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The Endogenous Opioid Spinorphin Blocks fMet-Leu-Phe-Induced Neutrophil Chemotaxis by Acting as a Specific Antagonist at the N-Formylpeptide Receptor Subtype FPR

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Spinorphin is an endogenous heptapeptide (leucylvalylvalyltyrosylprolyltryptophylthreonine), first isolated from bovine spinal cord, whose sequence matches a conserved region of α-hemoglobin. Also referred to as LVV-hemorphin-4 and a member of the nonclassical opioid hemorphin family, spinorphin inhibits enkephalin-degrading enzymes and is analgesic. Recently, spinorphin was reported to block neutrophil activation induced by the chemotactic N-formylpeptide N-formylmethionylleucylphenylanalanine (fMLF), suggesting a potential role as an endogenous negative regulator of inflammation. Here we use both gain- and loss-of-function genetic tests to identify the specific mechanism of spinorphin action on neutrophils. Spinorphin induced calcium flux in normal mouse neutrophils, but was inactive in neutrophils from mice genetically deficient in the fMLF receptor subtype FPR (N-formylpeptide receptor). Consistent with this, spinorphin induced calcium flux in human embryonic kidney 293 cells transfected with mouse FPR, but had no effect on cells expressing the closely related fMLF receptor subtype FPR2. Despite acting as a calcium-mobilizing agonist at FPR, spinorphin was a weak chemotactic agonist and effectively blocked neutrophil chemotaxis induced by fMLF at concentrations selective for FPR. Spinorphin did not affect mouse neutrophil chemotaxis induced by concentrations of fMLF that selectively activate FPR2. Thus, spinorphin blocks fMLF-induced neutrophil chemotaxis by acting as a specific antagonist at the fMLF receptor subtype FPR. The Journal of Immunology, 2001, 167: 6609–6614.

The generation of opioid peptides from endogenous proteins has been characterized in milk protein, mitochondrial cytochrome b, and hemoglobin (1–3). Hemorphins are nonclassical opioid peptides found in human pituitary gland, cerebrospinal fluid, adrenal gland, blood, and bronchoalveolar lavage fluid as well as bovine brain that have amino acid sequences identical with a conserved region of the β-chain of bovine and human hemoglobin (4–10). Peritoneal macrophages (11) and proteolytic enzymes such as aspartic endopeptidase (12) and cathepsin D (13) have been implicated in hemorphin processing and production. Hemorphins have been reported to function as agonists at AT4 angiotensin receptors in brain and as antagonists at enkephalins and angiotensin-converting enzyme (14–17), suggesting potential modulatory roles in memory, pain, and blood pressure control.

Spinorphin, or LVV-hemorphin-4, has the amino acid sequence leucyl-valyl-valyl-tyrosyl-prolyl-tryptophyl-threonine, which matches positions 32–38 of human β-hemoglobin (5). Like other hemorphins, spinorphin is an angiotensin-converting enzyme antagonist, and inhibits enkephalin-degrading enzymes such as neutral endopeptidase (NEP)/CD10, aminopeptidase, and dipeptidyl aminopeptidase (18). Spinorphin has been identified in human cerebrospinal fluid and bovine spinal cord (10, 18). Concentrations in vivo are not known, but high levels (115–300 nM) of the related hemorphin LVV-hemorphin-7 have been measured in cerebrospinal fluid from a patient with cerebrovascular bleeding (9). Spinorphin has been shown to have analgesic activity for morphine-resistant pain pathways (19).

Expression of the spinorphin targets NEP and aminopeptidase N (CD13) on blood phagocytes (20–24) has suggested a potential role for spinorphin in modulating inflammatory responses. NEP degrades and inactivates the potent phagocyte chemoattractant fMLF (25), suggesting that spinorphin might act to potentiate responses to this peptide. In fact, the opposite is the case; spinorphin inhibits fMLF-induced calcium flux, chemotaxis, exocytosis, and superoxide production in human neutrophils (26). The mechanism appears to involve competition for fMLF binding to surface receptors. Spinorphin has also been shown to block carrageenin-induced polymorphonuclear neutrophil accumulation in mouse air pouches (27).

Here we address the molecular basis of spinorphin action by testing its specificity for known fMLF receptors. Two human neutrophil fMLF receptor subtypes have been cloned, N-formylpeptide receptor (FPR) and FPR-like 1 receptor (FPRL1R) (28–32). These receptors are members of the G protein-coupled receptor superfamily and have 69% amino acid identity. Compared with FPR, FPRL1R binds fMLF with lower affinity and has additional agonists, including lipoxin A4, and serum amyloid A (33, 34). In the mouse the FPR homologue has been clearly identified (35), but two other related receptors have been cloned that are equally homologous to human FPRL1R (36). To date one has been characterized as a lipoxin A4 receptor and is known as LXA4R (37), whereas the other, known as FPR2, is a functional receptor for fMLF (low affinity compared with mouse FPR) and serum amyloid peptide.
A (38, 39). Here we use gene transfected cell lines and FPR knockout mice to prove that spinorphin blocks fMLF-induced chemotaxis of neutrophils by specifically antagonizing FPR.

Materials and Methods

Peptides

fMLF was purchased from Sigma (St. Louis, MO). Spinorphin was synthesized by the Peptide Synthesis and Analysis Unit, Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). HPLC analysis indicated that the peptide was 93% pure. Both fMLF and spinorphin were dissolved in DMSO, and 10 mM stocks were kept at −20°C until use.

Mouse leukocyte purification

Development of an FPR knockout mouse has been reported previously (40). Mice used in this study were littermates from FPR−/− and FPR2−/−. Leukocytes were harvested from the peritoneal cavity after thioglycollate irritation, as described previously (40). Cells obtained after 3 h were >90% neutrophils, as determined by the morphologic appearance of Diff-Quick-stained preparations.

Cell lines

Human embryonic kidney (HEK) 293 cell lines expressing mouse FPR and mouse FPR2 have been previously described (37). Cells were cultured in DMEM plus 10% FBS and 1 mM HEPES. Spinorphin and fMLF were loaded at varying concentrations in the lower compartment of a 48-well plate, supplemented with 1% BSA and 20 mM HEPES. Spinorphin and fMLF were continuously stirred. Cells that migrated through the filter were then removed, washed, and stained. Cells that migrated through the filter were counted microscopically.

Intracellular Ca2+ measurements

Cells were incubated in PBS (107 cells/ml) containing 2.5 μM fura-2/AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in the dark. Cells were washed twice and resuspended in HBSS. Cells (3 × 104), continuously stirred in a 2-ml cuvette at 37°C, were stimulated by fMLF or spinorphin in a fluorometer (Photon Technology, South Brunswick, NJ). The data were recorded every 200 ms as the relative ratio of fluorescence emitted at 510 nm following sequential excitation at 340 and 380 nm.

Chemotaxis

Leukocytes were resuspended at 1.5 × 106 cells/ml in RPMI 1640 supplemented with 1% BSA and 20 mM HEPES. Spinorphin and fMLF were loaded at varying concentrations in the lower compartment of a 48-well microchemotaxis chamber (NeuroProbe, Cabin John, MD). The chamber was soaked in 1% SDS overnight and washed before each experiment. A 3-μm pore size polycarbonate-free polycarbonate filter was used. Fifty microliters of 1.5 × 106 cells/ml was loaded in the upper compartment, and the chemotaxis chamber was incubated at 37°C, 100% humidity, and 5% CO2 for 1 h. The filter was then removed, washed, fixed, and stained. Cells that migrated through the filter were counted microscopically under high power. All conditions were tested in triplicate.

Results

Spinorphin is a partial agonist at mouse FPR

To identify specific spinorphin receptors, we used a calcium flux assay to test as candidates the two known mouse N-formylpeptide receptor subtypes (FPR and FPR2), expressed in HEK 293 cells by stable transfection with appropriate plasmids. Untransfected HEK 293 cells do not normally express these receptors or respond to fMLF and did not respond to spinorphin (not shown). As expected, each cell line responded specifically to fMLF with a typical rise in the intracellular Ca2+ concentration, followed by a rapid return to resting levels (Fig. 1). As previously reported (38), each receptor could be distinguished pharmacologically by the EC50 of fMLF for activation and by reactivity to selective agonists (data not shown). Neither cell line responded to stimulation with irrelevant chemoattractant agonists, such as the chemokine macrophage inflammatory peptide-1β.

Spinorphin also induced calcium flux in FPR-transfected cells, but not in cells expressing FPR2 (Fig. 1); the EC50 was 128 ± 24 μM (n = 3; Fig. 2). To confirm that spinorphin and fMLF share the same signaling pathway via FPR, we performed real-time sequential stimulation experiments, again monitoring calcium flux. A saturating concentration of spinorphin added first (500 μM) caused a marked reduction in the response of the cells to 5 μM fMLF given second, and 5 μM fMLF added first almost completely desensitized the cells to 300 μM spinorphin given second (Fig. 3). This interference pattern suggests usage of the same signaling pathway by these two agonists

FPR is a functional neutrophil receptor for spinorphin

We previously reported that FPR and FPR2 are both expressed in primary mouse neutrophils (38). To test whether our results from
In sequential stimulation experiments, 100 μM fMLF given second. This concentration of fMLF induces a maximal response in HEK 293 cells transfected with mouse FPR. Conversely, 200 nM fMLF abolished the response of the cells to 100 μM spinorphin given second (Fig. 4C).

These results are consistent with the idea that FPR functions as a shared calcium-mobilizing mouse neutrophil receptor for spinorphin and fMLF. Moreover, the failure of 100 μM spinorphin to induce calcium flux in FPR<sup>−/−</sup> neutrophils or to block the response of FPR<sup>−/−</sup> neutrophils to 10 μM fMLF, which is the EC<sub>50</sub> for activation of FPR2, strongly suggests that spinorphin is neither an agonist nor an antagonist of endogenous FPR2 (Fig. 4C). Thus, there is agreement between the gain- and loss-of-function genetic tests of these receptors in transfected HEK 293 cells and primary mouse neutrophils.

**Spinorphin acts as an antagonist at FPR to block fMLF induction of neutrophil chemotaxis**

We next tested whether spinorphin can affect neutrophil chemotaxis. In control experiments, fMLF was approximately 100-fold more potent in inducing chemotaxis of thioglycolate-elicited peritoneal neutrophils from FPR<sup>−/−</sup> mice compared with cells from FPR<sup>+/−</sup> mice (Fig. 5), which confirms our previous report (38). fMLF appears to induce chemotaxis of FPR<sup>−/−</sup> neutrophils via FPR2, since both these cells and FPR2-transfected HEK 293 cells respond chemotactically to fMLF over a similar dose range. To understand the spinorphin results that follow, it is important to realize that the dose-response curve for fMLF induction of chemotaxis in wild-type neutrophils has two optima at 1 and 100 μM (Fig. 5A), reflecting the well-known bell shape of chemotaxis dose-response curves and the responsiveness of FPR and FPR2 in these cells at low and high concentrations of fMLF, respectively (38).

Although spinorphin was an effective calcium-mobilizing agonist for neutrophils from FPR<sup>−/−</sup> mice, it was an extremely poor direct chemotactant of these cells (Fig. 5), which agrees with previously reported results using human neutrophils (26) and provides an additional example of the nonequivalence of calcium flux and chemotaxis pathways in neutrophils. The maximal chemotactic index was consistently <2 at optimal spinorphin concentrations (10–50 μM; Fig. 5A). Spinorphin-induced chemotaxis was not observed in neutrophils from FPR<sup>−/−</sup> mice, indicating that the activity, albeit weak, was specifically mediated by FPR (Fig. 5B). In contrast to this weak intrinsic agonist activity, spinorphin specifically and effectively antagonized fMLF-induced chemotaxis of

**FIGURE 3.** Desensitization of FPR by spinorphin. HEK 293 cells expressing mouse FPR were stimulated sequentially with spinorphin and fMLF at the times and concentrations indicated, and calcium flux was measured as the relative change in cell fluorescence. Data are from a single experiment repeated at least six times.

**FIGURE 4.** Spinorphin is an agonist at endogenous FPR expressed in mouse neutrophils. Calcium mobilization in thioglycolate-elicited peritoneal neutrophils obtained from wild-type (A and C) and FPR-knockout (B) mice. A. Dose response of spinorphin in FPR<sup>+/+</sup> neutrophils. B. Specificity of spinorphin for endogenous FPR. C. Cross-desensitization of calcium flux responses by fMLF and spinorphin in FPR<sup>+/+</sup> neutrophils. Data are from a single representative experiment representative of three independent experiments.
neutrophils from FPR knockout mice (Fig. 6). This agrees with the reported ability of spinorphin to block fMLF-induced chemotaxis of human neutrophils (26). Selectivity for FPR was suggested by the ability of 100 μM spinorphin to inhibit chemotaxis of wild-type neutrophils over the full chemotactic concentration range of fMLF (10–1000 nM) for FPR (Fig. 6A) but not at 10 μM fMLF, which induces chemotaxis via FPR2, not FPR (38), as well as by the inability of spinorphin to affect chemotaxis of wild-type mouse neutrophils to an unrelated agonist, the chemokine SDF-1 (Fig. 6B).

When an fMLF concentration that is both selective for FPR over FPR2 and optimal for FPR-mediated neutrophil chemotaxis was used to stimulate neutrophils from FPR knockout mice, spinorphin inhibited the activity in a dose-dependent manner. The 50 IC value was approximately 30 μM (Fig. 7A). At the highest concentration of spinorphin tested (100 μM) the inhibitory efficacy was about 70%. In contrast, when an fMLF concentration that is optimal for FPR2-mediated neutrophil chemotaxis was used to stimulate neutrophils from FPR knockout mice, the activity was unaffected by spinorphin throughout the concentration range tested (Fig. 7B). This is consistent with the calcium flux experiments in mouse receptor-transfected HEK 293 cells and FPR knockout mouse neutrophils and supports the conclusion that spinorphin is highly selective for mouse FPR compared with FPR2.

Spinorphin has also been reported to block human neutrophil oxidant production triggered by fMLF (26). However, fMLF induction of oxidant production in mouse neutrophils was weak, and spinorphin quenched the Diogenes reagent that is most sensitive for detecting superoxide protection; therefore, analysis of spinorphin modulation of this pathway could not be accomplished.

**Discussion**

In this study we have used genetic gain- and loss-of-function criteria to prove that the hemorphin family member spinorphin blocks fMLF induction of mouse neutrophil chemotaxis by acting as a specific antagonist at the fMLF receptor subtype FPR. Spinorphin also exhibited partial agonist activity at FPR, since it was able to directly induce calcium flux and to weakly induce chemotaxis in wild-type mouse neutrophils, but not in FPR knockout neutrophils. Our results are consistent with and provide a specific molecular mechanism for the findings of Yamamoto et al. (26), who reported that spinorphin could inhibit fMLF induction of human neutrophil chemotaxis, superoxide generation, and exocytosis and could inhibit fMLF binding to human neutrophils. That result did not specify a molecular mechanism of spinorphin action because of the existence of at least one other fMLF receptor subtype, FPR2, which we now show does not appear to interact with spinorphin, and because of the possibility that spinorphin could cross-desensitize fMLF receptors through an independent pathway.

FPR is the first membrane receptor identified for spinorphin. Other members of the hemorphin family have been shown to bind angiotensin receptors (14, 15), which, like FPR, are members of
the peptidergic group of G protein-coupled receptors (41). However, interaction of spinorphin with angiotensin receptors and interaction of other hemorphins with FPR have not been reported. FPR ligands were originally thought to be restricted to formylated peptides potentially derived from bacterial and mitochondrial proteins (42), but are now known to also include several nonformylated peptides, all of which, with the exception of spinorphin, are synthetic peptides derived from random peptide libraries or HIV proteins (43, 44). These peptides imply that there may also exist endogenous antagonists of chemoattractant receptors. We have identified spinorphin as a putative endogenous antagonist of fMLF-induced chemotaxis. To date very few examples of endogenous antagonists of chemoattractant receptors have been identified. Apart from spinorphin no others have been reported for FPR and FPR2. The approximately 50 known members of the chemokine family typically have strong agonist activity, although two endogenous chemokine antagonists active at CXCR3 have recently been reported (49).

Although the role of endogenous antagonists in the regulation of the inflammatory response remains undefined in vivo, the identification of specific spinorphin antagonism at FPR increases the biological plausibility of such a mechanism. Based on the specificity of FPR for phagocytes and the fact that spinorphin is cleaved from β-hemoglobin, such a mechanism is most likely to operate in the setting of acute inflammation and may be of general importance rather than restricted to a specific tissue type.

References