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Liver X Receptor and Retinoid X Receptor Agonists Inhibit Inflammatory Responses of Microglia and Astrocytes

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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-1 β 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- κ B) DNA-binding activity. These agonists also blocked LPS-induction of I κ B- α protein degradation in microglia, suggesting a mechanism by which these agonists modulate NF- κ B 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- κ B-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.

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; McFarlin and McFarland, 1982b). 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

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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., 2003b). 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 interleukin-1 β (IL-1 β) (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, 2001a). 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- κ B- α and suppress NF- κ B 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- κ B 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). 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-gial fibrillary 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 deVellis (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- μ m 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 hours 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 fibrillary 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 μ M 2-mercaptoethanol. Cells were seeded into 96-well plates (5 X 10⁴ cells/well) and incubated overnight at 37°C /5% CO₂. 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 hour, and then stimulated with LPS for 24 hours. 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 NaNO₂ 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-1 β 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 media were determined from standards containing known concentrations of the proteins.

2.6 Western blot analysis

Total cellular protein was isolated from untreated, LPS stimulated, and TO-91317 pre-treated then LPS stimulated cells plated in 6-well plates (2 X 10⁶ cells/well). Protein was then quantitated by BCA Protein Assay as described by the manufacturer (Pierce, Rockford, IL, USA). Cellular proteins (20 μ g) were separated electrophoretically on 4–15%-HCl Ready-gels obtained from Bio-Rad (Hercules, CA) containing the denaturant sodium dodecyl sulfate (SDS). Proteins were transferred electrophoretically to nitrocellulose membranes (NitroBind; MSI, Westborough, MA, USA) and then incubated with polyclonal rabbit anti-human I κ B- α antibody (Calbiochem, La Jolla, CA, USA) for 1 h at room temperature at a 1:750 dilution. Blots were then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature at a 1:5000 dilution.

I κ B- α protein was detected by ChemiGlow Western Blotting Detection Reagents as described by the manufacturer (Alpha Innotech Corporation, San Leandro, CA, USA), followed by exposure in BioRad ChemiImager.

2.7 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from untreated cells, cells stimulated with LPS, or cells treated with 60 μ M TO-91317 for 1 hour then stimulated with LPS for 24 h as previously described (Drew et al., 1993). A combination of the protease inhibitors, 0.5mM dithiothreitol, 10 μ g/ml leupeptin & aprotinin, 10 μ g/ml soy bean trypsin inhibitor, and 200mM PMSF & pepstatin A were used during preparation of nuclear extracts. Oligonucleotide probes used in gel mobility shift assay were end-labeled using T4 polynucleotide kinase (Invitrogen, Calsbad, CA, USA) as described previously (Drew et al., 1993). Binding assays were performed in a volume of 20 μ l containing 20mM Tris-HCl (pH 7.5), 0.2mM EDTA, 5% glycerol, 1mM MgCl₂, 1mM dithiothreitol, 100mM NaCl, 4 μ g poly dI-dC, 4 μ g nuclear extracts and 10⁵cpm of γ ³²P-ATP (Perkin Elmer, Wellesley, CA, USA) labeled double stranded oligonucleotide probe. Double-stranded oligonucleotide probes used in the analysis were the NF- κ B element of the mutated HIV promoter (5'-AGTTGAGGGGACTTTCCAGGC-3') and the murine leukemia virus UCR element (5'-CTGCAGTAACGCCATTTTGCAAGGCATGAA-3'). The latter is bound by a ubiquitously expressed factor that generally is unaltered by experimental treatment and thus was used to control for equal loading and integrity of individual nuclear protein extracts as previously described (Dhib-Jalbut et al., 1995; Drew et al., 1993). Radiolabeled DNA-protein complexes were separated from free probes by electrophoresis through 6% polyacrylamide gels in the presence of 0.4% tris-borate-EDTA for 1.5 hour at 150V. The gels were dried and visualized by autoradiography. For competition assays, the indicated unlabeled oligonucleotides were included in 100-fold molar excess relative to radiolabeled probe in the binding assays.

2.8 Statistics

Data were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test to determine the significance of difference.

3. RESULTS

3.1 Effect of LXR agonists on NO production by N9 microglial cells

Activated microglia produce NO that may be toxic to oligodendrocytes and neurons, cells which are compromised in MS. In the present studies we demonstrated that LPS increased nitrite production by N9 mouse microglia. The LXR agonists, TO-901317 (Figure 1A) and APD (Figure 1C), both inhibited LPS-induced nitrite production in a dose-dependent manner. The LXR agonists were not toxic to the cells at the concentrations examined as demonstrated by MTT reduction assays (Figure 1B and D). Thus, TO-901317 and APD inhibition of NO production was not due to effects on cell viability.

3.2 Effect of LXR agonists on NO production by primary mouse microglia

Untreated primary microglia expressed undetectable levels of NO, and LPS significantly increased NO production by these cells (Figure 2). The LXR agonists TO-901317 (Figure 2A) and APD (data not shown) significantly suppressed LPS-induction of NO by primary microglia but did no effect cell viability (Figure 2B). Interestingly, slightly higher concentrations of TO-901317 were required to inhibit LPS induction of NO production in primary microglia than N9 microglial cells.

3.3 Effect of LXR agonists on cytokine and chemokine production by microglia

Activated microglia produce a variety of inflammatory cytokines and chemokines, which may modulate MS. In the present study, LPS induced the secretion of cytokines IL-1 β and IL-6, and the chemokine MCP-1 (Figure 3). Pre-treatment with TO-901317 suppressed the production of IL-1 β (Figure 3A), IL-6 (Figure 3B) and MCP-1 (Figure 3C) in a dose-dependent manner.

3.4 Combined effect of LXR and RXR agonists on microglia function

LXR and RXR are capable of physically interacting to form heterodimers. These heterodimers bind liver X response elements (LXREs) present in the promoter elements of LXR responsive genes. The present studies demonstrate that LXR agonist TO-901317 and the RXR agonist 9-cis RA independently inhibited NO production by LPS-stimulated microglia. In addition, these agonists in combination inhibited NO production more strongly than the RXR or the LXR agonist alone (Figure 4A). These agonists did not significantly decrease microglia cell viability in these studies (Figure 4B). These studies suggest that LXR and RXR agonists act cooperatively in inhibiting NO production by activated microglia.

3.5 Effect of LXR agonist on NF- κ B DNA-binding activity

NF- κ B plays an important role in the activation of a variety of genes which encode pro-inflammatory proteins. In the present study, we demonstrated that NF- κ B DNA-binding activity was increased following stimulation of microglia with LPS. Importantly, the LXR agonist TO-901317 significantly inhibited LPS-induction of NF- κ B binding activity in these cells (Figure 5). This suggests that LXR agonists may inhibit production of pro-inflammatory molecules through a mechanism involving inhibition of NF- κ B binding to the promoters of genes which encode these molecules.

3.6 Effect of LXR agonist on I κ B- α expression

Inhibitor- κ B (I κ B) binds to NF- κ B masking its nuclear localization signal and thus sequestering this transcription factor complex in the cytoplasm. A variety of stimuli have been demonstrated to phosphorylate I κ B resulting in the release and nuclear translocation of NF- κ B. In the present study, we demonstrated that LPS treatment of primary microglia decreased the expression of I κ B- α and that the LXR agonist TO-901317 counteracted the effect of LPS. This suggests that TO-901317 prevented the degradation of I κ B levels in microglia, thus blocking NF- κ B translocation to the nucleus and LPS induction of NF- κ B genes including iNOS which regulates the production of NO, as well as genes that encode pro-inflammatory cytokines and chemokines. Collectively, these results suggest a mechanism by which the LXR agonist TO-901317 inhibits LPS induction of NF- κ B DNA-binding activity in primary microglia.

3.7 Effect of LXR agonist on NO production by primary astrocytes

Like microglia, activated astrocytes can also produce NO which can be toxic to oligodendrocytes thus contributing to the demyelination seen in MS patients. In the present study, the synthetic LXR agonist, TO-901317, was demonstrated to significantly reduce the production of NO by primary astrocytes activated by LPS in a dose-dependent manner (Figure 7A). This agonist was not toxic to astrocytes at the concentrations examined as determined by the MTT reduction assay (Figure 7B). Thus TO-901317 inhibition of NO production by astrocytes was not due to cell toxicity.

3.8 Effect of LXR agonists on cytokine and chemokine production by astrocytes

Like microglia, activated astrocytes produce a variety of inflammatory cytokines and chemokines which may modulate MS. In the present study, LPS induced the secretion of the

cytokines IL-1 β and IL-6 and the chemokine MCP-1 (Figure 8). Pre-treatment with TO-901317 suppressed the production IL-1 β (Figure 8A), IL-6 (Figure 8B) and MCP-1 (Figure 8C) in a dose-dependent manner.

3.9 Combined effect of LXR and RXR agonists on astrocyte function

The LXR agonist TO-901317 and the RXR agonist 9-cis RA inhibited NO production by LPS-stimulated astrocytes. In addition, a combination of these agonists more potently inhibited NO production in these cells than either agonist alone (Figure 9A). These agonists did not significantly decrease astrocyte viability (Figure 9B). The studies suggest that LXR and RXR agonists act cooperatively in inhibiting NO production by activated astrocytes.

4. DISCUSSION

MS is a chronic neuroinflammatory disease thought to be initiated by activated T cells that recognize self antigen (Martin and McFarland, 1995; Xiao and Link, 1999). These activated T cells are capable of migrating into the CNS where they trigger the activation of CNS glia. Upon activation, glia, including microglia and astrocytes, produce a variety of cytokines and chemokines which may contribute to the pathology associated with MS. For example, NO is abundantly produced by activated glia. In addition, cytokines including IL-1 β , and IL-6, and the chemokine MCP-1 are produced by activated glia. These pro-inflammatory molecules are associated with the induction and effector phase of EAE, an animal model of MS (Wang et al., 2002). Therefore, agents that suppress the production of these molecules may be effective in the treatment of MS.

LXRs, like PPARs, are nuclear receptors that play an important role in lipid and glucose metabolism. Previously, we and others have demonstrated that PPARs also are important modulators of inflammation (Kielian and Drew, 2003b; Ricote et al., 1998). For example, PPAR- γ agonists including the naturally occurring prostaglandin metabolite 15d-PGJ2 and synthetic ligands including thiazolidinediones which are used commonly for the treatment of type II diabetes, suppressed immune function of cells including macrophages T cells, and CNS glia (Clark et al., 2000; Drew and Chavis, 2001a; Jiang et al., 1998; Ricote et al., 1998; Storer et al., 2005a; Storer et al., 2005b). PPAR- γ ligands also suppress inflammation and pathology in animal models of atherosclerosis, arthritis, inflammatory bowel disease and MS (Diab et al., 2002; Feinstein et al., 2002; Kawahito et al., 2000; Li et al., 2000; Natarajan and Bright, 2002; Niino et al., 2001; Su et al., 1999). Like PPAR- γ agonists, PPAR- α agonists suppress the function of T cells and CNS glia in vitro (Lovett-Racke et al., 2004; Xu et al., 2006a; Xu et al., 2005b). In addition, these agonists are effective in the treatment of EAE (Lovett-Racke et al., 2004). Relatively little is known concerning the role of LXR agonists in modulating inflammation. Recently, however, these agonists were demonstrated to suppress the activation of macrophages in vitro and mouse models of atherosclerosis and contact dermatitis in vivo (Fowler et al., 2003; Joseph et al., 2004a; Joseph et al., 2003b; Joseph et al., 2002c). The present studies indicate that LXR agonists suppress the production of NO, as well as the pro-inflammatory cytokines IL-1 β and IL-6, and the chemokine MCP-1. These molecules are believed to contribute to the development of EAE and MS. Thus, these studies suggest that LXR agonists may be effective in the treatment of these disorders.

Our studies demonstrated that the LXR agonist TO-091317 blocked LPS induction of NF- κ B DNA-binding activity in microglia. NF- κ B is an important modulator of a variety of genes which respond to immune stimuli, including iNOS, as well as many of the genes encoding the cytokines and chemokines examined in the current studies. We further demonstrated that TO-901317 inhibits NF- κ B DNA binding activity, at least in part, by suppressing LPS-induced degradation of I κ B, which blocks the nuclear translocation of NF- κ B. LPS is known to stimulate NF- κ B by engaging the toll-like receptor TLR4 through a MyD88-dependent

pathway (Kielian, 2006a). Future studies will investigate additional potential mechanisms by which LXR agonists modulate MyD88-dependent signaling and the activation of NF- κ B.

The current studies demonstrated that RXR agonist 9-cis RA and the LXR agonist TO-091317 act cooperatively to inhibit the production of NO by primary mouse microglia and astrocytes. LXRs are known to physically interact with RXRs to form heterodimers. Maximal receptor activity requires ligand binding by both of these receptors, suggesting a mechanism for the combined effects of LXR and RXR agonists on microglia and astrocyte function in the present study. Interestingly, RXRs also physically interact with PPAR ligands. We have previously demonstrated that PPAR and RXR ligands act cooperatively in suppressing inflammatory responses (Lovett-Racke et al., 2004; Xu et al., 2006a; Xu et al., 2005b). Future studies will compare the profile of genes regulated by PPAR relative to LXR agonists. The role of specific co-activators and co-repressors that regulate gene expression in response to PPAR and LXR agonists will also be evaluated.

In summary, we have demonstrated that LXR agonists were capable of blocking the production of NO by LPS-stimulated primary mouse microglia and astrocytes. These ligands also inhibited the production of pro-inflammatory cytokines IL-1 β and IL-6 and the chemokine MCP-1. Furthermore, we demonstrate that LXR agonists inhibit LPS-induced degradation of I κ B- α and block NF- κ B DNA-binding activity. This suggests that LXR agonists may suppress the expression of these pro-inflammatory molecules, at least in part, by altering NF- κ B signaling pathways. In addition, the RXR agonist 9-cis RA and the LXR agonist TO-091317 acted cooperatively to inhibit NO production by both astrocytes and microglia. These studies suggest that LXR agonists, perhaps in combination with RXR agonists, may be effective in the treatment of neuroinflammatory disorders including MS.

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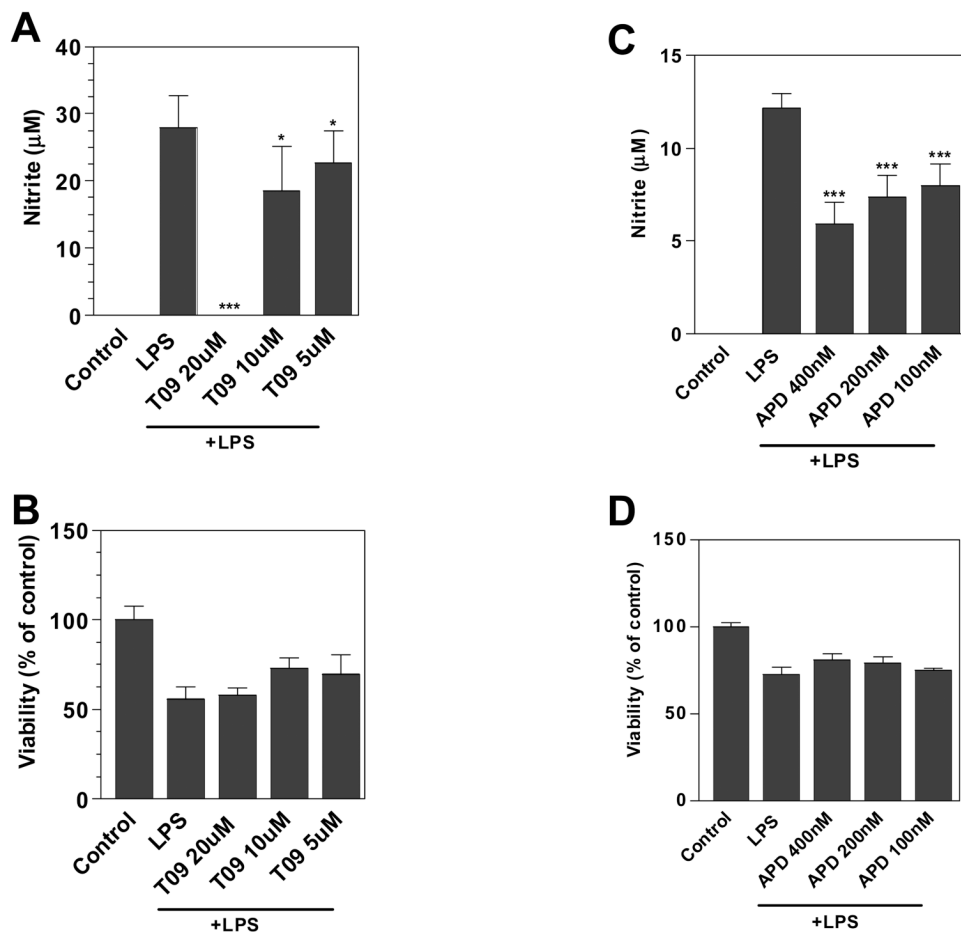


Figure 1. Effects of LXR agonists on LPS induction of NO in N9 microglia

Cells were pre-treated for 1 h with the indicated concentrations (μM) of LXR agonists (APD; TO-901317 = T99). Cells were then stimulated by LPS (1 μg/ml) and 24 h later, the level of nitrite in the culture medium was determined by Griess reaction (A and C). Cell viability was determined by MTT assay (B and D). Representative results from a single experiment are shown and at least three independent experiments were performed. Values represent the mean ± s.d. from one experiment performed in triplicate. *p<0.05 and ***p<0.001 vs. LPS-treated cultures.

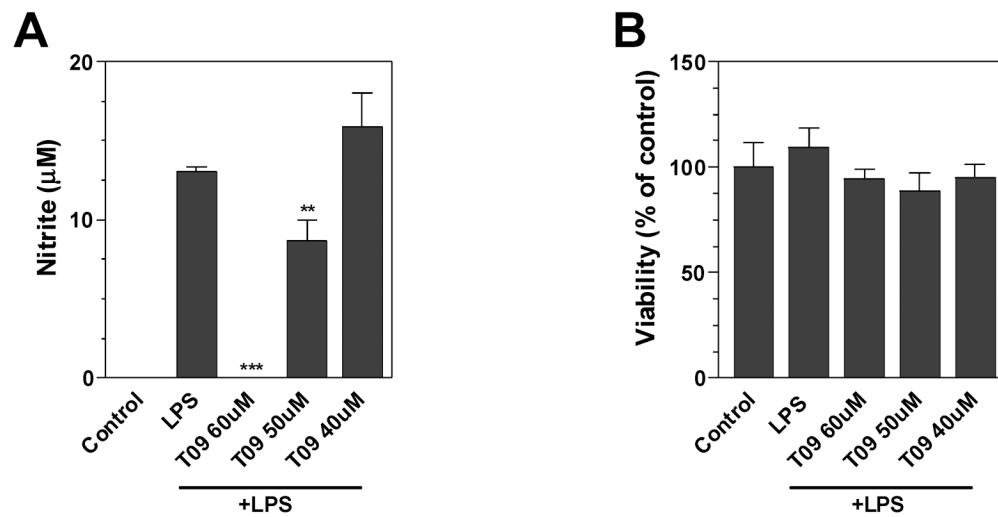


Figure 2. Effects of LXR agonist on LPS induction of NO in mouse primary microglia

Cells were pre-treated for 1 h with the indicated concentrations of LXR agonist TO-901317. Cells were then stimulated by LPS (1µg/ml) and 24 h later, the level of nitrite in the culture medium was determined by Griess reaction (A). Cell viability was determined by MTT assay (B). Representative results from a single experiment are shown and at least three independent experiments were performed. Values represent the mean \pm s.d. from one experiment performed in triplicate. **p<0.01 and ***p<0.001 vs. LPS-treated cultures.

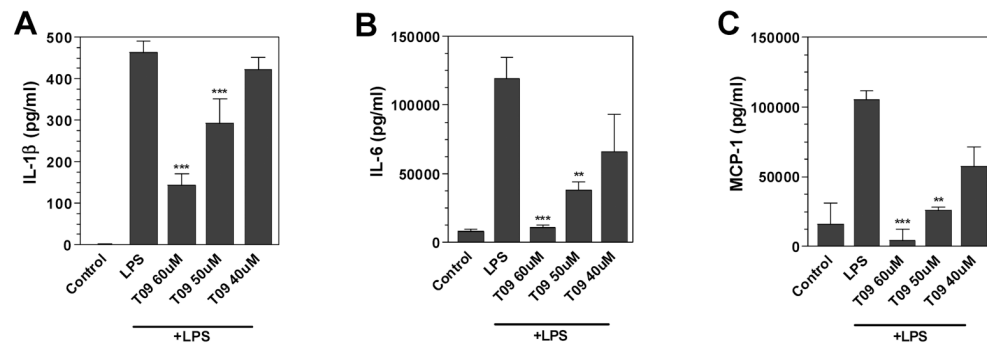


Figure 3. Effects of LXR agonist on cytokine and chemokine production by mouse primary microglia

Cells were pre-treated for 1 h with the indicated concentrations of LXR agonist, TO-901317. LPS (1μg/ml) was added as indicated, and 24 h later, IL-1β (A), IL-6 (B), and MCP-1 (C) levels were measured in the culture medium by ELISA. Representative results from a single experiment are shown and at least three independent experiments were performed. Values represent the mean ± s.d. from one experiment performed in triplicate. **p<0.01 and ***p<0.001 vs. LPS-treated cultures.

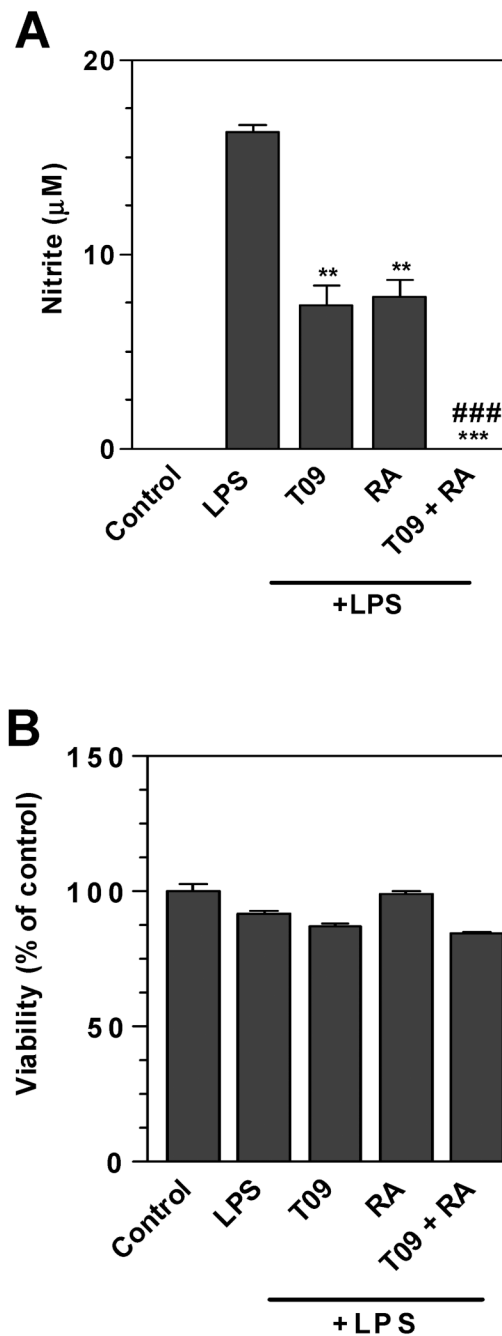


Figure 4. Combined effects of LXR and RXR agonists on nitrite production by mouse primary microglia

Cells were pre-treated for 1 h with TO-901317 (60 μM), 9-cis RA (0.75 μM), or their combination. LPS (1 $\mu\text{g}/\text{ml}$) was added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Representative results from a single experiment are shown and at least three independent experiments were performed. Values represent the mean \pm s.d. from one experiment performed in triplicate. *** $p < 0.001$ and ** $p < 0.01$ vs. LPS-treated cultures. ### $p < 0.001$ vs. LPS plus TO-91317 and vs. LPS plus 9-cis RA-treated cultures.

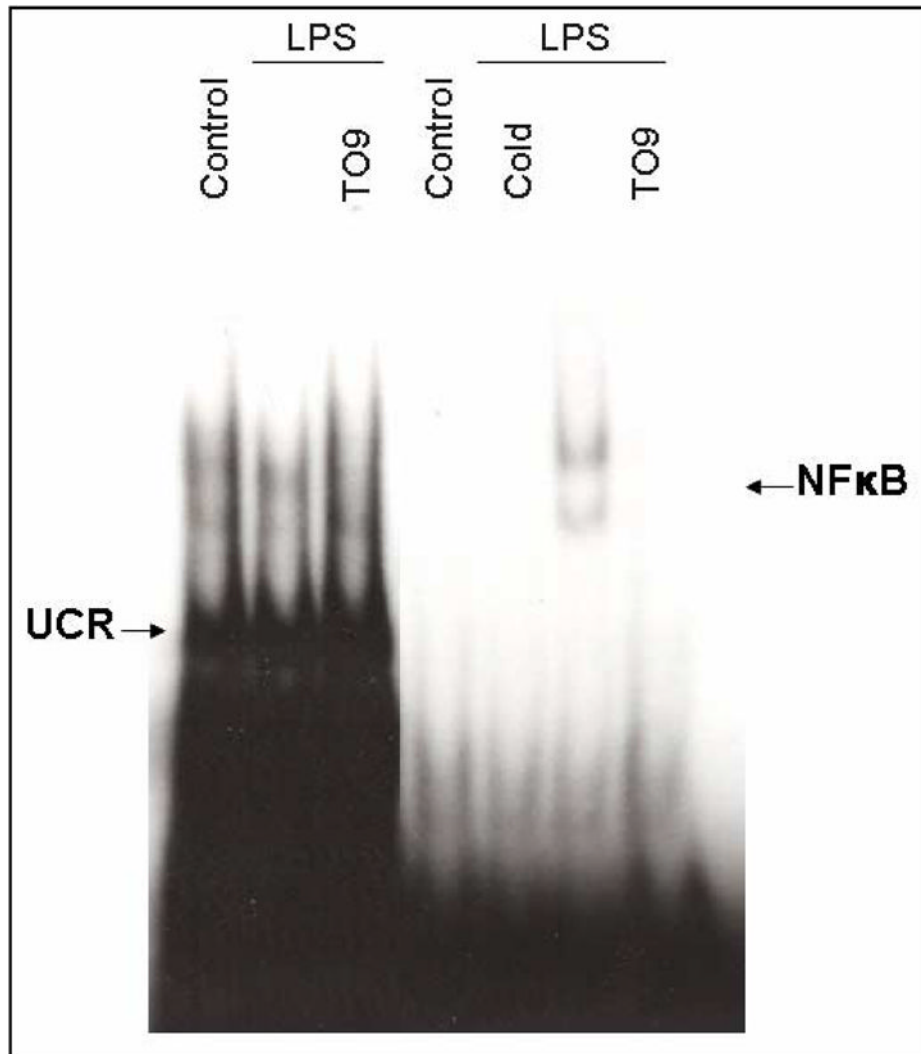


Figure 5. Effects of LXR agonist on NF- κ B binding activity in primary mouse microglia
Cells were pre-treated for 1 h with TO-901317 (60 μ M), then LPS (1 μ g/ml) was added, and 24 h later, nuclear extracts were isolated. The effect of TO-901317 on the DNA binding activity of NF- κ B was determined by EMSA. A 100-fold excess of unlabeled (cold) NF- κ B probe was added to the EMSA binding mixture as a control for non-specific DNA binding. The EMSA figure represents a typical example from five independent experiments. Similar results were observed when cells were stimulated with LPS for 6 h instead of 24 h (data not shown).

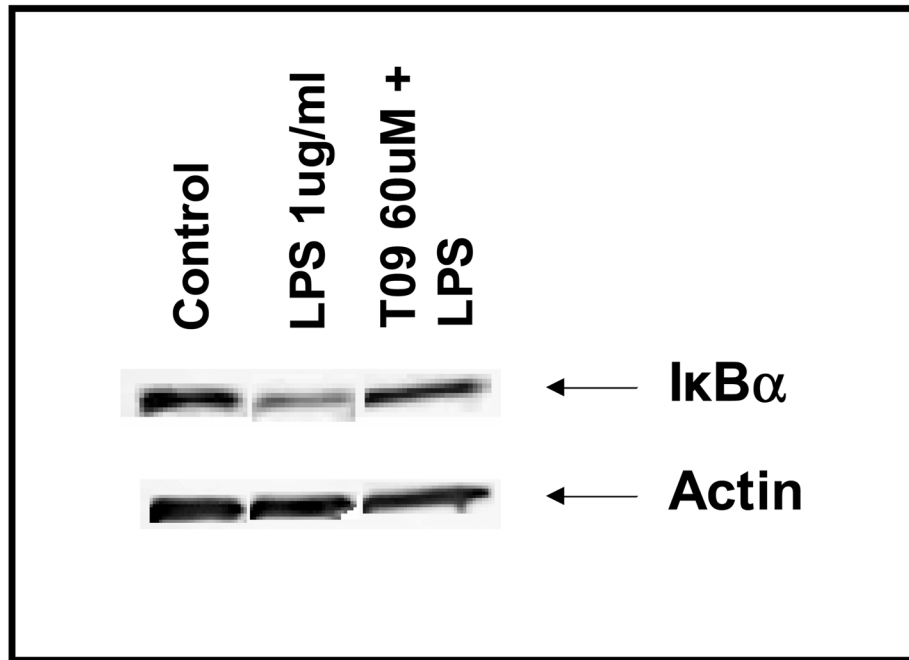


Figure 6. Effects of LXR agonist on IκB-α protein expression

Primary mouse microglia were untreated, stimulated with LPS (1 μg/ml), or pre-treated with TO-901317 (60 μM) for 1 h prior to LPS stimulation. Following 45 min of LPS stimulation, nuclear protein was isolated, and IκB-α protein levels were determined by western blot analysis. Representative results from a single experiment are shown and at least three independent experiments were performed.

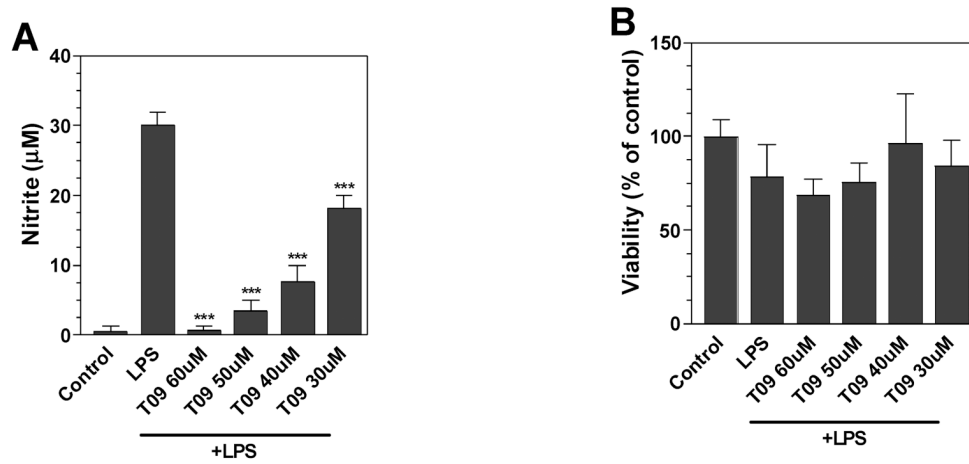


Figure 7. Effects of LXR agonist on LPS induction of NO in mouse primary astrocytes
 Cells were pre-treated 1 h with the indicated concentration of LXR agonist TO-901317. LPS (2µg/ml) was added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Values represent the mean \pm s.d. for a single experiment performed in triplicate. At least three independent experiments were conducted. *** $p < 0.001$ vs. LPS-treated cultures.

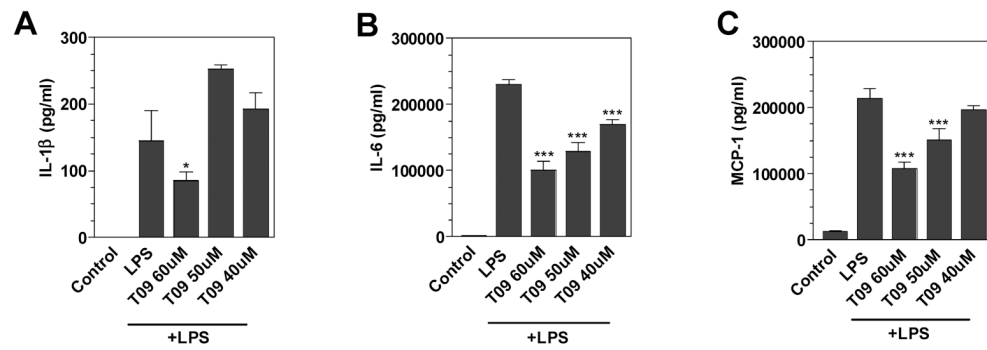


Figure 8. Effects of LXR agonist on cytokine and chemokine production by mouse primary astrocytes

Cells were pre-treated for 1 h with the indicated concentrations of LXR agonist, TO-901317. LPS (2 μ g/ml) was added as indicated, and 24 h later, IL-1 β (A), IL-6 (B), and MCP-1 (C) levels were determined in culture medium by ELISA. Values represent the mean \pm s.d. from a representative experiment performed in triplicate. At least three independent experiments were conducted. * $p < 0.05$ and *** $p < 0.001$ vs. LPS-treated cultures.

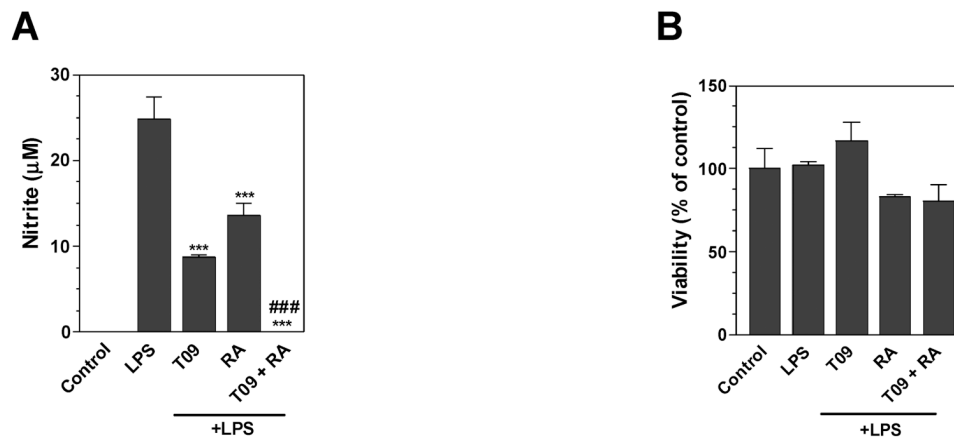


Figure 9. Combined effects of LXR and RXR agonists on nitrite production by mouse primary astrocytes

Cells were pre-treated for 1 h with the indicated concentration of T0-91317, 9-cis RA, or their combination. LPS (2µg/ml) was added as indicated, and 24 h later, the concentration of nitrite in the culture medium was determined (A). Cell viability was determined by MTT assay (B). Values represent the mean \pm s.d. from a representative experiment performed in triplicate. At least three independent experiments were conducted. *** $p < 0.001$ vs. LPS-treated cultures. ### $p < 0.001$ vs. LPS plus T0-91317 and vs. LPS plus 9-cis RA-treated cultures.