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LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-γ-independent manner by a JNK signaling pathway in primary murine microglia

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ABSTRACT

Inflammation-induced activation of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) causes depressive-like behavior in mice following acute activation of the innate immune system by lipopolysaccharide (LPS). Here we investigated the mechanism of IDO expression induced by LPS in primary cultures of microglia derived from neonatal C57BL/6J mice. LPS (10 ng/ml) induced IDO transcripts that peaked at 8 h and enzymatic activity at 24 h, resulting in an increase in extracellular kynurenine, the catabolic product of IDO-induced tryptophan catabolism. This IDO induction by LPS was accompanied by synthesis and secretion of the proinflammatory cytokines TNFα and IL-6, but without detectable IFNγ expression. To explore the mechanism of LPS-induced IDO expression, microglia were pretreated with the c-Jun-N-terminal kinase (JNK) inhibitor SP600125 for 30 min before LPS treatment. We found that SP600125 blocked JNK phosphorylation and significantly decreased IDO expression induced by LPS, which was accompanied by a reduction of LPS-induced expression of TNFα and IL-6. Collectively, these data extend to microglia the property that LPS induces IDO expression via an IFNγ-independent mechanism that depends upon activation of JNK. Inhibition of the JNK pathway may provide a new therapy for inflammatory depression.

1. Introduction

Toll-like receptors (TLRs) are important mediators of neuroinflammation and tissue damage during infectious and non-infectious diseases of the central nervous system (CNS) (Carpentier et al., 2008). Microglia are known to be key cellular mediators of neuroimmune responses, that constitutively express a wide complement of TLRs (Bsibsi et al., 2002). Microglia are activated in most pathological conditions of the CNS and play an important role in sensing and propagating inflammatory signals in response to activation of the peripheral innate immune system (Hanisch and Kettenmann, 2007). In the absence of inflammatory stimuli, microglia are quiescent even though they are actively involved in immune surveillance (Nimmerjahn et al., 2005; Soulet and Rivest, 2008). Once activated, microglial cells display macrophage-like capabilities including phagocytosis, antigen presentation and inflammatory cytokine production (Garden and Möller, 2006).

Chronic inflammation is often associated with clinical depression (Evans et al., 2005; Adler et al., 2008; Dantzer et al., 2008a,b). Recent studies have focused on potential mechanisms that might link inflammation-induced depression to tryptophan metabolism, particularly in the brain, where a reduction in the bioavailability of tryptophan could affect serotoninergic neurotransmission and play a synergistic role in the induction of depressive symptoms (Widner et al., 2002; Neumeister, 2003; Fitzgerald et al., 2008). A pivotal protein that has recently been shown to be required for development of inflammation-induced depressive-like behavior in mice is indoleamine 2,3-dioxygenase (IDO), the first rate-limiting tryptophan-degrading enzyme in the kynurenine pathway (Raison et al., 2006; O'Connor et al., 2009a,b). Activation of this enzyme by inflammatory signals leads to an increase in the kynurenine/tryptophan ratio in plasma and the generation of neuroactive mediators, including 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) (Guillemin et al., 2005). High levels of 3-HK and QUIN induce neuronal damage via oxidative stress (Lehrmann et al., 2008) and over stimulation of N-methyl-D-aspartate (NMDA)

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receptors (Guillemin et al., 2005; Spalletta et al., 2006). In animal studies, inhibition of IDO abrogates depressive-like behaviors induced by acute (O'Connor et al., 2009a) or chronic inflammation (O'Connor et al., 2009b). IFNγ is the predominant cytokine implicated in the induction of IDO (O'Connor et al., 2009c). However, subsequent studies have identified IFNγ-independent pathways, including TNFα and lipopolysaccharide (LPS), which are capable of inducing IDO activity (Fujigaki et al., 2006; Jung et al., 2007; Connor et al., 2008). The IFNγ-independent up-regulation of IDO was first described in the LPS model of immune activation in human acute monocyte leukemia cell line THP-1 (Fujigaki et al., 2001). However, it is not known if this property of IDO induction extends to primary microglia. Furthermore, the signal transduction mechanisms responsible for IFNγ-independent induction of IDO following exposure to LPS require further investigation.

In dendritic cells, Jung et al. (2007) recently reported that LPS induces IDO expression via an IFNγ-independent mechanism. In this system, c-Jun-N-terminal kinase (JNK) was required for LPS to induce IDO in the absence of IFNγ. Sickness behavior always precedes development of depressive-like behavior induced by an acute inflammatory stimulus, and we previously demonstrated that infusion of a specific JNK inhibitor i.c.v. completely blocks TNFα-induced sickness behavior (Palin et al., 2008). Since IDO is primarily expressed by activated microglia in the brain, we queried whether JNK is also involved in LPS-induced expression of IDO in these cells. These experiments establish that the JNK pathway plays a critical role in the microglial induction of IDO expression and activity following LPS stimulation via an IFNγ-independent mechanism.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS; <0.25 EU/ml endotoxin), 0.25% trypsin, Dulbecco's modified Eagle's medium/high glucose (DMEM) containing 0.584 g/l glutamine and 4.5 g/l glucose, sodium pyruvate and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) were purchased from HyClone (Logan, UT). Nylon cell strainers (70 μm) were obtained from BD Falcon (Bedford, MA) and the CytoTox^{96} non-radioactive cytotoxicity kit (cat# G1781) was from Promega Corporation (Madison, WI). Enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Wiesbaden, DE). Rabbit polyclonal antibodies (IgG) specific for JNK (cat# 9252) and phosphorylated JNK (p-JNK, cat# 9251) were purchased from Cell Signaling Biotechnology (Danvers, MA), whereas the secondary horseradish peroxidase (HRP)-linked donkey anti-rabbit antibody (NA934V) was purchased from GE Healthcare Biosciences (Piscataway, NJ). The JNK inhibitor SP600125 (cat# 420119) was obtained from Calbiochem (USA). Purity of primary microglia was confirmed with a rat FITC-labeled anti-mouse CD11b (IgG2b, cat# 557396) using a FITC-labeled rat IgG2b isotype antibody as a control (cat# 553988; BD Biosciences, Pharmingen, USA). Protein was measured with a standard Bradford assay kit (cat# 500–0113, 0114, 0115) and Immun-Blot polyvinylidene difluoride (PVDF, cat# 162-0177) membranes were from Bio-Rad (Hercules, CA). ECL Western blotting detection reagents (cat# RPN2106V1 and RPN2106V2) were from GE Healthcare Little Chalfont (Bucks, UK). TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA). Ambion (cat# 1710) reverse transcriptase kit, Ambion's DNA-free™ DNase treatment and removal reagents (cat# AM1906), RT-PCR primers for TNFα (cat# Mm00443258_m1), IL-6 (cat# Mm00446190_m1), IFNγ (cat# Mm00801778_m1), IDO (cat# Mm00492586_m1), KMO (cat#Mm00505511_m1), KYNU (cat# Mm0051012_m1), KAT (cat# Mm00496169.m1), HAO (cat# 005177945_m1), IL-1β (cat# Mm00434228_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cat# Mm999999_g1) were all obtained from Applied Biosystems. The protease inhibitor cocktail (cat# P2714), lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (cat# L-3137), poly-L-lysine (cat# P4832) and other reagents and chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). L-cell conditioned medium used to culture primary microglia was obtained from L-929 cells obtained from American Type Culture Collection (ATCC, cat# CCL-1™, Manassas, VA).

2.2. Preparation of primary murine microglia

Primary mixed glial cultures were established from brains of <2-day-old neonatal C57BL/6J mice. After removal of the meninges, brains were mechanically minced and dissociated with 0.25% trypsin/0.5 mM EDTA. After inactivation of trypsin, the tissue suspension was passed through a 70 μm nylon cell strainer. This cell suspension was centrifuged at 100*g* for 15 min. Cell pellets were resuspended in DMEM supplemented with 10% heat-inactivated FBS and plated in poly-L-lysine pre-coated culture flasks. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, with the culture medium being changed twice weekly.

Cells became confluent by 15–20 days at which time microglial cells were separated from astrocytes by shaking the flasks for 1 h at 37 °C on an Orbital platform shaker (Model Annova 2000; New Brunswick Scientific, Edison, NJ) at 150 rpm. Isolated microglia were collected and cultured in 20% (v:v) L-929-cell conditioned medium (LCCM) for 7–10 days. The LCCM provided a source of colony stimulating factors. Purity of microglia was confirmed as >95% CD11b+ cells, as verified by flow cytometry using previously described techniques (Liu et al., 1999; Shen et al., 2002).

Primary microglia were treated with 10 ng/ml LPS, the optimal concentration to stimulate expression of IDO, in DMEM supplemented with 2% FBS. At various times following addition of LPS, supernatants were collected and stored at −80 °C for measurement of kynurenine and cytokines. Cells were washed twice with cold PBS and stored at −80 °C for isolation of mRNA, Western blot and IDO enzymatic activity. For inhibition of JNK, cells were pre-incubated with SP600125 for 30 min and then treated with 10 ng/ml LPS for 0, 15, 30 and 60 min. Cell viability was evaluated using both trypan blue staining and the amount of lactate dehydrogenase (LDH) released into the culture medium by CytoTox^{96} non-radioactive cytotoxicity kit.

2.3. RNA extraction and reverse transcription

Total RNA was extracted from cultured microglial using TRIzol reagent as previously described (O'Connor et al., 2009b). Total mRNA (1–2 μg) was reverse transcribed using reverse transcriptase kits from Ambion according to the manufacturer's description. Briefly, RNA (1–2 μg) was pretreated with DNA-free™ DNase treatment at 37 °C for 20–30 min. RNA samples were incubated with a mixture containing a mixture of dNTPs, random primers, 1× first-strand buffer, a rRNase inhibitor, MMLV reverse transcriptase and water to a final volume of 20 μl at 44 °C for 1 h, followed by 10 m at 92 °C to inactivate the reverse transcription reagents.

2.4. Real-time RT-PCR

Real-time RT-PCR was used to quantify mRNA as the number of target gene cycle amplifications, as described previously (O'Connor et al., 2009a).

2.5. Enzyme-linked immunosorbent assays (ELISAs)

IL-6 and TNFα were measured with validated specific ELISA assays according to the manufacturer's instructions. Briefly, adding