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Participation of the GABA/benzodiazepine receptor and the NO-cyclicGMP pathway in the "antinociceptive-like effects" of diazepam

Guadalupe Jiménez-Velázquez, Francisco Javier López-Muñoz, Alonso Fernández-Guasti *

Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados—Sede Sur, Calz. de los Tenorios 235, Col. Granjas Coapa, C.P. 14330, México D.F., Mexico

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ABSTRACT

The mechanism of action underlying the "analgesic activity" of diazepam remains unclear. In this study, the possible participation of the GABA/benzodiazepine receptor and the nitric oxide-cyclic GMP (NO-cGMP) pathway was assessed utilizing the *pain-induced functional impairment model in the rat* (PIFIR). Nociception was induced by an intra-articular injection of 15% uric acid. Diazepam (1 and 2 mg/kg, i.p.) reversed the dysfunction induced by uric acid. Flumazenil (10 mg/kg, i.p.), a GABA/benzodiazepine receptor antagonist, abolished the "antinociceptive-like effect" of diazepam (at 2 mg/kg). The "antinociceptive-like effect" of diazepam (at 2 mg/kg) was antagonized by the non-selective nitric oxide synthase (NOS) inhibitor, N^{ω}-L-nitro-arginine methyl ester hydrochloride (L-NAME, 5 mg/kg, s.c.) (but not by its inactive isomer), and by the selective neuronal NOS inhibitor, 7-nitroindazole (7-NI, 1 mg/kg, i.p). While, the NO precursor, L-arginine (125 mg/kg, s.c.), but not D-arginine (125 mg/kg, s.c.), increased the "antinociceptive-like effect" of a non-effective dose of diazepam (1 mg/kg). Methylene blue (10 mg/kg, i.p.), a guanylate cyclase inhibitor, also prevented the "antinociceptive-like effect" of diazepam (at 2 mg/kg). These results suggest that the GABA/benzodiazepine receptor and the NO-cGMP pathway participate in the "antinociceptive-like effect" of diazepam.

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1. Introduction

Diazepam is a benzodiazepine widely used to treat various anxiety disorders, epilepsy, some symptoms of the abstinence syndrome to alcohol withdrawal, muscle tension and certain neurological diseases. Moreover, diazepam is used as an adjuvant agent along with anesthetic drugs during surgical procedures (Gries et al., 2005) and in the treatment of several specific painful states, such as: chronic or acute pain associated with anxiety, pain due to muscle injury and spasm or neuropathic pain (Reddy and Patt, 1994). Although diazepam is widely utilized in the treatment of pain, its role on nociception is not clear; some reports show that it lacks an effect (Jiménez-Velázquez et al., 2006; Rodgers and Randall, 1987; Rosland et al., 1987; Talarek and Fidecka, 2002), others suggest hyperalgesic responses (Rosland et al., 1987; Tatsuo et al., 1999) and a third set establish putative antinociceptive actions (Golombek et al., 1991; Haas et al., 1982; Jiménez-Velázquez et al., 2006; Oliveira and Prado, 1994; Palaoglu and Ayhan, 1986; Rosland et al., 1987; Talarek and Fidecka, 2002; Zambotti et al., 1991). The variability of these results may be consequence of the experimental conditions, the routes of administration, the dosages and most importantly the type of nociception evaluated. Interestingly, some authors have suggested that the sedative and/or anxiolytic properties of diazepam influence nociception. Thus, there is a possibility that this drug may lack a specific antinociceptive action and may modify nociception by altering anxiety (Jiménez-Velázquez et al., 2006; Rosland et al., 1987). The interaction between anxiety and nociception is supported by multiple clinical (Anbar and Geisler, 2005; Das et al., 2005; Deng and Cassileth, 2005; Hadjistavropoulos et al., 2003; Janssen and Arntz, 1999; Ploghaus et al., 2001; Schanberg et al., 2000) and preclinical (Kavaliers and Innes, 1988; Nunes-De-Souza et al., 2000; Rodgers and Shepherd, 1989; Tershner and Helmstetter, 2000; Teskey et al., 1984; Vendruscolo et al., 2004) studies. Indeed, the perception of painful stimuli is profoundly influenced by emotional variables. However, the neurobiological and biochemical basis of this relationship remains unclear. At a neuropharmacological level diazepam exerts most of its actions by enhancing the γ-aminobutyric acid (GABA)-ergic neurotransmission (Baur and Sigel, 2005; Rudolph et al., 1999) since it binds to the benzodiazepine receptor coupled to the $GABA_A$ receptor. This receptor is specifically antagonized by flumazenil (File and Pellow, 1986; Li et al., 2006).

Nitric oxide (NO) is an unconventional transmitter involved in a wide variety of physiological and pathological processes (Prast and Philippu, 2001), such as nociception (Budziński et al., 2000; Fernández and Assreuy, 2004; Hoheisel et al., 2005; Kawabata et al., 1993; López-Muñoz et al., 1996; Moore et al., 1991; Salvemini et al., 1996) and

* Corresponding author. Departamento de Farmacobiología, Cinvestav-Sede Sur, Calz. de los Tenorios 235, Col. Granjas Coapa, C.P. 14330, México D.F., Mexico. Tel.: +52 5483 28 56/5483 28 70; fax: +52 5483 28 63.
E-mail address: jfernand@cinvestav.mx (A. Fernández-Guasti).

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anxiety (Baretta et al., 2001; Kurt et al., 2004; Struffaldi et al., 2004). NO is synthesized from the amino acid L-arginine and molecular oxygen in a reaction catalyzed by nitric oxide synthase (NOS). At least three NOS isoforms have been identified: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). The neural and endothelial forms are constitutively expressed and require elevated intracellular Ca^{2+} for their activity (Alderton et al., 2001; Gorren and Mayer, 2007). One main target of NO is the activation of the soluble guanylate cyclase (sGC) that converts guanosine 5′-triphosphate (GTP) to cyclic guanosine 3′,5′-monophosphate (cGMP) (Bredt and Snyder, 1992), which is a second messenger implicated in diverse cellular processes either by activation of protein kinases or acting directly on ionic channels (Collier and Vallance, 1989; Mayer and Koesling, 2001). There is evidence showing that NO plays a modulatory role in several effects of benzodiazepines, including their antinociceptive- (De Oliveira et al., 1997; Fidecka, 2003; Lazzarini et al., 1996, 2001, 2006; Talarek and Fidecka, 2002) and anxiolytic- (Caton et al., 1994; Quock and Nguyen, 1992; Volke et al., 1998) like effects. However, the mechanism of action is not well known.

We recently showed that diazepam produced a clear "antinociceptive-like action" by using the *pain-induced functional impairment model in the rat* (PIFIR model) (Jiménez-Velázquez et al., 2006). In the present study we aim to evaluate some of the possible mechanisms involved in this "antinociceptive-like activity". We hypothesized that the GABA/benzodiazepine receptor and the NO-cGMP pathway are involved. To study the putative participation of the GABA/benzodiazepine receptor we tested the effect of a selective antagonist, flumazenil, on the "antinociceptive-like action" of diazepam. To analyze the NO-cGMP pathway in the "antinociceptive-like actions" of diazepam, three pharmacological strategies were performed: a) impairment of endogenous nitrergic transmission by administering a non-selective and a selective nNOS inhibitor, N^{ω}-L-nitro-arginine methyl ester hydrochloride (L-NAME) and 7-nitroindazole (7-NI), respectively; b) enhancement of this transmission using the enzymatic precursor for the synthesis of NO, L-arginine and c) inhibition of the soluble guanylate cyclase (sGC), which synthesizes cGMP, by the non-selective agent, methylene blue.

2. Materials and methods

2.1. Animals

Male adult Wistar rats weighing 180–200 g were used in this study. Animals were housed in a room under controlled conditions of temperature (22 °C) and 12 h light-dark cycles, with free access to food and water before the experiments. All experimental procedures followed the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Covino et al., 1980) and the Guidelines on Ethical Standards for investigations of Experimental Pain in Animals (Zimmermann, 1983). The protocol was approved by the local Animals Ethics Committee and was consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, revised 1985). The number of experimental animals was kept to a minimum, they were used only once, and following the end of the study, rats were euthanized by CO_2 overdose.

2.2. Drugs

The following drugs, obtained from Sigma (Chemical Co., St. Louis, MO, USA), were used in this study: flumazenil (Flu, GABA/benzodiazepine receptor antagonist), N^{ω}-L-nitro-arginine methyl ester hydrochloride (L-NAME, a non-selective NOS inhibitor), N^{ω}-D-nitro-arginine methyl ester hydrochloride (D-NAME, inactive isomer of L-NAME), 7-nitroindazole (7-NI, nNOS inhibitor), L-2-amino-5-guanidinovaleric acid (L-arginine, NO enzymatic precursor), D-2-amino-5-guanidinovaleric acid (D-arginine, inactive isomer of L-arginine), methylene blue (MB, a non selective guanylate cyclase inhibitor) and uric acid (UA). Diazepam (Dz, GABA/benzodiazepine receptor agonist) was obtained from Hoffman–La Roche, Mexico City, Mexico. All drugs were freshly prepared, diazepam was dissolved in a 15% propilenglycol solution, flumazenil was dissolved in saline solution with a few drops of Tween-80, 7-NI was dissolved in 35% dimethylsulfoxide (DMSO) and uric acid was suspended in mineral oil. L-NAME, D-NAME, L-arginine, D-arginine and methylene blue were dissolved in 0.9% saline solution.

2.3. Nociception test

Nociception was assessed using the PIFIR model (López-Muñoz et al., 1993). The animals were anaesthetized with ether in an anesthesia chamber (Pyrex glass dryer saturated with ether vapor), before pain induction by a 50 µl injection of 15% uric acid into the knee joint of the right hind limb. An electrode was attached to the plantar surface of each hind paw, between the plantar pads. Rats were allowed to recover from anesthesia and placed on a cylinder that rotated at 4 rpm for periods of 2 min every 28 min during 5.5 h. The time of contact between each electrode on the paws of the rat and the cylinder was recorded with a computer. The functionality index (FI%), defined as the time of contact of the injected foot divided by the time of contact of the control left foot and multiplied by 100, was calculated from the collected data. Each time course was graphed to obtain the area under the curve (AUC). Initially, the dose–response curve for diazepam was assessed in rats that received an intra-articular (i.a.) injection of 15% uric acid (Jiménez-Velázquez et al., 2006). The "antinociceptive-like effect" of diazepam was defined as the recovery of contact time of the injured limb.

2.4. Participation of GABA/benzodiazepine receptor in the "antinociceptive-like effect" of diazepam

The participation of the GABA/benzodiazepine receptor in the "antinociceptive-like effect" of diazepam was assessed. Groups containing six to eight rats were pre-treated by i.p. injection of flumazenil (10 mg/kg) or its vehicle (saline solution 0.9% plus Tween 80). Diazepam, at an anxiolytic dose of 2 mg/kg, or its vehicle (propilenglycol 15%) was i.p. administered 15 min later. Fifteen min after the administration of diazepam, the animals were i.a. injected with 50 µl of 15% uric acid and thereafter tested in the nociception paradigm. After each treatment, the functionality index was recorded over a period of 5.5 h and the AUC calculated from the time courses.

2.5. Participation of NO-cGMP in the "antinociceptive-like effect" of diazepam

In the second set of experiments, the participation of the NO-cGMP pathway in the "antinociceptive-like effect" of diazepam was assessed. Independent groups of eight to ten rats were administered with L-NAME (5 mg/kg, s.c.), D-NAME (5 mg/kg, s.c.), 7-NI (1 mg/kg, s.c.), methylene blue (10 mg/kg, i.p.) or their respective vehicles (35% DMSO for 7-NI and saline solution 0.9% for L-NAME, D-NAME and methylene blue). Fifteen minutes later, diazepam, at an anxiolytic dose of 2 mg/kg, or its vehicle (propilenglycol 15%) was i.p. administered. Fifteen minutes after of the administration of diazepam, the animals were i.a. injected with 50 µl of 15% uric acid and tested for nociception. In another set, independent groups of rats (6–9 animals) were i.p injected with L-arginine (125 mg/kg, s.c.), D-arginine (125 mg/kg, s.c.) or their vehicle (saline solution 0.9%); followed 15 min later by diazepam at a non-effective dose of 1 mg/kg or its vehicle (propilenglycol 15%). Fifteen minutes after the administration of diazepam, the animals were i.a. injected with 50 µl of 15% uric acid; thereafter the animals were tested in the nociception test. After each treatment, the functionality index was recorded over a period of 5.5 h and the AUC calculated from the time courses.

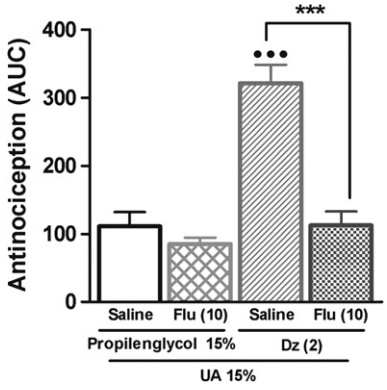

Fig. 1. Effect of flumazenil (Flu) (10 mg/kg, i.p.) on the "antinociceptive-like action" of diazepam (Dz) (2 mg/kg, i.p.) in rats treated with i.a. uric acid (UA). Tukey test, ●●●$P<0.001$ vs. its control group treated with propilenglycol, ***$P<0.001$ vs. treatment with flumazenil. The columns represent the "antinociceptive-like effect" expressed as the area under the curve (AUC) of the time courses. The plot shows the means ± S.E.M. of 6 to 8 rats.

2.6. Statistical evaluation

Data are expressed as means ± S.E.M. For each treatment, the corresponding time course was determined and the cumulative effect of each treatment expressed as the AUC calculated by the trapezoidal rule (Rowland and Tozer, 1989). The data were compared using a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test.

3. Results

3.1. Participation of GABA/benzodiazepine receptor in the "antinociceptive-like effect" of diazepam

Fig. 1 shows the "antinociceptive-like effects" of diazepam alone and in combination with flumazenil expressed as the AUC. As previously reported (Jiménez-Velázquez et al., 2006), diazepam (2 mg/kg, i.p.) produced a clear "antinociceptive-like effect" by increasing the AUC. Treatment with flumazenil (10 mg/kg, i.p.) antagonized the effect of diazepam (One-way ANOVA $F_{3,24}=29.80$, $P<0.001$), without modifying the functionality index by itself ($P>0.05$).

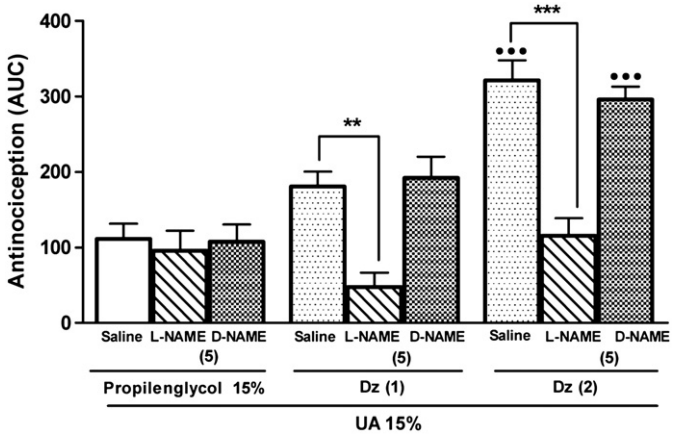

Fig. 2. Effect of N^{ω}-L-nitro-arginine methyl ester hydrochloride (L-NAME, 5 mg/kg, s.c.) or N^{ω}-D-nitro-arginine methyl ester hydrochloride (D-NAME, 5 mg/kg, s.c.) on the "antinociceptive-like effect" of diazepam (Dz) (1 or 2 mg/kg, i.p.) in rats treated with i.a. uric acid (UA). Tukey test, ●●●$P<0.001$ vs. its respective control group treated with propilenglycol, *** $P<0.001$ and **$P<0.01$ vs. treatment with L-NAME. The columns represent the "antinociceptive-like effect" expressed as the area under the curve (AUC) of the time courses. The plot shows the means ± S.E.M. of 6 to 8 rats.

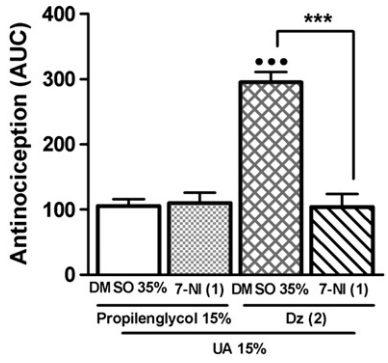

Fig. 3. Effects of 7-Nitroindazole (7-NI) (1 mg/kg, i.p.) on the "antinociceptive-like effect" of diazepam (Dz) (2 mg/kg, i.p.) in rats treated with i.a. uric acid (UA). Tukey test, ●●●$P<0.001$ vs. its control group treated with propilenglycol, *** $P<0.001$ vs. treatment with 7-NI. The columns represent the "antinociceptive-like effect" expressed as the area under the curve (AUC) of the time courses. The plot shows the means ± S.E.M. of 6 to 7 rats.

3.2. Participation of NO in the "antinociceptive-like effect" of diazepam

Figs. 2 and 3 show the effect of the systemic administration of NOS inhibitors on the "antinociceptive-like effect"of diazepam. Diazepam dose-dependently increased the AUC, suggesting an "antinociceptive-like effect". Treatment with the non-selective NOS inhibitor, L-NAME (5 mg/kg, s.c.), but not with its inactive isomer, D-NAME (5 mg/kg, s.c.), significantly blocked the "antinociceptive-like effect" of diazepam at both doses (one-way ANOVA $F_{8,55}=17.22$, $P<0.01$ and $P<0.001$, Fig. 2). Similarly, the treatment with the selective nNOS inhibitor, 7-NI (1 mg/kg), also blocked the "antinociceptive-like effect" induced by 2 mg/kg of diazepam (one-way ANOVA $F_{3,22}=35.12$, $P<0.001$, Fig. 3). L-NAME, D-NAME, or 7-NI produced similar effects to the saline solution in that they did not modify the functionality index by themselves ($P>0.05$, Figs. 2 and 3).

Diazepam at a low dose of 1 mg/kg produced a feeble increase in the AUC that did not reach statistical significance (Fig. 4). The NO enzymatic precursor, L-arginine (125 mg/kg, s.c.), but not its inactive isomer, D-arginine (125 mg/kg, s.c.), produced a clear increase in the "antinociceptive-like effect" of this dose of diazepam. The one-way ANOVA was statistically significant ($F_{5,40}=7.489$, $P<0.05$, Fig. 4). These drugs at these doses did not produce effects by themselves ($P>0.05$, Fig. 4).

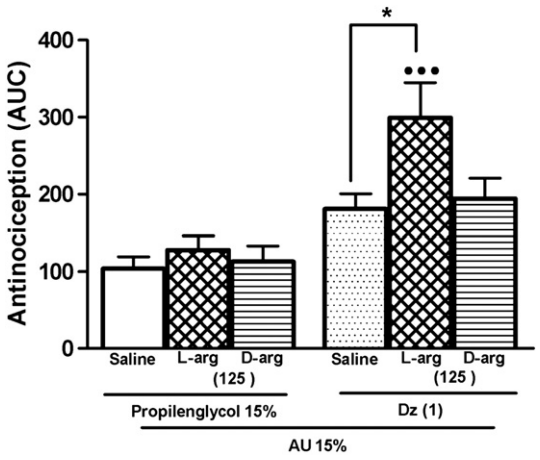

Fig. 4. Potentiating effects of *L*-arginine (*L*-arg) (125 mg/kg, s.c.) or *D*-arginine (*D*-arg) (125 mg/kg, s.c.) on the "antinociceptive-like effect" of diazepam (Dz) (1 mg/kg, i.p.) in rats treated with i.a. uric acid (UA). Tukey test, ●●●$P<0.001$ vs. its control group treated with propilenglycol, *$P<0.05$ vs. treatment with L-arginine. The columns represent the "antinociceptive-like effect" expressed as the area under the curve (AUC) of the time courses. The plot shows the means ± S.E.M. of 6 to 9 rats.

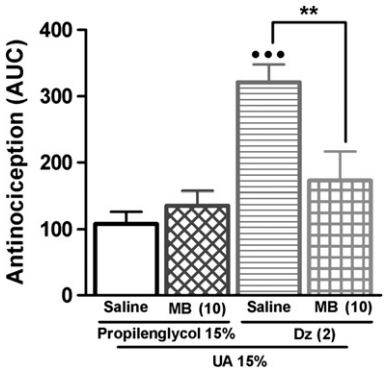

Fig. 5. Effects of the administration of methylene blue (MB) (10 mg/kg, i.p) on the "antinociceptive-like effect" of diazepam (Dz) (2 mg/kg, i.p.) in rats treated with i.a. uric acid (UA). Tukey test, ●●●$P<0.001$ vs. its control group treated with propilenglycol, **$P<0.01$ vs. treatment with MB. The columns represent the "antinociceptive-like effect" expressed as the area under the curve (AUC) of the time courses. The plot shows the means ± S.E.M. of 7 to 9 rats.

3.3. Effects of cGMP on the "antinociceptive-like effect" of diazepam

The effect of the systemic administration of an inhibitor of the sGC, methylene blue (10 mg/kg, i.p.), is shown in Fig. 5. Methylene blue administered alone did not modify the functionality index ($P>0.05$); however, it effectively blocked the "antinociceptive-like effect" of 2 mg/kg of diazepam (one-way ANOVA, $F_{3,28}=11.75$ $P<0.01$).

4. Discussion

The main conclusion derived from this study is that the GABA/benzodiazepine receptor and the NO-cGMP pathway participate in the "antinociceptive-like effect" of diazepam.

4.1. Participation of GABA/benzodiazepine receptor in the "antinociceptive-like effect" of diazepam

The results showed that the administration of flumazenil antagonized the "antinociceptive-like effect" of diazepam in the PIFIR model, indicating that the GABA/benzodiazepine receptor mediates the action of this compound. In agreement, it has been shown that flumazenil also antagonizes the "antinociceptive-like effects" of diazepam, chlordiazepoxide and clonazepam in the writhing test performed on mice (Talarek and Fidecka, 2002). Since the "antinociceptive-like effects" of diazepam in this model are mediated by the flumazenil-sensitive-benzodiazepine receptor, which occurs almost exclusively in the central nervous system (Sieghart, 1994), it could be proposed that such action is exerted at a central level, possibly via reducing anxiety. In this line, a previous study (Jiménez-Velázquez et al., 2006) showed that diazepam induced a decrease in experimental anxiety and prevented nociception only when administered before the nociceptive stimulus. Moreover, recent unpublished studies of our group demonstrate that the administration of diazepam into the basolateral amygdala and the dorsal periaqueductal grey induce parallel anxiolytic- and "antinociceptive-like effects" in the same subjects tested in the rat burying behavior and the PIFIR models.

4.2. Participation of NO-cGMP pathway in the "antinociceptive-like effect" of diazepam

The results showed that pre-treatment with the non-selective NOS inhibitor, L-NAME (but not with D-NAME), or with the nNOS selective inhibitor, 7-NI, abolished the "antinociceptive-like effects" of diazepam; while co-administration of the NO enzymatic precursor, L-arginine (but not of its inactive isomer, D-arginine) increased the feeble action of a non-effective dose of diazepam. Additionally, the administration of methylene blue (a non selective sGC inhibitor) also blocked the "antinociceptive-like effect" of diazepam. These results indicate that endogenous NO plays a role in this effect of diazepam.

The role of NO on the "antinociceptive-like effect" of benzodiazepines has been poorly explored. It has been found that diazepam, at very high doses (10 and 20 mg/kg), produces an anti-inflammatory effect in the rat carrageenan-induced paw edema (CIPE) (Lazzarini et al., 1996, 2001), that was increased by L-NAME and reverted by L-arginine (but not by D-arginine) (Lazzarini et al., 2006). These data suggest an important role of NO on the effects of diazepam on the CIPE that most likely involve a direct action of diazepam on the peripheral-type benzodiazepine receptor (PBR) present in the microvascular endothelium and/or on immune/inflammatory cells (Lazzarini et al., 2006). This peripheral action of diazepam would lead to decreased NO, thus reducing inflammation. However, this effect of diazepam was only observed after very high doses.

In the mouse writhing test, the systemic administration of the non-selective NOS inhibitor, L-NAME and of the relatively selective nNOS inhibitors, TRIM (1-(2-trifluoromethylphenyl)–imidazole) and 7–NI, but not of 3–Br–7–NI (3–bromo–7–nitroindazole) (also a selective nNOS–inhibitor), increased the "antinociceptive-like actions" of non-effective doses of diazepam and clonazepam, while L-arginine reverted these actions (Fidecka, 2003; Talarek and Fidecka, 2002). These data contrast with the present results showing that the NOS inhibitors prevented the "antinociceptive-like effects" of diazepam. The reason for this difference is yet unknown, but may be partly due to the "antinociceptive-like effects" produced by these NOS inhibitors *per se* (Fidecka, 2003), which may mask the actions of the benzodiazepines. In the present report, none of the NOS inhibitors showed effects *per se*.

Several studies have proposed that NO participates in the nociceptive process, both at central (Budziński et al., 2000; Hoheisel et al., 2005) and peripheral (Budziński et al., 2000; Déciga-Campos and López-Muñoz, 2004; Fernández and Assreuy, 2004; López-Muñoz et al., 1996; Salvemini et al., 1996; Ventura-Martínez et al., 2004) levels. However, the results of these investigations are contradictory showing that NO activation produces either antinociception (Budziński et al., 2000; Kawabata et al., 1993) or pro-nociception (Moore et al., 1991). These discrepancies may be due to different factors, such as: doses, administration routes, pharmacokinetic characteristics, animal species (mice or rats) or the nociception test utilized. Another important factor is that the various NOS isoforms are differentially localized and require diverse signals for their activation. Thus, nNOS is activated in the early inflammation phase induced by carrageenan (Salvemini et al., 1996), while an increase in the activity of iNOS is observed in a later phase (Budziński et al., 2000). In the present study, the peripheral intra-articular administration of uric acid produced an inflammatory process in which NO, possibly synthesized by the iNOS, plays a part (López-Muñoz et al., 1996). However, our results suggest that the NO that is participating in the "antinociceptive-like effects" of diazepam is mainly synthesized by the eNOS or nNOS, since both L-NAME and 7-NI similarly blocked the action of the benzodiazepine. As aforementioned, L-NAME inhibits all NOS isoforms (Osborne and Coderre, 1999) while 7-NI is a relatively selective nNOS inhibitor, suggesting that the "antinociceptive-like effect" of diazepam could be exerted at a central level and that such effect could be associated to its anxiolytic-like property. This hypothesis is further supported by several studies showing that NO, besides its involvement in nociception, also plays a role in modulating the anxiolytic-like effects of benzodiazepines (Caton et al., 1994; Quock and Nguyen, 1992; Volke et al., 1998). Thus, for example, it has been found that systemic pre-treatment with the NOS inhibitor, NG-nitro-L-arginine (L-NOARG), antagonized the anxiolytic-like effects of chlordiazepoxide, while the administration of L-arginine was able to restore these effects (Quock and Nguyen, 1992). The present results agree with this report. The mechanisms by which NO is mediating the effects of benzodiazepines

are still unknown. Interestingly, diazepam does not modify NOS activity directly (Volke et al., 1998), suggesting that other systems may be involved in the "antinociceptive-" or anxiolytic-like effects of this benzodiazepine. In this line, some studies have proposed that the anxiolytic actions of diazepam are partly mediated through the modulation of the endogenous opioid system (Primeaux et al., 2006; Tsuda et al., 1996), while others do not support this idea (Britton et al., 1981). The role of the opioid system in the "antinociceptive-like action" of benzodiazepines has also been assessed and the results are also contradictory. Thus, some reports indicate that the opioid antagonist, naloxone, blocks diazepam-induced analgesia in humans (Haas et al., 1982) and diazepam-induced antinociception in mice (Golombek et al., 1991); whereas others argue against this idea since naloxone fails to modify the "antinociceptive-like effect" of diazepam and chlordiazepoxide in the tail flick (Zambotti et al., 1991) and writhing (Talarek and Fidecka, 2002) tests.

One of the main targets of NO is the sGC, which subsequently elevates the intracellular concentration of cGMP derived from GTP (Bredt and Snyder, 1992). The present results showing that the "antinociceptive-like effects" of diazepam were antagonized by methylene blue suggest the involvement of a cGMP-dependent mechanism. However, methylene blue can inhibit both the sGC (Doyle and Hoekstra, 1981) and the NOS (Volke et al., 1999). As aforementioned, other NOS inhibitors prevented the "antinociceptive-like effects" of diazepam (*vide supra*). Thus, the actions of methylene blue may be mediated via the inhibition of the sGC or the NOS. Future studies with specific sGC inhibitors or cGMP analogues are necessary to study the role of cGMP in this effect of diazepam and of other benzodiazepines.

Studies *in vivo* and *in vitro* suggest that NO plays a modulatory role on GABAergic neurotransmission increasing the release of GABA from the pre-synaptic terminal through Ca^{2+}- and cGMP-dependent mechanisms (Guevara-Guzman et al., 1994; Kuriyama and Ohkuma, 1995; Prast et al., 1998; Trabace and Kendrick, 2000). Other reports show that NO modulates $GABA_A$ receptors by inhibiting their activity (Fukami et al., 1998; Zarri et al., 1994) through a cGMP-dependent mechanism (Robello et al., 1996) or may act directly at the $GABA_A$ receptor (Fukami et al., 1998). From the present results it could be suggested that NO modulates the actions of diazepam possibly by increasing GABA release. Such release could take place in brain areas associated to anxiety and nociception, such as the dorsolateral periaqueductal gray, the amygdala and the hypothalamic paraventricular nucleus where the distribution of nNOS is co-localized with GABA (De Oliveira et al., 1997; Guimarães et al., 2005; Vincent and Kimura, 1992). This hypothesis, however, needs experimental support.

In conclusion, these results indicate that diazepam produces "antinociceptive-like effects" via activation of the GABAA/benzodiazepine receptor and the endogenous NO-cGMP pathway. Moreover, the results also suggest that the "antinociceptive-like activity" of this benzodiazepine could be mediated by its anxiolytic-like properties.

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