Effect of Gender on Anti-Inflammatory and Analgesic Actions of Two \( \kappa \)-Opioids

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ABSTRACT

The higher incidence of inflammatory and painful disorders in women and recent reports that have emphasized the importance of gender in nociceptive sensitivity and responsiveness to analgesics prompted us to investigate gender as a factor in the variability in response to opioids. We studied the anti-inflammatory and antinociceptive effects of two \( \kappa \)-opioid agonists in adjuvant-induced arthritis, one that acts both peripherally and centrally (PNU50488H; 20 mg/kg/day), the other which is peripherally selective (asimadoline; 5 mg/kg/day). Both drugs had equally powerful anti-inflammatory effects in both male and female rats (reducing measures by 60–80%). In contrast, there were gender-based heterogeneities in their analgesic actions, contingent on the method of stimulation (mechanical or thermal); males were insensitive to the analgesic effects of asimadoline with thermal but not mechanical noxious stimuli. We also sought evidence for gender influences on the joint content of Substance P (SP), a peptide suggested to have a role in producing inflammation and found that levels were higher in the untreated arthritic females, although there were no gender differences in disease sensitivity or nociception in arthritic animals receiving no drugs. Paradoxically, both drugs elevated SP concentrations in the joints, perhaps as a consequence of an action of \( \kappa \)-opioids to suppress SP release from peripheral nerves, but the gender differences remained. Further experiments are required to determine exact mechanisms responsible for the gender distinction in analgesic response to \( \kappa \)-opioids that may involve differential activation of primary afferents.

Many chronic inflammatory diseases show a higher incidence in females than in males, a fact that not only has implications for therapy but also raises significant questions about gender differences in the development and persistence of inflammation and pain and their responsiveness to drugs. Whereas gender does not seem to be a factor in the anti-inflammatory efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) (Walker et al., 1994), it does seem relevant in the analgesic effects of this drug class (Walker and Carmody, 1998).

In fact, the matter of gender differences between male and females in the perception of painful stimuli has interested scientists for some time (Otto and Dougher, 1985) and the consensus is that males show greater tolerance than females (Woodrow et al., 1972; Rollman and Harris, 1984; Robin et al., 1987; Lautenbacher and Strian, 1991; Walker and Carmody, 1998). Other studies, in contrast, have found either no sex differences (Neri and Agazzani, 1984) or differences that depend on the stimuli used and the nature of the chosen endpoint (Lautenbacher and Strian, 1991; Lautenbacher and Rollman, 1993). However, less work seems to have been done on the question of gender and analgesia but it is beginning to become clear that gender is also a relevant matter in this respect. For example, Cicero et al. (1996) reported that male rats were strikingly more sensitive to the antinociceptive action of morphine than females and as well showed some pharmacokinetic differences. Gear et al. (1996) have found the \( \kappa \)-opioid pentazocine produced greater dental analgesia in females, whereas Walker and Carmody (1998) have found males but not females to show an analgesic response to an NSAID (ibuprofen) with essentially no differences in pharmacokinetics. Gender is also relevant to other aspects of nociception and its modulation, e.g., stress analgesia (Kavali
ters and Innes, 1987, 1988; Carmody and Cooper, 1996; Mogil and Belknap, 1997; Mogil et al., 1993).

Animal studies in our laboratory have demonstrated that opioid drugs, in particular \( \kappa \)-agonists, powerfully reduce the severity of adjuvant-induced arthritis (Walker et al., 1995, 1996, 1997; Wilson et al., 1996; Binder and Walker, 1998; Binder et al., 1999), although to date we had not investigated a gender influence on this action. Because this could mean that \( \kappa \)-agonists have a potential in the clinical management of human inflammatory disease, because of the female predominance in those conditions, and because of the gender influences on opioid actions, we have performed experiments in male and female rats with adjuvant-induced arthritis.

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; SP, substance P; DA, Dark Agouti; PSI, pooled severity index.

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First, we have examined the animals for gender-based differences in disease severity and have sought evidence for gender influences on the occurrence of Substance P (SP) in the joint tissue, which is suggested to have a role in producing inflammation. Second, we have investigated the anti-inflammatory and analgesic actions of two \( \kappa \)-opioid agonists (PNU50488H that acts on the central nervous system as well as at peripheral locations, and asimadoline that has essentially peripheral actions), seeking gender influences on their effects.

**Materials and Methods**

**Experimental Animals.** Adjuvant arthritis was induced in equal numbers of male and female Dark Agouti rats (DA; Animal Resource Center, Perth, Australia) weighing \(-200 \text{ g (150–240 g)\). In our hands, DA rats reliably develop polyarthritis with an incidence of 100\% (Binder and Walker, 1998; Wilson et al., 1996). The rats were housed in groups of 10 in large cages lined with cellulose bedding (Fibercycle Pty Ltd, Mudgeeraba Queensland, Australia) and shredded paper. They were kept in a temperature-controlled room (22 ± 1°C) with a 12-h alternating light/dark cycle and were given rat chow and water ad libitum. For 2 weeks before the study, the animals were handled regularly to accustom them to the experimenter and the procedures. All of the experiments and animal husbandry were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia.

**Induction of Arthritis.** To induce adjuvant arthritis, rats were anesthetized (on day 0) with a mixture of ketamine (50 mg/kg) and xylazine (5 mg/kg) and inoculated intradermally at the base of the tail with 100 \( \mu \text{g\) of Freund’s complete adjuvant (10 mg/ml heat-killed and dried Mycobacterium butyricum suspension in paraffin oil and mannide mono-oleate; Difco Laboratories, Detroit, MI). Nonarthritic controls received similar injections of Freund’s incomplete adjuvant (paraffin oil and mannide mono-oleate; Difco Laboratories).

**Drugs.** The following drugs were used: asimadoline (EMD 61753, donated by Dr. Andrew Barber, Merck, KGaA, Darmstadt, Germany) and PNU50488H (donated by Dr. Montford Piercey, Pharmacia & Upjohn, Kalamaoo, MI). All drugs were dissolved in 30\% poly-ethelene glycol in sterile normal saline (vehicle) and were administered by i.p. injection b.i.d. (at 9:00 AM and 5:00 PM) throughout the duration of the experiment; control animals were given injections of the glycol vehicle in the same regime. The SP antibody for the radioimmunoassay was a gift from Dr. Roger Murphy (Department of Pharmacology, University of Melbourne, Australia).

**Assessment of Arthritic Damage.** All experiments were conducted in a double blind manner and for consistency the same trained observer performed all measurements throughout the study. The disease progression was monitored from the induction of arthritis (day 0) until day 21 when the rats were sacrificed (60 mg of pentobarbitone i.p.). Three indices of arthritic damage were used: edema, radiology, and histology. On days 3, 7, 13, 17, and 21 post-adjuvant, edema was measured by plethysmometry (Ugo Basile, Comerio, Italy) in both the left and right ankle and on day 21 the left foot was removed for quantitative radiologic (fixed tissue) and histological examination to assess ankle joint damage (Binder and Walker, 1998).

**Anti-Inflammatory Actions.** The possible influences of gender on the development of adjuvant arthritis and on the antiarthritic actions of the peripherally selective \( \kappa \)-opioid agonist asimadoline and the generally (peripheral and central) acting \( \kappa \)-opioid agonist PNU50488H were assessed. Asimadoline (5 mg/kg/day; \( n = 10 \) per group) and PNU50488H (20 mg/kg/day; \( n = 10 \)) were administered to arthritic male and female DA rats; these doses were chosen on the basis of dose-response studies in our laboratory (Wilson et al., 1996; Binder and Walker, 1998). The anti-inflammatory effects of these drugs are fully reversed by the specific opioid antagonist MR2266, indicating that they act entirely via the \( \kappa \)-opioid receptor at such doses (Wilson et al., 1996; Binder and Walker, 1998). In addition, there were 10 male and 10 female vehicle-treated arthritic controls.

**Nociception.** Mechanical and thermal pain thresholds were measured under the same conditions with an analgesymeter and an apparatus that tests thermal thresholds (both Ugo Basile); nociception was measured at 11:00 AM, 2 h after drug administration (\( t_{1/2} \) for these drugs being approximately 2 h as determined in our laboratory).

The analgesymeter applied a linearly increasing force (16 g/s) to the hind paw, between the third and fourth metatarsals, until the rat withdrew its paw; two sequential measurements, 1 min apart, were made on each paw. Rats were trained on this equipment for 1 week before the start of the experiment. The thermal threshold was measured by placing an unrestrained rat in a perspex chamber where it was given time to acclimatize. An infrared light source was then shone underneath the two hind paws in random order and the paw withdrawal latency automatically recorded. The protocol required that a rat should be removed from the apparatus if it did not respond within 30 s but no animals fell into this category.

**SP Concentration.** SP levels were assessed after the rats were sacrificed at day 21 (Binder et al., 1999). Right ankle joints were removed and immediately boiled in 2 M acetic acid with 4% EDTA to inactivate peptidases. The samples were then homogenized with an electric pestle and centrifuged at 7000 rpm (Koolspin, KM18211) for 15 min. All samples were assayed simultaneously: 50-\( \mu \text{l\) aliquots of the supernatant of each sample or standard (containing SP (Ausepp, Parkville, Victoria, Australia) at a range of concentrations, 0.25–50 ng for construction of a standard curve) were placed in assay tubes with 100 \( \mu \text{l\) of SP antiserum (Ausepp). These solutions were incubated for 24 h and [\( ^{32}\text{P} \)] Tyr–SP (100 \( \mu \text{l\) was then added. Unbound material (radio-labeled and unlabeled SP) was precipitated 72 h later, counted in a Packard Cobra 5005 gamma counter, and analyzed. Standard curves to determine SP concentrations were generated with nonlinear regression techniques and all values of SP-like immunoreactivity were expressed as nanograms per gram of joint tissue.

**Data Analysis.** Raw scores for left and right paw volumes were individually normalized as percentage of change from their values at day 0, then averaged; nociceptive scores were scaled on the same basis. These normalized data also were time-averaged over the entire course of the experiments. All treated data were then analyzed with two-factor (treatment \( \times \) time) repeated-measures ANOVA. The three indices of arthritic damage (time-averaged paw swelling, radiology, and histology) were individually expressed as percentages of the values from vehicle-treated arthritic (i.e., control) rats, defined as 100\%, and summed to obtain a “pooled severity index” (PSI; the value in control animals is 300). In addition, these normalized PSI, radiology, and histology data were individually subjected to ANOVA. The ANOVA and subsequent multiple comparisons were all performed with the Number Cruncher Statistical System, version 6.06 (NCSS, Kaycee, UT). Data in the figures are presented as means ± S.E.

**Results**

**Time Course**

Figure 1 shows that, in our hands and with edema as the criterion, this disease begins to be diagnosable only after >7 days has elapsed postadjuvant and reaches a peak between 18 and 21 days. The time course of hyperalgesia follows a very similar pattern when the mechanical test is used (Fig. 2A). We observed no gender differences in severity of the adjuvant-induced arthritis either in its inflammatory aspects (see vehicle results in Figs. 1B, 3, and 4) or in the painful...
state (hyperalgesia) that resulted (vehicle results in Figs. 2 and 5).

Antiarthritic Actions

Next we examined the results for evidence of an influence of gender on the susceptibility of the disease to the therapeutic actions of the two \( \kappa \)-opioid agonists when they were administered at their optimal anti-inflammatory doses (Wilson et al., 1996; Binder and Walker, 1998), which are in excess of the antinociceptive doses as specified by Barber et al. (1994; see Discussion).

Figure 1A shows that both asimadoline (5 mg/kg/day) and PNU50488H (20 mg/kg/day) powerfully attenuated the edema of experimental arthritis, and to the same extent in both male and female rats (Fig. 1B). The quantitative measures of radiological and histological severity show a similar pattern (Fig. 3). When these measures are conflated into the PSI, which we have used and validated previously (Walker et al., 1996; Wilson et al., 1996; Binder and Walker, 1998), it is clear that the drugs were highly efficacious anti-inflammatory agents and that there were no differences in the outcomes between males and females (Fig. 4).

Nociception

Vehicle Treatment. Both mechanical and thermal nociceptive thresholds were significantly decreased in vehicle-treated arthritic animals. There were no differences in the outcomes between the males and the females (\( P > .05 \)), but there were strikingly different patterns in the two test paradigms. When the mechanical test is used, the animals become progressively more pain sensitive in the affected paws, the pattern essentially paralleling the development of inflammatory edema (with a reduction in stimulus threshold of \( \sim 75\% \) by day 21; Fig. 2A). In contrast, when the thermal test is used, hyperalgesia is apparent even earlier (day 3), before the indicators of inflammation have shown any change (1 week; Fig. 2A), but remains at this level (threshold reduced by \( \sim 20\% \)) throughout the disease (Fig. 2B). The differences in algesic magnitude and pattern are significant in these two tests (mechanical versus thermal, 75 versus 20%; \( P < .05 \)).

\( \kappa \)-Opioid Treatment. Mechanical nociception. When the animals are treated with the peripherally selective drug asimadoline and assessed with the mechanical algometer, the drug is effective as an analgesic throughout the course of
the disease (Fig. 6A), with no apparent gender distinction. The rats that were treated with PNU50488H show a consistent mechanical analgesia in the early stages of the disease but a gender difference later: analgesia persists in females but declines markedly in males after day 13 [although in comparison with untreated males (Fig. 2) there is still a clear analgesia: threshold reduced by 20 cf. 75%; Fig. 6B]. The time-averaged results confirm that both drugs are effective mechanical analgesics in this inflammatory arthritis but that PNU50488H is, overall, more potent in female rats than the males (Fig. 5A).

**Thermal nociception.** The pattern is different with the use of the thermal nociceptive stimuli, as it was in the control arthritic animals. Both drugs produce analgesia in female rats (Fig. 7, A and B). In males, however, asimadoline has no analgesic action at all (Fig. 7A), whereas, intriguingly, PNU50488H lacks any analgesia before day 13 but (as a quasi-mirror image of the result of the mechanical test; Fig. 6B cf. 7B) is analgesic only in the latter stages of the disease (Fig. 7B). The time-averaged data show these gender differences (Fig. 5B): overall asimadoline has an analgesic action only in the females, whereas PNU50488H is analgesic in both sexes.
SP Concentration

We have previously shown (Binder et al., 1999) that SP concentrations in ankle joints increase as arthritis develops and although marked inflammation is apparent by 12-days postadjuvant, the increase in SP content takes virtually the full 21 days to develop. The present experiments confirm this increase in SP content but reveal a marked gender disparity, with these levels twice as high in females as in males (Fig. 8). Paradoxically, treatment with both drugs produced significant increases in the SP content of the ankles and the gender differences persisted, the female rats having higher concentrations. In addition, those animals treated with asimadoline showed significantly higher SP concentrations within their joints than those treated with PNU50488H (Fig. 8).

Discussion

Despite the well documented female preponderance in human inflammatory arthritis, the present study has demonstrated that there are no gender-based differences in the severity or the time course of adjuvant-induced inflammation nor in the magnitude of the hyperalgesia in the ankle joints of the diseased rats. Interestingly, the animals show hyperalgesia (Figs. 1 and 2) that develops sooner than the inflammation, again without any gender differences, but with patterns of mechanical and thermal hyperalgesia that were distinct, perhaps because different afferent fiber classes are involved (Aδ and C, respectively; Zimmermann, 1979).

Our results show, as well, that in this disease, two κ-opioid agonists (the prototypical PNU50488H, which acts on the central nervous system as well as at peripheral locations, and asimadoline, which has essentially peripheral actions) have powerful and equal anti-inflammatory actions in both male and female animals (edema reduced by 60–80%; Fig. 1). This result is consistent with previous work by Walker et al. (1994) who injected urate crystals s.c. in healthy human volunteers and found no gender differences in the anti-inflammatory effects of ibuprofen.

As the inflammation becomes chronic, and overt joint damage occurs, the lack of gender difference persists, both in disease severity and in the efficacy of these opioids as judged
by the use of the PSI, which we have used and validated previously (Walker et al., 1996, Wilson et al., 1996; Binder and Walker, 1998). Furthermore, this therapeutic action must be peripheral because these two drugs, acting at different loci, are equivalent in the doses we used (Figs. 3 and 4): if, for example, the action were central, then asimadoline would not be efficacious because it is essentially without central action at this dose (Barber et al., 1994). These results therefore support our earlier findings that PNU50488H exerts its anti-inflammatory actions via peripheral $\kappa$-receptors (Wilson et al., 1996).

In contrast, there are response heterogeneities in the analgesic actions of these $\kappa$-opioids that have a gender basis. As with the hyperalgesia in vehicle-treated rats (Fig. 2), the analgesic response patterns are also dependent on the method of stimulation (Figs. 5–7). Could it be argued that these gender differences are spurious because the analgesic dosage is suboptimal? This is unlikely because Barber et al. (1994) found that the analgesic $ED_{50}$ ranged from 0.08 to 3.2 mg/kg for asimadoline, whereas our dose-response studies indicated the need for a higher dosage (5 mg/kg/day) to ensure the maximal anti-inflammatory action in chronic arthritis, which we used in this study ($ED_{50} \sim 1$ mg/kg/day; Binder and Walker, 1998). Likewise, earlier dose-response studies in our laboratory (Wilson et al., 1996) found the anti-inflammatory $ED_{50}$ for i.p. PNU50488H to be $\sim 20$ mg/kg/day, whereas the maximal analgesic dose has been reported to be much lower, viz. 5–10 mg/kg (Stein et al., 1988). Either the (putatively peripheral) sites of the anti-inflammatory action are less accessible to both drugs or they are intrinsically less potent as anti-inflammatory agents. This principle is also true of the NSAIDs with which the analgesic dosage is generally lower than what is required to suppress inflammation (Seideman, 1993). Insufficient analgesic dosage cannot, therefore, be advanced as a plausible theory for the gender differences observed herein so the disparity of the anti-inflammatory and analgesic effects suggests that there are different targets for these agents, presumably the inflammatory and neural cells that have been activated by their various peptide mediators.

Certainly there is support in the literature for gender differences in nociception. Kepler et al. (1991) reported striking gender and hormonal influences on centrally mediated opioid analgesia and on the expression of $N$-methyl-$d$-aspartate (glutamate) receptors in the central nervous system (Weiland, 1992) that are known (Lipa and Kavaliers, 1990; Akinc and Johnson, 1993, 1994) to be involved in opioid analgesia as well as in the responses to inflammation.

In arthritic rats that had received no drug treatment there was a progressive increase in the animals’ sensitivity to noxious mechanical stimulation. Such a methodology is most likely to activate receptors in the joints themselves compared with the thermal method that will activate receptors in the skin. Those joint receptors, probably capsular nerve-endings, are likely to be akin to the “sleeping nociceptors” that were discovered by Robert Schmidt and his collaborators (Schailbe and Schmidt, 1988), their “awakening” seemingly increasing throughout the course of the disease (Fig. 2A). However, the process appears to be different with thermal stimulation that is confined to the overlying skin (Fig. 2B): hyperalgesia occurs earlier and to a much lesser, but consistent, degree.

The precise mechanisms for the different patterns observed with both types of hyperalgesia are not known. It might occur in two distinct anatomical sites in the periphery, involving different afferents ($\alpha$ and $C$ fibers) and it is likely to also involve the hyperexcitability and receptive field expansion that are induced in the spinal cord by peripheral inflammation (Schaible and Grubb, 1993). These central effects involve the up-regulation of inflammatory mediators, such as SP and calcitonin gene-related peptide, and excitatory amino acids, such as glutamate (Schaible and Grubb, 1993), the magnitude of which might be differentially sensitive to analgesic drugs or differentially expressed, dependent on the animals’ gender.

With the mechanical stimulation, both asimadoline and PNU50488H abolish the disease-induced hyperalgesia (Fig. 5A; time-averaged data), restoring the prepathology sensitivity; PNU50488H is significantly more effective in the females than in the males. With thermal stimulation, the males are completely unresponsive to asimadoline, whereas this drug produces analgesia in the females; PNU50488H is extremely potent in both genders (Fig. 5B). The time course plots reveal some interesting additional detail (Fig. 7). With asimadoline, the males remain unresponsive to the drug throughout the entire treatment period, the pattern essentially replicating that in untreated animals (Fig. 2B); with PNU50488H, in contrast, the males are unresponsive for the first one-third of treatment but thereafter their sensitivity to the drugs equals that of the females’. Both of these time course patterns, it should be noted, are different from the picture with mechnoceptor (Fig. 6). Our results herein are in agreement with those of Gear et al. (1996) in patients with postoperative dental pain. In their work, the analgesic response to the $\kappa$-opioid pentazocine was significantly greater in females irrespective of the menstrual phase (Gear et al., 1996). In contrast, our results differ from those of Cicero et al. (1996) who, with both thermal stimuli and the abdominal-constriction test, found male rats to be more sensitive to the $\mu$-agonist morphine. Thus, gender differences in response to opioids may be receptor dependent. The results suggest that, although the present experiments ran over several estrus cycles, further experiments to examine the importance of the stages of the estrous cycle in determining the females’ responses might well be profitable in the light of the report of Kayser et al. (1996) highlighting this issue. Further studies are required to determine the precise mechanisms responsible for the delayed analgesic response or overall insensitivity in the males.

SP, with its vasodilator actions and its effects on microvascular permeability is known to be involved in inflammation (for review, see Schaible and Grubb, 1993). It is synthesized in dorsal root ganglion cells and then secreted from their peripheral and central terminals. Given that (see above) hyperalgesia in arthritis probably follows, in part, from the release of such mediators, the existence of gender differences in SP concentration within inflamed joints is striking because there are no concomitant differences in disease severity. Our nociceptive and inflammatory findings cannot, therefore, have a simple relation to SP and they argue against an integral peripheral role for SP in this pathology, especially when the increase in joint content of SP lags behind the development of the disease (Binder et al., 1999). Further studies are required to elucidate the role of central SP in this model. Recalling that there are no gender differ-
ences in the anti-inflammatory actions of the κ-opioids, yet there is a marked sex difference in their analgesic efficacy, it is noteworthy that SP levels in female rats with untreated disease are substantially higher than those in males and that, furthermore, these levels are increased by drug treatment although the gender differences very clearly remain. It is possible that the increased levels after treatment are a consequence of an action of the κ-opioids to suppress SP release from peripheral terminals of afferent nerves (Walker et al., 1997) or even from nonneuronal sources (Cerinic et al., 1998), although we are unaware of any reports of gender influences on this process. Clearly, the spectrum of SP action in inflammation and nociception is diverse, involving a complex network of interaction between neuronal and nonneuronal SP as well as other mediators (for review, see Cerinic et al., 1998).

In summary, both the peripherally selective asimadoline and the centrally active PNU50488H have powerful anti-inflammatory effects that are equal in both male and female rats. In contrast, there are clear gender-based heterogeneities in their analgesic actions, contingent on the method of stimulation. Strikingly, both drugs also elevate SP concentrations in the joints, although there are gender differences here, too.

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