Peripheral Morphine Administration Blocks the Development of Hyperalgesia and Allodynia after Bone Damage in the Rat

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Background: The current study aimed to assess whether local administration of morphine could block the development of hyperalgesia and allodynia in a rat model of osteotomy or bone damage.

Methods: Withdrawal responses to mechanical and thermal stimuli applied to the plantar surface of the hind paw were measured before and after bone damage. The bone was injured by drilling a 1-mm hole through the tibia during short-lasting general anesthesia. In separate groups of rats, the effects of administering morphine (20–80 μg), either into the marrow cavity or systemically, on the development of hyperalgesia and allodynia after bone damage were assessed. In an additional group of rats, a selective μ-opioid receptor antagonist, clonixinamox (0.15 mg), was administered into the marrow cavity before the administration of morphine (40 μg).

Results: In animals that received no drug treatment, hyperalgesia and allodynia peaked 2 h after injury. Injection of morphine (40 and 80 μg) into the marrow cavity immediately after bone injury prevented the development of hyperalgesia and allodynia. Cocoinamox (0.15 mg) injected into the marrow cavity before administration of morphine blocked the antihyperalgesic effect of morphine.

Conclusion: This study shows that local application of a low dose of morphine effectively blocks the development of hyperalgesia and allodynia in a rat model of bone damage through μ-opioid receptor action. These findings provide further evidence that local application of morphine at the time of orthopedic surgery, bone graft, or bone marrow harvesting may reduce the amount of postoperative pain. (Key words: Opioid receptors; osteotomy; pain.)

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Materials and Methods

General

All experiments were approved by the Institutional Animal Care and Use Committee. The animals were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain issued by the International Association for the Study of Pain.

Eighty adult male Sprague-Dawley rats were used in this study (weight, 220–420 g). The animals were
housed in groups of three in conventional Lucite cages with soft bedding with a 14-h light/10-h dark cycle. At the end of the protocol, the rats were killed with an overdose of pentobarbital (100 mg/g given intraperitoneally).

**Bone Injury**
Seventy-two rats were anesthetized with a short-lasting anesthetic agent, sodium methohexitol (60 mg/kg given intraperitoneally). The lateral side of the tibia was palpated, and a hand drill (drill bit diameter, 1 mm; Plastic One, Inc., Roanoke, VA) was used to bore a hole through the skin, muscle, and both laminae of the tibia at a site 3 mm below the patella (fig. 1A). Care was taken not to injure soft tissue other than that overlying the puncture site.

**Behavioral Testing**
Each animal was tested for responses to mechanical and cooling stimuli. The mechanical stimuli were applied before the cooling stimulus.

**Foot Withdrawal to Graded Mechanical Stimuli.**
To quantify mechanical sensitivity of the foot, frequency of foot withdrawals in response to graded mechanical stimuli were measured. Von Frey filaments with calibrated bending forces (10, 30, and 90 mN) were used to deliver punctate mechanical stimuli. The thin, nylon von Frey filaments were calibrated (mN) using an electronic balance. The rats were housed in small Lucite cubicles (20 × 8 × 8 cm) on an elevated metal wire mesh floor, and von Frey filaments were applied from underneath the wire mesh to the plantar surface of the foot. The filaments were placed on the skin overlying the head of the metatarsal bone, including the tori (fig. 1B), because it has been demonstrated that stimulation in this region results in a strong limb withdrawal. Each filament was applied 10 times, perpendicularly to the paw, until it started to bend for ≈1 s, with an interstimulus interval of ≈5 s. The number of brisk paw withdrawals was counted and expressed as the percent response frequency, e.g., 2 paw withdrawals observed of 10 filament applications was expressed as a 20% response frequency. The times chosen for von Frey testing were based on a preliminary investigation to coincide with maximum hyperalgesia or allodynia. Baseline (control) behavioral responses were measured immediately before any treatments (0 h) and then at 2, 3, and 4 h after the treatment.

**Foot Withdrawal to a Cold Stimulus.** A drop of acetone (80%) was delivered to the plantar surface of each hind paw using a 1-ml syringe without a needle attached. Acetone was applied three times to each paw with 5-min intervals between applications. As the acetone evaporated, there was rapid cooling of the skin. In rats with cold allodynia, foot withdrawal was observed in response to this stimulus. Again, the number of brisk paw withdrawals was counted and expressed as the percent response frequency. Acetone was applied immediately before any treatment (0 h) and 2, 3, and 4 h after the treatment.
Experimental Protocols

Control, Bone Damage Only. Eight rats were used to establish the effect of drilling a hole through the bone on the withdrawal responses to mechanical and cold stimuli. The withdrawal responses to the graded von Frey filaments and acetone were established before a hole was drilled through the tibia. Withdrawal responses to each stimulus were then measured at 2, 3, and 4 h after the bone was damaged.

Administration of Morphine. Twenty-four rats were pretreated (baseline, 0 h) to measure the withdrawal responses to von Frey filaments and acetone as described previously. The hole was drilled in the tibia, and a Hamilton syringe needle was advanced into the marrow cavity via this hole. Morphine in doses of 20, 40, or 80 µg (2, 4, or 8 µl) was then administered directly into the marrow cavity (n = 8 per group; fig. 1A). Withdrawal responses to the von Frey filaments and acetone were then measured at 2, 3, and 4 h after injection of morphine.

Intramuscular Morphine Control. Because it was possible that morphine may have leaked from the marrow cavity and acted locally on opioid receptors in the muscle of the hind limb, a separate group of animals (n = 8) was used to establish whether low doses of morphine acting at opioid receptors in the muscle tissue could inhibit hind limb withdrawal responses. Withdrawal responses to the graded von Frey filaments and acetone were measured before morphine (80 µg) was injected into the biceps femoris muscle of the hind limb. Again, withdrawal responses were measured at 2, 3, and 4 h after injection of morphine.

Systemic Morphine Control. To control for any leakage of morphine into the systemic circulation, and therefore the central nervous system, 24 additional rats were used to measure the effect of the same doses of morphine applied systemically. The animals were pretreated with von Frey filaments and acetone before a hole was drilled through the tibia, and the morphine was then administered (20, 40, and 80 µg given intraperitoneally; n = 8 per group). Withdrawal responses to the same stimuli were tested and compared with control animals and animals given injections of morphine into bone marrow.

Further, to compare effective doses of morphine administered interosseously and intraperitoneally, additional intraperitoneal doses of morphine were used. Sixteen animals were pretreated with von Frey filaments and acetone before a hole was drilled through the tibia, and the morphine was administered intraperitoneally (160, 320, 640, and 1,280 µg; n = 4 per group). Withdrawal responses to the same stimuli were tested.

Administration of Clofamine. To establish whether the effects of morphine were mediated via µ-opioid receptors, withdrawal responses were measured (n = 8), and then a hole was drilled through the tibia 3 mm below the patella. An irreversible µ-opioid antagonist, clofamine, was dissolved in 20% dimethyl sulfoxide (DMSO; 0.15 mg; 10 µl) and then injected into the marrow cavity using a 10-µl Hamilton syringe. After 5 min, morphine (40 µg) also was injected into the marrow cavity. Withdrawal responses were measured at 2, 3, and 4 h after the drugs were injected.

Clofamine Only. In a separate group of rats (n = 8), clofamine (0.15 mg; 10 µl) was administered after a hole was drilled through the tibia without subsequent administration of morphine to assess the effects of clofamine alone and to determine whether endogenous opioid peptides were involved in determining the level of hyperalgesia after bone injury. Withdrawal responses were measured at the same time points (0, 2, 3, and 4 h).

Vehicle Control. Because clofamine was dissolved in 20% DMSO, another group of rats (n = 8) was used to establish if this vehicle had any effect on the withdrawal responses. The rats were pretreated with the von Frey filaments and acetone before a hole was drilled through the tibia, during anesthesia with sodium methohexitol. Dimethyl sulfoxide (10 µl) was then injected into the marrow cavity, and withdrawal responses were measured at 2, 3, and 4 h after injection of DMSO.

Drugs
Preservative-free morphine sulfate (10 mg/ml) was purchased from Astra USA, Inc. (Westborough, MA). Clofamine mesylate was purchased from Tocris Cookson (St. Louis, MO) and dissolved in 20% DMSO (Fisher Scientific, Fair Lawn, NJ).

Statistical Analysis
The results of the behavioral testing were not normally distributed (Kolmogorov-Smirnov test) and thus were analyzed nonparametrically. To assess whether the withdrawal responses changed over time, Friedman's test was used. When Friedman's test was significant (P < 0.05), pairwise comparisons were made using Wilcoxon's signed rank test. When between-group comparisons were made at certain time points, a nonparametric analysis of variance (ANOVA) procedure
LOCAL ADMINISTRATION OF MORPHINE REDUCES BONE PAIN

(Kruskal-Wallis) was used first, followed by a Mann-Whitney U test for pairwise comparisons when the ANOVA results were significant ($P < 0.05$). Data are expressed as medians and ranges in the text and plotted as vertical boxes with error bars showing median, 10th, 25th, 75th, and 90th percentiles in the figures. Statistical tests were performed using Statistica software (Jandel Corporation, San Rafael, CA).

Results

Bone Damage

There was no visible bruising or swelling after a hole was drilled through the tibia. Postmortem examination of the tibia revealed a discrete hole ≈ 1 mm in diameter. Drilling a hole through the tibia resulted in the development of secondary mechanical hyperalgesia and allodynia and cold allodynia (fig. 2). Drilling the tibia caused a statistically significant increase in the incidence of withdrawal reactions to the 10-, 30-, and 90-mN filaments (Freidman’s ANOVA, $P < 0.02$, 0.01, and 0.03, respectively; fig. 2A–C). The response to acetone applied to the plantar surface of the paw was also significantly increased after a hole was drilled through the tibia (Freidman’s ANOVA, $P < 0.01$; fig. 2D).

Morphine Administered into the Marrow Cavity

Low doses of morphine (40 and 80 µg) administered directly into the marrow cavity, at the time the hole was drilled through the tibia, blocked the development of mechanical hyperalgesia and allodynia and cold allodynia (fig. 3, left). When the withdrawal responses to graded von Frey filaments were compared, 2 h after the tibia was drilled, it was found that of the four treatment groups (control and 20, 40, and 80 µg morphine) there was a significant difference (Mann-Whitney U test, $P < 0.02$) in the withdrawal responses of the rats that received 40 or 80 µg morphine compared with the rats that received no morphine (control). In contrast, the 20-µg dose of morphine had no significant effect on the development of hyperalgesia and allodynia after the bone was injured.

Intramuscular Morphine

Morphine (80 µg given intramuscularly) administered to the muscle tissue adjacent to the bone hole in healthy rats did not alter the withdrawal responses to the graded von Frey filaments or to the acetone applied to the plantar surface of the paw (data not shown). For example, before the morphine was injected, the 90-mN filament evoked withdrawal responses to a median of 30% (range, 10–60%) of the presentations. Two hours after the morphine was injected intramuscularly, the same filament evoked withdrawal responses to a median of 25% (range, 0–50%) of the presentations.

Intraperitoneally Administered Morphine

When injected into the abdomen, none of the low doses of morphine (20, 40, or 80 µg) affected the development of mechanical hyperalgesia or allodynia (fig. 3, right) or cold allodynia that normally develop in this model of bone injury (fig. 4, right). For example, 2 h after a hole was drilled through the tibia, there was no difference between the withdrawal responses evoked by the graded von Frey filaments in rats given no morphine (control rats, fig. 3, right) and those evoked in rats given 80 µg morphine intraperitoneally (fig. 3, right). Of the higher doses tested (160, 320, 640, and 1,280 µg; data not shown), only the 1,280-µg dose was effective in reducing the mechanical hyperalgesia and allodynia. This dose is similar to effective systemic doses for morphine published in the literature. For example, the 90-mN filament evoked withdrawal responses to a median of 45% of the presentations in the control but only to a median of 5% of the presentations (range, 0–10%) 2 h after intraperitoneal administration of 1,280 µg morphine. This was a statistically significant difference (Mann-Whitney U test, $P < 0.02$).

Administration of the µ-Opioid Antagonist, Clonixinamox, into the Marrow Cavity before Morphine

Secondary mechanical hyperalgesia and allodynia was again present after bone injury in rats in which the irreversible µ-opioid receptor antagonist, clonixinamox (0.15 mg), was administered into the marrow cavity before morphine (40 µg; fig. 5). For example, a statistically significant increase (Wilcoxon’s signed rank test, $P < 0.02$) in the withdrawal responses to the 30-mN filament was observed 2 h after bone injury and administration of clonixinamox and morphine (fig. 5B). Cold allodynia also was observed in these rats after bone injury (fig. 5D).

When the withdrawal responses from control rats, rats given 40 µg morphine injected into the marrow cavity, and rats given 0.15 mg clonixinamox followed by 40 µg morphine injected into the marrow cavity

Anesthesiology, V 89, No 1, Jul 1998
Fig. 2. Percent withdrawal responses evoked by three strengths of von Frey filament. Filled bars represent (A) 10, (B) 30, and (C) 90 mN filaments. (D) Open bars represent the number of responses to acetone application of three trials (80%), applied to the plantar surface of the paw, before (baseline) and after a hole was drilled through the tibia. The data are plotted as vertical boxes with error bars showing the median and the 10th, 25th, 75th, and 90th percentiles (n = 8). *Significant difference (Wilcoxon’s signed rank test, P < 0.05) from values obtained before the bone was damaged (baseline).

Fig. 3. Effect of morphine (0 [control], 20, 40, and 80 μg) administered into the marrow cavity (filled bars, left) or intraperitoneally (open bars, right) on the withdrawal responses evoked by three strengths of von Frey filament (10, 30, and 90 mN) applied to the plantar surface of the paw 2 h after bone damage. The data are plotted as vertical boxes (25th–75th percentile) with bars showing the median and the 10th and 90th percentiles (n = 8). *Significant difference (Mann-Whitney U test, P < 0.05) from the responses recorded in rats without administration of morphine (control). +Significant difference (Mann-Whitney U test, P < 0.05) between bone marrow and systemic administration.
LOCAL ADMINISTRATION OF MORPHINE REDUCES BONE PAIN

A. 10 mN

B. 30 mN

C. 90 mN

Withdrawal response (%)

Dose of morphine (µg)

control 20 40 80

control 20 40 80

control 20 40 80

Anesthesiology, V 89, No 1, Jul 1998
were compared, it was found that the cloccinamox blocked the anti-hyperalgesic actions of morphine (Kruskal-Wallis, \( P < 0.04 \); fig. 6). That is, the withdrawal responses recorded after administration of cloccinamox and morphine were comparable to, and not significantly different from, those recorded after bone injury in rats in which no drugs were administered (control). The response evoked by the 90-mN filament 2 h after bone damage in rats injected with 40 \( \mu \)g morphine into the marrow cavity was significantly less than the response evoked by this same filament in rats with no drug treatment (control rats; Mann-Whitney U test, \( P < 0.015 \); fig. 6C). This protective effect was blocked in rats injected with 0.15 mg cloccinamox before administration of 40 \( \mu \)g morphine into the marrow cavity. The response evoked by the 90-mN filament 2 h after bone damage in rats injected with both cloccinamox and morphine was also significantly greater than the response evoked by this same filament in rats injected with 40 \( \mu \)g morphine only (Mann-Whitney U test, \( P < 0.02 \); fig. 6C).

Cloccinamox had no effect on the amount of hyperalgesia and allodynia that developed after bone injury. In rats in which cloccinamox was administered without subsequent administration of morphine, there was a statistically significant increase in the incidence of withdrawal reactions to the 10-, 30-, and 90-mN filaments (Freidman's ANOVA, \( P < 0.01 \), 0.01, and 0.04, respectively; data not shown). There was no significant difference, however, when comparisons were made between the increased withdrawal responses of the cloccinamox-only group and the increased responses observed in rats with bone damage and no drug administration.

**Dimethyl Sulfoxide Vehicle Injected into the Marrow Cavity**

It was noted that the vehicle, DMSO, in which cloccinamox was dissolved did not have any significant effect on the development of mechanical hyperalgesia or allodynia or cold allodynia after bone injury (data not shown). For example, an increase in withdrawal responses to the 10-mN filament was observed 2 h after a hole was drilled through the tibia and DMSO injected into the marrow cavity. The response observed at 2 h was significantly different from the baseline response (Wilcoxon's signed rank test, \( P < 0.02 \)). The responses observed 2 h after bone damage in the DMSO treatment group, however, were not significantly different from the withdrawal responses evoked in rats 2 h after a hole was drilled but in which no drugs were administered (Mann-Whitney U test, \( P > 0.40 \)).
LOCAL ADMINISTRATION OF MORPHINE REDUCES BONE PAIN

A. 10 mN

B. 30 mN

C. 90 mN

D. acetone

Fig. 5. Effect of clocinnamox (0.15 mg) followed by morphine (40 μg) administered into the marrow cavity on the withdrawal responses evoked by three strengths of von Frey filament. Filled bars represent (A) 10-, (B) 30-, and (C) 90-mN filaments. (D) Open bars represent the three trials of acetone application (80%), applied to the plantar surface of the paw, before (baseline) and after a hole was drilled through the tibia. The data are plotted as vertical boxes (25th–75th percentiles) with bars showing the median and the 10th and 90th percentiles (n = 8). *Significant difference (Wilcoxon’s signed rank test, P < 0.05) from values obtained before drilling (baseline).

Discussion

In the current study, it was found that the development of mechanical hyperalgesia and allodynia after drilling a hole in bone can be prevented by application of low doses of the opiate, morphine, to the site of damage. This effect is probably mediated by μ-opioid receptors located in the bone, as the effect of morphine can be blocked by the irreversible μ-opioid receptor antagonist, clocinnamox, injected into the marrow cavity.

In the current study, secondary mechanical hyperalgesia and allodynia were measured using a range of graded, calibrated von Frey filaments applied repeatedly.

Anesthesiology, V 89, No 1, Jul 1998
Fig. 6. Effect of no drug (control), morphine (40 μg), and clocinnamox (0.15 mg) followed by morphine (40 μg) injected into the marrow cavity on the withdrawal responses evoked by three strengths of von Frey filament. Filled bars represent (A) 10-, (B) 30-, and (C) 90-mN filaments. (D) Open bars represent the response evoked by acetone (80%), applied to the plantar surface of the paw 2 h after osteotomy. The data are plotted as vertical boxes (25th–75th percentile) with bars showing the median and the 10th and 90th percentiles (n = 8). *Significant difference (Mann-Whitney U test, P < 0.05) from the responses recorded in rats without administration of morphine (control). + Significant difference (Mann-Whitney U test, P < 0.05) from the responses recorded in rats with morphine (40 μg) injected into the marrow cavity.

to a site distant to the injured tissue. The range of filament strengths chosen for the study was such that the two lower strength filaments (10 and 30 mN) rarely evoked a withdrawal reflex, whereas the highest strength von Frey filament (90 mN) commonly evoked a brisk withdrawal reflex response. Lecm and colleagues demonstrated that the 10-mN filament used in the current study does not activate A or C nociceptors, whereas a stimulus strength of 90 mN activates A and C nociceptors. Therefore, increases in the withdrawal responses to the 10- and 90-mN filaments can be described as allodynia and hyperalgesia, respectively, because allodynia is defined as a response to a stimulus that does not normally evoke pain and hyperalgesia as

Anesthesiology, V 89, No 1, Jul 1998
an increased response to a stimulus that previously was normally painful. Because the 30-nM filament is close to the strength of fiber thought to activate nociceptors, however, repeated application of this filament may result in activation of nociceptors via temporal summation. Therefore, it is more difficult to classify increased responses to this filament strength as either mechanical allodynia or secondary mechanical hyperalgesia. Von Frey filaments have been used to study mechanical hyperalgesia and allodynia in other experimental pain models and have been shown to produce reliable results. The rats' paw withdrawal frequency to acetone, as a measure of cold allodynia, was also tested before and after bone injury, because this is a stimulus that activates only receptors in the pain sensitivity range.

After injury, there was an increased withdrawal of the paw to the mechanical stimuli (mechanical allodynia and secondary mechanical hyperalgesia) and the application of acetone (cold allodynia), which often was accompanied by more complex pain-related behaviors, including licking of the paw or the damaged limb and continued elevation of the paw for prolonged periods, indicating an organized, voluntary, purposeful act requiring supraspinal sensory processing. It was shown in a previous study that the administration of the anesthetic agent sodium methohexital alone, or puncture of the tissue overlying the bone without damage to the bone, does not result in hyperalgesia or alldynia. Therefore, the changes in paw withdrawal responses after bone damage are presumably attributable to events occurring at the site of bone damage and not to damage of the skin or muscle overlying the bone.

Secondary hyperalgesia and allodynia are mediated by changes within the central nervous system as a result of increased afferent barrage from the site of injury; however, allodynia arises from a change in the modality of the sensation evoked by low-threshold mechanoreceptors from touch to pain, whereas mechanical hyperalgesia is an increase in the magnitude of the pain sensations evoked by mechanically sensitive nociceptors. The neurophysiologic mechanisms of secondary hyperalgesia and allodynia are not fully understood and warrant further investigation. The development of hyperalgesia and allodynia after bone injury in rats is consistent with clinical observations in which patients report pain after injury or surgery to their bones.

The opiate morphine was used in the current study because it is commonly used for pain relief in patients. It was found that a low dose of morphine administered directly into the marrow cavity could block the development of mechanical hyperalgesia and allodynia and cold allodynia. The same dose of morphine, however, administered to the adjacent muscle or systemically (in this study, intraperitoneally) affected neither the development of secondary mechanical hyperalgesia nor mechanical and cold allodynia. Further, the action of morphine injected into the marrow cavity was blocked by pretreatment with the irreversible μ-opioid receptor antagonist naltrexone. Therefore, it can be concluded that the morphine administered into the marrow cavity after the bone was drilled was acting on local μ-opioid receptors. Historically, opioid analgesia has been associated with the activation of opioid receptors in the central nervous system. Recently, however, several studies have demonstrated that opioid agonists can elicit pronounced antinociceptive effects via activation of opioid receptors in peripheral tissues. Such effects have been shown to occur primarily in inflamed tissue, in animal studies and in the clinical setting in patients, but also have been described recently in another rat model of pain, in which inflammation was not present. Further, opioid receptors are present in peripheral afferents. Although the presence of opioid receptors has not been demonstrated in bone, it has been known for some time that bone is innervated by myelinated and unmyelinated nerve fibers that are sensory and autonomic in nature, including nociceptors. Nerve fibers are abundant in the bone marrow and are thought to convey sensory information, because deep pain is often described by patients when the bone marrow is punctured. The nerves innervating bone are branches of the nerves that innervate muscle and skin. Therefore, it is conceivable based on the current study that opioid receptors are also present on the afferent fibers that are present in the bone marrow, and activation of these receptors by morphine prevents the cascade of events that results in hyperalgesia and allodynia.

Morphine did not affect the responses to the graded mechanical stimuli in healthy animals when injected into the biceps femoris of the hind limb. Because disruption of the perineurium is thought to be important for the analgesic action of opioids in the periphery, the current study suggests that μ-opioid receptors on nerves disrupted within the bone are responsible for generation of the nociceptive changes induced in this model. This study demonstrated that low doses of morphine, when applied directly to the site of bone damage, can
block the development of hyperalgesia and allodynia because the effective dose locally was at least an order of magnitude less than the effective systemic dose. This study provides further support for the efficacy of locally administered opioids at the time of bone damage and the concomitant reduction in postoperative analgesic administration. Further work needs to be undertaken, however, to assess whether the local administration of opioid peptides could cause a greater risk of infection after orthopedic surgery, which often involves the insertion of foreign objects (e.g., screws, plates) because of suppression of the immune system.14

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Anesthesiology, V 89, No 1, Jul 1998