Published in final edited form as:

Brain Res. 2008 June 27; 1217: 1–9. doi:10.1016/j.brainres.2008.03.093.

Nuclear Factor Kappa-B Mediates Selective Induction of Neuronal Nitric Oxide Synthase in Astrocytes During Low-Level Inflammatory Stimulation with MPTP

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Abstract

Recent advances in understanding the progression of Parkinson's disease (PD) implicate perturbations in astrocyte function and induction of constitutively expressed neuronal nitric oxide synthase (NOS1) in both human PD and in the MPTP model of the disease. Transcriptional regulation of NOS1 is complex but recent data suggest that nuclear factor kappa B (NF-κB) is an important transcription factor involved in inducible expression of the gene. The data presented here demonstrate that mild activation of primary astrocytes with low or 'sub-optimal' concentrations of MPTP (1 μM) and the inflammatory cytokines tumor necrosis factor alpha (10 pg/ml) and interferon gamma (1 ng/ml) results in selective induction of *Nos1* mRNA and protein, increased production of nitric oxide (NO), and a significant elevation in global protein nitration. This mild inflammatory stimulus also resulted in activation and recruitment of p65 to a putative NF-κB response element located in the *Nos1* promoter region flanking exon 1. A role for NF-κB in MPTP-dependent induction of NOS1 was confirmed through overexpression of a mutant IκBα super repressor of NF-κB that prevented induction of NOS1. The data presented here thus demonstrate a role for NF-κB in selective induction of NOS1 during early inflammatory activation of astrocytes stimulated by low-dose MPTP and inflammatory cytokines.

Keywords

Parkinson's disease; neurodegeneration; astrocyte; nitric oxide; nuclear factor-kappa B

1. Introduction

Parkinson's disease (PD) is a progressive and incurable neurodegenerative disorder characterized by irreversible loss of dopaminergic neurons in the substantia nigra pars compacta (DeLong, 2000). While the causes of PD remain unclear, recent evidence strongly supports a role for perturbations in astrocyte physiology and altered expression of the nitric oxide synthase (NOS; E.C. 1.14.13.39) isoforms in progression of the disease (Hirsch et al.,

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2003; Pekny and Nilsson, 2005; Teismann and Schulz, 2004). Studies exploring pathophysiologic expression of NOS have typically focused on the inducible isoform (NOS2) but induction of the constitutively expressed neuronal nitric oxide synthase (NOS1) has emerged as an underlying component of a diverse array of neurologic disorders, including ischemic cerebral injury (e.g. stroke) (Moro et al., 2004), substance-induced neurological dysfunction including alcohol and methamphetamine abuse (Imam et al., 2001), as well as idiopathic neurodegenerative disorders including amyotrophic lateral sclerosis, Alzheimer's disease, and PD (Catania et al., 2001; Chabrier et al., 1999; Eve et al., 1998). Underscoring a role for this enzyme in the progression of PD is the observation that ablation of the *Nos1* gene in the 1-methyl-MPTP model of parkinsonism completely prevents neurotoxicity (Przedborski et al., 1996), as well as additional studies demonstrating protection of dopaminergic neurons in MPTP-treated mice by the NOS1-specific inhibitor 7-nitroindazole (7-NI) (Watanabe et al., 2004). However, despite the suggested role for NOS1 in the progression of PD, the mechanism behind induction of this enzyme during states of glial inflammation remains poorly understood.

Because NOS1 is thought to play a crucial role in the progression of multiple neurological pathologies, suppression of this protein represents a potential therapeutic target for halting the progression of disorders such as PD. Unfortunately, the complexity of the *Nos1* gene has impeded our understanding of the transcriptional mechanisms by which it is regulated. The NF- κ B signaling pathway has recently been linked to induction of human *Nos1* in studies reporting a putative κ B-like response element in the 5' promoter region flanking the first exon (Hall et al., 1994). This report was followed by a study documenting an association between p65, the active nuclear component of NF- κ B, with the promoter region of human *Nos1* in human neuroblastoma cells (Li et al., 2007). Although the report by Li et al. (2007) documented a role for NF- κ B in the induction of *Nos1*, the study was conducted in an immortalized human neuronal cell line rather than primary neurons and did not consider potential differences in regulatory mechanisms that could exist between neurons and astrocytes.

The work presented here describes the selective induction of NOS1 in primary astrocytes treated with low concentrations of MPTP and the inflammatory cytokines TNF- α and IFN- γ , and tests the hypothesis that this induction occurs primarily through an NF- κ B-mediated mechanism. These data represent, to our knowledge, the first report of a functional role for NF- κ B in MPTP-induced expression of NOS1 in astrocytes and provide insight into signaling mechanisms that may mediate the deleterious effects of this enzyme during degeneration of dopaminergic neurons.

2. Results

The effect of low-level inflammatory stimulation by TNF- α and IFN- γ with or without the mitochondrial toxin MPTP on induction of *Nos1*, *Nos2*, and *Nos3* was measured in astrocytes using RT-PCR (Figure 1A). Only induction only of *Nos1* was observed (Lane 3). Neither *Nos2* nor *Nos3* mRNA was detected under control conditions or following exposure of astrocytes to the combination of MPTP and TNF- α /IFN- γ . Either whole brain (*Nos1* and *Nos3*) or LPS-activated astrocyte (*Nos2*) RNA subject to the RT reaction in the absence (-rt; Lane 4) or presence (wb; Lane 5) of reverse transcriptase were included as negative or positive PCR controls, respectively, to confirm PCR efficacy. Because *Nos1* was induced by the cytokine and MPTP insult, semi-quantitative real-time RT-PCR was employed to further explore the induction of this gene relative to saline-treated control astrocytes in the presence or absence of cytokines and/or MPTP (Figure 1B). Although treatment of the astrocytes with cytokines alone resulted in significant induction of *Nos1* (4-fold over control), treatment with only MPTP had no significant effect on levels of mRNA. However, MPTP strongly potentiated induction of *Nos1* (7-fold over control) when used in combination with TNF- α and IFN- γ . To further examine the specificity of NOS1 isoform expression during low-dose exposure to

MPTP and cytokines, expression of *Nos2* was determined by semi-quantitative real time PCR (Figure 1C). No significant induction of *Nos2* was observed at the levels of MPTP and cytokines that were effective in stimulating inducible expression of NOS1.

The induction of *Nos1* mRNA observed in Figure 1 was mirrored by immunoblot experiments (Figure 2A) demonstrating selective expression of NOS1, but not NOS2 or NOS3, in astrocytes exposed to MPTP and TNF- α /IFN- γ (Lane 3). In all cases, membranes were stripped of antibody and re-probed against β -Actin as a loading control. Although all three NOS isoforms were probed simultaneously, independent experiments measuring only NOS2 or NOS3 were performed to confirm selective induction of NOS1 (Figure 2A, lower panels). Potentiation of NOS1 induction was demonstrated through densitometric analysis of band intensities (Figure 2B). To confirm that induction of NOS1 correlated with expression of functionally active protein, steady state production of NO was determined by live-cell fluorescence imaging using the cell-permeable NO-sensitive dye, DAF-FM. Co-treatment of astrocytes with MPTP and TNF- α /IFN- γ resulted in an increase in NO production (Figure 2C). To confirm the involvement of NOS1 in elevated NO production, astrocytes were also treated with MPTP and TNF- α /IFN- γ in the presence of the selective NOS1 inhibitor, 7-nitroindazole (7-NI), resulting in complete suppression of NO production in both control and treated astrocytes (Figure 2C). Interestingly, 7-NI reduced NO to levels less than those observed in untreated control cells. DMSO, used as a vehicle control for 7-NI, resulted in no measurable suppression of NO production. Finally, to rule out any interference from induction of NOS2, astrocytes were co-treated the cytokine and MPTP combination as well as the specific NOS2 inhibitor AMT, which did not suppress NO production, thus confirming that NOS2 was not responsible for elevated intracellular NO concentrations (Figure 2D).

Although NOS1 induction has been implicated in multiple neurological disorders, the functional contribution of enhanced expression of this enzyme is not well understood. While induction of this protein may not produce a sufficient quantity of NO to result in direct cytotoxicity to neighboring cells, the possibility exists that abnormal nitration of intracellular astrocytic proteins could result in dysregulation of critical cellular signaling or trophic functions of astrocytes. Total cellular tyrosine nitration (e.g. 3-nitrotyrosine), an indicator of peroxynitrite formation, was therefore measured by whole-cell immunofluorescence (Figure 3). Representative images (Figure 3A–C) demonstrate that treatment of astrocytes with the MPTP and cytokines increased intracellular 3-nitrotyrosine protein adducts (Figure 3B) over levels observed in saline-treated astrocytes (Figure 3A) and that this effect was suppressed by co-treatment with the NOS1 inhibitor 7-NI (Figure 3C). Quantitative analysis of total cellular fluorescence for 3-nitrotyrosine protein adducts demonstrated significant increase in protein nitration over control levels in cells treated with MPTP and TNF- α /IFN- γ that was restored to basal levels by 7-NI (Figure 3D). DMSO, used as a vehicle control, had no suppressive effect on nitration. The results of Figure 3 thus demonstrate that treatment of primary cultured astrocytes with low levels of MPTP and TNF- α /IFN- γ resulted in increased protein nitration and that this effect was mitigated by pharmacologic inhibition of NOS1 with 7-NI.

Previous studies have implicated NF- κ B in regulation of *Nos1* through the identification of a putative kappa-B response element (Hall et al., 1994), as well as through measuring p65 binding to the promoter of the human gene in neuroblastoma cells (Li et al., 2007). Functional activation of NF- κ B was therefore measured in live astrocytes following treatment with MPTP and TNF- α /IFN- γ using cells isolated from a transgenic mouse possessing a reporter construct comprising three high-affinity HIV NF- κ B *cis*-acting promoter elements driving expression of enhanced green fluorescent protein (EGFP) (Figure 4A) (Magnez et al., 2004). Real-time imaging of transgenic astrocytes by fluorescence microscopy revealed activation of NF- κ B in response to treatment with MPTP and TNF- α /IFN- γ (Figure 4B). To determine a direct association between NF- κ B activation and induction of *Nos1*, binding of p65 to a putative NF-

κ B response element identified in the 5'-flanking region of exon 1 from mouse *Nos1* was determined by the ChIP assay. The putative NF- κ B response element was identified based on an alignment of the 5' promoter region flanking exon 1 in the mouse gene with a previously reported putative kappa-B response element (Hall et al., 1994) in a similar location on the human gene (Figure 4C). Slight constitutive binding of p65 was detected in control astrocytes that was strongly enhanced upon treatment with MPTP and TNF- α /IFN- γ (Figure 4D). PCR amplification of mock pull-downs with IgG yielded no contaminating bands and 10% ChIP input controls confirmed the loading efficiency of the PCR reactions (Figure 4D).

To determine the functional role of NF- κ B in MPTP-mediated induction of NOS1, wildtype astrocytes were transfected with an adenoviral vector containing a mutant (S32-36A) form of I κ B α that prevents nuclear translocation of p65 (Figure 5). Immunoblot analysis revealed significant induction NOS1 in astrocytes infected with empty vector (Figure 5A, Lanes 2–5), whereas both constitutive and inducible expression of NOS1 was substantially reduced following treatment with cytokines and MPTP in astrocytes expressing the NF- κ B “super-repressor” (Figure 5A, Lanes 6–9). Quantitative determination of band intensities indicated a significant reduction of NOS1 in astrocytes expressing mutant I κ B α (Figure 5B).

3. Discussion

Astrogliosis is a pathologic feature associated with the progression of neurodegeneration in PD. Activated astrocytes release inflammatory cytokines, prostaglandins, and NO that potentiate neuronal dysfunction in states of neurologic injury and disease (Hirsch and Hunot, 2000; McGeer and McGeer, 1998). The inducible NOS isoform, NOS2, has received considerable attention due to the capacity of this enzyme to produce levels of NO at concentrations that are cytotoxic to surrounding neurons and because deletion of the *Nos2* gene confers partial neuroprotection in the MPTP model of PD (Liberatore et al., 1999). However, studies also indicate the importance of the constitutively expressed neuronal isoform, NOS1, in the progression of human PD (Eve et al., 1998) and in MPTP-induced neuropathology in animal models (Przedborski et al., 1996; Watanabe et al., 2004). Unlike *Nos2*, deletion of *Nos1* in mice confers nearly complete protection against MPTP neurotoxicity (Przedborski et al., 1996) but the underlying reason for this observation remains to be determined. Because NOS1 produces substantially less NO than NOS2, it is questionable whether induction of this enzyme would lead to direct pathology in neighboring cells. An alternate hypothesis is that increases in intracellular NO may exacerbate astrocyte dysfunction following MPTP treatment, possibly through enhanced post-translational S-nitrosylation or nitration of proteins critical to normal astrocyte trophic activities, such as modulation of excitatory neurotransmission and regulation of cerebral blood flow (Haydon and Carmignoto, 2006). Interestingly, conflicting studies report that NO can either inhibit (Levrant et al., 2005) or stimulate (Riganti et al., 2007) NF- κ B signaling, raising the question of whether early induction of NOS1 may represent an attempt by the astrocyte to mitigate inflammation. Despite the possibility of early inhibition of NF- κ B by low levels of NO, 24 hrs of low-level inflammatory stimulation globally elevated nitration of intracellular proteins (Figure 3), suggesting that inappropriate protein nitration may be an important sequela of NOS1 induction. Indeed, studies characterizing the effects of increased protein nitration have documented deleterious consequences including conformational changes (Cassina et al., 2000) and prevention of phosphorylation resulting in abnormal loss or gain of function, such as that reported for glutamine synthetase (Berlett et al., 1996) and protein kinase C (Hink et al., 2003). Studies documenting the nearly complete resistance of mice lacking the *Nos1* gene to MPTP-induced lesions suggest that induction of this gene represents an early event in the development of MPTP-induced neuropathology (Przedborski et al., 1996). The data presented here support such a model, as treatment of astrocytes with sub-optimal concentrations of inflammatory cytokines and MPTP results in a moderately activated phenotype characterized by selective induction of NOS1 (Figure 1 and

Figure 2), an increase in steady-state production of NO (Figure 2), and increased protein nitration (Figure 3).

Despite a recognized association between *Nos1* and the progression of multiple neurological disorders, the regulation of this gene remains incompletely characterized due to its inherent complexity. Indeed, human *Nos1* is distributed over 160 kb among 29 exons and 28 introns, with nine recognized splice variants in the neuronal tissue cluster (*Nos1a-i*) regulated by alternate promoter usage in the 5' untranslated region flanking the first exon (Wang et al., 1999; Xie et al., 1995). Of these, *Nos1f* and *Nos1g* are predominantly expressed in brain (Wang et al., 1999). Although similar in both size and organization, mouse *Nos1* has been much less studied than the human gene and far less is known regarding regulation of inducible expression of different splice variants. Specifically, only three splice variants (*Nos1a-c*) of the *Nos1* cluster expressed in neurons have been characterized in mice, of which *Nos1a* and *Nos1b* have documented homology with human variants *Nos1f* and *Nos1g* respectively, whereas mouse *Nos1c* is not homologous to any known human splice variant (Sasaki et al., 2000). Furthermore, three splice variants (e.g. *Nos1a*, β , γ) involving the second exon in both the human and mouse *Nos1* result in deletion of the second exon in variants β and γ , which are reportedly induced in astrocytes in human ALS (Catania et al., 2001), and of which only the β variant retains catalytic activity (Huang et al., 1993; Kavya et al., 2006).

To date, few *cis*-activating transcription factors have been characterized that mediate inducible expression of mouse *Nos1*. Depolarization-induced Ca^{2+} influx in cortical neurons through L-type voltage-sensitive calcium channels is reported to induce *Nos1* through a CREB responsive pathway at the second exon (Sasaki et al., 2000) and steroidogenic factor 1 (SF-1) is also reported to mediate inducible expression of mouse *Nos1* (Wei et al., 2002). Involvement of NF- κ B in regulation of mouse *Nos1* is suggested by a separate report characterizing the organization of human *Nos1* in which the presence of an NF- κ B response element was documented in the promoter region flanking the first exon (Hall et al., 1994). This site was later demonstrated to interact with the active p65 NF- κ B subunit in neuroblastoma cells (Li et al., 2007). Although interaction with the putative *Nos1* NF- κ B binding site in this latter study was shown by electrophoretic mobility shift and ChIP assays, neither gene silencing nor dominant-negative approaches were performed to confirm a direct role for NF- κ B in inducible expression of the protein. Given these previous data, we performed an alignment of the human and mouse *Nos1* 5' UTR flanking the first exon and found the presence of a similar site (Figure 4C). While transactivation of NF- κ B responsive genes typically occurs in response to p65/p50 heterodimer translocation to the nuclear response element, binding of the p50/p50 homodimer has been reported to actually suppress induction (Ghosh et al., 1998). Therefore, binding of p65 to the *Nos1* promoter in the region identified as a potential NF- κ B response element following alignment with the human was measured through ChIP, demonstrating binding of this transcription factor to the predicted response element following treatment of primary astrocytes with MPTP and TNF- α /INF- γ (Figure 4D). Together with findings that NF- κ B was activated following this mild inflammatory insult in transgenic reporter astrocytes (Figure 4B) and that overexpression of an NF- κ B "super repressor" prevented MPTP- and cytokine-induced expression of *Nos1* (Figure 5), these data demonstrate direct involvement of NF- κ B in the induction of *Nos1* during MPTP-dependent potentiation of inflammatory signaling.

Collectively, the data presented here demonstrate that NOS1 is selectively induced in primary astrocytes during mild inflammatory stimulation with cytokines and MPTP. The capacity of low levels of MPTP to potentiate the effects of inflammatory cytokines on inducible expression of NOS1 may provide insight into the consequences of mitochondrial dysfunction evident in PD on the inflammatory phenotype of astroglia. Selective induction of NOS1 was confirmed by the absence of detectable mRNA for *Nos2* or *Nos3*. Although a faint immunoreactive band was inconsistently detected at a slightly smaller molecular weight than NOS1, this likely

represents a NOS1 splice variant, because immunoblots for NOS2 and NOS3 did not detect the presence of these isoforms (Figure 2A, lower panels). Additional studies using the NOS2 inhibitor AMT further demonstrated selective induction of functional NOS1, as this inhibitor had no effect on intracellular NO concentrations following low-dose exposure to MPTP and TNF- α /IFN- γ (Figure 2D). The absence of NOS2 induction supports the conclusion that the sub-optimal concentrations of inflammatory cytokines and MPTP used in these studies result in a phenotype that might be observed in the early stages of PD, before a full-scale inflammatory response and ensuing astrogliosis has occurred. Furthermore, the identification of NF- κ B as the predominant pathway by which *Nos1* is induced in this model reveals a potential target for suppressing the induction of this gene. Together, the results presented here provide a basis for further dissection of the signaling pathways regulating inducible expression of *Nos1* in astrocytes.

4. Experimental Procedures

4.1. Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The NOS2 inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) was purchased from Calbiochem (San Diego, CA). Cell culture media, antibiotics, and fluorescent antibodies and dyes were purchased from Invitrogen (Carlsbad, CA). Monoclonal antibodies against NOS1-3 were purchased from BD biosciences (San Jose, CA). Horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Boston, MA). For immunofluorescence studies, antibodies against GFAP and nitrosylated tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO) and Chemicon (Temecula, CA), respectively.

4.2. Astrocyte Isolation

Cortical astrocytes were isolated from day-1 old C57Bl/6 or transgenic mouse pups according to procedures described previously (Aschner and Kimelberg, 1991), and purity confirmed through immunofluorescent staining using antibodies against GFAP (astrocyte; approximately 98% cells per field) and Iba1 (microglia; approximately 1% per field). Briefly, pups were euthanized by decapitation, cortices rapidly dissected out, and meninges removed. Tissue was subject to digestion with Dispase (1.5 U/ml), and selection of astrocytes was performed by changing media 24 hrs. after plating to remove non-adherent microglial cells. Astrocyte cultures were maintained at 37°C and 5% CO₂ in minimum essential media supplemented with 10% heat-inactivated fetal bovine serum and a penicillin (0.001 mg/ml), streptomycin (0.002 mg/ml), and neomycin (0.001) antibiotic cocktail. Cell media was changed 24 hrs prior to all treatments to minimize interference from serum shock. All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee, and were conducted in accordance with published NIH guidelines.

4.3. RT and Real-Time RT-PCR

Astrocytes were treated with a sub-maximal pathologic insult (TNF- α , 10 pg/ml; IFN- γ , 1 ng/ml; MPTP, 1 μ M). These doses of inflammatory cytokines were based upon previous studies from our laboratory demonstrating little to no induction of *Nos2* at the concentrations used (Liu et al., 2005). RNA was isolated using an RNEasy Mini kit (Qiagen, Valencia, CA), and purity and concentration determined using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Following purification, 1 μ g of RNA was used as template for reverse transcriptase (RT) reactions using the iScript RT kit (BioRad, Hercules CA). The resulting cDNA was immediately profiled for expression of *Nos* using primers specific for *Nos1* (forward: 5'-TGCTACAACCTCGCTACTATT-3'; reverse: 5'-

ACATCGTCAGCCTGTATTCG-3'), *Nos2* (forward: 5'-TCACGCTTGGGTCTTGTT-3'; reverse: 5'-CAGGTCACCTTTGGTAGGATTT-3'), or *Nos3* (forward: 5'-GGCATCACCAGGAAGAAGAC-3'; reverse: 5'-GGGACACCACATCATACTCAT-3'). Real-time RT-PCR was used to measure relative *Nos1* or *Nos2* expression in response to cytokine and/or MPTP exposure using β -*Actin* as a housekeeping gene (forward: 5'-GCTGTGCTATGTTGCTCTAG-3'; reverse: 5'-CGCTCGTTGCCAATACTG-3') according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

4.4. Western Blotting

Astrocytes were treated with the cytokines TNF- α and IFN- γ (10 pg/ml and 1 ng/ml, respectively) and or MPTP (1 μ M) for a period of 24 hrs prior to harvesting. Following treatment, cells were lysed using standard RIPA buffer supplemented with CompleteTM protease inhibitor (Roche, Indianapolis IN). Protein was quantified using the BCA assay (Pierce, Rockford IL), and 20 μ g (NOS1) or 50 μ g (NOS2 and NOS3) of protein were separated by SDS-PAGE using a 6% slab gel (BioRad, Hercules CA) followed by semi-dry transfer to polyvinylidene fluoride (PVDF) membrane (Pall Corp., Penscola, FL). All blocking and antibody incubations were performed in 5% non-fat dry milk in tris-buffered saline containing 0.2% Tween-20. Positive controls consisting of 0.5 μ g activated macrophage lysate (NOS2; BD Biosciences, San Jose, CA) or 10 μ g mouse brain homogenate (NOS1 and NOS3) were included to confirm results. Protein was visualized on film using enhanced chemiluminescence (Pierce, Rockford, IL), and densitometry was performed using ImageQuant v5.3 (GE Healthcare, Sunnyvale, CA).

4.5. Quantitation of Intracellular Nitric Oxide

Astrocytes were grown to confluence in 4-chamber glass-bottom slides (Nalge Nunc, Rochester, NY), and treated for 24 hrs with saline or the cytokine and MPTP combination. Co-treatment or treatment with the NOS1 inhibitor 7-NI (10 μ M) or the NOS2 inhibitor AMT (25 nM) were included to demonstrate selective induction of NOS1. Following treatment, the fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) was added at 5 μ M and intracellular de-esterification allowed to proceed for 10 min at 37°C. Cell media was washed twice with serum and phenol-red-free MEM supplemented with 10 mM HEPES, and DAF-FM intensity recorded via fluorescence microscopy as described above at 2 min. intervals over the course of 30 min. using a Zeiss Axiovert 200M microscope (Thornwood, NY) equipped, 20 $\times$ air PlanApochromat objective and ORCA-ER cooled, interline charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan). Intracellular NO is reported as final over initial fluorescence (F/F_0) at 490 nm_{ex}/520 nm_{em}. Images were acquired and analyzed using Slidebook software (v4.1, Intelligent Imaging Innovations, Inc., Denver, CO). A minimum of 500 – 800 cells were imaged per treatment group for studies of NO over no less than 3 independent experiments.

4.6. Immunofluorescence

Primary astrocytes were grown to confluence on 20 mm serum-coated glass coverslips, and treated with saline or the TNF- α , IFN- γ , and MPTP combination in the presence or absence of the NOS1 inhibitor 7-NI (10 μ M) for 24 h. Blocking and antibody hybridization was conducted in 1% BSA (w/v) in PBS, while all washes were conducted in PBS. Images were acquired using a Zeiss 20X air PlanApochromat objective and 6 – 8 microscopic fields were examined per treatment group over no less than three independent experiments. Fluorescent secondary antibodies were used to detect GFAP (488 nm_{ex}/519 nm_{em}) and nitrosylated protein (647 nm_{ex}/668 nm_{em}), respectively, while DAPI (360 nm_{ex}/460 nm_{em}) mounting medium was used to stain nuclei.

4.7. NF- κ B reporter assays in cis-NF- κ B^{EGFP} transgenic astrocytes and expression of mutant I κ B α

To measure activation of NF- κ B in live cells, astrocytes were isolated from a unique transgenic mouse expressing a reporter construct consisting of three HIV NF- κ B consensus elements inserted 5' to a minimal c-fos promoter that drives expression of enhanced green fluorescent protein (EGFP) (Magness et al., 2004) (Provided by Dr. Christian Jobin, University of North Carolina at Chapel Hill). NF- κ B activity was determined by live-cell imaging and reported as percent activated cells following treatments. A phosphorylation-deficient mutant of I κ B α , I κ B α -(S32,36A)-HA, was overexpressed in primary astrocytes using an adenoviral vector, delivered for 24 hrs at 2×10^6 viral particles per ml of culture medium, with a multiplicity of infection of 1×10^3 virions per cell shown previously by us to result in expression of the mutant protein by over 99% of the astrocytes (Barhoumi et al., 2004). Parallel control experiments utilized the same adenoviral construct lacking the insert. Following incubation with the mutant I κ B α construct for 24 hrs, astrocytes were washed with PBS to remove viral particles and cultured in fresh medium for 24 hrs prior to gene expression studies or co-incubation with PC12 neurons. Images were acquired using a Zeiss 20X air PlanApochromat objective and 6 – 8 microscopic fields were examined per treatment group over three independent experiments.

4.8. Chromatin Immunoprecipitation (ChIP) Assay

ChIP procedures were adapted from a previously published report (Weinmann and Farnham, 2002). Astrocytes were grown to confluence in 60 mm plates (Approx. 1.2×10^6 cells) and were treated for 4 h with saline or the cytokine and MPTP combination. DNA was sheared into 500 bp fragments by three ten-second pulses using a Tekmar Sonic Disrupter (Tekmar Co., Cincinnati, OH) set at 30% output, and lysate was precleared for one hour at 4° C using salmon sperm-blocked protein-A agarose (Upstate Cell Signaling Solutions, Temecula, CA). The beads were then removed by centrifugation, 10% input controls were collected, and 2 μ g precipitating antibody (p65, Santa Cruz Biotechnologies) or control IgG (Calbiochem, San Diego, CA) was added to the remaining lysate. Immune complexes were allowed to form overnight at 4° C with gentle agitation, followed by the addition of salmon sperm-blocked protein-A agarose for an additional 60 min. Immunopurified DNA was isolated via phenol/chloroform extraction and subject to PCR using primers designed around a potential NF- κ B binding region in the mouse *Nos1* promoter which was located following Clustal v1.8 (Thompson et al., 1994) alignment of the mouse and human *Nos1* promoter regions (forward: 5'-ATC AGG CAT CCT TTC CAG AAC GTC-3'; reverse: 5'-ACT GGA TGT TAG TGA CCA CAG CGG-3'). Amplicons were separated by agarose gel electrophoresis and stained with ethidium bromide.

4.9. Statistical Analysis

Comparison of two means was performed by Student's *t*-test, while comparison of more than three means was performed using one-way ANOVA followed by the Tukey-Kramer multiple comparison *post-hoc* test using Prism software (v4.0c, Graphpad Software, Inc., San Diego, CA). For all experiments, $p < 0.05$ was considered significant, although the level of significance was often much greater. Statistically different groups are identified in the figures by the assignment of a unique letter (e.g. a, b, c).

Acknowledgements

The authors thank Dr. Christian Jobin at the University of North Carolina for providing NF- κ B reporter mice. This work was supported by R01ES012941 (RBT), NS055632 (DLC), and an individual research grant from the American Parkinson Disease Association (RBT).

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Abbreviations

NO, nitric oxide; NOS, nitric oxide synthase; TNF- α , tumor necrosis factor alpha; IFN- γ , interferon gamma; NF- κ B, nuclear factor-kappa B..

Fig. 1.

(A) Measurement of *Nos1-3* message in response to the cytokine (1 pg/ml TNF- α , 10 ng/ml IFN- γ) and MPTP (10 μ M) treatment paradigm resulted in expression of *Nos1*, while both *Nos2* and *Nos3* remained undetected. (B) Semi-quantitative real-time RT-PCR was used to further measure expression of *Nos1* following treatment with cytokines and or MPTP, demonstrating potentiation of *Nos1* expression by MPTP. (C) To eliminate the possibility that *Nos2* levels were below the detection limits of standard RT-PCR, semiquantitative real-time RT-PCR was employed to measure detection of this gene, demonstrating appearance of *Nos2* only following treatment with concentrations of MPTP 10-fold greater than those used in the present studies.

Fig. 2.

(A) Simultaneous immunoblot against NOS1-3 demonstrating selective induction of NOS1 following treatment of astrocytes with cytokine (1 pg/ml TNF- α , 10 ng/ml IFN- γ) and/or MPTP (10 μ M). Lower panels represent individual experiments probing only NOS3 or NOS2, confirming no detectable presence of these isoforms. (B) Quantitation by densitometry reveals potentiation of NOS1 induction following treatment with both cytokines and MPTP. (C) Catalytic activity of NOS was measured by quantifying intracellular NO with the fluorescent indicator DAF-FM, and an exclusive role for NOS1 in elevating NO levels was demonstrated through use of the inhibitor 7-NI (10 μ M). (D) Further intracellular NO measurement using DAF-FM in astrocytes co-treated with the NOS2 inhibitor AMT (25 nM) in addition to the cytokine/MPTP combination confirms that NOS2 is not responsible for elevations in intracellular NO.

Fig. 3.

Representative images demonstrating an increase in global tyrosine nitration within astrocytes following treatment with the cytokine (1 pg/ml TNF- α , 10 ng/ml IFN- γ) and/or MPTP (10 μ M) combination (Panel 3B) compared to saline-treated controls (Panel 3A), and suppression of this effect using the NOS1 inhibitor 7-NI (Panel 3C). (D) Quantitation of immunofluorescent signal demonstrating a significant increase in protein nitration following treatment of astrocytes with the cytokine/MPTP combination, and suppression of nitration following co-treatment with the NOS1 inhibitor 7-NI. The DMSO vehicle control had no suppressive effect.

Fig. 4.

(A) Live cell imaging using astrocytes isolated from transgenic mice which express a GFP reporter construct for NF- κ B activation (B) reveals induction of this signaling pathway by the cytokine (1 pg/ml TNF- α , 10 ng/ml IFN- γ) and MPTP (10 μ M) treatment. (C) Alignment of the human and mouse *Nos1* promoters reveals a potential NF- κ B binding domain which was used for the design of ChIP primers. (D) Recruitment of p65 to the NF- κ B-like site in the *Nos1* promoter region following treatment with the cytokine and MPTP combination was demonstrated by ChIP assay.

Fig. 5.

(A) Western blot of NOS1 in astrocytes expressing an NF- κ B “super repressor” (Lanes 6–9) demonstrating partial suppression of NOS1 induction following treatment with the cytokine (1 pg/ml TNF- α , 10 ng/ml IFN- γ) and MPTP (10 μ M) combination in comparison to astrocytes infected with empty vector (Lanes 2–5). Lane 1 represents a positive control from whole-brain homogenate. (B) Densitometry of the western blot demonstrates approximately six-fold induction of NOS1 by the cytokine/MPTP combination in astrocytes treated with the empty adenoviral vector, while astrocytes expressing the super repressor demonstrated slightly less basal expression of NOS1 and markedly less induction of this protein following treatment with the cytokine/MPTP combination.