Anti-inflammatory Effects of Fentanyl and Morphine on LPS-induced TLR4 Neuroinflammatory Signaling

L.K. Figueroa-Hall, Ph.D., Department of Pharmacology & Physiology, Oklahoma State University Center for Health Sciences, Tulsa, OK

C.W. Stevens, Ph.D., Department of Pharmacology & Physiology, Oklahoma State University-Center for Health Sciences, Tulsa, OK

R.L. Davis, Ph.D., Department of Pharmacology & Physiology, Oklahoma State University-Center for Health Sciences, Tulsa, OK

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Abstract
Opioid receptors located in the central nervous system and periphery are activated by opioids, which are classified as analgesics and non-analgesics. While investigations show opioids may have more severe consequences on inflammation and neuroinflammation due to down-regulation of cellular functions, these findings remain debatable. With these controversial implications at the forefront, we chose to investigate the fentanyl and morphine-mediated effects on LPS-induced TLR4 neuroinflammatory signaling. CHME-5 microglial cells treated with LPS induced *mu* opioid receptor gene expression. Co-treatment with LPS and fentanyl or morphine significantly decreased LPS-induced IκBα activation, while only fentanyl decreased NF-κB binding activity. Furthermore, treatment with naltrexone did not reverse the fentanyl-mediated down-regulation of NF-κB binding activity. These findings indicate that fentanyl, and to a lesser extent morphine, display anti-inflammatory effects on LPS-induced TLR4 signaling.

Keywords: opioids, neuroinflammation, LPS, fentanyl, morphine

Abbreviations: *mu* opioid receptor (MOR), central nervous system (CNS), Toll-like receptor 4 (TLR4), interleukin-1β (IL-1β), Lipopolysaccharide (LPS), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), polyvinylidene fluoride (PVDF), serum-free media (SFM), bovine serum albumin (BSA), inhibitor of kappa light chain alpha (IκBα), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(**MTT), one-way analysis of variance (ANOVA), unstimulated (US)

Introduction
Opioids agonists are the most effective analgesics in use today[1,2]. Morphine, which is the prototypical agonist at the *mu* opioid receptor (MOR), is used clinically for both acute and chronic pain management[1]. Fentanyl, also with predominant activity at MOR[3], is extremely potent (100× more potent than morphine), very lipid soluble, and used clinically for pain management. While opioids are clinically effective, the risk of addiction is high, and several adverse effects are associated with opioids, including immunosuppression[4,5]. For instance, opioids modulate immunity by suppressing humoral and cell-mediated responses including inhibition of antimicrobial resistance, antibody production, cytokine expression, natural killer cell activity, monocyte-mediated phagocytosis, and neutrophil and monocyte chemotaxis[6,7]. Additionally, both acute and chronic administration

of opioids increase susceptibility of animals to bacterial and viral infections and decrease survival in tumor-bearing animals[8]. Immune suppression by opioids may also predispose individuals to opportunistic infections, such as bacterial pneumonia, tuberculosis, hepatitis, central nervous system (CNS) infections, abscesses, endocarditis, and AIDS[6,9]. Morphine, for example, modulates both the innate and adaptive immune systems, and the effects on innate immunity may have more severe consequences due to immune-depression of cellular functions including delay in leukocyte migration, decrease proliferative capacity of macrophage progenitor cells, suppression of macrophage phagocytosis, as well as inhibition of oxidative burst activity and chemotaxis[10-12].

While much is known about the effects of opioids on peripheral immune function, less is known about how immune function in the CNS is affected by opioids. Indeed, there is increasing evidence that opioids may induce neuroinflammation[13,14] . For example, morphine induces neuroinflammation via an extracellular toll-like receptor (TLR) 4 event, and not via the classic opioid receptors[14,15]. Morphine also induced an increase in interleukin-1β (IL-1β) in BV-2 murine microglial cells; this induction was reduced with administration of a TLR4 or MD2 small molecule inhibitor. These studies suggest that morphine and possibly other opioid agonists have the potential to cause neuroinflammation through a TLR4-dependent mechanism. In contrast, others demonstrated morphine-mediated suppression of lipopolysaccharide (LPS)-induced IL-6, TNFα, and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), which was not reversible with naloxone, suggesting the involvement of a non-classical opioid receptor site[10,16]. More recently, our group used HekBlue-hTLR4 cells to show that LPS-induced NF-κB activation was inhibited by opioid agonists, morphine and fentanyl, but these inhibitory effects were not reversed by opioid antagonists, naltrexone and beta-funaltrexamine[17,18]. Moreover, LPS-induced TLR4 activity was decreased in response to increasing doses of morphine and fentanyl[18]. Taken together, these studies indicate the potential for opioid agents, both agonists and antagonists, to impart anti-inflammatory properties through inhibition of pro-inflammatory immune functions.

There is evidence that microglia, along with other cells of the CNS, differentially express TLRs and opioid receptors, but the precise mechanisms by which opioids such as morphine and fentanyl modulate TLR signaling are not well understood, and, the effect of opioids on neuroinflammation remains debatable. Therefore, defining the effects of fentanyl and morphine on TLR4 signaling mechanisms in microglial cells remains critical and has the potential to advance therapeutic strategies for neuroinflammatory processes involved in injury, neurodegenerative diseases, and cancer-associated pain. With these controversial implications at the forefront, we chose to investigate the opioid-mediated effects on LPS-induced TLR4 neuroinflammatory signaling using an immortalized microglial cell line, CHME-5. Based on our previous studies[17-19], we hypothesized that fentanyl and morphine would down-regulate LPS-induced TLR4 neuroinflammatory signaling in CHME-5 microglial cells.

Materials and Methods

Cells

CHME-5 rat microglia cells were obtained from Dr. Pierre Talbot, Quebec, Canada. As previously described[20], early passage (#4-6) CHME-5 cells were stored in liquid nitrogen and maintained in growth media at 37ºC, with 5% CO_2 after plating. For experimental assays, cells were plated in sterile 6-well culture plates at a concentration of 80,000 cells per well. Cells were passaged twice before being stimulated for experimental assays (cells were not used after passage 15). Once cells were ready, growth medium was aspirated from cells and replaced with serum-free media (SFM) for no less than 16 h at 37ºC. CHME-5 cells were stimulated with 1 μg/ml LPS *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO) or LPS (1 μg/mL) plus fentanyl citrate-1, 3, 10, 30, and 100 μM (Sigma-Aldrich), or morphine sulfate-1, 3, 10, 30, and 100 μM (Sigma-Aldrich), and/or naltrexone HCl-10, 30, and 100 μM (NIH-National Institute on Drug Abuse) and incubated at 37ºC for: RT-

PCR (3, 6, and 18 hours), p-IκBα immunoblot analyses, NF-κB binding assays, and MTT assays (10 minutes). Reagents were reconstituted as follows: LPS in sterile phosphate buffered saline (PBS), and fentanyl citrate, morphine sulfate, and naltrexone hydrochloride in sterile endotoxin-free water. For stimulation, all reagents were diluted in SFM for experimental treatments, and SFM was added to control wells.

Quantitative Real Time-Polymerase Chain Reaction (q RT-PCR) mRNA Expression

Total RNA was extracted as previously described[20] using the Trizol method. RNA concentrations were determined with a Nanodrop Spectrophotometer 1000 (ThermoFischer Scientific, Waltman, MA), validated with the 260/280 ratios between 1.8 and 2.0, and RNA reverse transcribed into complementary DNA (cDNA) using the Silverscript IV VILO Master Mix (Invitrogen). The cDNA was used as the template for qRT-PCR reaction using SYBR Green PCR Master Mix (ThermoFisher Scientific). The thermocycler (StepOne Real-Time PCR System; Applied Biosystems) settings for RT-PCR were as follows: 50ºC-2 min, 95ºC-2 min, (95ºC-15 sec, 60º-1 min for 40 cycles), 95ºC-15 sec, 60ºC-1 min, 95ºC-15 sec. *MOR* primers were as follows: *MOR*-Forward: 5'-CTC AGT TAC AGC CTA CCT AGT CCG C-3', 60.5ºC; Reverse: 5'-CCA TCA ACG TGG GAC AAG TTG AGC-3', 60.5ºC and *β-actin*-Forward: 5'-GAA GGA TTC CTA TGT GGG CGA CGA-3', 60.5ºC; Reverse: 5'-GAG CCA CAC GCA GCT CAT TGT AG-3', 60.3ºC. The relative amount of message was extrapolated using delta delta C_T values normalized to control (unstimulated) cells.

Immunoblot Analysis

Protein expression was determined with either whole cell lysates or cytoplasmic and nuclear extracts as previously described[20]. Protein extracts (50 μg protein) were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Polyvinylidene fluoride (PVDF) membranes were used for protein transfer and then membranes were rinsed and blocked in 5% bovine serum albumin (BSA) in 1× Tris-buffered saline-Tween for 2 h at 25ºC with rocking. Primary antibodies (phospho-IκBα-Ser32/36 (sc-847), total IκBα (sc-101713) purchased from Santa Cruz) were diluted in 5% BSA (1:1000) and membranes incubated at 4ºC, overnight. The alkaline phosphatase-linked rabbit antibody diluted in blocking buffer (1:5000) was added to membranes and rocked for 2 h at 25ºC and then washed three times with TBST for 20 min. Enhanced Chemifluorescence substrate (GE Healthcare Amersham, Pittsburgh, PA) was used to image blots using the Typhoon Scanner 9410.

NF-κB p65 Binding Activity

NF-κB activation was measured using the NF-κB p65 Transcription Factor Kit (ThermoFischer Scientific) according to manufacturer's instructions and as previously described[20]. Briefly, binding buffer was added to each well, which was pre-coated with the NF-κB binding consensus sequence and 10 μl of nuclear extract was added to each well, in duplicate. Following incubation for 1 h at 25ºC with mild agitation, wells were washed with wash buffer, primary antibody (1:1000) was added to each well, followed by a one-hour incubation at 25ºC, without agitation. Wells were washed and secondary antibody (1:10,000) was added to each well, followed by 1 h incubation at 25ºC, without agitation. Finally, wells were washed four times with chemiluminescent substrate added to each well. Chemiluminescence was measured immediately with a Nikon plate reader.

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine cell viability after treatment. Briefly, as previously described[20], fresh SFM (1 ml) was added to each well followed by addition of MTT. Cultures were then incubated at 37ºC for 45 min. Media was aspirated from each

well and 1.5 ml dimethyl sulfoxide added followed by rocking at 25ºC for 30 min. Absorbance was read at 492 nm with the Synergy 2 plate reader (Biotek Instruments).

Statistics
Image J Software (National Institutes of Health) was used to obtain the mean grey intensity of all immunoblots and the mean was taken for each time point. GraphPad Prism 7.0 was used for transformation, quantification, and graphing of all data. For statistical analysis of mRNA, protein expression, binding activity, and cell viability, one-way analysis of variance (ANOVA) with Dunnett's or Tukey's multiple comparison tests were used, unless otherwise stated in the results section. Kruskal-Wallis and Dunn's multiple comparison's non-parametric tests were used for data that failed the Bartlett's test and two Y=Log (Y) transformations. Significance was determined at $p<0.05$.

Results

To investigate LPS-induced mu opioid receptor (MOR) gene expression in CHME-5, cells were stimulated with LPS for 3, 6, and 18 hours. MOR gene expression in CHME-5 cells increased in response to LPS at 3 and 6 hours compared to unstimulated cells (Figure 1).

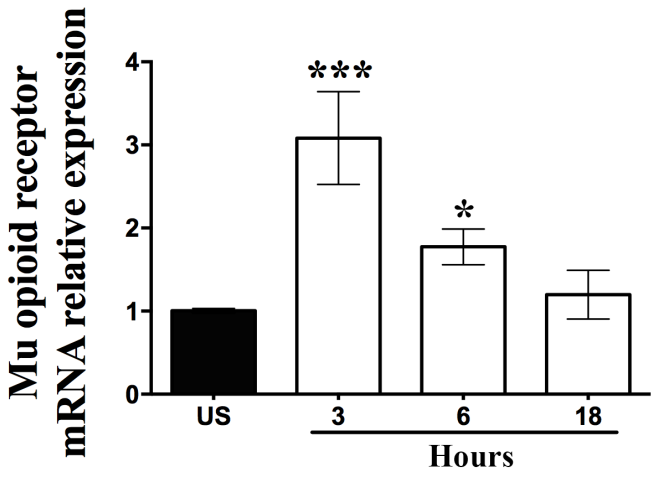


Figure 1. LPS-induced *mu* opioid receptor expression in CHME-5 cells
CHME-5 cells were stimulated with LPS (1 µg/ml) and incubated at 37ºC for 3, 6, and 18 hours. MOR mRNA expression was significantly increased at 3 h ($p<0.01$) and 6 h ($p<0.05$) compared to un-stimulated cells as analyzed by Kruskal-Wallis and Dunn's multiple comparison tests. Image is representative of three independent experiments (n=3) for each treatment group. Bars for all experiments are presented as mean ± SEM. ** $p<0.01$, * $p<0.05$ vs. unstimulated cells

IκBα activation is necessary for NF-κB translocation into the nucleus. Therefore we investigated the effects of fentanyl or morphine on LPS-induced IκBα activation. IκBα as measured by phosphorylation, was significantly decreased in cytoplasmic lysates with 100 µM fentanyl (Figure 2A-2B) and 1, 3, 10, 30, and 100 µM morphine (Figure 3A-3B), as compared to cells treated with LPS alone.

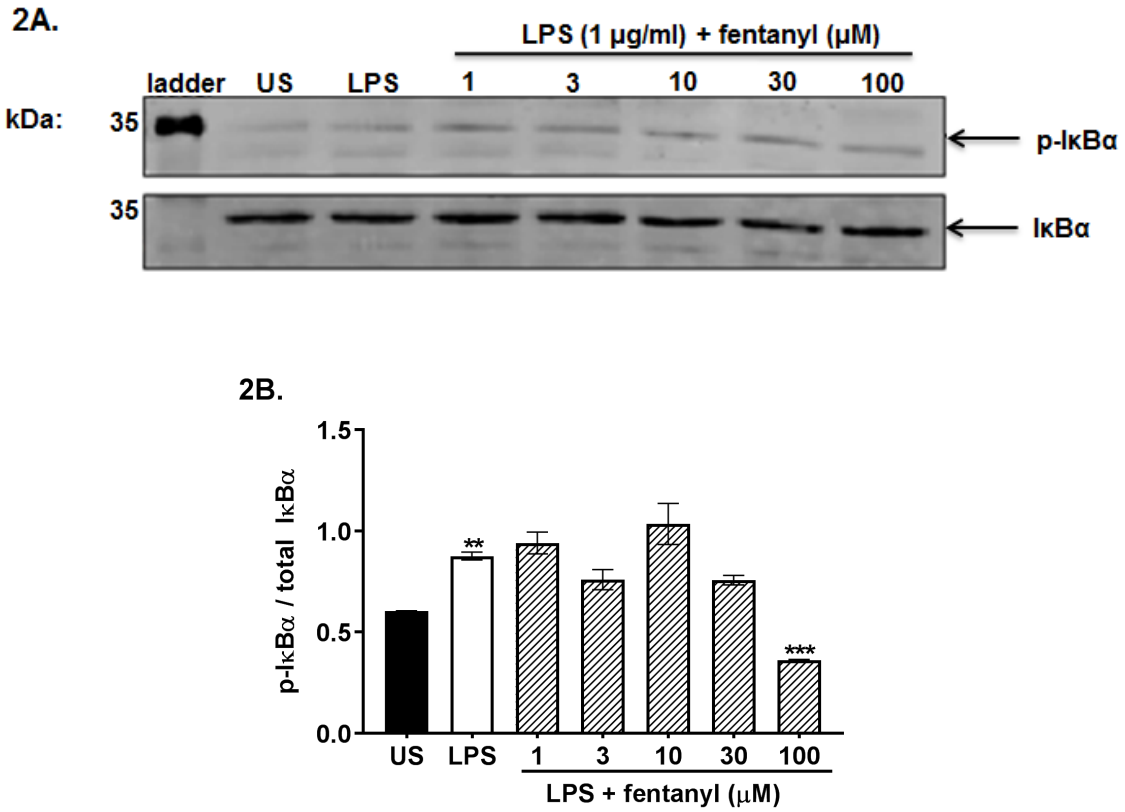
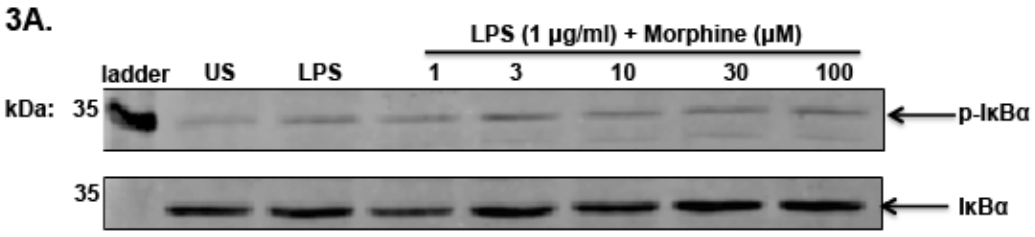


Figure 2. Fentanyl down-regulates LPS-induced IκBα activation in CHME-5 cells
CHME-5 cells were stimulated with LPS (1 µg/ml) and fentanyl (1, 3, 10, 30, or 100 µM) for 10 min. **A.** Cytoplasmic lysates were subjected to SDS-PAGE and immunoblotted with p-IκBα (1:1000), IκBα (1:1000), and β-tubulin (1:1000) antibodies. **B.** phospho-IκBα was quantified with IκBα and ANOVA and Dunnett's multiple comparison tests revealed a significant decrease with fentanyl at 100 µM ($p<0.001$). Images are representative of three independent experiments (n=3) for each treatment group. Bars for all experiments are presented as mean ± SEM. *** $p<0.001$, ** $p<0.05$ vs. cells treated with LPS alone



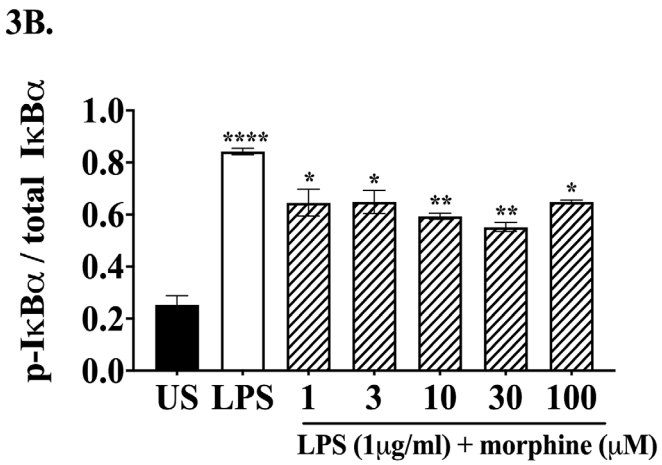
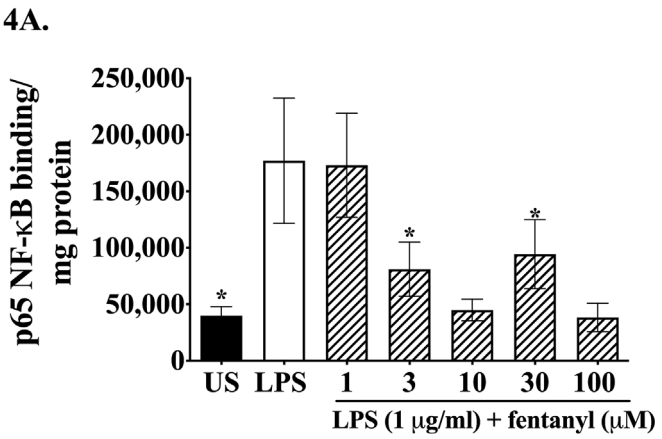


Figure 3. Morphine decreases LPS-induced IκBα activation in CHME-5 cells
CHME-5 cells were stimulated with or without LPS (1 μg/ml) and morphine (1, 3, 10, 30, or 100 μM) for 10 min. **A.** Cytoplasmic lysates were subjected to SDS-PAGE and immunoblotted with p-IκBα (1:1000), IκBα (1:1000), and β-tubulin (1:1000) antibodies. **B.** phospho-IκBα was quantified with IκBα and ANOVA and Dunnett's multiple comparison tests revealed a significant decrease in LPS-induced IκBα activation with morphine at 1 μM ($p<0.01$), 3 μM ($p<0.01$), 10 μM ($p<0.01$), 30 μM ($p<0.001$), and 100 μM ($p<0.01$). Images are representative of four independent experiments (n=4) for each treatment group. Bars for all experiments are presented as mean ± SEM. **** $p<0.0001$, ** $p<0.01$, * $p<0.05$ vs. cells treated with LPS alone

Upon LPS stimulation, NF-κB undergoes transactivation, which is necessary for the production of pro-inflammatory mediators. Therefore, we investigated the effects of fentanyl and morphine on LPS-induced p65 NF-κB binding activity. Fentanyl (10 μM or 100 μM) significantly decreased LPS-induced NF-κB p65 binding activity in nuclear lysates (Figure 4A), while there was no difference in response to morphine (Figure 4B).



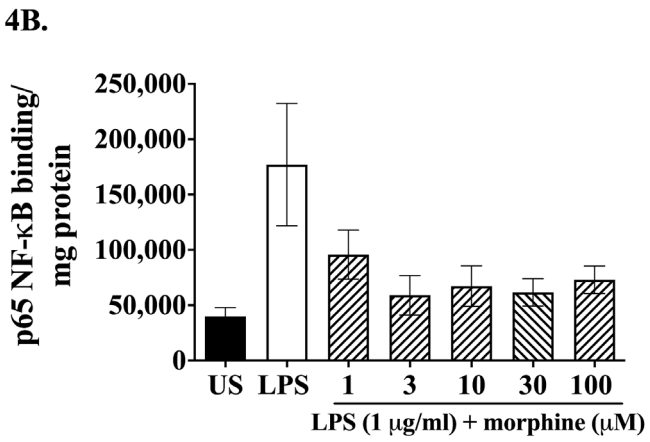


Figure 4. Fentanyl, not morphine, down-regulates LPS-induced NF-κB activity in CHME-5 cells
CHME-5 cells were stimulated with LPS (1 μg/ml) and fentanyl (1, 3, 10, 30, or 100 μM) for 10min. **A.** ANOVA and Dunnett's multiple comparison tests revealed significant decreases in NF-κB p65 binding activity in nuclear lysates with fentanyl at 10 μM ($p<0.05$) and 100 μM ($p<0.05$), as compared to cells treated with LPS alone. Image is representative of six independent experiments (n=6) for each treatment group. **B.** CHME-5 cells were stimulated with LPS (1 μg/ml) and morphine (1, 3, 10, 30, or 100 μM) for 10 min. ANOVA revealed no difference in NF-κB p65 binding activity ($p=1.00$) in nuclear lysates. Image is representative of six independent experiments (n=6) for each treatment group. Bars for all experiments are presented as mean ± SEM. *$p<0.05$ vs. cells treated with LPS alone

NF-κB p65 binding activity after treatment with LPS, fentanyl, and naltrexone led to a significantly decreased response with LPS and fentanyl co-treatment compared to cells treated with LPS alone, and no difference with naltrexone treatment compared to cells co-treated with LPS and fentanyl (Figure 5).

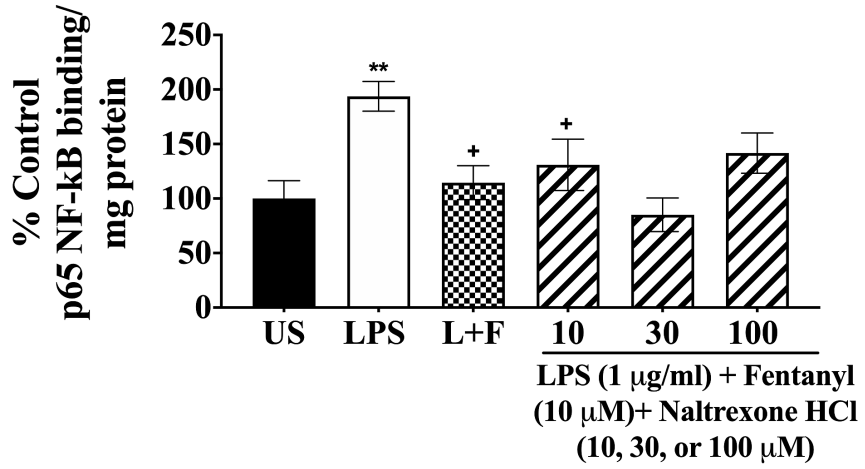


Figure 5. Naltrexone does not reverse fentanyl-mediated effect in CHME-5 cells
CHME-5 cells were stimulated with LPS (1 μg/ml), and fentanyl (10 μM), and naltrexone (1, 3, 10, 30, or 100 μM) for 10min. **A.** Nuclear extracts were analyzed for NF-κB p65 binding activity. ANOVA with Tukey's multiple comparison tests revealed a 1-fold decrease in LPS/fentanyl co-treated cells ($p<0.01$) and no difference in cells co-treated with naltrexone (10 μM: $p=1.0$; 30 μM: $p=0.90$; 100 μM; $p=0.30$), as compared to LPS/fentanyl co-treated cells. Image is representative of four independent experiments

(n=4) for each treatment group. Bars for all experiments are presented as mean ± SEM. ** $p<0.01$ vs. un-stimulated cells, ✚ $p<0.04$ vs. cells treated with LPS alone

To ensure that experimental treatments were not toxic to CHME-5 cells, cell viability assays were performed under different experimental conditions. The MTT assays revealed no significant differences between control and 1) LPS/fentanyl (Figure 6A), 2) LPS/morphine, (Figure 6B), and LPS/fentanyl/naltrexone (Figure 6C) co-treated cells.

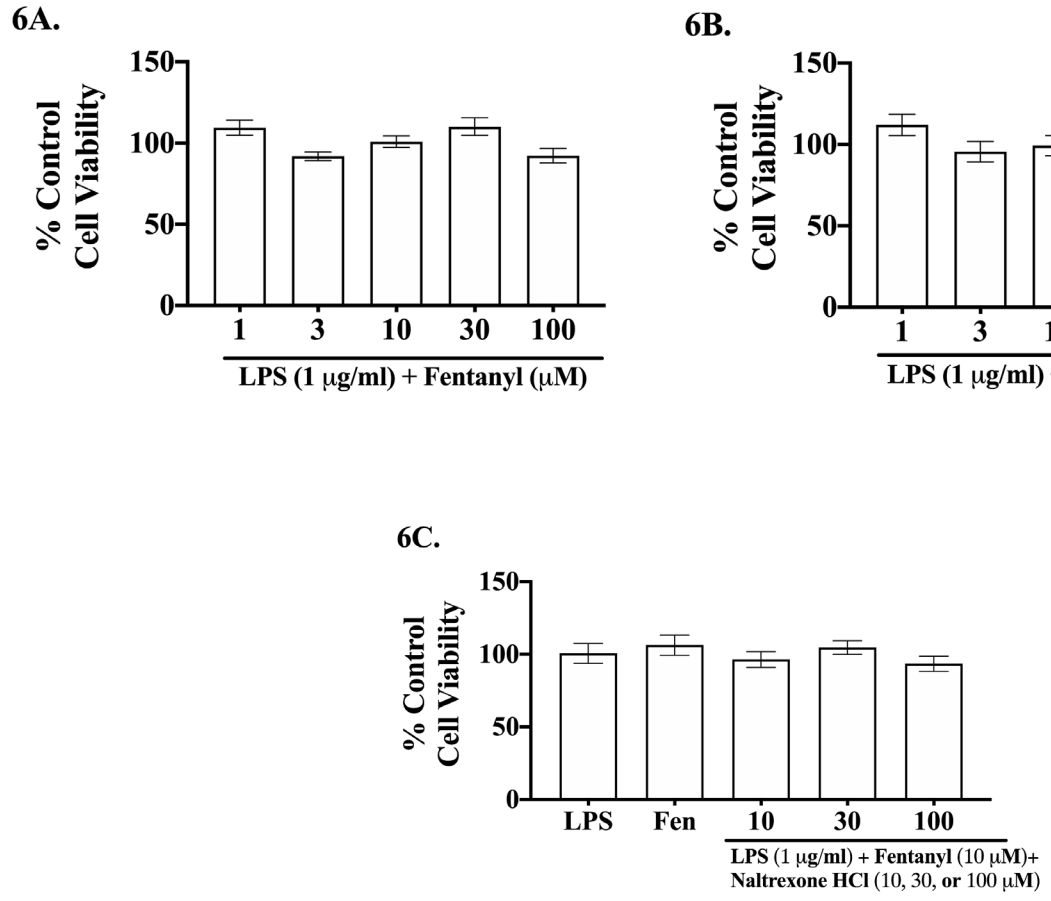


Figure 6. MTT cell viability assays for LPS and fentanyl or morphine in CHME-5 cells.
A. Following cell stimulation with LPS (1μg/ml) and fentanyl (1, 3, 10, 30, or 100 μM), MTT cell viability absorbance was measured at 492 nm. ANOVA revealed no significant difference between control and LPS/fentanyl co-treated cells, $p=0.54$. B. Following stimulation with LPS (1μg/ml) and morphine (1, 3, 10, 30, or 100 μM), MTT cell viability absorbance was measured at 492 nm. ANOVA revealed no difference between control and LPS/morphine co-treated cells, $p=0.70$. C. Following stimulation with LPS (1μg/ml), fentanyl (10 μM), and naltrexone HCl (10, 30, or 100 μM), MTT cell viability absorbance was measured at 492 nm. ANOVA revealed no significant difference between control and LPS/fentanyl/naltrexone co-treated cells, $p=0.94$. Data is presented as % control cell viability. Three experiments were performed in duplicate; n=3 for each treatment group. Bars for all experiments are presented as mean ± SEM.

Discussion

Opioids remain the gold standard of analgesia for treatment of postoperative and chronic pain, including pain associated with cancer. Opioids are also indicated for pain associated with neurodegenerative disorders including multiple sclerosis, Alzheimer's disease, and Parkinson's disease; all of which display inflammatory

components[21,22]. Therefore, it is important to understand the exact mechanisms occurring during treatment with opioid agonists and antagonists to determine the impact of opioids on inflammation.

Microglia, the "macrophages" of the CNS, are integral to innate immunity and inflammatory signaling in the CNS[23]. Determination of MOR expression in the CHME-5 microglial cell line was important due to the use of the MOR agonists, fentanyl and morphine, in our experimental design. To our knowledge, this is the first report of *MOR* gene expression in CHME-5 cells, which was upregulated in response to LPS. These findings are consistent with previous reports of MOR gene and protein expression in microglia[24-27]. Other reports have also shown a 2-fold increase in *MOR* mRNA expression in response to LPS (10 μg/ml) in rat peritoneal macrophages, and a 1.5-fold difference with LPS (5 μg/ml) at 24 h in TPA-HL-60 macrophage-like human cells[28-30].

Upon LPS-induced TLR4 activation, there are several downstream signaling events that occur, including activation of IκBα and NF-κB nuclear translocation[31]. Similarly, we demonstrated that LPS induced IκBα activation in CHME-5 cells and this induction was inhibited by fentanyl and morphine. LPS-induced NF-κB p65 activation in CHME-5 cells was also downregulated by fentanyl, but not morphine. The lack of a significant effect of morphine on LPS-induced NF-κB p65 binding activity may simply reflect the high degree of variability in this response. Alternatively, the differential effects between fentanyl and morphine may be attributed to their potency as MOR agonists; fentanyl being 100× more potent than morphine. The inhibitory effects of fentanyl are seemingly in contrast to the effects of opioids on primary murine microglia, in which LPS-induced p-IκBα immunoreactivity and NF-κB p65 activation were in increased in the presence of morphine (10 μM) co-treatment for 15 min[26]. These differences highlight the importance of considering species origin and *in vitro* conditions when assessing opioid-mediated effects on microglia. Nonetheless, both fentanyl and morphine can modulate TLR4-mediated downstream signaling in microglia.

We also investigated whether fentanyl-mediated down-regulation of LPS-induced NF-κB p65 binding activity was occurring through MOR or through a non-classical opioid site. Co-treatment with naltrexone, a MOR antagonist, did not prevent the fentanyl-mediated effect on LPS-induced NF-κB activation in CHME-5 cells, which is consistent with our previous finding that naltrexone failed to reverse the fentanyl or morphine-mediated effects on LPS-induced TLR4 signaling[18]. Together, these findings suggest that fentanyl-mediated effects on LPS-induced NF-κB p65 activation in CHME-5 cells are MOR-independent. Importantly though, follow-up experiments in microglia with MOR knockout are warranted to further substantiate the MOR-independent nature of fentanyl effects on LPS-induced TLR4 signaling.

Others have reported that opioid agonists and antagonists mediate their effects on TLR4 by interacting with the MD2 binding pocket[14,15,32]. Several studies showed that morphine interacts with the MD2 binding pocket, induces TLR4 oligomerization, and mediates MD2 conformational changes, thereby stabilizing the TLR4/morphine heterotetrameric complex, all of which are similar characteristics of LPS binding[14]. The possibility that naltrexone did not reverse the fentanyl-mediated effect may be due to lower affinity for the TLR4/MD2 pocket compared to LPS (Wang et al. unpublished data), or, naltrexone did not have sufficient incubation time to mediate an effect. Additionally, naltrexone was added after LPS/fentanyl co-treatment, therefore naltrexone may not have bound to the MD2 docking site before LPS and fentanyl; this may be important because computer modeling showed that pre-docking of naltrexone disrupted the preferred binding sites of morphine, which led to displacement of opioids outside the LPS binding pocket [33].
We also demonstrated that co-treatment with LPS and fentanyl or morphine, along with addition of naltrexone was not cytotoxic to the CHME-5 microglial cells, which is consistent with data from other cell viability assays

in our laboratory showing that increasing doses of opioid agonists and antagonists are not cytotoxic[19]. This data suggests that down-regulation in response to opioid co-treatments was not due to cytotoxicity of the opioid compound (s).

This work focused on the opioid-mediated effects in microglia. Fentanyl, and to a lesser extent morphine, displayed anti-inflammatory actions on several crucial signaling molecules in the TLR4 MyD88-dependent pathway. Fentanyl and morphine are prototypical ligands for the MOR and were expected to mediate their effects through this classical opioid receptor. What we discovered was that treatment with the opioid antagonist, naltrexone, failed to reverse the opioid-mediated effects on LPS-induced neuroinflammatory signaling. Therefore, these data suggest the opioid-mediated effects in CHME-5 cells are not occurring through the MOR, but through a non-classical opioid site. Furthermore, these effects occur through down-regulation of LPS-mediated IκBα activation and NF-κB binding activity. These insights are expected to further our understanding of opioid effects on inflammatory signaling and CHME-5 may serve as useful tool in these studies.

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