Neuromelanin inhibits CXCL10 expression in human astroglial cells

Neda Saffarian Tousi[1], **Daniel J. Buck**[1], **Luigi Zecca**[2], and **Randall L. Davis**[1,*]

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

[2]Institute of Biomedical Technologies - Italian National Research Council, 20090 Segrate, Milano, Italy

Abstract

Increasing evidence indicates neuroinflammation is instrumental in the pathogenesis of Parkinson's disease (PD). In PD, there is selective degeneration of neuromelanin (NM)-containing dopamine neurons. Neuromelanin is predominantly cytoprotective within dopaminergic neurons, whereas, NM released from damaged neurons activates microglia. However, the effects of NM on astroglial cells remain largely unknown. Astroglia are essential to neuronal homeostasis and responsive to injury, in part, through secretion of chemokines, including interferon γ inducible protein-10 (CXCL10). Thus, we used an *in vitro* approach to identify the effects of NM on TNFα-induced CXCL10 expression in human astroglial cells. TNFα-induced CXCL10 expression was inhibited in NM exposed cells. Additionally, TNFα-induced NF-κB activation was inhibited by NM. Given that CXCL10 expression is NF-κB-dependent in human astroglial cells, these findings suggest that NM may inhibit CXCL10 expression, in part, through an NF-κB-dependent mechanism. While the *in vivo* consequences of NM mediated effects on astroglial CXCL10 expression remain to be fully elucidated, insights obtained in this study further our understanding of the effects of NM on inflammatory signaling in human astroglial cells.

Keywords

Parkinson's disease; chemokine; astrocytes; neuroinflammation; NF-κB

Parkinson's disease (PD) affects the pigmented nigrostriatal dopaminergic neurons and results in a progressive neurodegeneration of these neurons. Parkinson's disease is distinguished as the second most common neurodegenerative disorder with a prevalence of 0.1% of the global population [39]. Clinical manifestations of PD include akinesia, bradykinesia, a rhythmic involuntary tremor at rest ('pill rolling movement'), postural instability, and extrapyramidal rigidity in which major muscle groups become stiff, collectively referred to as parkinsonism. The appearance of parkinsonism develops when loss of at least 50% of the dopaminergic neurons in the substantia nigra (SN) pars compacta (SNpc) occurs, leading to a reduction of over 80% in dopamine levels in the striatum [39].

Corresponding Author: Randall L. Davis, Oklahoma State University Center for Health Sciences, 1111 West 17th Street, Tulsa, Oklahoma 74107, Tel: 918-561-8408; Fax: 918-561-5798 **For proofs and reprint requests:** randall.davis@okstate.edu.

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Impairment of dopaminergic neurotransmission and parkinsonism are also observed in HIV infected subjects with HIV dementia [24]. Similarly, significant neuronal degeneration is observed in the substantia nigra of HIV infected individuals [21].

The exact mechanism responsible for the neuronal loss in PD and HIV dementia is not completely understood; however, oxidative stress [1,29], mitochondrial dysfunction [29] and neuroinflammation [16,20] are involved. In terms of neuroinflammation, levels of the proinflammatory cytokine TNFα are markedly elevated in the SN and cerebral spinal fluid (CSF) of individuals with PD [6,28] and HIV-1 infection [37,38]. Likewise, certain chemokines are up-regulated in the CNS of those with neuropathologies associated dopamine neurons, including PD [31], animal models of PD [22] and HIV-1 infection [23]. Importantly, increasing evidence points to activated astrocytes as contributors to the loss of dopaminergic neurons [19,27,43]. Elevated TNFα is likely one of several factors which activate astroglia during states of neuroinflammation, resulting in release of proinflammatory and neurotoxic molecules [36,40]. Chemokines are among the astroglial derived products thought to contribute to neuropathogenesis [20,31,43]. We are particularly interested in CXCL10, a chemokine which is known to be neurotoxic [33] and in a murine PD model has been reported to be elevated in the striatum and the ventral midbrain [22]. CXCL10 is also chemoattractant for activated T cells [35], monocytes/macrophages [35], and microglia [17], has antimicrobial activity [11], and induces astrocyte proliferation [17]. Thus, depending on the temporal, regional and magnitude of expression in the brain, CXCL10 may be neuroprotective or neurotoxic. Likewise, while dopamine neurons containing the pigment neuromelanin (NM) selectively degenerate in PD, the extent to which NM contributes to neurodegeneration versus neuroprotection remains unclear. Indeed, there is increasing interest in understanding the role of extracellular NM in neuropathogenesis [5,42,49]. Based on these collective insights, the objective of this study was to identify the effects of NM on TNFα-induced CXCL10 expression in human astroglial cells. These findings are expected to further our understanding of the role of astroglia and chemokines in the neuropathology involving dopaminergic neurons.

The human A172 astroglial cell line (ATCC #CRL-1620; American Type Culture Collection, Manassas, VA) is well documented as a useful model in pharmacology- and neurochemistry-based studies [14]. A172 cells were maintained in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 50 U/ml penicillin, 0.05 mg/ml streptomycin and 2 μg/ml amphotericin B. Cell cultures were maintained in a humidified incubator at 37°C, 5% CO_2 and 95% air with the medium changed every 48–72 h. Experimental cultures were seeded at a cell density to provide 80–90% confluence at the time of treatment.

Neuromelanin was isolated as previously described [47] from SN samples obtained during autopsies of subjects who died without any known neurological or psychiatric disorders. The purity of the NM was assessed by elemental analysis, amino acid analysis and electron paramagnetic resonance spectroscopy as previously described [44,45]. Shortly before the experiments were initiated a stock suspension of NM was prepared in sterile water (0.5 mg/ml) and stored at 4°C. Just prior to initiating the experiment, the NM suspension was diluted to desired concentrations (0.02-15 μg/ml) in serum-free culture medium and incubated at room temperature for 2 h before adding to cell cultures. The biological relevance of this concentration range of NM was based on the *in situ* concentrations found in the SN of normal subjects [46].

A172 cells were cultured in serum free medium for 48 h in the presence or absence of NM (0.02-15 μg/ml). Cells were then co-exposed to human recombinant TNFα (5 ng/ml; Peprotech, Rocky Hill, NJ) for an additional 24 h. While TNFα concentrations in human

brain tissue are not well established, 5 ng/ml is within the range typically used for *in vitro* stimulation of glia and immune cells [41,50]. Following the treatment period, levels of secreted CXCL10 protein in the culture media were quantitated using a standard dual-antibody solid phase immunoassay (Human CXCL10/IP-10 ELISA Development Kit, Peprotech, cat# 900-K39), according to the manufacturer's instructions as previously described [12]. Absorbance was read at 450 nm (with wavelength correction set at 650 nm) on a BIOTEK HT spectrophotometer.

To assess mitochondrial integrity/cell viability, parallel experiments were performed followed by performance of the MTT assay according to a modified version of the procedure described by [8]. Following, the 72 h treatment period, culture media was replaced with fresh serum free medium containing 0.55 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide (MTT) and cultures returned to the humidified incubator (37°C, 5% CO_2 and 95% air) for 45 min. Media was removed from the cultures, cells dissolved in 1 ml dimethyl sulfoxide and absorbance measured at 492 nm using a BIO-TEK HT spectrophotometer.

To assess NM effects on NF-κB activation, cells were treated as described above, except the cells were only exposed to TNFα for the final 0.5 h of the 72 h NM exposure period. Nuclear protein fractions were obtained as described in our previous report [15]. NF-κB p65- and NF-κB p50-DNA binding activity was determined using a Transcription Factor kit (Thermo Scientific) according to manufacturer's instructions. Luminescence of the labeled NF-κB-DNA product was measured using a BIO-TEK HT spectrophotometer. Additionally, total cell protein/well was determined using the bicinchoninic acid (BCA) protein assay as previously described [13] in order to normalize data when appropriate.

Prism™ version 4.0 software (GraphPad Inc.) was used for figure presentation, percent transformations and statistical analysis. Analyses included one-way analysis of variance (ANOVA) with Dunnett's comparison post hoc test. Data from 2-5 independent experiments are presented as mean + S.E.M. 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.

Based on the MTT assay, cell viability was not significantly affected by TNFα or TNFα + NM (Fig. 1). Similarly, NM alone did not alter cell viability (data not shown). Constitutive expression of CXCL10 protein was relatively low, whereas, TNFα significantly induced CXCL10 expression (Fig. 2). NM alone had no effect on CXCL10 expression at any concentration tested (data not shown). However, NM exposure (≥ 5 μg/ml) significantly inhibited TNFα-induced CXCL10 expression in human astroglial cells (Fig. 2).

NF-κB activation, as determined by nuclear levels of NF-κB p65 and p50 protein, was relatively low in untreated controls and cells exposed to NM alone (Fig. 3). Conversely, TNFα significantly enhanced nuclear levels of both NF-κB p65 and p50 protein (Fig. 3). TNFα-induced NF-κB p65 activation was inhibited by ≥ 0.6 μg/ml NM, whereas, NF-κB p50 activation was not significantly altered by NM (Fig.3).

Inhibition of astroglial CXCL10 expression by NM may be particularly important in our efforts to elucidate the role of NM in certain neuropathologies, including PD. It is known that in the SN of patients affected by PD a significant amount of extracellular NM is present and around these NM granules was observed an accumulation of activated microglia [4,25,26]. While there is increasing evidence that NM is involved in PD neuropathogenesis, it remains unclear exactly to what extent NM contributes to neurodegeneration versus neuroprotection. NM synthesis prevents accumulation of cytosolic catecholamines, thereby

providing protection to dopaminergic neurons [34]. Furthermore, NM can be cytoprotective in dopaminergic neurons through chelation of redox active metals, and a variety of other toxic metals and compounds [48]. In contrast to intracellular NM, NM released from damaged dopaminergic neurons results in microglial activation and production of neurotoxic molecules which further damage dopaminergic neurons [42,47,49].

Chemokines, including CXCL10, appear to be associated with dopaminergic neurodegeneration. For instance, in the 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) model of murine PD, CXCL10 mRNA expression was up regulated in the striatum and the ventral midbrain; however, the cellular source of CXCL10 was not determined [22]. Similarly, evidence points to the involvement of CXCL10 in HIV-1 associated neuropathology [10,32].

The consequences of altered chemokine (i.e, CXCL10) expression in dopaminergic associated neuropathology have not been fully elucidated. Modulation of astroglial CXCL10 expression by NM, could potentially have important implications in neuropathologies associated with dopaminergic neurons. Astroglial chemokine expression is of particular interest given these cells are the most prevalent cell type in the human CNS and are essential for neuronal homeostasis, response to injury, maintenance of the blood-brain barrier and a major source of chemokines in the brain [3,9,30].

In terms of direct NM mediated effects, we did not observe NM induced cytotoxicity in A172 cells, but did in human SK-N-SH neuronal cells (data not shown). Together, these findings are consistent with the concept that glial cells in the brain can survive under conditions where neuronal cells degenerate. Also, we provided *in vitro* evidence that extracellular NM may also exert anti-inflammatory actions. That is, TNF-induced CXCL10 expression in human astroglial cells was inhibited in NM exposed cells. The specific *in vivo* consequences of NM mediated inhibition of astroglial CXCL10 expression remain to be determined. However, under conditions where CXCL10 is inducing neurotoxicity, NM would potentially be neuroprotective. Conversely, in a microenvironment where CXCL10 is providing neuroprotection (i.e., immune responsiveness or neurotrophic support) inhibition of CXCL10 by NM may be detrimental to neurons in the region. It is also important to note that while this report is focused on a single chemokine, CXCL10, preliminary studies indicate CCL2 expression is also inhibited by NM (data not shown).

Activation of the transcription factor, NF-κB, is associated with dopaminergic associated neuropathology. For instance, within the ventral midbrain of PD patients, NF-κB p65 levels are elevated in microglia and astrocytes as compared to age-matched controls [18]. Similarly, in a mouse model of PD, MPTP significantly induced NF-κB activation in astrocytes of the SN [2]. Numerous inflammatory responses in astrocytes, including chemokine expression, are transcriptionally regulated by NF-κB [7,36]. In particular, we previously utilized functionally distinct inhibitors of NF-κB activation to demonstrate that TNFα-induced CXCL10 expression in human A172 astroglial cells is NF-κB-dependent [12]. Interestingly, the data presented in this manuscript suggest that NM may inhibit TNF-induced CXCL10 expression in human astroglial cells, in part, through an NF-κB p65 dependent mechanism. The importance of NM mediated inhibition of NF-κB activation likely extends beyond just inhibition of CXCL10 expression. That is, the expression of many inflammatory molecules in astrocytes is transcriptionally regulated by NF-κB, thus, NM may inhibit transcription of multiple inflammatory molecules in astroglia. Seemingly in contrast, in rat microglia, NM induces inflammatory signaling including NF-κB activation, and expression of inducible nitric oxide synthase and TNFα [42,49]. In previous work [49], NM injected into the rat SN induced microgliosis and astrogliosis. It was speculated that astrocytosis was a protective response consequent to the neurodegeneration induced by

activated microglia. However, based on the present data we can suggest also a direct effect of NM on astrocytes which would counteract the neurodegenerative effect caused by microglia. Together, these findings suggest that NM modulation of inflammatory signaling may differ among glial cell types. However, to our knowledge, this is the first report of NM effects on NF-κB activation and CXCL10 expression in human astroglial cells.

Therefore, this study provides novel insights into the effects of NM on inflammatory signaling in human astroglial cells. Further investigation is needed to fully elucidate the mechanism by which NM inhibits inflammatory signaling in astroglial cells and determine the *in vivo* consequences of these NM mediated effects in astroglia.

Acknowledgments

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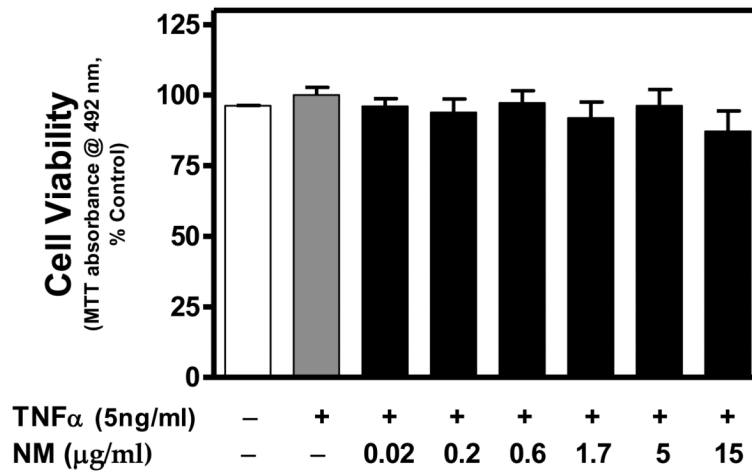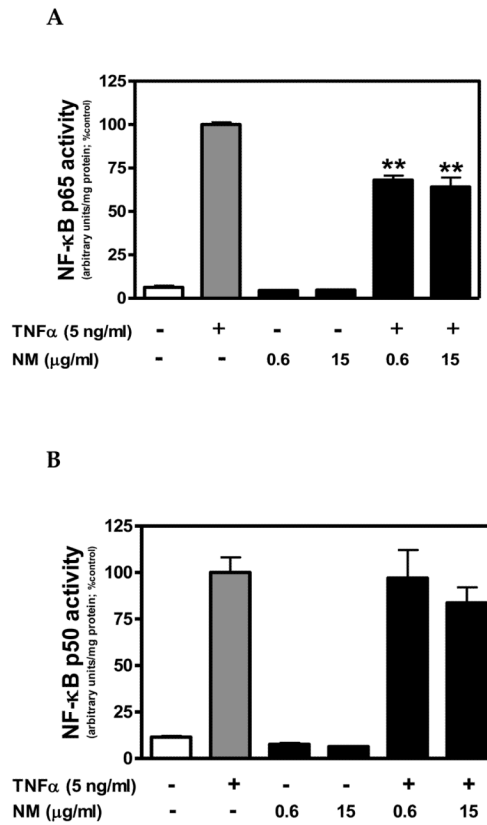
Figure 1. Neuromelanin is not cytotoxic to TNFα-stimulated human A172 astroglial cells
A172 cells were cultured in serum free medium for 48 h in the presence or absence of NM
(0.02-15 μg/ml), then co-exposed to human recombinant TNFα (5ng/ml) for an additional 24
h. To assess mitochondrial integrity/cell viability, the MTT assay was performed. Data were
normalized as percent control (TNFα alone) within each experiment and presented as the
mean + S.E.M. of triplicate measures from 2-3 independent experiments. No significant
differences ($P < 0.05$) among treatments were detected by one-way ANOVA.
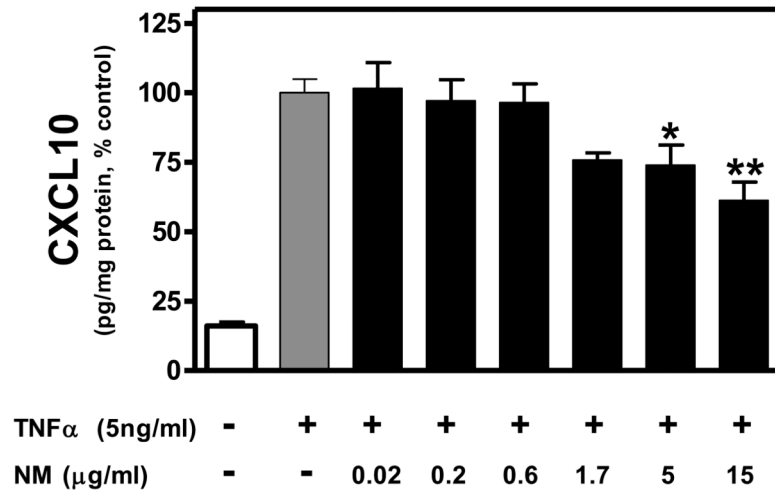
Figure 2. Neuromelanin inhibits TNFα-induced CXCL10 protein expression in human A172 astroglial cells

A172 cells were cultured in serum free medium for 48 h in the presence or absence of NM (0.02-15 μg/ml), then co-exposed to human recombinant TNFα (5ng/ml) for an additional 24 h. Secreted CXCL10 protein in the culture media was then quantitated using a standard dual-antibody solid phase immunoassay, according to the manufacturer's instructions as previously described [12]. Absorbance was read at 450 nm (with wavelength correction set at 650 nm) on a BIOTEK HT spectrophotometer. Data were normalized as percent control (TNFα alone) within each experiment and presented as the mean + S.E.M. of triplicate measures from 3-4 independent experiments. Statistical differences determined by one-way ANOVA with Dunnett's post-hoc comparisons. *$P < 0.05$ vs TNFα; **$P < 0.01$ vs TNFα.

A

B

Figure 3. Neuromelanin inhibits TNFα-induced NF-κB activation in human A172 astroglial cells
A172 cells were cultured in serum free medium for 72 h in the presence or absence of NM (0.6 or 15 μg/ml). Cells were co-exposed to human recombinant TNFα (5ng/ml) for the final 0.5 h of the 72 h NM exposure period. Nuclear protein fractions were obtained and NF-κB p65- and NF-κB p50-DNA binding activity was determined using a Transcription Factor kit, according to manufacturer's instructions. Binding activity was determined by luminescence of the labeled NF-κB-DNA product as measured using a BIO-TEK HT spectrophotometer. Binding activity was normalized to total nuclear protein and presented as arbitrary units. Data represent the mean + S.E.M. of duplicate measures from 2 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. **$P < 0.01$ vs TNFα.