Anti-hyperalgesic effect of systemic magnesium sulfate in carrageenan-induced inflammatory pain in rats: influence of the nitric oxide pathway

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Abstract. This study investigated whether systemic magnesium sulfate (an antagonist at the glutamate subtype of N-methyl-D-aspartate receptor) affects inflammatory pain, and whether the nitric oxide pathway is involved. Carrageenan (0.5%, 0.1 mL, intraplantar)-induced mechanical hyperalgesia was evaluated using the electronic von Frey test in male Wistar rats. Magnesium sulfate had no effect when injected locally into the inflamed rat paw. However, subcutaneous magnesium sulfate, at doses of 0.5, 5, 15 and 30 mg/kg, reduced the hyperalgesia by 44.4 ± 8.8, 68 ± 8.4, 24.6 ± 6.9 and 45.3 ± 6.7% respectively. N-nitro-L-arginine methyl ester hydrochloride (L-NAME) (3 and 5 mg/kg, intraperitoneal), a non-selective nitric oxide synthase inhibitor, significantly reduced the effects of magnesium sulfate. Also, L-arginine (0.4 mg/kg, subcutaneously) significantly reversed the effect of L-NAME in the magnesium sulfate-treated rats. A selective inhibitor of neuronal or inducible nitric oxide synthase, N-ω-Propyl-L-arginine hydrochloride (L-NPA) (0.5, 1 and 2 mg/kg, intraperitoneal) and S-methylisothiourea (SMT) (0.005, 0.01 and 0.015 mg/kg, intraperitoneal) reduced the effect of magnesium sulfate significantly only at the highest doses tested. When given alone, L-NAME (3 and 5 mg/kg) L-NPA (2 mg/kg) and SMT (0.015 mg/kg) did not have any influence on carrageenan-induced hyperalgesia. The present study revealed that magnesium sulfate is effective against inflammatory pain after systemic, but not after local peripheral administration, and activation of the nitric oxide pathway is probably involved in the anti-hyperalgesic effect of magnesium sulfate. Low doses of systemic magnesium sulfate given as a pretreatment or a treatment might have a beneficial effect in patients with inflammatory somatic pain.

Key words: inflammatory pain, carrageenan, magnesium sulfate, nitric oxide

Magnesium is the fourth most abundant ion in the human body and plays a fundamental role in many cellular functions, such as storage, metabolism, energy utilization, etc, and thus there is increasing interest in its role in clinical medicine [1]. Magnesium acts as an antagonist at the glutamate subtype of N-methyl-D-aspartate (NMDA) receptor and blocks NMDA-induced currents in a voltage-dependent manner by blocking the receptor channel effects [2]. Several lines of evidence indicate that magnesium enhances the analgesic effects of opioids, and general and
local anesthetics [3-6]. As the sole drug, magnesium demonstrated analgesic efficacy against neuropathic pain [7, 8], but little is known about the effect of magnesium on inflammatory pain. Studies using the formalin test in rats provided controversial data [9-11]. In addition, accumulated evidence suggests a link between inflammation and magnesium deficiency [12].

NMDA receptor activation is involved in the induction and maintenance of sensitization processes that characterize inflammatory pain states [13-16]. Activation of NMDA receptors by excitatory amino acids induces an increase in intracellular calcium, resulting in the production of nitric oxide (NO) [17]. A number of studies suggest that the activation of calcium-dependent nitric oxide synthase and the subsequent production of nitric oxide triggered by glutamate through the activation of NMDA receptors play an important role in central and peripheral modulation of nociception [18, 19]. In accordance with this, it is possible that magnesium, an antagonist of NMDA receptors, may have an influence on NO production.

The present study had two objectives. First, to determine whether magnesium sulfate (MS) affects inflammatory pain, using a carrageenan-induced hyperalgesia model. Second, to determine the potential involvement of the NO pathway in the effect of magnesium by assessing the effect of N-nitro-L-arginine methyl ester hydrochloride (L-NAME), a non-selective NOS inhibitor, N-ω-Propyl-L-arginine hydrochloride (L-NPA), a selective inhibitor of neuronal NOS, S-methylisothiourea (SMT), a selective inhibitor of inducible NOS, and L-arginine, an endogenous donor of nitric oxide, on magnesium’s effect on nociception.

Methods and materials

Animals

Adult, male Wistar rats (n = 204), weighing 230-290 g, were used. Researchers obtained the permission from the Ethics Committee for Animal Research and Welfare of the Faculty of Medicine, University of Belgrade (permit N° 4946/2). All experiments were approved by the Ethical Council for the Protection of Experimental Animals of the Ministry of Agriculture, Forestry and Water Management the Republic of Serbia, which operates in accordance with the Animal Welfare Law of our country, and IASP (International Association for the Study of Pain) Guidelines for the Use of Animals in Research. The animals were housed in groups of three in home cages (42.5 × 27 × 19 cm) under standard conditions: temperature of 22 ± 1°C, relative humidity of 60%, and a 12/12 h light/dark cycle. Food and water were freely available, except during the experimental procedures. The animals were fed with standard rat pellet feed obtained from the Veterinary Institute Subotica, Serbia. Prior to each experiment the animals were habituated to the handling and experimental procedures for at least three consecutive days. Rats were randomized into experimental groups consisting of six animals per group. The experiments were conducted by the same experimenter, on consecutive days, and always at the same time of the day, between 8:00 and 16:00 h to avoid diurnal variation in behavioral tests, and under relatively constant laboratory conditions (i.e., temperature, light, humidity, quietness). Each animal was used only once and was sacrificed at the end of the experiments with an intraperitoneal injection of sodium thiopental (200 mg/kg).

Experimental protocol for carrageenan-induced peripheral inflammation and the electronic pressure-meter test

The animals were acclimatized for 60 min prior to control measurements of paw withdrawal threshold (PWT) to mechanical stimuli. For the duration of the experiment, the rats were unrestrained and kept in individual Plexiglas boxes, raised on a special rack of steel mesh (mesh stand for mice and rats, IITC Life Science, Woodland Hills, USA). After control measurements, and a break of 30 min, peripheral hyperalgesia was induced by intraplantar injection of 0.1ml of carrageenan (0.5%) into the right hind paw. Hind paw, mechanical withdrawal thresholds were assessed by measuring the withdrawal response to von Frey filament stimulation, using an electronic version of the von Frey test (electronic von Frey Anesthesiometer, Model 2390, IITC Life Science, Woodland Hills, USA). The von Frey filament was applied to the plantar surface of the tested paw until paw withdrawal occurred, provoking a flexion reflex. The electronic pressure-meter
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automatically recorded the intensity of the stimulus when the paw was withdrawn. Before paw stimulation, the animals had to be quiet, without exploratory or toilet movements, and not resting over the paws. Measurements were performed three to five times in each rat, and the average of the middle three values was calculated. The intensity of hyperalgesia was quantified as the differences in pressures \( d \) (g) applied before and after injection of carrageenan (control d), or before and after injection of carrageenan plus drugs (test d). Analgesic activity (AA %) for each rat was calculated according to the formula [20]:

\[
\%AA = \frac{(\text{control group average } d - \text{test } d)}{(\text{control group average } d)} \times 100
\]

If test \( d \) was greater than the control group average \( d \), a value of 0% AA was assigned. The percentage inhibition (%I) of the antinociceptive effect with NOS inhibitor pretreatment was expressed as follows:

\[
\%I = 100 - \left( \frac{\%AA \text{ (in the presence of NOS inhibitor and MS)}}{\%AA \text{ (in the presence of MS)}} \right) \times 100
\]

Onset and offset of treatment effect were defined as the first time point and last time point, respectively, when a statistically significant difference between treatment groups and the control group (carrageenan/saline) occurred for the PWT. In order to examine whether drugs tested have any effect on the mechanical PWT in intact (not treated with carrageenan) rats, the highest doses tested were dissolved in saline and administered in a separate group of rats. Control rats received the corresponding volume of saline. Hind paw withdrawal threshold to mechanical stimuli was measured at 0, 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h after intraplantar injection of carrageenan.

**Drugs and drug administration**

Magnesium sulfate (Magnesio Solfato; S.A.L.F. Spa-Cenate Sotto-Bergamo, Italy), N-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich, St. Louis, MO, USA), L-arginine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), N-ω-Propyl-L-arginine hydrochloride (L-NPA, Tocris Bioscience, Bristol, UK) S-methylisothiourea (SMT, Tocris Bioscience, Bristol, Great Britain), α- carrageenan (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline and injected subcutaneously (MS and L-arginine), or intraperitoneally (L-NAME, L-NPA, SMT) at a final volume of 2 mL/kg, and intraplantar (carrageenan and MS) at a final volume of 0.1 mL per paw.

MS (0.5, 5, 15 and 30 mg/kg) was administered subcutaneously (s.c.), five minutes before (pretreatment) or two hours after (treatment) injection of carrageenan. Local peripheral effects were evaluated by intraplantar co-administration of carrageenan and MS. To exclude systemic effects, the same doses of MS were given in the contralateral paw. L-NAME (3 and 5 mg/kg), L-NPA (0.5, 1 and 2 mg/kg) or SMT (0.005, 0.01 and 0.015 mg/kg) was administered intraperitoneally (i.p.) 10 min before MS. Simultaneously, L-arginine (0.4 mg/kg, s.c.) and MS were administered intrathecally.

**Statistical analysis**

The data are presented as mean difference in pressure \( d \) (g) ± standard error of the mean (SEM) obtained in six rats. A difference between corresponding means in PWT was verified using analysis of variance (one-way ANOVA), followed by Tukey’s HSD post hoc test. A P value of less than 0.05 was considered statistically significant.

**Results**

**Carrageenan-induced hyperalgesia**

As expected, the carrageenan injection induced inflammation in the injected hind paw, as demonstrated by edema and erythema. After the carrageenan injection, PWT to mechanical stimuli was maximally reduced by 28.6 ± 3 \( (\text{figures } 1A,B) \), 26.8 ± 1.2 \( (\text{figure } 2) \), 35.8 ± 1.7 \( (\text{figures } 3A-B) \) and 33 ± 1.6 g \( (\text{figures } 4A,B) \) at time points 3 and 4 h. PWT did not change for the contralateral paw, indicating the lack of secondary hyperalgesia (not shown). There were no significant differences in the baseline (before carrageenan) PWTs between the groups tested (ranged between 49.9 ± 0.8 and 51.5 ± 0.5 g, not shown).

**Effects of magnesium sulfate given as a pretreatment on the mechanical PWT**

MS at dose of 5 mg/kg, given 5 min before carrageenan (pretreatment) significantly \( (p<0.05) \)
Figure 1. Time-response curves for the effect of magnesium sulfate (MS 0.5, 5, 15 and 30 mg/kg, s.c.) administered 5 min before (A) or 2 h after (B) the injection of carrageenan (Carr, i.pl.). (A) Statistical significance (*p < 0.05, **p < 0.01) was determined by comparison with the curve for saline. Statistical significance was found between MS 5 or 30 and 0.5 (*p < 0.05) and between MS 5 and 15 (*p < 0.05, **p < 0.01). (B) Statistical significance (*p < 0.05, **p < 0.01) was determined by comparison with the curve for saline. Small graph represents log dose-response curve for the analgesic effect of magnesium sulfate (MS) at time of peak effect (1 or 2 h after carageenan injection). s.c., subcutaneous. i.pl. intraplantar.
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Figure 2. Time-response curve for the effect of intraplantar magnesium sulfate (MS) 0.05, 0.1 and 0.5 mg/paw co-injected with carrageenan (Carr) at final volume of 0.1 mL/paw. Statistical significance was not found at any time point (p > 0.05). i.pl. intraplantar.

Reduced the hyperalgesia by 68 ± 8.4, 52 ± 5.7 and 45 ± 6.8% at 1, 2 and 3 h, respectively (figure 1A). The effect lasted up to 3 h. Lower dose of 0.5 mg/kg of MS reduced hyperalgesia by 44.4 ± 8.8 and 24.4 ± 6.8% at 1 and 2 h, respectively (figure 1A). Higher doses of 15 and 30 mg/kg of MS produced weaker antihyperalgesic effect with a maximum of 24.6 ± 6.9 (p > 0.05) and 45.3 ± 6.7% (p < 0.01), respectively, obtained at time point 2 h (figure 1A). Measurements taken for the contralateral (non-inflamed) paw with the highest dose of MS (30 mg/kg) did not show any effect on PWT suggesting that it did not disturb the behavioral assessment of the nociceptive sensitivity in rats (not shown).

Effects of magnesium sulfate given as a treatment on the mechanical PWT

MS at dose of 5 mg/kg given as a treatment (2 h after the induction of inflammation) produced a significant (p < 0.01) anti-hyperalgesic effect of 43.7 ± 6.1%. The effect was obtained one hour after drug administration (at time point 3 h) and

Figure 3. Time-response curve for the effect of L-NAME (3 and 5 mg/kg i.p.) and L-arginine (L-arg, 0.4 mg/kg s.c.) on the effect of magnesium sulfate (MS, 5 mg/kg, s.c) on carrageenan (Carr)-induced hyperalgesia in rats. (A) Statistical significance was found between L-NAME 3+MS and MS (*p < 0.05, **p < 0.01) and L-arg+L-NAME 3+MS and L-NAME 3+MS (**p < 0.01). (B) Statistical significance was found between L-NAME 5+MS and MS (*p < 0.05) and L-arg+L-NAME 5+MS and L-NAME 5+MS (***p < 0.01). s.c., subcutaneous, i.p., intraperitoneal, i.pl. intraplantar.
Effect of local peripheral magnesium sulfate on the mechanical PWT

*Figure 2* shows that MS at doses of 0.05, 0.1 and 0.5 mg/paw, co-injected with carrageenan had no influence on hyperalgesia (p>0.05). A dose of 0.1 mg/kg injected into the contralateral (non-inflamed) paw had no effect on carrageenan-induced hyperalgesia (not shown).

The effect of L-NAME and L-arginine on the anti-hyperalgesic effect of magnesium sulfate in carrageenan-treated rats

L-NAME at a dose of 3 mg/kg abolished (p<0.01) the anti-hyperalgesic effect of MS (5 mg/kg) at 0.5, 1, 2 and 3 h time points, while higher dose (5 mg/kg) significantly (p<0.05) reduced the effect by 84.1%, at time point 0.5 h (*figures 3A,B*). L-arginine (0.4 mg/kg, s.c.) significantly (p<0.01) reduced the effect of L-NAME at 3 and 5 mg/kg on the effect of MS by 57.8 and 72.9%, respectively. The effects peaked at 0.5 h (*figures 3A,B*). Given alone, L-NAME (3 and 5 mg/kg) and L-arginine had no effect on carrageenan hyperalgesia (not shown).

The effect of L-NPA or SMT on the anti-hyperalgesic effect of magnesium sulfate in carrageenan-treated rats

L-NPA, a selective inhibitor of neuronal NOS, at a dose of 2 mg/kg, significantly (p<0.01) reduced the anti-hyperalgesic effect of MS (5 mg/kg) by 75% at time point 0.5 h, while lower doses of 1 and 0.5 mg/kg had a weak or no effect on the anti-hyperalgesic effect of MS (*figure 4A*). SMT, a potent and selective inhibitor of inducible NOS, at doses of 0.005 and 0.01 mg/kg, had no effect on the anti-hyperalgesia induced by MS. However, the lower dose of 0.015 mg/kg significantly (p<0.01) reduced the effect of MS (5 mg/kg) by 98.5%, at time point 0.5 h (*figure 4B*). When given alone, L-NPA (2 mg/kg) and SMT (0.015 mg/kg) had no effect on carrageenan hyperalgesia (not shown).

Discussion

The present study showed that pretreatment with systemic MS (0.5, 5, 15 and 30 mg/kg, s.c.) resulted in a dose-independent increase in the mechanical
PWT in carrageenan-induced inflammatory pain in rats. There are a very few data about the antinociceptive effect of magnesium in inflammatory pain and even these are often controversial. Begon et al. [21] showed that systemic MS (30 and 90 mg/kg, i.p.) had no effect on phase 2 of the formalin test [9]. However, Takano et al. [11] and Ishizaki et al. [10] reported that intrathecal injection of magnesium was able to decrease the phase 2 responses to the formalin test in a dose-dependent manner. The reported disagreement could be explained by differences in the pro-inflammatory compounds used, pain stimulus, sites of injections, doses and/or species differences. The late phase of the formalin test and carrageenan hyperalgesia appear to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord, which involves NMDA receptors [9, 14, 22]. In experimental models of neuropathic pain, systemic MS and MK-801 (also an NMDA receptor antagonist) were shown to have a comparable effect [7]. However, in that study, a much higher doses of MS (150 mg/kg, i.p. b.i.d.) was used, which suggests that MS is more potent against inflammatory than against neuropathic pain. As regards our findings, the absence of any effect of MS following local peripheral administration, and the presence of an effect after systemic administration, might suggest that its effect is mediated by central mechanisms. Given as a treatment, systemic MS also provided anti-hyperalgesic effects, suggesting that it is efficacious in the late phase of inflammation too.

Our finding that MS did not modify the mechanical PWT on the contralateral (non-inflamed) paw, provided evidence that MS does not disturb the behavioral assessment of nociceptive sensitivity in rats. This is supported by previous findings that low doses of MS do not induce any adverse effects in rats [7, 23, 24].

The present study suggested the participation, at least partially, of NO pathway activation in the antinociceptive effect of MS. Although L-NAME displays a synergistic antinociceptive interaction with morphine [25], L-NAME did not change the antinociceptive activity of a morphine–magnesium sulfate combination [26] in an acute thermal pain test. In light of the present finding, this might have been a net effect of the increased antinociceptive effect of morphine and a decreased effect of magnesium in the presence of L-NAME.

As it is known that magnesium is a non-competitive NMDA receptor antagonist, its antinociceptive effect in the present study might be the result of NMDA receptor antagonism. If the NMDA receptors that are blocked by magnesium are also linked to NO synthesis and release, then it is reasonable to predict that inhibition of NOS in the central nervous system would enhance the analgesia produced by magnesium [27]. However, in the present study, systemic administration of L-NAME reduced rather than enhanced the antinociceptive effect of MS, and L-arginine re-established the effect of L-NAME. Our findings also show that the selective inhibitors of neuronal or inducible NOS, L-NPA and SMT, reduced the effect of MS. These results suggest that activation of the NO pathway may have an important role in the antinociceptive effects of systemic MS. This discrepancy might be explained by considering that NO is a controversial molecule with dual effects, depending on its concentration, NOS isoforms, redox balance and the function of neuron subpopulations [27, 28]. On the one hand, increased NO production has been shown to produce antinociception [29-33], while NOS inhibitors elicit antinociception in some models of acute and persistent pain [34-36]. In agreement with our findings, it has been reported that ketamine, a non-competitive NMDA antagonist, also activates the NO pathway allowing it to exert its central analgesic effects [30].

Alternatively, the observed activation of NOS by magnesium may result from non-NMDA mechanisms. Magnesium has been shown to decrease not only NMDA receptor activity, but also the activity of other presynaptic and postsynaptic calcium channels, and it modulates the release of neurotransmitters [1, 37].

In conclusion, the present results suggest that systemic, but not local administration of magnesium sulfate produced an anti-hyperalgesic effect in carrageenan-induced inflammatory pain in rats, and that activation of the NO pathway might play a role in the mechanism of action. Our results suggest that pretreatment or treatment with low doses of systemic MS might have a beneficial effect in patients with inflammatory somatic pain.

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