Effects of endogenous and synthetic opioid peptides on neutrophil function in vitro

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Background. Opioid peptides released from immunocytes during inflammation and stress in critically ill patients are associated with an altered immune response. Moreover, concentrations of opioid peptides are increased in peripheral blood and at the sites of inflammatory reactions.

Methods. Using flow cytometric assay of whole human blood, we investigated direct effects of endogenous and synthetic opioid peptides on surface expression of complement receptors CD35 and CD11b/CD18 and Fca receptor III CD16, and superoxide anion generation of neutrophils.

Results. The endogenous opioid peptides ß-endorphin1-31 and met-enkephalin, representing the N-terminal fragment of ß-endorphin1-31, and the synthetic δ opioid receptor agonists D-Ala²-D-Leu⁵-enkephalin and D-Pen²-enkephalin produced concentration-dependent stimulation of neutrophil activity. Incubation with met-enkephalin 10⁻⁷ M or ß-endorphin1-31 10⁻⁷ M led to an increase in receptor expression of up to 10% (met-enkephalin) and 15% (ß-endorphin1-31). After incubation with D-Ala²-D-Leu⁵-enkephalin or D-Pen²/⁵-enkephalin, receptor expression was increased by up to 30%. This correlated with concentration-dependent stimulation of the production of reactive oxygen intermediates, as shown by an increase of up to 40% in oxidative burst activity. All effects were abolished after preincubation with naloxone or with the selective δ opioid antagonist naltrindole, whereas the selective µ receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ showed only partial inhibitory effects.

Conclusions. Our data suggest a δ opioid receptor-mediated stimulatory effect on neutrophil function. ß-Endorphin27-31, the C-terminal fragment of ß-endorphin1-31, did not alter neutrophil function, indicating that ß-endorphin1-31 mediates its effect on neutrophils via the N-terminal fragment. This study may contribute to a better understanding of neuroimmune interaction.

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Inflammatory processes induce migration of leucocytes containing opioid peptides to inflamed tissue, while opioid receptors on primary afferents are upregulated.¹ ² Endogenous pain relief is achieved through the local secretion of opioid peptides by immunocytes, activating peripheral opioid receptors.³ Locally produced corticotropin releasing hormone seems to induce endogenous analgesia by releasing opioid peptides from leucocytes, relieving pain by interacting with peripheral opioid receptors.³ Immunosuppression abolishes endogenous analgesia, emphasizing the functional importance of leucocytes in this process.⁴ Because of their ability to recognize, opsonize, phagocytose and kill microorganisms, polymorphonuclear neutrophils (PMN) and monocytes play a crucial role in defence against bacterial infections.⁵ In circulating blood, pathogens are rapidly coated by opsonins, such as antibodies and complement fragments. Recognition of opsonized bacteria is achieved by complement and immunoglobulin receptors,
which are expressed on the cellular surface of leucocytes. Binding of opsonized particles to these receptors initiates phagocytosis and triggers an oxidative burst and degranulation. However, there is little information dealing with the consequences of a local increase in opioid peptide concentrations for leucocyte function in whole blood assays, leaving leucocytes in their physiological medium. In this study, we sought to determine the direct effects of the endogenous opioid peptides β-endorphin1–31 and met-enkephalin on neutrophil function. We also focused on two synthetic δ opioid receptor agonists, D-Ala2-D-Leu5-enkephalin (DADLE) and D-Pen2,5-enkephalin (DPDPE), which are not cleaved by endopeptidases, thus offering a potential therapeutic option in the treatment of inflammatory imbalances.

We examined the expression of complement receptors 1 (CD35) and 3 (CD11b/CD18) and the Fcγ receptor III (CD16) on the surface of neutrophils. We also determined changes in oxidative burst after incubation of whole blood with opioid peptides using a flow cytometric assay.

Materials and methods

After informed consent had been given, 10 ml of venous blood from 12 healthy male volunteers was collected, using lithium heparinate for anticoagulation. None of the donors had a history of infection or allergy; none were smokers or undergoing immunosuppressive therapy. Four functional variables of polymorphonuclear neutrophils (PMN) were determined: expression of CD11b/CD18, CD35, expression of FcγRIII receptor CD16, and oxidative burst.

In a whole-blood assay, 100 µl blood was incubated with different concentrations of β-endorphin1–31, met-enkephalin, β-endorphin27–31, DADLE or DPDPE. NaCl 0.9% was added to control tubes. As neutral endopeptidase (NEP) cleaves opioid peptides in whole blood after 15 min, we incubated β-endorphin1–31, met-enkephalin and β-endorphin27–31 for 10 min at 37°C. The synthetic opioid peptides DADLE and DPDPE were incubated for 10–150 min. To investigate naloxone reversibility, naloxone was added 10 min before opioid treatment. To assess any receptor-specific effect of these opioids, the selective δ-opioid receptor antagonist naltirindole and the selective µ-opioid receptor antagonist d-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 (CTOP) was preincubated for 10 min at 37°C before addition of the different opioid peptides. Control tubes were incubated with saline solution (0.9%).

Staining for cell surface antigens

After incubation with the above-mentioned drugs, whole blood samples were stained with mouse-derived monoclonal antibodies. Single-colour reagents were fluorescein isothiocyanate (FITC)-conjugated CD11b/CD18 (clone ICRF 44), CD35 (clone E11) and CD16 (clone LNK16) (Camon/Serotec, Heidelberg, Germany). Non-fluorescent antibodies against CD11b/CD18, CD16 and CD35 were used to determine optimal flow cytometer (Facscalibur®; Becton Dickinson, Heidelberg, Germany) conditions. Cell surface staining was performed by incubating 100 µl blood with 10 µl of the appropriate antibody reagent for 15 min in the dark at room temperature. Lysing solution (Facs® Brand Lysing Solution; Becton Dickinson) was used for red cell lysis. Samples were washed twice with phosphate-buffered saline (PBS) and stored on ice in the dark until flow cytometric analysis. After staining of cell nuclei with propidium iodide, flow cytometric analysis was performed within 30 min.

Oxidative burst assay

Oxidative burst was determined by the hydrogen peroxide- and peroxidase-dependent conversion of non-fluorescent dihydorhodamine 123 to green fluorescent rhodamine 123. Neutrophils were primed with unlabelled bacteria (Escherichia coli). Whole blood 100 µl was incubated with either bacteria solution 100 µl or with PBS buffer as a control. Samples and controls were incubated at 37°C with steady shaking and cooling on ice, followed by incubation with dihydrohodamine 123, 20 µl, under the same conditions. Lysis of erythrocytes and propidium iodide staining were performed as described above.

Flow cytometric analysis

Flow cytometric analysis was performed using a Facscalibur flow cytometer with argon ion laser excitation at 488 nm; 15 000 events of each sample were measured. Data were acquired and processed using Cellquest® software (Becton Dickinson, Heidelberg, Germany). Neutrophils and monocytes were identified and gated by their special characteristics in forward angle light scatter and 90° side scatter. Cell debris and aggregation artefacts were excluded by DNA staining with propidium iodide. We determined medians of fluorescent intensities (MFI) of antibody-stained granulocytes and monocytes as a measure of complement or FcγRIII receptor expression, and the percentage expression of receptor-positive cells (Fig. 1). Oxidative burst activity was expressed as the MFI of granulocytes that had converted dihydorhodamine 123 to rhodamine 123.

Statistical analysis

All experimental results are expressed as mean values with standard deviations. Statistical analysis was performed with SPSS 9.0.1 software (SPSS, Chicago, IL, USA), using Friedman’s test followed by the Wilcoxon–Wilcox procedure, allowing multiple comparisons against a control group. P values <0.05 were considered significant.
Results

A significant concentration-dependent increase in complement and Fcγ receptor expression was observed after treatment with different β-endorphin₁₋₃₁ and met-enkephalin concentrations (Table 1). A peak effect was observed at 10⁻⁷ M, as indicated by a significant increase in MFI. Incubation with 10⁻⁷ M met-enkephalin or β-endorphin₁₋₃₁ led to an increase of up to 23% in receptor expression, whereas preincubation with the unspecific opioid receptor antagonist naloxone completely abolished this effect. This correlated with a concentration-dependent increase in the production of reactive oxygen intermediates, as shown by a 10% (met-enkephalin) and 15% (β-endorphin₁₋₃₁) increase in MFI (Table 1). To clarify opiate receptor specificity further, neutrophils were incubated with the µ-selective antagonist CTOP or the δ-selective antagonist naltrindole before opioid peptides. CTOP reversed none of the opioid peptide-induced stimulatory effects on neutrophil function, whereas naltrindole antagonized these effects completely, indicating a δ-opiate receptor-mediated effect on neutrophils. In control experiments, incubation with naloxone, naltrindole or CTOP alone had no effect on surface receptor expression or neutrophil function (data not shown). To elucidate the binding domain of β-endorphin₁₋₃₁ on neutrophils, we incubated whole blood with different concentrations of the C-terminal fragment β-endorphin₂₇₋₃₁. This fragment did not alter neutrophil function (data not shown).
The synthetic δ opioid receptor agonists DADLE and DPDPE produced an increase in neutrophil receptor expression, with a peak effect at 10^{-7} M (Tables 2 and 3). After incubation with DADLE or DPDPE, receptor expression was increased by up to 30%. This correlated with concentration-dependent stimulation of the production of reactive oxygen intermediates, as shown by an increase of up to 40% in oxidative burst activity (Tables 2 and 3). After 2.5 h incubation, receptor expression and the oxidative burst showed a small, non-significant increase in fluorescence compared with 10 min incubation. The δ opioid receptor-selective antagonist CTOP showed a partial inhibitory effect, whereas naltrindole and naloxone completely reversed the stimulation of receptor expression, indicating a δ opioid receptor-mediated effect.

**Discussion**

We demonstrated the effects of synthetic and endogenous opioid peptides on human neutrophils in a flow cytometric assay. Earlier studies revealed an effect of opioid peptides on granulocyte chemotaxis and phagocytosis using isolated cells. As we used a whole blood assay, preactivation of leukocytes by cell separation was avoided. Whereas it is not possible to determine interactions between different cell populations and the influence of plasma proteins in isolated cells, the whole blood assay provides physiological environment. CD16 and complement receptors are of special significance in opsonophagocytosis and the subsequent triggering of the oxidative burst. Apart from its involvement in phagocytosis of opsonized bacteria, CD11b/CD18 contributes to neutrophil aggregation and adhesion to the endothelial surface. CD16, a low-affinity IgG Fc receptor, is found in two forms, a transmembrane FcγRIIIa expressed by NK cells and monocytes and a phosphatidylinositol-linked FcγRIIIb present on neutrophils. CD11b/CD18, a β2-integrin acts as a tether for intercellular adhesion, together with changes in cytokines, chemokines and molecules upregulated on the surfaces of endothelial cells and other sites of inflammation. CD11b/CD18 was significantly stimulated after 10 min of incubation with the endogenous opioids β-endorphin, and met-enkephalin. The increase in complement receptor expression was completely abolished after preincubation with naloxone or naltrindole, suggesting a δ opioid receptor-mediated mechanism. Our data indicate that increased receptor expression may contribute substantially to opioid peptide-induced activation of neutrophil phagocytic and oxidative activity.
β-endorphin$_{1-31}$ did not alter neutrophil function, indicating that the effect of β-endorphin$_{1-31}$ on neutrophils is mediated via the N-terminal fragment, represented by met-enkephalin. Binding studies on monocytic U937 cells showed an affinity constant of 10$^{-8}$ M for the endogenous opioid peptide β-endorphin$_{1-31}$. In our study, the effect of met-enkephalin and β-endorphin$_{1-31}$ on neutrophil function occurred from 10$^{-7}$ to 10$^{-11}$ M, which correlates with this affinity constant. Neutral endopeptidase (CD10, NEP) is a zinc-metalloendopeptidase that cleaves a variety of substrates, such as enkephalins, substance P and bradykinin, after 10–15 min in vivo. For this reason we did not extend incubation time with the endogenous opioid peptides further than 10 min. Synthetic δ-opioid agonists are not cleaved through NEP, so we incubated whole blood with DADLE and DPDPE for up to 2.5 h. Both peptides showed a concentration-dependent stimulatory effect on the oxidative burst and complement receptor expression on human neutrophils. This could be abolished by preincubation with naltrindole or naloxone, indicating that the effects of met-enkephalin, β-endorphin$_{1-31}$, DADLE and DPDPE on neutrophils are mediated, at least in part, through δ rather than μ opioid receptors. However, the μ-selective antagonist CTOP partly inhibited the stimulatory effects of DADLE and DPDPE, whereas incubation with the endogenous peptides met-enkephalin and β-endorphin$_{1-31}$ had no effect. One could hypothesize that this may have been caused by non-selective effects of DADLE and DPDPE on neutrophil μ-receptors. Both receptor types are known to be expressed by leucocytes, and their differential ability to interact with chemokine receptors suggests the possibility of using specific receptor agonists as potential modulators of inflammation. Recent data have shown that synthetic opioid peptides may have beneficial effects, e.g. in cardiac ischaemic preconditioning and infarction. Though the results of our experiments are statistically significant and highly reproducible, we are well aware that the numerical increases in neutrophil receptor expression and oxidative burst are rather small. We hypothesize that the increase of 10–40% in neutrophil activation does reflect a physiologically relevant situation in humans. One should not expect an all-or-nothing phenomenon with an overwhelming response to elevated opioid peptide concentrations. This scenario can be observed in patients suffering from a massive bacterial invasion leading to severe immunological dysfunction, e.g. a systemic inflammatory response syndrome. In contrast to previous studies, we measured baseline levels of neutrophil surface receptor expression without prestimulation. In our study, oxidative burst activity was induced using vital E. coli bacteria, representing a naturally occurring pathogen. Earlier studies used very potent but synthetic stimuli (e.g. fMLP, PMA) for neutrophil activation. Therefore we believe that the present data, obtained by the use of a whole blood assay and vital bacteria for stimulation, represents a physiological model of neutrophil interaction. Further research on the clinical use of synthetic opioid peptides and their effect on immunocytes may contribute to better understanding of the pathophysiology of inflammation.

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