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In vitro and *in vivo* induction and activation of nNOS by LPS in oligodendrocytes

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ABSTRACT

There are currently four known isoforms of nitric oxide synthase (NOS). Of these, neuronal NOS (nNOS) is known to be present exclusively in neurons, endothelial NOS (eNOS) in vascular endothelium, while the inducible form of NOS (iNOS) is known to be activated in oligodendrocytes, astrocytes and microglia. The fourth isoform, mitochondrial NOS (mtNOS), represents a post-translational modification of nNOS. Using western blotting and real time-PCR, we show induction and activation of nNOS following culture of oligodendrocyte progenitor cells (OPC) with lipopolysaccharide (LPS). Activation of nNOS results in accumulation of peroxynitrite and tyrosine nitration of proteins in oligodendrocytes resulting in reduced cell viability. Injection of LPS in vivo into the corpus callosum of rats leads to the development of extensive demyelination of the white matter tracts. Immunostaining of regions close to the injection site shows the presence of nNOS, but not iNOS, in oligodendrocytes. Neither iNOS nor nNOS was seen in astrocytes in areas of demyelination. These studies suggest that activation of nNOS in oligodendrocytes leads to oligodendrocyte injury resulting in demyelination.

1. Introduction

Oligodendrocytes are the myelin producing cells of the central nervous system (CNS) (Bradl and Lassmann, 2009a; Bradl and Lassmann, 2009b). Inability of oligodendrocytes to support the integrity of myelin can result from metabolic, infectious and immune causes. Destruction of oligodendrocytes is a central feature of a number of human demyelinating diseases, the most common of which is multiple sclerosis (MS) (Compston and Coles, 2008).

A number of mutually overlapping mechanisms of oligodendrocyte cell death have been proposed in inflammatory demyelinating diseases of the CNS. Demyelination can occur as a consequence of humoral and cell-mediated cytotoxicity or due to activation of apoptotic pathways induced by local cytokines or due to their susceptibility to excitotoxic death. Cytokines such as tumor necrosis factor (TNF) and gamma interferon (IFN-γ) mediate cell death in oligodendrocytes (Akassoglou et al., 1998). The development of demyelinating lesions in transgenic mice that over express interleukin 6 (IL-6), TNF-alpha and interleukin-12 (IL-12) and evidence of oligodendrocyte toxicity following direct injection of inflammatory cytokines support a role for cytokine-mediated demyelination (Campbell, 1998; Cua et al., 1999; Horwitz et al., 1997).

More recently, the free radical NO (nitric oxide) is proposed to be an important mediator of oligodendrocyte death (Ghafourifar and Sen, 2007; Smith et al., 2001; Smith and Lassmann, 2002). NO is produced by the activation of NOS (nitric oxide synthase) and currently four isoforms of NOS are known (Pacher et al., 2007). Neuronal NOS (nNOS) is constitutively present in neurons and endothelial NOS (eNOS) is present in endothelial cells. The inducible form of NOS (iNOS) is only seen in glial cells, including oligodendrocytes, following stimulation with bacterial cell wall products or cytokines (Giulivi, 2003). Recently, a fourth isoform of NOS, mitochondrial mtNOS, has been reported. mtNOS is a post-translational modified product of nNOS that is myrisylated and phosphorylated and is localized to the inner membrane of the mitochondria (Ghafourifar and Cadenas, 2005; Haynes et al., 2004; Kanai et al., 2001; Pearce et al., 2002). At present there are no reagents that will differentiate mtNOS from nNOS and NOS activity in mitochondria is inferred to result from activation of mtNOS. nNOS and eNOS require calcium/calmodulin for activation, while the activity of iNOS is calcium independent. Although by itself nontoxic, NO can be converted to a number of more reactive derivatives, referred to as reactive nitrogen species (RNS) that can impair cellular function. Most importantly, NO can combine with the free radical superoxide, O_2^-, to produce peroxynitrite, which can nitrite or oxidize other molecules. Peroxynitrite accumulation has been thought to play a role in a number of inflammatory and degenerative diseases (Pacher and Szabo, 2008; Radi et al., 2002; Wizemann et al., 1994).

In inflammatory demyelinating diseases, the presence of NO derived compounds has been recognized. Increased levels of nitrite and nitrate levels in cerebrospinal fluid (CSF) of MS patients have argued for a role of reactive nitrogen species (RNS) in MS (Giovannoni, 1998; Rejdak

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et al., 2004a; Rejdak et al., 2004b). Since nNOS and eNOS were thought to be present in neurons and endothelial cells, the source of derivatives of reactive nitrogen species in brain and CSF in inflammatory disorders was thought to result from activation of iNOS (Brown and Bal-Price, 2003). Demyelination and loss of oligodendrocytes are thought to result from collateral damage from the production of iNOS in inflammatory cells.

nNOS was initially described to be present exclusively in neurons. Subsequent studies have shown constitutive expression of nNOS in astrocytes, but the presence and activation of nNOS in oligodendrocytes have thus far not been demonstrated (Kugler and Drenckhahn, 1996; Tolias et al., 1999). Although there are reports on the induction of iNOS in immature oligodendrocytes (OC), there are no reports on either the constitutive or inducible activity of nNOS in OC (Baud et al., 2004; Boullerne and Benjamins, 2006). We have recently shown the induction and activation of nNOS in a human hybrid oligodendroglial cell line (Yao et al., 2009). These studies showed that not only do MO3.13 cells express receptors for Toll ligands, but also that MO3.13 cells activate nNOS in respond to LPS that result in mitochondrial injury and cell death.

We chose to examine the activation and expression of nNOS in non-transformed rat oligodendrocytes and in the in vivo model of demyelination induced by intracerebral injection of LPS. The pathological features of the demyelination have been shown to resemble one of the types of demyelinating syndromes seen in a sub population of MS patients. In both the in vitro and in vivo systems of LPS-induced stimulation, we show that the activation of nNOS was associated with dysfunction of oligodendrocytes in vitro and correlated with development of demyelination in vivo.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (O55:B5), nNOS inhibitor 7-nitroindozale (7-NI), iNOS inhibitor L-Canavanin and MTT assay kit were purchased from Sigma (St. Louis, MO). The following antibodies were used in Western blots: anti-nNOS, anti-iNOS, anti-β actin and peroxidase conjugated secondary antibodies, (Santa Cruz Laboratories, CA) and anti-nitrotyrosine antibodies (Millipore, CA); NOS activity assay kit was purchased from Cayman Chemical. [^{3}H]-L-Arginine was purchased from Amersham. For immunostaining, the following antibodies were used: O4 (MAB345), CNPase (MAB326), Olig1 (MAB5540), GFAP (MAB360), nNOS (AB5380), and anti-nitrotyrosine (AB5532) (Millipore, CA); anti-iNOS (sc-650) and Iba1(sc-32725) (Santa Cruz, CA) and Alexa Fluor 488 and 594 conjugated antibodies (Invitrogen, Oregon). HRP conjugated antibody, Peroxidase block and AEC substrate were purchased commercially (Envision System, DAKO, CA). Alkaline phosphatase conjugated antibody was purchased through Sigma, MO and NBT/BCIP substrate from Boehringer-Mannheim. Antigen unmasking solution and Vecta-Shield mounting medium with DAPI were purchased from Vector Laboratories, CA.

2.2. Preparation of rat primary oligodendrocyte progenitor cells (OPC)

Oligodendrocytes were isolated using previously published protocol (Colello and Sato-Bigbee, 2001). Two-day-old Sprague–Dawley rat pups were anesthetized, decapitated, and the brain was removed using sterile technique. The neural cells were mechanically dissociated in ice-cold Hank's balanced salt solution (HBSS) with 25 mM HEPES and passed through a 70 μm strainer, and washed and subjected to Percoll gradient centrifugation. The isotonic Percoll solution was prepared and mixed with the cell suspension (5.7:8.5). The cells that were loaded on the Percoll gradient were spun at 19,000 rpm for 30 min. The oligodendrocyte band was harvested, washed and pelleted. The pelleted cells were placed in Petri dishes for 1 h to remove contaminated microglia and astrocytes and the non attached cells were predominantly oligodendrocytes. Suspended cells were collected and re-placed in Petri dishes one more time. The cells were cultured in poly-lysine coated dishes in medium consisting of 500 ml DMEM containing 0.1% BSA, 2.5 mg insulin, 25 mg transferrin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone, 4 mM L-glutamine, 1 mM sodium pyruvate, 5 μg PDGF, 5 μg b-FGF and antibiotics for 5–7 days. After three to five days in culture, the cells were assessed for purity by immunostaining with anti CNPase, GFAP and Iba1 antibody and used in experimental procedures. With the above protocol we were able to achieve 85% purity of oligodendrocytes. The rest of the cell population consisted of 15% astrocytes and rare microglia and neurons. The cells were Olig2+, Olig1+, CNpase+ but weakly expressed myelin basic protein suggesting that they were mainly oligodendrocyte progenitor cells (OPC) that had not yet expressed myelin membranes. MO3.13 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Mediatech, Inc USA, # 35-015-CV endotoxin<10U) and penicillin–streptomycin (100 u/ml, and 100 μg/ml respectively). Purified preparations of astrocytes were a gift from the laboratory of M. Aschner, Vanderbilt Medical Center and cultured in DMEM/10% FBS/antibiotics.

2.3. In vivo injection of LPS into corpus calossum

We established the demyelination model system in the corpus calossum in a manner similar to that seen following injection of LPS into the dorsal columns of rat spinal cord. Two-month old rats were injected with LPS or saline and were sacrificed on days 2, 7, 14, 21 and 28 respectively (eight rats in each group). Rats were anesthetized and positioned in a small-animal stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) to conform to the brain atlas (Pellagrino, 1979 #278). Microinjection of LPS (*Escherichia coli* serotype 055:B5) into the corpus callosum was performed with a 32-gauge needle through a dentist's burr hole. To perform the injection into the corpus callosum, the following coordinates were used: 1 mm posterior from Bregma, 1 mm lateral from the sagittal suture, and 3.3–3.5 mm below the dura mater. LPS-treated rats received 5 μl of PBS containing 5 μg of LPS and injection was done using a microinjection pump over 15 min and needle was held for an additional 10 min after injection. Saline control received 5 μl of PBS solution.

2.4. Immunostaining of paraffin embedded brain tissue and cultured glial cells from neonatal rat brains

For immunostaining of paraffin embedded brain tissue, animals were perfused using 4% paraformaldehyde/4% sucrose in PBS at 2, 7, 14, 21 and 28 days after injection with LPS or saline as injection control. Brains were removed and postfixed in 10% formaldehyde for

Table 1
Primer sequences of TLR for RT-PCR.

Name		Sequences	Products size	GeneBank accession no#
Human				
TLR2	FW	5′-TCC GGA GGC TGC ATA TTC CAA AGG-3′	295	NM_004264
	RV	5′-CAG AGT GAG CAA AGT CTC TCC GGT-3′		
TLR3	FW	5′-TCC GTT GAG AAG AAG GTT TTC GGG-3′	321	NM_003265
	RV	5′-ATA TCC TCC AGC CCT CCA AGT GGA-3′		
TLR4	FW	5′-AGG ACT GGG TAA GGA ATG AGC TAG-3′	432	NM_138554
	RV	5′-GTA CCC ACT GGT CCT TCT GGA TTC-3′		
Rat				
TLR2	FW	5′-CCA CAG GAC TCA AGA GCA T-3′	120	NM_198769
	RV	5′-AGA ATG GCC TTC CCT TGA-3′		
TLR3	FW	5′-AAC TTG ATT TTC TTG GCA ATT CT-3′	137	NM_198791
	RV	5′-GAG GTT CAG TTG GGC ATT-3′		
TLR4	FW	5′-GGA AAA GCC TTG AAT CCA GA-3′	137	NM_019178
	RV	5′-GCA GAA ACC CAG ATG AAC T-3′		