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# Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes

Cindy X. Zhang-Gandhi, Paul D. Drew *

*Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, Arkansas, 72205, USA*

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**Abstract**

Liver X receptors (LXRs) are nuclear receptors previously identified to be important in lipid metabolism. Recent reports suggest that LXR agonists also exhibit anti-inflammatory properties in mouse models of atherosclerosis and contact dermatitis. In the present study, we investigated the effects of LXR agonists on mouse microglia and astrocytes. When chronically activated, these resident-CNS glia have been implicated in the pathology of neuroinflammatory disorders including multiple sclerosis (MS). Our studies demonstrated for the first time that LXR agonists inhibited the production of nitric oxide, the pro-inflammatory cytokines IL-1β and IL-6 and the chemokine MCP-1 from LPS-stimulated microglia and astrocytes. Furthermore, LXR agonists inhibited LPS-induction of nuclear factor-kappa B (NF-κB) DNA-binding activity. These agonists also blocked LPS-induction of IκB-α protein degradation in microglia, suggesting a mechanism by which these agonists modulate NF-κB DNA-binding activity. These studies suggest that LXR agonists suppress the production of pro-inflammatory molecules by CNS glia, at least in part, by modulating NF-κB-signaling pathways. Retinoid X receptors (RXRs) physically interact with LXR receptors, and the resulting obligate heterodimer regulates the expression of LXR-responsive genes. Interestingly, a combination of LXR and RXR agonists additively suppressed the production of NO by microglia and astrocytes. Collectively, these studies suggest that LXR agonists may be effective in the treatment of neuroinflammatory diseases including MS.
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*Keywords:* LXR; Microglia; Astrocytes; Cytokine; Chemokine; Multiple sclerosis

## 1. Introduction

Multiple sclerosis (MS) affects approximately 400,000 people in the United States (Anderson et al., 1992). This disorder commonly strikes young adults and can profoundly affect quality of life (McFarlin and McFarland, 1982a,b). Multiple factors have been proposed to contribute to the development of MS, including viral infections, environmental factors and genetic background (Gilden, 2005; Marrie, 2004). MS is thought to be an autoimmune disease characterized by immune mediated myelin destruction and axonal transection. Currently available treatments for MS are modestly efficacious, and there is no cure (Cohen et al., 1999; Wekerle, 2002). Therefore, a great need exists for developing novel MS therapies.

Liver X receptors (LXRs) are nuclear receptors involved in the regulation of lipid metabolism (Cao et al., 2004; Edwards et al., 2002). Two LXR receptor subtypes exist; LXR-α, which is expressed on cells including macrophages and hepatocytes, and LXR-β, which is ubiquitously expressed (Janowski et al., 1999). Recent studies indicate that LXR agonists also modulate inflammatory responses. For example, LXR agonists suppressed inflammation in mouse models of atherosclerosis and contact dermatitis (Fowler et al., 2003; Joseph et al., 2003). This suggests that LXR agonists may be effective in the treatment of neuroinflammatory disorders including MS.

Microglia are immune cells that reside in the CNS. They are antigen presenting cells and upon activation are capable of phagocytosis and the production of various pro-inflammatory molecules such as nitric oxide (NO) and

* Corresponding author. University of Arkansas for Medical Sciences, Department of Neurobiology and Developmental Sciences, Slot 846, Biomed II Bldg., Rm. 563-2, 4301 W. Markham St., Little Rock, AR 72205. Tel.: +1 501 296 1265; fax: +1 501 526 6756.

*E-mail address:* drewpauld@uams.edu (P.D. Drew).

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interleukin-1β (IL-1β) (Benveniste, 1997). These molecules are capable of destroying pathogens, but can also be toxic to neurons and myelin-producing oligodendrocytes, cells which are compromised in MS (Drew and Chavis, 2001). The secretion of C–X–C and C–C chemokines by activated microglia may also contribute to the recruitment of leukocytes from the periphery, as well as to the migration of microglia to sites of CNS inflammation (Aarum et al., 2003). Like microglia, chronically activated astrocytes are believed to contribute to MS through production of NO and various pro-inflammatory cytokines and chemokines (De Keyser et al., 2003; Holley et al., 2003; Raine, 1997; Storer et al., 2005a; Trapp et al., 1998; Zeinstra et al., 2003). Therefore, agents that block the activation of microglia and astrocytes may be effective in the treatment of MS.

The current studies demonstrate that LXR agonists alone, or in combination with RXR agonists, suppress the production of various pro-inflammatory mediators by activated microglia and astrocytes. Furthermore, these studies demonstrate that LXR agonists block the degradation of I-κB-α and suppress NF-κB DNA-binding activity in LPS-stimulated microglia. This suggests that LXR agonists may modulate the production of pro-inflammatory mediators, at least in part, through effects on NF-κB signaling pathways. These results support the growing potential of LXR agonists in the treatment of MS and other neuroinflammatory diseases.

## 2. Materials and methods

### 2.1. Reagents

The LXR agonist TO-901317 was obtained from Cayman Chemical Company (Ann Arbor, MI) and acetyl-podocarpic dimer (APD) was synthesized at Merck (Whitehouse Station, NJ), and kindly provided by Dr. C. Sparrow. The RXR agonist 9-*cis*-retinoic acid, lipopolysaccharide, and L-Leucine methyl ester hydrochloride (L-LME) were obtained from Sigma (St. Louis MO). Lectin, *Griffonia simplicifolia*, was obtained from Sigma and anti-glial fibrilary acidic protein (GFAP) antibody was obtained from Dako (Carpinteria, CA). DMEM media, glutamine, trypsin, and antibiotics used for tissue culture were obtained from BioWhittaker (Walkersville, MD). OPI medium supplement was obtained from Sigma (St Louis, MO). GM-CSF was obtained from BD Pharmingen (San Diego, CA). Fetal bovine serum (FBS) from Hyclone (Logan, UT) was used for primary cultures and FBS used for N9 microglia cultures was obtained from Sigma. C57BL/6 mice were obtained from Harlan (Indianapolis, IN), and bred in house.

### 2.2. Cell culture

Primary mouse microglia were obtained through a modification of the McCarthy and de Vellis (1980) protocol. Briefly, cerebral cortices were excised from 1–3 day-old C57BL/6 mice and the meninges removed. Cortices were minced into small pieces and cells were separated by trypsinization and trituration of cortical tissue, and cellular debris was removed by filtration through a 70-μm cell strainer. Cells were plated into tissue culture flasks and allowed to grow to confluence (~1 week) in complete DMEM media containing 10% FBS, 1.4 mm L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, OPI medium supplement, and GM-CSF. Flasks were shaken overnight (200 rpm at 37 °C) in a temperature-controlled shaker to loosen microglia and oligodendrocytes from the more adherent astrocytes. These non-adherent cells were plated for 2 h and then lightly shaken to separate oligodendrocytes from the relatively more adherent microglia. Primary astrocytes were prepared as described above, with the exception that GM-CSF was omitted from the culture media. These panning procedures were repeated, as necessary, to obtain primary microglia or astrocytes of greater than 95% purity as determined by immunocytochemical staining with lectin or anti-glial fibrilary acidic protein (GFAP) antibody, respectively. The N9 microglial cell line is derived from *myc*-immortalized mouse microglia (Corradin et al., 1993), and was graciously provided by P. Ricciardi-Castagnoli (U. Milan, Italy). N9 cells were cultured in MEM medium containing 10% FBS, 1.4 mM glutamine, and 20 μM 2-mercaptoethanol. Cells were seeded into 96-well plates ($5 \times 10^4$ cells/well) and incubated overnight at 37 °C/5% $CO_2$. Cells were treated with the LXR agonist TO-91317 or APD, or LXR agonist in combination with the RXR agonist, 9-*cis*-retinoic acid for 1 h, and then stimulated with LPS for 24 h. Finally, tissue culture supernatants and cells were collected for nitrite assay, enzyme-linked immunosorbent assay (ELISA) and MTT reduction assay.

### 2.3. Nitric oxide production

Levels of the NO derivative nitrite were determined in the culture medium by Griess reaction as described previously (Drew and Chavis, 2001). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 550 nm. A standard curve using $NaNO_2$ was generated for each experiment for quantitation.

### 2.4. Cell viability assays

Cell viability was determined by MTT reduction assay as described previously (Drew and Chavis, 2001). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm. Results were reported as percent viability relative to untreated cultures.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Cytokine (IL-1β and IL-6) and chemokine (MCP-1) levels in tissue culture media were determined by ELISA as described by the manufacturer (OptEIA Sets, Pharmingen, San Diego, CA). Optical densities were determined using a Spectromax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. Cytokine and chemokine concentrations in

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