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The opioid antagonist, β -funaltrexamine, inhibits NF- κ B signaling and chemokine expression in human astrocytes and in mice

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Abstract

Opioid-immune crosstalk occurs when opioid drugs alter the activity of the immune system. In this study, the opioid antagonist β -funaltrexamine (β -FNA) decreases the expression and release of an inflammatory chemokine, interferon- γ inducible protein-10 (CXCL10) from normal human astrocytes stimulated by interleukin 1 β (IL-1 β). β -FNA decreased CXCL10 by an unknown action that did not involve the mu opioid receptor (MOR). As IL-1 β acts through its receptor to activate NF- κ B/MAPK signaling pathways which leads to CXCL10 expression and release, key steps in the IL-1 β signaling pathways were examined following β -FNA treatment. IL-1 β -induced activation of p38 mitogen-activated protein kinases (p38 MAPK) was inhibited by β -FNA as shown by decreased p38 MAPK phosphorylation in treated cells. β -FNA also decreased the levels of activated subunits of NF- κ B (p50 and p65) in treated astrocytes. The impact of β -FNA was also observed in proteins that act to negatively regulate NF- κ B signaling. IL-1 β upregulated the expression of A20, a ubiquitin (Ub)-editing enzyme that dampens NF- κ B signaling by altering ubiquitination patterns on IL-1 receptor second messengers, and the increase in A20 was significantly inhibited by β -FNA treatment. Inhibition of the Ub-activating enzyme E1 by the inhibitor PYR41 also decreased CXCL10 release, like β -FNA, and concurrent treatment with both PYR41 and β -FNA inhibited CXCL10 more than did either agent alone. In mice, lipopolysaccharide-induced CXCL10 expression in the brain was inhibited by treatment with β -FNA. These findings suggest that β -FNA exerts an anti-inflammatory action in vitro and in vivo that is MOR-independent and possibly due to the alkylating ability of β -FNA.

Keywords

opioid; β -FNA; cytokine; chemokine; astrocyte; IL-1 β ; NF- κ B

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1. Introduction

Interactions between the opioid and immune systems ('crosstalk') is a growing area of research, given the tremendous use of opioid drugs across the world and the potential for therapeutic intervention in immune dysfunction using opioid agents (Hutchinson and Watkins, 2014; Watkins et al., 2009). Our work focuses on the discovery that the opioid receptor antagonist, β -funaltrexamine (β -FNA), inhibits the expression and release of the pro-inflammatory chemokine interferon- γ inducible protein-10 (CXCL10) in astroglial cells (Davis et al., 2007). Chemokine production in astroglial cells was stimulated by the application of tumor necrosis factor- α (TNF α), signaling through the NF- κ B pathway. Inhibition of CXCL10 production also occurred after treatment of astroglial cells with the opioid agonist, fentanyl, but fentanyl was not as potent in inhibiting CXCL10 production as was β -FNA. The opioid inhibition of chemokine CXCL10 was not mediated through the classical mu opioid receptor (MOR) or other opioid receptors as the effects of the opioid agents were not altered by the general opioid receptor antagonist, naltrexone (Davis et al., 2007).

1.1 Chemokine release and neuroinflammation

Pro-inflammatory chemokines, such as CXCL10, are released from activated astrocytes in response to injury and diseases involving neuroinflammation (John et al., 2005; Moynagh, 2005; Skaper, 2007). CXCL10 is a small secreted protein involved in physiological and pathological processes, including chemoattraction of monocytes/macrophages and microglia (Flynn et al., 2003; Taub et al., 1993). Furthermore, CXCL10 induces astroglial proliferation and is directly neurotoxic (Flynn et al., 2003; Sui et al., 2006). The pro-inflammatory cytokine interleukin-1 β (IL-1 β) is one of the mediators of astrocyte activation implicated in neuroinflammation (Emanuele et al., 2010; Holmin and Hojeberg, 2004; Lucas et al., 2006; Soderlund et al., 2011; Xing et al., 2009). The expression and release of CXCL10 from astrocytes has been observed following activation with IL-1 β (Rivieccio et al., 2005).

1.2 β -FNA and inhibition of pro-inflammatory pathways

The discovery that TNF α -induced CXCL10 protein expression in human astroglial cells was dose-dependently inhibited by the selective, MOR antagonist, β -FNA (Davis et al., 2007) was further investigated using different activating agents in normal human astrocytes (NHA). Interferon- γ (IFN γ) + HIV-1 Tat-induced CXCL10 expression in NHA also was inhibited by β -FNA (Davis et al., 2013). Importantly, neither the MOR-selective antagonist, D-Phe-Cys-Tyr-D-Trp-Arg-Pen-Thr-NH₂ (CTAP) nor the nonselective opioid receptor antagonist, naltrexone inhibited IFN γ +HIV-1Tat-induced CXCL10 expression. These findings confirmed that the inhibitory actions of β -FNA were mediated through a MOR-independent mechanism (Davis et al., 2007). In other studies from our laboratory, β -FNA was shown to non-competitively inhibit toll-like receptor (TLR) 4 signaling in a MOR-independent manner (Stevens et al., 2013).

Herein, we expand our studies to examine the effect of β -FNA on chemokine CXCL10 expression in an in vitro model of neuroinflammation using NHA. The pro-inflammatory cytokine, IL-1 β , was used to stimulate chemokine expression; and key steps in NF- κ B and

MAPK signal pathways were examined (in the presence or absence of β -FNA). In addition, for the first time, the anti-inflammatory effects of β -FNA were assessed in vivo using C57BL/6J mice treated with LPS and measurement of CXCL10 expression in the brain.

2. Materials and Methods

2.1 Cell culture and chemokine stimulation

Normal human astrocytes (cat# HA1800; ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured using reagents and procedures provided by the supplier. Experimental cultures (passages 1–3) were seeded at a cell density that provided 80–90% confluence at the time of treatment, and medium was replenished every 48 h. Growth medium in cultures was replaced with serum-free medium containing human recombinant IL-1 β (3 ng/ml; Peprotech, Rocky Hill, NJ, USA) to trigger NF- κ B signaling in NHA. The duration of stimulation was 24 h for induction of CXCL10 protein expression, 8 h for CXCL10 mRNA expression and 10 min to 4.5 h for NF- κ B and p38 MAPK activation, and A20 expression. However, in the IL-1 β washout experiment, NHA cells were only exposed to IL-1 β for 30 or 360 min (see Fig. 4 for further details).

2.2 Drugs and Reagents

The MOR-selective antagonist, beta-funaltrexamine (β -FNA) was obtained from the NIDA reagent supply program (Bethesda, MD, USA). The concentration of β -FNA ranged from 0.3–100 μ M and for the majority of the experiments was added to cell cultures at the time of chemokine stimulation. The exceptions were in the experiment where cells were pre-treated for 60 min followed by drug washout (Fig. 3) and the experiment where β -FNA was added after IL-1 β stimulation and washout (Fig. 4). Naltrexone, a non-selective opioid receptor antagonist (Sigma-Aldrich, St. Louis, MO, USA) was used at a concentration of 10 μ M and added to cell cultures at the time of chemokine stimulation in the one set of experiments where it was used (Fig. 2).

2.3 CXCL10 protein and RNA expression

A standard dual-antibody solid phase immunoassay (ELISA Development Kit; Peprotech) was used for quantitation of secreted CXCL10 in NHA cell supernatants, and in mouse plasma and whole brain homogenates, according to the manufacturer's instructions and as previously described (Davis et al., 2007). CXCL10 mRNA levels were assessed by real-time PCR using the SYBR Green detection method as previously described (Davis et al., 2008). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RT-PCR primer pair sets were obtained from Invitrogen and amplification from first strand cDNA was performed as instructed by the supplier. Expression of CXCL10 mRNA was normalized to the expression of the endogenous control, GAPDH mRNA. The primer sequences for CXCL10 were AACCTCCAGTCTCAGACCATGAA (forward) and AGGTACAGCGTAAGGTTCTAGAGAG (reverse); the primer pairs for GAPDH were GAGTCAACGGATTTGGTCGT (forward) and TTGATTTTGGAGGGATCTCG (reverse). Relative quantification of gene expression was evaluated using the comparative cycle threshold (C_T) method and fold change relative to unstimulated controls calculated using 2^{-C_T} (Hettinger et al., 2001).

2.4 NF- κ B/p38 MAPK signal pathway assays

To assess the role of NF- κ B in IL-1 β -induced CXCL10 expression, NHA were stimulated in the presence of SN50 (Biomol; Farmingdale, NY), a cell permeable inhibitory peptide containing the nuclear localization sequence of NF- κ B p50, which prevents nuclear translocation of the active NF- κ B complex (Lin et al., 1995). As done previously (Yang et al., 2005), cells were pre-incubated with either 50 μ M SN50 or 50 μ M of the inactive mutant peptide SN50M, for 1 h prior to stimulation.

The nuclear translocation of NF- κ B p50 and p65 was determined by first obtaining nuclear protein as previously described (Davis and Syapin, 2004). NF- κ B activation, as determined by increased levels of the active (DNA-binding) forms of NF- κ B p50 and p65 in the nucleus, was then assessed using the p50 and p65 Transcription Factor kits (Thermo Scientific, Rockford, IL, USA) as previously described (Tousi et al., 2010).

Phospho-p38 levels, as a measure of p38 activation, were determined by western blot as described earlier (Das et al., 2011). Briefly, after experimental treatments, NHA were washed twice with PBS and cytosolic extracts prepared using Cytoplasmic Extraction Reagent (Thermo Scientific). Fifty micrograms of total protein were loaded on 10% SDS-PAGE gel, electrophoresed and then transferred to PVDF membrane. Antibodies included anti-Phospho-p38 and anti-p38 (both from Cell Signaling, Danvers, MA, USA) and were used in the dilution range of 1:1000. The blots were scanned in a phosphoimager Typhoon 9410 (GE Healthcare, Uppsala, Sweden) using enhanced chemifluorescence reagent (GE Healthcare, UK) and Image J software (National Institute of Health, Bethesda, MD, USA) was used for densitometric analysis.

2.5 Determination of A20 expression and inhibition of the ubiquitination pathway

A20 protein levels were determined in cytosolic extracts by western blot as described above. Anti-A20 antibody (Cell Signaling) was used at 1:1000 dilution and anti- β -tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:200 dilution as a loading control. The involvement of the ubiquitination pathway in IL-1 β -induced CXCL10 expression in NHA was assessed using PYR41 (4 nM, Tocris Bioscience, Minneapolis, MN) a cell permeable inhibitor of ubiquitin (Ub)-activating enzyme E1, to block signaling through this pathway. Thus, cells were co-exposed to IL-1 β and PYR41 for 24 h, followed by measurement of CXCL10 in media by ELISA.

For all studies, total cellular/nuclear protein levels were determined using the bicinchoninic acid (BCA) protein assay to normalize data when appropriate (Davis et al., 2002).

2.6 Animal procedures and LPS-induced sickness behavior

Seven-week old male C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME) were housed onsite in USDA approved facilities at Oklahoma State University Center for Health Sciences (OSU-CHS) and acclimated for 1–2 weeks prior to experimental treatment. Mice were housed individually in plastic cages (10 $\times$ 17 $\times$ 28 cm) containing pine chip bedding and paper towel tubes to provide hiding places and some level of environmental enrichment, and had ad libitum access to food and water. The ambient

temperature of the animal room was maintained at 21°C with a 12:12 light:dark cycle. All experimental manipulations and animal handling procedures were approved by the OSU-CHS Institutional Animal Care and Use Committee.

This is the initial investigation of the potential anti-inflammatory effects of β -FNA in vivo, so we selected a simple, well-established and biologically relevant model of neuroinflammation (Qin et al., 2007; Dantzer et al., 2008; Thompson et al., 2008). In this model, 6 h after LPS administration, there is both sickness behavior and significant chemokine expression in the brain. Thus, mice were injected (i.p., 0.2 ml total volume) with saline (n = 7), LPS (*Escherichia coli* O55:B5; Sigma; 5 mg/kg; n = 5), β -FNA (28 mg/kg; n = 3), or LPS + β -FNA (n = 5). The dose of β -FNA was based on reports in the literature in which β -FNA was administered i.p. in mice (Choi et al., 2003; Ramachandra et al., 2011). After 6 h treatment, mice were tested in open-field arenas for quantitative evidence of sickness behavior (e.g., reduced locomotion). Each mouse was placed alone into an open field arena (40×40 cm) and behavior was digitally recorded and scored using Ethovision software. The a priori dependent measure was distance traveled (cm).

Immediately after openfield testing, mice were euthanized by CO₂ inhalation, after which mice were decapitated and trunk blood and brain were collected on water-ice. Plasma was collected by centrifugation and stored at -80°C. Whole brain tissue was washed one time by adding 0.5 mL ice cold PBS, centrifugation (500 × g, 3 min, 4° C), and aspiration. Tissue was homogenized in 4.7 mL ice cold triple-detergent lysis buffer (Curtis et al., 2011) using a Sonic Dismembrator Model 100 (Fisher Scientific), then centrifuged (20,000 × g, 30 min, 4° C). The aqueous phase was collected and stored at -80° C until total protein levels were determined and ELISA performed.

2.7 Statistical analysis

Prism™ version 6.0 software (GraphPad Inc., San Diego, CA) was used for figure presentation and statistical analysis. Analyses included nonlinear regression (Hill slope not constrained), linear regression, unpaired t-test, one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test, and two-way ANOVA followed by Bonferroni's post-hoc test. Data are presented as mean ± S.E.M. and a probability (P) of < 0.05 indicated statistically significant differences between groups.

3. Results

3.1 Effects of β -FNA on IL-1 β -induced CXCL10 expression

Initially we were interested in confirming the inhibitory effects of β -FNA on CXCL10 protein expression in NHA. CXCL10 is not constitutively expressed by NHA. However, following 24 h exposure to IL-1 β (3 ng/ml), levels of secreted CXCL10 into the cell supernatant exceeded 50,000 pg/mg total protein (Fig. 1A). IL-1 β -induced CXCL10 expression was inhibited in a concentration-dependent fashion by co-exposure to β -FNA, with an EC₅₀ = 7.6 μ M (95% CI = 3.0–19.4). Also, the treatments did not affect cell viability, as determined by the MTT reduction assay (data not shown). Consistent with CXCL10 protein expression, constitutive CXCL10 mRNA levels were negligible, whereas IL-1 β treatment resulted in a significant increase in CXCL10 mRNA expression 8 h post-

stimulation (Fig. 1B). However, co-exposure to β -FNA significantly inhibited IL-1 β -induced CXCL10 mRNA expression as indicated by an unpaired t-test (Fig. 1B).

β -FNA is an alkylating derivative of naltrexone that covalently binds lysine²³³ of the MOR, resulting in irreversible antagonism (Chen et al., 1996), whereas, naltrexone is a non-alkylating, reversible, non-selective opioid receptor antagonist (Rothman et al., 1988). To further demonstrate that the inhibition of CXCL10 expression by β -FNA is not mediated by the classical MOR, we compared the effects of equimolar concentrations (10 μ M) of these two compounds on IL-1 β -induced CXCL10 expression in NHA. One-way ANOVA ($F_{2,30} = 5.093$, $P < 0.02$) and Dunnett's post-test ($P < 0.05$ vs. IL-1 β alone) indicated that β -FNA significantly inhibited IL-1 β -induced CXCL10 expression, whereas naltrexone had no effect (Fig. 2).

Pre-treatment with β -FNA, followed by drug washout, inhibited IL-1 β -induced CXCL10 expression. Limited exposure (60 min) to β -FNA was sufficient to inhibit subsequent IL-1 β -induced CXCL10 expression and the level of inhibition was similar to that produced when β -FNA was present for the entire 24 h IL-1 β treatment (Fig. 3). Thus, β -FNA effects were persistent, and not amenable to washout, leading to continued inhibition of CXCL10 expression after β -FNA was removed from the NHA.

The next experiment was designed to determine the extent to which β -FNA could inhibit IL-1 β -induced CXCL10 expression when added after IL-1 β stimulation was initiated and excess IL-1 β washed out, thus assessing the effects of β -FNA on signaling events downstream of the IL-1 receptor (IL-1R). Stimulation of NHA with IL-1 β for a limited period of time, 0.5 h, resulted in minimal CXCL10 expression at 24 h and the level of expression was 5% of that in cells exposed to IL-1 β for 24 h (Fig. 4). Two-way ANOVA (β -FNA treatment $\times$ time) indicated a significant effect of time ($F_{1,33} = 70.02$, $P < 0.0001$), β -FNA treatment ($F_{1,33} = 6.10$, $P < 0.02$) and a significant interaction ($F_{1,33} = 7.17$, $P < 0.02$). Thus, the addition of β -FNA after the 0.5 h IL-1 β stimulation period and subsequent IL-1 β washout had no effect on CXCL10 expression. However, a 3 h IL-1 β stimulation period was sufficient to induce appreciable levels of CXCL10 expression at 24 h (CXCL10 expression levels were 30% of those in cells exposed to IL-1 β for 24 h). Furthermore, as determined by Bonferroni's post-test, addition of β -FNA after the IL-1 β was washed out resulted in significant inhibition of CXCL10 expression that was induced by the 3 h IL-1 β stimulation period (Fig. 4).

3.2 Effects of β -FNA on NF- κ B signaling proteins in IL-1 β -induced CXCL10 expression

To confirm that IL-1 β -induced CXCL10 expression is NF- κ B-dependent in NHA, we utilized SN50, a well characterized inhibitor of NF- κ B nuclear translocation (Lin et al., 1995). Pretreatment of NHA with SN50 significantly inhibited IL-1 β -induced CXCL10 protein expression as indicated by one-way ANOVA ($F_{2,24} = 5.79$, $P < 0.009$) and Dunnett's post-test ($P < 0.05$ vs. IL-1 β alone) (Fig. 5A). However, pretreatment with the mutated form of this inhibitory molecule (SN50 Mut), did not significantly inhibit IL-1 β -induced CXCL10 protein expression (Fig. 5A).

After confirming that IL-1 β -induced CXCL10 protein expression is NF- κ B-dependent in NHA, we determined whether β -FNA inhibits NF- κ B activation. IL-1 β induced a time-dependent increase in NF- κ B activation (Fig. 5B and C). IL-1 β -induced NF- κ B p50 activation increased steadily over the 270 min stimulation period (Fig. 5B). IL-1 β -induced NF- κ B p65 activation increased over the first 30 min of stimulation and appeared to be declining by 270 min (Fig. 5C). For NF- κ B p50, two-way ANOVA (β -FNA treatment $\times$ time) indicated a significant effect of time ($F_{3, 35} = 92.92$, $P < 0.0001$) and β -FNA treatment ($F_{1, 35} = 6.92$, $P < 0.02$). However, there was not a significant interaction ($F_{3, 35} = 0.66$, $P = 0.58$). Similarly for NF- κ B p65, two-way ANOVA indicated a significant effect of time ($F_{3, 40} = 49.11$, $P < 0.0001$) and β -FNA treatment ($F_{1, 40} = 5.57$, $P < 0.03$); again, there was no significant interaction ($F_{3, 40} = 0.66$, $P = 0.58$).

Activation of p38 MAPK occurs following pro-inflammatory stimuli that induce the expression of the chemokine CXCL10 in astrocytes (Davis et al., 2013). Therefore, activated p38 MAPK (phospho-p38) was determined in β -FNA treated and control NHA. IL-1 β induced p38 MAPK activation as indicated by significantly increased levels of phospho-p38 following 10 min of stimulation (Fig. 6). Co-exposure to β -FNA significantly inhibited IL-1 β -induced p38 MAPK activation as indicated by lower levels of phospho-p38 at 10 and 30 min post-stimulation. Two-way ANOVA (β -FNA treatment $\times$ time) indicated a significant effect of time ($F_{3, 16} = 328.6$, $P < 0.0001$) and β -FNA treatment ($F_{1, 16} = 27.35$, $P < 0.0001$). The interaction only approached significance ($F_{3, 16} = 3.19$, $P = 0.052$).

The ubiquitin-editing enzyme A20 is a well-characterized negative regulator of NF- κ B activation (Vereecke et al., 2009). We hypothesized that β -FNA treatment would decrease A20 expression in NHA due to less feedback up-regulation resulting from β -FNA inhibition of NF- κ B activation. Constitutive expression of A20 was below the level of detection by western blot. IL-1 β induced a time-dependent increase in cytosolic A20 expression that was detectable at 30 min and that increased 150-fold by 270 min (Fig. 7). Co-exposure to β -FNA significantly inhibited IL-1 β -induced A20 expression as indicated by lower levels of A20 at 90 and 270 min post-stimulation. Two-way ANOVA (β -FNA treatment $\times$ time) indicated a significant effect of time ($F_{3, 16} = 326.4$, $P < 0.0001$) and β -FNA treatment ($F_{1, 16} = 73.92$, $P < 0.0001$). There was also a significant interaction ($F_{3, 16} = 33.98$, $P < 0.0001$).

The activity of the Ub-activating enzyme E1 is essential for NF- κ B signaling and the small molecule PYR41 was the first cell-permeable agent discovered to inhibit E1 (Yang et al., 2007). PYR41 inhibited IL-1 β -induced chemokine CXCL10 expression in NHA by 50%, as did β -FNA (Fig. 8). Furthermore, one-way ANOVA ($F_{3, 20} = 25.12$, $P < 0.0001$) and Dunnett's post-hoc test ($P < 0.01$) indicated that the combination treatment of PYR41 and β -FNA caused even further inhibition of chemokine expression compared to that of either agent alone.

3.3 Effects of β -FNA on LPS-induced sickness behavior and chemokine expression in C57BL/6J mice

In order to test the anti-inflammatory effects of β -FNA in vivo, we used an established model in which a single i.p. injection of LPS induces neuroinflammation and sickness behavior in C57BL/6J mice. Then, 6 h after injection, CXCL10 protein levels in the brain of

LPS-treated mice were significantly higher (8-fold) than in mice injected with saline or β -FNA alone as indicated by one-way ANOVA ($F_{3,15} = 32.53$, $P < 0.0001$) (Fig. 9A). Furthermore, co-treatment with β -FNA resulted in significantly reduced CXCL10 in the brains of LPS-treated mice as indicated by Dunnett's post-test ($P < 0.05$). Levels of CXCL10 were also increased in plasma following LPS treatment, however, co-treatment with β -FNA did not alter plasma CXCL10 levels in LPS-treated mice (Fig. 9B).

Using the open-field test immediately following the 6 h treatment period, we determined that LPS-treated mice displayed profound sickness behavior (limited distance traveled) compared to mice injected with saline or β -FNA alone (Fig. 10). Linear regression analysis of all 4 treatment groups indicated that distance moved was inversely related to CXCL10 levels in the brain ($P < 0.0001$, $R^2 = 0.7443$; Fig. 10A). While mice co-treated with LPS and β -FNA tended to display greater locomotor activity than mice treated with LPS alone (Fig. 10B), the difference did not reach the level of statistical significance ($P = 0.14$ by unpaired t-test).

4. Discussion

Opioid-immune system crosstalk characterized by opioid modulation of immune or inflammatory pathways can occur through non-classical opioid receptor targets. For example, naloxone is well characterized as a general opioid receptor antagonist; however, naloxone reduces neuroinflammation via mechanisms that do not require binding to classical opioid receptors (Liu and Hong, 2003). Opioid receptor-independent actions of naloxone include prevention of bacterial LPS-binding to microglia and reduced microglial superoxide production (Liu et al., 2000; 2002). Various opioids and their stereoisomers modulate signaling through the TLR4 receptor without action at classical opioid receptors (Hutchinson and Watkins, 2014; Stevens et al., 2013).

We are particularly interested in the anti-inflammatory actions of β -FNA and were the first to report that β -FNA inhibits inflammatory signaling in human astroglial cells via a MOR-independent action (Davis et al., 2007). We discovered that TNF α -induced CXCL10 expression in A172 astroglial cells was inhibited by the MOR agonist, fentanyl. However, neither the general opioid antagonist, naltrexone, nor the selective MOR antagonist, β -FNA, blocked the fentanyl-mediated inhibition of CXCL10 expression (Davis et al., 2007). In fact, β -FNA was a more potent inhibitor of CXCL10 expression in A172 cells than was fentanyl. Recently, we performed a series of experiments to further address the potential involvement of opioid receptors, including MOR, kappa opioid receptor (KOR) and nociceptin/orphanin FQ receptor (ORL). β -FNA actions were not blocked by concurrent treatment with selective opioid antagonists targeting their respective opioid receptors (Davis et al., 2013).

Based on our published reports, it is clear that β -FNA inhibits inflammatory signaling regardless of the pro-inflammatory stimulus or cell type, as determined using several endpoints (Davis et al., 2007; 2008, 2013; Stevens et al., 2013). Continuing our efforts to identify the mechanism of action for the anti-inflammatory effects of β -FNA, the present study focused on IL-1 β -induced signaling in human astrocytes. Concurrent treatment of astrocytes with β -FNA and IL-1 β inhibited CXCL10 expression compared to IL-1 β alone at

both mRNA and protein levels (Fig. 1), which is consistent with our previous findings of transcriptional inhibition by β -FNA (Davis et al. 2007; 2008). Naltrexone had no effect on IL-1 β -induced CXCL10 expression. The inhibition of chemokine expression by β -FNA was observed when β -FNA was given as a pretreatment (Fig. 3) or after astrocyte activation by IL-1 β (Fig. 4).

Based on both behavioral (Ward et al., 1982) and in vitro assays (Liu-Chen et al., 1990), the mechanism of action of β -FNA was shown to be an initially reversible kappa-opioid receptor (KOR) agonism followed by irreversible MOR antagonism. As an alkylating agent, β -FNA covalently binds Lys²³³ of MOR resulting in irreversible antagonism (Chen et al., 1996) although other residues on MOR are needed for optimal covalent binding of β -FNA (DiMattio et al., 2015). Given that β -FNA exerted its anti-inflammatory effect through a non-opioid receptor mechanism, it is possible that covalent modification of NF- κ B/MAPK signaling protein(s) accounts for the anti-inflammatory effect of β -FNA (see below). This is consistent with the ability of β -FNA to inhibit chemokine expression when applied and washed out before IL-1 β administration, and when applied following washout of IL-1 β administration.

IL-1 β administration alone stimulates CXCL10 expression, which is significantly blocked by co-administration of SN50 but not the SN50 mutant protein (Fig. 5A). SN50 is a small molecule inhibitor which prevents nuclear translocation of the activated NF- κ B complex (Lin et al., 1995). β -FNA had a small but significant effect of reducing levels of NF- κ B p65 in the nucleus of activated astrocytes. It remains to be determined whether β -FNA prevents nuclear translocation directly or inhibits upstream events in the NF- κ B signaling pathway. We recently determined that IFN γ + HIV-1 Tat-induced CXCL10 expression in NHA was also inhibited by β -FNA, but NF- κ B activation was not (Davis et al., 2013). It may be that β -FNA effects on NF- κ B signaling in astrocytes are, in part, stimulus-dependent. Together, these findings indicate that β -FNA can inhibit CXCL10 expression via a mechanism other than just the disruption of NF- κ B nuclear translocation.

Activation of p38 MAPK is instrumental in inflammatory signaling in glia, including induction of CXCL10 (Williams et al., 2009). β -FNA inhibited IL-1 β -induced p38 MAPK activation in NHA (Fig. 6) and this is consistent with our recent observation that IFN γ + HIV-1 Tat-induced p38 MAPK activation was inhibited by β -FNA (Davis et al., 2013). MAPK signaling intersects with NF- κ B pathways as p38 MAPK is needed for phosphorylation and activation of NF- κ B transcription factors (Wagley et al., 2013). Additionally, p38 MAPK activates the transcription factor, activator protein-1 (AP-1), which is also involved in pro-inflammatory responses (Hayashi et al., 2013). In cases where the anti-inflammatory effect of β -FNA was not shown to occur by the alteration of NF- κ B signaling proteins, β -FNA may be acting via MAPK pathways, p38 MAPK and AP-1, to inhibit pro-inflammatory responses of astrocytes.

The expression of A20, a cytoplasmic, ubiquitin-modifying enzyme, is instrumental in the negative regulation of NF- κ B (Verstrepen et al., 2010). The expression of A20 is induced via an NF- κ B-dependent mechanism providing a negative feedback loop for NF- κ B signaling (Verstrepen et al. 2010). A20 disrupts NF- κ B signaling downstream from multiple

receptors including TLRs, tumor necrosis factor receptor 1 and the IL-1 receptor (Shembade and Harhaj 2012). A20 interacts with multiple protein substrates to regulate NF- κ B signaling. For example, A20 inhibits IL-1R-mediated NF- κ B signaling by cooperating with Tax1 binding protein 1 to block TNF receptor associated factor 6 (TRAF6) ubiquitination (Shembade and Harhaj, 2012). The present study is the first to report A20 expression in human astrocytes, and consistent with the majority of cell types, IL-1 β -induces a time-dependent increase in A20 expression that occurs subsequent to NF- κ B nuclear translocation (Fig. 7). Additionally, the IL-1 β -induced A20 expression is inhibited by β -FNA. There also is other evidence that A20 expression is dependent upon p38 MAPK activation as SB202190, a selective inhibitor of p38 MAPK activation, inhibited A20 mRNA expression in LPS-treated primary human monocytes (Manthey et al. 1998). Together, these findings suggest that the inhibition of IL-1 β -induced A20 expression in NHA following β -FNA treatment is due, at least in part, to decreased NF- κ B and/or p38 MAPK activation.

The activity of the ubiquitin-activating enzyme E1 marks I κ B α for proteasomal destruction, releasing its inhibition of NF- κ B activation (Dyson and Komives, 2012). The small molecule inhibitor of ubiquitin-activating enzyme E1, PYR41, attenuates cytokine-mediated NF- κ B activation by inhibiting ubiquitination and proteasomal degradation of I κ B α (Yang et al., 2007). PYR41 also prevents ubiquitination at Lys residues in TRAF6. In the present study, PYR41 + β -FNA inhibited IL-1 β -induced CXCL10 expression to a greater extent than did either agent alone (Fig. 8). Thus, it may be that covalent modification of lysine(s) by β -FNA could prevent ubiquitination and potentially other protein interactions integral to signal transduction in the NF- κ B pathway.

For the initial investigation of the anti-inflammatory actions of β -FNA in vivo, we used an animal model in which a single i.p. injection of LPS is sufficient to induce neuroinflammation and sickness behavior (Qin et al., 2007). Consistent with our in vitro studies in NHA, we observed that LPS-induced CXCL10 expression in the brain was inhibited by β -FNA (Fig. 9). Interestingly, β -FNA did not affect CXCL10 levels in the plasma highlighting its potential as a neuroprotective agent rather than exerting a ‘global’ anti-inflammatory effect. It may be that astrocytes (and/or microglia) are more sensitive to β -FNA than are peripheral monocytes; yet, an expanded time course may reveal that chemokine expression is reduced in the plasma at a point beyond 6 h. LPS-induced sickness behavior, as measured by distance traveled, was correlated with CXCL10 expression as mice with higher levels of CXCL10 in the brain displayed less locomotor activity (Fig. 10). However, β -FNA did not significantly reduce LPS-induced sickness behavior at the group level. Thus, while CXCL10 expression is inhibited by β -FNA, all of the critical signaling molecules, cell types and/or brain regions involved in sickness behavior may not be uniformly affected by β -FNA. It is also possible that a higher dose of β -FNA is necessary to inhibit sickness behavior.

The inhibition of chemokines has received considerable attention as a means of reducing neuroinflammatory insults (Kiguchi et al. 2012; O’Sullivan et al. 2010; Semple et al. 2010). Neuroinflammation occurs in a wide range of brain disorders, from neurodegenerative diseases and central nervous system infection to major depressive disorders, autism, and traumatic brain injury (Glass et al., 2010; Khansari and Sperlagh, 2012; Kolb et al., 1999;

Vargas et al., 2005; Ziebell and Morganti-Kossmann, 2010). The opioid antagonist, β -FNA crosses the blood-brain barrier (Labuz et al. 2007) and is well-tolerated (Egashira et al. 2012; Jang and Yoburn 1991; Valle et al. 2001). Although the mechanism of action for the anti-inflammatory effects of β -FNA are unknown, we hypothesize that β -FNA causes covalent modification of Lys residues in signal proteins or interferes with the ubiquitination regulation of NF- κ B/MAPK pathways. Preliminary bioinformatic analysis of short sequences surrounding the Lys²³³ that β -FNA alkylates in MOR yielded a number of matches with signaling proteins in the NF- κ B pathway (unpublished observations). Further sequence- and structural-based analyses are needed to pursue potential targets for possible covalent modification by β -FNA.

5. Conclusions

The opioid antagonist, β -FNA, exhibits anti-inflammatory properties and inhibits the release of pro-inflammatory chemokines from normal human astrocytes. Chemokines are inflammatory mediators that are instrumental in response to CNS insults and elevated chemokine levels are frequently associated with neuropathology (Cinque et al. 2005; Xia et al. 2000). While the precise mechanism of action of the anti-inflammatory effects of β -FNA are unknown, it is hypothesized that the alkylating ability of β -FNA produces interference within the NF- κ B/MAPK signaling pathway or ubiquitination regulation. The opioid antagonist, β -FNA, does not interact with classical opioid receptors to produce an anti-inflammatory effect. Further research and development of β -FNA, or related analogs, for the treatment of neuroinflammation is warranted given the novel findings of an anti-inflammatory effect of β -FNA in human astrocytes.

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Fig. 1. β -FNA inhibits IL-1 β -induced CXCL10 expression in NHA. A) NHA were co-exposed to human recombinant IL-1 β (3 ng/ml) and β -FNA (0.3–100 μM) for 24 h. CXCL10 protein levels in the media were then measured by ELISA. The data represent mean $\pm$ S.E.M (n=11–15). B) NHA were treated with IL-1 β (3 ng/ml) alone or co-exposed to IL-1 β and β -FNA (10 μM) for 8 h. Total RNA was isolated and CXCL10 and GAPDH mRNA assessed by real-time PCR. The data represent mean $\pm$ S.E.M. (n=5–6). *P < 0.05.

Fig. 2.

Naltrexone does not inhibit IL-1 β -induced CXCL10 expression in NHA. NHA were exposed to IL-1 β (3 ng/ml) alone or co-exposed to IL-1 β and β -FNA (10 μ M) or naltrexone (10 μ M) for 24 h. CXCL10 protein levels in the media were then measured by ELISA. Data represent mean $\pm$ S.E.M. (n=9–12). *P < 0.05 vs. IL-1 β alone.

Fig. 3.

Inhibitory effects of β -FNA persist after drug washout. NHA were unstimulated, exposed to human recombinant IL-1 β (3 ng/ml) alone or exposed to IL-1 β + β -FNA (10 μ M) for 24 h. In another treatment group, cells were pre-treated with β -FNA for 60 min. followed by drug washout. Cells were then exposed to IL-1 β for 24 h. CXCL10 protein levels in the media then were measured by ELISA. The data represent mean $\pm$ S.E.M (n=11–12). *P < 0.05 vs. IL-1 β alone.

Fig. 4.

β -FNA actions are downstream of IL-1R. NHA were exposed to human recombinant IL-1 β (3 ng/ml) for 0.5 or 3 h, followed by washout of stimulus. Cells were then exposed to β -FNA (10 μ M) for 21 h. CXCL10 protein levels in the media then were measured by ELISA. The data represent mean $\pm$ S.E.M. (n=5–14) and are presented as percent control (24 h IL-1 β stimulation). *P < 0.05 vs. 3 h control.

Fig. 5.

β -FNA inhibits IL-1 β -induced NF- κ B activation. NF- κ B activation was induced in NHA by stimulating with human recombinant IL-1 β (3 ng/ml) for 0.5 h. A) IL-1 β -induced-CXCL10 expression was confirmed to be NF- κ B-dependent in NHA. An inhibitor of NF- κ B nuclear translocation, 50 μ M SN50 (or 50 μ M of the inactive control peptide, SN50 Mut), was added 1 h before stimulation. The data represent mean $\pm$ S.E.M. (n=9). *P < 0.05 vs. IL-1 β . B) and C) β -FNA inhibits IL-1 β -induced NF- κ B activation. NHA were either untreated or co-exposed to IL-1 β (3 ng/ml) and β -FNA (10 μ M) for 10–270 min. Levels of the active (DNA-binding) forms of NF- κ B p50 and p65 in the nucleus were determined using a NF- κ B Transcription Factor kits (Thermo Scientific). The data represent mean $\pm$ S.E.M. (n=3–6).

*P < 0.05 vs. IL-1 β at 10 min. and IL-1 β + β -FNA at 10 min; #P < 0.05 vs. IL-1 β + β -FNA at 30 min.; &P < 0.05 vs. IL-1 β and IL-1 β + β -FNA at 10, 30 and 90 min.

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Fig. 6.

β -FNA inhibits IL-1 β -induced p38 activation in NHA. A) NHA were either untreated or stimulated with human recombinant IL-1 β (3 ng/ml) in the presence or absence of β -FNA (10 μ M) for 10–270 min. Western blot was used to determine levels of total p38 and phosphorylated-p38 (p-p38) in the cytosolic fraction. B) Data for the densitometric analysis represent mean $\pm$ S.E.M. (n=3). Integrated density values are presented as a ratio of p-p38/total p38 and expressed as fold change relative to unstimulated control. **P < 0.01 and *P < 0.05 for IL-1 β vs. IL-1 β + β -FNA at 10 and 30 min, respectively.

Fig. 7. β -FNA inhibits IL-1 β -induced A20 expression in NHA. A) NHA were either untreated or stimulated with human recombinant IL-1 β (3 ng/ml) in the presence or absence of β -FNA (10 μ M) for 10–270 min. Western blot was used to determine levels of A20 and β -tubulin in the cytosolic fraction. B) Data for the densitometric analysis represent mean $\pm$ S.E.M. (n=3). Integrated density values are presented as a ratio of A20/ β -tubulin and expressed as fold change relative to unstimulated control. **P < 0.01 and ***P < 0.001 for IL-1 β vs. IL-1 β + β -FNA at 90 and 270 min, respectively.

Fig. 8.

Inhibition of the ubiquitination pathway is likely involved in the anti-inflammatory actions of β -FNA. NHA were exposed to human recombinant IL-1 β (3 ng/ml) alone, IL-1 β + β -FNA (5 μ M), IL-1 β + PYR41 (4 nM; inhibitor of Ub-activating enzyme E1), or, IL-1 β + β -FNA + PYR41 for 24 h. CXCL10 protein levels in the media then were measured by ELISA. The data represent mean $\pm$ S.E.M. (n=6). **P < 0.01, ***P < 0.001 vs. IL-1 β + β -FNA.

Fig. 9.

β -FNA inhibits LPS-induced CXCL10 protein levels in brain, but not plasma. C57BL/6J mice were injected (i.p.) with saline ($n = 7$), LPS (5 mg/kg; $n = 5$), β -FNA (28 mg/kg; $n = 3$), or LPS + β -FNA ($n = 5$). At 6 h post-injections, plasma and whole brain were collected on ice. CXCL10 protein levels in plasma and whole brain tissue homogenates were determined by ELISA. Data represent the mean $\pm$ S.E.M.; * $P < 0.05$ vs. LPS.

Fig. 10.

C57BL/6J mice were injected (i.p.) with saline ($n = 7$), LPS (5 mg/kg; $n = 5$), β -FNA (28 mg/kg; $n = 3$), or LPS + β -FNA ($n = 5$). At 6 h post-injections, mice were tested in open-field arenas for quantitative evidence of sickness behavior (e.g., reduced locomotion). Each mouse was placed alone into an open field arena (40×40 cm) and distance traveled (cm) was digitally recorded and scored using Ethovision software. A) Distance traveled; B) linear regression analysis of distance traveled and CXCL10 levels in brain. Data represent mean $\pm$ S.E.M.