의학 석사학위 논문

Anti-Inflammatory Mechanism By Oxysterols In Cultured Rat Brain Astrocytes

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의 학 과

이 창 석

Anti-Inflammatory Mechanism By Oxysterols In Cultured Rat Brain Astrocytes

by

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이창석의 의학 석사학위 논문을 인준함.

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-ABSTRACT-

Anti-Inflammatory Mechanism By Oxysterols In Cultured Rat Brain Astrocytes.

Cholesterols are enriched in the brain and can be oxidized to oxysterols by spontaneous or enzyme-mediated processes. Oxysterols are transport forms of cholesterols across cell membranes and the blood-brain barrier. Here, to elucidate the roles of oxysterols in brain inflammation, I treated lipopolysaccharides(LPS)- or interferon gamma(IFNγ)-stimulated rat brain astrocytes with two oxysterols, 7-ketocholesterol and 22(R)-hydroxycholesterol. In LPS-stimulated astrocytes, both oxysterols suppressed inducible nitric oxide synthase expression and nitric oxide release as well as upstream signaling molecules including interferon-β, phosphorylated signal transducer and activator of transcription 1/3, and interferon regulatory factor-1. And oxysterols also inhibited interleukin-6 and tumor necrosis factor α transcripts, and monocyte chemoattractant protein-1 release.

Oxysterols are known as nuclear receptor liver X receptor agonists, and these inhibitory effects were observed with synthetic agonists of liver X receptor and retinoid X receptor in a similar manner. In addition, *ATP-binding cassette transporter a1* transcription, LXR-induced target gene, was increased by addition of either oxysterol or LXR agonist. Thus, I conclude that these effects are mediated by LXR/RXR heterodimers.

Next, these inhibitory effects of oxysterols also appeared and were

potentiated by RXR agonist in IFNγ-stimulated astrocytes. Furthermore, iNOS expression was inhibited by oxysterols and synthetic LXR ligands in LPS-stimulated primary microglia as well as in LPS- or IFNγ-stimulated primary astrocytes. I conclude that oxysterols suppress inflammatory gene expression through LXR/RXR activation in activated rat brain glial cells and these results provide new insights into the roles of oxysterols in brain inflammation.

Key words: oxysterols, LXR, iNOS, IFN-β, STAT, IRF-1, astrocytes

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ABBREVIATION

ABCA1 : ATP-binding cassette transporter a 1

COX : Cyclooxygenase

HC : Hydroxycholesterol

IFN : Interferon

IL : Interleukin

iNOS : Inducible nitric oxide synthase

IRF : Interferon regulatory factor

JAK : Janus Kinase

KC : Ketocholesterol

LPS : Lipopolysaccharide

LXR : Liver X Receptor

MA : Methoprene acid

MCP : Monocyte Chemoattractant Protein

RXR : Retinoid X Receptor

STAT : Signal Transducer and Activator of Transcription

TNF : Tumor Necrosis Factor

I. INTRODUCTION

A. Cholesterol in brain

Cholesterol is highly enriched in the brain compared to other tissues. In mammals, the brain comprises less than 10% of total body mass yet ~25% of total body cholesterol resides in the brain. The majority of cholesterol present in the Central Nervous System (CNS) is believed to reside in two pools : one is represented by the myelin sheaths (oligodendroglia) and the other by the plasma membranes of astrocytes and neurons (Snipes and Suter, 1997). It has been estimated that 70~80% of the cholesterol in the brain of adult animals is present in myelin. Consequently, since ~90% of the cells of the brain are glial cells, neurons contribute only a small fraction of the total cholesterol in the brain (Dietschy and Turley, 2004). In a mammalian cell, 50 to 90% of the cholesterol resides in plasma membrane (Lange et al., 1989) where cholesterol is concentrated in detergent-resistant microdomains (lipid rafts) that are also enriched in sphingomyelin and glycosphingolipids.

According to in vitro studies with cultured cells, astrocytes synthesize at least 2- to 3-fold more cholesterol than neurons and fibroblasts. Evidence has been presented that oligodendrocytes, the cells responsible for myelinization, have an even higher capacity for cholesterol synthesis than astrocytes (Saito et al., 1987). Neurons, which specialize in the generation of electrical activity, may reduce or even abandon cholesterol synthesis. Experimental evidence for a cholesterol shuttle from astrocytes

to neurons in an in vitro system has been presented (Mauch et al., 2001), and cultured neurons from mammalian CNS appear to require glia-derived cholesterol to form numerous and efficient synapses (Pfrieger, 2003).

The major source of cholesterol in most mammalian cells is de novo synthesis (Dietschy et al., 1993;Dietschy and Turley, 2002;Spady and Dietschy, 1983) although cholesterol can also be acquired from exogenously supplied plasma lipoproteins. Cholesterol metabolism in the CNS appears to be distinct from that in other tissues, most likely because the CNS and plasma compartments are separated by the blood-brain barrier(BBB). Several studies have demonstrated that essentially all of the cholesterol required for development of the brain and spinal cord is derived from endogenous synthesis within the CNS (Jurevics and Morell, 1995;Turley et al., 1998). There is no convincing evidence that cholesterol derived from HDLs and LDLs is transferred from the plasma, across the blood-brain barrier, and into CNS during either fetal or postnatal development (Turley et al., 1996). These and other observations imply that plasma lipoproteins do not across the blood-brain barrier and do not deliver significant amounts of cholesterol to the CNS. In addition to limiting the entry of plasma lipoproteins into the brain, the blood-brain barrier also restricts the egress of cholesterol from the CNS. In rat brain, cholesterol has a long half-life of 4 to 6 months. However, when the rate of cholesterol synthesis in the CNS exceeds that required for cholesterol homeostasis there is a net export of cholesterol from the CNS to the plasma. The most important mechanism by which cholesterol is excreted from the brain is by conversion to 24(S)-hydroxycholesterol via cholesterol 24-

hydroxylase (Bjorkhem et al., 1997;Lund et al., 2003).

Furthermore, changes in net cholesterol balance across the whole animal may, indirectly, alter sterol balance across the CNS and also contribute to the development of neurodegeneration. Indeed, it has recently been reported that treating humans with agents that block cholesterol synthesis in every organ, including the brain, markedly reduces the incidence of Alzheimer's disease (Wolozin et al., 2000). These findings illustrate how important it is to have a better understanding of the mechanism that control cholesterol balance, both across blood-brain barrier between the plasma and cerebrospinal fluid(CSF) and within the CNS between the various neurons and glial cells.

B. Oxysterol metabolism

Oxysterols are mono-oxygenated derivatives of cholesterol (or precursors to cholesterol) that are important as intermediates and end products in cholesterol excretion pathways. The rapid degradation and excretion of oxysterols is facilitated by their physical properties, allowing them to pass lipophilic membranes and to be redistributed in the body at a much faster rate than cholesterol itself (Lange et al., 1995). Oxysterols are typically found in conjunction with cholesterol in almost all biological locations, though at significantly lower concentrations (10-100 thousand fold less). As they may be formed by spontaneous or enzyme mediated processes they tend to be unevenly distributed across different tissues. The spontaneous oxidation of cholesterol is a well-recognized phenomenon generally termed auto-

oxidation (Smith, 1987). This process is a non-enzymatic reaction that can be initiated by diverse compounds including various radical species, lipid peroxides and divalent metal cation (such as Cu2+). In addition to these auto-oxidative processes some oxysterols are formed by the action of specific enzymes, most of which belong to the cytochrome P-450 superfamily (Russell, 2000). The products of such enzymatic reactions represent the majority of oxysterols present in circulation.

The introduction of an oxygen function in the cholesterol molecule makes it much more polar, drastically reduces its half-life and directs it to excretion or to oxidation to water-soluble bile acids. In general, oxysterols are more toxic than cholesterol itself, and very efficient mechanism have evolved for transport, detoxification and elimination of these compounds. The quantitatively most important catabolic pathway is the conversion of the oxysterol into a water-soluble bile acid that can be rapidly eliminated in bile. Whereas the primary formation of the oxysterol may occur in many tissues and organs, the final conversion of the oxysterol into a bile acid is always restricted to the liver. It is thus evident that oxysterols are obligatory intermediates in bile acid synthesis. In view of their rapid transport over cell membranes and the blood-brain barrier, they may also be regarded as transport forms of cholesterol.

In addition to these well established roles of oxysterols, they have been ascribed a number of more uncertain roles in connection with regulation of cholesterol turn over, atherosclerosis, apoptosis, necrosis, inflammation, immunosuppression and development of gallstones (Brown and Jessup, 1999;Guardiola et al.,

1996;Schroepfer, Jr., 2000;Wang and Afdhal, 2001). Among all these suggested roles, regulation of cholesterol homeostasis has the best experimental support.

7-Ketocholesterol (7-KC) is one of the primary oxysterols produced by radicals of oxidized LDL (Aviram, 1993;Dyer et al., 1995), and 22(R)-hydroxycholesterol (22(R)-HC) is an intermediate in the CYP270 enzymatic pathway during pregnenolone formation from cholesterol. Both of these oxysterols are known to activate LXRs.

C. LXRs in cholesterol metabolism and inflammation

In 1996, Janowski et al. (Janowski et al., 1996) reported that certain oxysterols activate transcription via the nuclear receptor LXRα. 22(R)-HC was reported to show the highest potency in activation of LXRα. A prerequisite for the transcriptional activation by LXR is that the receptor forms a heterodimer with retinoid X receptor (RXR) (Mangelsdorf and Evans, 1995). The LXRs (LXRα and LXRβ) play a broad role in the control of lipid homeostasis (Peet et al., 1998;Schultz et al., 2000). The LXRs are able to regulate the expression of a number of genes involved in cholesterol metabolism (Chawla et al., 2001;Repa and Mangelsdorf, 2000b;Russell, 1999). Many LXR target genes appear to be involved in the pathway of reverse cholesterol transport, in which excess cholesterol is transferred in HDL particles from peripheral cells to the liver for excretion in bile. Consistent with these targets, synthetic LXR ligands promote reverse cholesterol transport and excretion in mice (Repa and Mangelsdorf, 2000a). In addition to its importance in lipid and glucose

metabolism, LXR activation has recently been demonstrated to regulate immune processes and to inhibit inflammatory gene expression in macrophage (Castrillo et al., 2003;Joseph et al., 2003). Synthetic LXR agonists have been demonstrated to prevent atherosclerosis in murine models and to inhibit inflammation (Joseph et al., 2003;Joseph et al., 2002). These observations suggest that LXR agonists may exert their antiathrogenic effects not only by promoting cholesterol efflux, but also by acting to limit the production of inflammatory mediators in the artery wall. Moreover, they suggest that LXR-dependent repression of inflammatory gene expression might be useful to control excess inflammation in a variety of human diseases.

Both LXRs are also expressed in the brain, but their roles in tissue remain to be clarified. Wang L et al. (Wang et al., 2002) examined the brains of mice in which the genes of both LXRα and LXRβ have been disrupted and found several severe abnormalities. One of the most striking features is that the lateral ventricles are closed and lined with lipid-laden cells. In addition, there are enlarged brain blood vessels, especially in the pars reticularus of the substantia nigra and in the globus pallidus. Other features of the brains are excessive lipid deposits, proliferation of astrocytes, loss of neurons, and disorganized myelin sheaths. Electron micrographs revealed that, as mice aged, lipid vacuoles accumulated in astrocytes surrounding blood vessels. Comparison of mRNA profiles in LXR knockout mice and wild-type littermates showed that expression of several LXR target genes involved in cholesterol efflux from astrocytes was reduced. Although both LXR subtypes are expressed in the brain, LXRβ, in particular, is broadly expressed in the developing

and adult rodent brain (Kainu et al., 1996). Little is known about the functions of the LXRs in the brain. In rat pheochromocytoma cells, the LXR agonist 22(R)-HC induces neuronal differentiation as measured by neurite outgrowth (Schmidt et al., 1999). In primary astrocyte cultures, but not in primary neuronal cultures, LXR agonists enhance cholesterol efflux and regulate several established LXR target genes. Induction of these target genes was also confirmed by treating animals with LXR agonists (Whitney et al., 2002). These findings show that LXRs have an important function in lipid homeostasis in the brain, and that loss of these receptors results in neurodegenerative diseases. Further characterization of the role of LXRs in the brain could lead to new insights into the etiology and treatment of some neurodegenerative disorders.

D. Aims of study

The brain is the most cholesterol rich organ and oxysterols are cholesterol oxidation products. In addition, oxysterols are known as liver X receptor (LXR) agonists and LXR has recently been demonstrated to act as a negative modulator in inflammatory diseases. Thus, to establish oxysterols profile of the brain in this study, I investigated effects of two oxysterols and synthetic LXR ligands and signaling pathways in cultured rat brain astrocytes.

II. MATERIALS AND METHODS

A. Reagents

Salmonella typhimurium LPS and 7-ketocholesterol (7-KC) were purchased from Sigma (St. Louis, MO). 22(R)-hydroxycholesterol (22(R)-HC) and TO901317 were purchased from Cayman (Ann Arbor, MI). Methoprene acid (MA) was purchased from BIOMOL (Plymouth Meeting, PA). GW3965 was kindly provided by GlaxoSmithKline (Stevenage, UK). Rat IFN-γ was purchased from Calbiochem. Antibodies against inducible NO synthase (iNOS) was purchased from Upstate Biotechnology (Lake Placid, NY), and Abs against cyclooxygenase-2 (COX2), actin, and IRF-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Tyr-701-phosphorylated STAT1, and Tyr-705-phosphorylated STAT3 were purchased from Cell Signaling Technology (Beverly, MA).

B. Cell Culture

Primary glial cells were cultured from 1- to 3-day-old Sprague-Dawley rats, as we have described previously (Pyo et al., 1999). Briefly, the cortices were triturated into single cells in MEM containing 10% fetal bovine serum (Hyclone, Logna, UT) and plated into 75 ㎠ T-flasks (0.5 hemisphere/flask) for 2 weeks. Then, microglia were detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes and cell clumps. Primary astrocytes were prepared using

trypsin after microglia were removed. Detached astrocytes were seeded in 6-well plates or 60㎜ dishes.

C. Western Blot Analysis

Cells were washed twice with cold phosphate-buffered saline and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.5 mM Na_3VO_4, and 2 mM EDTA). The lysates were centrifuged for 10 min at 12,000 × *g* at 4 ℃, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies and peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) and then visualized using an enhanced chemiluminescence system (Sigma-Aldrich).

D. Determination of NO Release

Media nitrite concentration was measured as an indication of NO release. Following the indicated cell incubations, 50 μl of culture medium was removed and mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine, 1% sulfanilamide, 2.5% H_3PO_4), and absorbance of the mixture at 540 nm was measured.

E. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using easy-BLUE from iNtRON (Seoul, Korea), and cDNA was prepared using reverse transcriptase that originated from avian meloblastosis virus (Takara, Shiga, Japan), according to the manufacturer's instructions. PCR was performed with 30 cycles of sequential reactions as follows: 94 °C for 60 s, 55 °C ~ 62 °C for 30 s, and 72 °C for 90 s. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). The sequences of PCR primers were shown on table 1. PCR products were separated by electrophoresis in a 1.5% agarose gel and detected under UV light.

Table 1. Primer Sequences for RT-PCR

Primers	Forward	Reverse
ABCA1	5'-GGGCGGGGGCCTGAAGATC-3'	5'-AGAGCCATTTGGGGACTGAACATC-3'
LXRα	5'-GCGAGGGCTGCAAGGGATTCT-3'	5'-ATGGGCCAAGGCGTGACGCG-3'
LXRβ	5'-GCCTTGCTTATCGCCATCAAC-3'	5'-AATGCAAAGACCTGCTCCGAG-3'
RXRα	5'-AGAGGACAGTACGCAAAGAC-3'	5'-GTGAAGAGCTGCTTGTCTG-3'
IFNβ	5'-ATGGCCAACAGGTGGACCCT-3'	5'-TCAGTTCTGGAAGTTTCTAT-3'
IL-6	5'-ACAAGAGACTTCCAGCCAGTTGC-3'	5'-TTGCCGAGTAGACCTCATAGTGACC-3'
TNFα	5'-GTAGCCCACGTCGTAGCAAA-3'	5'-CCCTTCTCCAGCTGGGAGAC-3'
GAPDH	5'-TCCCTCAAGATTGTCAGCAA-3'	5'-AGATCCACAACGGATACATT-3'

F. Enzyme-linked immunosorbent assay (ELISA)

MCP-1 levels in cell culture media were determined by ELISA as described by the manufacturer (OptEIA Sets, Pharmingen, San Diego, CA). MCP-1 concentrations in the media were determined by spectrophotometer and calibrated

from standards containing known concentrations of the cytokines.

G. Data analysis

Data were expressed as mean ± S.E.M. Analysis of variance followed by Dunnett's multiple comparison tests were used for statistical comparisons.

III. RESULTS

A. Oxysterols suppress iNOS, COX2 expression and NO production in LPS-stimulated rat brain astrocytes.

To test the effect of two oxysterols, 7-ketocholesterol (7-KC) and 22(R)-hydroxycholesterol (22(R)-HC), astrocytes from rat brain were treated for 18 h with oxysterols. Then I observed the expression of inflammatory key enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2). Oxysterols itself had no effect on iNOS expression and COX2 expression at indicated doses. (Fig. 1A). However, at relatively high doses of oxysterols, iNOS and COX2 expression were increased and finally cells appeared death (data not shown).

A lipopolysaccharide (LPS) is a major suprastructure of Gram-negative bacteria that induces inflammatory responses. Thus, I treated primary astrocytes with 10 ng/ml LPS for 18 h after cells were pre-incubated with oxysterols for 1 h. In these results, both oxysterols were observed dose-dependently to suppress LPS-induced iNOS and COX2 expression (Fig. 1B), and NO production (Fig. 1C). Thus, I find that these oxysterols inhibit iNOS and COX2 expression at indicated doses without toxic effects in LPS-stimulated primary astrocytes though they are known as toxic agents.

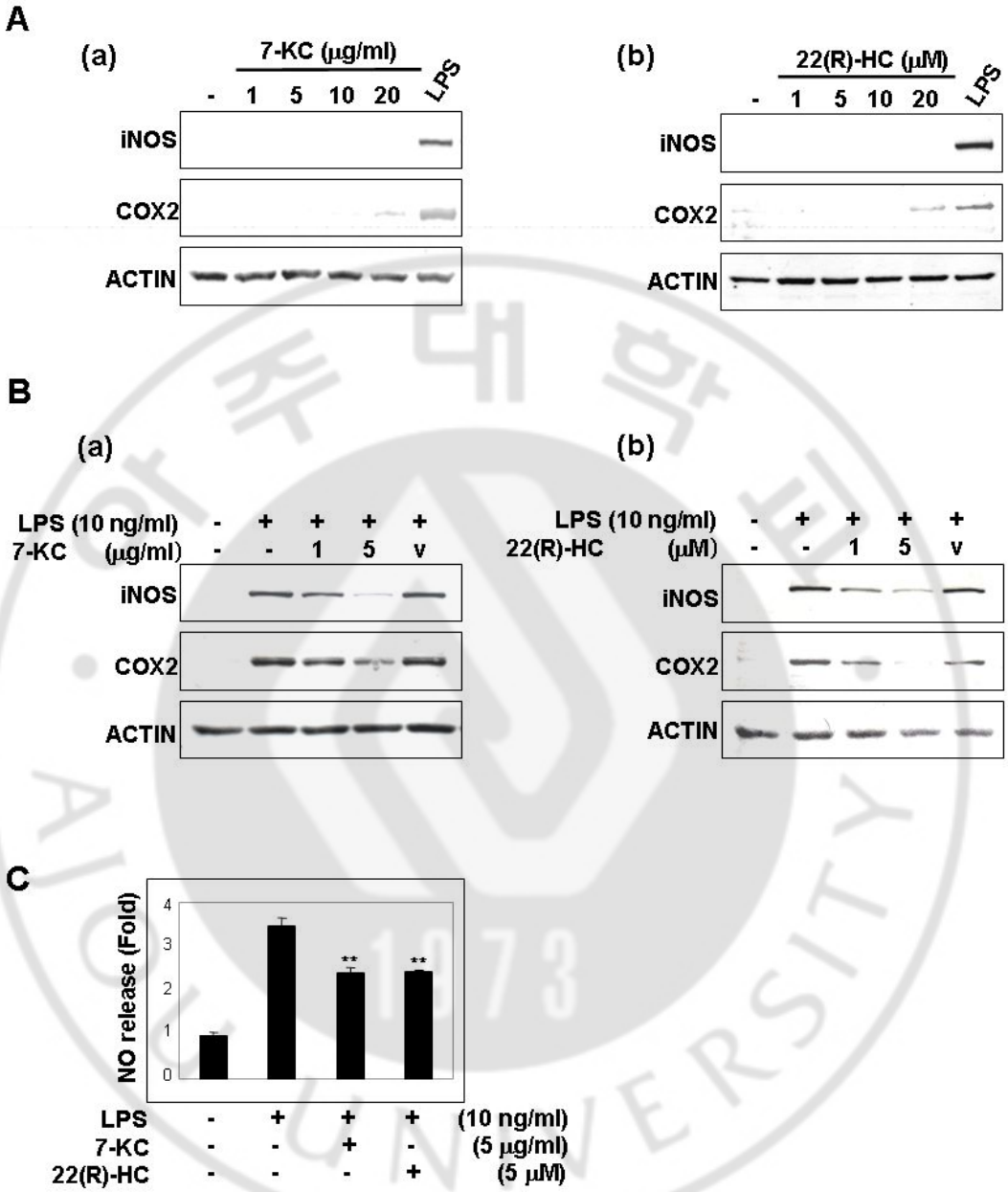

A
(a)
7-KC (μg/ml)
- 1 5 10 20 LPS
iNOS
COX2
ACTIN
(b)
22(R)-HC (μM)
- 1 5 10 20 LPS
iNOS
COX2
ACTIN
B
(a)
LPS (10 ng/ml) - + + + +
7-KC (μg/ml) - - 1 5 v
iNOS
COX2
ACTIN
(b)
LPS (10 ng/ml) - + + + +
22(R)-HC (μM) - - 1 5 v
iNOS
COX2
ACTIN
C
NO release (Fold)
0 1 2 3 4
**
**
LPS - + + + (10 ng/ml)
7-KC - - + - (5 μg/ml)
22(R)-HC - - - + (5 μM)

Fig. 1. Inhibition of iNOS, COX2 expression and NO release by oxysterols in LPS-stimulated astrocytes. (A) Primary astrocytes were treated for 18 h with each oxysterol at indicated doses. LPS (10 ng/ml) was treated for positive control of iNOS and COX2 expression. (B) Primary astrocytes were incubated for 18 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated doses of 7-KC or 22(R)-HC. Then, protein extracts were prepared and subjected to Western blotting using antibodies against iNOS, COX2 and ACTIN (loading control). (C) Primary astrocytes were incubated for 24 h with 10 ng/ml LPS following pretreatment for 1 h with 7-KC or 22(R)-HC. Then, the amount of NO was determined by measuring nitrite concentrations in the media. Data are representative of three independent experiments and presented as mean ± SEM (**P<0.01, comparison with LPS-treated cells). v, vehicle for agent solution.

B. Suppression of iNOS expression by oxysterols occurs in a LXR/RXR heterodimer-dependent manner.

To elucidate the inhibition of iNOS expression and NO release by oxysterols in LPS-stimulated astrocytes, I tested whether oxysterols function via activation of liver X receptors (LXRs), since these compounds are known to be endogenous agonists of LXRs. First, I examined whether synthetic LXR ligands suppress iNOS, COX2 expression and NO production. I observed that synthetic LXR agonists, TO901317 and GW3965, dose-dependently suppressed iNOS, COX2 expression (Fig. 2A) and NO release (Fig. 2B) in a similar manner in LPS-stimulated astrocytes.

To further clarify that these inhibitory effects occur via LXR activation, I also examined the expression of LXRs and RXR. LXRα, LXRβ, and RXRα transcripts were detected in control and stimulated astrocytes, but transcript levels did not appear to change in the presence of oxysterols or LXR agonists (Fig. 3A). In contrast, *ATP-binding cassette transporter a1* (*Abca1*), a representative LXR-induced target gene, transcription was efficiently induced by addition of either oxysterol or LXR agonist (Fig. 3A), which reveals that LXRs function in astrocytes. Next, as LXRs form heterodimers with RXRs, I used methoprene acid (MA), an RXR agonist, additionally. Then, I found that addition of 10 μM MA to LPS-stimulated astrocytes which are incubated with oxysterols or synthetic LXR agonists potentiated their inhibitory effects (Fig. 3B), thereby providing evidence that these inhibitory effects occur via activation of LXR/RXR.

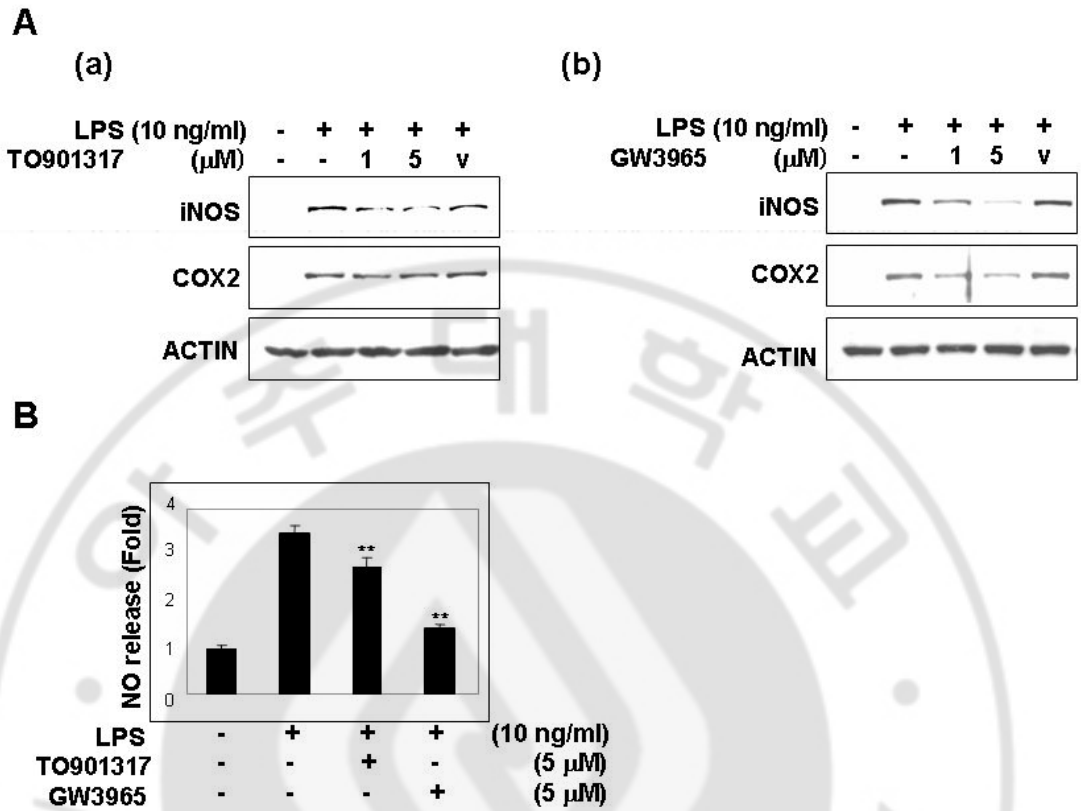


Fig. 2. Inhibition of iNOS, COX2 expression and NO release by synthetic LXR ligands in LPS-stimulated astrocytes. Primary astrocytes were incubated for 18 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated doses of the LXR agonists TO901317 or GW3965. (A) Protein extracts were prepared and subjected to Western blotting as described in Fig. 1. (B) NO release was determined as described in Fig. 1C. Data are representative of three independent experiments and presented as mean ± SEM (**P<0.01, comparison with LPS-treated cells). v, vehicle for agent solution.

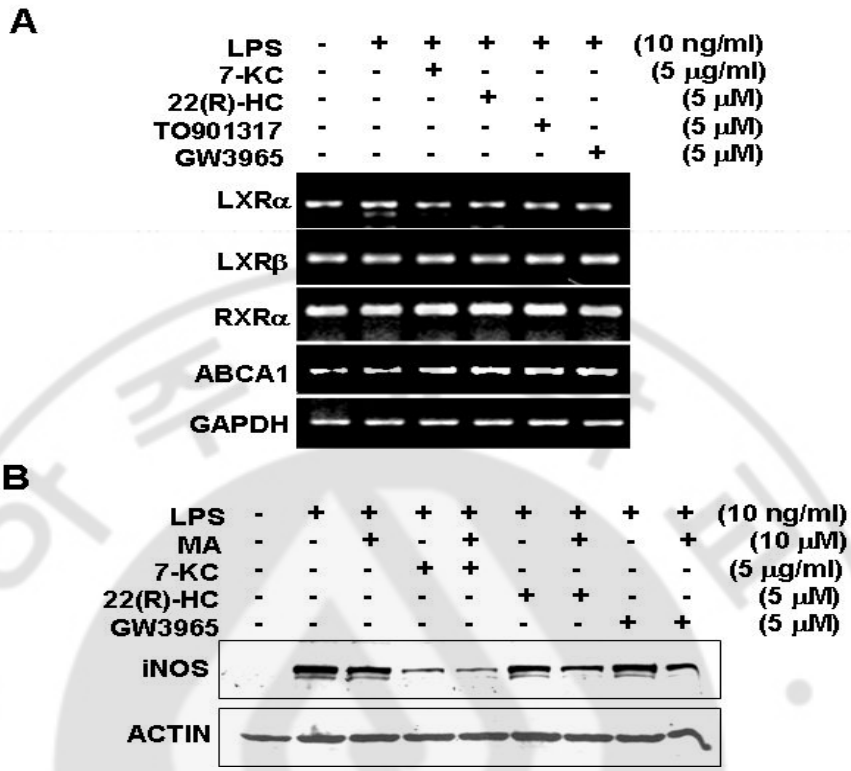


Fig. 3. Inhibition of iNOS expression by oxysterols through a LXR/RXR heterodimer activation. (A) Cells were incubated for 8 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated drugs. Total RNA was then isolated and an RT-PCR–based analysis was performed using the primers described in Table 1. Transcript levels were normalized against those of GAPDH. (B) Cells were incubated for 18 h with 10 ng/ml LPS following pretreatment for 1 h with the respective indicated oxysterols with or without MA (10 μM). Western blotting was then performed as described in Fig. 1.

C. Oxysterols and synthetic LXR agonists suppress the expression of IFN-β transcripts, phosphorylated STATs, and IRF-1 expression in LPS-stimulated primary astrocytes.

Recent reports show that iNOS can be induced via inflammatory signaling events by LPS. LPS stimulates STATs signaling indirectly through IFN-β induction. And activation of STATs affects an expression of IRF-1, which is important to iNOS expression.

To detect these inflammatory signaling events, I examined IFN-β expression after addition of LPS in oxysterols-incubated astrocytes. Both oxysterols and synthetic LXR agonists suppressed IFN-β mRNA expression (Fig. 4A). Then, for detection of IFN-β downstream signaling, I observed the effects of oxysterols and LXR agonists on phosphorylated STAT1/3 and IRF-1 expression. Similarly, both types of agents reduced STAT1/3 phosphorylation as well as IRF-1 expression (Fig. 4B). These results revealed that oxysterols suppress iNOS expression through inhibition of up-stream signaling IFN-β/phosphorylated STATs/IRF-1 expression.

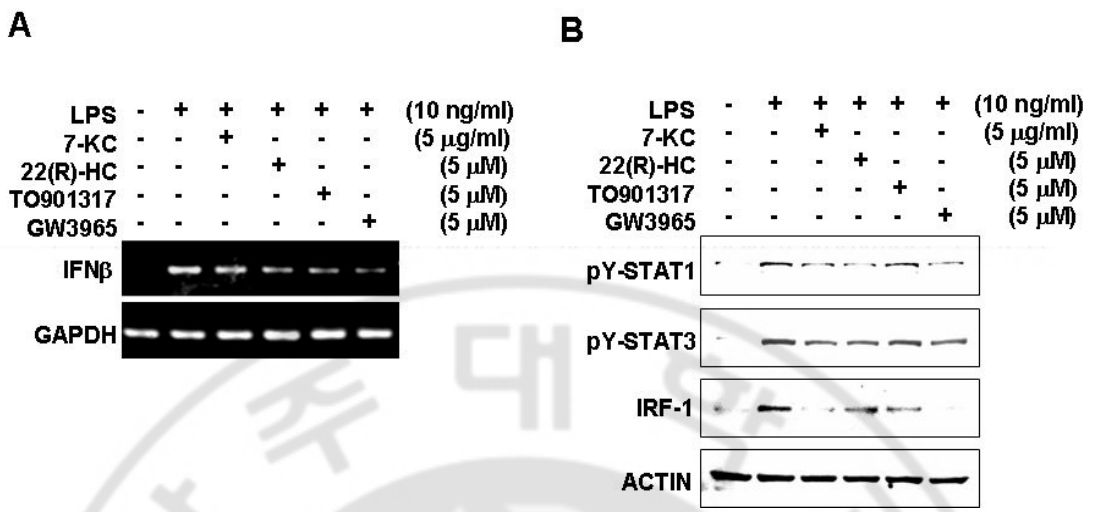


Fig. 4. Inhibition of up-stream signaling molecules of iNOS expression by oxysterols and synthetic LXR ligands in LPS-stimulated astrocytes. (A) Cells were incubated for 1.5 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated drugs. Total RNA was isolated and used in an RT-PCR assays to determine IFN-β transcript levels. (B) Primary astrocytes were incubated for 2 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated doses of oxysterols and synthetic LXR agonists. Protein extracts were prepared and subjected to Western blot analysis using antibodies against Tyr-701–phosphorylated STAT1, Tyr-705-phosphorylated STAT3, or IRF-1.

D. Oxysterols and synthetic LXR agonists suppress not only iNOS expression but the expression of IL-6 and TNFα transcripts, and MCP-1 release in LPS-stimulated primary astrocytes.

As LPS induce a number of pro-inflammatory molecules, I examined expression of other inflammatory mediators such as IL-6, TNFα and MCP-1. Two oxysterols and TO901317 suppressed IL-6 and TNFα transcripts in LPS-stimulated astrocytes treated with oxysterols (Fig. 5A). Moreover, they inhibited MCP-1 release in the same condition (Fig. 5B). Thus, I revealed that two oxysterols and synthetic LXR ligands inhibited the expression of pro-inflammatory cytokine (IL-6) and chemokines (TNFα and MCP-1) as well as iNOS, COX2 expression and NO production in LPS-stimulated rat brain astrocytes.

Together, these findings demonstrate that oxysterols and LXR agonists inhibit LPS-induced IFN-β expression, STAT1/3 activation, and IRF-1 expression, in addition to suppressing iNOS expression and NO production through LXR/RXR activation in rat primary astrocytes. Furthermore, they inhibit pro-inflammatory mediators such as IL-6, TNFα and MCP-1 expression in LPS-stimulated astrocytes (Fig. 6).

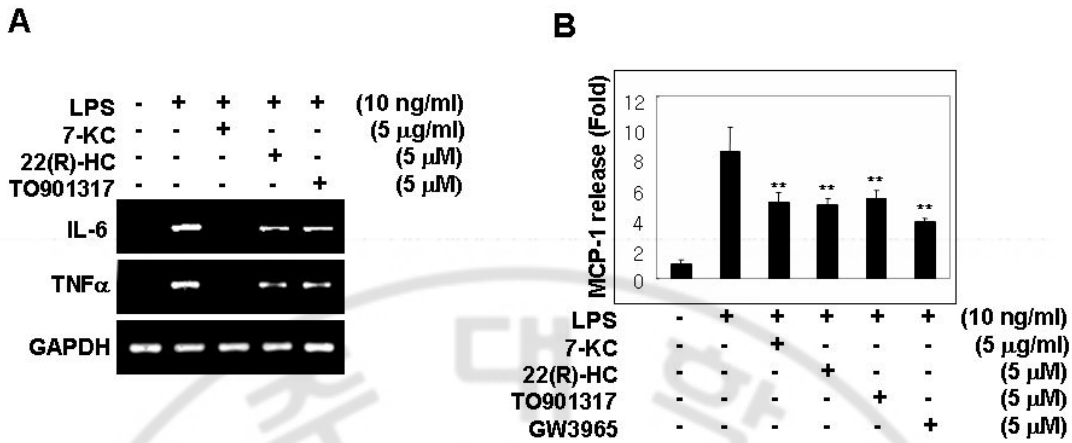


Fig. 5. Inhibition of other inflammatory mediators by oxysterols and synthetic LXR ligands in LPS-stimulated astrocytes. (A) Cells were incubated for 3 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated drugs. Total RNA was isolated and an RT-PCR–based analysis was performed using the primers described in Table 1. (B) Release of MCP-1 was determined by ELISA. Cells were incubated for 12 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated drugs. Data represent the mean ± S.E.M. of three independent experiments. (**P<0.01, comparison with LPS-treated cells).

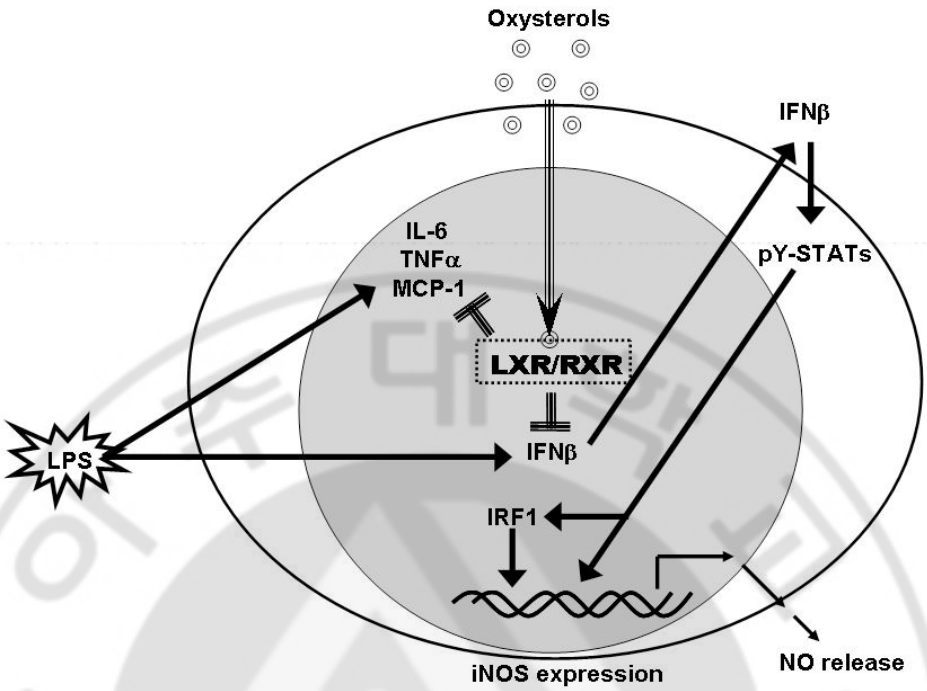


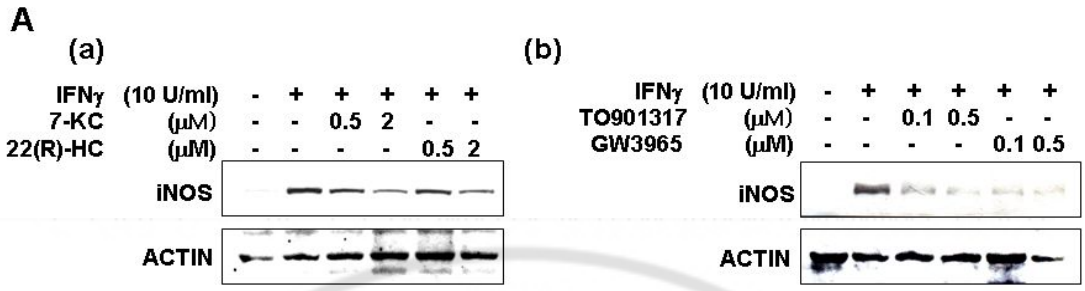
Fig. 6. Model for suppression of the expression of pro-inflammatory molecules by oxysterol-activated LXR in LPS-stimulated rat brain astrocytes.

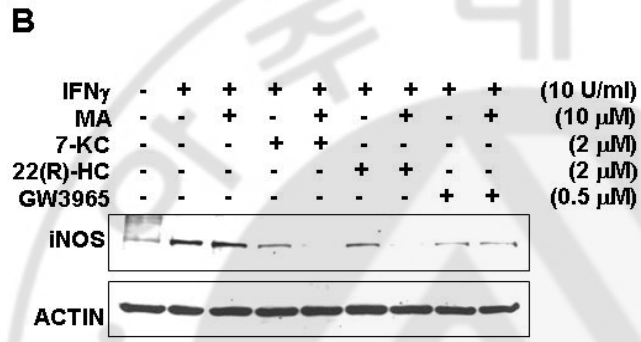
E. Oxysterols and synthetic LXR agonists suppress also iNOS expression in IFNγ-stimulated primary astrocytes and LPS-stimulated primary microglia.

To find whether these inhibitory effects of oxysterols appear in specific to LPS-stimulated astrocytes, I treated oxysterols and synthetic LXR ligands to IFNγ-stimulated astrocytes (Fig. 7A) and LPS-stimulated microglia (Fig. 8). Interferon gamma (IFNγ) is a natural chemical (cytokine) produced by the body to help fight infections. Its major functions are to activate macrophages and to increase the expression of class II MHC on APC. And microglia are the brain's normally resting tissue macrophages. They show high structural as well as functional plasticity and become activated in traumatic, inflammatory, degenerative and neoplastic diseases.

In these tests, oxysterols and synthetic LXR ligands inhibited iNOS expression in all cases. And Methoprene acid, RXR agonist, potentiated their inhibitory effects (Fig. 7B). Furthermore, I detected IRF-1 expression by oxysterols in IFNγ-stimulated astrocytes because IFNγ-induced gene expression for iNOS had been shown to be regulated by IRF-1. In results, IRF-1 was decreased by oxysterols and synthetic LXR ligands.

Finally, I concluded that oxysterols may have an anti-inflammatory effect via LXR/RXR activation in LPS- or IFNγ-activated rat brain glial cells, astrocytes and microglia.


A
(a)
IFNγ (10 U/ml) - + + + + +
7-KC (μM) - - 0.5 2 - -
22(R)-HC (μM) - - - - 0.5 2
iNOS
ACTIN
(b)
IFNγ (10 U/ml) - + + + + +
TO901317 (μM) - - 0.1 0.5 - -
GW3965 (μM) - - - - 0.1 0.5
iNOS
ACTIN


B
IFNγ - + + + + + + + + (10 U/ml)
MA - - + - + - + - + (10 μM)
7-KC - - - + + - - - - (2 μM)
22(R)-HC - - - - - + + - - (2 μM)
GW3965 - - - - - - - + + (0.5 μM)
iNOS
ACTIN

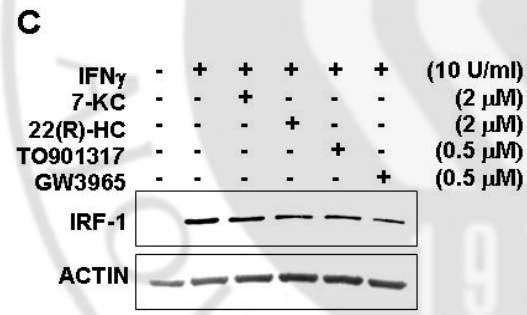

C
IFNγ - + + + + + (10 U/ml)
7-KC - - + - - - (2 μM)
22(R)-HC - - - + - - (2 μM)
TO901317 - - - - + - (0.5 μM)
GW3965 - - - - - + (0.5 μM)
IRF-1
ACTIN

Fig. 7. Inhibition of iNOS expression by oxysterols and synthetic LXR ligands in IFNγ-stimulated astrocytes. (A) Primary astrocytes were incubated for 18 h with 10 U/ml IFNγ following pretreatment for 1 h with the indicated doses of oxysterols and synthetic LXR ligands. Protein extracts were prepared and subjected to Western blotting as described in Fig. 1. (B) Cells were incubated for 18 h with 10 U/ml IFNγ following pretreatment for 1 h with the respective indicated oxysterols with or without MA (10 μM). Western blotting was then performed as described in Fig. 1. (C) Cells were incubated for 12 h with 10 U/ml IFNγ following pretreatment for 1 h with the respective indicated oxysterols and synthetic LXR ligands. Protein extracts were prepared and subjected to Western blot analysis using antibodies against IRF-1.

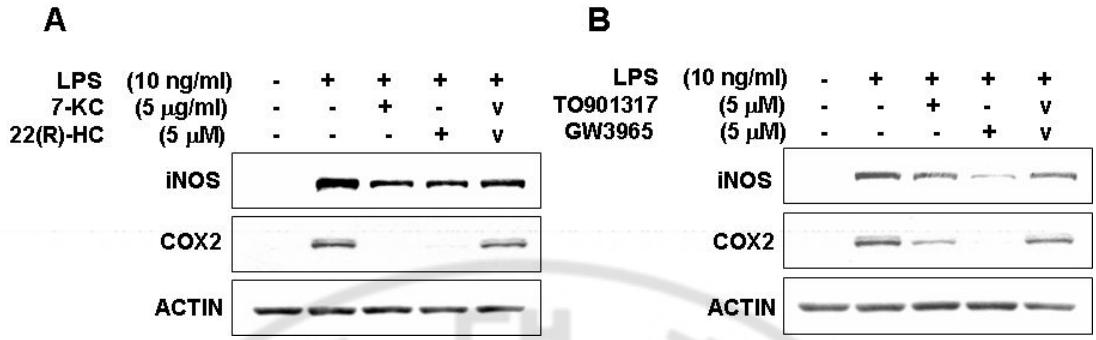


Fig. 8. Inhibition of iNOS and COX2 expression by oxysterols and synthetic LXR ligands in LPS-stimulated primary microglia. Primary microglia were incubated for 18 h with 10 ng/ml LPS following pretreatment for 1 h with the indicated doses of oxysterols (A) and synthetic LXR ligands (B). Protein extracts were prepared and subjected to Western blotting as described in Fig. 1.

IV. DISCUSSION

Recent reports show that activators of the nuclear receptors PPARα and PPARγ suppress janus kinase (JAK)-STAT signaling in activated brain glial cells (Lee et al., 2005a;Park et al., 2003). In the present study, I showed that oxysterols can suppress JAK-STAT-mediated iNOS expression in LPS-stimulated astrocytes and that this is mediated through the activation of LXRs.

Although oxysterols are present in tissues at very low concentrations, they are potent regulators of diverse cellular functions in addition to lipid metabolism. 7-ketocholesterol (7-KC) is one of the primary oxysterols produced by radicals of oxidized LDL (Aviram, 1993;Dyer et al., 1995), and 22(R)-hydroxycholesterol (22(R)-HC) protects neuronal cells from ß-amyloid-induced cytotoxicity by binding to ß-amyloid peptide (Yao ZX et al., 2002). Both of these oxysterols are known to activate LXRs (Janowski et al., 1999). Consistent with previous reports that LXRs act as negative regulators of the expression of inflammatory genes such as iNOS, IL-6, and MMP-9 in macrophages (Castrillo et al., 2003;Joseph et al., 2003), I found herein that LXR activation via two synthetic agonists, TO901317 and GW3965, also suppresses the expression of iNOS and release of nitric oxides in LPS-stimulated rat brain astrocytes.

There are reports (Jacobs and Ignarro, 2001;Lee et al., 2005b;Lee et al., 2005a) that LPS-induced iNOS expression is mediated by the induction of IFN-β.

Here, oxysterols suppressed IFN-β transcript levels, phosphorylation of STAT1/3, and IRF-1 protein expression in LPS-stimulated primary astrocytes. These inhibitory effects were also observed following addition of TO901317 and GW3965. Furthermore, the addition of the RXR agonist MA potentiated the inhibitory effect of oxysterols and LXR agonists in LPS-stimulated astrocytes, providing further evidence that the inhibitory effects of oxysterols are most likely mediated through activation of LXR/RXR heterodimers.

LXR activation can be demonstrated by an increase in the mRNA levels of several target genes, including *Abca1* (Repa and Mangelsdorf, 2002). Despite reports that LXRα gene expression itself is up-regulated by its agonist binding via auto-regulatory positive feedback in THP-1 cells, it is not induced by LXR agonists in most cell types (Whitney et al., 2001). My data revealed that oxysterol- and LXR agonist-induced LXR activation is accompanied by an increase in *Abca*1 mRNA levels, but not by an increase in LXRα/β expression. These results suggest that oxysterols suppress LPS-induced iNOS expression and NO release via LXR/RXR activation without an increase of LXR/RXR expression.

In addition to inhibition of iNOS expression by oxysterols, oxysterols and LXR agonists suppress other inflammatory molecules, including COX2, IL-6, TNFα, and MCP-1 expression in LPS-stimulated astrocytes. Also, oxysterols inhibit iNOS expression in IFNγ-stimulated astrocytes and LPS-stimulated microglia. These results raise the possibility that oxysterols may be new target for regulation of activated glial cells in brain inflammation.

V. CONCLUSION

The present data demonstrate that two oxysterols, 7-KC and 22(R)-HC, suppress LPS-induced iNOS expression and NO release via activation of LXR/RXR. With respect to signaling upstream of iNOS expression, I observed that IFNβ transcripts, STAT1/3 phosphorylation, and IRF-1 expression are inhibited by both of these oxysterols via LXR activation in rat brain astrocytes. Additionally, oxysterols reduce IL-6, TNFα and MCP-1 expression in LPS-stimulated astrocytes. Also, these inhibitory effects were detected in IFNγ-stimulated astrocytes and LPS-stimulated microglia.

REFERENCES

1. Aviram M. 1993. Modified forms of low density lipoprotein and atherosclerosis. *Atherosclerosis* 98:1-9.

2. Bjorkhem I, Lutjohann D, Breuer O, Sakinis A, Wennmalm A. 1997. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with 18O2 techniques in vivo and in vitro. *J Biol Chem* 272:30178-30184.

3. Brown AJ, Jessup W. 1999. Oxysterols and atherosclerosis. *Atherosclerosis* 142:1-28.

4. Castrillo A, Joseph SB, Marathe C, Mangelsdorf DJ, Tontonoz P. 2003. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. *J Biol Chem* 278:10443-10449.

5. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science* 294:1866-1870.

6. Dietschy JM, Turley SD. 2002. Control of cholesterol turnover in the mouse. *J Biol Chem* 277:3801-3804.

7. Dietschy JM, Turley SD. 2004. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J Lipid Res* 45:1375-1397.

8. Dietschy JM, Turley SD, Spady DK. 1993. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res* 34:1637-1659.

9. Dyer RG, Hetherington CS, Alberti KG, Laker MF. 1995. Simultaneous measurement of phytosterols (campesterol and beta-sitosterol) and 7-ketocholesterol in human lipoproteins by capillary column gas chromatography. *J Chromatogr B Biomed Appl* 663:1-7.

10. Guardiola F, Codony R, Addis PB, Rafecas M, Boatella J. 1996. Biological effects of oxysterols: current status. *Food Chem Toxicol* 34:193-211.

11. Jacobs AT, Ignarro LJ. 2001. Lipopolysaccharide-induced expression of

interferon-beta mediates the timing of inducible nitric-oxide synthase induction in RAW 264.7 macrophages. *J Biol Chem* 276:47950-47957.

12. Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ. 1999. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci U S A* 96:266-271.

13. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383:728-731.

14. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med* 9:213-219.

15. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. 2002. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A*

99:7604-7609.

16. Jurevics H, Morell P. 1995. Cholesterol for synthesis of myelin is made locally, not imported into brain. *J Neurochem* 64:895-901.

17. Kainu T, Kononen J, Enmark E, Gustafsson JA, Pelto-Huikko M. 1996. Localization and ontogeny of the orphan receptor OR-1 in the rat brain. *J Mol Neurosci* 7:29-39.

18. Lange Y, Swaisgood MH, Ramos BV, Steck TL. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J Biol Chem* 264:3786-3793.

19. Lange Y, Ye J, Strebel F. 1995. Movement of 25-hydroxycholesterol from the plasma membrane to the rough endoplasmic reticulum in cultured hepatoma cells. *J Lipid Res* 36:1092-1097.

20. Lee JH, Joe EH, Jou I. 2005a. PPAR-alpha activators suppress STAT1 inflammatory signaling in lipopolysaccharide-activated rat glia. *Neuroreport* 16:829-833.

21. Lee JH, Park EJ, Kim OS, Kim HY, Joe EH, Jou I. 2005b. Double-stranded RNA-activated protein kinase is required for the LPS-induced activation of STAT1 inflammatory signaling in rat brain glial cells. *Glia* 50:66-79.

22. Lund EG, Xie C, Kotti T, Turley SD, Dietschy JM, Russell DW. 2003. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. *J Biol Chem* 278:22980-22988.

23. Mangelsdorf DJ, Evans RM. 1995. The RXR heterodimers and orphan receptors. *Cell* 83:841-850.

24. Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW. 2001. CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294:1354-1357.

25. Park EJ, Park SY, Joe EH, Jou I. 2003. 15d-PGJ2 and rosiglitazone suppress Janus kinase-STAT inflammatory signaling through induction of suppressor of cytokine signaling 1 (SOCS1) and SOCS3 in glia. *J Biol Chem* 278:14747-14752.

26. Peet DJ, Janowski BA, Mangelsdorf DJ. 1998. The LXRs: a new class of

oxysterol receptors. *Curr Opin Genet Dev* 8:571-575.

27. Pfrieger FW. 2003. Role of cholesterol in synapse formation and function. *Biochim Biophys Acta* 1610:271-280.

28. Pyo H, Joe E, Jung S, Lee SH, Jou I. 1999. Gangliosides activate cultured rat brain microglia. *J Biol Chem* 274:34584-34589.

29. Repa JJ, Mangelsdorf DJ. 2000b. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* 16:459-481.

30. Repa JJ, Mangelsdorf DJ. 2000a. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* 16:459-481.

31. Repa JJ, Mangelsdorf DJ. 2002. The liver X receptor gene team: potential new players in atherosclerosis. *Nat Med* 8:1243-1248.

32. Russell DW. 2000. Oxysterol biosynthetic enzymes. *Biochim Biophys Acta* 1529:126-135.

33. Russell DW. 1999. Nuclear orphan receptors control cholesterol catabolism. *Cell*

97:539-542.

34. Saito M, Benson EP, Saito M, Rosenberg A. 1987. Metabolism of cholesterol and triacylglycerol in cultured chick neuronal cells, glial cells, and fibroblasts: accumulation of esterified cholesterol in serum-free culture. *J Neurosci Res* 18:319-325.

35. Schmidt A, Vogel R, Holloway MK, Rutledge SJ, Friedman O, Yang Z, Rodan GA, Friedman E. 1999. Transcription control and neuronal differentiation by agents that activate the LXR nuclear receptor family. *Mol Cell Endocrinol* 155:51-60.

36. Schroepfer GJ, Jr. 2000. Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol Rev* 80:361-554.

37. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. 2000. Role of LXRs in control of lipogenesis. *Genes Dev* 14:2831-2838.

38. Smith LL. 1987. Cholesterol autoxidation 1981-1986. *Chem Phys Lipids* 44:87-

125.

39. Snipes GJ, Suter U. 1997. Cholesterol and myelin. *Subcell Biochem* 28:173-204.

40. Spady DK, Dietschy JM. 1983. Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *J Lipid Res* 24:303-315.

41. Turley SD, Burns DK, Dietschy JM. 1998. Preferential utilization of newly synthesized cholesterol for brain growth in neonatal lambs. *Am J Physiol* 274:E1099-E1105.

42. Turley SD, Burns DK, Rosenfeld CR, Dietschy JM. 1996. Brain does not utilize low density lipoprotein-cholesterol during fetal and neonatal development in the sheep. *J Lipid Res* 37:1953-1961.

43. Wang DQ, Afdhal NH. 2001. Good cholesterol, bad cholesterol: role of oxysterols in biliary tract diseases. *Gastroenterology* 121:216-218.

44. Wang L, Schuster GU, Hultenby K, Zhang Q, Andersson S, Gustafsson JA. 2002. Liver X receptors in the central nervous system: from lipid homeostasis to

neuronal degeneration. *Proc Natl Acad Sci U S A* 99:13878-13883.

45. Whitney KD, Watson MA, Collins JL, Benson WG, Stone TM, Numerick MJ, Tippin TK, Wilson JG, Winegar DA, Kliewer SA. 2002. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. *Mol Endocrinol* 16:1378-1385.

46. Whitney KD, Watson MA, Goodwin B, Galardi CM, Maglich JM, Wilson JG, Willson TM, Collins JL, Kliewer SA. 2001. Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. *J Biol Chem* 276:43509-43515.

47. Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. 2000. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch Neurol* 57:1439-1443.

48 .Yao ZX, Brown RC, Teper G, Greeson J, Papadopoulos V. 2002. 22R-Hydroxycholesterol protects neuronal cells from beta-amyloid-induced cytotoxicity by binding to beta-amyloid peptide. *J Neurochem.* 83(5):1110-9.

- 국문요약 -

성상교세포에서 옥시스테롤의 항염증반응에 대한 연구

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콜레스테롤은 뇌에 다량으로 존재하며 자발적 내지 효소의존적인 산화과정을 통하여 옥시스테롤로 전환될 수 있다. 옥시스테롤은 세포벽과 blood-brain barrier(BBB)를 통과할 수 있는 콜레스테롤의 이동가능한 형태이다. 본 논문에서는 뇌염증반응에서의 옥시스테롤 역할을 규명하기 위하여 lipopolysaccharides(LPS)또는 interferon gamma(IFNγ)로 뇌성상교세포에 자극을 주어 in vitro상에서 염증반응을 유발시킨 뒤 두가지 옥시스테롤(7-ketocholesterol 과 22(R)-hydroxycholesterol)을 처리하여 그 반응을 살펴보았다. LPS에 의해 자극된 성상교세포에서 두가지 옥시스테롤은 염증반응에서 대표적으로 유도되는 효소인 inducible nitric oxide synthase (iNOS)발현 및 이로 인한 nitric oxide(NO)생성을 억제하였으며, 뿐만 아니라 iNOS발현 신호전달과정에 관여하는 interferon-β, 인산화된 Signal Transducer and Activator of Transcription 1/3, 그리고 interferon regulatory factor-1등을 모두 억제하였다.

옥시스테롤은 핵수용체인 liver X receptor(LXR)작용제로 알려져 있고, 위와 같은 억제효과는 인위적으로 합성된 LXR작용제에서도 동일하게 나타났다. 또한, 옥시스테롤을 처리한 성상교세포에서 LXR발현에는 변화가 없었지만 LXR 활성화에 의해 대표적으로 유도되는 ATP-binding cassette transporter a 1 (ABCA1)의 발현이 증가하고, LXR과 함께 heterodimer로 작용하는 retinoid X receptor ligands(RXR)작용제를 같이 처리하였을 때 억제효과가 더 강하게 나타나는 것을 미루어 보아 이러한 옥시스테롤의 iNOS억제반응은 LXR/RXR을 통해 조절된다는 결론을 얻을 수 있었다. 그 외에도 옥시스테롤은 LPS에 의해 유도된 cyclooxygenase2, interleukin-6, tumor necrosis factor α, monocyte chemoattractant protein 1의 발현을 줄임으로써 염증반응에 관여하는 매개물질들을 전반적으로 잘 억제하는 것으로 나타났다. 마지막으로, 옥시스테롤은 LPS로 자극된 성상교세포뿐만 아니라 IFNγ로 자극된 성상교세포와 LPS에 자극된 소교세포에서도 유사한 결과를 나타냄으로써 본 논문은 이러한 옥시스테롤의 항염증효과가 뇌염증반응을 조절하는 새로운 기작으로 부각될 수 있음을 보여주고 있다.

핵심어: 옥시스테롤, LXR, iNOS, IFN-β, STAT, IRF-1, 성상교세포