

ORIGINAL ARTICLE

Inhibitory effect of opiates on LPS mediated release of TNF and IL-8

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Abstract

Most patients with advanced cancer experience severe pain and are often treated with opiates. Cancer patients are especially susceptible to opportunistic infections due to treatment with immunosuppressive and cytostatic drugs. Since opiates have been demonstrated to have immunomodulatory effects, it is of clinical importance to evaluate potential differences between commonly used opiates with regard to their effect on the immune system. The aim of this study was to evaluate the effect of morphine, tramadol, fentanyl and ketobemidone on the functioning of the immune system with special reference to TNF and IL-8 release. *Method.* U-937 cells were preincubated with different concentrations of opioids followed by stimulation with LPS 100 µg/ml for three hours. The effect of opioids on the levels of cytokine mRNA was studied using RT-PCR. Erk and Akt phosphorylation was also measured by Western blot. *Results.* All opioids with the exception of fentanyl were capable of inhibiting TNF release from U-937 cells. Morphine had no effect on IL-8 release but the effect of other opiates was almost the same as the effect on TNF. All opioids with the exception of fentanyl were capable of inhibiting production of mRNA for TNF and IL-8. The observed effects of opiates were not always reversible by naloxone, suggesting that the effects might be mediated by other receptors or through a non-receptor mediated direct effect. Although preliminary evidence suggests the involvement of Erk and Akt pathways, further studies are needed to unravel the intracellular pathways involved in mediating the effects of opiates. Our data suggests that the order of potency with regard to inhibition of cytokine release is as follows: tramadol > ketobemidone > morphine > fentanyl. *Conclusion.* Further studies are needed to understand the clinical implications of the observed immunosuppressive effects of tramadol and ketobemidone and to improve opioid treatment strategies in patients with cancer.

Many patients with advanced cancer experience severe pain. Roughly 40–70% of cancer patients have chronic pain, often due to the cancer itself, e.g. as a result of tumor infiltration of a nerve plexus but also as an adverse effect of the anticancer therapy. Inflammation is mediated by a variety of soluble factors that are involved in acute inflammation such as IL-1, IL-2,

TNF- α , IL-6, IL-11, IL-8, GCSF, and GM-CSF. Animal models suggest that cytokines, especially tumor necrosis factor (TNF), play an important role in mediating inflammation and are also involved in promoting pain and increasing pain severity [1]. TNF is involved in many inflammatory responses and mediates its effect through activation of members of

the tumor necrosis factor receptor (TNFR) superfamily. TNF is thereby involved in a variety of physiological processes including hematopoiesis, immune surveillance and tumor regression [2]. TNF- α also triggers synthesis, surface expression and repositioning of several adhesion molecules. Furthermore, TNF acts as a strong chemoattractant for neutrophils, and enhances resorption of bone and articular cartilage [3]. Chemokines are responsible for the chemotactic migration and activation of leukocytes, especially neutrophils and are therefore important mediators of acute and chronic inflammation [4]. IL-8, recently renamed as CXCL8, a member of the CXC family of chemokines, is produced by a variety of cells including epithelial cells, macrophages and endothelial cells. There is now ample evidence suggesting that pro-inflammatory cytokines and chemokines like TNF and IL-8 have a role in carcinogenesis by promoting tumor growth, angiogenesis, metastasis and influx of inflammatory cells [5].

Cytokines bind to specific cell-surface receptors on target cells and initiate a series of intracellular signal transduction pathways. Erk and Akt kinases are among those signaling pathways that are important for many processes in immune responses [6]. Akt protein kinases are able to induce protein synthesis pathways and Erk kinases are involved in all aspects of immune responses, from the initiation phase of innate immunity, to the activation of adaptive immunity and finally to cell death.

Opiates, more specifically morphine, are the mainstay of current pain relief. Opiates have various immunomodulatory effects and influence cytokine expression, phagocytic activity, natural killer cell activity and lymphocyte activity [7]. Studies *in vitro* have shown that micromolar concentrations of morphine can inhibit release of TNF and interferon from stimulated leukocytes [8]. Morphine also inhibits the phagocytic capacity of neutrophils, decreases the migration of macrophage by diminishing chemotaxis, and modulates macrophage function by leading it to apoptosis [9]. Other studies have demonstrated that administration of intrathecal morphine in patients undergoing hysterectomy had an inhibitory effect on natural killer (NK) cell activity [10].

Fentanyl has been reported to suppress cytokine production and decrease T-cell cytotoxic activity, B cell proliferation and NK cell activity [11]. In another study, intravenous fentanyl was reported to increase NK cell cytotoxicity and circulatory CD16 (+) lymphocytes in humans [12]. The effect of fentanyl on the immune response seemed to differ *in vitro* and *in vivo* [13].

Tramadol, in contrast to morphine, did not suppress cellular immune functions. Studies suggest that tramadol increases NK activity, lymphocyte

proliferation, interleukin-2 production and the number of phagocytes [14] but had no effect on phagocytic capacity [15].

Ketobemidone is synthetic opioid that is often used as an alternative to morphine in the Scandinavian countries. To our knowledge there is no documentation so far regarding the effect of ketobemidone on inflammatory components.

It is of significant clinical importance to evaluate potential differences between commonly used opiates with regard to their effect on the immune system. Cancer patients are especially susceptible to opportunistic infections immediately after a cytostatic cure due to severe leukopenia caused by high doses of cytostatic drugs. There are conflicting reports regarding the extent of immunosuppression induced by opioids. It is also not clear if all opioids induce the same degree of immunomodulation. According to a review opioids such as codeine, fentanyl and morphine seem to be potent immunosuppressive agents whereas tramadol, oxycodone and buprenorphine seem milder in comparison [16]. Although the analgesic effect is of paramount importance in choosing the right drug for pain relief, the effects of opiates on the immune system could also be taken into consideration in the future before choosing between equipotent alternatives.

The objective of this study is to evaluate the effect of opiates (morphine, tramadol, fentanyl and ketobemidone) on the functioning of the immune system with special reference to TNF and CXCL8 release. We also wanted to gain insights into the potential mechanisms involved in opioid induced immunomodulation.

Material and methods

Chemicals drugs and antibodies

Tradolan® (tramadol), Morfin® PEU (morphine), Ketobemidon HCl® PEU (ketobemidone) and Fentanyl® (fentanyl) are registered drugs and were obtained from the Hospital Pharmacy, University Hospital, Linköping, Sweden. Naloxon HCl (naloxone) powder Eur Kval D was obtained from Apoteket Produktion & Laboratorier (APL). Stock solutions were created in sterile physiological saline and serial dilutions were prepared in sterile water. The solutions were stored at +4°C until use.

PMA (phorbol 12-myristate 13-acetate) was diluted in sterile dimethyl sulphoxide (DMSO; Sigma, Stockholm, Sweden) to a stock concentration of 1 mM and was stored in aliquots at -20°C. Freeze-dried LPS extracted from *Salmonella typhimurium* (kindly provided by Karl-Eric Magnusson, Institute of Medicine and Surgery, Linköping University,

Sweden) was dissolved in DMSO and stock solutions of 4 mg/ml were prepared in sterile water and stored refrigerated. Lipopolysaccharide purified by trichloroacetic acid extraction [(LPS, L7261, Sigma-Aldrich, St. Louis, MO, USA) Sigma #L7261] was also purchased for later experiments. This was dissolved in water to a concentration of 5 mg/ml and stored refrigerated until use. The polyclonal antibodies specific to Akt, phospho-Akt (Ser 473), Erk1/2 (p44/42 MAP kinase), phospho-Erk1/2 (Tyr202/Tyr204) and the HRP-conjugated secondary polyclonal goat anti-rabbit antibody (#7074) were from Cell Signalling Technology Inc. (Beverly, MA, USA).

Cells and culture conditions

A serum-dependent sub clone (U-937) of the wild type human histiocytic lymphoma U-937 cell line was used in all experiments and kindly provided by Dr. K. Nilsson, Department of Genetics and Pathology, Uppsala University, Sweden. The human monocytoid cell line U-937 displays monocytic characteristics and has served as a robust in vitro model for the study of various aspects of monocyte and macrophage differentiation, intracellular signaling pathways and cytokine release. A recent study has confirmed that TNF and IL-8 are secreted by U-937 cells and more specifically that IL-8 secreted by U-937 cells seems to be involved in fibronectin expression in breast cancer cells by stimulating the PI3K/Akt pathway [17]. Cells were cultivated in 25 cm²-cell culture flasks (Costar, Cambridge, MA, USA) in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 nM L-glutamine, penicillin (50 units/ml) and streptomycin (50 µg/ml). Cells were incubated at 37°C in humidified air containing 5% CO₂ and sub-cultivated twice weekly at a seeding density of 3×10^5 cells/ml. Viability of the cells as assessed by the exclusion of 0.4% trypan blue (Sigma Aldrich, Stockholm, Sweden).

Cell stimulation before ELISA analysis

Cells were always preincubated for one hour with fentanyl, ketobemidone, morphine or tramadol followed by stimulation with LPS for three hours. Naloxone was added one hour prior to treatment with opioids and LPS. In experiment with IL-4, cells were incubated with IL-4, for three and 24 hours.

Seeding density was always 4×10^5 cells and the volume of cell suspension 2 ml. After incubation, cell culture suspensions were transferred into test tubes for centrifugation at $212 \times g$ at 4°C for 10 minutes. Supernatants were stored in eppendorf tubes at -70°C until analysis.

TNF- ELISA

TNF release from U-937 cells was analyzed using an ELISA-kit for human TNF α from Mabtech AB (Stockholm, Sweden, # 3510-1H-20) according to the manufacturer's instructions. Tween was obtained from Bio-RAD (Hercules, CA) and Bovine Serum Albumin (BSA) from Saveen Werner (Malmö, Sweden). Substances were all dissolved in milliQ-water. Washing solution used was PBS-Tween (0.1% BSA, 0.05% Tween). Phosphate-buffered saline (PBS, pH 7.45, 137 mM NaCl, 2.7 mM KCl and 8.1 mM Na₂HPO₄·2H₂O), incubation buffer/blocking buffer (PBS-Tween containing 0.1% BSA) were prepared using milliQ-water. The substrate used for streptavidin-horse radish peroxidase was Enhanced K-blue®, a ready to use substrate from Neogen Corporation (Lansing, MI, product # 308175), and stop solution was HCl (1 M), according to enclosed ELISA protocol.

Optical density was read at 450 nM in the ELISA plate reader (VERSAmix tunable microplate reader, Molecular Devices, Union City, CA, USA) using SOFTmax® PRO software v. 3.1 (Molecular Devices). According to the manufacturer this kit has a standard range of TNF α at 13–13000 pg/ml and a limit of detection at 8 pg/ml.

IL-8- ELISA

IL-8 release from U-937 cells was measured with an ELISA kit from R&D Systems (Minneapolis, USA, Cat. No DY208). Substrate and stop solution used was the same as were used in ELISA for TNF. Reagent diluent contained 0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20 nM Trizma base, 150 mM NaCl, pH 7.4, 0.2 µM filtered). Substances were all dissolved in milliQ-water and 0.2 µM filtered.

Optical density was read at 450 nM (with correction at 540 nM) using the same plate reader as mentioned above.

Real Time RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Cell stimulation before PCR. Cells were preincubated for one hour with fentanyl, ketobemidone, morphine or tramadol followed by LPS stimulation for three hours. The highest concentration of opioid used was morphine 1.5 mM, ketobemidone 1.75 mM, tramadol 4.2 mM and fentanyl 2.3 µM. Naloxone concentration was twice as much as respective opioid. Naloxone was added one hour prior to treatment with opioids and LPS. Seeding density was always 10^6 cells in 10 ml cell suspension.

RNA-extraction. RNA from U-937 cells were isolated using QIAamp RNA Blood Mini Kit (cat. No 74104) following the instructions given by the manufacturer. After incubation, cell culture suspensions were transferred into test tubes for centrifugation at $212 \times g$ at 4°C for 10 minutes. Pellets were washed in 10 ml of cold PBS ($212 \times g$, 4°C , 10 minutes), followed by wash in 1 ml cold PBS (13000 rpm, 4°C , 10 minutes).

PBS was removed and the pellet was resuspended in 350 μl RLT buffer in order to lyse the cells. Isolated RNA was stored at -70°C until future use.

cDNA synthesis. cDNA was reverse transcribed from total RNA samples using random primers from the High-Capacity cDNA Archive Kit (Applied Biosystems, 4368813). A mixture of $10 \times \text{RT-buffer}$ (5 μl), $25 \times \text{dNTP}$ (2 μl), $10 \times \text{Random hex}$ (5 μl), Multiscribe (2.5 μl), Nuclease free water (10.5 μl) and 1 μg RNA (25 μl) were prepared for each sample. The mixture was gently mixed and placed in Eppendorf Mastercycler personal. Amplification conditions were 25°C for 10 minutes followed by 37°C for 120 minutes. The cDNA was stored at -20°C until RT-PCR.

Quantification of mRNA expression. Before cDNA synthesis the amount of isolated RNA was measured using the NanoDrop ND-1000 Spectrophotometer (Saveen Werner AB, Malmö, Sweden). The ratio of absorbance was from 260 nM to 280 nM. One microgram of RNA was reverse transcribed in a total volume of 25 μl . The Applied Biosystems 7500 Fast Real-Time PCR System was used for real-time PCR.

The gene expression assays had a FAM receptor dye at the 5' end of the TaqMan[®] MGB probe and a non-fluorescent quencher at the 3' end of the probe. The TaqMan[®] MGB probes and primers was $20 \times \text{mix}$. TaqMan primer pairs for TNF (art. Nr. Hs00174128_m1), IL-8 (art. nr. Hs00174103_m1), OPRM1 (art. Nr. HS 01053957_m1), and human GAPDH (art. Nr. 4333764F) were used. GAPDH was selected as the internal standard for the target gene. Relative quantification of TNF and IL-8 mRNA was accomplished using the standard-curve method, comparing it with the gene expression of the housekeeping gene GAPDH. Five serial dilutions of untreated samples in duplicate were used to plot standard curves for both TNF and IL-8. Fifteen microliters mixture containing 7.5 μl of a commercially available master mix ($2 \times \text{TaqMan universal PCR Mastermix}$, Applied Biosystems Inc.), 0.75 μl of $20 \times \text{target genes mix}$ (Taqman gene expression assay), 5.55 μl RNAase-free water and 1.2 μl cDNA of samples

were quantified in each well. All samples were assayed in triplicate in a 96-well optical plate which was covered with ABI PRISMTM optical caps. In addition, a non-template control (NTC) was included in triplicate for both genes on every plate, containing all constituents except the samples. The amplification conditions were the same for target genes and GAPDH: 2 minutes at 50°C , 10 minutes at 95°C , 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C . The log-input amount ($\times$) for unknown samples was calculated for both TNF and IL-8 using the equation from the standard curve. The equation was $y = mx + b$, where b is the Y-intercept of the standard curve, m for the slope of the standard curve and Y is the CT value of the sample.

Experimental settings for kinase experiments

For the cell stimulation assay U-937 cells were seeded in 10-cm dishes at a concentration of 0.4×10^6 cells/ml and incubated with morphine (1.55 mM), ketobemidone (1.75 mM), tramadol (4.20 mM) or fentanyl (2.25 mM) for one hour. LPS (0.1 mg/ml) was then added and incubated for 15 minutes. At the end of the incubation periods, cells were washed three times with ice-cold TBS [10 mM Tris-HCl (pH 7.5) and 100 mM NaCl]. They were then lysed for 20 minutes at 4°C in RIPA buffer [65 mM Tris-HCl (pH 7.5), 155 mM NaCl, 2.5 mM sodiumdeoxycholate, 1% NP40, 1 mM EDTA, 60 μM NaF, and 60 μM Na3Va4] supplemented with protease inhibitor cocktail (Sigma-Aldrich Inc.: 2 mM AEBSF, 1.6 μM aprotinin, 42 μM leupeptin, 78 μM bestatin, 29 μM pepstatin and 27 μM E-64). The cell lysate was collected, passed 10 times through a 16-gauge needle and then cleared by centrifugation three times at $13\,000 \times g$ for 5 minutes each at 4°C . Total protein concentration was determined by colorimetric detection and quantification using the Pierce[®] BCA Protein Assay Kit (Thermo Scientific, USA).

Western blot analysis

Protein extracts were denatured by boiling for 4 minutes in the presence of Laemmli sample buffer, separated by electrophoresis in 12% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride 0.45 μM transfer membranes (Pall Corporation, Pensacola, FL, USA). The membranes were blocked in TBST (TBS containing 0.1% Tween 20) with 5% non-fat dry milk for one hour at room temperature and then incubated overnight at 4°C with TBST 5% BSA containing antibodies directed towards phospho-Akt (1:1000),

Akt (1:1000), phospho-Erk1/2 (1:1000), Erk1/2 (1:1000). Incubation with primary antibody was ended by washing the membranes six times for 10 minutes each at room temperature with TBST. The following incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) was performed for one hour at room temperature in TBST with 5% non-fat milk. The membranes were then washed six times in TBST for 10 minutes each, and immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Bioscience, Little Chalfont Buckinghamshire, UK).

Statistics

The mean score of TNF and IL-8 values was compared before and after treatment with each opioid in different concentrations. Each experiment was repeated three times in duplicate.

Distilled water was used as control. We used a general linear model one way ANOVA to investigate the difference within and between groups. $p < 0.05$ was considered to be statistically significant.

Results

Opioid induced inhibition of TNF release analysed by ELISA (Figure 1)

Earlier experiments showed that stimulation of U-937 cells with LPS 100 $\mu\text{g/ml}$ resulted in TNF release which peaked after three hours of incubation (data not shown). Cells were preincubated with four concentrations of commonly used opiates – ketobemidone, morphine, tramadol or fentanyl. Viability of the cells assessed in 0.4% trypan blue after incubation with opioids for one hour, was always equal to or more than 95% (data not shown). All opioids tested, except fentanyl, were capable of inhibiting TNF release from U-937 cells (Figure 1). There was a dose dependent inhibition of TNF release by ketobemidone, morphine and tramadol but not by fentanyl. The highest concentration of morphine tested (1.5 mM) significantly diminished TNF release (86% reduction, $p < 0.0001$) compared to samples incubated with LPS. The highest concentration of ketobemidone (1.75 mM) and tramadol (4.2 mM) almost abrogated TNF release reducing it by 96% ($p < 0.0001$) and

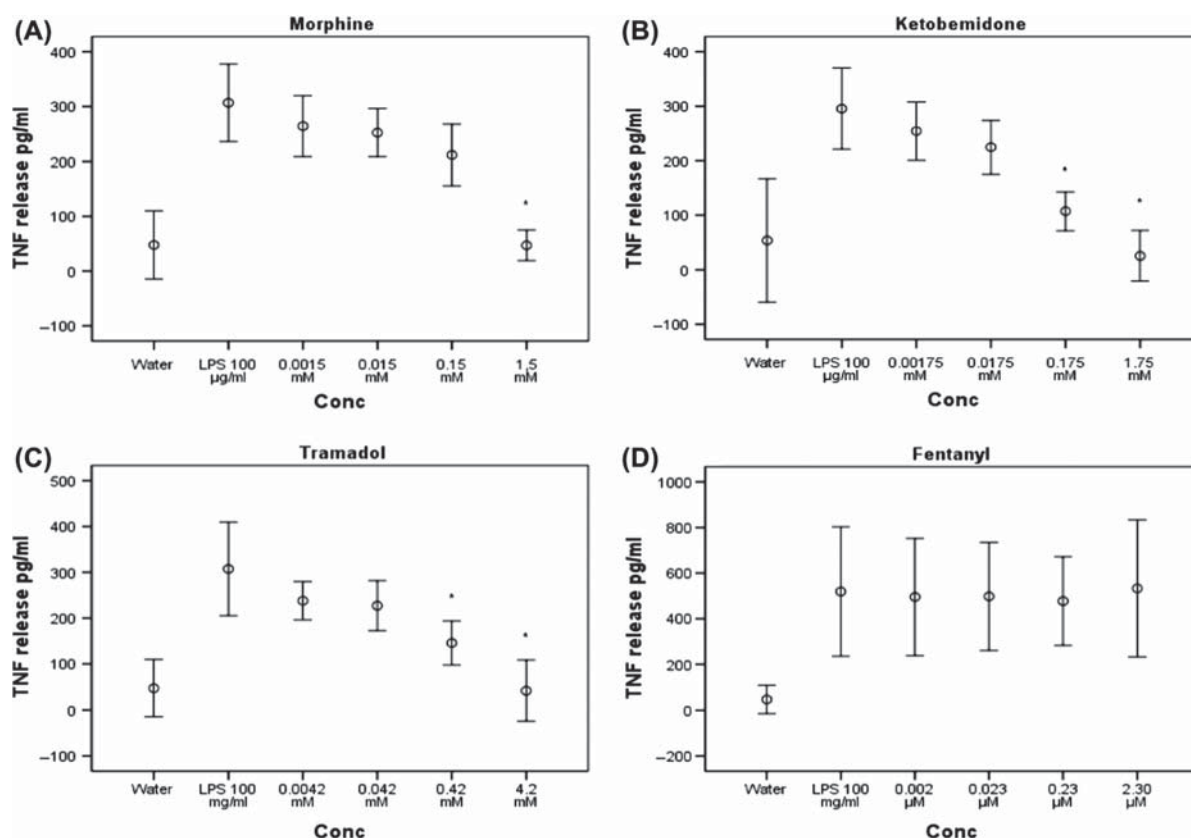


Figure 1. Dose-dependent effect of morphine (A), ketobemidone (B), tramadol (C) and fentanyl (D) on LPS dependent TNF release from U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 3 hours. TNF release was analyzed by ELISA. The value represents as a mean of six observations $\pm$ 95% CI. Two observations at each measurement time point and treatment group for all patients. P-value is shown in those cases that the difference between LPS and opioids was statistically significant. * $p < 0.05$ (+).

93% ($p < 0.0001$), respectively. Further, both, ketobemidone and tramadol showed significant inhibition of TNF release even at lower concentrations ($p < 0.0001$ and $p < 0.005$, respectively).

Opioid induced inhibition of IL-8 release analysed by ELISA (Figure 2)

Cell incubations with various agents were performed as described above. Although, IL-8 release upon LPS stimulation was also inhibited by opioids, there were distinct differences (Figure 2). Morphine had no effect on LPS stimulated IL-8 release unlike TNF-release. Similarly, fentanyl had no effect on effect on LPS stimulated IL-8 release, which was in line with results observed regarding TNF release. Both, ketobemidone and tramadol induced a dose dependent inhibition of IL-8 release. Although, both ketobemidone and tramadol did inhibit IL-8 release, the extent of inhibition was lower with the highest concentrations reducing IL-8 release by 47% and 51%,

respectively. LPS stimulated IL-8 release was significantly inhibited by the highest concentration of tramadol (4.2 mM, $p < 0.0001$) and ketobemidone (1.75 mM, $p < 0.0001$). Ketobemidone showed a significant reduction of IL-8 release at lower concentrations as well (0.175 mM, $p < 0.0001$ and 0.0175 mM, $p < 0.007$).

Comparison of potency with equivalent doses analyzed by ELISA (Figure 3)

Since adequate analgesia is observed with equipotent doses of all opioids tested, we wanted to further understand the relative potency of morphine, ketobemidone, tramadol and fentanyl with respect to cytokine release and potential differences in immunosuppression.

It was, however, difficult to obtain reliable data regarding equipotent doses. A therapeutic concentration in blood of respective opioid is essential to obtain adequate pain relief. According to Micro-medex, an established evidence-based clinical

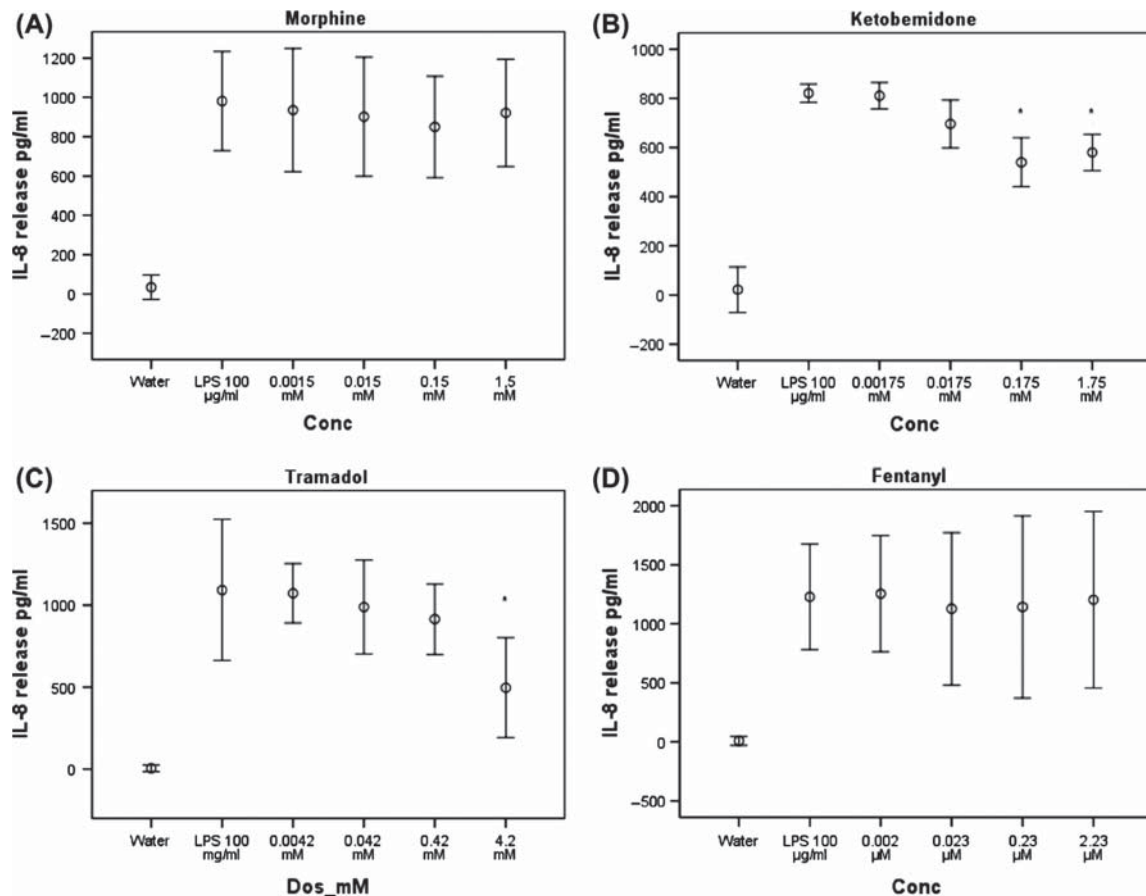


Figure 2. Dose-dependent effect of morphine (A), ketobemidone (B), tramadol (C) and fentanyl (D) on LPS dependent IL-8 release from U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 3 hours. IL-8 release was analyzed by ELISA. The value represents as a mean of six observations $\pm$ 95% CI. P-value is shown in those cases that the difference between LPS and opioids was statistically significant. * $p < 0.05$.

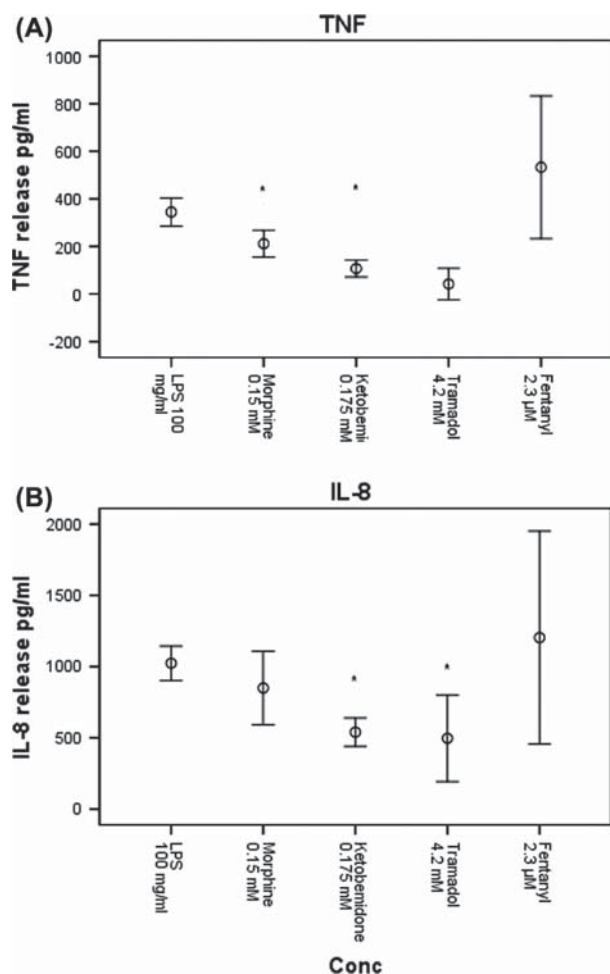


Figure 3. Effect of comparable concentration of morphine, ketobemidone, tramadol and fentanyl on LPS dependent TNF release (A) IL-8 release (B) from U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 3 hours. IL-8 release was analyzed by ELISA. The value represents as a mean of six observations $\pm$ 95% CI. P-value is shown in those cases that the difference between LPS and opioids was statistically significant. * $p < 0.05$.

reference database, 5–20 ng/ml (15–62 nM) for morphine and ketobemidone, 3–5 ng/ml (0.006–0.009 nM) for fentanyl and 100–600 ng/ml (0.3–2 μ M) for tramadol are normal therapeutic concentrations in blood [18]. Lack of reliable information regarding equipotent doses of the above opioids, forced us to use the relationships between concentrations of these drugs in blood as a measure of equipotency and have tried to compare the substances in the same concentration ratio as in vivo, as shown in Figure 3. We are aware that our approach has its limitations but might still be useful in forming a more objective opinion regarding relative potency. Our data suggests that the order of potency is similar for both cytokines and is as follows: tramadol > ketobemidone > morphine > fentanyl.

Intracellular effects of opioids on TNF and IL-8- mRNA

To further understand the molecular mechanisms involved in opioid induced inhibition of cytokine release we studied the effect of opioids on the levels of mRNA for both TNF and IL-8 using real-time RT-PCR.

RT-PCR analysis showed (Figures 4 and 5) that all opioids were capable of inhibiting production of mRNA for TNF. For morphine (1.5 mM), the inhibition was around 40%, for ketobemidone (1.75 mM) 27%, for tramadol (4.2 mM) nearly 50% and for fentanyl (2.3 μ M) the production of TNF-mRNA was paradoxically raised by 13%. Preincubation with naloxone (twice as much as respective opioids) resulted in a synergistic increase in the inhibition of TNF mRNA transcription for all opioids. Preincubation with naloxone gave a significant transcriptional inhibition more than 95% for Tramadol ($p < 0.01$). Similarly naloxone accentuated and resulted in a total inhibition of approximately 80% ($p < 0.06$) and 76% ($p < 0.08$) for morphine and ketobemidone, respectively. Naloxone reinforced inhibition of TNF mRNA very marginally only about 6%.

The mRNA production of IL-8 was also inhibited by all opioids but the reduction was almost the same for morphine, ketobemidone and tramadol, but lower compared to the inhibition seen for TNF mRNA. The reduction of mRNA production for IL-8 was around 34% for morphine, 38% for ketobemidone, and 36% for tramadol. Surprisingly fentanyl also decreased the mRNA production for IL-8 by 13%, a decrease which was not statistically significant. In contrast with the results from TNF-experiments, naloxone had divergent effects with respect to mRNA production for IL-8. On one hand, it reversed the inhibition of mRNA production when co-incubated with ketobemidone and morphine. On the other hand, it significantly reinforced the inhibition of mRNA production even for IL-8 when co-incubated with tramadol and fentanyl.

The immunosuppressive effects of glucocorticoids such as dexamethasone are well established. Dexamethasone has broad anti-inflammatory effects on multiple components of cellular immunity. In a separate series of experiments we compared the effect of these opioid with dexamethasone simultaneously, in order to further understand the relative effect of these opioids on the immune system.

The results (Figure 6) showed that dexamethasone induced a more potent inhibition and reduced the mRNA production of TNF by 80%, and IL-8 by 63%. It can be observed that morphine, ketobemidone and tramadol had roughly half of the effect of dexamethasone. Co-incubation of cells treated with

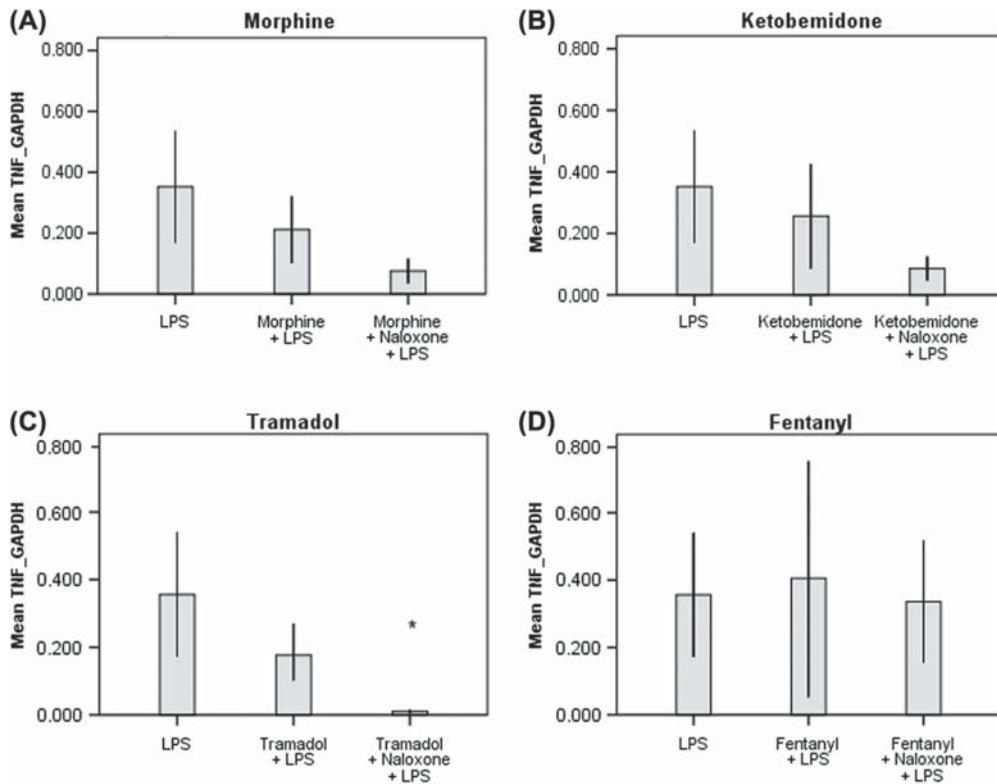


Figure 4. Real-time RT-PCR for TNF mRNA isolated from U-937-cells. The figure shows effect of morphine 1.5 mM (A), ketobemidone 1.75 mM (B), tramadol 4.2 mM (C) and fentanyl 2.3 μ M (D) on LPS stimulated U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 3 hours. The experiments were performed in triplicate on six different occasions. Values are represented as mean value $\pm$ 95% CI. Cells were preincubated with naloxone for 1 hour before LPS stimulation in experiments involving naloxone. Data are given as mean $\pm$ SE. P-value is shown in those cases that the difference between LPS and opioids was statistically significant.

morphine and ketobemidone with naloxone accentuated the inhibition of mRNA production for TNF as observed earlier. The combined effect was almost comparable to the effect of dexamethasone. Tramadol and naloxone together had reduced mRNA for TNF more than dexamethasone. Similar effects were observed for IL-8 mRNA production.

Stimulation with IL-4

It has been suggested that unstimulated U-937 cells do not express μ -opioid receptors, but if the cells are stimulated with 5 ng/ml IL-4 for 24 hour, μ -opioid receptor transcription is strongly induced. We incubated U937-cells with IL-4 for three and 24 hours and measured opioid receptor mRNA by real-time PCR. The samples were analyzed according the same protocol as was used for TNF and IL-8 analysis. The results showed no detectable amount of opioid receptor mRNA. To eliminate the unlikely possibility that U937 cells lack the gene for the opioid receptor, we genotyped the U937 cells. DNA for μ -receptor in U937-cells was genotyped by pyrosequencing method of an accredited laboratory at the Agency of Forensic Medicine which

showed that the gene for this receptor was present in the genome.

Opiates and intracellular signaling through Erk and Akt kinases

We studied the activation of Erk and Akt kinases to further understand the downstream intracellular signaling systems associated with opioid induced inhibition of cytokine mRNA production. Western blot experiments indicated that stimulation with LPS for 15 minutes reduced the phosphorylation of Erk whereas the phosphorylation of Akt was slightly increased during the same time period (Figure 7). There were distinct differences between the opioids with respect to their effect on Erk and Akt phosphorylation. Pre-incubation with morphine did not affect the LPS induced reduction of Erk phosphorylation. Tramadol, ketobemidone and fentanyl seemed to reverse the LPS induced reduction of Erk phosphorylation, albeit to different degrees. The pattern of phosphorylation was completely different with respect to Akt phosphorylation. Morphine and fentanyl had no effect on the increased Akt phosphorylation induced by LPS. This was in sharp contrast

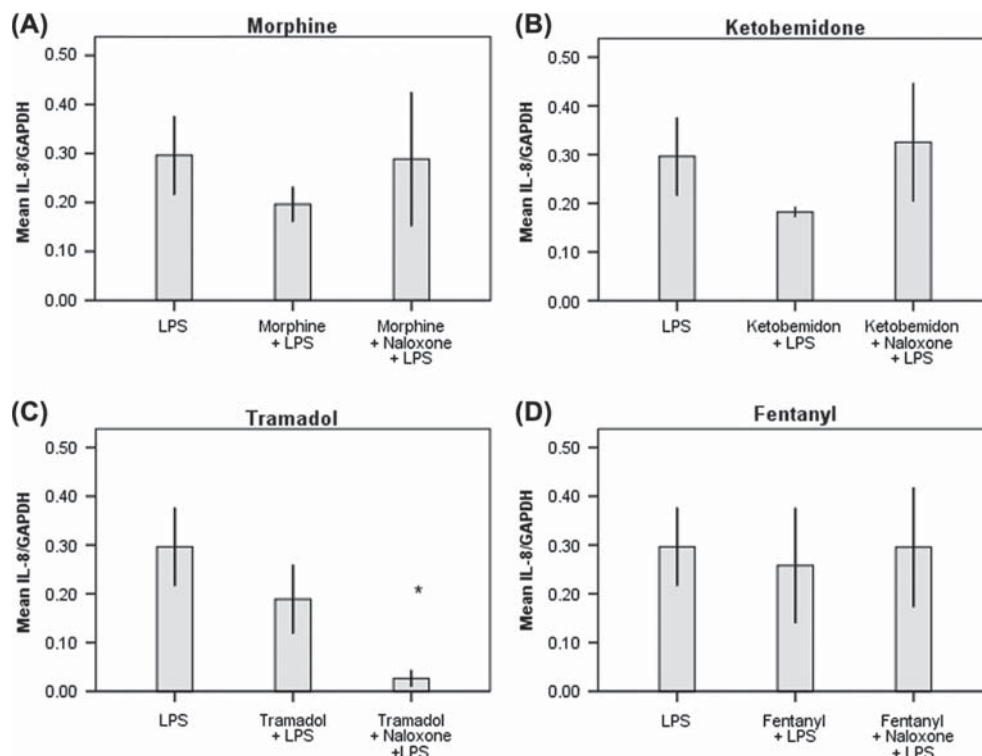


Figure 5. Real-time RT-PCR for IL-8 mRNA isolated from U-937-cells. The figure shows effect of morphine 1.5 mM (A), ketobemidone 1.75 mM (B), tramadol 4.2 mM (C) and fentanyl 2.3 μ M (D) on LPS stimulated U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 3 hours. In case Naloxone was used, the cells were preincubated with naloxone for 1 hour before LPS stimulation. The experiments were performed in triplicate on six different occasions. Values are represented as mean value $\pm$ 95% CI. P-value is shown in those cases that the difference between LPS and opioids was statistically significant.

to a clear and detectable inhibition of Akt phosphorylation induced by ketobemidone and Tramadol. Taken together, the data suggests that although LPS induces opposing effects on Erk and Akt phosphorylation, the opiates exhibit somewhat similar effects. Morphine had no detectable effect on changes in neither Erk nor Akt phosphorylation induced by LPS, whereas tramadol and ketobemidone were able to reverse the effects induced by LPS. Fentanyl reversed the LPS induced effect on Erk phosphorylation but had no effect on LPS induced changes in Akt phosphorylation.

Discussion

Opioids are the drug of choice for pain relief in a multitude of conditions including postoperative care and cancer. There are however significant differences in both effects and adverse effects of opiates in individual patients. Opiates have been demonstrated to have immunomodulatory effects. Further, the immunosuppressive effect of opiates have been suggested to be independent of the nociceptive effect but related to the specific structural modifications of the morphine molecule [1]. Since cancer patients are especially susceptible to opportunistic infections immediately after

a cytostatic course, due to severe leucopenia caused by high doses of cytostatic drugs, it is of significant clinical importance to evaluate potential differences between commonly used opiates with regard to their effect on the immune system. Very few studies have made simultaneous comparisons of commonly used analgesics in clinically relevant doses in the same experimental system.

In the present study we have focused on four of the most commonly used analgesics in Sweden – morphine, tramadol, fentanyl and ketobemidone. We have evaluated the effect of these opiates on the functioning of the immune system by studying their effect on LPS stimulated synthesis and release of TNF and IL-8. Preliminary experiments were performed using U-937 cells which were preincubated with different concentrations of ketobemidone, morphine, tramadol or fentanyl followed by stimulation with LPS 100 μ g/ml for three hours. Stimulation with 100 μ g/ml LPS showed a peak in TNF release after three hours of incubation (results not shown) and this time frame was used for stimulation in all subsequent experiments. All opioids, with the exception of fentanyl, were capable of inhibiting TNF release from U-937 cells. There was a clear, statistically significant, dose-response relationship with a maximum inhibition of

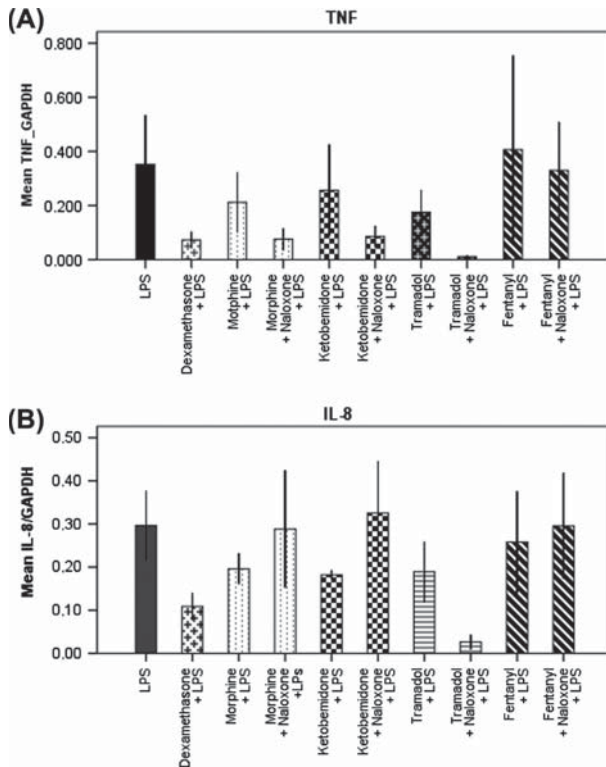


Figure 6. Real-time RT-PCR mRNA isolated from U-937-cells. The figure shows effect of dexamethasone and monensine on A) TNF mRNA, B) IL-8 mRNA on LPS stimulated U-937 cells compared with opioids. Cells were preincubated for 1 hour with the opioid, dexamethasone, monensine followed by LPS for 3 hours. In the case naloxone was used the cells were preincubated with naloxone for 1 hour before LPS stimulation. The experiments were performed in triplicate on six different occasions. Values are represented as mean value $\pm$ 95% CI.

about 80% occurring at millimolar levels of tested substances. The effect of opiates on IL-8 release was slightly different than for TNF release. Morphine and fentanyl had no significant effect on IL-8 release whereas ketobemidone and tramadol resulted in a

significant reduction of IL-8 release. Our data suggests that the order of potency is similar for both cytokines and is as follows: tramadol > ketobemidone > morphine > fentanyl. We are not aware of any study that has compared the efficacy of these drugs at the same time. The relative potency tables presented in the literature are almost always based on indirect comparison of drugs or case-report based experiences of switching between drugs. In clinical practice, although the relative potency tables provide a rational basis for selecting the appropriate starting dose, there is always an associated risk for under and over dosage. Adjustment of doses after switching opioids according to these tables is not unusual. Lack of reliable information regarding equipotent doses of the above opioids, forced us to use the relationships between concentrations of these drugs in blood as a measure of equipotency and we have tried to compare the substances in the same concentration ratio as in vivo. Despite the limitations in our approach, an objective appraisal of relative potency is, in our opinion, both relevant and useful.

Previous studies have demonstrated that the immune modulating effects of opioids are a direct result of interaction of opiates with the opioid receptors – μ (μ), δ (δ) and κ (κ) [19]. However, later studies have demonstrated that other receptors such as serotonin, dopamine and NMDA receptors also play an important role in the regulation of the immune responses [19–21]. Both enhancement and reduction of immune response have been related to the activation of noradrenergic system [22]. In our study tramadol had the strongest effect on both TNF and IL-8 release. The mechanism of action of tramadol is not fully understood, but is believed to work through modulation of serotonin and norepinephrine in addition to its relatively-weak μ -opioid receptor agonism. This plausible double action on both μ -, serotonin- and norepinephrine receptors might explain the observed potency. Ketobemidone, a poorly documented opiate also acts through multiple receptors. It is structurally similar to other μ -opioid receptor agonists but is also postulated to have affinity for other opioid receptors such as δ - and κ -opioid receptors and an inhibitory effect on NMDA receptor as well [23]. Since both, fentanyl and morphine are highly selective for the μ -opioid receptors, they should theoretically exert similar effects on the immune system. However, the results of our study do not support this theory, but instead suggest the existence of an alternative mechanism of action. There is some evidence about the existence of a low-affinity, naloxone-insensitive morphine-binding site on human peripheral blood macrophages, granulocytes and monocytes, [24]. This receptor is apparently activated by morphine but not fentanyl [24],

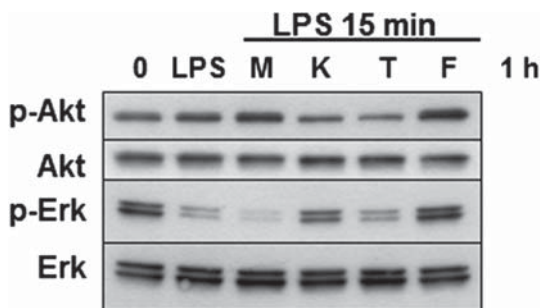


Figure 7. Effect of opiates on Erk and Akt kinases phosphorylation analyzed by Western blot. The figure shows effect of morphine 1.5 mM (M), ketobemidone 1.75 mM (K), tramadol 4.2 mM (T) and fentanyl 2.3 μ M (F) on LPS stimulated U-937 cells. Cells were preincubated for 1 hour with the opioid, followed by LPS for 15 minutes.

which could partly explain the lack of an inhibitory effect of fentanyl on TNF and IL-8 release observed in this study.

In order to further understand the intracellular activity of tested opiates we measured the mRNA production of TNF and IL-8 by RT-PCR. We used dexamethasone, a potent synthetic member of glucocorticoid, as an internal control. Dexamethasone inhibits signaling through the mitogen-activated protein kinase pathways (MAPK) that mediate the expression of numerous inflammatory genes [25]. Our results showed that morphine, ketobemidone and tramadol had an inhibitory effect on production of mRNA. In congruence with our earlier finding regarding release of cytokines, fentanyl had no effect on mRNA production for TNF or IL-8. We used naloxone, a stereospecific, competitive antagonist to opioid receptors, to further understand the intracellular pathways and the receptors involved in mediating the effect of opiates. Naloxone is used on a routine basis to reverse the effects of opioids in the clinics. In our experiments, naloxone failed to reverse the observed effects of opiates and paradoxically exhibited a synergistic effect on mRNA production for both TNF and IL-8. The synergistic inhibitory effect of naloxone and tramadol on cytokines mRNA production was equivalent to the effect observed with dexamethasone alone. There is considerable structural similarity between naloxone and other opioids which could account for the observed synergistic effect. It has been proposed that the μ -receptor is not expressed in U937-cells but can be upregulated by IL-4 [26]. Genotyping demonstrated that U937-cells do possess the gene for μ -receptor but we were not able to detect any mRNA for the receptor. Stimulation with IL-4 did not result in an amplification of opiate receptor mRNA. Taken together, it could be speculated that the effects observed in our study are mediated by other naloxone-insensitive receptor/s or through a direct non-receptor mediated effect of these substances.

Binding of opioids specifically morphine to the μ -receptor initiates a cascade of G-protein signaling events that lead to inhibition of cAMP which shuts down the protein kinase activity and decrease phosphorylation. Western blot experiments were performed to further understand the intracellular signaling pathways that might be involved in mediating the observed intracellular effects of opiates. A previous study suggested that activation/phosphorylation of Erk could be involved in mediating TNF release from stimulated U-937 cells [27]. LPS exhibited diametrically different effects on Erk and Akt phosphorylation. However, the effects of opiates were somewhat similar but not completely congruent. The present study suggests that Erk and Akt pathways

might be involved in mediating the effects of opiates. However, further carefully designed experiments involving specific signaling enhancement and inhibition are needed to dissect the intracellular pathways involved in mediating the effects of opiates including Erk and AKT pathways.

The results from his study need to be verified in other cancer cell lines and experiments involving human peripheral blood cells. The effect of opioids on other mediators of inflammation may be a subject for future studies.

Conclusions

The results of this study showed that tramadol and ketobemidone have an inhibitory effect on the release of TNF and IL-8 in LPS stimulated U937-cells. Fentanyl had no effect on either TNF or IL-8 release. Morphine had an inhibitory effect on TNF-release only. The order of potency with regard to inhibition of cytokine release is as follows: tramadol > ketobemidone > morphine > fentanyl. We have shown that opiates exert an intracellular effect at the mRNA level. However, since these effects were not always reversible by naloxone, we could postulate that these effects could be mediated by naloxone-insensitive receptor/s or through a direct non-receptor mediated effect of these substances. We have also presented preliminary evidence suggesting the involvement of Erk and Akt pathways in intracellular signaling. It is too early to conclude if these observed effects *in vitro* can be extrapolated to clinical practice. Further studies on other cell lines and other inflammatory factors are needed.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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