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Peripheral antinociceptive effects of μ- and δ-opioid receptor agonists in NOS2 and NOS1 knockout mice during chronic inflammatory pain

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ABSTRACT

The aim of this study is to investigate the involvement of nitric oxide synthesized by the inducible (NOS2) or neuronal (NOS1) nitric oxide synthases in the local antinociceptive effects produced by μ- and δ-opioid receptor agonists during chronic inflammatory pain. Peripheral inflammatory pain was induced in NOS2 and NOS1 knockout mice and their wild type littermates by the subplantar administration of complete Freund's adjuvant (30 μl). The presence of paw inflammation, mechanical allodynia and thermal hyperalgesia induced by complete Freund's adjuvant were assessed by measuring paw diameter and using the von Frey filaments and plantar tests, respectively. During chronic inflammation, NOS2 deficient mice have a more rapid recovery of paw edema and a reduced thermal hyperalgesia compared to wild type. In contrast, a reduced paw edema and mechanical allodynia, as well as a modest rapid recovery from thermal hyperalgesia were observed in NOS1 knockout mice compared to wild type. The thermal hyperalgesia induced by complete Freund's adjuvant was not completely reversed by the subplantar administration of morphine (days 4 and 7) or [D-Pen ^{2,5}] enkephalin (DPDPE) (days 1 and 4) in NOS2 knockout mice as occurs in wild type mice. Moreover, the local administration of morphine or DPDPE also failed to reverse the decrease of ipsilateral paw withdrawal latency induced by complete Freund's adjuvant in NOS1 knockout mice throughout 10 days of peripheral inflammation. These results indicate the different roles played by nitric oxide synthesized by NOS2 or NOS1 in the maintenance of mechanical allodynia and thermal hyperalgesia induced by chronic inflammatory pain as well as, in the antinociceptive effects produced by μ- and δ-opioid receptor agonists during peripheral inflammatory pain.

1. Introduction

Several studies have demonstrated that opioids administered either locally or systemically during peripheral inflammation have powerful antinociceptive effects (Stein, 1995; Fernández-Dueñas et al., 2007). These effects are mainly induced by the activation of opioid receptors located in the peripheral nervous systems whose expression increases during inflammatory pain (Pol and Puig, 2004).

Nitric oxide produced by the three nitric oxide synthase (NOS) isoforms: neuronal (NOS1), inducible (NOS2) and endothelial (NOS3) is a neurotransmitter in the central and peripheral nervous systems. Inflammatory pain increases the expression of NOS2 and NOS1 in the spinal cord and paw (Gühring et al., 2000; Chu et al., 2005; De Alba et al., 2006) and accordingly the nitric oxide levels. This neurotransmitter has been described either as pro- or antiinflammatory. Thus, treatment with specific and nonspecific NOS inhibitors has been reported to ameliorate (De Alba et al., 2006) and not alter (Sakaguchi et al., 2004) or exacerbate (Tedesco et al., 2002) the symptoms of joint inflammation. The administration of nitric donors or precursors could also exert an anti- and a proinflammatory effect in the presence and absence of peripheral inflammation (Fujii et al., 1999; Fernandes and Assreuy, 2004).

Nitric oxide has also been implicated at various levels of the nociceptive neural pathways and could produce both pro- and antinociceptive effects. Therefore, the administration of specific or unspecific NOS inhibitors may attenuate (De Alba et al., 2006) and not alter (Tao et al., 2003) or increase (Budzinski et al., 2000) the hyperalgesia induced by inflammatory pain. Likewise, the administration of nitric donors may have opposing effects like nociception (Tassorelli et al., 2006) or antinociception (Da Rocha et al., 2002) depending on the dose and administration site.

Numerous studies show that the peripheral antinociception induced by opioids is produced by the stimulation of the L-arginine-nitric oxide-cGMP pathway (Ferreira et al., 1991; Pacheco and Duarte, 2005). Accordingly, after acute peripheral inflammation, opioid receptor agonist induced antinociception was significantly reduced by the local administration of NOS or guanylate cyclase inhibitors (Ortiz et al., 2005;

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Pacheco et al., 2005) and increased by the local administration of substances capable of inhibiting cGMP degradation (Mixcoatl-Zecuatl et al., 2000; Amarante and Duarte, 2002) or nitric oxide donors (Nozaki-Taguchi and Yamamoto, 1998; Tasatargil and Sadan, 2004). This indicates that the peripheral antinociceptive effects of opioids in acute inflammation are mediated by the activation of the L-arginine-nitric oxide-cGMP pathway. However, the role of the nitric oxide in the antinociceptive effects produced by opioids during chronic inflammatory pain is not known.

In a murine model of peripheral inflammation induced by complete Freund's adjuvant we evaluate the involvement of the nitric oxide synthesized by NOS2 or NOS1 in the development and expression of chronic inflammatory pain and in the local antinociceptive effects produced by μ and δ-opioid receptor agonists during peripheral inflammation by using knockout mice.

2. Materials and methods

2.1. Animals

In this study, male NOS2-deficient mice (C57BL/6J background, Jackson Laboratories, Bar Harbor, ME, USA), NOS1-deficient mice (C57BL/6J background, Jackson Laboratories, Bar Harbor, ME, USA) and their wild type littermates (C57BL/6J; Harlam Laboratories, France), weighing 21 to 25 g were used. Mice were housed under 12-h/12-h light/ dark conditions in a room with controlled temperature (22 °C) and humidity (66%). Animals had free access to food and water and were used after a minimum of 6 days acclimatization to the housing conditions. All experiments were conducted between 9:00 AM and 5:00 PM.

The study protocol was approved by the Committee of Animal Use and Care of the Autonomous University of Barcelona, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.2. Induction of inflammation

Paw inflammation was induced in wild type, NOS2 and NOS1 knockout mice by the subplantar (s.p.) injection of 30 μl of complete Freund's adjuvant (Sigma) into the right hind paw. These animals developed a local inflammatory reaction that remained confined to the injected paw. The presence of inflammation was assessed by measuring paw diameter (Fine Science Tools, Heidelberg, Germany) and nociceptive behaviour (see below). Experiments were performed at 1, 4, 7, 10, 14 and 20 days after complete Freund's adjuvant injection and at the same time points in control mice (non-injected). However, since the results obtained in control mice and in the contralateral paw were similar, we used the latter as a true control in all subsequent testing. Body weight was also measured for the duration of the study.

2.3. Nociceptive behavioural tests

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. In brief, animals were placed in a Plexiglas® box (20 cm high, 9 cm diameter) with a wire grid bottom through which the von Frey filaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose, CA, USA) were applied by using a modified version of the up–down paradigm, as previously reported (Chaplan et al., 1994). The filament of 0.4 g was used first. Then, the strength of the next filament was decreased or increased according to the response. The threshold of response was calculated from the sequence of filament strength used during the up–down procedure by using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that includes curve fitting of the data. Clear paw withdrawal, shaking or licking of the paw were considered nociceptive-like responses. Both ipsilateral and contralateral hind paws were tested. Animals were allowed to habituate for 1 h before testing in order to allow an appropriate behavioural immobility.

Thermal hyperalgesia was assessed as previously reported by Hargreaves et al. (1988). Paw withdrawal latency in response to radiant heat was measured using the plantar test apparatus (Ugo Basile, Italy). Briefly, mice were placed in Plexiglas boxes (20 cm high×9 cm diameter) positioned on a glass surface. The heat source was positioned under the plantar surface of the hind paw and activated with a light beam intensity, chosen in preliminary studies to give baseline latencies from 8 to 10 s in control mice. A cut-off time of 15 s was used to prevent tissue damage in the absence of response. The mean paw withdrawal latencies from the ipsilateral and contralateral hind paws were determined from the average of 3 separate trials, taken at 5 min intervals to prevent thermal sensitization and behavioural disturbances. Animals were habituated to the environment for 1 h before the experiment to become quiet and to allow testing.

2.4. Experimental protocol

In a first set of experiments we assessed the influence of NOS2 or NOS1 deletion in the development and expression of inflammatory pain by using knockout mice. Animals were habituated for 1 h to the environment of the different experimental tests during 4 days. After the habituation period, baseline responses were established in the following sequence: paw volume, von Frey filaments and plantar test. After baseline measurements, inflammatory pain was induced as previously described. NOS2 and NOS1 knockout mice, and their wild type littermates were tested in each paradigm on days 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant injection by using the same sequence as for baseline responses.

In a second set of experiments, we investigated the involvement of the nitric oxide (synthesized by NOS2 or NOS1) in the local antinociceptive effects of μ and δ-opioid receptor agonists during peripheral inflammatory pain by using knockout mice. Due to in knockout mice (NOS2 and NOS1) the paw withdrawal responses to a thermal stimulus at 14 and 20 days after complete Freund's adjuvant injection were similar comparing ipsilateral vs. contralateral paws, the antinociceptive effects of morphine and [D-Pen ^{2,5}] enkephalin (DPDPE) on the thermal hyperalgesia induced by inflammatory pain in knockout and wild type mice were only evaluated at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. Thus, animals received one dose of 100 μg of morphine, DPDPE or saline (control group), subplantarly administered into the ipsilateral paw (right hind paw), on days 1, 4, 7 or 10 after complete Freund's adjuvant injection. Behavioural testing started 30 min after drug or saline administration. Finally, in order to exclude any possible systemic effect of the drugs, 100 μg of morphine, DPDPE or saline were subplantarly administered into the contralateral (left) paw.

The doses of opioid receptor agonists (morphine and DPDPE) used in this study were selected based upon their high efficacy in inhibit the thermal hyperalgesia induced by peripheral inflammation in rodents (Whiteside et al.; 2005; Pacheco and Duarte, 2005).

2.5. Drugs

Morphine–HCl was obtained from Alcaiber S.A. (Madrid, Spain) and DPDPE ([D-Pen ^{2,5}]-enkephalin) from Sigma-Aldrich (St. Louis, MO). Both drugs were dissolved in 30 μl of saline solution (0.9% NaCl) for local administration. For each group treated with a drug, the respective control group received the same volume of saline.

2.6. Statistical evaluation

Data are expressed as mean±S.E.M. For each knockout mice, data obtained from paw volume, plantar test and von Frey filament stimulation model were compared on each experimental day by using

a two-way ANOVA repeated measures on the factor paw and genotype as between factor of variation followed by the corresponding Student's *t* test to compare between paws and genotypes when appropriated. For each knockout mice, the effects of morphine or DPDPE treatment were compared on each experimental day by using a three-way ANOVA on the factor genotype, paw and drug as between factor of variation followed by the corresponding Student's *t* test to compare between paws when appropriated. A value of $P<0.05$ was considered significant.

3. Results

3.1. Local inflammatory reaction induced by the subplantar injection of complete Freund's adjuvant in NOS2 knockout mice and their wild type littermates

The two-way ANOVA revealed a significant effect of the paw ($P<0.001$) at 1, 4, 7, 10, 14 and 20 days after complete Freund's adjuvant as well as, a significant effect of the genotype ($P<0.042$) and the interaction between genotype and paw ($P<0.042$) on days 10, 14 and 20 after complete Freund's adjuvant injection.

Thus, the subplantar injection of complete Freund's adjuvant induces a marked and long lasting increase of paw volume in the ipsilateral paw of wild type mice from days 1 to 20 after complete Freund's adjuvant injection (Table 1). Indeed, a significant effect of peripheral inflammation was observed on day 1 ($P<0.001$), day 4 ($P<0.001$), day 7 ($P<0.001$), day 10 ($P<0.001$), day 14 ($P<0.001$) and day 20 ($P<0.001$) after complete Freund's adjuvant injection (ipsilateral vs. contralateral paw; paired Student's *t* test). The same degree of paw edema was developed in NOS2 knockout mice during the first 7 days of peripheral inflammation but it was significantly diminished from day 10 to 20 after complete Freund's adjuvant injection (Table 1). Genotype differences were observed on days 10, 14 and 20 after complete Freund's adjuvant injection, when NOS2 knockout mice showed a significant lower volume of the ipsilateral paw than wild type littermates ($P<0.045$; Student's *t* test). Even though a significant increase of the ipsilateral paw volume as compared to the contralateral was observed in NOS2 knockout mice at days 1 ($P<0.001$), 4 ($P<0.001$), 7 ($P<0.001$), 10 ($P<0.005$), 14 ($P<0.001$) and 20 ($P<0.008$) after complete Freund's adjuvant injection (paired Student's *t* test).

No significant changes in paw volume were seen on the contralateral hind paw in both NOS2 knockout and wild type mice after complete Freund's adjuvant injection. In both genotypes, paw volume was generally 3 mm before complete Freund's adjuvant injection.

3.2. Inflammatory pain induced by the subplantar injection of complete Freund's adjuvant in NOS2 knockout mice and wild type littermates

3.2.1. von Frey filament stimulation (mechanical allodynia)

The subplantar injection of complete Freund's adjuvant led to a profound decrease of the threshold for evoking withdrawal of the ipsilateral hind paw to a mechanical stimulus in both NOS2 knockout and wild type mice (Table 1). Baseline values (before complete Freund's adjuvant injection) were similar in both genotypes with withdrawal thresholds to von Frey filaments between 1.3 and 1.5 g.

The two-way ANOVA revealed a significant effect of the paw ($P<0.001$) at days 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant and a significant effect of the genotype ($P<0.011$) on day 4 after complete Freund's adjuvant injection. That is, in both genotypes peripheral inflammation induced by complete Freund's adjuvant led to a significant decrease of the threshold for evoking withdrawal of the ipsilateral hind paw to a mechanical stimulus from the first day after complete Freund's adjuvant and persisting during the whole duration of the experiment. In fact, a significant effect of peripheral inflammation was observed on day 1 ($P<0.004$), day 4 ($P<0.001$), day 7 ($P<0.001$), day 10 ($P<0.001$), day 14 ($P<0.006$) and day 20 ($P<0.004$) after complete Freund's adjuvant injection in both genotypes (ipsilateral vs. contralateral paw; paired Student's *t* test). Genotype differences were only observed on day 4, when NOS2 knockout mice showed a significantly lower withdrawal of the ipsilateral hind paw to mechanical stimulus than wild type littermates ($P<0.018$; Student's *t* test).

No significant changes in paw withdrawal responses to mechanical stimulus were seen on the contralateral hind paw in both NOS2

Table 1
Local inflammatory reaction (edema, mechanical allodynia and thermal hyperalgesia) induced by the subplantar administration of complete Freund's adjuvant in NOS2 knockout mice (NOS2-KO) and wild type littermates (WT) at days 0, 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant injection

Time of evaluation (days)							
Tests	0	1	4	7	10	14	20
Paw edema: paw volume (mm)							
NOS2-KO							
Contralateral	2.98±0.02	3.02±0.02	3.02±0.02	3.01±0.02	3.02±0.01	3.00±0.01	3.00±0.01
Ipsilateral	2.98±0.01	3.50±0.06[a]	3.57±0.08[a]	3.41±0.08[a]	3.35±0.09[a]	3.32±0.07[a]	3.20±0.06[a]
WT							
Contralateral	2.98±0.02	3.01±0.02	3.02±0.01	3.03±0.02	3.02±0.02	3.00±0.01	3.03±0.02
Ipsilateral	2.99±0.02	3.45±0.05[a]	3.65±0.08[a]	3.55±0.08[a]	3.58±0.06[a b]	3.55±0.07[a b]	3.51±0.08[a b]
Mechanical allodynia: von Frey filaments strength (g)							
NOS2-KO							
Contralateral	1.43±0.12	1.44±0.14	1.27±0.08	1.36±0.11	1.31±0.13	1.32±0.12	1.33±0.08
Ipsilateral	1.42±0.11	0.60±0.10[a]	0.41±0.04[a]	0.70±0.05[a]	0.65±0.08[a]	0.74±0.11[a]	0.89±0.11[a]
WT							
Contralateral	1.34±0.12	1.22±0.14	1.41±0.09	1.46±0.08	1.34±0.08	1.36±0.09	1.47±0.12
Ipsilateral	1.32±0.14	0.63±0.07[a]	0.71±0.10[a b]	0.69±0.07[a]	0.70±0.05[a]	0.71±0.05[a]	0.88±0.06[a]
Thermal hyperalgesia: paw withdrawal latency (s)							
NOS2-KO							
Contralateral	10.24±0.69	10.27±0.82	9.86±0.81	9.15±0.50	9.41±0.31	9.59±0.82	8.84±0.95
Ipsilateral	10.22±0.93	4.95±0.45[a]	5.70±0.64[a]	6.54±0.76[a]	7.39±0.75[a]	8.83±0.76	8.77±0.41
WT							
Contralateral	10.10±0.81	9.51±0.54	9.47±0.32	8.66±0.50	10.29±0.55	9.88±0.45	9.75±0.70
Ipsilateral	10.69±0.75	3.33±0.28[a b]	3.61±0.55[a b]	4.52±0.54[a b]	5.45±0.52[a b]	6.32±0.58[a b]	6.70±0.81[a b]

Results are shown as mean values of 10 animals±SEM. For each test and time tested the letter a, indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's *t* test) and the letter b, indicates significant differences when compared wild type vs. NOS2 knockout mice ($P<0.05$, Student's *t* test).

knockout and wild type mice after complete Freund's adjuvant injection.

3.2.2. Plantar test (thermal hyperalgesia)

The two-way ANOVA revealed a significant effect of the paw ($P<0.045$) on days 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant injection. Thus, the subplantar injection of complete Freund's adjuvant in wild type mice led to a significant ($P<0.018$) decrease of the withdrawal latency of the ipsilateral hind paw to thermal stimulus from the first day after complete Freund's adjuvant and persisting during the whole duration of the experiment (20 days; Table 1). In NOS2 knockout mice a decreased withdrawal latency of the ipsilateral hind paw to a thermal stimulus was only present during the first 10 days after complete Freund's adjuvant injection. Thus, NOS2 knockout mice injected with complete Freund's adjuvant only showed thermal hyperalgesia on day 1 ($P<0.001$), day 4 ($P<0.001$), day 7 ($P<0.013$) and day 10 ($P<0.042$) after complete Freund's adjuvant injection (ipsilateral vs. contralateral paw; paired Student's *t* test).

The two-way ANOVA also revealed a significant effect of the genotype on days 1, 4 and 7 after complete Freund's adjuvant ($P<0.050$) as well as an interaction between genotype and paw on days 10, 14 and 20 after complete Freund's adjuvant injection ($P<0.050$). Genotype differences were observed on day 1 ($P<0.008$), day 4 ($P<0.025$), day 7 ($P<0.045$), day 10 ($P<0.048$), day 14 ($P<0.017$) and day 20 (0.035) after complete Freund's adjuvant injection, when NOS2 knockout mice showed a significantly lower withdrawal latency of the ipsilateral paw than wild type littermates (Student's *t* test).

Baseline values (before complete Freund's adjuvant injection) were similar in both genotypes with withdrawal latencies to a thermal stimulus between 10 and 10.7 s. Subplantar injection of complete Freund's adjuvant did not produce significant changes in paw withdrawal responses to thermal stimulation on the contralateral paw of NOS2 knockout or wild type mice (Table 1).

When these parameters were evaluated in animals without inflammation (non-injected with complete Freund's adjuvant), no significant changes were observed in any of the parameters when compared to the contralateral paw (results not shown). Therefore, all experiments were performed with the contralateral paw as a control to reduce the number of used animals.

3.3. Local inflammatory reaction induced by the subplantar injection of complete Freund's adjuvant in NOS1 knockout mice and their wild type littermates

The two-way ANOVA revealed a significant effect of the paw ($P<0.001$) at 1, 4, 7, 10, 14 and 20 days after complete Freund's adjuvant injection, a significant effect of the genotype ($P<0.02$) at 4, 7, 10, 14 and 20 days after complete Freund's adjuvant and a significant interaction ($P<0.010$) between genotype and paw on days 10, 14 and 20 after complete Freund's adjuvant injection. Results showed that similarly to occur in wild type animals, NOS1 knockout mice also developed paw edema at first day of peripheral inflammation but this edema was significantly diminished compared to wild type mice from 4 to 20 days after complete Freund's adjuvant injection ($P<0.040$; Student's *t* test; Table 2). Even though a significant increase of the ipsilateral paw volume as compared to the contralateral was observed on days 1 ($P<0.007$), 4 ($P<0.001$), 7 ($P<0.001$), 10 ($P<0.001$) and 14 ($P<0.001$) after complete Freund's adjuvant injection in NOS1 knockout mice (paired Student's *t* test).

No significant changes in paw volume were seen on the contralateral hind paw in both NOS1 knockout and wild type mice after complete Freund's adjuvant injection. In both genotypes, paw volume was generally 3 mm before complete Freund's adjuvant injection.

3.4. Inflammatory pain induced by the subplantar injection of complete Freund's adjuvant in NOS1 knockout mice and wild type littermates

3.4.1. von Frey filament stimulation (mechanical allodynia)

The effect of NOS1 deletion on the mechanical allodynia induced by inflammatory pain was evaluated in NOS1 deficient mice by measuring the hind paw withdrawal response to von Fey filament stimulation at 1, 4, 7, 10, 14 and 20 days after complete Freund's adjuvant injection (Table 2).

Table 2
Local inflammatory reaction (edema, mechanical allodynia and thermal hyperalgesia) induced by the subplantar administration of complete Freund's adjuvant in NOS1 knockout mice (NOS1-KO) and wild type littermates (WT) at days 0, 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant injection

Time of evaluation (days)							
Tests	0	1	4	7	10	14	20
Paw edema: paw volume (mm)							
NOS1-KO							
Contralateral	2.95±0.02	2.97±0.02	2.95±0.02	2.97±0.02	2.99±0.02	3.00±0.01	3.01±0.02
Ipsilateral	2.96±0.02	3.32±0.09[a]	3.41±0.07[a]	3.34±0.09[a]	3.26±0.04[a]	3.24±0.04[a]	3.10±0.05
WT							
Contralateral	2.99±0.19	3.00±0.02	2.98±0.02	3.03±0.02	3.01±0.02	3.02±0.01	3.02±0.02
Ipsilateral	2.99±0.02	3.45±0.04[a]	3.65±0.07[a b]	3.59±0.07[a b]	3.58±0.06[a b]	3.52±0.08[a b]	3.51±0.08[a b]
Mechanical allodynia: von Frey filaments strength (g)							
NOS1-KO							
Contralateral	1.47±0.12	1.30±0.10	1.20±0.07	1.33±0.07	1.41±0.09	1.38±0.05	1.52±0.07
Ipsilateral	1.39±0.10	0.44±0.06[a]	0.61±0.06[a]	0.97±0.10[a]	0.96±0.08[a]	1.04±0.09[a]	1.13±0.04[a]
WT							
Contralateral	1.33±0.12	1.32±0.14	1.41±0.08	1.45±0.07	1.35±0.08	1.36±0.09	1.47±0.11
Ipsilateral	1.33±0.14	0.63±0.07[a b]	0.70±0.10[a]	0.68±0.07[a b]	0.69±0.05[a b]	0.72±0.05[a b]	0.87±0.06[a b]
Thermal hyperalgesia: paw withdrawal latency (s)							
NOS1-KO							
Contralateral	10.31±0.45	9.23±0.55	9.30±0.88	9.10±0.58	9.80±0.69	9.55±0.86	9.59±0.89
Ipsilateral	10.20±0.94	2.69±0.25[a]	2.85±0.40[a]	5.32±0.58[a]	5.79±0.66[a]	7.57±0.66	8.31±1.05
WT							
Contralateral	10.10±0.81	9.08±0.70	9.77±0.40	8.67±0.51	10.30±0.55	9.86±0.44	10.26±0.90
Ipsilateral	10.09±0.70	3.33±0.30[a]	3.61±0.60[a]	4.51±0.55[a]	5.71±0.58[a]	6.31±0.57[a]	6.44±0.60[a]

Results are shown as mean values of 10 animals±SEM. For each test and time tested the letter a, indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's *t* test) and the letter b, indicates significant differences when compared wild type vs. NOS1 knockout mice ($P<0.05$, Student's *t* test).

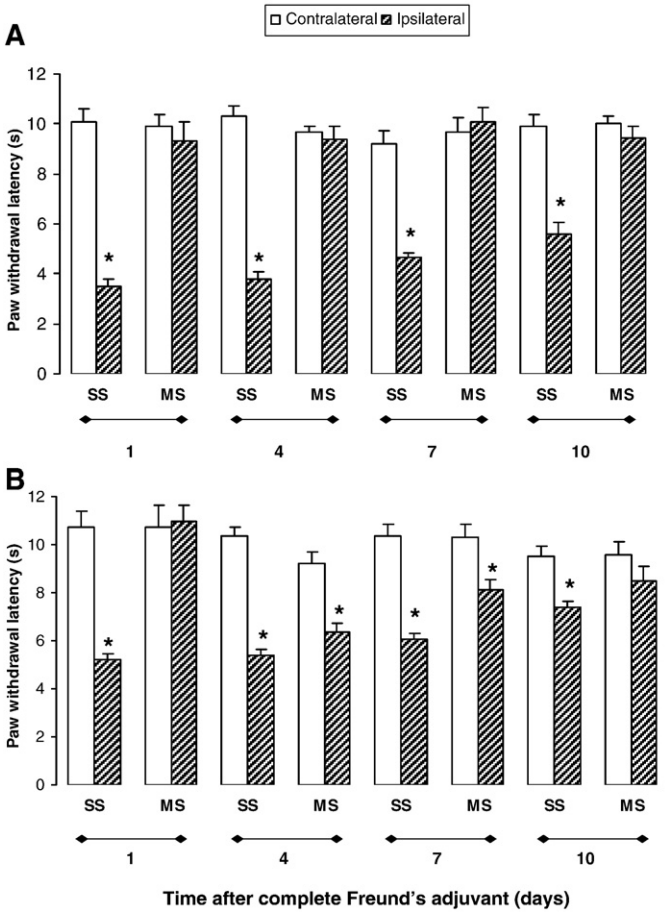

Fig. 1. Effect of the subplantar administration of 100 μg of morphine in the ipsilateral and contralateral paw withdrawal latencies to a thermal stimulus in wild type (A) and NOS2 knockout mice (B) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. For each time, * indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's t test).

The two-way ANOVA revealed a significant effect of the paw ($P<0.001$) at days 1, 4, 7, 10, 14 and 20 after complete Freund's adjuvant, a significant effect of the genotype ($P<0.05$) at days 10, 14 and 20 after complete Freund's adjuvant injection as well as, a significant interaction between genotype and paw at 7 and 14 days of peripheral inflammation (Table 2).

The same decrease of the threshold for evoking withdrawal of the ipsilateral hind paw to a mechanical stimulus observed in wild type mice on day 4 after complete Freund's adjuvant injection was also observed in NOS1 knockout mice but it was significantly increased on day 1 and diminished from days 7, 10, 14 and 20 after peripheral inflammation in NOS1 knockout mice when compared to wild type mice ($P<0.045$; Student's t test). Although an allodynic response on day 1 ($P<0.001$), day 4 ($P<0.001$), day 7 ($P<0.011$), day 10 ($P<0.008$), day 14 ($P<0.014$) and day 20 ($P<0.002$) after complete Freund's adjuvant injection was observed in NOS1 knockout mice comparing the ipsilateral vs. contralateral paw (paired Student's t test).

No significant changes in paw withdrawal responses to mechanical stimulus were seen on the contralateral hind paw in both NOS1 knockout and wild type mice after complete Freund's adjuvant injection. Baseline values (before complete Freund's adjuvant injection) were similar in both genotypes with withdrawal thresholds to von Frey filaments between 1.3 and 1.5 g.

3.4.2. Plantar test (thermal hyperalgesia)

The two-way ANOVA revealed a significant effect of the paw ($P<0.006$) from days 1 to 20 after complete Freund's adjuvant injection, as a result of the significant decrease of the withdrawal latency of the ipsilateral hind paw to thermal stimulus observed in wild type mice during peripheral inflammation ($P<0.004$, paired Student's t test).

The subplantar injection of complete Freund's adjuvant in NOS1 knockout mice also led to a significant decrease of the withdrawal latency of the ipsilateral hind paw to thermal stimulus which persists during 10 days (Table 2). In fact, a significant effect of peripheral inflammation was observed on day 1 ($P<0.001$), day 4 ($P<0.001$), day 7 ($P<0.001$) and day 10 ($P<0.005$) after complete Freund's adjuvant injection in NOS1 knockout mice (ipsilateral vs. contralateral paw; paired Student's t-test). The genetic disruption of NOS1 enzyme did not change the significant decrease of the withdrawal latency of the ipsilateral hind paw to thermal stimulus observed in wild type mice, as revealed by the absence of significant effects of the genotype on the two-way ANOVA ($P>0.05$).

Baseline values (before complete Freund's adjuvant injection) were similar in both genotypes with withdrawal latencies to a thermal stimulus between 10 and 10.7 s. Subplantar injection of complete Freund's adjuvant did not produce significant changes in paw withdrawal responses to thermal stimulation on the contralateral paw of knockout or wild type mice.

When these parameters were evaluated in animals without inflammation (non-injected with complete Freund's adjuvant), no significant changes were observed in any of the parameters when compared to the contralateral paw (results not shown). Therefore, all experiments were performed with the contralateral paw as a control to reduce the number of used animals.

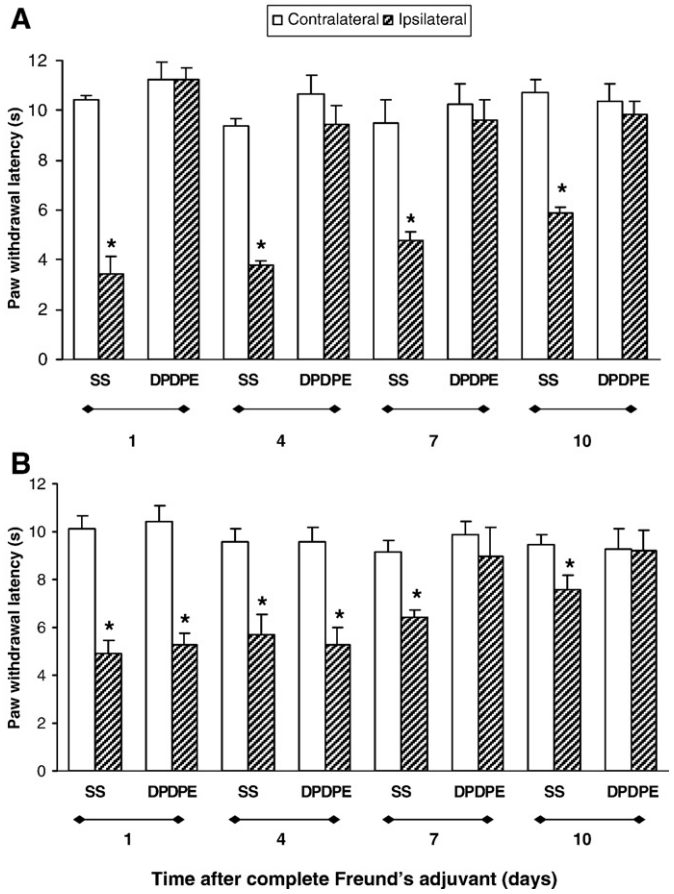

Fig. 2. Effect of the subplantar administration of 100 μg of DPDPE in the ipsilateral and contralateral paw withdrawal latencies to a thermal stimulus in wild type (A) and NOS2 knockout mice (B) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. For each time, *indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's t test).

The body weight in NOS2 and NOS1 knockout mice as well as in their littermates at different time points after complete Freund's adjuvant injection (0, 1, 4, 7, 10, 14 and 20 days) was also assessed. The one way ANOVA did not show significant differences between genotypes from 0 (before complete Freund's adjuvant) to 20 days after complete Freund's adjuvant injection which values vary between 22 and 25 g.

3.5. Effects of morphine and DPDPE during complete Freund's adjuvant induced inflammatory pain in NOS2 knockout mice and wild type littermates

The antihyperalgesic effects of subplantar morphine or DPDPE in the ipsilateral and contralateral paws of NOS2 knockout mice and their wild type littermates at 1, 4, 7 and 10 days after complete Freund's adjuvant injection are shown in Figs. 1 and 2, respectively.

For morphine, the three-way ANOVA reveals a significant effect of the paw ($P<0.001$) and drug ($P<0.002$) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection, a significant interaction between genotype and drug ($P<0.040$) after 4, 7 and 10 days of peripheral inflammation, a significant interaction between paw and drug ($P<0.004$) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection as well as, a significant interaction between genotype, paw and drug ($P<0.050$) at 4, 7 and 10 days after complete Freund's adjuvant injection.

That is, whereas in wild type mice the subplantar administration of morphine completely reversed the decrease of ipsilateral paw withdrawal latency induced by peripheral inflammation at 1, 4, 7 and 10 days after complete Freund's adjuvant injection (Fig. 1A). In NOS2 knockout mice, the thermal hyperalgesia induced by peripheral inflammation was only completely reversed by morphine at days 1 and 10 but not at 4 and 7 after complete Freund's adjuvant injection ($P<0.030$; ipsilateral vs. contralateral paw; paired Student's t test; Fig. 1B).

For each time evaluated, the effects of morphine in the contralateral paws of NOS2 knockout mice and wild type littermates were similar and non significant differences were observed when compared each of them to their contralateral site on NOS2 knockout mice or their littermates treated with saline.

Regarding DPDPE, the three way ANOVA reveals a significant effect of the paw ($P<0.002$) and drug ($P<0.020$) as well as their interaction ($P<0.050$) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. A significant interaction between genotype and drug ($P<0.001$) as well as between genotype, paw and drug ($P<0.020$) were also revealed on days 1 and 4 after peripheral inflammation. This is related to the fact that whereas the subplantar administration of DPDPE completely reversed the decreased ipsilateral paw withdrawal latency induced by peripheral inflammation from 1 to 10 days after complete Freund's adjuvant injection (Fig. 2A). In NOS2 knockout mice the decrease ipsilateral paw withdrawal latency induced by inflammatory pain was only completely reversed by the δ-opioid receptor agonist at 7 and 10 but not at 1 and 4 days after complete Freund's adjuvant injection ($P<0.004$, paired Student's t test; ipsilateral vs. contralateral paw; Fig. 2B).

For each time evaluated, the effects of DPDPE in the contralateral paws of NOS2 knockout mice and wild type littermates were similar and non significant differences were observed when compared each of them to their contralateral site on NOS2 knockout mice or their littermates treated with saline.

3.6. Effects of morphine and DPDPE during complete Freund's adjuvant induced inflammatory pain in NOS1 knockout mice and wild type littermates

The antihyperalgesic effects of subplantar morphine or DPDPE in the ipsilateral and contralateral paws of NOS1 knockout mice and their littermates at 1, 4, 7 and 10 days after complete Freund's adjuvant injection are shown in Figs. 3 and 4, respectively.

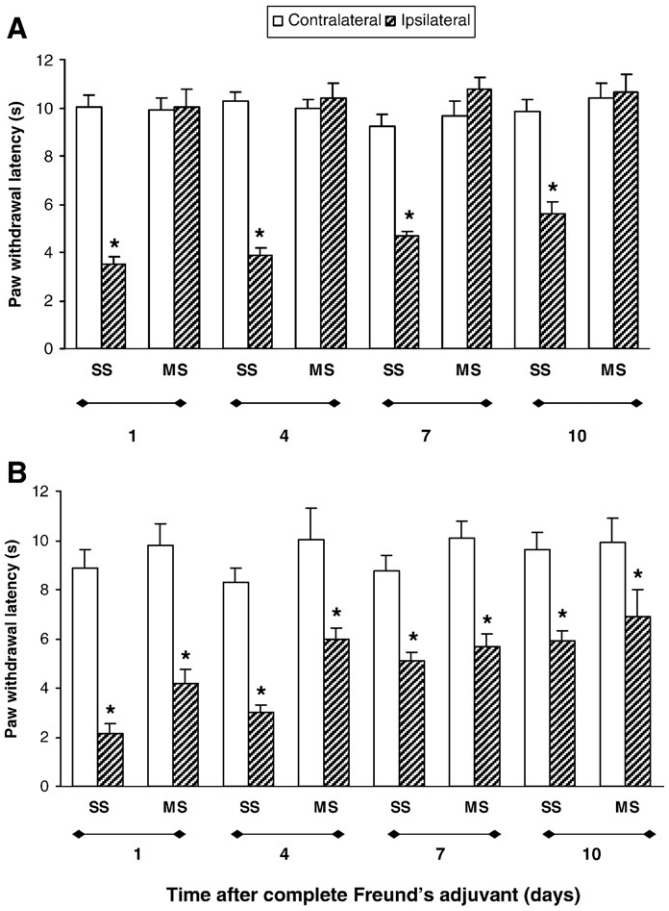

Fig. 3. Effect of the subplantar administration of 100 μg of morphine in the ipsilateral and contralateral paw withdrawal latencies to a thermal stimulus in wild type (A) and NOS1 knockout mice (B) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. For each time, *indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's t test).

For morphine, the three-way ANOVA reveals a significant effect of the genotype ($P<0.003$) at 1, 4 and 7 days after complete Freund's adjuvant injection. A significant effect of the paw ($P<0.001$) and drug ($P<0.004$) as well as their interaction ($P<0.025$) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection has been also demonstrated. The three way ANOVA also reveals a significant double interaction between genotype and paw ($P<0.050$) and a triple interaction between genotype, paw and drug ($P<0.002$) at 1, 4 and 7 days after complete Freund's adjuvant injection. And finally, a significant interaction between genotype and drug ($P<0.050$) at 1, 7 and 10 days of peripheral inflammation has been also established.

These results are related to the fact that in NOS1 knockout mice the decrease of ipsilateral paw withdrawal latency induced by peripheral inflammation was not completely reversed by the subplantar administration of morphine, as occurs in wild type mice, at 1, 4, 7 and 10 days after complete Freund's adjuvant injection (Fig. 3A and B; $P<0.030$; ipsilateral vs. contralateral paw; paired Student's t test).

For each time evaluated, the effects of morphine in the contralateral paws of NOS2 knockout mice and wild type littermates were similar and non significant differences were observed when compared each of them to their contralateral site on NOS2 knockout mice or their littermates treated with saline.

For DPDPE, the three-way ANOVA reveals a significant effect of the genotype ($P<0.050$), paw ($P<0.001$) and drug ($P<0.025$) on days 1, 4, 7 and 10 after complete Freund's adjuvant injection. A significant interaction between genotype and paw ($P<0.030$) at 1, 4 and 7 days

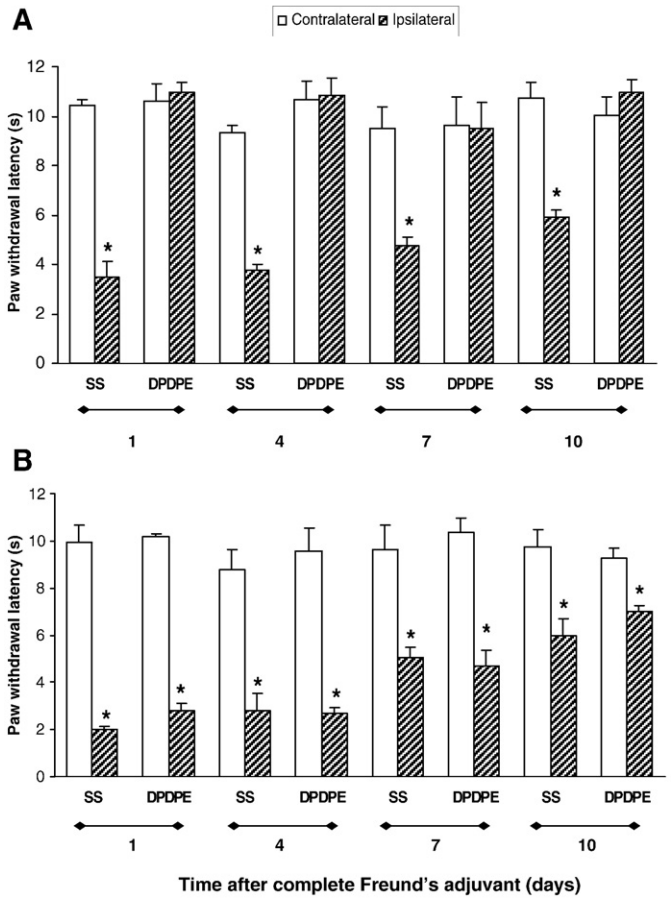

Fig. 4. Effect of the subplantar administration of 100 μg of DPDPE in the ipsilateral and contralateral paw withdrawal latencies to a thermal stimulus in wild type (A) and NOS1 knockout mice (B) at 1, 4, 7 and 10 days after complete Freund's adjuvant injection. Each column represents the mean value of 6–8 animals and the vertical bars indicate the S.E.M. For each time, *indicates significant differences when compared ipsilateral vs. contralateral paw ($P<0.05$, paired Student's t test).

after complete Freund's adjuvant injection has been demonstrated. Moreover, a significant interaction between genotype and drug ($P<0.030$) and between paw and drug ($P<0.012$) at 1, 4 and 10 days after complete Freund's adjuvant injection has been also established. The three-way ANOVA also revealed a significant triple interaction between genotype, paw and drug ($P<0.020$) at 1, 4, 7 and 10 days of peripheral inflammation.

Thus similarly that occurs with morphine, whereas in wild type mice the administration of DPDPE completely reversed the decreased ipsilateral paw withdrawal latency induced by inflammatory pain on days 1, 4, 7 and 10 after complete Freund's adjuvant injection (Fig. 4A). In NOS1 knockout mice, the decrease ipsilateral paw withdrawal latency induced by inflammatory pain from 1 to 10 days after complete Freund's adjuvant injection was not completely reversed by the subplantar administration of the δ-opioid receptor agonist ($P<0.008$, paired Student's t test; ipsilateral vs. contralateral paw, (Fig. 4B).

For each time evaluated, the effects of DPDPE in the contralateral paws of NOS1 knockout mice and wild type littermates were similar and non significant differences were observed when compared each of them to their contralateral site on NOS1 knockout mice or their littermates treated with saline.

4. Discussion

We investigated the role played by nitric oxide synthesized by NOS2 or NOS1 in the development and expression of peripheral inflammatory pain induced by complete Freund's adjuvant as well as in the local antinociceptive effects produced by μ and δ-opioid receptor agonists during peripheral inflammation by using knockout mice.

Thus, in a murine model of chronic inflammatory pain induced by the administration of 30 μl of complete Freund's adjuvant which produces paw edema, allodynia to mechanical stimuli and hyperalgesia to noxious thermal stimuli that persists more than 20 days we have demonstrated that: 1) NOS2 deficient mice have a more rapid recovery of paw edema and a reduced thermal hyperalgesia compared to wild type, 2) NOS1 knockout mice have reduced paw edema and mechanical allodynia as well as a modest rapid recovery from thermal hyperalgesia compared to wild type and 3) the nitric oxide synthesized from NOS2 and NOS1 during peripheral inflammation induced by complete Freund's adjuvant is differentially involved in the antinociceptive effects produced by μ- and δ-opioid receptor agonists during inflammatory pain.

Nitric oxide plays an important role in the control of inflammation and pain processes. Inflammatory pain increases the nitric oxide levels and accordingly the expression of NOS2 and NOS1 in the spinal cord and paw (Gühring et al., 2000; Chu et al., 2005; De Alba et al., 2006). This neurotransmitter has been described either as a pro-inflammatory or as anti-inflammatory. In our study, the administration of complete Freund's adjuvant (30 μl) induced a local inflammatory response that persists for 20 days in wild type mice. Our results showed that although NOS2 knockout mice have a more rapid recovery of paw edema compared to wild type, the targeted disruption of the NOS1 gene significantly reduced paw edema from 4 to 20 days after complete Freund's adjuvant injection. Indicating a differential pro-inflammatory role played by nitric oxide synthesized from NOS2 or NOS1 during complete Freund's adjuvant-induced inflammatory pain in mice. Our results are consistent with previous studies reporting that pharmacological inhibitors of NOS and ablation of the gene NOS2 reduce the development of the inflammatory response (Cuzzocrea et al., 2002; De Alba et al., 2006) and provide new data about the pro-inflammatory role played by nitric oxide synthesized by NOS1 during complete Freund's adjuvant-induced chronic paw inflammation in mice.

Nitric oxide has been also implicated at various levels of the nociceptive neural pathways, however the exact role of nitric oxide synthesized by NOS2 or NOS1 in the chronic inflammatory pain remain controversial. Our results reveal that whereas NOS1 deficient mice show an enhanced (68.3 vs. 52.6%) and a similar (56.1 vs. 47.3%) mechanical allodynia compared to wild type at 1 and 4 days after complete Freund's adjuvant injection, respectively. This mechanical allodynia was significantly reduced compared to wild type on days 7 (30.2 vs. 48.8%), 10 (30.9 vs. 48.1%), 14 (25.2 vs. 45.8%) and 20 (18.7 vs. 34.5%) after peripheral inflammation. In contrast, NOS2 deficient mice only reveal a significant enhanced mechanical allodynia comparing to wild type on day 4 after complete Freund's adjuvant injection (71.1 vs. 46.2%). Thus, the analogous mechanical allodynia observed in wild type and NOS2 knockout mice following 7 days of peripheral inflammation suggests that nitric oxide synthesized by NOS2 enzyme is not critically involved in the expression of mechanical allodynia during chronic inflammatory pain. In contrast, mechanical allodynia induced by complete Freund's adjuvant was progressively reduced from day 7 to 20 after complete Freund's adjuvant injection in NOS1 knockout mice, suggesting that the nitric oxide synthesized by NOS1 enzyme plays a critical role in the expression of mechanical allodynia induced by complete Freund's adjuvant. Our results are in agreement with the critical role played by nitric oxide synthesized from NOS1 in attenuating mechanical allodynia during the maintenance of chronic inflammatory pain evaluated during 7 days after the subplantar injection of 20 μl of complete Freund's adjuvant in NOS1 knockout mice (Chu et al., 2005) and with the pharmacological studies performed in rodents demonstrating that the administration of

specific or unspecific NOS inhibitors attenuate the mechanical allodynia induced by inflammatory pain (De Alba et al., 2006). Additionally, the expression of NOS1 increases in the spinal cord of rodents with chronic monoarthritis (Infante et al., 2007). Taken together, all of these results suggest that the nitric oxide synthesized by NOS1 play a critical role in the regulation of mechanical allodynia during chronic inflammatory pain.

Our results also show that whereas the NOS2 deficient mice have a reduced thermal hyperalgesia compared to wild type, only a modest quick recovery from thermal hyperalgesia induced by complete Freund's adjuvant was observed in NOS1 knockout mice. The fact that thermal hyperalgesia induced by complete Freund's adjuvant that lasted at least 20 days in wild type mice was significantly reduced in NOS2 knockout mice indicates that nitric oxide synthesized by NOS2 is critically involved in the development and expression of chronic inflammatory pain. This is in accordance with the decisive role played by nitric oxide synthesized by NOS2 in the thermal hyperalgesia during the development (first day) of chronic inflammatory pain evaluated during 7 days after the subplantar injection of 3 mg/ml of zymosan in NOS2 knockout mice (Gühring et al., 2000) but in contrast with the non involvement of nitric oxide synthesized by NOS2 in the thermal hyperalgesia during the development and expression of inflammatory pain assessed during 4 days after the subplantar injection of 20 µl of carrageenan in NOS2 knockout mice (Tao et al., 2003). This apparent contradictory result may be probably explained by the different inflammatory agents used to induce peripheral inflammation that represents distinct pain conditions.

Our results also showed for the fist time that the NOS1 knockout mice have a modest rapid recovery from thermal hyperalgesia compared to wild type starting in day 14 after complete Freund's adjuvant injection. In agreement with our results, another study showed that the NOS1 deletion failed to attenuate the development and maintenance of complete Freund's adjuvant-induced thermal hyperalgesia during 7 days after the subplantar injection of 20 µl of complete Freund's adjuvant (Chu et al., 2005). Therefore, our results indicate that the nitric oxide synthesized by NOS1 is necessary for the maintenance of long lasting thermal hyperalgesia induced by the injection of a high (but not small) dose of complete Freund's adjuvant (30 µl).

In our study similar baseline withdrawal thresholds to mechanical and thermal stimuli were found in wild type and mutant mice suggesting that nitric oxide synthesized by NOS1 or NOS2 do not seems to tonically modulate the mechanical and thermal nociceptive sensitivity (Chu et al., 2005; Boettger et al., 2007).

Nitric oxide is also implicated in several pathophysiological processes such as inflammation and regulation of gene expression (Pol, 2007). Accordingly, we have demonstrated that nitric oxide derived from NOS2 was involved in the enhanced antitransit and antiexudative effects of µ and δ-opioid receptor agonists as well as in the augmented µ-opioid receptor gene transcription during chronic intestinal inflammation in mice (Pol et al., 2005; Jiménez et al., 2006). Several data also showed that the peripheral inhibition of inflammatory hyperalgesia induced by opioids is produced by the stimulation of the L-arginine-nitric oxide-cGMP pathway (Ferreira et al., 1991; Granados-Soto et al., 1997). Thus, after acute peripheral inflammation opioid induced antinociception was significantly reduced by the local administration of nitric oxide synthase or guanylate cyclase, inhibitors (Ferreira et al., 1991; Tasatargil and Sadan, 2004; Pacheco et al., 2005; Menéndez et al., 2007) and increased by the administration of substances capable of inhibiting cGMP degradation (Mixcoatl-Zecuatl et al., 2000). This indicates that nitric oxide/cGMP system mainly improves the peripheral antinociceptive action of opioids during acute peripheral inflammation. Nonetheless, the specific role played by nitric oxide synthesized from NOS2 or NOS1 in the antinociceptive effects produced by opioids during acute and chronic inflammatory pain remains to be elucidated.

To study the involvement of nitric oxide in the peripheral antinociception induced by opioids during chronic inflammatory, the antinociceptive effects of locally administered morphine or DPDPE on the thermal hyperalgesia induced by inflammatory pain in knockout and wild type mice were evaluated. Initially, we tested if the antinociceptive effects produced by the subplantar administration of 100 µg of µ- and δ-opioid receptor agonists during peripheral inflammation are not due to a systemic effect. The possibility that morphine or DPDPE had a systemic effect at the dose of 100 µg was excluded since its administration into the contralateral paw (non inflamed paw) did not elicit antihyperalgesic effects in the ipsilateral paw (inflamed paw) of mice (data not shown).

Our results also showed that whereas NOS2 modulates the antinociceptive effects of morphine at 4 and 7 days after complete Freund's adjuvant injection, NOS1 participates in the antinociceptive effects produced by morphine at 1, 4, 7 and 10 days after peripheral inflammation. Indicating that the antinociceptive effects of morphine at 4 and 7 days of peripheral inflammation induced by complete Freund's adjuvant are, at least, partly mediated by the nitric oxide synthesized from both NOS2 and NOS1 isoenzymes. While the antinociceptive effects of the µ-opioid receptor agonist at 1 and 10 days of complete Freund's adjuvant-induced peripheral inflammation were principally mediated by NOS1-derived nitric oxide.

Regarding δ-opioid receptor agonist, we demonstrated that the antinociceptive effects of DPDPE during the first four days of peripheral inflammation induced by complete Freund's adjuvant are, at least, partly mediated by the nitric oxide synthesized from both NOS2 and NOS1 isoenzymes. However, the antinociceptive effects of DPDPE at 7 and 10 days of peripheral inflammation were mainly mediated by nitric oxide derived from NOS1.

The present study provides the first evidence for the differential role played by nitric oxide synthesized by NOS2 or NOS1 isoenzymes in the peripheral antinociceptive effects of µ- and δ-opioid receptor agonists during chronic inflammatory pain. This study also demonstrates that NOS1 is more involved in the maintenance of mechanical allodynia while NOS2 is more implicated in the maintenance of thermal hyperalgesia induced by complete Freund's adjuvant, in a chronic model of inflammatory pain.

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