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Translational Research

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Evidence for nociceptin/orphanin FQ (NOP) but *not* μ (MOP), δ (DOP) or κ (KOP) opioid receptor mRNA in whole human blood

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Abstract

Background: While it is well known that opioids depress the immune system, the site(s) of action for this depression is highly controversial. Immune modulation could occur directly at the immune cell or centrally via the hypothalamic-pituitary-adrenal axis. In a number of studies using individual enriched immune cell populations we have failed to detect classical μ (MOP), δ (DOP) and κ (KOP) receptors. The non-classical nociceptin/orphanin FQ (N/OFQ) receptor (NOP) is expressed on all cells examined thus far. Our hypothesis was that immune cells do not express classical opioid receptors and that using whole blood would definitively answer this question.

Methods: Whole blood (containing all immune cell types) was incubated with opioids (morphine and fentanyl) commonly encountered in anaesthesia and with agents mimicking sepsis [lipopolysaccharide (LPS) and peptidoglycan G (PepG)]. Opioid receptor mRNA expression was assessed by endpoint polymerase chain reaction (PCR) with gel visualisation and quantitative PCR.

Results: Classical MOP, DOP, and KOP receptors were not detected in any of the samples tested either at rest or when challenged with opioids, LPS or PepG. Commercial primers for DOP did not perform well in quantitative PCR, so the absence of expression was confirmed using a traditional gel-based approach. NOP receptors were detected in all samples; expression was unaffected by opioids and reduced by LPS/PepG combinations.

Conclusions: Classical opioid receptors are not expressed on circulating immune cells.

Key words: immune cells; morphine; nociceptin; opioid receptors; PCR

Opioid receptors are classified as both classical [μ (MOP), δ (DOP), and κ (KOP)] and non-classical [nociceptin/orphanin FQ peptide (NOP)] receptors. A causal link between opioids and immune function has been historically presumed, based on observations of opioid addicts having an increased incidence and severity of infections.[1 2] More recent evidence highlights how opioids can have an effect on endocrine and immune function.[3] However, the immunomodulatory effects of opioids have not been determined precisely, with various opioid drugs having different effects on immune function, despite targeting a single receptor subtype, the MOP receptor.[4–6] Moreover, elucidating the mechanism(s) by which opioids modulate immune function is confounded by interspecies differences in their immunomodulatory effects. Hence opioid immunological interactions are both drug and species dependent. In MOP receptor knockout animal studies, no opioid immune modulation is

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Editor's key points

- The site of action of opioids in depressing immune function remains controversial.
- Authors incubated whole blood with opioids or the mediators of sepsis.
- Classical MOP, DOP, and KOP receptors were not detected in any of the samples tested either at rest or when challenged with opioids or the mediators of sepsis.
- The study provides strong evidence that classical opioid receptors are not expressed on circulating immune cells.

seen, providing robust evidence that this receptor is the biological target.[7 8]

One of the proposed mechanisms for the immunomodulatory action of opioids is a direct action on immunocytes; however, there is huge controversy as to whether classical opioid receptors are expressed on these cells. While there is still a strong belief that peripherally mediated immunomodulatory effects of opioids are facilitated through receptors expressed on immunocytes, and several studies have determined the presence of opioid receptors on peripheral blood mononuclear cells (PBMCs), our group has been unable to detect classical opioid receptor expression on immunocytes in several settings and using a series of detection methodologies, including fluorescent/immunofluorescent labelling, quantitative polymerase chain reaction (PCR) and western blotting.[9–13] Previous studies have focused on individual populations of immune cell types. In one study, NOP receptor mRNA and mRNA for the precursor protein of N/OFQ (ppNOC) was differentially detected in granulocytes, monocytes, and lymphocytes; MOP receptor mRNA was not detected in any cell type.[11–15] We therefore aimed to take a more systematic approach using several PCR methodologies to investigate opioid receptor expression in unstimulated blood from healthy volunteers. It is possible that opioid treatment or the presence of sepsis might upregulate expression, so we additionally treated whole blood with supraclinical concentrations of morphine and fentanyl and lipopolysaccharide (LPS) and peptidoglycan G (PepG), agents commonly used to mimic sepsis.

Our hypothesis was that immune cells do not express classical opioid receptors and that using whole blood would definitively answer this question.

General methodology

Blood collection

Venous blood was collected from 10 healthy volunteers from the Department of Cardiovascular Sciences, Division of Anaesthesia, Critical Care and Pain Management, with approval from the University of Leicester (volunteer) Research Ethics Committee and with informed consent. Up to 30 ml of venous blood was collected using EDTA-monovette® tubes and divided into sterile 2 ml tubes to which LPS from *Escherichia coli* 0111:B4 (Sigma, Dorset, UK) 5 μg ml^{-1}, *Staphylococcus aureus* PepG (Sigma, Dorset, UK) 20 μg ml^{-1}, fentanyl 10 μM (Tocris Bioscience, Bristol, UK), and morphine 10 μM (Sigma, Dorset, UK) were added separately and in combinations as indicated in the results. Samples were incubated for 24 h at 37.0°C in 5% CO_2 humidified air.

Tissue culture

Positive control samples were prepared using Chinese hamster ovary cells (CHO) expressing human KOP, DOP, and NOP receptors and human embryonic kidney (HEK) cells expressing human MOP. $CHO_{hKOP/DOP}$ cells were cultured in Hams F12, CHO_{hNOP} in DMEM/Hams F12 (1:1), and HEK_{hMOP} cells in MEM, and all media were supplemented with 10% FCS, penicillin (100 IU ml^{-1}), Fungizone (2.5 μg ml^{-1}), and streptomycin (100 μg ml^{-1}). For maintaining expression of inserted genes, stock cultures of cells were additionally supplemented with 200 μg ml^{-1} of G418 for classical opioid receptors MOP/KOP/DOP, and 200 μg ml^{-1} of G418 and Hygromycin B for CHO_{hNOP}. Raji cells (lymphoblast-like cell) were used as an additional cell expressing DOP and cultured in RMPI 1640 supplemented with 10% FCS, Penicillin (100 IU ml^{-1}), Fungizone (2.5 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and glutamine (2 mM). All cells were cultured at 37.0°C in 5% CO_2 humidified air. All tissue culture media were from Sigma (UK) and supplements were from Thermo Fisher, Paisley (UK).

RNA extraction, copy DNA (cDNA) synthesis, and quantitative PCR (qPCR)

Total RNA was purified from whole blood samples using a RiboPure™ RNA Purification Kit (Thermo Fisher Scientific, UK) as per the manufacturer's instructions and from CHO cells and Raji cells using a preparatory RNA isolation kit (*mir*Vana, Thermo Fisher Scientific) in which 1 ml of *mir*Vana lysis buffer was used for extraction of RNA from confluent 25 cm^2 flasks for adherent cells or 1×10^7 for non-adherent cells. In all cases, final RNA samples were resuspended in PCR-grade water, the mass of RNA determined using a NanoDrop 2000 (LabTech) and purity assessed from the 260/280 nm ratio, which was >1.8. Isolated RNA was processed using a Turbo DNA-*free*® kit for the removal of possible genomic DNA (gDNA) contamination before reverse transcription and production of cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK). For some samples, additional production of cDNA using the Quantitect Reverse Transcription Kit (Qiagen, UK), which included an additional genomic DNA elimination reaction, took place. For all samples, a non-template control (in which the reverse transcriptase enzyme was omitted from reverse transcription reactions), was included during PCRs/qPCRs.

Statistical analysis

qPCR experimental data are expressed as cycle threshold (C_t; one cycle representing a doubling of starting material) relative to the geometric mean of two housekeeper gene C_t values; human β_2-microglobulin (β_2M) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) representing the ΔC_t. The effect of treatments on mRNA expression was determined by normalizing the target gene expression level using the equation $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ (treated)$-\Delta C_t$ (control). Data were analysed using Kruskal–Wallis analysis of variance (ANOVA) with Dunn's multiple comparison test. *P*-values <0.05 were considered statistically significant.

Methods and results

Volunteers included four females and six males, with a mean age of 40.6 years (range 32–54), mean weight 86.4 kg (range 55–110) and mean height 174.9 cm (range 153–196).

MOP/KOP

Whole blood was collected and incubated overnight with the inflammatory mediators LPS and PepG in the absence and presence