Evaluation of opioid receptors in synovial membranes of horses

Barbara E. Powers, DVM, MS; Dean A. Hendrickson, DVM, MS; Kathy R. Magnusson, DVM, PhD; Jenifer G. Sheehy, BS; Peter W. Hellyer, DVM, MS; Ginger E. Sammonds, MS; Khursheed R. Mama, DVM

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From the Professional Veterinary Medicine Program (Sheehy), and the Departments of Clinical Sciences (Hellyer, Mama, Hendrickson), Anatomy and Neurobiology (Sammonds, Magnusson), and Pathology (Powers), College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80524.

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The authors thank Lu Brevard for technical assistance. Address correspondence to Dr. Hellyer.

Objective—To evaluate μ-opioid receptors in synovial membranes of horses and determine whether these receptors are up-regulated in nerve endings during inflammation.

Sample Population—Synovial tissue obtained from 39 client-owned horses during arthroscopy and 14 research horses during necropsy; brain and synovial tissues were obtained during necropsy from 1 horse, and control tissues were obtained from a mouse.

Procedure—Horses were classified into 7 groups on the basis of histologically determined degree of inflammation. Binding of primary rabbit antibody developed against μ-opioid receptors in equine synovial tissue was studied, using western blot analysis. Synovial membranes were tested for μ-opioid receptors by immunohistochemical staining, using a diaminobenzidine-cobalt chloride chromogen. Homogenates of synovial membranes were evaluated by use of radioligand binding.

Results—Examination of western blots of equine thalamus revealed that rabbit antibody developed against μ-opioid receptors yielded a band (molecular weight, 55 kd) that corresponded with that of other opioid receptors. Use of immunohistochemical staining of synovial tissue revealed considerable staining in the proliferative lining layer and in regions surrounding vascular structures. Specific radioligand binding of tissue homogenates was found in all groups. We did not detect significant differences in binding between horses with inflammation and horses without inflammation.

Conclusions and Clinical Relevance—Results of immunohistochemical analysis and radioligand binding of tissue homogenates suggest that there are opioid receptors in synovial membranes of horses. Our results support the practice of intra-articular administration of opioids to relieve pain after arthroscopic surgery in horses. (Am J Vet Res 2001;62:1408–1412).

Arthroscopy has become an increasingly important tool in the assessment of horses with signs of joint pain. Arthroscopic surgery is a minimally invasive pro-

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another research project. Samples of thalamus, spinal cord, and synovial tissue of the stifle joint were obtained during necropsy from a healthy 6-year-old Quarter Horse that was euthanatized because of an acetalubar fracture. Harvested synovial tissues were flash-frozen on dry ice and stored at -70 C until analyzed. The Colorado State University Animal Care and Use Committee approved the protocol for the study.

**Light microscopy**—Samples of synovial tissues from the 39 client-owned and 14 research horses were slowly thawed to prevent cellular damage, using an initial temperature of -20 C followed by a temperature of -4 C. Tissues then were fixed in alcohol-buffered formalin and embedded in paraffin. Tissues were sectioned at a thickness of 14 µm, mounted on slides, and stained with H&E. To determine the degree of inflammation and to ascertain quality of tissue preservation, a pathologist (BEP) evaluated slides of H&E-stained sections from each piece of tissue. Tissue samples were classified into 7 groups on the basis of histologic grade. The grading scheme used was based on subjective assessment of the amount of hyperplasia in the synovial villi and number of inflammatory cells. Groups were as follows: 1, noninflamed synovium (n = 9); 2, noninflamed synovium (9); 3, mildly inflamed (grade-1) synovium (8); 4, noninflamed (7); 5, noninflamed (7); 6, noninflamed (7); and 7, mildly inflamed (grade-1) synovium (6).

**Immunohistochemical analysis**—Other paraffin-embedded sections from each of these tissue samples were immunohistochimically stained. Sections were cut to a thickness of 14 µm and mounted on slides. Mounted sections were deparaffinized in xylene and rinsed twice (3 min/rinse). Sections were rehydrated in successive dilutions of ethanol (100%, 2 rinses at 3 min/rinse; 95%, 2 rinses at 3 min/rinse; and 70%, 2 rinses at 3 min/rinse). Sections then were rinsed in PBS solution (pH 7.2) and encircled with a petroleum product to form a reservoir. Following rinses, sections were incubated with 0.3% H2O2 for 1 hour. Wills for determination of nonspecific binding contained 100 µl of tissue (100 µl) of 50 mTris buffer, and 100 µl of 10 nM n-allyl-2,3-[3H]-naloxone was added to each well. Wells for determination of nonspecific binding contained 100 µl of tissue, 100 µl of cold naloxone (200 nM), and 100 µl of 10 nM n-allyl-2,3-[3H]-naloxone. Plates were incubated on a shaker at 25 C for 40 minutes. Wells were drained by vacuum until nearly dry and then rinsed 3 times with 300 µl of ice-cold 50 mTris buffer. After the last rinse, wells were again drained by vacuum and then dried with an air dryer. Each membrane filter was placed in a scintillation vial, vortexed, and counted on a scintillation counter.

**Statistical analysis**—A 2-sample t-test was performed on results for noninflamed versus inflamed tissues (groups 1, 2, 4, 5, and 6 vs groups 3 and 7) to establish whether receptors were up-regulated during inflammation. A 2-sample t-test was performed on results for groups 1 and 2, 4, 5, and 6 and groups 3, 4, and 5 to compare results for samples with high nonspecific binding to those with lower nonspecific binding among noninflamed tissues. Values of P < 0.05 were considered significant.

**Results**

**Immunohistochemical analysis**—The amount of stain was substantially greater in all tissue sections in which primary antibody was applied, compared with those in which primary antibody was not applied (Fig 1). Intensity of stain was particularly high in regions surrounding vessels and in the synovial lining (Fig 2). Examination of western blots of thalamus obtained from a horse revealed that rabbit anti-µ-opioid receptor antibodies produced a band (molecular weight, 53 kDa) that corresponded with that of other opioid receptors.

**Radioligand binding of tissue homogenates**—Tissue homogenates were incubated with 100 µl of 10 nM [3H]-naloxone. Tissue homogenates for each of the 7 groups of horses and 2 samples of cortex from a
mouse had concentrations for specific binding of 0.0684, 0.393, 0.174, 0.603, 0.642, 0.622, 0.740, 1.602, and 1.830 fmol/mg of protein, respectively.

Average counts per minute of the wells for nonspecific binding represented approximately 30 to 50% of the total counts in groups 3 to 7 (Fig 3).

Results did not differ significantly (P = 0.08) between samples of noninflamed and inflamed tissues (groups 1, 2, 4, 5, and 6 vs groups 3 and 7). Results also did not differ significantly (P = 0.44) between samples with high nonspecific binding and those with lower nonspecific binding among noninflamed tissues (groups 1 and 2 vs groups 3, 4, and 5).

Discussion

Opioids have been used for millennia in the treatment of pain. During recent years, the use of opioids has continually been refined, allowing for more specific and, thus, more effective treatments for pain. Among these refinements has been the local administration of opioids in peripheral tissues in hopes of minimizing negative systemic effects such as respiratory depression and dysphoria.14,15 We conducted the study of opioid receptors in the peripheral tissues of horses because of the unusual challenges horses pose during the postoperative recovery period. Systemic administration of opioids in horses is often discouraged because of the excitatory and dysphoric effects.16 If efficacy of intra-articular administration of opioids can be documented, such use would allow practitioners to take advantage of this long-lasting effective class of analgesics.

Consistent with our hypothesis, we found opioid receptors in the synovial membranes of horses. Analysis of western blots clearly revealed binding of
our anti-opioid receptor antibody to equine opioid receptors. In all groups of horses, immunohistochemical analysis revealed a greater density of stain in synovial tissues than in tissue sections stained without the application of primary antibody. Radioligand binding of tissue homogenates provided quantitative evidence for receptor binding with specific-to-nonspecific binding ratios of greater than 2.1 in 5 of 7 groups. We did not detect significant (P = 0.08) differences in staining or binding characteristics between inflamed and noninflamed tissues. These findings provide a rationale for the practice of intra-articular injection of opioids following arthroscopy and arthrotomy, although the efficacy of intra-articularly administered opioids for the relief of pain in horses following arthroscopy was not determined in the study reported here. Our data are consistent with those of other investigators who attempted to identify opioid receptors in cats, rats, and humans that could be targeted for local administration to induce analgesia.2,3

Several investigations have attempted to correlate expression of opioid receptors with degree of inflammation.4-12,19,20 documented an increase in receptor delivery to the ends of peripheral nerves in rats with iatrogenically induced acute inflammation, compared with noninflamed control rats. Those studies primarily involved severe inflammation, whereas the horses included in our study had, at most, mild synovitis, which did not correlate with an increase in number of receptors. This degree of inflammation may not have been sufficiently severe to detect a quantifiable difference in receptor binding from that of clinically normal horses. It also is possible that through the nature of sectioning and homogenization, we exposed receptors that had not yet been transported to the plasma membranes. Additionally, we found considerable discrepancies between the degree of inflammation determined by a veterinary pathologist and inflammation graded by veterinary surgeons during arthroscopy. This has been reported in another study.19

Opioids are chemicals related to or derived naturally or synthetically from morphine, and they can produce potent antinociceptive effects by binding to local opioid receptors on nervous tissues.10 The µ-opioid receptor belongs to the pertussis-sensitive guanine nucleotide regulatory protein (ie, G-protein) superfamily. Binding of an opioid agonist evokes hyperpolarization secondary to increased conductance of potassium ions. This increased conductance effectively makes it more difficult to depolarize neuronal membranes to the action-potential threshold, thus decreasing synaptic transmission of nociceptive signals.11

Identification of receptors can be accomplished by various methods. We chose immunohistochemical analysis and radioligand binding, because many other investigators have documented the efficacy of these methods for use in specifically isolating receptors.22-24 Immunohistochemical analysis can identify cell types that are associated with receptors and can provide regional context. Additionally, weak signals associated with low numbers of receptors can be difficult to detect in western blots, and this problem can potentially be solved with immunohistochemical amplification. The main disadvantage of immunohistochemical analysis is that it is not a quantitative technique. By using radioligand binding of tissue homogenates, we were able to quantitate receptor binding to provide a more detailed picture of receptor density.

Our data support the identification of opioid receptors in the synovial membranes of horses, consistent with reports in other species of animals. Identification of opioid receptors is a major step in supporting the use of intra-articular administration of opioids by clinicians for control of pain after surgery. Additional studies, including cloning of the equine µ-opioid receptor and determination of the effects of inflammation, would help to elucidate the mechanisms of opioid antinociception in horses. Specifically, study of animals with chronic inflammation and arthritis would allow us to more thoroughly assess inflammatory effects on expression of opioid receptors. Clinical studies to assess the quality of recovery following surgery and efficacy of analgesia when opioids are administered intra-articularly are indicated.

References


