Endomorphins in rheumatoid arthritis, osteoarthritis and experimental arthritis: clinical prospects

The opioid peptides endomorphins have the potential to act as anti-inflammatory agents for the treatment of chronic inflammatory diseases such as rheumatoid arthritis. Since endomorphins in their native form are susceptible to proteolytic degradation, if they are to be considered as viable therapeutic agents it is necessary to modify their structure in order to confer proteolytic resistance while maintaining bioactivity. Endomorphin analogs have only been previously tested for analgesic activity and some of these compounds have been found to be more potent than the native forms. It is important to test these compounds for anti-inflammatory activity and also to identify intracellular and cytokine release mechanisms underlying their anti-inflammatory activity. Further work towards the identification and characterization of an endomorphin analog(s), optimized for efficacy and biostability, may lead to potential therapeutic application of endomorphins in low doses to patients as an anti-inflammatory drug.

KEYWORDS: anti-inflammatory agents ■ arthritis ■ endomorphins ■ interleukins ■ opioid receptors ■ peptidase-resistant analogs ■ substance P ■ tolerance

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According to the patient support group, Arthritis Care, there are nine million individuals with various forms of arthritis in the UK. Chronic inflammatory diseases, such as rheumatoid arthritis (RA), have been treated very effectively over the past 50 years with glucocorticoids, DMARDs or nonsteroidal drugs such as aspirin and COX-2 inhibitors. However, these are associated with a range of side effects and a significant number of patients do not respond to treatment, although some success has been reported with low doses of prednisone in a timed-release preparation [1]. Little in the way of novel anti-inflammatory compounds has recently come to the market other than TNF-α blockers. These drugs, while they are undoubtedly effective in treating severe RA, are expensive, require repeated injection, have serious nonspecific immunocompromising effects and are ineffective in approximately a third of cases. Therefore, there is a pressing need for a new generation of anti-inflammatory drugs to treat RA. The ideal drug to treat chronic inflammatory diseases, particularly in those accompanied by neurogenic pain such as RA, would be a relatively inexpensive, easily administered, stable compound that is potent at low doses with minimal side effects. Analgesic properties would add value.

Endomorphin (EM)-1 (Tyr–Pro–Trp–Phe–NH_2) and EM-2 (Tyr–Pro–Phe–Phe–NH_2) are opioid peptides that were first isolated from bovine brain [2,3]. The biochemistry and pharmacology of EM-1 and EM-2 has been described in detail [4,5]. The unique characteristics of EM-1 and EM-2 are their high affinity and selectivity for the μ-opioid receptor (MOR) [2]. EM-1 and EM-2 are widely distributed within the mammalian brain and spinal cord [6–8] and have also been located in human and rodent immune tissues [9–12]. Following reversed-phase HPLC, undegraded peaks of EM-1 and EM-2 were identified within human peripheral blood mononuclear cells [13], whereas multiple degraded forms were found in plasma [14]. This suggests that, while EMs are subjected to proteolytic degradation in plasma, they can be transported intact in the blood within lymphocytes for sequestration and targeted action within inflamed tissues, consistent with the mechanism proposed for β-endorphin [15,16]. EM-1, and to a lesser extent EM-2, levels were elevated in synovial tissue from the hindpaws of rats in which arthritis had been induced, whereas EM-1 and EM-2 could not be detected in the nonarthritic controls [12]. In a rat model of localized inflammation, the number of cells staining positive for EM-1 and EM-2 were significantly increased in inflamed paw tissue and lymph nodes, with concomitant increased expression of MOR [17]. In synovial tissues from patients with RA or osteoarthritis (OA), EM-positive cells were located in the sublining area and vessel walls of the synovium and were particularly evident in the highly inflamed lining area [12]. Macrophages, T cells and fibroblasts stained positive for EM-1 and EM-2. The

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synovial density of EM-positive cells was higher in patients with OA than in those with RA, suggesting that the turnover of EM synthesis and secretion may be higher in RA, possibly in response to increased pain and inflammation.

Anti-inflammatory actions

In addition to the well-documented analgesic effects of EM-1 and EM-2, anti-inflammatory actions have been reported in *in vivo* rodent models of acute inflammation. An acute, localized inflammatory response to electrical stimulation or substance P in rats was inhibited by EM-1, albeit at relatively high doses [18]. Intraplantar injection of EM-1 was effective in reducing acute carrageenin-induced oedema [19], while perfusion with EM-1 reduced carrageenin-induced synovial vascular permeability in rats [20]. Destruction of peripheral afferent nociceptive (PAN) neurons by capsaicin partially blocked the anti-inflammatory effects of EM-1, suggesting a mechanism of EM-1 action through MOR expressed on PAN. In a rat model of adjuvant-induced arthritis, EM-1 injected intraperitoneally, daily for 5 days at doses of 0.1 or 1 µmol, significantly reduced hindpaw volume [12]. In this model of chronic inflammation, doses of EM-1 as low as 100 fmol were also effective in reducing hindpaw volume when injected intraplantarly at the site of the ankle joint [Jessop DS, Harbuz MS, University of Bristol, UK, Unpublished data]. EM-1 was effective in reducing neurogenic rat hindpaw inflammation and mouse ear oedema [21] in a dose-dependent manner.

Possible mechanisms for these anti-inflammatory effects of EM-1 are the inhibition of substance P and calcitonin gene-related peptide (CGRP) release from PAN [21], and the inhibition of the secretion of proinflammatory cytokines, such as TNF-α, from lymphocytes [22]. MOR and opioid peptides are widely distributed throughout the immune system [23–26] and in PAN [27]. Therefore, EMs, alone in the family of opioids, have the potential to act as highly effective anti-inflammatory compounds through high affinity and specificity for MOR, which permits them to be utilized at doses low enough to maximize efficacy while minimizing systemic leakage and consequent side effects. Morphine and β-endorphin fail both the affinity and specificity tests and the synthetic agonist (D-Ala^2, *N*-MePhe^4, Gly-ol)-enkephalin (DAMGO), while it has a similar affinity to the EMs for MOR, fails the specificity test as well since it also binds to δ-opioid receptors [2].

Mechanisms of action

The molecular mechanisms of action of EMs in immune cells are not well understood. In immune cells, as in other tissues, EMs act via MOR, which are cell surface transmembrane receptors that signal primarily via G-protein-coupled receptors (GPCRs) [28]. Upon GPCR activation, both G-protein and subunits mediate a range of cytoplasmic effector mechanisms, including the inhibition of adenylyl cyclases and voltage-gated calcium channels, stimulation of phospholipase C and activation of nuclear transcription factors such as CREB. MOR-mediated effects of EMs on cytokine secretion have been reported, although non-MOR-mediated immune effects have also been observed [29]. EM-1 has been reported to stimulate secretion of the proinflammatory cytokine IL-8 from cultured cells [30], while a strong inhibition of IL-8 secretion from superfused human synovial tissue from RA patients was observed in response to low doses of EM-1 or EM-2 [12]. These paradoxical effects may be due to differences in dose and target cells. EM-1 or EM-2 were also effective inhibitors of the pleiotrophic proinflammatory cytokine IL-6 in this superfusion system. EM-1 and EM-2 have been reported to inhibit secretion of a range of cytokines from macrophages including TNF-α, IL-10 and IL-12, while potentiating IL-1β production [22,31]. Therefore, although anomalies exist, a picture is emerging of a consistent effect of EMs inducing an anti-inflammatory profile of cytokine secretion. It is of interest that opioid peptides, including EMs have been reported to be effective in a range of immune functions at very low doses, many orders of magnitude below the MOR association constant [10,29]. It has been proposed that receptor heterodimerization may underly this phenomenon [32,33]; for a general review on heterodimerization of GPCRs as drug targets, see reference [34].

µ-opioid receptor-mediated activation of nitric oxide synthase 2 activity, and concomitant downregulation of expression by EM-1 (but not EM-2) has been reported [35]. Involvement of the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in regulating the anti-inflammatory properties of EMs has not been studied in any detail, with the single exception of a report where EMs potentiated NF-κB binding to DNA in macrophage-like cells [31]. Inhibition of NF-κB in macrophages decreases production of the proinflammatory cytokines IL-1, IL-6 and IL-8, while production of the anti-inflammatory cytokines IL-10 and IL-11 is not affected [35]. Inhibition of the

MAPK subtype p38 decreases production of TNF-α, IL-6 and IL-8 from human monocytes and macrophages [36]. MAPK and NF-κB have been reported to modulate inflammatory processes in human arthritic chondrocytes [37,38]. Therefore, NF-κB and MAPK pathways are important targets for anti-inflammatory compounds. Very little is known about NF-κB and MAPK involvement in the anti-inflammatory actions of EMs. Nevertheless, it is clear that any (or all) of these pathways have the potential to influence cytokine secretion from immune cells (or substance P release from PAN), either by upstream effects on the major intracellular pathways controlling cytokine production and secretion or by interacting with one or more of the transcriptional signals that control cytokine gene-enhancer/promoter regions. Our current knowledge of the mechanisms underlying anti-inflammatory actions of EMs is summarized in Figure 1.

Clinical prospects

The current therapeutic arsenal of antirheumatic drugs is largely composed of glucocorticoids, DMARDs and biological agents such as TNF-neutralizing compounds. Chronic treatment with these drugs is associated with a wide range of toxic effects such as osteoporosis, glaucoma, gastrointestinal bleeding and diabetes mellitus [39], TB and other opportunistic fungal and bacterial infections [40]. Therefore, there is an unmet clinical need for a new generation of safer anti-inflammatory drugs to treat RA and other autoimmune diseases.

In noncancer studies that have used systemic morphine to treat pain, there has been no significant clinical improvement and a high drop-out rate owing to the unpleasant side effects [41]. Therefore, any study proposing the use of opioid compounds as analgesic or anti-inflammatory agents should focus on topical application at the site of inflammation to maximize local efficacy and minimize the risk of leakage into the circulation and consequent adverse effects within the CNS, respiratory and cardiovascular systems. Low doses of the high-potency opioid compound EMs targeted to sites of inflammation should obviate any problems of nonspecific systemic effects. It has been reported that intraplantar injection of the MOR agonist fentanyl, prior to induction of inflammation in rat paws, has a potent analgesic effect at doses that are systemically ineffective [42]. In addition, in a mouse model of experimental OA [43], treatment with naloxone led to an onset of pain 4 weeks earlier than in control animals and to upregulation of MOR in sensory neurons innervating joints. This is evidence for the activation of local opioid systems in response to joint damage, with clinical potential to alleviate peripheral pain and inflammation by 'topping up' production of endogenous opioids.

If EMs are to provide a useful contribution to the treatment of chronic inflammatory diseases, one major problem to be addressed is biostability. The proline residue at position 2 of EM-1 and EM-2 creates an ideal substrate for degradation by the membrane-bound serine protease dipeptidyl-peptidase (DPP)-IV [44]. Therefore, modified EM analogs that are resistant to DPP-IV will be more stable than the native form [45]. However, EM analogs resistant to DPP-IV have only been characterized for analgesic properties and the effects of modification on anti-inflammatory activity are unknown. The assumption that since both anti-inflammatory and analgesic actions are mediated through MOR, analogs will be equally effective in both conditions may not be warranted since analgesia and inflammation may be mediated through differing intracellular mechanisms. Indeed, agonist-specific desensitization and/or internalization of MOR are important determinants of MOR function [46,47]. A number of modified forms of EM-1 and EM-2 have potential for testing as anti-inflammatory agents on the basis of their demonstrated equi- or enhanced potency in analgesic tests compared with native EMs, and their potential for resistance to proteolytic degradation. No solubility problems in aqueous solution have been reported for any of these modified peptides.

D-proline substitution of L-proline at position 2

D-Pro-EM-2 is completely resistant to degradation by DPP-IV [48]. The D-proline substitution does not compromise bioactivity in tail-flick latency or jump threshold analgesia tests compared with native EM-2, effects that could be reversed by naltrexone [48], suggesting that affinity for MOR is not attenuated by modification.

Substitution of tyrosine at position 1 with 2′6′-dimethyl-L-tyrosine

2′6′-dimethyl-L-tyrosine (Dmt)EM-2 exhibited increased MOR affinity and analgesic activity by five- and 30-fold, respectively, compared with native EM-2 [49,50]. (Dmt)EM-1 given intracerebroventricularly was even more potent than (Dmt)EM-2 in the tail-flick and hot-plate tests [51]. The analgesic effects of (Dmt)EM-1 administered

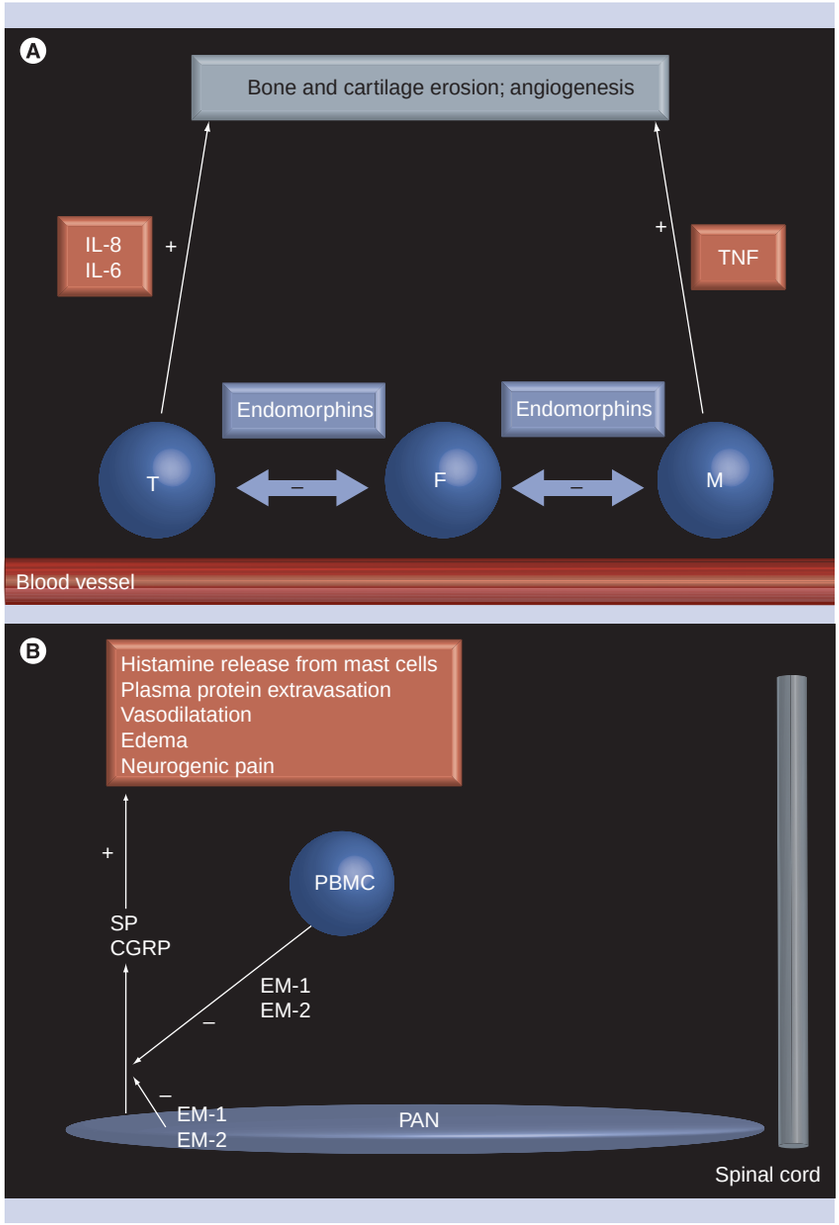

Figure 1. Demonstrates the current knowledge of mechanisms underlying the anti-inflammatory actions of endomorphins in an inflamed arthritis joint. **(A)** T cells (T) and macrophages (M) are sequestrated from blood into inflamed tissues in response to chemokines and other local signals. A range of proinflammatory cytokines are secreted from lymphocytes, which induce chronic inflammation and tissue damage. In addition to cytokines, endomorphins are released from T and M, and also fibroblasts (F), to act at μ-opioid receptor (MOR) on lymphocytes in an autocrine or paracrine manner to inhibit secretion of IL-6, IL-8 and TNF-α. Therefore, endomorphins contained within circulating T and M have the potential to be delivered to target tissues to act as anti-inflammatory mediators, as proposed for β-endorphin [64]. **(B)** Endomorphins secreted from PBMC and nerve terminals of PAN within inflamed joint tissue inhibit the release of SP and CGRP through MOR expressed on PAN. EM-2 is colocalized with SP in PAN [65] and may be cosecreted from axonal terminals. PAN innervation and MOR expression are increased in inflamed tissues in rheumatoid arthritis. Therefore, endomorphins may play a crucial role in limiting secretion of SP and CGRP in rheumatoid arthritis.
+: Stimulatory; -: Inhibitory; CGRP: Calcitonin gene-related peptide; EM: Endomorphin; F: Fibroblasts; M: Macrophages; PAN: Primary afferent nociceptive neurons; PBMC: Peripheral blood mononuclear cells; SP: Substance P; T: T cells.

peripherally in mice lasted over 2 h [51], much longer than expected for native EM-1, suggesting a degree of proteolytic resistance.

Substitution of proline with an N-methylated glycine at position 2

This substitution of proline with an N-methylated glycine at position 2 to create the analog (Sar^2)EM-2 was highly resistant to degradation by carboxypeptidase Y, aminopeptidase M and rat brain homogenates in which DPP-IV is the main proteolytic enzyme [52]. (Sar^2)EM-2 has a similar affinity to native EM-2 for MOR and was slightly more potent than EM-2 in inducing analgesia in the hot-plate test when administered centrally [53]. When injected intraperitoneally, (Sar^2)EM-2 induced significant analgesia while EM-2 was ineffective [53].

Methods of analog administration could include direct injection or controlled release from drug-impregnated synthetic beads implanted within synovial tissue. A strategy for virally mediated gene transfer for delivery of opioid peptides, including EM-2, to alleviate inflammatory pain has also been proposed [54] although the principal difficulty with this approach is generating release of clinically significant amounts of the peptide.

A further caveat that must be addressed in the therapeutic use of EMs is the possible downregulation of MOR and consequent development of tolerance. Downregulation of MOR in dorsal root ganglia (DRG) and the concomitant loss of analgesic effects have been reported following chronic administration of EM-1 [55]. This raised a question over the long-term utility of opioids as analgesic agents [56], although few studies into any concomitant loss of anti-inflammatory effects have been performed. However, receptors that are expressed in low amounts on resting lymphocytes can be upregulated by activation during chronic inflammation [17,57] and upregulation of MOR has been reported in DRG of PAN neurons in a rat model of monoarthritis [58], a similar model of chronic arthritis to that used by Li *et al.* [55]. Receptor affinity was not attenuated. In a rat model of neurogenic inflammation, a single dose of EM-1 reduced hindpaw plasma protein extravazation and repeated doses of EM-1 over 10 days did not induce desensitization [21]. Upregulation of MOR in human lymphocytes has been observed following morphine administration [59]. In an immunohistochemical study of MOR in human synovial tissue, increased staining of MOR was observed in PAN neurons in tissues from patients with RA compared with OA or joint trauma [24]. This suggests a correlation

with inflammatory activity. Since PAN neuronal infiltration of synovial tissue increases during chronic inflammation, the target for opioid therapy may be commensurately enhanced. In contrast to MOR, κ- and δ-opioid receptors are downregulated in synoviocytes from RA patients [60], which may indicate that MOR agonists have selective utility as therapeutic opioid agents in chronic inflammatory conditions.

Perhaps the most encouraging outlook for the potential for opioids in the treatment of chronic inflammatory conditions is provided by the recent demonstration that chronic use of morphine induced peripheral tolerance to pain in control rats, but in a rat model of chronic inflammatory pain, morphine did not result in tolerance [42]. This is important evidence that the phenomenon of tolerance, which compromises the use of opioids as analgesics in non-inflammatory conditions, may not be a barrier to their use in treating chronic inflammatory pain. Treatment with morphine synchronous with the induction of inflammation was associated with internalization of MOR in DRG, sustained G-protein coupling and decreased intracellular cAMP. The phenomenon of tolerance is closely associated with MOR endocytosis within lysosomes [61]. It is possible that during the onset of inflammation, mechanisms are activated that direct MOR away from lysosomal degradation and into a recycling pathway, resulting in more functionally active MOR on DRG neurons, and possibly, also on PAN nerve terminals. Highly specific lysosomal sorting pathways have been described for GPCRs, involving ubiquitin tagging and other covalent and noncovalent signals, which are instrumental in controlling GPCR trafficking and membrane recycling [62]. Elucidation of cytoplasmic mechanisms that are potentially upregulated during inflammation, which control the intracellular destiny of MOR through endocytosis or recycling, may be instrumental to our understanding of how opioid tolerance can be overcome in the treatment of inflammatory disease. It should be mentioned that there have been reports of opioid tolerance in peripheral inflammation [55,63] but in these studies, in contrast to the report by Zöllner *et al.* [42], opioid treatment was commenced following the onset of inflammation. Therefore, the timing of administration may be an important factor in avoiding tolerance.

Conclusion

In spite of the caveats presented above, there are excellent prospects for the clinical application of EM analogs for the treatment of arthritis. It is quite clear that the native peptides are an important endogenous component in the immune arsenal in chronic disease and pharmacological 'topping up' of this protective system with doses of maximum efficacy targeted at sites of inflammation will be beneficial. Targeted local delivery of anti-inflammatory drugs is not a common strategy in treating arthritis and perhaps deserves more consideration, particularly where the number of affected joints is small. Localized delivery of small doses of EMs will have the triply beneficial effect of maximizing efficacy, minimizing systemic side effects and reducing cost to health service providers.

Future perspective

Current compounds in the treatment of chronic inflammatory diseases such as RA are administered systemically and are nonspecifically immunocompromising. The next generation of anti-inflammatory drugs will be compounds with associated analgesic properties and be targeted to the site of inflammation. The relatively low cost of stable opioid peptide analogs will be an attractive proposition for the future treatment of arthritis, as well as consequent improvement in quality of life in an increasingly aging population.

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Executive summary

- Endomorphins are endogenous ligands for the μ-opioid receptor.
- Mechanisms of inhibition of inflammation are through cytokines and peptides.
- Intracellular molecular mechanisms are not well resolved.
- G-protein-coupled receptors and intracellullar trafficking are important pathways for the specific actions of opioids.
- The phenomenon of opioid tolerance is not a problem in peripheral inflammation.
- Analogs resistant to proteolysis must be developed for clinical application.
- Targeted delivery of endomorphin analogs to sites of inflammation has potential clinical benefit.

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