Opioid-induced Hyperalgesia in a Murine Model of Postoperative Pain

Role of Nitric Oxide Generated from the Inducible Nitric Oxide Synthase

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Background: Opioid-induced delayed hyperalgesia and allodynia have been reported in human and animal models. The authors evaluated the influence of different opioids used during clinical anesthesia on nociceptive sensitivity and incisional pain in mice. The role of the inducible nitric oxide synthase on surgical pain and opioid-induced pronociception also was investigated.

Methods: CD1 mice were used to study the efficacy of opioids inducing pronociception and enhancing incisional pain. The implication of nitric oxide generated from the inducible nitric oxide synthase was investigated using knockout mice (C57/BL6) for its gene. Mice underwent right hind paw surgery under sevoflurane anesthesia combined with subcutaneous administration of saline or the opioids fentanyl (0.05 mg/kg), alfentanil (1 mg/kg), and remifentanil (0.04 mg/kg). Nociception was evaluated daily for 7 days using paw-pressure, plantar, and von Frey tests.

Results: The antinociceptive effect of opioids was followed by long-lasting thermal hyperalgesia and mechanical allodynia (each lasting between 2 and 7 days), but not mechanical hyperalgesia. Intraoperative infusion of opioids significantly enhanced incisional pain in all tests. The most prominent effects were observed with remifentanil. The inducible nitric oxide synthase gene deletion attenuated both remifentanil- and incision-induced pronociceptive effects. In mutant mice for the inducible nitric oxide synthase gene, remifentanil was still efficient in enhancing incisional pain, but the global pronociceptive effect was attenuated significantly as compared with wild-type mice.

Conclusions: The authors' study demonstrates that the intraoperative administration of fentanyl or remifentanil enhances the extent and duration of postoperative pain. The results suggest a role of the nitric oxide systems in the cause of acute postoperative pain and opioid-induced pronociception.

ALTHOUGH opioids are among the most effective analgesics in humans, there is growing evidence demonstrating that they also induce abnormal and prolonged pain states after acute or chronic administration.[1,2] As a result, the question of the clinical use of opioids during surgery and its consequences on the management of postoperative pain has been raised recently in the anesthesia literature.[3–9] It has been proposed that an opioid activation of pronociceptive systems may be responsible for the increased pain scores and analgesic requirements in the postoperative period.[5,10,11] In this context, the development of animal models of postoperative pain (roughly reproducing the clinical conditions) has been proven useful in the study of the mechanisms implicated in opioid-induced hyperalgesia, providing relevant information to improve postoperative pain management in humans. Of special relevance is the introduction of a murine model of postoperative pain, which allows the use of knockout mice for specific target proteins.[12]

In behavioral studies performed in nonmanipulated rodents (no surgical injury), opioid-induced pain sensitization has been demonstrated after the antinociceptive effect of opioids, such as heroin and fentanyl, or precipitated by naloxone in animals that previously received morphine.[1,2] In addition, opioids have been shown to exacerbate nociceptive responses in several models of acute and chronic inflammatory pain, such as in the formalin[13,14] and carrageenan tests.[15] In a rat model of postoperative pain,[16] it has been demonstrated that morphine-induced pain sensitization is additive with the hyperalgesia and allodynia resulting from surgical injury. More recently, fentanyl administration has been shown to induce exaggerated hyperalgesia after plantar incision in rats.[9] Based on the results obtained in all these studies, it has been proposed that postoperative pain in patients receiving opioids during surgery could result not only from the nociceptive input related to tissue damage, but also from opioid-induced pain sensitization.[1,15,17]

Complex interactions between inflammatory mediators, including prostaglandins, cytokines, and nitric oxide, have been implicated in the inflammatory response to surgery.[18,19] Nitric oxide is a free radical that, among other functions, behaves as an intracellular and intercellular messenger in the nervous system.[20,21] It is synthesized by nitric oxide synthase, of which three isoforms have been characterized. The neuronal and endothelial isoforms are constitutively expressed in the central nervous system, whereas the third form is inducible and found in macrophages and inflammatory cells.[22] The inducible nitric oxide synthase (iNOS) isoform has been identified as a key enzyme responsible for sustained

nitric oxide production under pathologic conditions, including neuropathic pain.[23,24] Moreover, recent studies suggest a possible implication of nitric oxide in acute nociception as well as the development of chronic pain.[25-28] The nitric oxide synthase/nitric oxide system also participates in the development of opioid tolerance and withdrawal,[29-32] although no reports exist in the literature regarding its possible role in the pronociceptive effects of opioids during incisional pain.

The aim of our study was to evaluate the effects on postoperative pain of three potent opioids currently used in clinical practice as intraoperative analgesics. For this purpose, we used a model that mimics the conditions in which these opioids are used in humans. We first studied the effectiveness of alfentanil, fentanyl, and remifentanil in inducing pain sensitization and enhancing incisional pain in mice. The possible role of iNOS on surgical pain and opioid-induced sensitization was investigated in knockout mice for this enzyme.

Materials and Methods

Animals

Male mice were used in all experiments. CD1 mice (25–30 g) were obtained from Charles River (CRIFFA, France) and wild-type (22–27g) and iNOS-mutant mice (B6.129P2-$Nos2^{tm1Lau}$/J) from the C57/BL6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in the animal quarters of our institution. The genotype of these mice was verified using polymerase chain reaction of tail-tip DNA. Mice were housed five per cage and were maintained at controlled temperature (21 ± 1°C) and humidity (55 ± 10%) in a room with a 12-h light-dark cycle (light between 8 AM to 8 PM). Food and water were available *ad libitum* except during behavioral evaluation. The investigator was blinded to the treatment and genotype of each experimental subject. All procedures and animal handling met the guidelines of the National Institutes of Health detailed in the *Guide for Care and Use of Laboratory Animals*[33] and the European Communities directive 86/609/EEC regulating animal research. The protocol used in the study was endorsed by the Local Ethical Committee of the institution (CEEA-IMAS, Comité Ético de Experimentación Animal IMAS, Barcelona, Spain)

Drugs

Fentanyl (Fentanest®; Spain Kern Pharma, Barcelona, Spain), alfentanil (Limifen®; Janssen-Cilag, Madrid, Spain), remifentanil (Ultiva®; GlaxoSmithKline, Madrid, Spain), and sevoflurane (Sevorane®; Abbott Laboratories SA, Madrid, Spain) were supplied by the Department of Anesthesiology of the Hospital del Mar (Barcelona, Spain). Fentanyl (0.05 mg/kg), alfentanil (1 mg/kg), and remifentanil (0.04 mg/kg) were dissolved in saline (NaCl 0.9%) and were infused subcutaneously over a period of 30 min using a Harvard Apparatus pump (Biosis S.L., Biologic Systems, Barcelona, Spain). The infusion rate was 0.8 ml/h. Control animals received the same volume of saline in identical conditions. The doses were selected on the basis of previous studies reported in the literature in rodents, demonstrating central effects of these opioids.[34-36]

Surgery

The incisional pain model was adapted from a previous study in rats.[37] Mice were anesthetized with sevoflurane delivered *via* a nose mask (induction, 3%; surgery, 1%) in a sterile operating room. A 0.7-cm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar surface of the right hind paw, starting 0.3 cm from the proximal edge of the heel and extending toward the toes. The underlying plantaris muscle then was elevated and incised longitudinally, keeping the muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was closed with two 5-0 nylon sutures and the wound was covered with povidone-iodine antiseptic ointment. After surgery, the animals were allowed to recover in their cages under a heat source. Control animals (nonoperated mice) underwent a sham procedure that consisted of the administration of anesthesia (sevoflurane ± opioid) without incision.

Behavioral Testing

Hyperalgesia to noxious thermal and mechanical stimuli and hyperalgesia to punctate stimuli (which will be referred as mechanical allodynia throughout the text) were used as outcome measures of nociception (pain). We evaluated the following parameters.

Thermal hyperalgesia was assessed as previously reported.[38] Briefly, paw withdrawal latency in response to radiant heat was measured using the plantar test equipment (Ugo Basile, Varese, Italy). Mice were placed in polymethyl methacrylate boxes (20 cm height, 9 cm diameter) positioned on a glass surface. Animals were habituated to the environment for 1 h before the experiment to become quiet and to allow testing. The heat source then was positioned under the plantar surface of the hind paw and activated with a light beam intensity that in preliminary studies gave baseline latencies of 9 to 11 s in control mice. A cutoff time of 20 s was established to prevent tissue damage in the absence of a response. The mean paw withdrawal latencies for both hind paws were obtained from the average of three separate trials, taken at 5- to 10-min intervals to prevent thermal sensitization and abnormal behavioral responses.

Mechanical hyperalgesia was evaluated by the paw-pressure test using the method of Randall and Selitto,[39] adapted to mice. Animals were held gently and progressive pressure was applied to the dorsal surface of the

hind paw using the LE 7306 analgesimeter (Panlab, Cornellà, Spain) until a flexor response of the toes was observed. A cutoff of 300 g was set to prevent tissue damage. Both hind paws were tested.

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. Animals were placed in a polymethyl methacrylate box (20 cm height, 9 cm diameter) with a wire grid bottom through which the von Frey filaments (bending force range, 0.008–2 g; North Coast Medical, Inc., San Jose, CA) were applied. Animals were allowed to habituate for 1 h before testing to achieve immobility. The threshold of response was evaluated using a modified version of the up–down paradigm, as previously reported.[40] The area tested was the midplantar right hind paw bordering the incision wound near the heel. 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 upper limit value (2 g) was recorded even if there was no withdrawal response to this force. 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 hind paws were tested.

Experimental Protocol and Groups of Experiments

In a first set of experiments, we assessed the pronociceptive effects of alfentanil, fentanyl, and remifentanil when administered in the absence and presence of incisional pain in male CD1 albino mice. For each opioid, we performed the experiments using five to eight animals per group. The opioids fentanyl (0.05 mg/kg), alfentanil (1 mg/kg), and remifentanil (0.04 mg/kg) were infused subcutaneously over a period of 30 min. In operated animals, opioid infusion was initiated at the moment of surgical incision, and the operating procedure was performed during the 30 min of opioid infusion. Control animals received the same volume of saline.

In a second set of experiments, we investigated the implication of iNOS in opioid and incision-induced hyperalgesia/allodynia using knockout and wild-type C57/BL6 mice (n = 5 animals per group). Wild-type and mutant animals were assigned to four experimental groups: remifentanil nonoperated, remifentanil-operated, and the control saline nonoperated and operated groups.

All experimental groups received the same inspired concentration of sevoflurane (1% in air) during 30 min. Special care was taken to reduce to a minimum interindividual variability and to use the smallest number of animals per group. After arrival, animals were kept undisturbed in the animal quarters for 5 days. Then, the animals were handled daily by the investigator and ha-

bituated to the experimental room environment for a period of 1week before beginning the experiments. All experiments were performed by the same investigator in a quiet test room close to the storage room. For the plantar, paw-pressure, and von Frey tests, the animals were familiarized with the special conditions of evaluation (nociceptive equipment, noise) in the absence of nociceptive stimulation. After the habituation period, baseline responses were obtained during 3 consecutive days for each paradigm in the following sequence: von Frey, paw-pressure, and plantar tests. The experiments (incision ± opioid administration) were performed 1 day later according to the protocol described above. Mice were tested in each paradigm on days 1, 2, 3, 4, and 7 after the surgical procedure using the same sequence as the evaluation of baseline responses. In the present investigation, the antinociceptive effects of the opioids were not evaluated.

Statistical Analysis

A general linear-mixed model[41] was used to estimate the overall (days 1–7) pronociceptive effects induced by the opioids, by the incision, and their combination. To determine the overall magnitude of the pronociceptive effect of each treatment or manipulation, the value obtained for each experimental group was subtracted from the value obtained in the corresponding saline control group. In this type of analysis, negative numbers indicate pronociceptive effects, whereas positive ones indicate antinociceptive effects (table 1). In the text, the results are expressed as a percentage of the overall decrease of nociceptive threshold taking the saline group as control; this allows the comparison of the results obtained in the three nociceptive tests.

All statistical analyses were performed with S-PLUS functions using the *nlme* library.[41] This model allows multiple between-group comparisons to asses the effect of the treatment, the surgery, the time, and the strain on the response. The analysis also includes the evaluation of the interaction between the different factors and the animal hind paws (left *vs.* right), a factor particularly influenced by the incision. The time effect assesses differences at any day with respect to baseline values determined before infusion and/or surgery. A similar model, including genotype variable, was used to establish differences between wild-type and mutant mice for the iNOS gene. Estimation of coefficients, and their associated *P* values, was based on restricted maximum likelihood. Comparison of models was based on likelihood ratio tests derived from model fits using a maximum likelihood fit. The models were checked by plotting the residuals *versus* fitted values. The variance function structure was used to model heterocedasticity in the within-days errors. A *P* value less than 0.05 was considered significant.

Table 1. Magnitude of the Delayed Hyperalgesia/Allodynia Induced by Alfentanil, Fentanyl, and Remifentanil in Operated and Sham Mice

	Saline CD1	Alfentanil CD1	Fentanyl CD1	Remifentanil CD1	Saline iNOS$^{-/-}$ C57B6	Remifentanil iNOS$^{-/-}$ C57B6
Opioid or saline						
Thermal hyperalgesia	0	−0.83	−2.4*	−2.6*	0	−1.3*
Mechanical hyperalgesia	0	−3.57	−9.0*	−0.29	0	−0.55
Mechanical allodynia	0	−0.08	−0.26*	−0.59*	0	−0.20*
Incision ± opioid or saline						
Thermal hyperalgesia	−3.6	−3.3	−5.3	−7.0*	−2.9	−5.3*
Mechanical hyperalgesia	−26.0	−27.0	−40.0*	−43.0*	−14.0	−23.0*
Mechanical allodynia	−0.39	−0.36	−0.56*	−0.76*	−0.11	−0.44*

Values represent the overall decrease of nociceptive thresholds compared with the respective saline control group. Nociceptive evaluation was performed for 7 days after surgery. For statistical analysis, we used the mixed-effects model, where negative values indicate pronociceptive effects, whereas positive values indicate antinociception (see Materials and Methods).

* Significant effect ($P < 0.05$) when comparing the effect of the opioid versus the control saline group.

iNOS = inducible nitric oxide synthase.

Results

Opioid-induced Delayed Hyperalgesia/Allodynia (Pain Sensitization)

We evaluated the pronociceptive effects induced by alfentanil, fentanyl, and remifentanil in sham-operated mice using the plantar, paw-pressure, and von Frey tests (fig. 1A and table 1). When compared with nonanesthe-tized control animals (data not shown), the administration of 1% sevoflurane for a period of 30 min to mice receiving a subcutaneous infusion of saline (fig. 1A, saline) did not produce significant changes in nociceptive thresholds in the plantar (mean values = 10.7 s), paw-pressure (mean values = 151 g), and von Frey (mean values = 1.36 g) tests.

Fig. 1. Time course of the effects of opioids on nociceptive thresholds in (*A*) control conditions (Sham) and (*B*) after surgery (Incision) in CD1 mice. Nociceptive thresholds of both hind paws were measured by the plantar, paw-pressure, and Von Frey tests and are expressed as mean values ± SEM. Fentanyl (0.05 mg/kg), alfentanil (1 mg/kg), remifentanil (0.04 mg/kg), or saline were infused subcutaneously in the absence or presence of surgery during a period of 30 min. Plantar incision was performed on the right hind paw. Number of mice per group was five to eight.
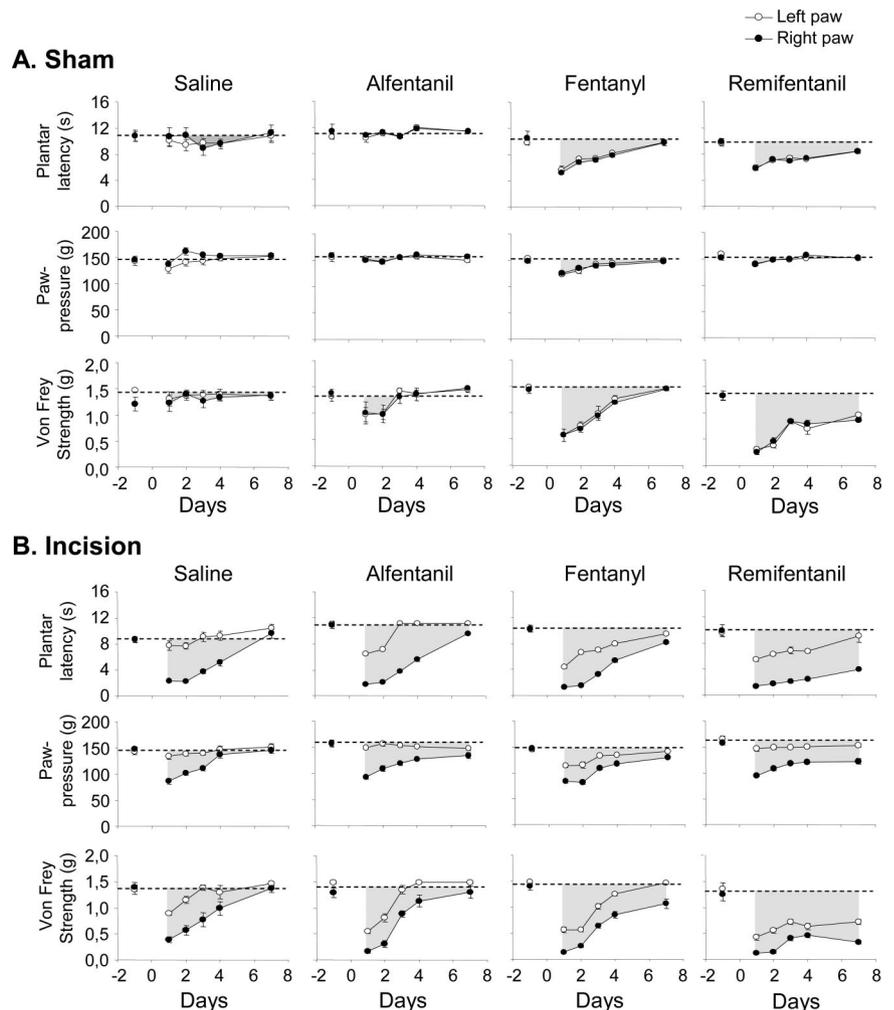
The intraoperative subcutaneous infusion of opioids in mice anesthetized with 1% sevoflurane induced differential delayed pronociceptive effects with the most robust effect observed with remifentanil. Overall, fentanyl produced delayed thermal hyperalgesia, mechanical allodynia, and mechanical hyperalgesia. This was manifested by a significant decrease in nociceptive thresholds below those observed in the saline group in the plantar (a 24% decrease in nociceptive threshold; $P < 0.001$), von Frey (19% decrease; $P < 0.001$), and paw-pressure (6% decrease; $P < 0.05$) tests, respectively. Significant thermal hyperalgesia (26% decrease; $P < 0.001$) and mechanical allodynia (43% decrease; $P < 0.001$) also were observed after remifentanil infusion. However, intraoperative alfentanil administration did not induce statistically significant pronociceptive effects in any of the tests used.

The pronociceptive effects induced by remifentanil were of greater magnitude (see Statistical Analysis for description) than those of fentanyl, but statistically significant differences were observed only in the von Frey test ($P < 0.001$). Regarding extent of the pronociceptive effects, a longer duration was observed for remifentanil in all the tests used. Thus, 28% of the initial thermal hyperalgesia (observed on day 1) and 55% of the mechanical allodynia were still present 7 days after surgery in the remifentanil group ($P < 0.001$ in both tests), whereas these had completely disappeared in the fentanyl group. In these animals, the opioid induced pronociceptive effects were similarly observed in the right and left hind paws in all the tests used.

Effects of the Intraoperative Administration of Opioids on Postincisional Pain

The impact of opioid-induced delayed pronociceptive effects on incisional pain was evaluated using the plantar, paw-pressure, and von Frey tests (fig. 1B and table 1). In saline-treated animals, the plantar incision performed under sevoflurane anesthesia induced significant thermal hyperalgesia (49% decrease in nociceptive threshold; $P < 0.001$), mechanical hyperalgesia (19% decrease; $P < 0.001$), and mechanical allodynia (29% decrease; $P < 0.001$) in the operated paw (fig. 1B, saline) when compared with nonoperated mice. No statistically significant effect was observed in the contralateral paw in any of the tests. Animals completely recovered from the pronociceptive effects of the incision 7 days after surgery in all the tests used.

In operated mice, the overall comparison of the groups that received opioids with those treated with saline showed that opioids enhanced nociception induced by the incision ($P < 0.001$), with remifentanil showing the greatest effect. Fentanyl enhanced the magnitude of the incision-induced mechanical hyperalgesia (a 26% decrease in nociceptive threshold when compared with saline; $P < 0.01$) and mechanical allodynia (41%; $P < 0.01$). Similarly, remifentanil enhanced mechanical hy-

peralgesia (28%; $P < 0.05$) and allodynia (56%; $P < 0.001$) induced by surgery. Incision-induced thermal hyperalgesia was also enhanced by remifentanil (65%; $P < 0.001$), but not by fentanyl. Alfentanil did not significantly modify the magnitude of the pronociceptive effects of the incision in any of the tests used.

The length or duration of postoperative incisional pain also was increased when opioids were infused during surgery. In the remifentanil group, 70% of the initial thermal hyperalgesia observed on day 1 was still present after 7 days ($P < 0.001$). Moreover, 56% and 83% of the initial mechanical hyperalgesia ($P < 0.001$) and mechanical allodynia ($P < 0.001$) also were present on day 7. In the fentanyl group, the remaining pronociceptive effect on day 7 (compared with the effect observed in day 1) was 28% in the plantar test ($P < 0.001$), 24% in the paw pressure test ($P < 0.001$), and 26% in the von Frey test ($P < 0.01$). In the alfentanil group, only the duration of incision-induced mechanical hyperalgesia was significantly increased, with a 37% of the initial thermal hyperalgesia still present on day 7 ($P < 0.05$).

When both factors (the administration of an opioid and the presence of incision) were assessed simultaneously, interaction was present in the plantar test ($P < 0.05$) but not in the paw pressure and von Frey tests. The results suggest that the effects of opioids and those of the incision on thermal hyperalgesia could be synergistic, whereas those observed on mechanical hyperalgesia and allodynia would be merely additive.

In operated animals, fentanyl and remifentanil induced a decrease in nociceptive thresholds in the contralateral paw, which was of the same magnitude than the observed in sham animals. In the alfentanil group, significant thermal hyperalgesia ($P < 0.05$) and mechanical allodynia ($P < 0.001$) was observed during 2 days in the contralateral paw. This finding suggests that the presence of the incision unmasks underlying latent effects of the opioid, which were not observed in basal conditions.

Effects of the Deletion of the iNOS Gene on Opioid-induced Sensitization and Incisional Pain

The implication of the iNOS gene in opioid and incisional-induced pronociceptive effects was investigated in knockout and wild-type mice from the C57/BL6 background (fig. 2 and table 1). In these experiments, we tested only remifentanil because it was the opioid that induced a more pronounced pronociceptive effect both by itself and when administered during surgery.

Comparison between Strains of the Effects of Remifentanil and the Incision (Individually and Combined)

The overall effects of remifentanil and/or surgery were similar in C57/BL6 wild-type (fig. 2A; iNOS$^{+/+}$) and CD1 mice (fig. 1). Thus, no strain differences were observed in the plantar and the paw pressure test (not significant).
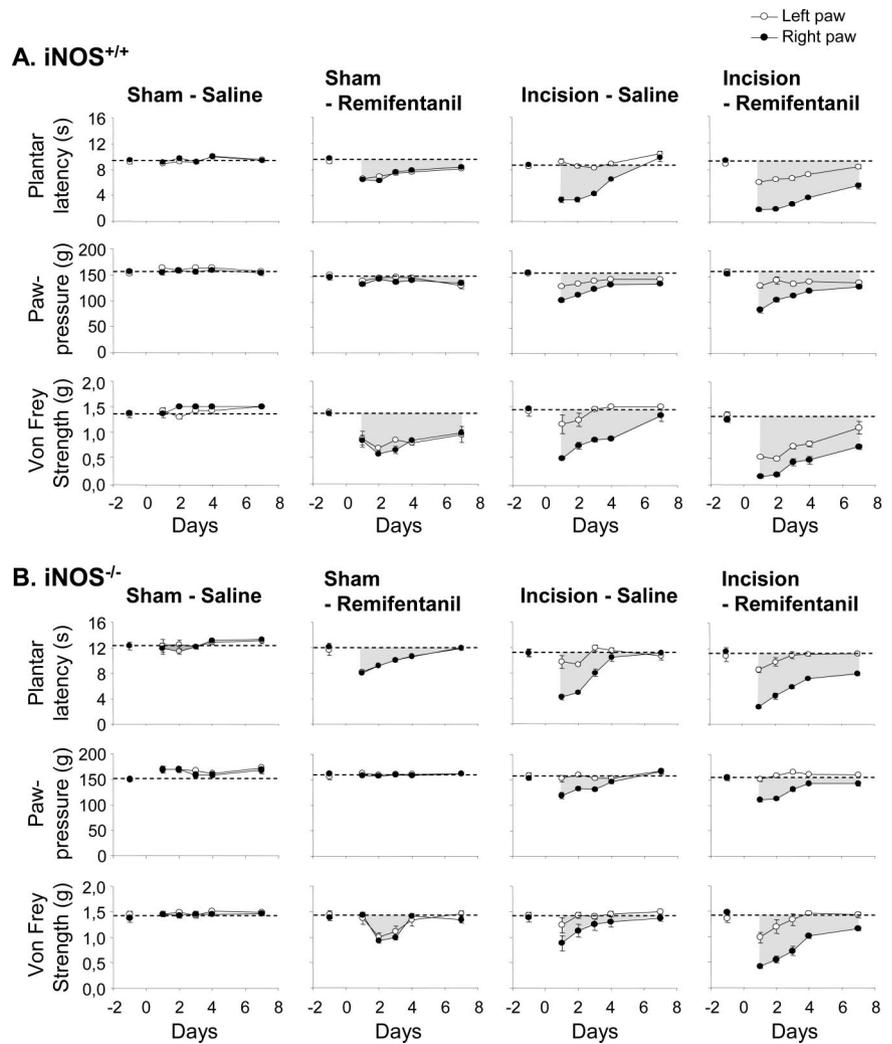
Fig. 2. Time course of the long-lasting effects of opioids on nociceptive thresholds in control conditions and after surgery in (*A*) wild-type (iNOS$^{+/+}$) and (*B*) iNOS knock-out mice (iNOS$^{-/-}$) in a C57/BL6 strain. Nociceptive thresholds of both hind paws were measured in the plantar, paw-pressure, and von Frey tests and are expressed as the mean values ± SEM. Remifentanil (0.04 mg/kg) and saline were infused subcutaneously in the absence or presence of surgery during a period of 30 min. Plantar incision was performed on the right hind paw. Number of mice per group was five.

However, an effect of the strain was found in the von Frey test; the analysis of the results shows that the allodynic effect of remifentanil (administered in the absence of surgery) was less pronounced in C57/BL6 wild-type mice during the first 2 days after infusion (*P* < 0.001).

Comparison of the Effects Induced by Remifentanil and the Surgical Incision (Individually and Combined) in iNOS$^{+/+}$ and iNOS$^{-/-}$ C57/BL6 Mice

In sham animals with iNOS deletion, the infusion of saline in combination with sevoflurane did not produce changes in nociceptive threshold in any of the tests used in the study (mean values, 12.3 s, 150 g, and 1.42 g in the plantar, paw-pressure, and von Frey tests, respectively; fig. 2B and table 1). In these animals, remifentanil induced significant thermal hyperalgesia (18% decrease in nociceptive threshold; *P* < 0.001) and mechanical allodynia (14% decrease; *P* < 0.001) when compared with saline. However, the overall effects were of a lesser magnitude than those observed in wild-type animals (*P* < 0.01). Specifically, the effects of remifentanil in

mutant mice were 50% and 66% lower in the plantar and von Frey tests, respectively, than in wild-type animals (table 1). Remifentanil did not induce mechanical hyperalgesia in knockout or wild-type animals.

In iNOS-mutant mice, the incision alone induced significant thermal (23% decrease in nociceptive threshold; *P* < 0.001) and mechanical hyperalgesia (9% decrease; *P* < 0.001) as well as mechanical allodynia (8% decrease; *P* < 0.001) when compared with saline. The overall effects also were of a lesser magnitude that those observed in wild-type animals (*P* < 0.05). Thus, the effects of remifentanil in mutant mice were 19% lower than those in wild-type mice in the plantar test, 46% lower in the paw pressure test, and 72% lower in the von Frey test (table 1).

In iNOS-mutant mice, the intraoperative infusion of remifentanil enhanced the magnitude of thermal hyperalgesia (43% decrease in nociceptive threshold; *P* < 0.001), mechanical hyperalgesia (15% decrease; *P* < 0.05), and mechanical allodynia (31% decrease; *P* < 0.01) induced by the incision. In these animals, the overall pronociceptive effect of the opioid plus surgery combi-

nation was attenuated in iNOS-mutant mice when compared with wild-type animals ($P < 0.001$ in all tests). Thus, the deletion of the iNOS gene diminished hyperalgesia by 13% and 22% (mechanical and thermal, respectively) and reduced allodynia by 25%.

Discussion

Opioid-induced pain sensitization (manifested by hyperalgesia/allodynia) has been widely documented in nonmanipulated animals and in several models of acute and chronic pain. In our study, we used a murine model of postoperative pain designed to establish whether the intraoperative administration of opioids would alter the nociceptive response to surgery. In this model, we attempted to reproduce the sequence of events occurring during the administration of general (balanced) anesthesia in humans. Thus, surgical anesthesia was maintained with a combination of sevoflurane (1%) and a fixed-rate infusion of an intravenous opioid (fentanyl, remifentanil, or alfentanil). Pain sensitization was evaluated daily for 7 days in the postoperative period. In our experiments, we used a single fixed dose of each opioid, because our aim was to assess the effects of opioids on postsurgical pain qualitatively. Because the pronociceptive effects of opioids have been shown to be dose related,[17] we used high doses of each opioid, which have been reported to have central nervous system effects. We did not attempt to generate dose-response relationships, because the protocol of the study was extremely time consuming and also because the dose range of opioids used in clinical practice is very wide. For fentanyl and remifentanil, the doses were selected on the basis of previous reports published in the literature in rodents showing that the opioids reduce the minimal alveolar concentration of inhalational anesthetics[36] or induce a loss of righting reflex that is predictive of clinical anesthesia.[34] For alfentanil, a wide range of doses have been reported in the literature to induce central effects in rats (range, 45 μg/kg–4 mg/kg), and the dose of 1 mg/kg was selected arbitrarily.[35] Thus, the doses of the opioids used in the study cannot be considered equianalgesic, and consequently, the comparison of the effects between drugs only applies to the specific doses tested.

Our results show that the infusion of μ-opioid agonists induces long-lasting delayed pronociceptive effects when administered in the absence of surgery. When the same opioids were given during surgery as part of a balanced general anesthesia, they significantly enhanced and prolonged incisional pain in the postoperative period for up to 7 days. The results confirm and expand recent findings showing that repeated subcutaneous injections of fentanyl result in exaggerated hyperalgesia after plantar incision in the rat.[9] Although the experimental design used in our study does not allow the

actual comparison between the three opioids, it is worth mentioning that the greater pronociceptive effects and enhancement of incisional pain were observed in animals receiving remifentanil. These results are in agreement with several clinical studies showing that the quality of postoperative pain may depend, at least partially, on the opioid infused during surgery.[42,43] For instance, patients receiving remifentanil during surgery have been reported to experience more pain in the immediate postoperative period and to require higher doses of analgesics in this period.[4,7,44–46]

It has been suggested, that the magnitude of opioid-induced hyperalgesia could be related to the pharmacokinetic characteristics of the drug[7,17] and that pronociception may develop more rapidly with fast-offset drugs like remifentanil than with longer-acting opioids.[7] Remifentanil is the only fentanyl derivate with an ester link that leads to a very rapid breakdown,[47] providing a rapid and predictable recovery, which is relatively independent of the dose and the length of infusion.[4] This fact could explain the more prominent pronociceptive effects observed after remifentanil administration in our study. However, other factors related to drug-specific interactions with the opioid receptors and signal transduction cannot be excluded at present. Moreover, the vehicle where remifentanil is dissolved for clinical use in humans, which is the same that we used in our study, also could play a role. A recent electrophysiological study using slices of rat spinal cord reported that glycine contained in the commercial preparation of remifentanil (Ultiva®; GlaxoSmithKline) directly activates *N*-methyl-D-aspartate receptors and enhances the excitatory effects of the opioid.[48] Because glycine is not present in the alfentanil and fentanyl preparations, the greater pronociceptive effect of remifentanil observed in our model could have a component related to the effect of glycine, an assumption that needs to be tested in further experiments. Glycine also could play a role in the decreased pronociceptive effects of remifentanil observed in iNOS knockout mice. Because nitric oxide is an important regulator of *N*-methyl-D-aspartate channel function in the central nervous system, it could be speculated that decreasing nitric oxide levels may impair the interaction between glycine and the *N*-methyl-D-aspartate receptor. However at present, this hypothesis is not supported by published data.

Opioid-induced thermal hyperalgesia and mechanical allodynia were readily demonstrated in our experimental conditions as compared with mechanical hyperalgesia. In control animals (no opioid), surgery (incision) induced both thermal hyperalgesia and mechanical allodynia as previously described by Pogatzki and Raja.[12] We also observed for the first time the presence of mechanical hyperalgesia in a murine model of incisional pain, a result that supports similar findings in rats.[49] Opioid-induced enhancement of incisional pain was observed

more readily on thermal hyperalgesia and mechanical allodynia than on mechanical hyperalgesia, suggesting that in our model, the effects of opioids increasing postoperative pain is related to their capacity to induce pronociceptive effects in nonoperated animals. Although we are aware that animal data cannot be accurately extrapolated to humans, our findings suggest that opioids may increase postoperative pain in humans and play an important role in the chronification of pain after surgery. Using the same experimental model, we are performing experiments to establish the possible role of intraoperative administration of opioids on the development of chronic pain after surgery.

The possible mechanisms involved in opioid-induced pronociception and incisional pain are not completely established. It has been proposed that the hyperalgesic states after opioid administration could be related to the activation of excitatory or facilitatory pathways, which would decrease the antinociceptive efficacy of opioids.[1,2,9,50,51] However, the development of acute tolerance described in humans after the administration of large doses of short-acting opioids[3,4,7,52] and/or opioid withdrawal after the abrupt interruption of their administration cannot be excluded. Several systems and intracellular pathways have been implicated in opioid-induced pain hypersensitivity, which could underlay the behavioral manifestations, and the glutaminergic/*N*-methyl-D-aspartate receptor systems and associated molecules such as protein kinase Cγ[1,2,14,15] seem to play an important role.

In our study, we show that the deletion of the iNOS gene attenuates both opioid- and incision-induced pronociceptive effects. Moreover, in iNOS mutant mice, remifentanil was still able to enhance incisional pain, but the overall pronociceptive effect was decreased significantly as compared with that in the wild-type mice. This suggests an implication of nitric oxide originated form iNOS in the overall pronociceptive effects observed. Other groups have reported reduced thermal hyperalgesia after zymosan injection[25] and in the carrageenan model[53] in knockout mice for the iNOS gene, supporting a role for iNOS in inflammatory pain. However in these experiments, possible compensatory events related to gene deletion as observed in mice knockout for the neuronal nitric oxide synthase gene[54] cannot be excluded. Additional experiments assessing the effect of selective iNOS inhibitors in this model would be helpful to clarify this point

From our experiments, the role of nitric oxide originated from iNOS at peripheral (wound), spinal, or supraspinal sites cannot be established. At present, considerable controversy exists regarding the role of spinal iNOS in different models of inflammatory pain. Peripheral inflammation upregulates the expression of neuronal nitric oxide synthase and induces the expression of iNOS in the spinal cord.[25,55-59] Also, interleukin 1β, a proinflammatory cytokine implicated in inflammatory and neuropathic pain, induced thermal hyperalgesia by activating the iNOS-nitric oxide cascade in the rat spinal cord.[27] However, pharmacological studies conducted in the incisional model of postoperative pain in rats show that the spinal nitric oxide system does not play a role in pain after incision. Indeed, the intrathecal administration of a nonselective antagonist of nitric oxide synthase (L-NAME) did not attenuate pain behavior after incision.[60] These experiments suggest that peripheral or supraspinal iNOS could be involved in postsurgical pain after incision. In fact, it has been shown that the peripheral perfusion of specific iNOS antagonists in the glabrous skin of the rat hind paw blocks the local release of nitric oxide induced by carrageenan.[61] Moreover, iNOS expression has been shown to be induced in inflammatory cells in the early phases of wound healing in different animal models, although its role is controversial at present.[62] These and other studies suggest that peripheral iNOS may play an important role in incisional pain.

Regarding opioid-induced pronociception, our study reports for the first time in the literature a possible implication of the iNOS enzyme in this effect, but the possible mechanisms explaining how the activation of the iNOS could be initiated after opioid administration should be further explored. The glutamate/*N*-methyl-D-aspartate receptor system may represent a possible link, because the hyperexcitability of *N*-methyl-D-aspartate receptors observed during inflammation seems to be related to the increased levels of nitric oxide and activation of its signaling pathways in the spinal cord.[55,63-65] In addition, *N*-methyl-D-aspartate receptor antagonists have been shown to block opioid-induced pronociceptive effects, acute and chronic tolerance to the analgesic effects of opioids, as well as opioid-induced exacerbation of preexisting pain in a wide variety of animal models, including the incisional pain model in the rat.[1,2,9,15] From these studies, we speculate that in the incisional pain model, peripheral iNOS plays an important role in postoperative hyperalgesia, whereas central nervous system iNOS is implicated in the pronociceptive effects of opioids.

In conclusion, although opioids provide adequate analgesia in patients with severe pain, their use as anesthetics during surgery may contribute to the development of neuronal plasticity underlying pain sensitization in the postoperative period. Our results corroborate clinical observations showing that relatively large dose of intraoperative remifentanil increase postoperative pain and morphine consumption in humans and support the use of a murine model to evaluate their effects on postoperative pain. Our study also suggest a role for iNOS in the cause of both acute inflammatory pain and opioid-induced hyperalgesia, suggesting that nitric oxide originating from iNOS plays an important role in pain sensitization.

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