

# Intrathecal Ketorolac Reverses Hypersensitivity following Acute Fentanyl Exposure

Yoo-Jin Kang, M.D.,\* Michelle Vincler, Ph.D.,† Xinhui Li, Ph.D.,‡ Dawn Conklin, B.A.,§, James C. Eisenach, M.D.||

OPIOIDS remain the mainstay for the treatment of acute and chronic severe pain, but their side effects often limit their utility. Besides well-known side effects such as respiratory depression and nausea, opioid therapy can also be followed by *increased* perception of pain, both to normally painful stimuli (hyperalgesia) and to normally innocuous ones (allodynia). This has been observed following the administration of chronic systemic or intrathecal opioids,<sup>1,2</sup> and as part of withdrawal symptoms from cessation of opioids after chronic use.<sup>3</sup> In addition, acute heroin administration in animals causes antinociception for a few hours, followed by several days of hypersensitivity.<sup>4</sup> Fentanyl also causes this delayed hypersensitivity in animals,<sup>5</sup> and clinical observations that high dose intraoperative opioid exposure is associated with increased postoperative pain and opioid use,<sup>6,7</sup> led us to speculate that acute opioid-induced hyperalgesia may be important in anesthesia practice.<sup>8</sup>

Opioid receptor stimulation increases glutamate synaptic effectiveness at *N*-methyl-D-aspartate (NMDA) receptors,<sup>9</sup> and delayed hypersensitivity after acute fentanyl exposure is blocked by administration of NMDA antagonists.<sup>5</sup> In addition,<sup>10</sup> intrathecal injection of a cyclo-oxygenase (COX) inhibitor decreases hypersensitivity during withdrawal from chronic opioids in rats,<sup>11</sup> suggesting a role for spinal prostaglandin synthesis in hypersensitivity associated with opioid therapy. We have completed animal toxicology screening and are examining in humans the safety of intrathecal injection of ketorolac, a selective COX-1 inhibitor.<sup>12</sup> The purpose of the current study was to determine whether intrathecal injection of ketorolac would reverse delayed hypersensitivity following acute opioid exposure. Expression of COX enzymes in the lumbar segment of the spinal cord were also

studied. Finally, since acute opioid-induced hypersensitivity has only been examined using one stimulus modality (paw pressure to the end-point of vocalization)<sup>5</sup> we further characterized hypersensitivity to other mechanical stimuli and to thermal testing.

## Materials and Methods

### Animal Preparation and Fentanyl Administration

Male Harlan Sprague-Dawley rats weighing 225-275 g were used, and all procedures were approved by the Animal Care and Use Committee. For intrathecal drug administration animals were anesthetized with halothane and a 32-gauge polyurethane catheter was inserted through a puncture of the atlanto-occipital membrane as previously described<sup>13</sup> and advanced caudally so that the tip of the catheter was at the level of the lumbar enlargement. Animals that showed neurologic deficits were excluded from the study and euthanized immediately. After surgery, animals were housed individually and allowed to recover for 1 to 2 weeks.

To induce hyperalgesia, fentanyl, 80 µg/kg, was injected subcutaneously four times at 15 min intervals resulting in a total dose of 320 µg/kg, which has been reported to produce a near maximal delayed hypersensitivity.<sup>5</sup> Animals were housed in an enclosed Plexiglas box with oxygen flow at greater than 2 l/min during injections and for 1 to 2 h thereafter.

### Behavioral Tests

Three types of nociceptive tests were used, all measuring a withdrawal threshold. For thermal testing we used a previously described method<sup>14</sup> in which animals were acclimated in a Plexiglas box on a glass surface maintained at 30°C. A lamp was positioned under the hind paw, and when activated, focused light and radiant heat on the surface of the glass under the paw. Latency to withdrawal was determined before fentanyl exposure, and lamp intensity was adjusted to result in withdrawal with a latency of 10-15 s. Animals were tested 1, 2, and 4 days after fentanyl or saline exposure using the same lamp intensity as before drug injection. A cutoff of 30 s was not exceeded to avoid tissue injury. For mechanical testing, we used two methods. First, we used a commercially available device (Analgesymeter, Ugo Basile, Rome, Italy) to apply increasing pressure on a hind paw of the rat until paw withdrawal. A cutoff of 250 g was not exceeded to avoid tissue injury. Second, we used punctate stimulation with von Frey filaments. For this, rats

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\* Visiting Research Scholar, † Post-Doctoral Fellow, ‡ Assistant Professor of Anesthesiology, § Laboratory Technician, || FM James, III Professor of Anesthesiology.

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Address reprint requests to Dr. Eisenach: Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina, 27157. Address electronic mail to: eisenach@wfbmc.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

were placed in a Plexiglas box over a smooth mesh surface and allowed to acclimate for 30 min. A series of calibrated, hand made von Frey filaments (0.9–27.9 g), all with the same diameter, were applied perpendicularly to the plantar surface of the left paw with a force to bend the filament for 5 s. Filaments of increasing force were applied until the rat withdrew its paw. Two minutes later, a filament of the next lesser force was applied, and threshold determined by the up-down method previously described.<sup>15</sup> As with thermal tests, mechanical tests were performed before and 1, 2, and 4 days after subcutaneous injections. Six rats were tested with both thermal and von Frey methods, and six were tested for paw pressure.

#### *Ketorolac Treatment*

Preliminary experiments demonstrated that after fentanyl exposure animals achieved cutoff levels of thermal mechanical stimulation for at least 3 h after injection, and had a maximal hypersensitivity to mechanical testing 1 day after fentanyl exposure. On the first day after fentanyl exposure, animals were randomized to receive intrathecal ketorolac, 5, 15, or 50  $\mu$ g, with von Frey filament testing before and at 30 min intervals for 2 h after intrathecal injection ( $n = 6$  per group). The investigator was blinded to the ketorolac dose.

#### *Immunocytochemistry*

Rats were deeply anesthetized with pentobarbital and perfused pericardially with buffer (0.01 M phosphate buffered saline + 1% sodium nitrite, 100 ml) followed by 4% paraformaldehyde (400 ml) either 24 or 96 h after fentanyl administration ( $n = 4$  at each time period). The L4–L6 portion of the spinal cord was extracted and submerged in 4% paraformaldehyde for 2 to 3 h followed by postfixation in 30% sucrose for 48–72 h at 4°C. Tissue was imbedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA) and cut transversely into 40  $\mu$ m sections on a cryostat.

Immunocytochemistry was performed on free-floating sections using standard biotin-streptavidin techniques. After 4 washes with 0.01 M phosphate buffered saline + 0.15% Triton 100X (PBS + T), sections were incubated in 0.3% hydrogen peroxide for 15 min. Sections were washed 4 times with PBS + T, incubated with 50% alcohol (45 min), washed 4 times with PBS + T and blocked with 1.5% normal serum. Section were incubated in primary antibody, COX-1 monoclonal (1:1000; Cayman Chemicals, Ann Arbor, MI) or COX-2 polyclonal (1:5000; Cayman Chemicals), 24–48 h at 4°C. Sections were washed 4 times with PBS + T then incubated for 1 h with biotinylated secondary antibodies (1:200) and finally with horseradish peroxidase (HRP) conjugated tertiary antibody (1:100). Antibodies were visualized using the glucose-nickel-diaminobenzidine method. Images were captured on a light microscope at 10 $\times$  magnifica-

tion. Positively labeled cells were identified for automated counting using SigmaScan Pro 5 (Jandel Scientific, Carlsbad, CA) at a preset intensity threshold. Labeling was examined in a standardized area of the outer laminae (I-II) with 6–10 slices examined per animal.

#### *Drugs*

The following drugs were used: fentanyl citrate (Abbott Laboratories, Chicago, IL), and ketorolac tromethamine (Allergan, Irvine, CA). Ketorolac was diluted with normal saline and injected intrathecally in a volume of 10  $\mu$ l over 30 s followed by 15  $\mu$ l saline flush.

#### *Statistics*

Data are presented as mean  $\pm$  SE. Behavioral data were analyzed by either one-way or two-way repeated measures analysis of variance (ANOVA), followed by Dunnett test. Quantification of COX isoenzymes was compared by one-way ANOVA followed by Dunnett test.  $P < 0.05$  was considered significant.

## **Results**

#### *Behavioral Characterization of Fentanyl-Induced Hypersensitivity*

Fentanyl, 320  $\mu$ g/kg, first caused antinociception, then reduced withdrawal threshold to both measures of mechanical testing, but did not affect withdrawal threshold to heat (fig. 1). Hypersensitivity to mechanical testing was maximum on the first day after fentanyl exposure, and was still present to punctate, but not pressure testing 4 days after exposure (fig. 1). Hypersensitivity was greater to von Frey testing than to paw pressure testing, when expressed as percent reduction (57% *vs.* 26%), but not when expressed as reduction in multiples of the SD of the baseline (3.1-fold in both cases).

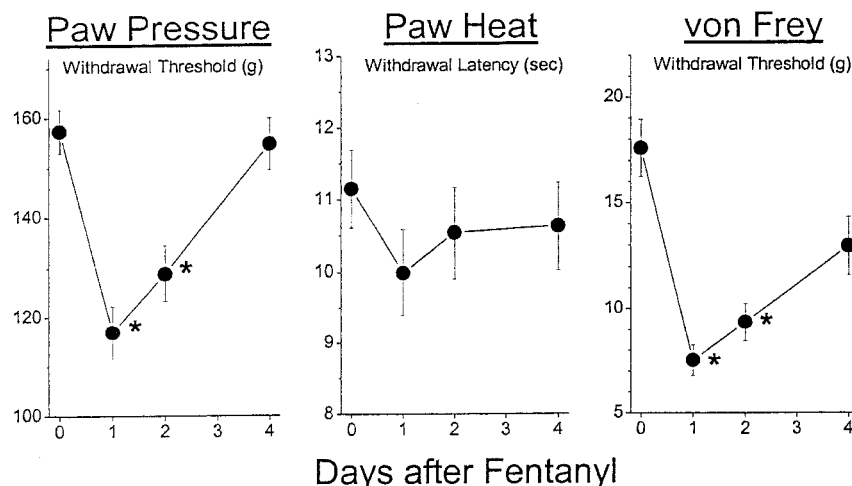
#### *Effects of Intrathecal Ketorolac*

Intrathecal ketorolac, 5  $\mu$ g, did not affect withdrawal threshold to von Frey filament testing, whereas 15, and 50  $\mu$ g ketorolac increased withdrawal threshold for 30–60 min after injection (fig. 2). Two-way repeated measures ANOVA revealed a highly significant ( $P < 0.001$ ) dose-dependent effect from ketorolac, with each dose differing from the other. Animals appeared calm after intrathecal injections, with no alterations in spontaneous behavior.

#### *Spinal COX Isoenzyme Expression*

COX-1 immunoreactivity (COX-1-IR) was localized exclusively within cells with glial morphology, and fentanyl administration did not alter this pattern of distribution. However, fentanyl administration significantly reduced the number of COX-1-IR cells at both 24 and 96 h (the number of labeled objects in laminae I and II

Fig. 1. Response to nociceptive testing before and after treatment with fentanyl, 320  $\mu$ g subcutaneously on Day 0. Data are represented as withdrawal threshold to positive airway pressure ( $P_{aw}$ ) (left), withdrawal latency to radiant heat (middle), or withdrawal threshold to punctate testing with von Frey filaments (right). Each symbol represents the mean  $\pm$  SE of 6 animals. \*  $P < 0.05$  versus Day 0.



per section was  $73 \pm 1.4$  in normal animals compared with  $53 \pm 3.2$  24 h after surgery, and  $55 \pm 6.7$  96 h after surgery;  $P < 0.05$  for both postsurgical times compared with normals).

COX-2 immunoreactivity was observed on the nuclei of neurons in the outer laminae with numerous perikarya being labeled throughout the dorsal horn. Motor neurons in the ventral horn were also immunoreactive. Fentanyl administration did not alter the immunoreactivity of COX-2 (number of COX-2 positive objects in laminae I and II in normals, animals at 24 h after surgery, and animals 96 h after surgery was  $225 \pm 30$ ;  $208 \pm 42$ , and  $263 \pm 55$ ;  $P > 0.05$ ).

## Discussion

Opioids are most commonly considered to induce hypersensitivity and pain during the withdrawal state after

abrupt cessation of chronic use,<sup>3</sup> but this also occurs following acute opioid exposure. Thus, single or very short-term exposure to opioids can result in acute tolerance<sup>16</sup> and hyperalgesia<sup>17</sup> in volunteers and that high dose intraoperative opioid exposure can increase post-operative pain.<sup>6,7</sup>

Although hypersensitivity following acute or chronic opioid exposure clearly involves an interaction with spinal NMDA receptors,<sup>9</sup> other studies suggest an involvement of spinal COX activation. For example, intrathecally administered COX inhibitors prevent tolerance from intrathecal morphine, and reverse tolerance from chronic morphine treatment in rats.<sup>18</sup> Naloxone precipitated withdrawal from chronic opioid exposure results in thermal hyperalgesia which is blocked by intrathecal injection of a COX inhibitor.<sup>11</sup> The mechanisms for this reversal by COX inhibitors is not certain. It may reflect an interaction with NMDA receptors, since intrathecally administered COX inhibitors block hypersensitivity induced by spinal glutamate<sup>19</sup> and by treatments that stimulate spinal glutamate release, such as peripheral formalin injection.<sup>20</sup>

Both COX-1<sup>21</sup> and COX-2<sup>22</sup> isoenzymes are constitutively expressed in the spinal cord in glia and neurons, respectively. Spinal COX-2 may be upregulated under several conditions, including inflammation,<sup>23</sup> although COX-1 is also capable of upregulation by growth factors and cytokines,<sup>24</sup> and spinal COX-1 expression is increased after peripheral inflammation.<sup>25</sup> If spinally synthesized prostaglandins are important to delayed hypersensitivity following fentanyl exposure, we hypothesized that COX enzyme expression might be increased. At least with the method of immunocytochemistry, we failed to support this hypothesis. Of course, enzyme activity can be altered without change in enzyme expression.

Ketorolac, although often mentioned as a nonselective COX inhibitor, is actually several hundred-fold selective for the COX-1 isoenzyme.<sup>12</sup> We observed a dose-dependent reversal of hypersensitivity following fentanyl by

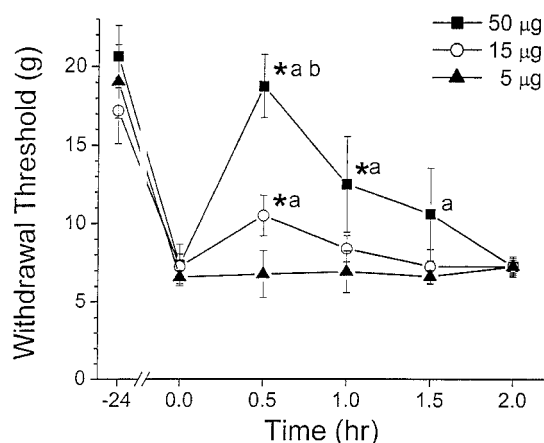


Fig. 2. Effect of intrathecal ketorolac on withdrawal threshold to punctate testing on Frey filaments. Subcutaneous fentanyl, 320  $\mu$ g, resulted in a large decrease in withdrawal threshold 24 h later. At time 0 animals received intrathecal ketorolac, 5  $\mu$ g (closed triangles), 15  $\mu$ g (open circles), or 50  $\mu$ g (closed squares). Each symbol represents the mean  $\pm$  SE of 6 animals. \*  $P < 0.05$  versus time 0 within each group. (A)  $P < 0.05$  versus 15  $\mu$ g dose. (B)  $P < 0.05$  versus 5  $\mu$ g dose.



ketorolac using a dose range shown to inhibit spinal COX activity and reduce hypersensitivity from other treatments.<sup>26</sup> Although ketorolac's effect in the current study could reflect a nonspecific action, these previous studies suggest it most likely reflects COX inhibition.

In summary, short-term exposure to a large dose of fentanyl results in delayed hypersensitivity to mechanical, but not thermal, stimuli in rats. This hypersensitivity is blocked in a dose-dependent manner by intrathecal injection of the COX-1 preferring inhibitor, ketorolac. Acute fentanyl exposure was not associated with an increase in number of COX-1 or COX-2 expressing elements in the spinal cord. These data suggest that prostaglandins participate in the prolonged hypersensitivity associated with acute opioid exposure, and suggest that intrathecal ketorolac, currently in clinical trials under Food and Drug Administration regulation, may transiently reduce the pain-enhancing effects occurring after large doses of opioids are administered, such as during surgery.

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