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## **$\beta$ -Funaltrexamine Inhibits Inducible Nitric-oxide Synthase Expression in Human Astroglial Cells**

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### **Abstract**

The inducible isoform of nitric-oxide synthase (iNOS) is involved in neuropathogenesis associated with infection and disease in the brain. Hence, there is considerable interest in the identification of therapeutic interventions to prevent iNOS-mediated pathology. Astroglia are a major site of iNOS expression during neuropathogenesis. To mimic a key component of neuroinflammation, human A172 astroglial cells were exposed *in vitro* to a cytokine mixture containing interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and interleukin- $1\beta$ , resulting in significant iNOS expression. Next, we assessed the effects of the *mu* opioid receptor antagonist,  $\beta$ -funaltrexamine ( $\beta$ -FNA), on cytokine induced iNOS expression in human astroglia.  $\beta$ -FNA dose-dependently inhibited iNOS expression.  $\beta$ -FNA transcriptionally (or pre-transcriptionally) inhibited cytokine-induced iNOS activation as indicated by a significant decrease in NOS2 messenger RNA expression. Further characterization of the novel, anti-inflammatory actions of  $\beta$ -FNA may provide insights for pharmacologic strategies to treat or prevent brain pathologies associated with neuroinflammation.

## Keywords

neuroinflammation; opioids; astrocytes; nitric oxide; fentanyl; morphine

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## Introduction

Increasing evidence suggests that the inducible isoform of nitric-oxide synthase (iNOS) is instrumental in the neuropathogenesis associated with infection (Poluektova et al. 2005) trauma (Louin et al. 2006) and neurodegenerative diseases (Marchetti et al. 2005). While not typically expressed in the central nervous system (CNS) under physiological conditions, iNOS is transcriptionally induced by various stimuli associated with pathologic insults including, hypoxia, bacterial components, viral proteins, and cytokines. Once expressed, iNOS catalyzes the nicotinamide adenine dinucleotide phosphate-dependent oxidation of L-arginine to nitric oxide (NO) and citrulline. Unlike other NOS isoforms, iNOS has continuously high and long-lasting activity and, depending on the temporal expression and cellular context, can either be neurotoxic or neuroprotective. Increased iNOS activation associated with infection is typically neuroprotective given the role of NO in the destruction of infectious organisms. Whereas, sustained iNOS activation in the CNS is predominantly detrimental resulting in neurotoxicity and cognitive deficit. Therefore, there is an interest in identifying therapeutic strategies to attenuate excessive iNOS expression and activity, thereby reducing further neuropathogenesis (Jafarian-Tehrani et al. 2005; Louin et al. 2006). For instance, administration of 1400W (a selective inhibitor of iNOS) within 18 h of traumatic brain injury reduces neuropathogenic insult (Jafarian-Tehrani et al. 2005). More recently, opioids such as morphine have gained attention for their potential neuroprotective actions (Zhao et al. 2006). However, very little is known about the specific effects of opioids on iNOS activation in astroglia. Additionally, the clinical use of *mu* opioid receptor (MOR) agonists such as morphine is associated with numerous adverse effects including respiratory depression, nausea, and dependence. Herein, we have assessed the effects of a non-addictive opioid agent, the MOR-selective antagonist,  $\beta$ -funaltrexamine ( $\beta$ -FNA), on human astroglial iNOS expression. We have demonstrated that cytokine-induced iNOS expression in human astroglial cells is dose-dependently inhibited by  $\beta$ -FNA. Evidence for a pretranslational mechanism of action is presented and discussed; to our knowledge, this is the first report of iNOS inhibition by  $\beta$ -FNA.

## Materials and methods

### Cell culture

The human astrocytoma cell line (A172, ATCC #CRL-1620; American Type Culture Collection, Manassas, VA, USA) was used to model a key component of neuroinflammation. A172 cells were maintained as previously described (Davis et al. 2002), and experimental cultures were seeded at a cell density ( $1 \times 10^4/\text{cm}^2$ ) to provide 80–90% confluence at the time of treatment.

### iNOS induction

Optimal induction of iNOS expression in A172 cells requires co-exposure to a mixture of cytokines (Davis et al. 2002). Thus, iNOS was induced by 8–24 h exposure to a cytokine mixture containing 100 ng/ml human recombinant IFN $\gamma$  (Peprotech Inc., Rocky Hill, NJ), 30 ng/ml human recombinant TNF $\alpha$  (Peprotech), and 5 ng/ml human recombinant IL-1 $\beta$  (Peprotech). To assess the effects of  $\beta$ -FNA on iNOS expression,  $\beta$ -FNA (0.05–33  $\mu\text{M}$ ; Sigma, St. Louis, MO) was added to cell cultures at the time of cytokine stimulation. Cell

viability was monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as previously described (Davis et al. 2007).

To quantitate iNOS activity, nitrite accumulation in the culture media was determined spectrophotometrically 24 h post-stimulation using the Griess reagent as previously described (Davis et al. 2002). Quantitative analysis of NOS2 gene expression was evaluated using the quantitative real-time polymerase chain reaction (qRT-PCR) and the SYBR green I reporter assay. Total cellular RNA was collected according to an established procedure (Davis and Syapin 2004). Primers, forward (5' GGAGCCAGCTCTGCATTATC 3') and reverse (5' TTTTGTCTCCAAGGGACCAG 3') were then developed for amplification of a 195-bp region of the iNOS gene (GenBank accession no. NM\_000625) using Primer 3 software. The PCR product generated was sequenced to confirm that the amplicon was iNOS. Two-step reverse transcriptase–polymerase chain reactions (RT-PCR) were performed. Complementary DNA (cDNA) synthesis was performed using 1 µg of total RNA using the QuantiTec reverse transcription kit (Qiagen, Valencia, CA, USA), which includes a DNA removal step to eliminate genomic contamination. Manufacturer's protocol was followed with one modification where the cDNA synthesis reaction was incubated for 30 min at 42°C instead of the 15 min described in the protocol. The quantitative gene expression analysis was performed using the FastStart SYBR green master kit (Roche Applied Science, Indianapolis, IN, USA) in a Biorad MyiQ detection system (Biorad, Hercules, CA, USA). A total reaction volume of 15 µl contained 400 nM forward primer, 400 nM reverse primer, 1× master mix, and 40 ng of cDNA. Thermal cycling conditions were 95°C for 10 min, followed by 45 additional cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a melting curve to ensure the amplification of a single product. Ribosomal 18S RNA (18S, RNA control kit, Eurogentec, Philadelphia, PA, USA) was assayed according to the manufacturer's protocol for each sample for normalization. Relative quantification of gene expression was evaluated using the comparative computed tomography (CT) method as described previously (Hettinger et al. 2001). The  $\Delta$ CT value was determined by subtracting the gene CT value of each sample from the corresponding sample ribosomal 18S CT value. Calculation of  $\Delta\Delta$ CT was carried out by subtracting the mean  $\Delta$ CT value obtained for the unstimulated samples from all other  $\Delta$ CT mean values. Fold changes in gene expression were then calculated from the  $\Delta\Delta$ CT values using the formula  $2^{-\Delta\Delta Ct}$ .

### 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 + SEM or mean IC<sub>50</sub> values. For analysis of iNOS gene expression, data were analyzed by the least square ANOVA using the Proc Mixed procedure of the Statistical Analysis Software (SAS 1988). The fixed effect of treatment was analyzed for iNOS gene expression using  $\Delta$ CT values. Results are presented as least square means ± standard error of the mean. A probability (*P*) of <0.05 was accepted as demonstrating statistically significant differences between groups. The number of replicate measures and independent experiments from which the data were obtained are provided in the individual figure legends.

## Results

### $\beta$ -FNA effects on iNOS activity

$\beta$ -FNA potently inhibited cytokine-induced iNOS activity in A172 cells (Fig. 1). The reduction in iNOS activity mediated by  $\beta$ -FNA was not a consequence of cytotoxicity, as no differences in viability were detected among the concentrations tested (Fig. 1). Also, control experiments confirmed that  $\beta$ -FNA did not interfere with the nitrite assay and had no effect on nitrite detection (data not shown).

### $\beta$ -FNA effects on NOS2 mRNA expression

Minimal NOS2 mRNA expression was detected in unstimulated control cells as revealed by qRT-PCR (Fig. 2). Conversely, 8 h cytokine exposure resulted in a marked induction of NOS2 mRNA expression. Cytokine-induced NOS2 mRNA expression was significantly inhibited in the presence of 11  $\mu$ M  $\beta$ -FNA (Fig. 2). Exposure to  $\beta$ -FNA alone for 8 h had no effect on NOS2 mRNA expression in A172 cells.

## Discussion

Astroglia are a major source of iNOS expression and NO production in the brain, and extensive and/or prolonged iNOS expression is presumably instrumental in neuropathogenesis (Louin et al. 2006). In the present study, iNOS activation was assessed through measurement of nitrite accumulation in the media (which only occurs in these cells after iNOS expression) and at the level of NOS2 mRNA expression. Details on the time course of cytokine-induced iNOS expression in A172 cells, at the level of mRNA and protein, have been discussed in a previous report (Davis and Syapin 2004). Likewise, cytokine-stimulated iNOS activity in A172 cells as measured by nitrite accumulation and the citrulline conversion assay has been characterized by our group (Davis et al. 2002; Davis and Syapin 2004).

Ablation of iNOS induction prevents neuropathogenesis (Jafarian-Tehrani et al. 2005; Louin et al. 2006). In particular, opioids such as morphine have neuroprotective properties (Zhao et al. 2006). While the mechanism for the neuroprotective effects of morphine are not completely understood, under certain conditions, morphine attenuates oxidative and proinflammatory stress (Lee et al. 2004). In our human astroglial model, we found that morphine ( $\geq 1$  mM) and the more potent MOR agonist, fentanyl ( $\geq 0.2$  mM) only inhibited iNOS expression at relatively high, biologically irrelevant concentrations (data not shown). Interestingly, the MOR antagonist,  $\beta$ -FNA failed to block the inhibitory actions of fentanyl (data not shown). However, this is the first report to demonstrate that  $\beta$ -FNA dose-dependently inhibits iNOS activity in astroglial cells. Inhibition of NOS2 mRNA expression by  $\beta$ -FNA suggests that iNOS activation is inhibited transcriptionally or pre-transcriptionally, yet the details remain to be elucidated. Given that the MOR selective antagonist,  $\beta$ -FNA, did not block fentanyl-mediated inhibition of iNOS activity suggests a MOR-independent mechanism.

The anti-inflammatory actions of  $\beta$ -FNA identified in this study support and add to the findings of others (Liu et al. 2000), which have identified MOR-independent, anti-inflammatory actions of the non-specific opioid receptor antagonist, naloxone. For example, both the MOR inactive stereoisomer (+)-naloxone and (-)-naloxone reduced inflammatory-induced damage of rat dopaminergic neurons, suggesting a potential therapeutic role in the treatment of Parkinson's disease (Liu et al. 2000). More recently, this same group determined that A $\beta$  (1–42)-induced neurotoxicity was reduced by naloxone in an opioid receptor-independent mechanism (Liu et al. 2002). It was determined that the

neuroprotective effects of naloxone were due to reduced microglial superoxide production (Liu et al. 2002).

Astroglia express all four opioid receptor types (*mu*, *kappa*, *delta*, and nociceptin/orphanin FQ), and these receptors are differentially expressed among brain regions (Ruzicka et al. 1995; Zhao et al. 2002). We recently determined that human MOR (hMOR) is expressed in A172 cells after TNF $\alpha$  exposure (preliminary data). Thus, it is possible that inhibition of iNOS induction results from  $\beta$ -FNA action at a receptor other than MOR. For instance, depending on the opioid receptor type,  $\beta$ -FNA can exhibit initial KOR agonist activity and then irreversibly bound antagonist activity at MOR (Qi et al. 1990).

Maximal induction of iNOS in A172 cells requires NF- $\kappa$ B activation, as demonstrated via pharmacologic inhibition of iNOS activation with two mechanistically distinct NF- $\kappa$ B inhibitors (MG-132 and helenalin, data not shown). We recently found that translocation of the p65 subunit of the transcription factor NF- $\kappa$ B into the nucleus is inhibited by  $\beta$ -FNA (Davis et al. 2007). Thus, it may be that  $\beta$ -FNA inhibits iNOS induction through modulation of NF- $\kappa$ B expression or function; however, further investigation is needed.

In summary, the neuroprotective properties of opioid compounds are not completely understood, but both MOR-dependent and MOR-independent mechanisms are likely involved. Regarding therapeutic potential,  $\beta$ -FNA is particularly attractive given it is a safe, non-addictive opioid agent that readily enters the CNS. A better understanding of the mechanism governing the anti-inflammatory actions of  $\beta$ -FNA may provide clues for development of therapeutic strategies to combat pathologies in the CNS associated with neuroinflammation.

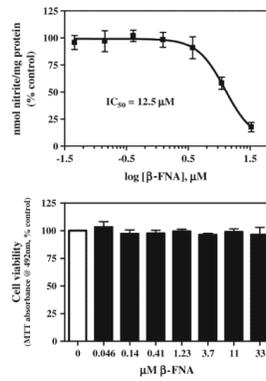
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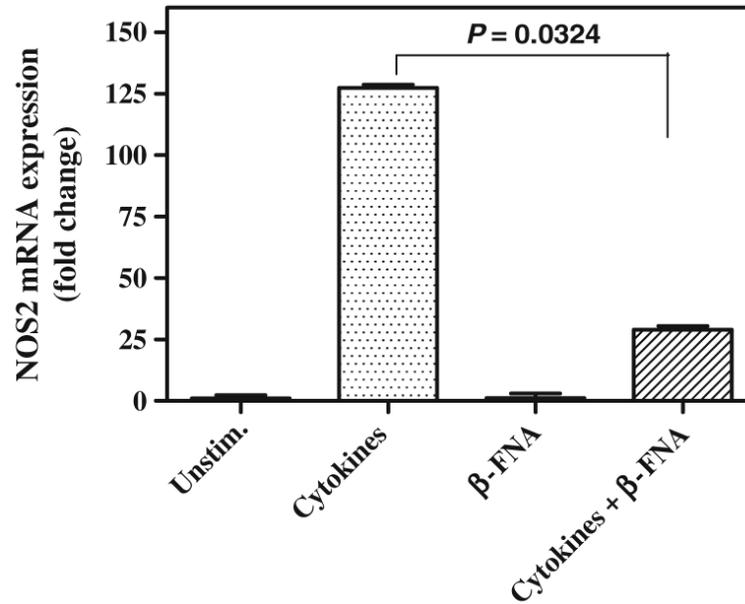
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**Fig. 1.**  $\beta$ -Funaltrexamine dose-dependently inhibits cytokine-stimulated nitrite production A172 cells. Astroglial cells were exposed for 24 h to a cytokine mixture containing IFN $\gamma$  (100 ng/ml), IL-1 $\beta$  (5 ng/ml), and TNF $\alpha$  (30 ng/ml) in the presence or absence of 0.05–33  $\mu$ M  $\beta$ -FNA. Nitrite accumulation (*top panel*) in the culture media was determined spectrophotometrically using the Griess reagent. Cell viability (*bottom panel*) was assessed using the MTT assay. The curve was generated by nonlinear regression in which the Hill slope was not constrained. Data points represent the mean  $\pm$  SEM of duplicate measures from four to eight independent experiments



**Fig. 2.**  $\beta$ -Funaltrexamine inhibits cytokine-stimulated NOS2 mRNA expression in A172 cells. Astroglial cells were exposed for 8 h to a cytokine mixture containing IFN $\gamma$  (100 ng/ml), IL-1 $\beta$  (5 ng/ml) and TNF $\alpha$  (30 ng/ml) in the presence or absence of 11  $\mu$ M  $\beta$ -FNA. NOS 2 mRNA expression was measured using qRT-PCR and is expressed as fold change. qRT-PCR was performed using SYBR green reporter assay. The mean  $\Delta$ CT value obtained after normalization with 18S for unstimulated cells was used as a baseline for comparing NOS2 gene expression among treatments. Results are presented as least square means + standard error of the mean