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The opioid antagonist, β -funaltrexamine, inhibits chemokine expression in human astroglial cells

Randall L. Davis^{†,*}, Daniel J. Buck[†], Neda Saffarian[†], and Craig W. Stevens[†]

[†] Department of Pharmacology/Physiology, Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma 74107

Abstract

Emerging evidence indicates that neuroinflammatory responses in astroglia, including chemokine expression, are altered by opioids. Astroglial chemokines, such as CXCL10, are instrumental in response to many neuropathological insults. Opioid mediated disruption of astroglial CXCL10 expression may be detrimental in opioid abusers or patients receiving acute opioid therapy. We have characterized the *in vitro* effects of opioids on CXCL10 protein expression in human astroglial (A172) cells. The proinflammatory cytokine, tumor necrosis factor (TNF) α induced CXCL10 expression in A172 cells. Using MG-132, helenalin and SN50 [inhibitors of the transcription factor, nuclear factor (NF)- κ B], we determined that NF- κ B activation is instrumental in TNF α induced CXCL10 expression in A172 astroglia. Morphine exposure during the 24 h TNF α stimulation period did not alter CXCL10 expression. However, fentanyl, a more potent *mu* opioid receptor (MOR) agonist, inhibited TNF α induced CXCL10 expression. Interestingly, neither the nonselective opioid receptor antagonist, naltrexone nor β -funaltrexamine (β -FNA), a highly selective MOR antagonist, blocked fentanyl mediated inhibition of TNF α induced CXCL10 expression. Rather, β -FNA dose dependently inhibited TNF α induced CXCL10 expression with a greater potency than that observed for fentanyl. Immunoblot analysis indicated that morphine, fentanyl and β -FNA each reduced TNF α induced nuclear translocation of NF- κ B p65. These data show that β -FNA and fentanyl inhibit TNF α induced CXCL10 expression via a MOR independent mechanism. Data also suggest that inhibition of TNF α induced CXCL10 expression by fentanyl and β -FNA is not directly related to a reduction in NF- κ B p65 nuclear translocation. Further investigation is necessary in order to fully elucidate the mechanism through which these two opioid compounds inhibit CXCL10 expression. Understanding the mechanism by which chemokine expression is suppressed, particularly by the opioid antagonist, β -FNA, may provide insights into the development of safe and effective treatments for neuroinflammation.

Keywords

astrocyte; brain injury; *mu* opioid receptor; neuroinflammation; tumor necrosis factor α

1. Introduction

Increasing evidence indicates that inflammatory mediators are involved in the neuropathogenesis associated with neurodegenerative diseases (Kadiu et al., 2005), viral

*Corresponding author: Randall L. Davis, Oklahoma State University Center for Health Sciences, 1111 W. 17th Street, Tulsa, OK 74107, Tel: 918-561-8408; Fax: 918-561-8276, E-mail: randall.davis@okstate.edu

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infections (Poluektova et al., 2005; Toborek et al., 2005), ischemic stroke (Wang et al., 2004), trauma (Vlodavsky et al., 2006) and neuropathic pain (Myers et al., 2006). Importantly, chemokines have emerged as key molecules involved in neuropathological events and depending on the cellular context can either be neurotoxic or neuroprotective. In particular, CNS levels of the chemokine CXCL10 (formerly referred to as interferon- γ inducible protein or IP-10) are elevated in Alzheimer's disease (Xia et al., 2000), HIV dementia (Cinque et al., 2005; Kolb et al., 1999), ischemic stroke (Wang et al., 1998; Wang et al., 2000) and following spinal cord injury (Gonzalez et al., 2003). We are primarily interested in CXCL10, which is a member of the CXC or α -chemokine family, all of which have four highly conserved cysteine residues with the first two cysteines separated by a single amino acid (Bajetto et al., 2002; Luster et al., 1985). CXCL10 can also be subclassified as ELR-negative given that it does not contain a conserved tripeptide motif, glutamate-leucine-arginine (ELR) at the N-terminus, before the CXC domain (Belperio et al., 2000). Importantly, chemokines are small secreted proteins that function in both physiological and pathological conditions. CXCL10 is well characterized as a chemoattractant for activated T cells (Taub et al., 1993), monocytes/macrophages (Taub et al., 1993), and microglia (Flynn et al., 2003). CXCL10 is also a potent angiostatic factor (Belperio et al., 2000) and induces astroglial proliferation (Flynn et al., 2003).

Astroglia appear to be a major source of CXCL10 in many neuropathologies. For instance, compared to controls, CXCL10 protein expression was markedly increased in a subpopulation of astrocytes from Alzheimer's disease brains (Xia et al., 2000). Astroglial expression of CXCL10 has also been observed in ischemic stroke. For example, after occlusion of the middle cerebral artery in rat, CXCL10 mRNA expression in cortical tissues peaked 6 h after occlusion, and a second induction of CXCL10 was noted from 10–15 d post-occlusion (Wang et al., 1998). Immunohistochemical analysis of the ischemic cortex indicated CXCL10 protein predominated in the astrocytes of the cortical, striatal and white matter regions surrounding the lesions, as indicated by co-localization of CXCL10 and glial fibrillary acidic protein (Wang et al., 1998).

In recent years, numerous investigators have focused on neutralization of CXCL10 as a therapeutic strategy for decreasing inflammatory-mediated neuropathogenesis (Glaser et al., 2004; Sorensen, 2004). For instance, in a murine model of spinal cord injury (SCI), anti-CXCL10 antibody increased angiogenesis and reduced SCI-induced tissue damage (Glaser et al., 2004). Others have utilized a broad spectrum chemokine inhibitor (NR58-3.14.3) to provide neuroprotection in a rat model of cerebral ischemia-reperfusion injury (Beech et al., 2001). While not specifically targeted to CXCL10, another intriguing strategy that has been employed to attenuate inflammation-mediated neuropathogenesis is treatment with naloxone (Liao et al., 2003; Liu et al., 2000; Liu and Hong, 2003; Liu et al., 2002). Naloxone is well characterized as a non-selective opioid receptor antagonist; however, it has been demonstrated that naloxone reduces neuroinflammation via mechanisms that do not require binding to opioid receptors, including prevention of bacterial lipopolysaccharide-binding to microglia (Liu et al., 2000) and reduced microglial superoxide production (Liu et al., 2002).

The *mu*-opioid receptor (MOR) agonist, morphine, has both neuroprotective (Peart et al., 2005; Rambhia et al., 2005) and neurotoxic (El-Hage et al., 2005; Khurdayan et al., 2004) properties. While the mechanism for the neuroprotective effects of morphine are not completely understood, there is evidence to suggest that under certain conditions, morphine attenuates oxidative and proinflammatory stress (Lee et al., 2004; Rambhia et al., 2005). In contrast, opioids exacerbate HIV neuropathogenesis, in part by enhancing astroglial release of proinflammatory mediators including chemokines (El-Hage et al., 2005). That is, morphine potentiates HIV-1 Tat-induced chemokine expression in murine astrocytes and attenuation of the morphine effects with the MOR specific antagonist β -funaltrexamine (β -FNA) indicates a

MOR dependent mechanism. More recently, these same investigators reported that morphine enhances MOR expression and potentiates HIV-1 Tat-induced chemokine production (El-Hage et al., 2006).

While the above studies demonstrate the role of opioid agents in neuroinflammation, herein, we provide the first characterization of opioid effects on CXCL10 expression in human astroglial cells. More specifically, we have demonstrated that TNF α induced CXCL10 protein expression in human astroglial cells is dose dependently inhibited by two different opioid compounds, fentanyl and β -FNA. Of particular interest is that while typically described as a MOR agonist and MOR antagonist, respectively, fentanyl and β -FNA each inhibit TNF α induced CXCL10 expression through a MOR-independent mechanism.

2. Materials and methods

2.1. Cell culture

The human astrocytoma cell line (A172, ATCC #CRL-1620; American Type Culture Collection, Manassas, VA) was utilized in various pharmacology- and neurochemistry-based studies (Guthikonda et al., 1998; Kubota et al., 2001; Zaheer et al., 1995). Additionally, we have previously characterized proinflammatory pathways in A172 cells (Davis et al., 2002; Davis and Syapin, 2004a; Davis and Syapin, 2004b). A172 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 50 U/ml penicillin, 0.05 mg/ml streptomycin and 2 g/ml amphotericin B. Cultures were maintained in a humidified incubator at 37°C, 5% CO₂ and 95% air and culture medium changed every 48–72 h. Experimental cultures were seeded at a cell density ($1 \times 10^4/\text{cm}^2$) in order to provide 80–90% confluence at the time of treatment.

2.2. Proinflammatory stimulation

In order to induce CXCL10 expression and nuclear translocation of NF- κ B p65, growth medium in astroglial cultures was replaced with serum free medium containing proinflammatory cytokines as previously described (Davis and Syapin, 2004b; Davis and Syapin, 2004c). The proinflammatory cytokines used were human recombinant TNF α , IL-1 β , and IFN- γ (5 ng/ml; Peprotech, Rocky Hill, NJ). The duration of cytokine exposure was 24 h for CXCL10 expression and 0.5 h for NF- κ B activation.

2.3. Inhibitors of NF- κ B activation

To assess the role of NF- κ B in TNF α -induced CXCL10 expression, A172 cells were stimulated in the presence of MG-132 (0.14 nM–2.0 M; carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Biomol, Plymouth Meeting, PA) or the sesquiterpene lactone, helenalin (2.7 nM–2.0 M; Biomol). MG-132 inhibits NF- κ B activation by blocking the activity of the 26S proteasome (Lee and Goldberg, 1996), and thereby prevents nuclear translocation of NF- κ B. The effects of helenalin are known to be further downstream where it prevents NF- κ B-DNA binding by specifically alkylating the p65 subunit (Lyss et al., 1998). MG-132 and helenalin were reconstituted in dimethyl sulfoxide at a stock concentration of 10 mM and stored at –80°C and –20°C, respectively (Sanchez et al., 2003). These inhibitors were added to cultures at the time of stimulation with TNF α . A third inhibitor of NF- κ B activation, SN50 (Biomol) was also used. SN50, a cell permeable inhibitory peptide containing the nuclear localization sequence of NF- κ B p50, prevents nuclear translocation of the active NF- κ B complex. Similar to others (Pahan et al., 2001; Yang et al., 2005), cells were preincubated for 1 h prior to stimulation with 50 M SN50 or 50 M of the inactive mutant peptide SN50M, used as a control (Biomol).

2.4. Opioid compounds

Opioids [morphine (0.05–150 M), fentanyl (1.2–150 M), β -FNA (0.14–100 M), naltrexone (33–150 M); all from Sigma, St. Louis, MO] were added to cell cultures at the time of TNF α stimulation, except in the pre-treatment experiments in which opioids were added 5–60 minutes prior to TNF α addition. In the pre-treatment experiments, residual opioids were rinsed from the cells prior to TNF α stimulation.

2.5. ELISA

A standard dual-antibody solid phase immunoassay (ELISA Development Kit, Peprotech) was used for quantitation of secreted CXCL10 in cell culture supernatants, according to the manufacturer's instructions. Briefly, 100 μ l of standards (0–2000 pg/ml) and cell culture supernatants were added to appropriate antibody-coated wells of a 96 well plate. Next, 100 μ l of antigen-specific biotinylated detection antibody was added to each well followed by a 2 h incubation at room temperature. Liquid contents were then aspirated and wells washed 3 $\times$ with wash buffer. Avidin-peroxidase conjugate (100 μ l) was then added to each well and incubated for 30 min at room temperature. Wells were then aspirated and washed 3 $\times$ as before, and 100 μ l of ABTS liquid substrate solution (Sigma cat. # A3219) was added to each well followed by 25 min incubation at room temperature. Absorbance was read at 450 nm (with wavelength correction set at 650 nm) on a BIO-TEK HT spectrophotometer.

2.6. Nuclear translocation of NF- κ B p65

Nuclear fractions were collected as described previously (Davis and Syapin, 2004c). For immunoblot analysis of nuclear levels of p65, 10 μ g protein was electrophoretically separated through a 8% polyacrylamide gel (Bio-Rad, Hercules, CA) in running buffer (25 mM Tris base, 190 mM L-glycine, 0.1% SDS) with the Mini-Protean $\text{\textcircled{R}}$ 3 Cell (Bio-Rad) for 25 min at 200 V. Proteins were electro-transferred to polyvinylidene difluoride membranes (Bio-Rad) in transfer buffer (25 mM Tris base, 150 mM L-glycine, 20% methanol) with the Mini Trans-Blot $\text{\textcircled{R}}$ Electrophoretic Transfer Cell (Bio-Rad) for 2 h at 87 V. Membranes were blocked for 1 h at room temperature in Tris sodium Tween 20 (TST) buffer (10 mM Tris base, 100 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) containing 5% non-fat powdered milk then incubated overnight at 4 $^{\circ}$ C with anti-p65 antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with TST buffer five times for 5 min each, incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:5,000; Sigma) and then washed with PBS (5 times for 5 min each). Bands were then visualized using SuperSignal TM West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography (Fuji Medical X-ray film).

2.7. Determination of total cellular protein content

Total cell protein/well was determined using the bicinchoninic acid (BCA) protein assay as previously described (Davis et al., 2002) in order to normalize data when appropriate (i.e., pg chemokine protein produced/mg total cellular protein).

2.8. Determination of cell viability

Cell viability was assessed using the MTT assay according to a modified version of the procedure described by Carmichael et al., (1987). Briefly, cells were incubated for 45 min in culture medium containing 0.55 mg/ml 3-[4,5-Dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide (MTT). Media was then removed and cells were dissolved in 1 ml DMSO and absorbance measured at 492 nm using a BIO-TEK HT spectrophotometer.

2.9. Statistical analysis

Prism™ version 4.0 software (GraphPad Inc., San Diego, CA) was used for figure presentation, curve fitting, percent transformations and statistical analysis. Analyses included one-way analysis of variance (ANOVA) with Neuman-Kuels multiple comparison post hoc test as well as nonlinear regression in which the Hill slope was not constrained. Data are presented as mean + S.E.M. or mean IC₅₀ values with their 95% confidence interval (CI). A probability (*p*) of < 0.05 was accepted as demonstrating statistically significant differences between groups; data points with any common letters above them are not statistically different from each other. The number of replicate measures and independent experiments from which the data were obtained are provided in the individual figure legends.

3. Results

3.1. Cytokine-induced CXCL10 expression

In pilot studies, we determined that CXCL10 protein expression is induced by a cytokine mixture containing IL-1 β (5 ng/ml) + TNF α (30 ng/ml) + IFN- γ (100 ng/ml). In the present study, we showed that TNF α and to a lesser degree, IL-1 β , are each sufficient to induce CXCL10 in A172 cells (Fig. 1). In fact, a relatively low concentration of TNF α (5 ng/ml) is stimulatory. Conversely, IFN- γ at 100 ng/ml does not induce CXCL10 in A172 cells.

3.2 Role of NF- κ B in TNF- α -induced CXCL10 expression

The role of NF- κ B in CXCL10 expression in human astroglial cells is not well defined. Therefore, we assessed the ability of mechanistically distinct NF- κ B inhibitors, MG-132, helenalin and SN50 to modulate CXCL10 expression. We determined that MG-132 and helenalin dose-dependently inhibit CXCL10 protein expression in human astroglial cells with IC₅₀ values of 26 and 969 nM, respectively (Fig. 2). Additionally, we have determined that blockade of the NF- κ B p50 nuclear recognition sequence with the peptide inhibitor SN50 prevents TNF α induced CXCL10 expression in A172 cells (Fig. 8).

3.3. Inhibition of CXCL10 protein expression by opioids

Exposure to morphine for 24 h did not significantly alter TNF α -induced CXCL10 expression in A172 cells (Fig. 3A). The more potent MOR agonist, fentanyl, dose-dependently inhibited TNF α -induced CXCL10 expression, albeit at relatively high concentrations (IC₅₀ = 328 M; Fig. 3B). The highly selective MOR antagonist, β -FNA, failed to prevent the inhibitory effects of fentanyl on CXCL10 expression. Interestingly, β -FNA was actually a relatively potent inhibitor of TNF α -induced CXCL10 expression (IC₅₀ = 7.6 M; Fig. 3C). The inhibitory effects of β -FNA and fentanyl were not a consequence of cytotoxicity as all four opioids were determined to be non-toxic under the experimental condition utilized (Fig. 4).

At a concentration of 100 M, the non-specific opioid receptor antagonist, naltrexone, did not prevent either fentanyl- or β -FNA-inhibition of TNF α -induced CXCL10 expression (Fig. 5). In contrast, naltrexone slightly inhibited TNF α -induced CXCL10 expression and actually potentiated 11M β -FNA-mediated inhibition of CXCL10 expression. Potentiation of β -FNA-mediated inhibition of CXCL10 expression by naltrexone occurred when cells were simultaneously exposed to naltrexone, β -FNA and TNF α for 24 h (Fig. 5). Similarly, potentiation of β -FNA-mediated inhibition of CXCL10 expression by naltrexone occurred in the 30 min pre-exposure paradigm in which cells are exposed to naltrexone and β -FNA for 30 min, followed by washout of the drug, prior to 24 h TNF α exposure (data not shown).

To further assess the inhibitory actions of β -FNA, the time-dependent effects on CXCL10 expression were determined. Pre-exposure to β -FNA for 5 – 60 min, followed by washout of the drug, prior to 24 h TNF α exposure, resulted in a time-dependent decrease in CXCL10

expression (Fig. 6). Exposure to both β -FNA and TNF α for 24 h resulted in > 95% inhibition of CXCL10 expression (Figs. 3C and 6); whereas, 5 min pre-exposure to β -FNA caused minimal inhibition ($\approx$ 20%) of CXCL10 expression, but only at 100 M (Fig. 6).

3.4. Effect of opioids on NF- κ B activation

Nuclear translocation of the p65 subunit was assessed as a measure of NF- κ B activation (Fig. 7). Very low levels of p65 protein were present in the nucleus of unstimulated (not exposed to TNF α) cells. In contrast, exposure to TNF α for 0.5 h resulted in a marked increase in p65 accumulation in the nucleus. When cells were stimulated with TNF α in the presence 33 M β -FNA (a concentration that produces remarkable inhibition of stimulated CXCL10 expression) there was an apparent decrease in nuclear p65 levels. Similarly, at this same concentration (33 M) fentanyl and to a greater degree, morphine seemed to prevent p65 nuclear translocation. However, statistical analyses did not reveal any differences among the opioid treated groups. There was no evidence of a naltrexone effect on p65 nuclear translocation. It should be noted that both the gel image and the graphic representation of the data suggest that when compared to control (TNF α -stimulated) cells, β -FNA reduces TNF α -induced p65 nuclear translocation. Yet, statistical conclusions can not be made given that the data are normalized to these control data. Additionally, combined effects of SN50 and opioids on CXCL10 expression were assessed. As noted in Fig. 8, SN50-inhibition of CXCL10 is not potentiated by either 11 M β -FNA or 100 M fentanyl.

4. Discussion

To our knowledge, this is the first report describing the effects of morphine or fentanyl on CXCL10 expression in astroglia. In recent years, there has been considerable interest in understanding the effects of opioids (primarily morphine) on chemokine expression and function in astroglia (El-Hage et al., 2005; El-Hage et al., 2006; Mahajan et al., 2005; Mahajan et al., 2002). For instance, morphine ($\geq$ 1 nM for 24 h) reduced constitutive expression of IL-8 (or CXCL2) and macrophage inhibitory protein 1 β (MIP-1 β or CCL4) genes and proteins in normal human astrocytes (Mahajan et al., 2002). Suppression of constitutive chemokine expression by morphine was reversed by β -FNA, indicating a MOR-dependent mechanism (Mahajan et al., 2002). More recently, this same group (Mahajan et al., 2005) showed that MIP-1 β and monocyte chemoattractant protein 1 (MCP-1 or CCL2) gene expression is down-regulated by morphine. Additionally, the non-selective opioid receptor antagonist, naloxone blocked morphine-induced inhibition of chemokine expression in these cells, suggesting an opioid receptor (likely MOR)-dependent mechanism.

Other investigators have also provided evidence of MOR-independent actions of morphine on astroglial chemokines expression. For instance, the HIV-1 Tat protein (Tat₁₋₇₂) increases MCP-1 and RANTES expression and morphine synergistically enhances Tat₁₋₇₂-induced expression of these chemokines (El-Hage et al., 2005). Interestingly, β -FNA reversed the synergistic action of morphine on Tat₁₋₇₂-induced chemokine expression but did not significantly reduce the stimulatory actions of morphine alone. Together these data indicate that the potentiation of Tat₁₋₇₂-induction of MCP-1 and RANTES by morphine is MOR-dependent, yet stimulation of these chemokines by morphine alone may be, in part, via MOR-independent actions. Therefore, with regard to morphine and chemokine expression in astrocytes there appear to be substantial species- and chemokine-specific effects.

Previously, we found that CXCL10 protein expression in A172 cells is strongly induced by either LPS (1 g/ml) + IL-1 β (10 ng/ml) or a cytokine mixture containing IL-1 β (5 ng/ml) + TNF α (30 ng/ml) + IFN- γ (100 ng/ml) (Davis and Syapin 2004b; unpublished data). In the present study we determined that TNF α alone, at a considerably lower concentration (5 ng/ml), significantly induced CXCL10 protein expression in A172 cells. Other studies have found

that opioids, such as morphine, suppress the expression of astroglial chemokines, including IL-8, MCP-1 and MIP-1 β (Mahajan et al., 2005; Mahajan et al., 2002). In the present study, morphine did not modulate CXCL10 protein expression whereas the more potent MOR agonist, fentanyl, suppressed TNF α -induced CXCL10 expression in A172 cells. In addition to the observation that morphine (≤ 150 M) failed to alter CXCL10 protein expression, there are several other factors to suggest that this reduction in CXCL10 expression may not be MOR-dependent. For instance, the concentration required for fentanyl inhibition of CXCL10 expression is relatively high and the highly MOR-specific antagonist, β -FNA did not reverse the effects of fentanyl. Additionally, the non-selective opioid receptor antagonist naltrexone did not block fentanyl-induced effects on CXCL10 expression. Given that β -FNA irreversibly binds to MOR, we predicted that if β -FNA was acting in a MOR-dependent manner, inhibition of CXCL10 expression would result even when β -FNA was added for only 5–60 min and subsequently removed prior to TNF α exposure. However, we observed a time- and dose-dependent effect of β -FNA on CXCL10 expression. One explanation for these findings is that β -FNA is acting through an alternate receptor and/or is modulating an intracellular target. Presumably, it would require more time (or higher concentrations) for β -FNA to reach intracellular targets. Failure of naltrexone to reverse β -FNA effects further suggests that β -FNA is acting independent of opioid receptors. Furthermore, naltrexone potentiated β -FNA-mediated inhibition of CXCL10 expression; yet by itself, naltrexone (100 M) only minimally reduced CXCL10 expression.

Interestingly, we have demonstrated that it is not the interaction of fentanyl or β -FNA with MOR that is causing the inhibition of TNF α -induced CXCL10 expression. This is very interesting given that fentanyl is typically described as a MOR agonist and β -FNA is best known as a MOR antagonist. Of particular interest is the finding that β -FNA inhibited TNF α -induced CXCL10 expression more potently than fentanyl. The anti-inflammatory actions of β -FNA have not been previously characterized and the mechanism responsible for the MOR independent actions of β -FNA (and fentanyl) have yet to be identified. However, others have found unexpected effects of β -FNA on chemokine (IL-8 and MIP-1 β) expression in human astrocytes (Mahajan et al., 2002). More specifically, these investigators reported that in addition to blocking morphine-suppression of constitutive chemokine expression, β -FNA alone up-regulated chemokine expression in these cells (Mahajan et al., 2002). These researchers postulated that β -FNA may be activating at another type of opioid family receptor such as the nociceptin (ORL) receptor or another as yet unidentified site. For instance, depending on the receptor type, β -FNA can exhibit agonist or antagonist activity (Holtzman, 1997; Sayre et al., 1984). Astroglia express all three opioid receptor types (δ and κ) and these receptors are differentially expressed among brain regions (Ruzicka et al., 1995; Stiene-Martin et al., 1998). Also, age-related and cell-cycle-dependent variations in opioid phenotype can be observed at the cellular level (Stiene-Martin et al., 1998). However, morphine functions predominantly through MOR (Baumhaker et al., 1993; Stefano et al., 1996). MOR is expressed on human astroglia, yet in normal human astrocytes and the U87 human astroglial cell line, expression levels are relatively low (Mahajan et al., 2005; Mahajan et al., 2002). MOR expression is up-regulated in human astroglial cells following morphine exposure (Mahajan et al., 2005; Mahajan et al., 2002). We recently determined that expression of the human *mu*-opioid receptor (*hMOR*) mRNA is up-regulated in A172 cells following TNF α exposure (unpublished data). TNF α also induces MOR expression in various cell types including peripheral immune effector cells, microvascular endothelial cells and SH SY5Y neuroblastoma cells (Borner et al., 2004; Kraus et al., 2003). Further characterization of opioid receptor expression in A172 cells is currently being investigated in our laboratory.

The anti-inflammatory actions of β -FNA identified in this study also support and add to the findings of others (Liu et al., 2000; Liu and Hong, 2003) which have identified MOR-independent, anti-inflammatory actions of naloxone (a non-specific, opioid receptor

antagonist). For example, addition of lipopolysaccharide (LPS)-stimulated microglia to rat mesencephalic mixed neuroglia cultures resulted in pronounced neuronal toxicity (Liu et al., 2000). However, co-exposure of these cultures to naloxone provided neuroprotection, reportedly by blocking LPS-binding to microglia (Liu et al., 2000). Additionally, the inactive stereoisomer, (+)-naloxone, was equally effective as (–)-naloxone (Liu et al., 2000). In a more recent study, this same group determined that A β (1–42)-induced neurotoxicity was reduced by naloxone in an opioid receptor-independent mechanism (Liu et al., 2002). In this study it was determined that the neuroprotective effects of naloxone were due to reduced microglial superoxide production (Liu et al., 2002). Together, these insights support a potential role of incorporating opioid agents in the treatment of neuroinflammation associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, HIV dementia and ischemic stroke. However, more mechanistic information is necessary before this therapeutic potential can be fully realized.

The therapeutic relevance of attenuating the transcription factor NF- κ B, with respect to neuroinflammation and neuropathogenesis, has increased in recent years. For example, the potential benefit of down regulating NF- κ B activation and CXCL10 expression in astroglial cells on recovery from spinal cord injury was recently highlighted (Brambilla et al., 2005). Use of the functionally distinct inhibitors of NF- κ B activation provided data to suggest that TNF α -induced CXCL10 protein expression is dependent in part, on proteasome degradation, NF- κ B p50 mediated nuclear translocation and NF- κ B p65-DNA binding. That MG-132 is a more potent inhibitor of CXCL10 expression compared to helenalin, suggests that NF- κ B activation in A172 cells is more sensitive to disruption of proteasome degradation than blockade of p65-DNA binding. It should be noted that as an inhibitor of proteasome degradation, MG-132 may also modulate other pathways that utilize the 26S proteasome. Importantly though, we have confirmed by immunoblot analysis that MG-132 reduces p65 translocation into the nucleus of cytokine-stimulated A172 cells (unpublished data).

Regarding the role of NF- κ B p65 in the anti-inflammatory effects of fentanyl and β -FNA, findings in this study indicate that the effects of these opioid compounds on TNF α induced CXCL10 expression are not directly related to decreased NF- κ B p65 nuclear translocation. Further analysis is needed to fully appreciate the importance of p65 (or other NF- κ B molecules) in fentanyl and β -FNA modulation of CXCL10 expression. Additionally, further investigation of the potential role of other opioid receptor types in β -FNA mediated inhibition of CXCL10 expression in A172 cells is warranted. It will also be important to identify and characterize the effects of opioids on CXCL10 expression and other inflammatory molecules in normal human astrocytes and *in vivo* models of neuropathogenesis. A better understanding of the mechanism governing the MOR-independent, anti-inflammatory actions of opioid agents, and especially the safe and non-addictive opioid antagonist, β -FNA, may result in important treatments for neuroinflammation.

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Fig 1.

Cytokine-induced CXCL10 protein expression in A172 cells. Astroglial cells were unstimulated (US) or exposed for 24 h to human recombinant TNF α , IL-1 β , or IFN- γ (5 ng/ml). A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data represent mean + S.E.M. of duplicate measures from 3 independent experiments. Data points with any common letters above them are not statistically different from each other as determined by one-way analysis of variance (ANOVA) with Neuman-Kuels' multiple comparisons.

Fig 2.

Inhibitors of NF- κ B activation reduce TNF α -stimulated CXCL10 protein expression in A172 cells. Astroglial cells were exposed for 24 h to human recombinant TNF α (5 ng/ml) in the presence or absence of MG-132 (0.14 nM-2.0 M; carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) or the sesquiterpene lactone, helenalin (2.7 nM-2.0 M). A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data points represent the mean + S.E.M. of duplicate measures from 2–7 independent experiments. Curves are from nonlinear regression in which the Hill slope was not constrained. The IC₅₀ value and 95% confidence interval (CI) for MG-132 are 26 nM (95% CI: 17–41 nM) and 969 nM (95% CI: 754 nM-1.25 M) for helenalin.

Fig 3.

Modulation of TNF α -stimulated CXCL10 protein expression in A172 cells by selected opioids. Astroglial cells were exposed for 24 h to human recombinant TNF α (5 ng/ml) in the presence or absence of morphine (0.05–150 M), fentanyl (1.2–150 M) or β -funaltrexamine (β -FNA; 0.14–100 M). A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data points represent the mean + S.E.M. of duplicate measures from 2–5 independent experiments. Curves are from nonlinear regression analysis in which the Hill slope was not constrained. The IC_{50} values are presented for each curve and the 95% confidence interval (CI) for each opioid are: morphine = not determined, fentanyl = 14.5–522.1 M and β -FNA = 2.1–27.5 M.

Fig 4.

Effects of opioids on viability of TNF α stimulated astroglial cells. A172 cells were exposed for 24 h to human recombinant TNF α (5 ng/ml) in the presence or absence of morphine (1.23–100 M), fentanyl (1.23–100 M), naltrexone (1.23–100 M) or β -funaltrexamine (β -FNA; 1.23–40 M). Cell viability was determined by the MTT assay. Data represent mean + S.E.M. of duplicate or triplicate measures from 3 independent experiments. One-way analysis of variance (ANOVA) with Neuman-Kuels' multiple comparisons indicated no significant opioid effects.

Fig 5.

Modulation of (A) fentanyl- and (B) β -FNA-mediated inhibition of TNF α -stimulated CXCL10 protein expression in A172 cells by naltrexone. Astroglial cells were exposed for 24 h to human recombinant TNF α (5 ng/ml) in the presence or absence of fentanyl (33, 100 and 150 M), β -funaltrexamine (β -FNA; 11 and 33 M) and naltrexone (100 M). A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data points represent the mean + S.E.M. of triplicate measures from 2 independent experiments (panel A) and quadruplicate measures from 3 independent experiments (panel B). Data points with any common letters above them are not statistically different from each other as determined by one-way analysis of variance (ANOVA) with Neuman-Kuels' multiple comparisons.

Fig 6.

Time-course effects of β -FNA on TNF α -stimulated CXCL10 protein expression in A172 cells. Astroglial cells were either co-exposed to β -funaltrexamine (β -FNA; 0.14–100 M) and human recombinant TNF α (5 ng/ml) for 24 h (●) or pre-exposed to β -FNA for 5 (▲), 30 (□) or 60 (○) min, followed by washout of the drug, and subsequent exposure to TNF α for 24 h. A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data for each experiment were transformed to percent control (TNF α stimulation in the absence of β -FNA). Data points represent the mean + S.E.M. of duplicate measures from 3 independent experiments. Curves are resultant from nonlinear regression analysis in which the Hill slope was not constrained.

Fig 7. Modulation of NF-κB p65 nuclear translocation by opioids. Astroglial cells were exposed for 0.5 h to human recombinant TNFα (5 ng/ml) in the presence or absence of 33 M morphine, naltrexone, fentanyl or β-funaltrexamine. Nuclear fractions were collected and NF-κB p65 protein levels assessed by immunoblot analysis of 10 g total protein. β-tubulin levels were measured and used as loading controls. Images of the blots were digitally captured, and then integrated density values (IDV) were obtained using ImageJ software (NIH). β-tubulin band densities were used to normalize the densities of the NF-κB bands. Data points represent the mean + S.E.M. of the IDV obtained from 4 independent experiments (normalized for each experiment as percent stimulated control); the blot is representative of the 4 independent experiments.

Fig 8.

Inhibition of TNF α stimulated CXCL10 expression by the NF- κ B inhibitor SN50 and selected opioids β -FNA and fentanyl. Astroglial cells were preincubated for 1 h with SN50 (50 M) or the mutant peptide SN50M (50 M). Cells were then stimulated with TNF α (5 ng/ml) in the presence or absence of β -FNA (11 M) or fentanyl (100 M) for 24 h. A standard dual-antibody solid phase immunoassay (ELISA) was used for quantitation of secreted CXCL10 in cell culture supernatants. Data points represent the mean + S.E.M. of duplicate measures from 3 independent experiments. Data points with any common letters above them are not statistically different from each other as determined by one-way analysis of variance (ANOVA) with Neuman-Kuels' multiple comparisons.