versus i.t. in combination with i.p. tramadol. Research conducted under physiologic conditions in vivo has shown that NO reacts with NE to produce 6-nitro-norepinephrine (6-nitro-NE) (de la Breteche et al. 1994). This molecule is known to inhibit NE reuptake by neurons (Chiari et al. 2000, Zhu et al.

2002), inhibit the activity of cathecol-O-methyltransferase, and inhibit NE transport into rat synaptosomes (Shintani et al. 1996). Based on these pieces of evidence, we hypothesized that tramadol (an inhibitor of NE re-uptake) facilitates release of NE from the spinal cord. In addition to inhibiting NE re-uptake, tramadol inhibits re-uptake of serotonin (5-HT) (Gobbi and Menini 1999). This agent is a racemic mixture of two synergistic enantiomers, with (+) tramadol producing greater 5-HT re-uptake inhibition, and (-) tramadol inhibiting NE re-uptake. Evidence also indicates that NE and 5-HT interact at the spinal cord level to produce more powerful antinociceptive effects (Cui et al. 1999). Studies have shown that NE reduces stimulation-induced release of substance P in spinal cord slices (Kuraishi et al. 1985), and reduces the excitability of spinal neurons (North and Yoshimura 1984). As a result, NE is an important endogenic neurotransmitter in antinociception (Kim and Chung 1992, Li et al. 2000).

Shintani and associates (1996) suggested that the 6-nitro-NE that is generated in nuclei containing both adrenergic and nitrergic neurons inhibits NE activation. They found that levels of 6-nitro-NE fell after i.p. injection of L-NAME, and that this decrease was reversed by coadministration of L-arginine. These results suggest that NOS is involved in the formation of 6-nitro-NE, which is a potential signal molecule that links the action of NE and NO. Studies of hippocampal slices have revealed that NO generators evoke [3H]NE release both directly from noradrenergic terminals and via release of glutamate (Lauth et al. 1995, Lonart and Johnson 1995). Kaye and colleagues (1997) showed that NO donors inhibit neuronal NE uptake. Xu and coworkers (1997) proposed the idea of positive feedback, whereby NO released from the spinal cord increases NE release. These results are basic information of the interaction of NO and NE in antinociception according to the tramadol.

As explained above, under physiological conditions, NO reacts with NE to form an adduct, 6-nitro-NE, which is suggested to inhibit NE reuptake. Similarly, systemic administration of tramadol has been shown to inhibit NE reuptake. This suggests that activation of the descending inhibitor pathway in the spinal cord by the noradrenergic system could be central to antinociception. In our study, the antinociceptive effects of tramadol (increased mechanical threshold) were reduced by an NOS inhibitor (L-NA), and then this effect was countered by administration of an NO donor (L-

arginine). These changes suggest that at least part of the mechanism underlying tramadol's analgesic action is affected by local NO.

In summary, the behavioral data in the literature are consistent with the theory that the antinociceptive action of tramadol is due to reduction of NE reuptake and the interaction of the accumulated NE with NO. The results of this study suggest that the effects of NOS inhibitors on mechanical thresholds in rats with CCI are similar when administered intraperitoneally or intrathecally, and the same holds true for NO donor substances. The specific mechanisms involved in these actions remain to be identified. On a clinical level, the findings indicate that coadministration of tramadol and NOS might be of value for treating neuropathic pain.

Acknowledgements

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