의학 박사학위 논문

# A Study on the Regulation of Microglial Inflammatory Responses by Gangliosides and Oxidized Low Density Lipoprotein


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# A Study on the Regulation of Microglial Inflammatory Responses by Gangliosides and Oxidized Low Density Lipoprotein

by

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**-ABSTRACT-**

# A Study on the Regulation of Microglial Inflammatory Responses by Gangliosides and Oxidized Low Density Lipoprotein

The brain is abundant in lipids, and lipid metabolism is clearly related to brain functions such as synaptogenesis, neuronal survival and signal transduction. Microglia are one type of glial cells in the brain and play a role as major immune effector cells. Because microglia-mediated brain inflammation participate in the initiation or progression of neurodegenerative disorders, it is important to understand the regulation of microglial inflammatory responses. In this study, I investigated whether lipid derivatives, gangliosides and oxidized low density lipoprotein (oxLDL), could modulate microglia-mediated brain inflammation.

In part 1, I examined whether gangliosides activate JAK-STAT pathway, an essential inflammatory signaling pathway in microglia. Neuronal cell membranes are particularly rich in gangliosides, which play important roles in brain physiology and pathology. In this study, I provide evidence that JAK-STAT inflammatory signaling mediates gangliosides-stimulated microglial activation. Both in rat primary microglia and murine BV2 microglial cells, gangliosides stimulated nuclear factor binding to GAS/ISRE elements, which are known to be STAT-binding sites. Consistent with this, gangliosides rapidly activated JAK1 and JAK2 and induced phosphorylation of STAT1 and STAT3. In addition, gangliosides increased

transcription of the inflammation-associated genes inducible nitric oxide synthase (iNOS), intracellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), which are reported to contain STAT-binding elements in their promoter regions. AG490, a JAK inhibitor, reduced induction of these genes, nuclear factor binding activity, and activation of STAT1 and STAT3 in gangliosides-treated microglia. AG490 also inhibited gangliosides-induced release of NO, an inflammation hallmark. Furthermore, AG490 markedly reduced activation of ERK1/2 MAPK, indicating that ERKs act downstream of JAK-STAT signaling during microglial activation. However, AG490 did not affect activation of p38 MAPK. I also reported that the sialic acid present on gangliosides may be one of the essential components in activation of JAK-STAT signaling. These results indicate that JAK-STAT signaling is an early event in gangliosides-induced brain inflammatory responses.

In part 2, I studied the effects of oxLDL on microglia-mediated brain inflammation. LDL is readily oxidized under certain conditions, resulting in the formation of oxLDL. Despite numerous in vitro reports that reveal the pathogenic role of oxidative stress, anti-oxidative strategies have underperformed in the clinic. In this study, I examined the role of oxLDL in brain inflammatory responses using cultured rat brain microglia. I demonstrate that oxLDL inhibits lipopolysaccharide (LPS)-induced inflammatory responses in these cells. It decreases LPS-induced expression of iNOS and production of NO, and reduces LPS-induced secretion of tumor necrosis factor-alpha (TNFα) and MCP-1. Oxysterols, known components of

oxLDL, can simulate the inhibitory effects of oxLDL in LPS-activated microglia. In addition, their inhibitory effects were mimicked by liver X receptor (LXR) agonists and potentiated by a retinoid X receptor (RXR) agonist, suggesting these molecules heterodimerize to function as oxysterol receptors. Taken together, these results demonstrate that oxLDL inhibits LPS-induced inflammatory responses in brain microglia and that these inhibitory effects are mediated by oxysterols and, at least in part, by the nuclear receptor LXR, suggesting an additional mechanism of action for oxidative stress that acts indirectly via modulation of inflammatory responses. Although further studies are needed, these results answer in part the question of why anti-oxidative strategies have not been successful in clinical situations. Moreover, as brain inflammation participates in the initiation and progression of several neurodegenerative disorders, the present data provide information that should prove useful for designing therapeutic strategies to combat oxidative brain diseases.

In summary, lipid derivates, gangliosides and oxLDL, could regulate microglial-mediated brain inflammation. Gangliosides induce microglial activation through JAK-STAT pathway and oxLDL suppress LPS-induced microglial activation.

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**Key words**: Microglia, Neuroinflammation, Gangliosides, OxLDL, Oxysterol, JAK-STAT, LXR

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# ABBREVIATION

AAPH: 2,2'-Azobis(2-amidimopropane) Dihydrochloride

AcLDL: Acetylated LDL

COX: Cyclooxygenase

ERK: Extracellular Signal-regulated Kinase

GAS: Gamma Interferon Activated Site

Gmix: Gangliosides Mixture

HC: Hydroxycholesterol

ICAM: Intercellular Adhesion Molecule

IFN: Interferon

iNOS: Inducible NO Synthase

ISRE: Interferon-Stimulated Regulatory Element

JAK: Janus Kinase

JNK: c-Jun N-terminal Kinase

KC: Ketocholesterol

LDL: Low Density Lipoprotien

LPO: Lipid Hydroperoxide

LPS: Lipopolysaccharide

LXR: Liver X Receptor

MA: Methoprene Acid

MCP: Monocyte Chemoattractant Protein

oxLDL: Oxidized LDL

REM: Relative Electrophoretic Mobility

RXR: Retinoid X Receptor

STAT: Signal Transducer and Activator of Transcription

TBARS: Thiobarbituric Acid Reacting Substance

TNF: Tumor Necrosis Factor

# I. INTRODUCTION

## A. Brain inflammation

Until recently, it has been thought that the brain is an immune-privileged organ, but there have been increasing numbers of reports of immune reactions in the brain. Glial cells, which include microglia and astrocytes, mainly play a role in immune reactions in the central nervous system (CNS). Microglia are a class of brain mononuclear phagocytes and are thought to be the principal immune cell resident to the CNS. Microglia have functions similar to those of other tissue macrophages, including phagocytosis, antigen presentation and production of cytokines, eicosanoids, complement components, excitatory amino acid (glutamate), proteinase and nitric oxide (Gehrmann et al., 1995; Streit et al., 1999). The inflammatory mediators from activated microglia protect brain from bacterial and viral infection. When the CNS is injured, microglia rapidly proliferate and migrate to the injured sites where they secrete inflammatory mediators. In this way, microglia are thought to protect neurons from external injuries. Of course, microglial activation can occur as a result of brain injury, and there is substantial evidence that microglial activation can aggravate brain injuries, resulting in neurodegenerative diseases. Activated microglia are observed in the brains of patients of Alzheimer’s disease (AD), multiple sclerosis, stroke and other neurodegenerative diseases (Benveniste, 1997; Breitner, 1996; Danton and Dietrich, 2003; Nelson et al., 2002). Because microglia-

mediated brain inflammation can participate in the initiation or progression of neurodegenerative disorders, it is important to understand not only their mechanisms of activation but also their functions.

## B. Gangliosides and oxidized low density lipoprotein (oxLDL)

### 1. Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids that are constituents of mammalian cell membrane. Gangliosides are particularly abundant in neuronal cell membrane and participate in various cellular events of nervous system (Riboni et al., 1997; Tettamanti and Riboni, 1994). The major types of gangliosides in the brain are GM1, GD1a, GD1b, GT1b and GQ1b, which differ in their profiles of sialic acid residues and carbohydrate moieties (Dreyfus et al., 1997; Tettamanti, 2004). Exogenously added gangliosides exert neuritogenic, neurotrophic and neuroprotective effects on a variety of cell systems of neural origin (Byrne et al., 1983; Facci et al., 1984). Several lines of evidence point to the importance of the brain-derived gangliosides in immune responses and pathogenesis of brain disease. There are reports that brain injury can cause release of gangliosides from damaged neuronal cells into the extracellular space, which may lead to pathophysiological conditions (Blennow et al., 1991; Gisslen et al., 1997; Michikawa et al., 2001). Gangliosides have been also reported to interact with β-amyloid ($A_\beta$), suggesting they play a role in $A_\beta$ toxicity as well as in the deposition of $A_\beta$ into senile plaques

associated with Alzheimer's disease (AD). In addition, gangliosides regulate the production of various inflammatory mediators, such as cytokines and inducible nitric oxide synthase (iNOS) (Ding et al., 1998; Oderfeld-Nowak and Zaremba, 1998). Despite the evidence of a role for gangliosides in brain pathology, little appears known regarding how gangliosides act.

## 2. OxLDL

### (A) LDL in brain

Lipoproteins are macromolecular complex containing an envelope of phospholipids, some free cholesterol and a core of triglycerides or cholesteryl esters (Steinberg, 2002). The lipoproteins vary in origin, size, density in an aqueous environment, lipid and apolipoprotein content. The brain contains almost 25% of total body cholesterol, and lipoproteins also are present in the brain (Dietschy and Turley, 2001). LDL is present in the brain, largely as the result of cholesterol metabolism (Pitas et al., 1987). Although the cell types that produce and release lipoproteins in the brain has not yet been determined, there have been increasing numbers of reports of possibilities of astrocyte and microglia (Fujita et al., 1999; Mori et al., 2004; Saura et al., 2003; Xu et al., 2000). LDL in the brain is also supplied by cellular uptake from the circulation as well as in peripheral tissues. Although, the blood-brain barrier prevents diffusion of large molecules at the level of tight junctional attachments between adjacent capillary endothelial cells, surprisingly,

it has been shown that brain endothelial cells have the potential to take up LDL cholesterol through luminal LDL receptors and translocation of LDL across the cell (Dehouck et al., 1994; Dehouck et al., 1997).

**(B) Oxidative modification of LDL**

LDL is rapidly oxidized under certain conditions, which results in the formation of oxLDL. Although the oxidants responsible for in vivo oxidation of LDL are unknown at present, there are several candidates for oxidants including 15-lipoxygenase (Kuhn et al., 1994), myeloperoxidase (Daugherty et al., 1994), endothelial nitric oxide synthase (White et al., 1994), or transition metals (Balla et al., 1991; Ehrenwald and Fox, 1996; Heinecke et al., 1984). LDL within the brain is highly vulnerable to oxidative modification. CNS is particularly sensitive to oxygen radical damage because of poor antioxidant defense and an abundant supply of transition metals (Coyle and Puttfarcken, 1993). There are reports that oxidative stress is associated with neurodegenerative disorders, including Parkinson’s disease (PD), AD and Amyotorphic laterlal sclerosis (ALS). Markers for lipid peroxidation, such as 4-hydroxynonal (4-HNE) and malondialdehyde (MDA), have been identified in the cortex and hippocampus of patients with AD (Butterfield et al., 2002), the substantia nigra of patients with PD (Dexter et al., 1989) and in spinal fluid from patients with ALS (Pedersen et al., 1998). In addition, ischemic injury to brain is associated with disruption of blood-brain barrier (BBB), increasing the possibility of exposing the CNS to plasma LDL or oxLDL (del Zoppo and Hallenbeck, 2000).

Actually Uno et al. have shown raised plasma oxLDL in acute cerebral infarction (Uno et al., 2003). Together, these findings suggest that oxLDL may contribute to pathophysiology of brain.

**(C) Biological functions of oxLDL**

OxLDL are believed to play a critical role in atherosclerosis and exert diverse biological effects on different cell types. OxLDL stimulates endothelial cells to produce MCP-1 which recruits circulating monocytes into the intima (Cushing et al., 1990). Macrophages in the intima bind and take up oxLDL, and subsequently change themselves into foam cells (Itabe, 2003). OxLDL also induces atherosclerosis by stimulating monocyte infiltration and smooth muscle cell migration and proliferation (Mertens and Holvoet, 2001).

In addition to its well-established role in atherosclerosis, oxLDL exerts complex effects on inflammation. OxLDL has been demonstrated to modulate the expression of inflammatory cytokines in cell types present in the vascular wall and this activity is believed to be an important mechanistic feature of its pathophysiological action. It is interesting to note that oxLDL can modulate inflammatory gene expression in both positive and negative fashions. On the one hand, oxLDL induces the expression of several inflammatory mediators (Cushing et al., 1990; Lin et al., 2003; Terkeltaub et al., 1994). On the other hand, oxLDL antagonizes certain inflammatory gene expression initiated after LPS stimulation (Chung et al., 2000; Fong et al., 1991; Hamilton et al., 1990). Among the variables

that determine the nature of the effect are the cell type and the magnitude of LDL oxidation.

**(D) Active components of oxLDL**

OxLDL contains a complex, variable, incompletely characterized mixture of oxidation products. Both the diversity of product formation and observed cellular responses depend on the method and time course of oxidation (Navab et al., 1996). OxLDL contains several lipid-derived bioactive molecules such as lysophosphatidylcholine (LPC), platelet-activating factor (PAF)-like bioactive lipids and oxysterols (Hajjar and Haberland, 1997; Heery et al., 1995; Huang et al., 1999). One well studied, stable end product includes LPC, which is generated by phospholipase $A_2$ ($PLA_2$) hydrolysis during cellular oxidative modification of LDL (Quinn et al., 1988). LPC has been demonstrated to be a chemoattractant to monocytes and T lymphocytes (McMurray et al., 1993). PAF-like bioactive lipids have previously been implicated to play roles in vascular cell activation. Binding of PAF by the G protein-coupled PAF receptor leads to activation of heterotrimeric G proteins linked to phosphoinositide–phospholipase C (PI-PLC) and triggers a broad array of biological actions. PAF receptor antagonists block the ability of PAF-like phospholipids extracted from highly oxLDL to induce mitogenesis of smooth muscle cells (Heery et al., 1995). The cholesterol oxidation products, termed oxysterols, that are found in oxLDL include 7-ketocholesterol (Jialal et al., 1991) and 22 (R)-hydroxycholesterol (Fowler et al., 2003). Oxysterols may induce apoptosis in a wide

variety of cells, making them resonable candidates for the apoptotic activity of oxLDL. Recent studies suggest that oxysterols possess anti-inflammatory properties (Fowler et al., 2003; Liu et al., 1998).

## C. Inflammatory signaling pathways

### 1. JAK-STAT pathway

JAK-STAT (Janus kinase-signal transducers and activators of transcription) signaling pathways have been reported to be involved not only in the immune response of numerous cytokines but also in the actions of primarily non-immune mediators such as growth factors and hormones. Specific subtypes of JAK and STAT molecules are activated by different signals, resulting in specificity of response (Igaz et al., 2001; Kishimoto et al., 1994). The binding of ligand to its receptor induces assembly of an active receptor complex and consequent phosphorylation of the receptor-associated JAKs (JAK1, JAK2, JAK3, TYK2). Phosphorylated JAKs lead to the activation of neighboring JAKs, receptor subunits and several other substrates. Phosphorylation of JAKs provides the docking sites for STATs, which in turn become phosphorylated on tyrosine and serine residues; phosphorylation of both amino acid species being required for full STAT activity. Phosphorylated STATs are released from the receptor complex and form dimers. These dimers translocate to the nucleus where they directly bind to IFNγ-activated sites (GAS) of specific target genes, thus regulating transcription of these genes such as ICAM-1 and MCP-1,

which are involved in immune responses (Ramana et al., 2000; Tessitore et al., 1998; Zhou et al., 2001). JAK-STAT signaling can specifically mediate the inflammatory pathways activated by various stimulators in the brain, and appropriate regulation of JAK-STAT intensity and duration can protect against inflammation-induced brain injury.

**2. Lipopolysaccharide (LPS)–induced signaling pathways**

LPS is an integral cell wall component of gram-negative bacteria that can provoke life-threatening inflammatory conditions through the sequential activation of intracellular signaling molecules (Aderem and Ulevitch, 2000; Ulevitch and Tobias, 1995). LPS binds to LPS-binding protein (LBP) in plasma and is delivered to the surface receptor CD14. Next, LPS is transferred to the transmembrane signaling receptor, toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS stimulation activates several intracellular signaling pathways that include the transcription factor NF-κB pathway and three mitogen-activated protein kinase (MAPK) pathways: extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38 (Guha et al., 2001; Guha and Mackman, 2001). Recently, LPS has been found to activate the STAT inflammatory signaling cascade in macrophages and glial cells, leading to the massive production of inflammatory cytokines and causing multiple organ system failure and death (Dell'Albani et al., 2001; Jacobs and Ignarro, 2001). LPS activates NF-κB, leading to the induction and secretion of interferon-β (IFNβ). The secreted IFNβ then bind to interferon receptor, thereby leading to

phosphorylation of STAT1. The activated STAT1 then bind to the promoter of inflammation-associated genes, leading to the secretion of newly synthesized cytokines and nitric oxide (NO) and the induction of various inflammatory responses.

**3. Liver X receptor (LXR) pathway as anti-inflammatory signaling pathway**

LXRs (LXRα and LXRβ) are members of the nuclear receptor superfamily and are activated by oxysterols and intermediates in the cholesterol synthetic pathway. LXRs form obligate heterodimers with retinoid X receptors (RXRs), which are members of the nuclear receptor superfamily that is regulated by 9-cis-retinoic acid (9cRA). Although originally identified as liver enriched transcription factors, the LXRs are now being intensely studied in various tissues as well as in liver. LXRs are also expressed in the brain, but their roles in tissue remain to be clarified. 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).

LXRs are able to regulate the expression of a number of genes involved in cholesterol metabolism (Mangelsdorf and Evans, 1995; Peet et al., 1998; Schultz et al., 2000). A recent report shows that LXRs have an important function in lipid homeostasis in the brain, and that loss of these receptors results in neurodegenerative diseases (Whitney et al., 2002). In addition to its importance in lipid 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). Also, Synthetic LXR agonists have been demonstrated to prevent

atherosclerosis in murine models and to inhibit inflammation (Joseph et al., 2003; Joseph et al., 2002).

## D. Aims of study

The brain is abundant in lipids, and lipid metabolism is clearly related to brain functions such as synaptogenesis (Mauch et al., 2001), neuronal survival (Xu et al., 2000) and signal transduction (Brown and London, 1998). In this study, I investigated whether lipid derivatives, gangliosides and oxLDL, could modulate microglia-mediated brain inflammation. Because microglia-mediated brain inflammation participates in the initiation or progression of neurodegenerative disorders, it is important to understand their mechanism of activation. In part 1, I tested whether gangliosides activate JAK-STAT pathway, an essential inflammatory signaling pathway in microglia. In part 2, I studied the effects of oxLDL on microglia-mediated brain inflammation.

# II. MATERIALS AND METHODS

## A. Reagents

Bovine brain gangliosides mixture, GM1 and GD1a, was purchased from Matreya (Pleasant Gap, PA). Asialogangliosides GM1 was from Sigma-Aldrich (St. Louis, MO). Human LDL was purchased from Calbiochem (La Jolla, CA) and Cu-oxLDL was purchased from Biomedical Technologies Inc. (Stoughton, MA). Rat IFN-γ, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG490), and PD98059 were from Calbiochem. *Anthrobacter ureafaciens* neuraminidase was purchased from Sigma. *Salmonella typhimurium* LPS and 7-ketocholesterol were purchased from Sigma-Aldrich. 22(R)-hydroxycholesterol and TO901317 were purchased from Cayman (Ann Arbor, MI). Methoprene acid was purchased from BIOMOL (Plymouth Meeting, PA). GW3965 was kindly provided by GlaxoSmithKline High Throughput Chemistry (Stevenage SG1 2NY, UK). Antibodies against STAT1, Tyr-701-phosphorylated STAT1, Ser-727-phosphorylated STAT1, and Tyr-705-phosphorylated STAT3 were from Cell Signaling Technology (Beverly, MA). Antibodies against phosphorylated ERK, phosphorylated JNK and phosphorylated p38 were from Calbiochem. Antibodies against phosphorylated JAK1 and -2 were from Affinity Bioreagents (Denver, CO). Antibodies (Abs) against inducible NO synthase (iNOS) was purchased from Upstate Biotechnology (Lake Placid, NY), and Abs against cyclooxygenase-2 (COX2) and actin were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA).

**B. Cell Culture**

Primary microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague-Dawley rats as described previously (Pyo et al., 1999). Briefly, the cortices were triturated into single cells in minimal essential medium (MEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) and plated in 75-$cm^2$ T-flasks (0.5 hemisphere/flask) for 2-3 weeks. Microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes. Cells were plated in 6-well plates ($7 \times 10^4$ cells/well), 60-mm dishes ($5 \times 10^5$ cells/dish), or 100-mm dishes ($10^6$ cells/well). One hour later, the cells were washed to remove unattached cells before being used in experiments. BV2 immortalized murine microglia cells were from Dr. E. J. Choi. The BV2 cell line was grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and supplemented with 5% FBS. Cells were serum-starved overnight before treatment with gangliosides.

**C. Electrophoretic Mobility Shift Assay (EMSA)**

Cells were harvested and suspended in 9 times packaged cell volume of a hypotonic solution (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) including 0.5% Nonidet P-40. Cells were centrifuged at $500 \times g$ for 10 min at 4 °C,

and the pellet (nuclear fraction) was saved. The nuclear fractions were resuspended in a buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, incubated on ice for 60 min with occasional gentle shaking, and centrifuged at 12,000 × *g* for 20 min. The crude nuclear proteins in the supernatant were collected and stored at −70 °C until used. EMSA was performed for 30 min on ice in a volume of 20 µl, containing 4 µg of nuclear protein extract in a reaction buffer containing 8.5 mM EDTA, 8.5 mM EGTA, 8% glycerol, 0.1 mM $ZnSO_4$, 50 µg/ml poly (dI-dC), 1 mM DTT, 0.3 mg/ml bovine serum albumin, 6 mM $MgCl_2$, and $\gamma$-$^{32}$P-radiolabeled oligonucleotide probe ($3 \times 10^4$ cpm), with or without 20-50-fold excess unlabeled probe. In supershift experiments, protein extracts were incubated with 0.2-0.5 µg of STAT1 and STAT3 antibodies (Santa Cruz Biotechnology) for 30 min prior to the addition of $^{32}$P-labeled probe. DNA-protein complexes were separated on 6% polyacrylamide gels in Tris/glycine buffer. The dried gels were exposed to x-ray film. The following double-stranded oligonucleotide was used in these studies: GAS/ISRE, 5'-AAG TAC TTT CAG TTT CAT ATT ACT CTA-3', 27 bp (Santa Cruz Biotechnology, sc-2537). 5'-end-labeled probes were prepared with 40 µCi of [$\gamma$-$^{32}$P] ATP using T4 polynucleotide kinase (Promega, Madison, WI) and were purified on Quick Spin Columns Sephadex G-25 (Roche Molecular Biochemicals, Mannheim, Germany).

**D. Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using RNAzol B (Tel-Test Inc., Friendswood, TX)

TABLE 1. Primer Sequences for PCR

| Genes | Primer sequences |
|---|---|
| ABCA1 | F: 5'-GGG CGG GGG CCT GAA GAT C-3'<br>R: 5'-AGA GCC ATT TGG GGA CTG AAC ATC-3' |
| Actin | F: 5'-GCC ATC TCC TGC TCG AAG TCT AG-3'<br>R: 5'-CAT GTT TGA GAC CTT CAA CAC CCC-3' |
| CD36 | F: 5'-TCA AGG TGT GCT CAA CAG CC-3'<br>R: 5'-AGG ATA AAA CAC ACC AAC TGT-3' |
| CD68 | F: 5'-TCC CTC TTG CTG CCT CTC ATC-3'<br>R: 5'-GGT GGC TTA CAC AGT GGA CTG G-3' |
| GAPDH | F: 5'-TCC CTC AAG ATT GTC AGC AA-3'<br>R: 5'-AGA TCC ACA ACG GAT ACA TT-3' |
| ICAM-1 | F: 5'-CTG GAG AGC ACA AAC AGC AGA G-3'<br>R: 5'-AAG GCC GCA GAG AGC AAA AGA AGC-3' |
| IFNβ | F: 5'-GCC TTCG CTT ATC GCC ATC AAC-3'<br>R: 5'-AAT GCA AAG ACC TGC TCC GAG-3' |
| iNOS | F: 5'-ACA ACC TTG GTG TTG AAG GC-3'<br>R: 5'-GCA GAA TGT GAC CAT CAT GG-3' |
| LOX-1 | F: 5'-GAC TGG ATC TGG CAT AAA GA-3'<br>R: 5'-CCT TCT TCT GAC ATA TGC TG-3' |
| LXRα | F: 5'-GCG AGG GCT GCA AGG GAT TCT-3'<br>R: 5'-ATG GGC CAA GGC GTG ACG CG-3' |
| LXRβ | F: 5'-GCC TTG CTT ATC GCC ATC AAC-3'<br>R: 5'-AAT GCA AAG ACC TGC TCC GAG-3' |
| MCP-1 | F: 5'-CTA GTT CTC TGT CAT ACT GG-3'<br>R: 5'-ATG CAG GTC TCT GTC ACG CT-3' |
| RXRα | F: 5'-AGA GGA CAG TAC GCA AAG AC-3'<br>R: 5'-GTG AAG AGC TGC TTG TCT G-3' |
| SRA | F: 5'-TTG GCT TCC CTG GAG GTC GAG-3'<br>R: 5'-ACA CAG GAA CCA ATG TCA TTT G-3' |

and cDNA was prepared using avian reverse transcriptase (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 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 on a 1.5% agarose gel and detected under UV light.

**E. 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 PMSF, 100 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml aprotinin, and 2 mM EDTA). The lysates were centrifuged for 10 min at 12,000 × *g* at 4 °C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred to a 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).

**F. 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.

### G. Enzymatic Digestion of Sialic Acid

Neuraminidase derived from *A. ureafaciens* was used for cleaving sialic acids residues from gangliosides. Gangliosides were dissolved in 10 mM sodium acetate buffer, pH 5.0, containing 1 µg of sodium cholate per µl and were incubated with *A. ureafaciens* neuraminidase (Sigma-Aldrich) at 37 °C for 2 h.

### H. Oxidation of LDL

I was prepared oxLDL using a standard method of 2,2'-azobis(2-amidimopropane) dihydrochloride (AAPH; Sigma-Aldrich)-mediated oxidation (Neuzil et al., 1998; Shie et al., 2004). Oxidation of LDL was performed at 37 °C under the following conditions to obtain different degrees of oxidation. First, LDL was oxidized by 10 mM AAPH for 2, 6, or 18 h. Second, oxidation of LDL was performed for 18 h at AAPH concentrations of 1, 5, or 10 mM. Produced oxLDLs were represented as "oxLDL-concentration of AAPH (mM): oxidation period (h)".

### I. Relative electrophoretic mobility (REM) assay

Electrophoretic mobility relative to LDL was measured by agarose gel (0.8% agarose in 0.08 mol/L Tris-HCl buffer, pH 8.3) electrophoresis and Coomassie Brilliant Blue R250 staining. This allows detection of changes in electric charge

induced by oxidation (Napoli et al., 1999; Sparks and Phillips, 1992).

**J. Thiobarbituric acid reacting substances (TBARS) assay**

200 μl of LDL/oxLDL (100 μg) was added to a test tube containing 200 μl SDS (8%, w/v), 400 μl acetic acid (20%, w/v), and 400 μl of thiobarbituric acid (0.8%, w/v). The mixture was vortexed well and boiled for 1 h. After cooling, the specimens were centrifuged (13,000 rpm, 10 min) and the absorbance of the supernatant was determined at 540 nm using a spectrophotometer. The amount of TBARS was determined by comparison to a standard of malondialdehyde (MDA) equivalents prepared using 1,1,3,3-tetraethoypropane (Sigma-Aldrich).

**K. Lipid hydroperoxide (LPO) assay**

The LPO level in oxLDL was determined by a LPO assay kit provided by Cayman. 100 μg LDL/oxLDL were used for LPO measurements. The absorbance at 500 nm was measured using a spectrophotometer (Amersham Pharmacia Biotech, San Francisco, CA).

**L. Enzyme-linked immunosorbent assay (ELISA)**

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

**M. 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

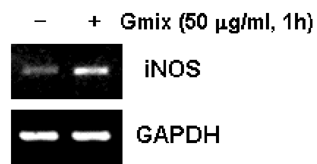
## A. JAK-STAT Signaling Mediates Gangliosides-induced Inflammatory Responses in Brain Microglial Cells

### 1. Gangliosides Induce Nuclear Factor Binding to GAS/ISRE Elements

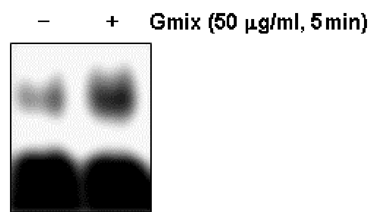
Functional GAS/ISRE elements are found in the promoter regions of several inflammation-related genes, such as iNOS, and these elements are known to bind the phosphorylated STAT dimer. In an attempt to explore the molecular mechanism of gangliosides on microglial activation, I investigated whether STATs could be involved in gangliosides-induced activation of microglia. I first examined the transcript level of iNOS in gangliosides-treated rat primary microglia. Gangliosides markedly induced iNOS mRNA within 1 h (Fig. 1A, a), suggesting that gangliosides directly regulate NO production at the level of transcription. This observation was subsequently evaluated by EMSA using a $\gamma$-$^{32}$P-labeled consensus GAS/ISRE oligonucleotides probe. After the cells were treated with 50 μg/ml brain-derived gangliosides mixture for the indicated times, nuclear extracts were prepared and then analyzed by EMSA. The specific binding complex was detected in nuclear extracts from gangliosides-treated rat primary and murine BV2 microglia (Fig. 1, A(b) and B). Time course analysis showed that gangliosides rapidly induced the nuclear factor binding within 5 min and that the binding activity was decreased to basal levels after

A

a)




b)




B

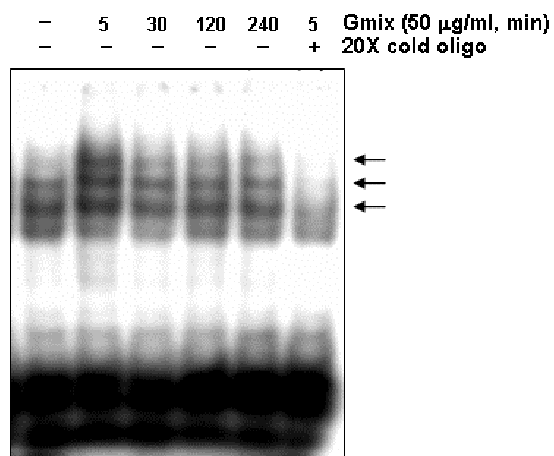


**Fig. 1. Gangliosides stimulate iNOS transcription and nuclear factor binding to GAS/ISRE elements in microglial cells.** (**A**) Rat primary microglial cells were treated with or without 50 μg/ml brain gangliosides mixture (Gmix) for 1 h, after which total RNA was isolated, and levels of iNOS mRNA were measured using an RT-PCR-based assay. The transcription of GAPDH was measured for normalization (**a**). Cells were treated with Gmix for 5 min, after which nuclear extracts were prepared and assayed for the amount of binding activity to GAS/ISRE oligonucleotides using EMSA (**b**). (**B**) BV2 cells were treated with 50 μg/ml Gmix for the indicated periods. Nuclear extracts were prepared, and binding activity to GAS/ISRE oligonucleotides was determined by EMSA.

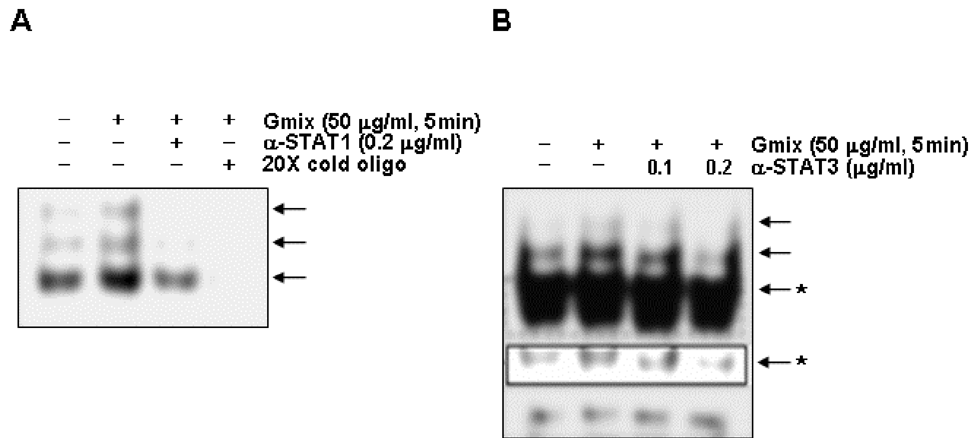



**Fig. 2. Gel shift assay using anti-STAT1 and anti-STAT3 in BV2 microglial cells.** After cells were treated with 50 µg/ml Gmix for 5 min, gel shift assays were performed as described in Fig. 1. with the exception that nuclear extracts were incubated with 0.2-0.5 µg of STAT1(**A**) and STAT3 (**B**) antibodies for 30 min prior to the addition of $^{32}$P-labeled probe. *, inset is a short-exposed autograph of the upper band (dotted box).

30 min in both microglial cell types (Fig. 1B). The specificity of the shifted bands was confirmed by competition assay using excess amounts of unlabeled oligonucleotides (cold oligo). In addition, gel shift assay showed that the binding complex was diminished by addition of anti-STAT1 and anti-STAT3, indicating that both STAT1 and STAT3 are constituents of the nuclear factor binding complex (Fig. 2). These results show that functional GAS/ISRE elements may be involved in gangliosides-induced activation of microglia.

**2. Gangliosides Induce the Phosphorylation of STAT1 and STAT3**

Essential roles for STAT signaling in brain inflammatory response have emerged. Because gangliosides rapidly induced the GAS/ISRE-nuclear factor binding, I examined whether gangliosides indeed caused phosphorylation of STAT proteins. Primary microglial cells were stimulated with 50 μg/ml gangliosides for the indicated times, and the levels of phosphorylated STAT1 were determined by Western blot analysis using antibodies against Tyr-701-STAT1 and Ser-727-STAT1. Both phosphorylations of STAT1 occurred within 5 min of gangliosides addition (Fig. 3A, a). Similar patterns of phosphorylation were observed in lysates from murine BV2 microglial cells, where incubation of cells with gangliosides resulted in STAT1 phosphorylation on tyrosine and serine residues, with phosphorylation levels returning to basal at 30 min (Fig. 3B). In addition to phosphorylation of STAT1, I detected gangliosides-induced phosphorylation of STAT3 in both microglial cell types. The pattern of STAT3 tyrosine phosphorylation appeared similar to that of

**Fig. 3. Gangliosides induce the phosphorylation of STAT1 and STAT3 in microglial cells.** Rat primary microglial cells (**A**) and mouse BV2 microglial cells (**B**) were serum-starved for 12 h and then stimulated with 50 µg/ml Gmix for the indicated times. Cell lysates were separated by 10% SDS-PAGE and Western blots probed with anti-pSTAT1 (Y), anti-pSTAT1 (S), or pSTAT3 (Y). The membrane was then stripped and analyzed with anti-STAT1 antibody to determine loading.

STAT1 phosphorylation (Fig. 3, A and B). The Western blotting data show that gangliosides trigger rapid phosphorylation of STAT1 and STAT3, suggesting their involvement in gangliosides-induced microglial activation. The phosphorylation patterns of both STAT1 and STAT3 determined by Western blotting correlate with the binding activity results from EMSA.

### 3. Gangliosides Induce Phosphorylation and Activation of JAK1 and JAK2

Phosphorylation of STATs depends on the activation of JAKs. JAKs both functionally and physically associate with cytokine signaling. In particular, activation of JAK1 and JAK2 provides a molecular explanation for cellular actions of a broad range of cytokines. Thus, I investigated whether JAK1 and JAK2 could be involved in gangliosides-induced STAT phosphorylation. Primary rat microglial cells were stimulated with 50 μg/ml gangliosides for the indicated times, and cell lysates were Western blotted using antibodies directed against phosphorylated JAK1 and JAK2. The data presented in Fig. 4 show that following addition of gangliosides to cells, phosphorylation of both JAK1 and JAK2 occurred within 5 min, after which phosphorylation levels returned to basal levels by 30 min. The involvement of JAK signaling in gangliosides-induced microglial activation was also shown using a second, independent approach. The pharmacological agent AG490 is known to inhibit the phosphorylation of both JAK1 and JAK2. I found that pretreatment of rat primary microglial cells with AG490 effectively reduced gangliosides-induced phosphorylation of STAT1 and STAT3 (Fig. 5A). In addition, AG490 inhibited the

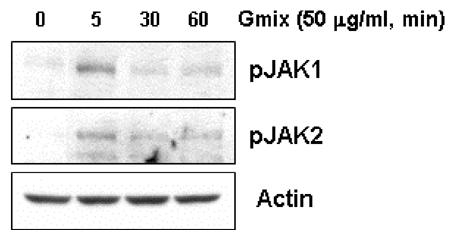



**Fig. 4. Gangliosides stimulate phosphorylation of JAK1 and JAK2 in rat primary microglial cells.** Cells were serum-starved for 12 h and then stimulated with 50 µg/ml Gmix for 5 min. The phosphorylation of JAK1 and JAK2 was determined by Western blot analysis using antibodies specific for phospho-JAK1 or -JAK2.

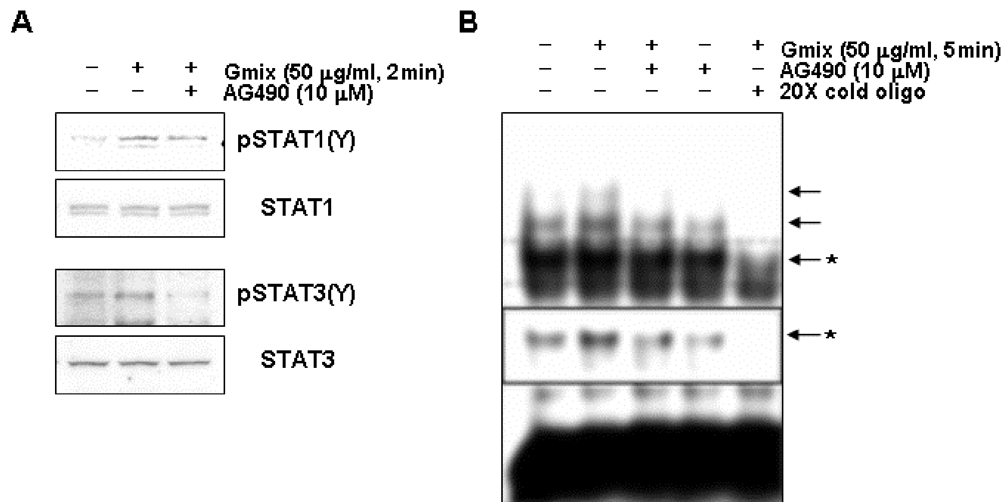



**Fig. 5. AG490, a specific inhibitor of JAK, reduce gangliosides-induced STAT phosphorylation and nuclear factor binding to GAS/ISRE.** (**A**) Cells were pretreated with 10 µM AG490 for 1 h and then stimulated with 50 µg/ml Gmix for 2 min. Western blots were probed with anti-pSTAT1 (Tyr-701) and pSTAT3 (Tyr-705). The membrane was subsequently stripped and probed with anti-STAT1 and STAT3 antibodies. (**B**) Cells were pretreated with 10 µM AG490 for 1 h and then stimulated with 50 µg/ml Gmix for 5 min. Nuclear extracts were prepared, and binding activity to GAS/ISRE oligonucleotides was determined by EMSA. *, inset is a short-exposed autograph of the upper band (dotted box).

nuclear factor binding to GAS/ISRE nucleotides in gangliosides-treated microglial cells (Fig. 5B). These results indicate that gangliosides induce phosphorylation and activation of STAT1 and STAT3 through phosphorylation and activation of JAK1 and JAK2.

### 4. Gangliosides Stimulate STAT-responsive Inflammatory Gene Expression

Brain inflammatory responses are coordinated by the production of cytokines, chemokines, and reactive oxygen species. The above data indicate that gangliosides-induced microglial activation may be mediated, at least in part, by JAK-STAT-dependent transcriptional responses. Therefore, I examined the transcript level of genes that have been reported previously to have functional GAS elements and act as mediators of inflammation, namely MCP-1 and ICAM-1. Rat primary microglial cells and BV2 cells were stimulated with 50 μg/ml gangliosides for 3 h, and total RNA was extracted for RT-PCR analysis. Addition of gangliosides rapidly increased the mRNA levels of both MCP-1 and ICAM-1, as did IFN-γ, which was included as a positive control (Fig. 6A). Pretreatment with AG490 significantly inhibited gangliosides-induced transcription of both genes (Fig. 6B). These findings demonstrate that gangliosides trigger STAT-dependent transcriptional activation of inflammatory genes in microglia.

### 5. AG490 Reduces Gangliosides-induced Release of NO

NO is known as an important physiological signaling molecule in the brain.

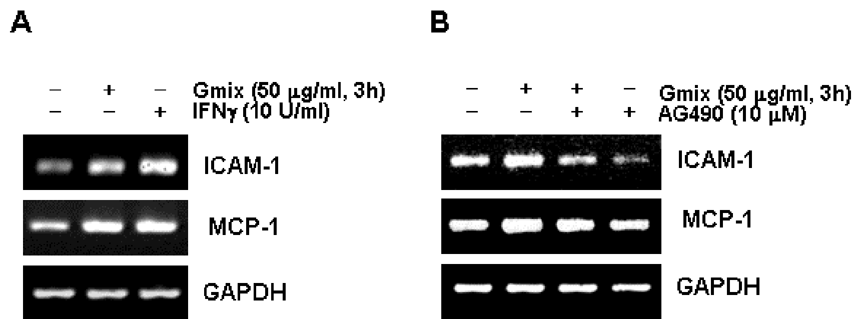



**Fig. 6. Gangliosides stimulate transcription of STAT-responsive inflammatory genes in microglial cells and AG490 suppresses this transcription.** (**A**) Cells were treated for 3 h with 50 μg/ml Gmix or 10 U/ml IFN-γ. Total RNA was isolated and analyzed for levels of MCP-1 and ICAM-1 mRNA using an RT-PCR-based assay. The transcription of GAPDH was measured for normalization. (**B**) Cells were pretreated with 10 μM AG490 for 1 h and then stimulated with 50 μg/ml Gmix for 3 h. mRNA expression of ICAM-1 and MCP-1 was detected using an RT-PCR-based assay.

Aberrant iNOS expression and excessive NO production are observed in various pathophysiological conditions. Previously, I showed that gangliosides-induced microglial activation was accompanied by induction of NO release. Thus, I tested whether gangliosides induced NO release via JAK-STAT signaling. First, I examined the effect of JAK inhibition on gangliosides-induced transcription of iNOS in rat primary microglial cells. RT-PCR analysis showed that the inhibitor AG490 reduced mRNA levels of iNOS (Fig. 7A). Second, I investigated the effect of AG490 on NO release. In these studies, the ERK inhibitor, PD98059, was also used since I have shown previously that it reduced gangliosides-induced NO release (Pyo et al., 1999). In the presence of AG490, microglial cells were treated with 50 μg/ml gangliosides for 48 h, and the amount of NO produced was determined by measuring the amount of nitrite converted from NO in the media. AG490 significantly reduced gangliosides-enhanced NO release, as did PD98059 (Fig. 7B). Compared with cells treated with gangliosides alone, NO release was reduced to 38.6 ± 4.3 and 25.2 ± 14% in cells co-treated with PD98059 and AG490, respectively. These results are consistent with the results shown in Figs. 5 and 6. The findings indicate that JAK-STAT signaling is required for NO release and provide evidence of the critical functional involvement of JAK-STAT signaling in gangliosides-induced microglial activation.

### 6. ERK Activity Appears to be Regulated by JAK Activation

There are several reports showing that the transcriptional activity of STATs

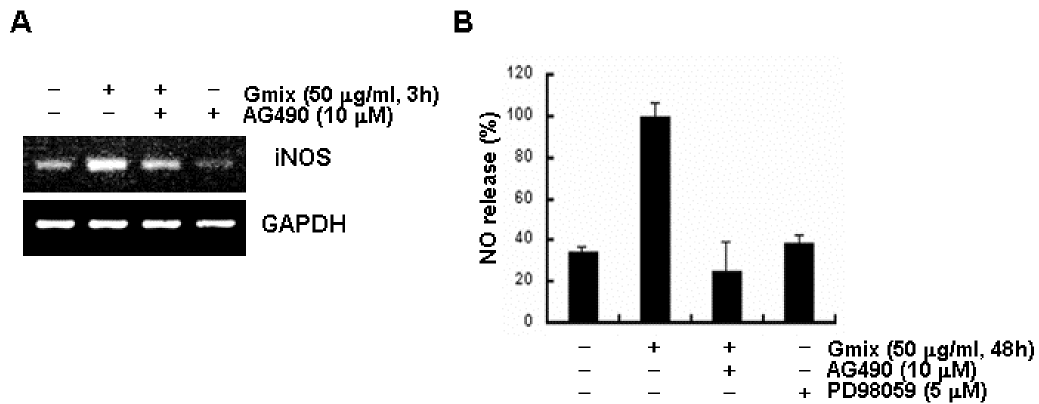



**Fig. 7. AG490 reduces gangliosides-stimulated NO in rat primary microglial cells.** (**A**) Cells were pretreated with 10 µM AG490 for 1 h and then stimulated with 50 µg/ml Gmix for 3 h. mRNA expression of iNOS was detected by RT-PCR analysis. The transcription of GAPDH was measured for normalization. (**B**) Cells were treated with 50 µg/ml Gmix for 48 h in the presence or absence of AG490 or PD98059. The amount of NO was determined by measuring the amount of nitrite in the media, as described under "Experimental Procedures.".

is regulated through mitogen-activated protein kinases (MAPKs). MAPKs are considered as common intracellular signaling molecules involved in microglial activation. Previous reports by others and us showed that gangliosides induced activation of MAPKs in microglia. In the present study, I used pharmacological inhibitors to examine possible cross-talk between the JAK-STAT and MAPKs signaling pathways. When primary rat microglial cells were pretreated for 2 h with the JAK inhibitor AG490, gangliosides-induced activation of ERK1/2 was significantly reduced compared with controls with no AG490 (Fig. 8). In contrast, no significant suppression of p38 was observed under this condition. However, in the presence of PD98059, an ERK inhibitor, not only ERK but also p38 activation was completely inhibited. These results indicate that gangliosides-stimulated JAK activation leads to activation of ERK in microglial cells. These pharmacological studies also indicate that gangliosides-stimulated activation of p38 may not be due to activation of ERK by JAK.

**7. Sialic Acid Residues Are Important for Gangliosides-induced Phosphorylation of STAT**

The major types of gangliosides in brain are GM1, GD1a, GD1b, GT1b, and GQ1b. These gangliosides differ with respect to the number and position of sialic acid residues attached to the carbohydrates. The approximate percentages of each gangliosides present in the brain gangliosides mixture used in the current study are 18% GM1, 55% GD1a, 15% GD1b, 10% GT1b, and 2% others. To address whether

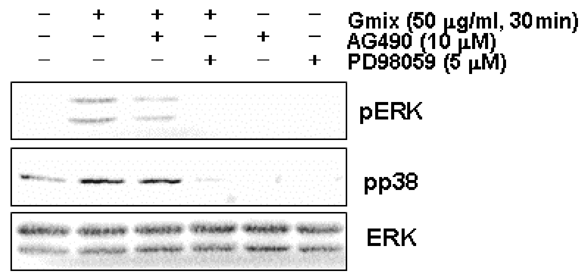



**Fig. 8. Activation of ERK1/2 follows JAK-STAT activation in gangliosides-treated primary microglial cells.** Primary microglial cells were pretreated with AG490 or PD98059 for 1 h and then treated with 50 μg/ml Gmix for 30 min. Cell lysates were separated by 10% SDS-PAGE and Western blots probed with anti-phospho-ERK and anti-phospho p38, respectively. The membrane was then stripped and probed with anti-ERK antibody. At least four experiments were independently performed, and representative data are shown in this figure.

the structural diversity of gangliosides affected activation of STAT, I compared the effect of GM1, which has one molecule of sialic acid, with GD1a, which has two molecules of sialic acid, on phosphorylation of STAT1. Primary microglial cells were treated with GM1 or GD1a for 2 min, and levels of phosphorylated STAT1 were determined by Western blot analysis using antibodies against Tyr-701-STAT1. The data in Fig. 9 show both GM1 and GD1a stimulated phosphorylation of STAT1 within 2 min. The level of STAT1 phosphorylation stimulated by either GM1 or GD1a was similar to that caused by the gangliosides mixture, suggesting that the number of sialic acid residues per gangliosides molecule has little effect on the phosphorylation of STAT1 in microglial cells (Fig. 9A). Because sialic acid residues are characteristic of gangliosides, I examined whether sialic acid residues were important for gangliosides-stimulated STAT phosphorylation. Gangliosides were preincubated with either 550 or 1000 units/ml *A. ureafaciens* neuraminidase, which is known to release sialic acid attached to an internal galactose in any gangliosides including GM1. Primary microglia cells were stimulated with gangliosides or neuraminidase-treated gangliosides (desialylated gangliosides) for 2 min, and levels of phosphorylated STAT1 were determined by Western blot analysis. The data presented in Fig. 9, B and C show a dose-dependent inhibitory effect of neuraminidase treatment on phosphorylation of STAT1, indicating that sialic acid residues are required for stimulation of JAK-STAT signaling. To rule out the possibility that these reductions are due to contaminating sialic acid or neuraminidase, I compared the effect of GM1 and asialo-GM1 (Sigma) on phosphorylation of

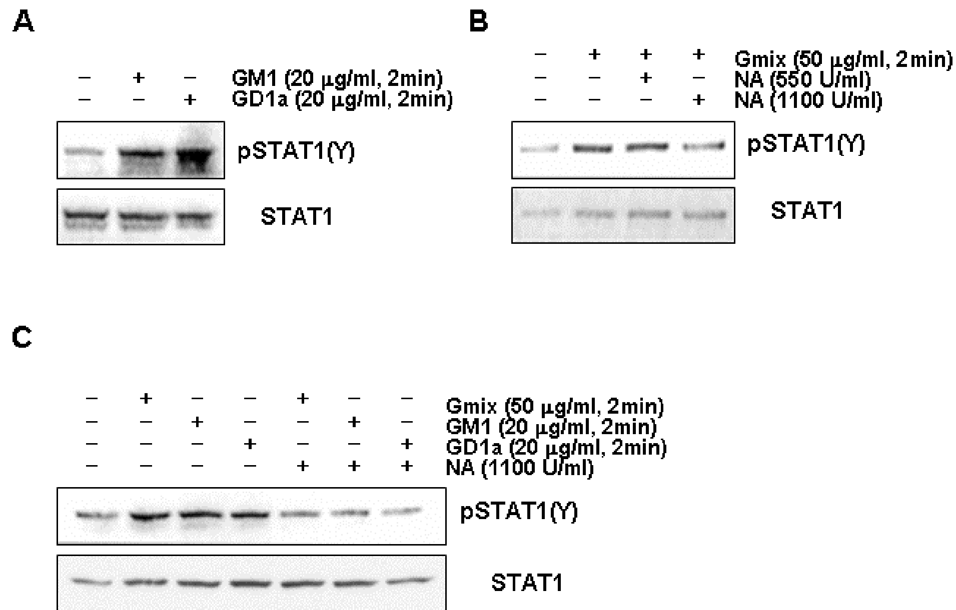



**Fig. 9. The sialic acid of gangliosides is necessary for activation of JAK-STAT signaling.** (**A**) Primary microglial cells were treated with 20 µg/ml GM1 or GD1a for 2 min. Cell lysates were subjected to Western blot analysis, and levels of phosphorylated STAT1 were determined using anti-pSTAT1 (Tyr-701). (**B** and **C**) To remove the sialic acid residue, gangliosides were preincubated with 550 or 1000 units/ml *A. ureafaciens* neuraminidase as described under "Experimental Procedures." Cells were treated with the indicated gangliosides or the desialylated gangliosides for 2 min, after which cell lysates were prepared, separated by 10% SDS-PAGE, Western blotted, and probed using anti-pSTAT1 (Tyr-701). The membrane was then stripped and probed with anti-STAT1 antibody.

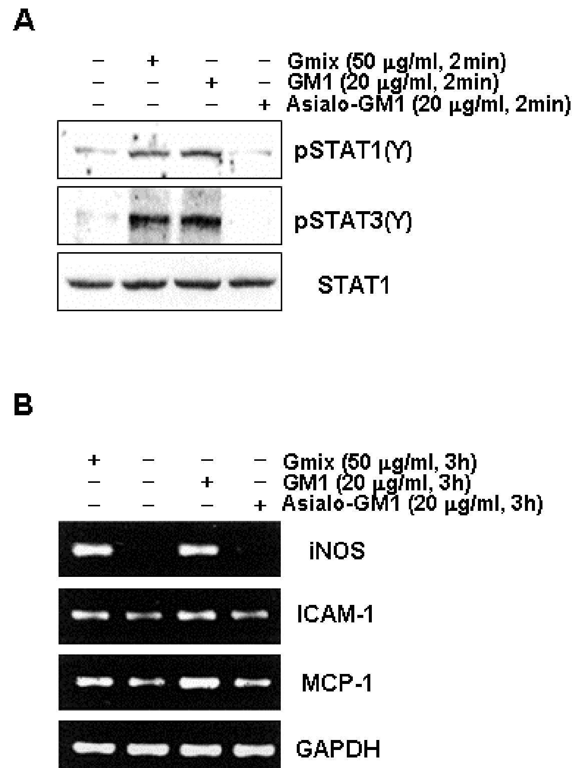



**Fig. 10. Asialo-GM1 does not activate the JAK-STAT signaling in primary microglial cells.** (**A**) Primary microglial cells were treated with 20 μg/ml of GM1 or asialo-GM1 for 2 min. Cell lysates were subjected to Western blot analysis, and levels of phosphorylated STAT were determined using anti-pSTAT1 and anti-pSTAT3. (**B**) Total RNA was isolated and analyzed for levels of iNOS, MCP-1, and ICAM-1 mRNA using an RT-PCR-based assay.

STAT and transcription of STAT-responsive genes. Consistent with Fig. 9, not only the phosphorylation of STAT1 and STAT3 but also the transcriptions of iNOS, MCP-1, and ICAM-1 were not induced in asialo-GM1-treated primary microglial cells (Fig. 10). Taken together, these results suggest that the presence of sialic acid residues is important for gangliosides-stimulated JAK-STAT signaling, although the number of sialic residues per gangliosides molecule may not influence phosphorylation.

**B. Oxidized Low Density Lipoprotein (oxLDL) Suppresses Lipopolysaccharide (LPS)–induced Inflammatory Responses in Microglia.**

**1. OxLDL Potently Inhibits LPS-induced Expression of iNOS and COX2 in Primary Microglia Cultured from Rat Brain.**

To investigate the role of oxLDL on neuroinflammation, I first examined the effect of LDL or modified LDLs on the expression of iNOS and COX2, key enzymes in inflammatory processes in activated microglia. For the experiment, I prepared oxLDL using a standard method of AAPH-mediated oxidation (Neuzil et al., 1998; Shie et al., 2004). LDL was oxidized under 10 mM AAPH for 18 hr at 37℃. LDL or modified LDLs treatment for 24hr itself had no effect on iNOS and COX2 expression in rat primary microglia. But pretreatment of oxLDL for 1hr, but not LDL and AcLDL, markedly suppressed LPS-induced iNOS and COX2 expression in microglia (Fig. 11A and Fig 12). These anti-inflammatory effects of oxLDL were dose dependent (Fig. 11B). The inhibition effect of oxLDL was also observed when microglia was activated by gangliosides (Fig. 11C). Although oxLDL has been reported to show cytotoxic effects in other cells(Han and Pak, 1999; Sugawa et al., 1997), 50 μg/ml or below oxLDL had little effect on cell viability in our experiments. Thus, I used 50 μg/ml oxLDL for subsequent experiments.

**2. OxLDL Suppresses the Expression and Release of Inflammatory Mediators in an Oxidation-dependent Manner.**

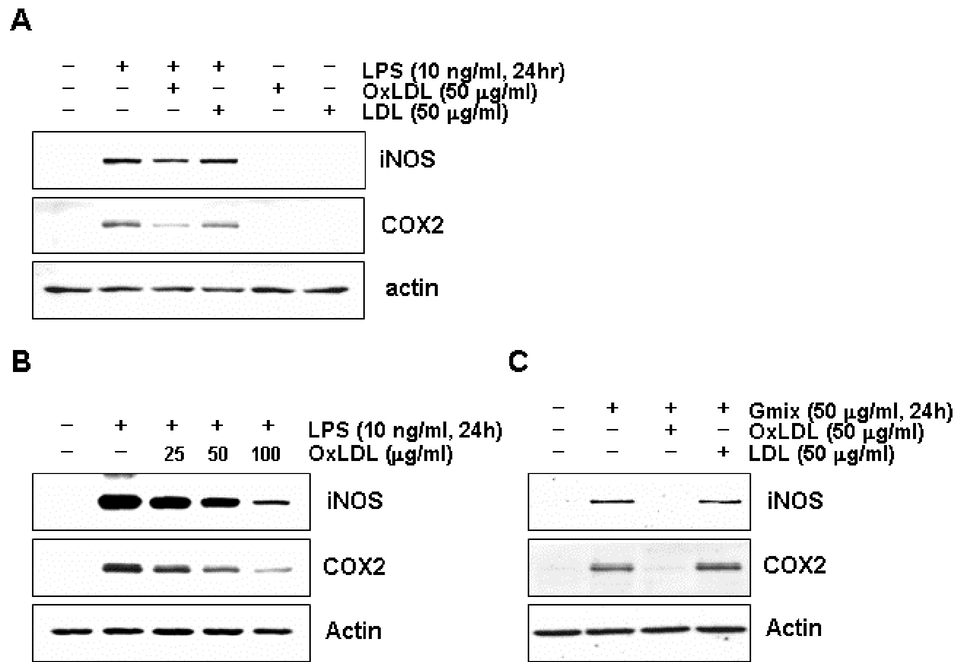



**Fig. 11. OxLDL suppressed iNOS and COX2 expression in LPS-activated microglia.** Rat primary microglia were first incubated with LDL or oxLDL (50 μg/ml) for 1h and then activated with LPS (10 ng/ml) (**A**) or Gmix (50 μg/ml) (**C**) for further 24h. (**B**) Microglia were pretreated with oxLDLs at indicated doses for 1h, and stimulated with LPS (10 ng/ml) for 24h. Then, cell lysates were separated using 10% SDS-PAGE, and Western blot analysis was performed using antibodies against iNOS and COX2. Actin was used as a loading control. Data shown are representative of three independent experiments.

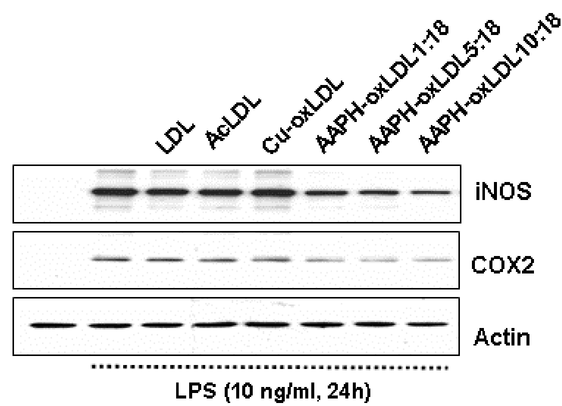



**Fig. 12. Effects of other modified LDLs on LPS-induced iNOS and COX2 expression in microglia.** Microglia were pretreated with indicated LDLs for 1h, and stimulated with LPS (10 ng/ml) for 24h. Then, cell lysates were separated using 10% SDS-PAGE, and Western blot analysis was performed using antibodies against iNOS and COX2. Actin was used as a loading control. Data shown are representative of three independent experiments. Ac-LDL: acetylated LDL; Cu-oxLDL: oxidized LDL by $Cu^{2+}$; AAPH-oxLDL: oxidized LDL by AAPH.

To better define the suppressive effect of oxLDL on brain inflammation, I tested the effects of oxLDLs with different degree of oxidation. LDLs were oxidized under various conditions to achieve different levels of oxidation, and I characterized the oxidation content of oxLDL using several methods. First, the oxidative modification of LDL was determined by REM assay. Alteration of electrophoretic mobility on the agarose gel reflects the increase of negative charge in LDL particle after oxidation. OxLDL not only migrates faster but is also less visible with Commassie Blue stain because of partial degradation of LDL protein. As the REM data in Fig. 13B show, the increase in electrophoretic mobility corresponded with the oxidation period (1.1, 1.4 and 2.3) and AAPH concentration (1.1, 1.7 and 2.3) in oxLDLs. Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA), the degradation products of polyunsaturated fatty acids (PUFAs) hydroperoxides (Esterbauer et al., 1991; Janero, 1990). As oxidation progressed, MDA content of oxLDL preparations increased gradually with oxidation period- and AAPH concentration-dependent manner as measured by TBARS assay (Table 2). The better parameters for LDL oxidation were the content of lipid hydroperoxides. Because TBARS assays use by-products as indicators of lipid peroxidation, I performed direct measurement of lipid hydroperoxide (Mihaljevic et al., 1996). As Table 2 show, LPO content of oxLDL preparations increased gradually with oxidation period- and AAPH concentration-dependent manner as measured by direct LPO assay.

I next determined the inhibitory effect of oxLDLs, which were different

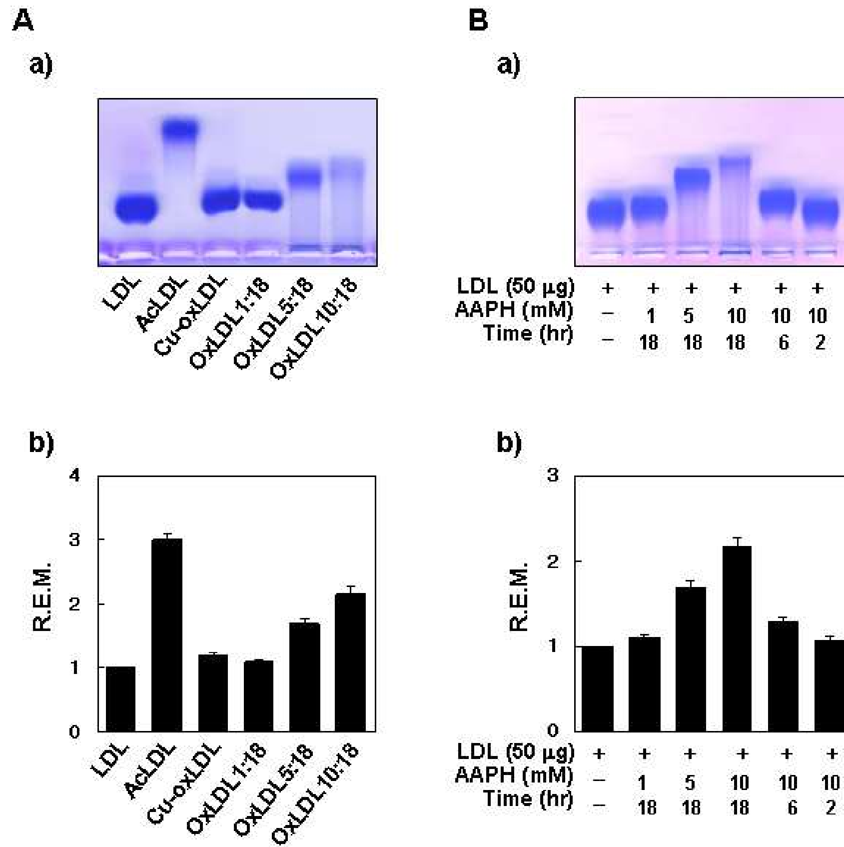



**Fig. 13. Relative agarose gel electromobility of modified LDLs.** LDLs were oxidized with various concentrations of AAPH for indicated time periods. After incubation, 50 µg of LDLs were loaded onto 0.8 % agarose gel for electrophoresis. The gel was stained with Coomassie brilliant blue R-250 (**a**), and the migration distance was measured (**b**). The stained gel is representative of three independent oxLDL preparations.

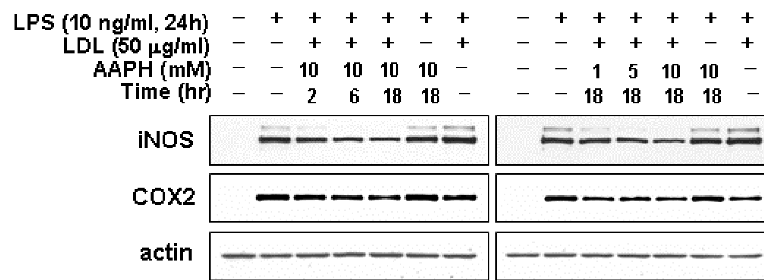
TABLE 2. Thiobarbituric acid-reacting substances and lipid hydroperoxide assay

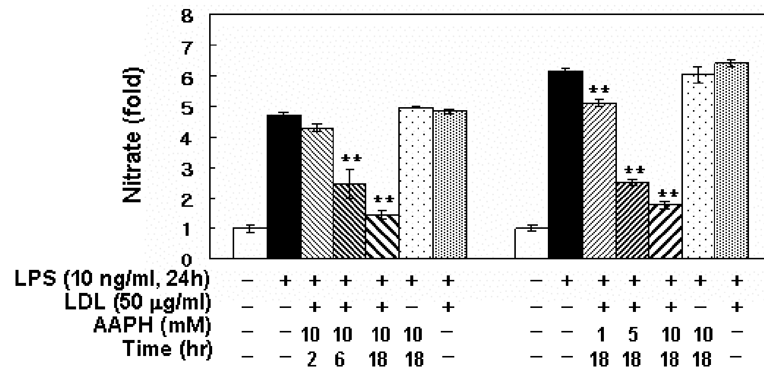
| | LDL | AcLDL | Cu-oxLDL | oxLDL 1:18 | oxLDL 5:18 | oxLDL 10:18 | oxLDL 10:6 | oxLDL 10:2* |
|---|---|---|---|---|---|---|---|---|
| TBARS (μM MDA /mg LDL) | 0.511 ± 1.016 | 3.071 ± 1.040 | 17.540 ± 0.768 | 7.938 ± 2.103 | 25.031 ± 2.266 | 32.394 ± 2.368 | 27.784 ± 2.464 | 21.382 ± 3.275 |
| LPO (nM LPO /mg LDL) | 5.310 ± 1.033 | 5.733 ± 0.634 | 5.289 ± 0.783 | 8.946 ± 0.735 | 17.159 ± 0.834 | 21.405 ± 0.627 | 18.709 ± 0.773 | 8.409 ± 2.054 |

* AAPH concentration (mM) : oxidized period (h)

levels of oxidation on LPS-induced inflammatory responses. First, LDLs were oxidized for three different time periods (2, 6 and 18 hr) in 10 mM AAPH (Fig. 14, left panel). As shown in Fig. 14A, oxLDL reduced LPS-induced iNOS and COX2 expression with oxidation period-dependent pattern. Also, LPS-mediated NO production was inhibited by oxLDLs pretreatment in oxidation period-dependent manner. The NO inhibition content of oxLDL-2hr, -6hr and -18hr were roughly 10, 50 and 70%, respectively (Fig. 14B). Next, I tested effects of oxLDLs on LPS-induced cytokine and chemokine secretion. As the ELISA data in Fig. 15A and 15B show, the LPS-induced secretion of both TNFα and MCP-1 also were reduced in the oxLDL pretreated microglia with oxidation period-dependent manner. LPS-induced TNFα secretions were inhibited by 13.7, 26.8 and 48.3%, respectively by the pretreatment of oxLDL-2hr, -6hr and -18hr (Fig. 15A). In the case of MCP-1, the inhibition content of oxLDL-2hr, -6hr and -18hr were 13.2, 37.1 and 67.5, respectively (Fig. 15B).

Second, I performed experiments as mentioned above using oxLDLs that oxidized for 18 hr in three different concentration of AAPH (1, 5 and 10 mM) (Fig. 14, right panel). OxLDLs suppressed LPS-induced iNOS and COX2 expression with AAPH concentration-dependent pattern (Fig. 14A). Also, NO production was inhibited by oxLDLs pretreatment in AAPH concentration-dependent manner. The NO inhibition content of oxLDL-1mM, oxLDL-5mM and oxLDL-10mM were roughly 20, 60 and 70%, respectively (Fig. 14B). LPS-induced TNFα secretions were inhibited by 9.6, 20.8 and 60.2%, respectively by the pretreatment of oxLDL-1mM, -


A
LPS (10 ng/ml, 24h) – + + + + + + – + + + + + +
LDL (50 µg/ml) – – + + + – + – – + + + – +
AAPH (mM) – – 10 10 10 10 – – – 1 5 10 10 –
Time (hr) – – 2 6 18 18 – – – 18 18 18 18 –
iNOS
COX2
actin


B
Nitrate (fold)
0
1
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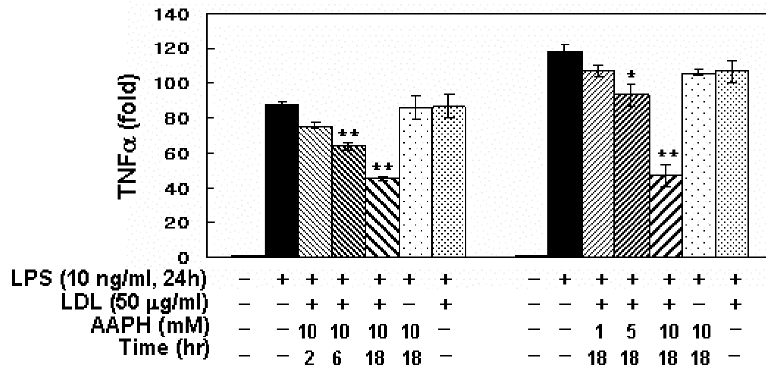
8
**
LPS (10 ng/ml, 24h) – + + + + + + – + + + + + +
LDL (50 µg/ml) – – + + + – + – – + + + – +
AAPH (mM) – – 10 10 10 10 – – – 1 5 10 10 –
Time (hr) – – 2 6 18 18 – – – 18 18 18 18 –

**Fig. 14. OxLDLs suppress LPS-induced iNOS expression and NO production in an oxidation-dependent manner.** Left panel, Rat primary microglia were pretreated with oxLDLs (50 μg/ml) that were oxidized with AAPH for indicated times and then stimulated with LPS (10 ng/ml) for 24h. Right panel, microglia were pretreated with oxLDLs (50 μg/ml) that were oxidized with indicated doses of AAPH for 18h and stimulated with LPS (10 ng/ml) for 24h. (**A**) Cell lysate was isolated and analyzed for levels of iNOS and COX2 protein using a Western blot analysis. (**B**) Media nitrite concentration was measured as an indication of NO release, using Griess reagent. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, *$P<0.05$; **$P<0.01$.

A

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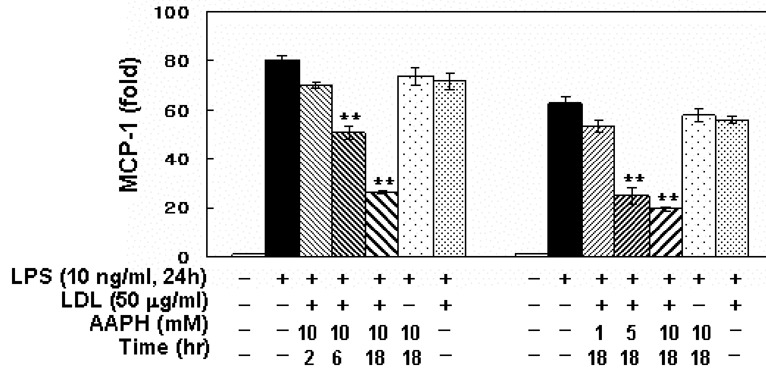


**Fig. 15. OxLDLs suppress LPS-induced TNFα and MCP-1 secretion in an oxidation-dependent manner.** Left panel, Rat primary microglia were pretreated with oxLDLs (50 μg/ml) that were oxidized with AAPH for indicated times and then stimulated with LPS (10 ng/ml) for 24h. Right panel, microglia were pretreated with oxLDLs (50 μg/ml) that were oxidized with indicated doses of AAPH for 18h and stimulated with LPS (10 ng/ml) for 24h. Release of TNFα (**A**) and MCP-1 (**B**) were determined by ELISA. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, *$P<0.05$; **$P<0.01$.

5mM and -10mM (Fig 15A). In the case of MCP-1, the inhibition content of oxLDL-1mM, -5mM and -10mM were 14.8, 60.2 and 68.9%, respectively (Fig.15B).

REM assay revealed 3-fold increase in electrophoretic mobility in AcLDL than in LDL (Fig. 13A). LPO production level was similar in AcLDL and LDL, although MDA production was found to be higher (about 6-fold) in AcLDL in TBARS assay (Table 2). Cu-oxLDL, another modified LDL which did not have a suppressive effect against the LPS-induced iNOS expression, showed no differences in electronegativity (Fig. 13A) and LPO content, while showed significant up-regulation of MDA content as shown in Table 2. The MDA content of Cu-oxLDL was 18 μM/mg, which was similar with oxLDL5:18 having sufficient suppressive effect. Based on these results, suppressive effect of AAPH-oxLDL is considered to be elicited by oxidation (especially on LPO content) increased in proportion to oxidation time and AAPH concentration.

**3. Inhibitory Effects of OxLDL are Mediated by Interference with LPS-induced NFκB Activation, IFNβ Release, and STAT1 activation in Brain Microglia.**

The next question I addressed was how oxLDL pretreatment inhibited LPS-induced inflammatory responses. The first possibility was that oxLDL simply blocked LPS binding on their receptor. This possibility was tested by adding oxLDL at times after treatment of LPS. I added oxLDL into cells at various time points, from 1 hr pretreatment to 1 hr after treatment of LPS, and incubated for indicated times. The suppressive effects of oxLDL on expression of iNOS and COX2 were

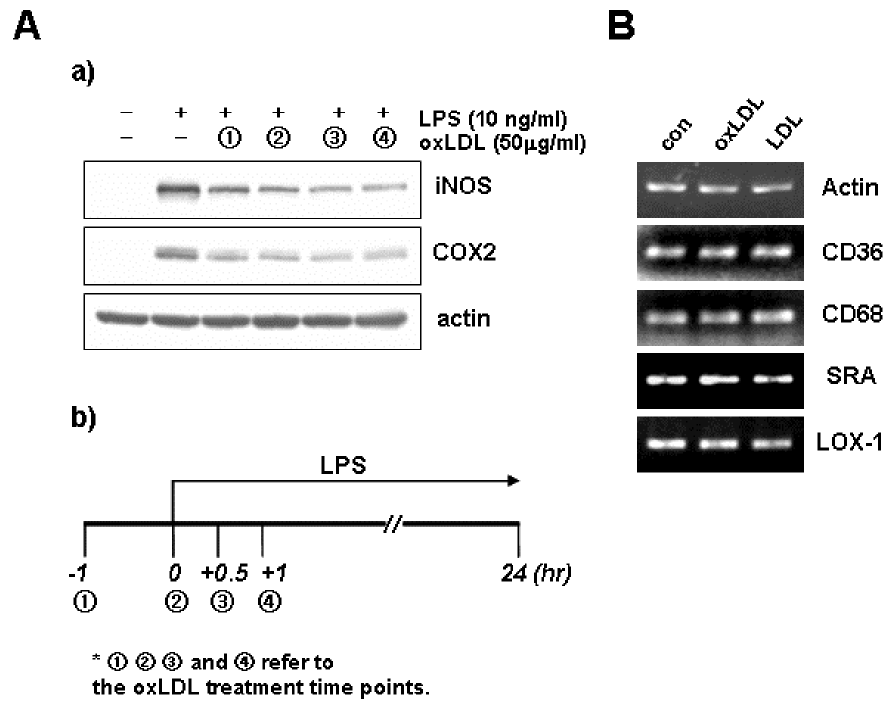



**Fig. 16. The inhibitory effects of oxLDL are not due to the displacement of LPS from their cellular receptor.** (**A**) OxLDL (50 µg/ml) was added to microglia 1h before, simultaneously with or after (0.5 or 1h) LPS (10 ng/ml). iNOS, COX2 and Actin were determined in microglia lysates by Western blot analysis. (**B**) Rat primary microglia were stimulated with LDL or oxLDL (50 µg/ml) for 8 hrs. Total RNA was isolated and analyzed for levels of genes of oxLDL receptors using an RT-PCR assay.

observed at all the times tested (Fig. 16A). These results indicate that the inhibitory effects of oxLDL are not due to the displacement of LPS from their cellular receptor. Actually, scavenger receptors that recognize oxLDL include CD36 (Endemann et al., 1993), CD68 (Ramprasad et al., 1996), SRA (Kodama et al., 1990) and LOX-1 (Yoshida et al., 1998) expressed in control microglia (Fig. 16B). LDL or oxLDL had no effect on mRNA levels of these receptors. Therefore, I suggest that oxLDL internalize into microglia via scavenger receptors and can affect intracellular signaling.

LPS induces inflammatory responses via NF-κB-IFNβ-STAT pathway (Jacobs and Ignarro, 2001). First, I investigated the effect of oxLDL pretreatment on LPS-induced NF-κB activation through IκB phosphorylaiton and degradation. Pretreatment of oxLDL inhibited LPS-induced IκB phosphorylaiton and reversed LPS-induced IκB degradation (Fig. 17A). I next determined the effect of oxLDL on LPS-induced IFNβ mRNA expression. IFNβ is critical and necessary component of the signaling processes leading to inflammation and LPS-induced delayed STAT1 activation (Gao et al., 1998). IFNβ mRNA expression and tyrosine phosphorylation of STAT1 by LPS was markedly suppressed in oxLDL-treated primary microglia (Fig. 17B and C). LPS also induces inflammatory responses via MAPK pathway (Lee et al., 2001). Pretreatment of oxLDL had no inhibitory effect on MAPK phosphorylation when LPS treated for 45 min (Fig. 18A) or 3h (Fig. 18B).

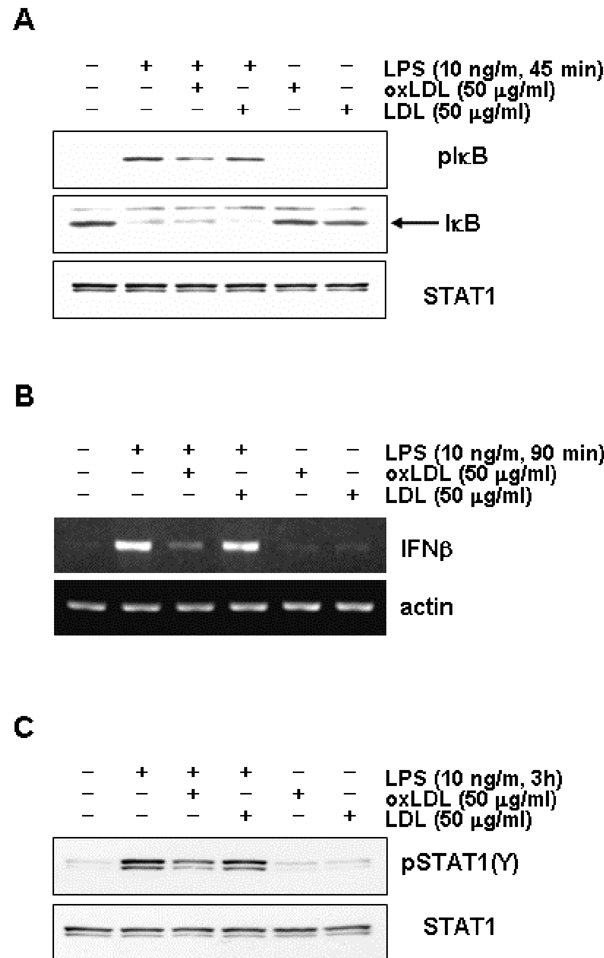



**Fig. 17. OxLDL inhibit LPS-induced intracellular signaling pathway in microglia.** Microglia were pretreated with LDL or oxLDL (50 µg/ml) for 1h and stimulated with LPS (10 ng/ml) for 45 min (**A**), 90 min (**B**) or 3h (**C**). Phosphorylation of signaling molecules were analyzed by Western blotting. Expression of IFNβ was analyzed by RT-PCR assay. Data shown are representative of three independent experiments.

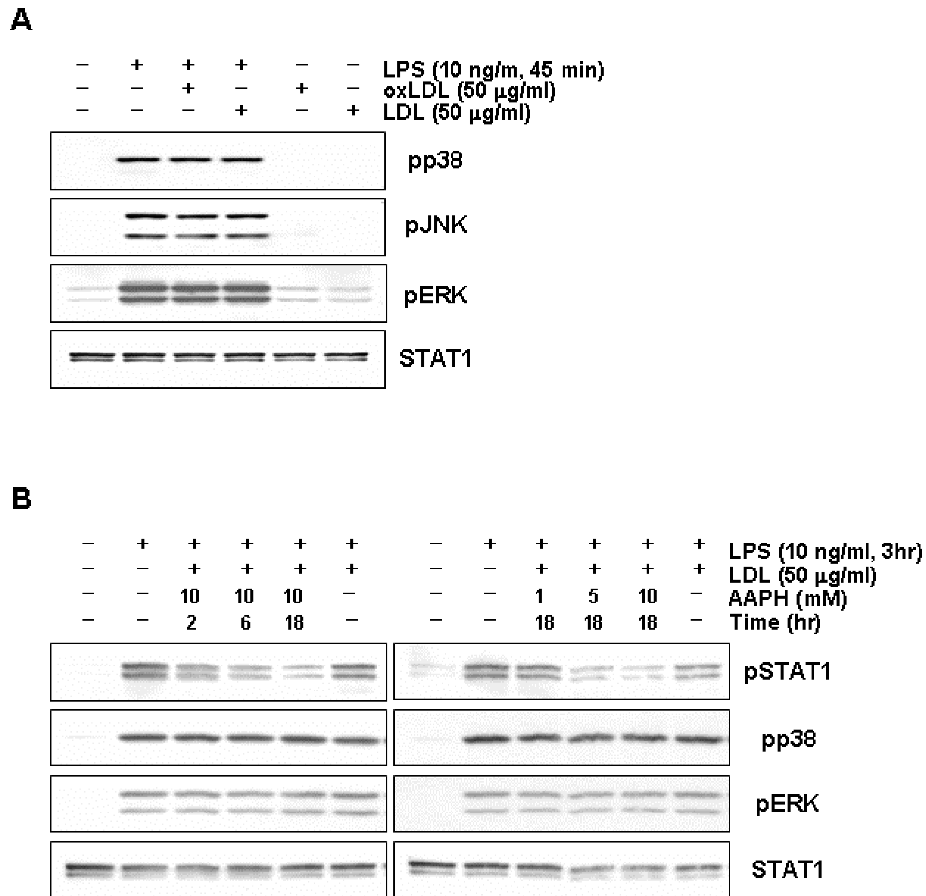


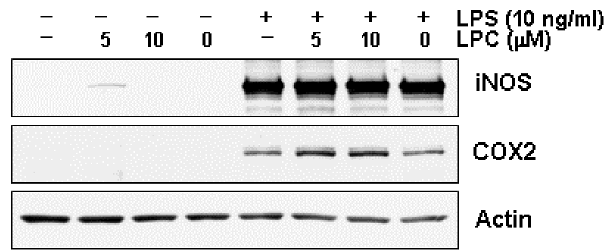

**Fig. 18. OxLDL dose not inhibit LPS-induced MAPK pathway in microglia.** Microglia were pretreated with LDL or oxLDL (50 µg/ml) for 1h and stimulated with LPS (10 ng/ml) for 45 min (**A**) or 3h (**B**). Phosphorylation of signaling molecules were analyzed by Western blotting. Data shown are representative of three independent experiments.

**4. Oxysterol/LXR Agonists Mimic Anti-inflammatory Effects of OxLDL on LPS-activated Microglia.**

In order to identify the active components of oxLDL that contribute to the suppression of inflammatory responses, the effects of lysophosphatidylcholine (LPC) and oxysterols were studied individually. Pretreatment of LPC did not suppress LPS-induced inflammatory responses (Fig. 19A). Trans-BTP, platelet-activating factor (PAF) receptor antagonist, had no effect on anti-inflammatory effect of oxLDL (Fig. 19B). Oxysterols, 7-ketocholesterol (7KC) and 22 (R) hydroxycholesterol (22(R)HC), markedly inhibited LPS-induced iNOS and COX2 expression (Fig. 19C). Oxysterol derived from internalized oxLDL or generated intracellularly through modification of cholesterol is known as natural LXR agonist (Janowski et al., 1996). Microglia expressed LXRα/β mRNA and LDL, oxLDL, oxysterol and LXR agonist had no effect on its expression. At the same condition, the level of mRNA of ATP binding cassette transporter-1 (ABCA-1) known LXR-regulated gene increased in oxLDL, oxysterol and LXR agonist treated microglia (Fig. 20A). These data suggest that LXR may have utility in oxLDL/oxysterol-modulated microglial inflammation. To investigate the relations of LXRs in anti-inflammatory effects of oxLDL/oxysterol, I tested the influence of LXR agonists on LPS-induced microglial responses. LPS-stimulated microglia pretreated with the synthetic LXR agonists(Edwards et al., 2002), TO901317 and GW3965 showed significantly reduced LPS-induced iNOS and COX2 expression (Fig. 20B). The inhibitory effects of oxysterol/LXR agonist on LPS-induced inflammatory responses, such as iNOS expression (Fig. 21A), NO

production (Fig. 21B), TNFα (Fig. 22A) and MCP-1 secretion (Fig. 22B), were investigated. LPS-mediated NO production and MCP-1 secretion was inhibited by oxysterol/LXR agonist in dose-dependent manner. LPS-induced TNFα secretion was inhibited by oxysterol in dose-dependent manner, but wasn't affected by LXR agonist. To further explore the role of LXRs involved in the inhibitory function of oxLDL, the effect of RXR agonist (methoprene acid; MA) was evaluated (Fig. 23 and Fig. 24). Pretreatment of MA potentiated inhibitory effect of oxLDL on LPS-induced iNOS expression and NO production (Fig. 23, A and B) and MCP-1 secretion (Fig. 24B). Inhibition effect of oxLDL on LPS-induced TNFα secretion wasn't affected by MA pretreatment (Fig. 24A). These observations suggest that inhibitions of inflammatory responses by oxLDL/oxysterols are mediated by LXR-dependent (NO production and MCP-1 secretion) and – independent pathway (TNFα secretion).

A

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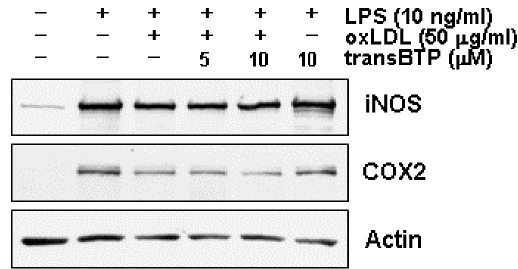


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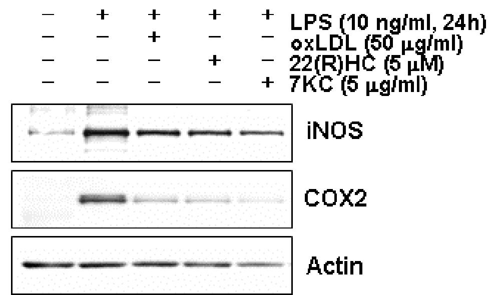


**Fig. 19. Individual components of oxLDL for their anti-inflammatory effects.** (**A**) Lysophosphatidylcholine (LPC) does not suppress LPS-induced iNOS and COX2 expression. Primary microglia were pretreated with indicated concentrations of LPC for 1h, and stimulated with LPS (10 ng/ml) for 24h. (**B**) Trans-BTP, platelet-activating factor (PAF) receptor antagonist, has no effect on anti-inflammatory effect of oxLDL. Trans-BTP treated for 30 min before oxLDL treatment. (**C**) Oxysterols suppress LPS-induced inflammatory responses. Rat primary microglia were pretreated with 7-ketocholesterol or 22-(R)-hydroxycholesterol and then stimulated with LPS (10 ng/ml) for 24h. Cell lysate was isolated and analyzed for levels of iNOS and COX2 protein using a Western blot analysis

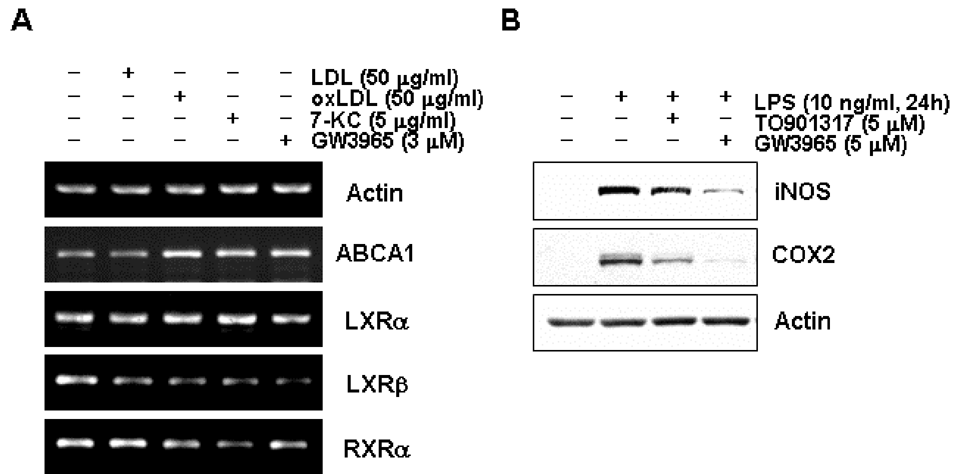



**Fig. 20. LXR agonists mimicked anti-inflammatory effect of oxLDL.** (**A**) Microglia were stimulated with the indicated dose of LDL, oxLDL, 7KC or GW3965 for 8 hrs. LXRα/β, RXRα, ABCA1 and Actin were determined in microglia RNA by RT-PCR analysis. (**B**) Microglia were pretreated with LXR agonists, TO901317 (5 μM) or GW3965 (5 μM) for 1h and then stimulated with LPS (10 ng/ml) for 24h. Expression of iNOS and COX2 were analyzed by Western blotting.

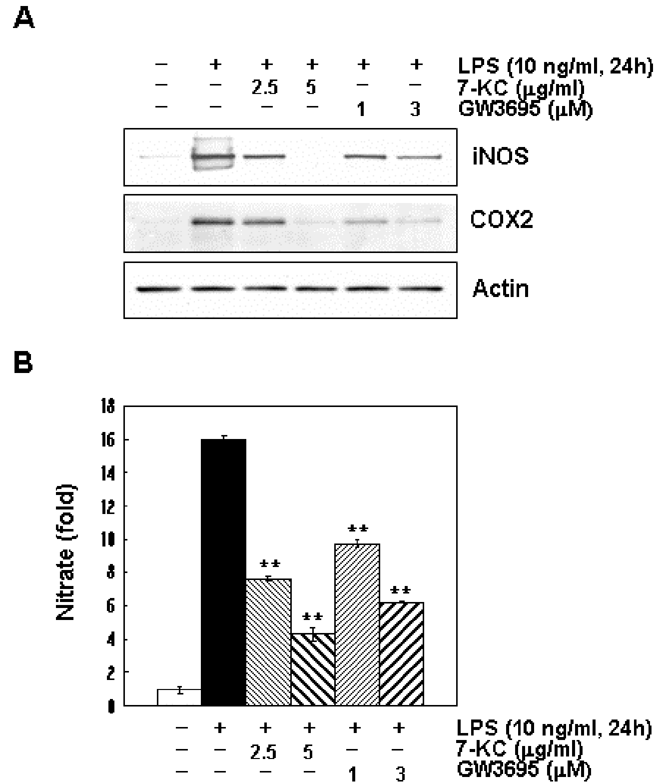



**Fig. 21. Oxysterols/LXR agonists suppress LPS-induced iNOS expression and NO production in microglia.** Microglia were pretreated with LXR agonists, TO901317 (5 μM) or GW3965 (5 μM) for 1h and then stimulated with LPS (10 ng/ml) for 24h. (**A**) Cell lysate was isolated and analyzed for levels of iNOS and COX2 protein using a Western blot analysis. (**B**) Media nitrite concentration was measured as an indication of NO release, using Griess reagent. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, **P<0.05; **P<0.01*.

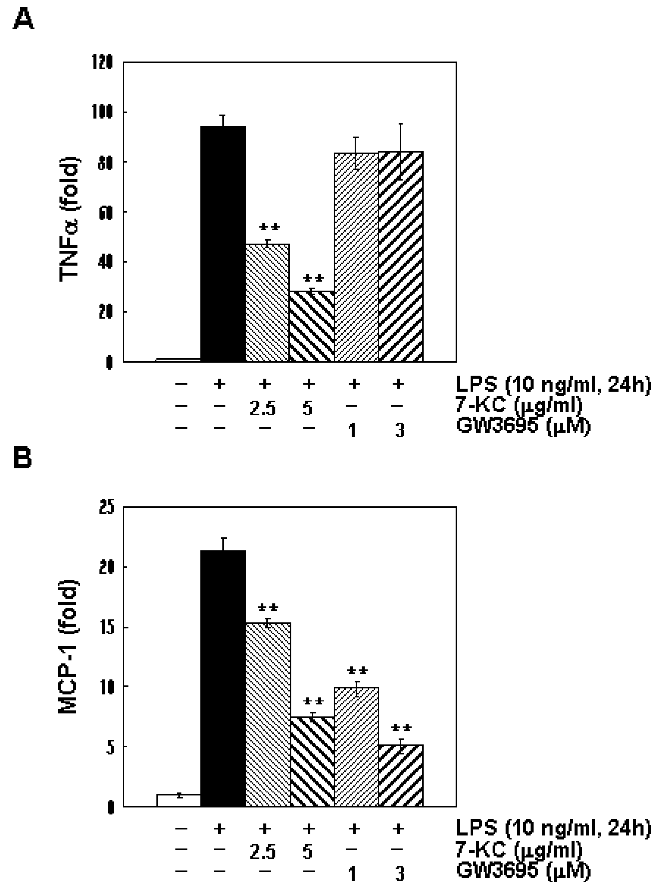
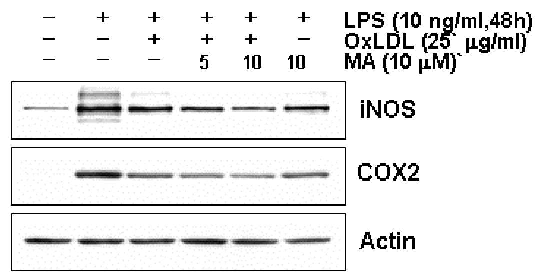



**Fig. 22. Oxysterols/LXR agonists suppress LPS-induced TNFα and MCP-1 secretion in microglia.** Microglia were pretreated with LXR agonists, TO901317 (5 μM) or GW3965 (5 μM) for 1h and then stimulated with LPS (10 ng/ml) for 24h. Release of TNFα (**A**) and MCP-1 (**B**) were determined by ELISA. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, **P<0.05; **P<0.01.*

A

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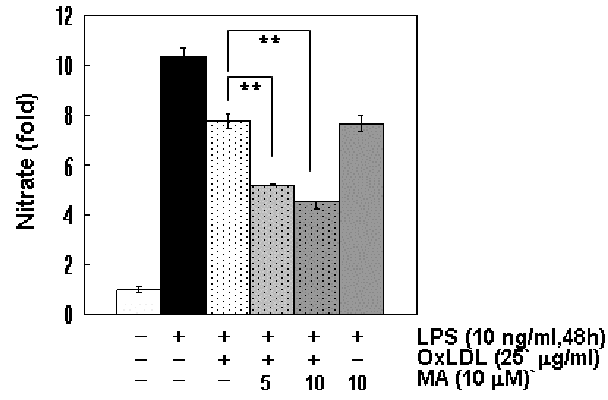

**Fig. 23. RXR agonist potentiates anti-inflammatory effects of oxLDL on LPS-induced iNOS expression and NO production in microglia.** RXR agonist, MA (10 μM), treated for 30 min before oxLDL treatment. Rat primary microglia were pretreated with oxLDL (25 μg/ml) for 1h and then stimulated with LPS (10 ng/ml) for 24h. (**A**) Cell lysate was isolated and analyzed for levels of iNOS and COX2 protein using a Western blot analysis. (**B**) Media nitrite concentration was measured as an indication of NO release, using Griess reagent. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, $^{*}P<0.05$; $^{**}P<0.01$.

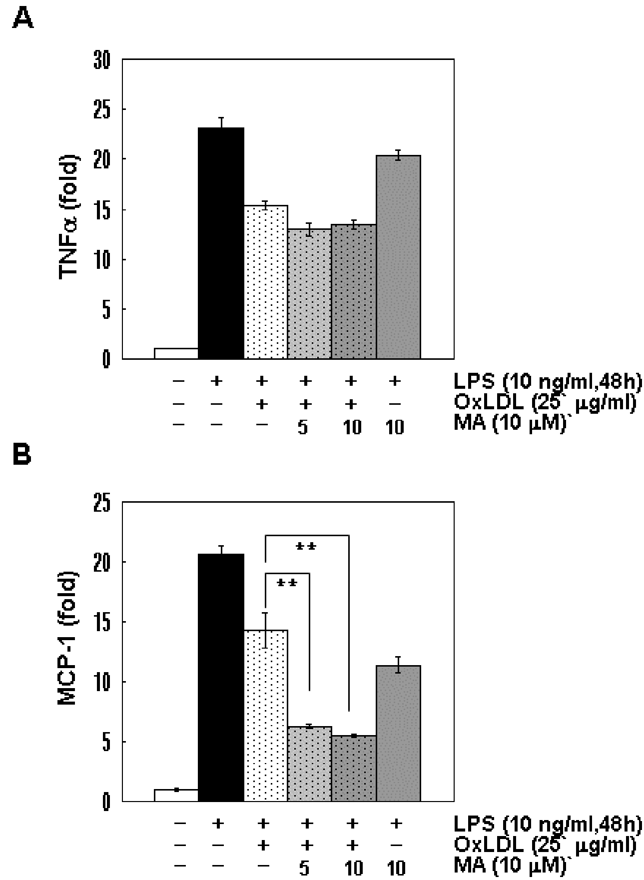


**Fig. 24. RXR agonist potentiates anti-inflammatory effects of oxLDL on LPS-induce MCP-1 secretion in microglia.** RXR agonist, MA (10 μM), treated for 30 min before oxLDL treatment. Rat primary microglia were pretreated with oxLDL (25 μg/ml) for 1h and then stimulated with LPS (10 ng/ml) for 24h. Release of TNFα (**A**) and MCP-1 (**B**) were determined by ELISA. Data represent the mean ± S.E.M. of three independent experiments. Significantly different from the LPS group by Dunnett's multiple range test, **P<0.05; **P<0.01.*

# IV. Discussions

## A. JAK-STAT Signaling Mediates Gangliosides-induced Inflammatory Responses in Brain Microglial Cells

Increasing evidence indicates that gangliosides act not only as mediators for cellular interactions but also as modulators of signal transduction in a variety of cellular events. These functions appear to occur simultaneously and influence each other (Hakomori, 1990). Identification of the precise mechanisms underlying how gangliosides regulate cellular responses has been the subject of many investigations, but it appears there is still much that is unknown (Gouni-Berthold et al., 2001; Kanda and Watanabe, 2001; Kasahara et al., 1997). In this study, I reveal that gangliosides directly induce the activation of JAK-STAT signaling, a key pathway in inflammation, which leads to the expression of several inflammation-associated genes.

JAK-STAT signaling has been reported to be closely involved in inflammation. Although STAT proteins were discovered during the course of analysis of interferon signaling, recent studies (Darnell, Jr., 1997; Fu et al., 1990; Kessler et al., 1990) have revealed that STAT signaling can account for various cellular responses to a number of cytokines, growth factors, and hormones. IL-4 and IL-13 stimulate enhanced expression of major histocompatibility complex class II, CD23, IL-4Ra chain, and Ig class switching to IgE and IgG via activation of STAT6

(Jiang et al., 2000; Nelms et al., 1999). The common cytokine receptor γ-chain, which is shared by receptors for IL-2, -4, -7, -9, and -15, associates with JAK3, thus resulting in STAT-dependent immune responses (Bacon et al., 1995; Johnston et al., 1994). Prolactin, erythropoietin, and growth hormone are all known to activate JAK2 (Imada and Leonard, 2000).

The functional association between cytokine signaling and JAK-STAT signaling prompted us to examine the involvement of STATs in gangliosides-induced inflammatory responses. Consistent with a connection between gangliosides and STAT activity, I found that gangliosides treatment of brain microglial cells increased the binding activity of a nuclear factor to a consensus GAS/ISRE element (Fig. 1). It has been reported that STAT1 and -3 are major STAT types that bind GAS and ISRE elements, and that they function to regulate the transcription of numerous genes (Ehret et al., 2001; Gao et al., 1997). By using supershift antibodies for STAT1 and STAT3, I determined that they are constituents of nuclear factor binding complex to the GAS/ISRE element in gangliosides-stimulated microglial cells (Fig. 2). At the present time, I cannot clearly identify the individual bands since all the three bands were reduced by addition of not only anti-STAT1 but also anti-STAT3. However, our EMSA data and Western blotting data convincingly proved the involvement of STAT1 and STAT3 in gangliosides-induced inflammatory responses. Next, I investigated whether gangliosides could induce the phosphorylation of these particular STATs. As expected, phosphorylation of STAT1 and -3 was induced within 1 min and then rapidly decreased to basal levels (Fig. 3). The kinetics of these

phosphorylation events were consistent with the timing of binding activity to GAS/ISRE elements. These results show that STAT1 and -3 may directly mediate gangliosides-induced microglial activation. NF-κB is also reported to be involved in gangliosides-induced microglial activation, but it is considered to be a pathway common to a range of microglial activators (Pyo et al., 1999). Thus, it may be that early activation of STATs by gangliosides is a specific mechanism underlying gangliosides-induced microglial activation.

Phosphorylation of JAKs leads to their activation, and activated JAKs phosphorylate and hence activate STATs. I examined the effect of gangliosides on JAK1 and JAK2 to determine the cause of STAT1 and STAT3 phosphorylation. I found that both JAK1 and JAK2 were phosphorylated within 5 min of the addition of gangliosides (Fig. 4). Furthermore, AG490, a JAK inhibitor, diminished both gangliosides-enhanced phosphorylation of STATs and nuclear factor binding activity. These data provide strong evidence to indicate that gangliosides activate STATs through activation of JAKs.

In general, inflammatory stimuli induce release of mediators such as cytokines, chemokines and cell adhesion molecules (Laskin and Pendino, 1995). Regulation of mediator release is important for controlling inflammation. I investigated the transcription of inflammation-associated genes that contain functional GAS elements in their promoters. RT-PCR analysis showed that gangliosides enhanced the transcript level of ICAM-1 and MCP-1 within 3 h, whereas pretreatment with AG490 inhibited this increase (Fig. 6). These results

provide further evidence for involvement of JAK-STAT inflammatory signaling in gangliosides-induced microglial activation. Moreover, AG490 significantly reduced gangliosides-induced NO release, indicating that NO production was partly dependent on JAK-STAT signaling (Fig. 7). Taken together, these results suggest that inflammatory mediators including MCP-1, ICAM-1, and NO may be induced in response to gangliosides through JAK-STAT signaling.

I next examined other signaling events that may be associated with JAK-STAT activation in gangliosides-treated microglial cells. Having previously observed that ERKs and p38 MAPKs were activated by gangliosides, I investigated whether gangliosides-stimulated JAK-STAT signaling was linked to activation of ERKs and p38 MAPKs. By using pharmacological inhibitors, I found that gangliosides-stimulated activation of JAK resulted in phosphorylation of ERK1/2 (but not p38) (Fig. 8), suggesting cross-talk between JAK and ERK pathways. Interestingly, inhibition of ERK by PD98059 resulted in complete inhibition of p38 activation, indicating cross-talk between different MAP kinase pathways. Because AG490 only partially reduced the phosphorylation of ERK, but PD98059 completely inhibited the activation of ERKs and p38, it is likely that activation of p38 is downstream of ERK activation and that any connection occurs via signaling molecules other than JAK-STATs. The activation profile of signaling molecules seems to depend on stimulators and/or cell types. Furthermore, upstream and downstream signaling molecules may be specifically affected by cross-talk and convergence in a particular environment. For example, Gouni-Berthold *et al.* (Gouni-Berthold et al., 2001) recently reported

that gangliosides had no effect on phosphorylation of p38 in vascular smooth muscle cells, and that platelet-derived growth factor-BB-induced phosphorylation of p38 was not influenced by PD98059. In contrast, both MAPKs are activated by lipopolysaccharides and gangliosides in primary glial cells (Bhat et al., 1998; Pyo et al., 1999). In view of the fact that gangliosides-induced activation of JAK-STAT signaling occurs more rapidly than activation of ERKs, and that inhibition of JAK activation reduced ERKs activation, it appears gangliosides-stimulated JAK-STAT signaling regulates ERKs activation in microglial cells. In contrast, p38 activation does not seem to be linked to JAK-STAT signaling, even though it too plays a role in microglial activation.

Gangliosides are amphipathic molecules that belong to a class of anionic glycosphingolipids. They contain sialic acid residues (*N*-acetylneuraminic acids), linked to the sugar residues of a ceramide oligosaccharide. It has been reported (Gouni-Berthold et al., 2001; Kopitz et al., 1998) that different types of gangliosides have distinct roles in several cell types. To gain insight into the mechanism underlying JAK-STAT activation by gangliosides, I investigate whether GM1 and GD1a, the major types of gangliosides mixture used in these studies (18 and 55%, respectively), had different effects on STAT activation. Despite containing different numbers of sialic acid residues per molecule, GM1 containing one and GD1a containing two, both gangliosides activated STAT1 to a similar extent, which was similar to the activation caused by the gangliosides mixture. Although the different number of sialic acid residues on gangliosides appeared to have no influence on

STAT phosphorylation, the presence of sialic acid on gangliosides was critical, since removal of sialic acid by neuraminidase resulted in reduced STAT1 phosphorylation (Fig. 9). These results are further confirmed by experiments to compare activities of GM1 and asialo-GM1. Unlike GM1, asialo-GM1 did not induce phosphorylation of STAT or expression of STAT-responsive genes including iNOS (Fig. 10) Thus, it appears sialic acid residues of gangliosides are important for STAT activation, and one residue per molecule is sufficient. However, I do not exclude the possibility that other moieties of gangliosides also importantly affect JAK-STAT signaling in microglial cells.

It is intriguing, but unresolved, as to how gangliosides stimulate phosphorylation of JAKs. One possibility is that gangliosides act by binding to specific cell surface receptors. However, although galectin-1 and -3 are reported to bind GM1, the specific receptors of gangliosides have not been clearly elucidated (Kopitz et al., 1998). Alternatively, gangliosides may act by modulating other signaling molecules. For example, there are reports (Garofalo et al., 1998; Gouy et al., 1994; Meuillet et al., 1996; Mitsuda et al., 2002) that incorporation of gangliosides into plasma membranes regulates CD4, growth factor receptors, and phospholipase C. It may be that the gangliosides-enriched membrane domains, assigned as lipid rafts, regulate intermolecular associations in the plasma membranes. Many transmembrane receptors are reported to be constitutively or inducible localized within lipid rafts (Kantakamalakul et al., 1999; Okuyama et al., 1979; Simons and Toomre, 2000). It has been reported that the Src family kinases Rho, FAK, Lyn, and

Lck are associated with gangliosides-enriched lipid rafts (Iwabuchi et al., 1998; Kasahara et al., 1997; Prinetti et al., 1999). Interestingly, a recent report (Takaoka et al., 2000) showed that JAK1 and JAK2 are exclusively localized in lipid rafts of mouse embryonic fibroblasts. Furthermore, it has been suggested that JAKs interact not only with STATs but also with several other proteins such as Grb2, Shc, Vav, Fyn, and c-Abl (Danial et al., 1995; Stark et al., 1998). Based on these reports and our current findings that JAK-STAT signaling is rapidly activated by gangliosides, it is possible that gangliosides physically and functionally associate with certain signaling molecules in membranes, thereby activating JAKs. In this regard, I am interested in the composition and functional organization of gangliosides in the membrane. Further studies are needed to clarify how gangliosides act to initiate JAK-STAT signaling. These studies will provide important information regarding microglial activation.

In conclusion, our findings suggest that gangliosides rapidly trigger JAK-STAT inflammatory signaling, resulting in brain microglial activation. Our studies indicate that gangliosides-stimulated JAK-STAT signaling mediates several important inflammatory events, including production of NO and transcription of inflammatory mediators. Gangliosides are now emerging as important factors in signaling pathways and neuronal diseases. Our data provide significant new information regarding the molecular mechanisms underlying gangliosides-induced microglial inflammation, and such knowledge will assist in the better understanding of the pathogenesis of brain disease.

## B. Oxidized Low Density Lipoprotein (oxLDL) Suppresses LPS–induced Inflammatory Responses in Microglia

Based on the controversial roles of LDL and oxLDL during cellular functioning, I explored their activity in the brain. Here, I demonstrate that oxLDL inhibits inflammatory responses in LPS-activated rat primary microglia. Since their anti-inflammatory effects are mimicked by oxysterols and at least in part by LXR agonists, I conclude that oxidative stress regulates inflammatory responses through oxidative modulation of LDL in an oxidation-dependent manner in brain microglia.

I prepared oxLDL according to the standard method of AAPH-mediated oxidation. Oxidative modification of LDL was essential for the observed anti-inflammatory effects, as no inhibitory effects were noted with native LDL and AcLDL. Moreover, the inhibitory effects of AAPH-oxLDL showed a positive correlation with oxidative stress. However, I did not observe any apparent inhibitory effects using Cu-oxLDL, which showed a significant increase in MDA as measured by TBARS assay, but not in direct measurements of LPO (Fig. 2B and Table 2). This finding is consistent with a previous report that the LPO content of oxLDL is responsible for its induction of cellular damage (Zorn-Pauly et al., 2005).

The biological effects of oxLDL are largely mediated by products formed during oxidation of the lipid component of LDL. Since the composition of oxLDL might be altered during the oxidation process and each component of oxLDL triggers its own intracellular responses, the degree of LDL oxidation might be one of the most important factors in determining its diverse biological functions. Oxidation of

LDL is a complex reaction that generates various lipid mediators, and the functional complexity of oxLDL is likely to reflect the relative level of these mediators in the particle. Among these lipid mediators, lysophosphatidylcholine (LPC) and oxysterols are of special interest because they are among the major constituents and display opposite effects on inflammation. As previously elucidated by other groups, I also observed a pro-inflammatory, not anti-inflammatory effect, in LPC-treated cells. The anti-inflammatory effects of oxLDL were simulated by two oxysterols, 7KC and 22(R)HC. Oxysterols, which are mono-oxygenated derivatives of cholesterols, may be formed by either spontaneous or enzyme-mediated processes. The spontaneous oxidation of cholesterol is a well-recognized phenomenon, generally termed auto-oxidation, which can be initiated by compounds including various radical species, lipid peroxides, and divalent metal cations such as copper ions. In general, oxysterols are known to be more toxic than cholesterol itself, and I also observed that oxysterols exhibit cytotoxic effects at higher doses but dramatic anti-inflammatory effects at the relatively low dose used in our experiments. Thus, the anti-inflammatory effects of AAPH-oxLDL are thought to be mediated by its oxysterol components.

Because oxysterols are natural ligands for LXRs, I tested whether LXRs were involved in the anti-inflammatory mechanism of oxLDL/oxysterols. A recent study showed that LXRs play critical roles in inflammation in addition to cholesterol metabolism and fatty acid homeostasis. Two LXR isoforms (LXRα and LXRβ) encoded by distinct genes, have been identified. LXRs form obligate heterodimers with RXRs, which are members of the nuclear receptor superfamily that can be

regulated by 9-cis-retinoic acid (9cRA) and the synthetic RXR agonist MA. To gain insight into the mechanism responsible for the anti-inflammatory effects elicited by oxLDL/oxysterols, I tested whether LXR activation mimicked the anti-inflammatory effects of oxLDL/oxysterols in LPS-activated microglia using the synthetic LXR agonists TO901318 and GW3965. While NO and MCP-1 release was suppressed by treatment with oxLDL, oxysterols, and LXR agonists, TNFα release was suppressed by oxLDL and oxysterols, but not by LXR agonists. The potentiation of inhibitory responses by MA was not observed in TNFα release, further suggesting the existence of an LXR-independent mechanism. Activators of PPARγ, another nuclear receptor that forms heterodimers with RXR, also antagonize inflammatory responses in both receptor-dependent and -independent manners. Moreover, I noted that MCP-1 and TNFα are differentially regulated, with activator protein (AP)-1 signaling performing a critical role in protein expression of MCP-1, but not of TNFα (Lee and Jou, unpublished data). Altogether, these results show that oxLDL/oxysterols are likely to have LXR-independent effects (Cader et al., 1997; Chawla et al., 2001; Park et al., 2003). Moreover, we noted that MCP-1 and TNFα are differentially regulated, with activator protein (AP)-1 signaling performing a critical role in expression of MCP-1, but not in TNFα (Lee and Jou, unpublished data). Altogether, these results show that oxLDL/oxysterols are likely to have LXR-independent effects.

How do oxLDL/oxysterols suppress inflammatory mediator release in LXR-dependent and -independent manners in LPS-stimulated microglia? I excluded the possibility that oxLDL interferes with LPS binding to its receptors extracellularly by

adding oxLDL at several time points before and after LPS treatment. The suppressive effects of oxLDL on cellular responses such as inflammation (Joseph et al., 2003) and apoptosis (Valledor et al., 2004) primarily occur through alteration in gene expression. In our system, however, inhibitory effects on inflammation were still observed even when oxLDL was added after LPS treatment. Thus, I suggest that oxLDL interferes with LPS-induced signal transduction. LPS activates a Toll-like receptor (TLR), which causes activation of NFκB. The gene encoding IFNβ is transcribed and translated, and the secreted product binds to interferon receptor (IFNR), which results in STAT1 phosphorylation (pSTAT1). Activation of MAPK-mediated pathways by LPS may represent an additional mechanism for STAT1 phosphorylation. NFκB, pSTAT1, and MAPK act in concert to promote expression of inflammatory genes (Jacobs and Ignarro, 2001), but in our experimental conditions, oxLDL inhibited LPS-induced signal transduction pathways via suppression of IFNβ, NFκB, or pSTAT1, but not MAPK signaling.

In spite of numerous reports that oxidative stresses are harmful to cellular physiology and thereby result in the pathogenesis responsible for diverse human diseases, anti-oxidant strategies have shown limited success in treating human disease. The present study shows that AAPH-oxLDL suppresses inflammatory mediator release in LPS-activated microglia and that these inhibitory effects can be simulated by oxysterols, particularly in doses lower than those that cause cytotoxicity. Oxysterols residing in biological membranes as well as lipoprotein are normally present in trace amounts; thus, it is biologically relevant that they can act at these

lower doses. Our study also reveals that oxLDL/oxysterols may have opposing cellular functions depending on the degree of oxidation. Most importantly, the present data reveal that oxidative stresses act indirectly through regulation of inflammatory responses, which may help explain why anti-oxidant strategies have thus far underperformed in clinical situations. Thus, the relationship between oxidative stress and inflammation needs to be further clarified and the signaling pathways involved need to be fully dissected for these molecules to be developed into effective treatments for various neurodegenerative diseases.

# V. CONCLUSION

In this study, I investigated whether lipid derivatives, gangliosides and oxLDL, could modulate microglia-mediated brain inflammation. In part 1, I tested whether gangliosides could activate JAK-STAT pathway, an essential inflammatory signaling pathway in microglia. Gangliosides rapidly activated JAK1 and JAK2 and induced phosphorylation of STAT1 and STAT3. In addition, gangliosides increased transcription of the inflammation-associated genes iNOS, ICAM-1, and MCP-1, which are reported to contain STAT-binding elements in their promoter regions. The present study indicates that JAK-STAT signaling is an early event in gangliosides-induced brain inflammatory responses. In part 2, I studied the effects of oxLDL on microglia-mediated brain inflammation. OxLDL decreases LPS-induced expressions of iNOS and production of NO. It also reduces LPS-induced secretion of TNFα and MCP-1. Oxysterols (7KC and 22(R)HC), showed similar inhibitory effects in LPS-activated microglia. In addition, their inhibitory effects were mimicked by LXR agonists (GW3965 and TO901317) and potentiated by RXR agonist (methoprene acid). Taken together these data demonstrate that oxLDL inhibit LPS-induced brain inflammation and these inhibitory effects of oxLDL are mediated at least in part by oxysterol via nuclear receptors.

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-국문요약-

# Gangliosides 와 oxidized low density lipoprotein (oxLDL) 에 의한 신경교세포의 활성조절 및 그 기전에 대한 연구

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Microglia는 중추신경계의 macrophage로서 다양한 자극에 의해 활성화되어 염증반응을 유도한다. 활성화된 microglia는 Alzheimer's disease (AD), multiple sclerosis, stroke등의 다양한 뇌질환의 발생과 진행과정에 중요한 역할을 담당할 것으로 생각되고 있다. 특히 brain은 다른 신체조직에 비해 지질이 풍부하게 존재하는 부위로, 이러한 지질유도체들에 의한 microglia의 활성조절을 연구하는 것이 중요할 것으로 여겨진다.

첫 번째 연구에서는 microglia의 활성에 대한 gangliosides의 효과를 조사하였다. 신경세포막에는 뇌의 생리기능과 병리작용에 중요한 역할을 하는 gangliosides가 풍부하게 존재한다. 본 연구에서 JAK-STAT 신호전달 과정이 gangliosides에 의한 신경교세포의 활성화에 관여한다는 것을 밝혔다. 일차 배양한 rat microglia와 murine BV2 microglial cell에서 gangliosides는 STAT 결합부위로

알려진 GAS/ISRE element와 nuclear factor의 결합을 증가시켰으며 JAK1과 JAK2를 활성화시키고 STAT1과 STAT3의 인산화를 유도하였다. 또한 gangliosides는 promoter 부위에 STAT 결합부위를 가지고 있으며 염증반응과 관련이 있는 유전자들인 iNOS, ICAM-1과 MCP-1등의 전사를 증가시켰다. Gangliosides를 처리한 microglia에 JAK의 억제제인 AG490을 처리한 결과, 위의 유전자들의 전사, nuclear factor의 결합활성, 그리고 STAT-1과 3의 활성화가 감소하는 것을 확인할 수 있었다. AG490은 또한, 염증반응에서 대표적으로 관찰되는 물질이며, gangliosides에 의하여 생성이 증가하는 NO의 분비를 감소시켰다. 그리고, AG490은 ERK1/2 MAPK의 활성화를 현저하게 감소시켰으며, 이는 ERK가 micoglia의 활성화에 있어 JAK-STAT 신호전달 과정의 하위에 위치하고 있음을 의미한다. 그러나, AG490은 p38 MAPK의 활성화에는 아무런 영향을 미치지 않았다. 또한, 본 연구에서 우리는 gangliosides의 sialic acid residue가 JAK-STAT 신호전달의 활성에 필수적인 요소임을 밝혔다. 이 연구는 JAK-STAT 신호전달 과정이 gangliosides에 의한 뇌 염증 반응의 초기에 관여함을 밝혔다.

두 번째 주제에서는 oxLDL의 microglia의 활성에 대한 효과를 조사하였다. Low density lipoprotein (LDL)은 재빠르게 산화되어 oxidized LDL (oxLDL)의 형태가 된다. 본 연구는 일차 배양한 microglia에서 oxLDL이 LPS에 의하여 유도된 염증반응을 저해한다는 것을 밝혔다. oxLDL은 LPS에 의하여 증가된 iNOS와 NO의 생성을 감소시켰으며 TNFα와 MCP-1의 방출 또한 감소시켰다. oxLDL의 이러한 항염증효과는 native LDL과 acetylated LDL에서는 관찰할 수 없었으며, 산화된 정도에 비례하여 관찰되었다. oxLDL의 대표적인 구성물인 Oxysterols (7-ketocholesterol, 22(R) hydroxycholesterol)과

lysophosphatidylcholine (LPC)를 처리한 결과 oxysterols은 oxLDL과 유사한 항염증효과를 나타낸 반면, LPC는 염증 저해효과를 전혀 나타내지 않았다. Oxysterols은 RXR과 heterodimer를 형성하여 작용하는 nuclear receptor인 LXR의 agonist로 항염증작용에 LXR의 관련성을 유추할 수 있었다. 합성 LXR agonists인 GW3965와 TO901317를 처리한 경우에도 항염증효과를 관찰할 수 있었다. LPS에 의해 증가된 iNOS와 NO 생성, MCP-1의 분비는 LXR agonists에 의해 저해되었지만 TNFα의 경우는 저해되지 않았다. LXR와 heterodimer를 형성하는 RXR의 agonist를 처리한 경우에도 TNFα의 생성은 다른 염증관련 물질의 경우와 달리 oxLDL의 저해효과가 증가되는 것을 관찰할 수 없었다. 위의 결과를 종합하면, oxLDL은 oxysterols을 통해 LPS에 의한 뇌 염증반응을 저해하며, 이러한 효과는 nuclear receptor인 LXR을 경유하여 일어나는 것으로 생각된다. OxLDL과 oxysterols의 염증 저해효과는 LXR-의존적인 경로 (NO, MCP-1)와 LXR-비의존적인 경로 (TNFα)를 모두 포함하고 있다. 이 연구 결과는 oxidative stress가 LDL의 산화변형을 통하여 염증반응을 조절함으로써, 간접적으로 세포기능에 관여하는 또 다른 기전을 제시한다.

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**핵심어**: 신경교세포, 뇌염증반응, Gangliosides, OxLDL, Oxysterols, JAK-STAT, LXR