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Sulfatide, A Major Lipid Component of Myelin Sheath, Activates Inflammatory Responses As an Endogenous Stimulator in Brain-Resident Immune Cells¹

Sae-Bom Jeon,*† Hee Jung Yoon,* Se-Ho Park,‡ In-Hoo Kim,28 and Eun Jung Park2*

Sulfatide, a major lipid component of myelin sheath, participates in diverse cellular events of the CNS, and its cellular level has recently been implicated in many inflammation-associated neuronal diseases. Herein, we report that sulfatide alone can trigger pathological inflammatory responses in glia, brain-resident immune cells. We show that sulfatide changed the morphology of primary microglia to their activated form, and it significantly induced the production of various inflammatory mediators in primary microglia and astrocytes. Moreover, sulfatide rapidly triggered the phosphorylation of p38, ERK, and JNK within 30 min, and it markedly enhanced the NF binding activity to NF-κB and AP-1 binding elements. However, nonsulfated galactocerebroside, another major lipid component of myelin, had no effect on activation of glia. We further reveal that CD1d did not contribute to sulfatide-stimulated activation of MAPKs, although its expression was enhanced by sulfatide and sulfatide-treated microglial cells actually stimulated type II NKT cells. Sulfatide significantly stimulated the phosphorylation of MAPKs in glia from CD1d-deficient mice, and the phosphorylation levels were similar to those in wild-type littermates. Sulfatide-triggered inflammatory events appear to occur at least in part through an L-selectin-dependent mechanism. L-selectin was dramatically down-regulated upon exposure to sulfatide, and inhibition of L-selectin resulted in suppression of sulfatide-triggered responses. Collectively, these results show that abnormally released sulfatide at demyelinated regions may act as an endogenous stimulator in the brain immune system, thus causing and further exacerbating pathological conditions in the brain. *The Journal of Immunology*, 2008, 181: 8077–8087.

ulfatide, or sulfated galactocerebroside, is a glycosphingolipid found at high concentrations exclusively in the brain, kidney, and spleen (1, 2). In the nervous system, sulfatide is especially enriched in myelin, which is a specialized multilamella membrane with a small number of specific proteins and lipids. More than 70% of myelin are composed of lipid constituents, and almost 30% of myelin lipids are comprised of sulfatide and its nonsulfated precursor galactocerebrosides (3). Increasingly, reports have suggested that sulfatide has important functions not only as a structural component of membranes but also in diverse biological processes, including developmental signaling, axon-myelin interactions, regulation of cell growth, protein trafficking, cell adhesion, and neuronal plasticity (4, 5).

In particular, sulfatide has been closely implicated in the etiology and pathogenesis of chronic inflammatory diseases of the CNS, including multiple sclerosis (MS),³ which are characterized by inflammation and tissue damage accompanying myelin destruc-

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tion (6–9). Several reports have shown the altered levels of sulfatide and anti-sulfatide Ab in patients with degenerative diseases, in the experimental autoimmune encephalomyelitis (EAE) model of MS, and in tumor cells (6, 10, 11). Additionally, there are reports that sulfatide content in postmortem brain samples from Parkinson's disease is higher than that in controls, but is dramatically depleted in Alzheimer disease (8, 12, 13). Kanter et al. recently demonstrated using lipid microarrays that Abs against sulfatide are increased in CSF samples from MS patients and in sera from mice with EAE, and they further revealed that immunization of sulfatide with myelin peptides worsens EAE (14).

Inflammation-associated neurological diseases are usually accompanied by abnormal activation of glial cells, which are resident immunoeffector cells in the CNS (15). They can serve as vigilant housekeepers, APCs, and phagocytes in the CNS (16, 17). Microglia and astrocytes are rapidly activated in response to pathological stimuli, and they lead to various immune and inflammatory processes. Upon activation, they change their morphology, immunophenotype, and expression pattern of inflammatory mediators, including cytokines and chemokines (18). Although transient activation of glial cells is beneficial for defense processes against pathological stimuli, either chronic activation or overactivation can cause or exacerbate a variety of brain disorders including neurodegenerative diseases and tumors (19). Indeed, recent reports have suggested the potent immunomodulatory roles for glial cells in CNS diseases (20, 21).

To date, despite the abundance of myelin lipids and their presumed roles in the pathophysiology of numerous CNS disorders, most studies on the structural and functional characteristics of myelin and its associated diseases have focused on the myelin

intensity; MOG, myelin oligodendrocyte glycoprotein; SOCS, suppressor of cytokine signaling.

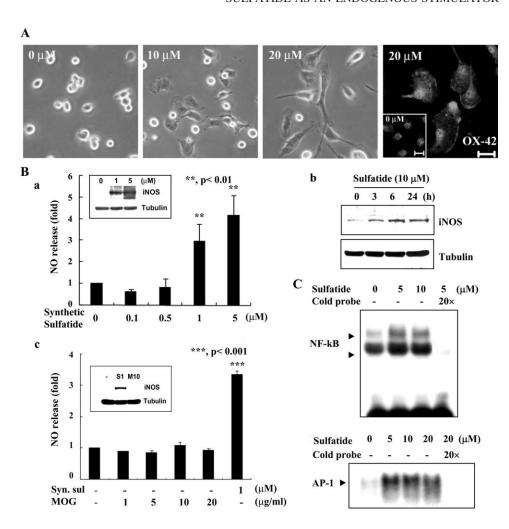
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³ Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; iNOS, inducible NO synthase; MFI, mean fluorescence

FIGURE 1. Primary microglial cells are activated upon exposure to sulfatide. A, Rat primary microglial cells were treated with brain-derived sulfatide for 18 h, and then photographed using phase and confocal microscopes. For confocal microscopy, the cells were stained with OX-42 Ab, a microglial marker. The inset shows the resting condition of primary microglia. B, Primary microglial cells were incubated with the indicated concentrations of synthetic sulfatide (a) or MOG_{35-55} (c) for 2 days, and the amount of NO produced was determined according to Materials and Methods. The inset shows iNOS expression in the same conditions. Western blot analysis was performed using iNOS and tubulin Abs (b). **, p < 0.01 and ***, p < 0.001when compared with mock-treated controls. C, Primary microglia were treated with sulfatide for 30 min, after which nuclear extracts were prepared and assayed for the amount of binding activity to NF-kB and AP-1 binding elements using EMSA. Data are representative of four independent experiments. Scale bar, 10 μ m.



proteins. Given its abundance in myelin and altered levels of sulfatide in several brain disorders, we attempted to define the properties and pathophysiological effects of sulfatide in the brain immune system. In the present study, we provide evidence that sulfatide alone significantly impacts the immune and inflammatory responses in brain-resident cells, and we suggest a possible mechanism for the pathological role of sulfatide in demyelinating brain. Our results propose that endogenous sulfatide released at sites of injured and/or inflamed brain lesions may contribute to the amplification and persistence of inflammatory reactions, thus causing chronic inflammatory CNS diseases.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6.CD1d-deficient mice (22) and wild-type littermates were maintained under specific pathogen-free conditions in the barrier facility at Korea University. Sprague-Dawley rats were purchased from SamTako Bio Korea. All animal procedures were performed according to the National Cancer Center guidelines for the care and use of laboratory animals.

Reagents

Sulfatide and synthetic sulfatide (3-sulfo-C12 GalCer) were purchased from Avanti Polar Lipids. Nonsulfated galactocerebroside and myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) were obtained from Sigma-Aldrich. MEM and G418 antibiotics were obtained from Invitrogen-BRL. DMEM and FBS were purchased from HyClone. SB203580, SP600125, and PD98059 were purchased from BIOMOL. Salmonella typhimurium LPS and polymyxin B sulfate were purchased from Sigma-Aldrich. TAPI-2 and myo-inositol were purchased from Calbiochem.

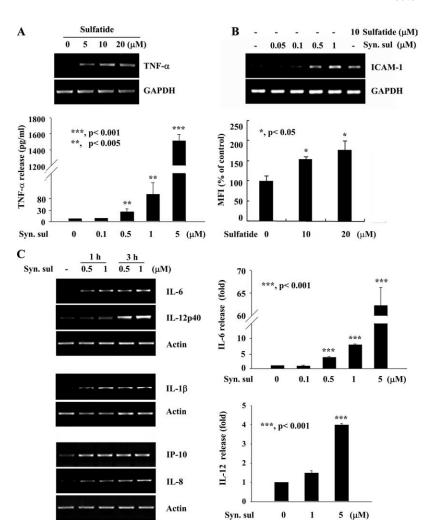
Glial cell cultures

Primary microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague-Dawley rats. Briefly, the tissues were triturated into single cells in MEM containing 10% FBS and plated in 75-cm² T-flasks (0.5 hemisphere/flask) for 2 wk. The microglia were detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes and cell clumps. Cells were plated in 6-well plates (5 \times 10 5 cells/well), 60-mm² dishes (8 \times 10 5 cells/dish), or 100-mm² dishes (2 \times 10 6 cells/dish) and washed after 1 h later to remove unattached cells before use in experiments. Following removal of the microglia, primary astrocytes were prepared by trypsinization. Cells were demonstrated to consist of $>\!95\%$ authentic microglia and astrocytes due to their characteristic morphology and the presence of the astrocyte marker glial fibrillary acidic protein and the microglial marker CD11b.

RT-PCR analysis

Total RNA was isolated using RNAzol B (Tel-Test) and cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (TaKaRa) according to the manufacturers' instructions. PCR was performed with 25 cycles of sequential reactions. Oligonucleotide primers were purchased from Bioneer. The sequences for the PCR primers used are as follows: forward (F) 5'-GTA GCC CAC GTC GTA GCA AA-3' and reverse (R) 5'-CCC TTC TCC AGC TGG GAG AC-3' for TNF-α; (F) 5'-AAA ATC TGC TCT GGT CTT CTG G-3' and (R) 5'-GGT TTG CCG AGT AGA CCT CA-3' for IL-6; (F) 5'-CCG ATG CCC CTG GAG AAA C-3' and (R) 5'-CCT TCT TGT GGA GCA GCA G-3' for IL-12p40; (F) 5'-TGA TGT TCC CAT TAG ACA GC-3' and (R) 5'-GAG GTG CTG ATG TAC CAG TT-3' for IL-1 β ; (F) 5'-CTG GAG AGC ACA AAC AGC AGA G-3' and (R) 5'-AAG GCC GCA GAG CAA AAG AAG C-3' for ICAM-1; (F) 5'-TCC AGA AGC ACC ATG AAC C-3' and (R) 5'-GCT GAA GAG ATT AGT ACC T-3' for IP-10; (F) 5'-GAA GAT AGA TTG CAC CGA TG-3' and (R) 5'-CAT AGC CTC TCA CAC ATT TC-3' for IL-8; (F) 5'-ATG CAG GTC TCT GTC ACG CT-3' and (R) 5'-CTA GTT

FIGURE 2. Sulfatide stimulates the production of several inflammatory cytokines and chemokines in primary microglia. A, Primary microglial cells were treated with the indicated doses of sulfatide for 3 h, and the message levels of TNF- α were determined by an RT-PCR-based assay (upper). After the cells were incubated with 10 μ M sulfatide for 18 h, the media was collected and assayed for TNF- α levels by ELISA (lower). The results shown represent the means \pm SD of triplicate wells and are representative of four individual experiments. B, The transcript and cell surface expression levels of ICAM-1 were measured by RT-PCR analysis (upper) and flow cytometric analysis using FITCconjugated ICAM-1 Ab (lower). The MFI values represent the means ± SD of three independent experiments. C, The mRNA levels of IL-6, IL-12p40, IL-1\beta, IP-10, and IL-8 were determined by RT-PCR (left), and the levels of IL-6 and IL-12 secreted into the media were measured by ELISA. Data are representative of four independent experiments. *, p < 0.05; **, p < 0.005; ***, p < 0.001 when compared with mocktreated controls.



CTC TGT CAT ACT GG-3' for MCP-1; (F) 5'-AAG CGC AGA AGT CGG AGC CG-3' and (R) 5'-GCA GGT ACG CAC ATT TGC AGT TGT G-3' for CD1d; (F) 5'-TTG AAG ACA AGG CAT GGC AT-3' and (R) 5'-TCT CCC AAG ATC AAC CGA TG-3' for TLR4; (F) 5'-ACC AGC GCC ACT TCT TCA CG-3' and (R) 5'-GTG GAG CAT CAT ACT GAT CC-3' for suppressor of cytokine signaling 3 (SOCS3); (F) 5'-TCC CTC AAG ATT GTC AGC AA-3' and (R) 5'-AGA TCC ACA ACG GAT ACA TT-3' for GAPDH; (F) 5'-CAT GTT TGA GAC CTT CAA CAC CCC-3' and (R) 5'-GCC ATC TCC TGC TCG AAG TCT AG-3' for actin.

Flow cytometry

Cells were treated with sulfatide in the presence of 5% serum for the indicated times. The cells were washed twice with PBS containing 1% FBS, collected, and stained with FITC-conjugated anti-mouse ICAM-1 (eBioscience), PE-conjugated anti-mouse TLR4/MD2 Ab (eBioscience), or CD1d mAb 19G11 (BD Biosciences) for 30 min at 4°C. After washing, the cells were analyzed using a FACSVantage or FACSCalibur flow cytometer (BD Biosciences). The data were processed using the CellQuest, WinMDI, or FlowJo program. The mean fluorescence intensity (MFI) was analyzed by CellQuest software, and the change in MFI of cells incubated with ICAM-1 Ab or CD1d Ab was calculated for sulfatide-treated and mock-treated cells after subtraction of the MFI obtained with isotype control Ab (eBioscience).

Western blot analysis

Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with Ab and visualized using an ECL system. Abs against phospho-JNK, phospho-p38, phospho-ERK, phospho-c-jun, total ERK, phospho-STAT1, and total STAT1 were purchased from Cell Signaling Technology. C-fos and tubulin Abs were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. The inducible NO synthase (iNOS) Ab was purchased from Upstate Biotechnology.

EMSA

Cells were stimulated in the presence of 2.5% serum, and cell extracts were then suspended in 9× 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 DTT, 0.5 mM PMSF, 0.5% Nonidet P-40) and centrifuged at 5000 rpm for 10 min at 4°C. The pellet (nuclear fraction) was resuspended in 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 13,000 rpm for 20 min. The crude nuclear proteins in the supernatant were collected and stored at -70°C. EMSA was performed for 30 min on ice in a volume of 20 µl containing 2 μg of nuclear protein extract in a reaction buffer containing 8.5 mM EDTA, 8.5 mM EGTA, 8% glycerol, 0.1 mM ZnSO₄, 50 μg/ml poly(dIdC), 1 mM DTT, 0.3 mg/ml BSA, 6 mM MgCl $_2$, and γ^{-32} P-radiolabeled oligonucleotide probe (3 \times 10⁴ cpm) with or without a 20- to 50-fold excess of unlabeled probe. DNA-protein complexes were separated on 6% polyacrylamide gels in Tris/glycine buffer, and the dried gels were exposed to x-ray film. The following double-stranded oligonucleotides were used in these studies: NF-kB gel-shift oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3', 22 bp; Santa Cruz Biotechnology sc-2505) and AP-1 oligonucleotides (5'-CGC TTG ATG ACT CAG CCG GAA-3', 21 bp; Santa Cruz Biotechnology sc-2501). The 5' end-labeled probes were prepared with 40 μ Ci of $[\gamma^{-32}P]$ ATP using T4 polynucleotide kinase (Promega) and purified on Sephadex G-25 quick spin columns (Roche Molecular Biochemicals).

ELISA

ELISA kits were used according to the manufacturers' protocols. After treatment with stimuli, $100~\mu l$ of conditioned media was collected and assayed using ELISA kits for rat TNF- α (eBioscience), IL-6, and IL-12 (BioSource International), according to the manufacturers' procedures.

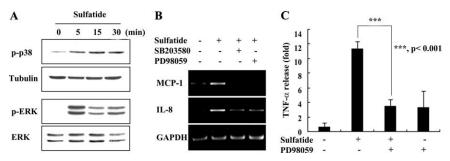


FIGURE 3. Sulfatide rapidly induces the phosphorylation of p38 and ERK. *A*, Primary microglia were treated with 10 μM sulfatide for the indicated times. Cell lysates were separated by 10% SDS-PAGE and Western blots were probed with Abs against phospho-p38 (p-p38) and phospho-ERK (p-ERK). The membranes were reprobed with α-tubulin or ERK Abs for loading control. *B*, Primary microglia were pretreated with SB203580, a p38-specific inhibitor, and PD98059, an ERK-specific inhibitor, for 1 h and stimulated with sulfatide for 3 h. Total RNA was isolated and analyzed for levels of MCP-1 and IL-8 mRNA using an RT-PCR-based assay. The transcription of GAPDH was measured for normalization. *C*, Primary microglia were treated with PD98059 for 1 h, and the cells were then incubated with 10 μM sulfatide for 18 h. The media was collected and assayed for levels of TNF-α protein by ELISA. The results shown represent the means \pm SD of triplicate wells and are representative of three individual experiments. ***, p < 0.001 when compared with mock-treated controls.

Measurements of NO release

The media nitrite concentration was measured as an indication of NO release. Following the indicated cell incubations, $50~\mu l$ of culture medium was removed and mixed with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, 2.5% H_3PO_4), and the absorbance of the mixture was measured at $540~\rm nm$.

Immunostaining and confocal microscopy

Primary microglia were plated onto round coverslips (Fisher Scientific) and fixed with ice-cold 100% methanol. Fixed microglia were permeabilized with PBS containing 0.1% Triton X-100 (PBS-T) and blocked with 1% BSA (Sigma-Aldrich). The product was then washed with PBS-T and incubated overnight at 4°C with OX-42 (1/100) or L-selectin (1/100) Abs. Samples were rinsed three times for 5 min with PBS-T. Each coverslip was mounted on slides with mounting solution, and the cells were observed with a Carl Zeiss LSM510 confocal microscope.

Ag presentation assay

Primary microglial cells were used as APCs, and 1C8.DC1 (CD1d-specific type II NKT hybridoma cell) and DN32.D3 (type I NKT hybridoma cell) (23) cells were used to detect Ag presentation by CD1d. Microglial cells were cultured with 10 μ M sulfatide for the indicated times in 24-well plates (8 × 10⁴/well), followed by removal of the media. IC8.DC1 cells or DN32.D3 cells (1 × 10⁶/well) were then added and cultured for 15 h. The efficiency of Ag presentation was quantified by measuring the amount of mouse IL-2 secreted into the supernatant using an IL-2 ELISA kit (eBioscience).

Endotoxin assay

Sulfatide and synthetic sulfatide preparations were tested for endotoxin contamination using the chromogenic *Limulus* amebocyte lysate assay according to the manufacturer's specifications (WinKQCL, BioWhittaker).

Statistical analyses

All data represent means \pm SD. The statistical significance of differences was assessed using ANOVA, followed by Student-Newman-Keuls multiple comparison tests (SPSS for windows, standard version).

Results

Primary microglia are rapidly activated upon exposure to sulfatide

Essential roles for lipids in immune regulation and the pathogenesis of several CNS diseases have recently been described (14, 24). In an effort to define the pathological action of abnormally released lipid components at sites of injured and/or inflamed brain lesions, we investigated the effect of sulfatide, a major lipid component of myelin, on brain-resident immune cells. We first examined the morphology of rat primary microglia after exposure to sulfatide,

since microglia have been shown to undergo morphological changes in response to inflammatory stimuli. Primary microglia were cultured from the cerebral cortices of 1-day-old Sprague-Dawley rats, and the cells were treated with the indicated concentrations of brain-derived sulfatide for 18 h. Intriguingly, we found that microglia became flat and enlarged when stimulated with brain-derived sulfatide (Fig. 1A), suggesting that treatment with sulfatide resulted in activation of microglia. To confirm this, we tested the effect of pure synthetic sulfatide, and similar changes were observed (data not shown).

Aberrant expression of iNOS and excessive production of NO are observed in various pathophysiological conditions (25). We thus determined whether sulfatide could induce the expression of iNOS and production of NO. Rat primary microglia were treated with sulfatide for the indicated times, and the amount of NO produced was determined by measuring the amount of nitrite converted from NO in the media. Compared with microglial cells

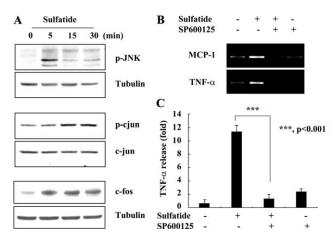
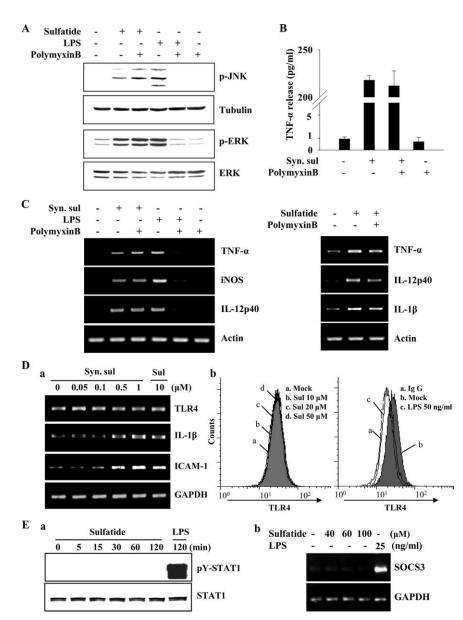


FIGURE 4. The JNK pathway also contributes to sulfatide-stimulated activation of microglia. A, Primary microglial cells were treated with 10 μ M sulfatide for the indicated times. The level of phosphorylated JNK was determined by Western blot analysis using phospho-JNK, phospho-c-jun, and c-fos Abs. The membranes were subsequently probed with α -tubulin Ab or c-jun Ab for normalization. B, Primary microglial cells were pretreated with SP600125, a JNK specific inhibitor, for 1 h, and stimulated with sulfatide for 3 h. Total RNA was isolated and analyzed for levels of MCP-1 and TNF- α mRNA using an RT-PCR-based assay. C, After exposure to 10 μ M sulfatide for 18 h, TNF- α release was assayed by ELISA. Data are representative of three independent experiments.

FIGURE 5. Sulfatide-induced inflammatory events do not occur as a result of endotoxin contamination during the preparation of sulfatide. A, Primary microglia were pretreated with 10 μg/ml of polymyxin B for 1 h and stimulated with 10 µM sulfatide for 30 min. Western blots were probed with Abs against phospho-JNK and phospho-ERK. The membranes were reprobed with α -tubulin Ab or ERK Ab as controls for protein loading. B, After primary microglial cells were incubated with 1 µM synthetic sulfatide for 18 h, the media was collected and assayed for TNF- α level by ELISA. The results shown represent the means \pm SD of triplicate wells and are representative of three individual experiments. C, Primary microglia were stimulated with 1 μ M synthetic sulfatide, 10 μ M brain-derived sulfatide, or 50 ng/ml LPS for 3 h in the presence or absence of 10 µg/ml of polymyxin B, and the mRNA levels of inflammatory mediators were determined by an RT-PCRbased assay. D, a, Primary microglial cells were mock treated or treated with the indicated concentrations of synthetic sulfatide and sulfatide for 3 h. Total RNA was then extracted for RT-PCR analysis. b, BV2 microglial cells were treated with sulfatide or LPS as a control for 18 h. Cell surface expression of TLR4 was analyzed by flow cytometric analysis using PEconjugated anti-TLR4 Ab. E, a, Primary microglial cells were treated with sulfatide or LPS for the indicated times. Western blots were probed with Abs against phospho-STAT1 and total STAT1. b, Primary microglial cells were treated with the indicated doses of sulfatide and 25 ng/ml of LPS for 3 h. The mRNA levels of SOCS were determined using an RT-PCR-based assay. The data are representative of more than three independent experiments.



treated with vehicle, NO release was rapidly increased in a dose-dependent manner in microglial cells treated with sulfatide (Fig. 1B). Additionally, sulfatide rapidly increased the expression level of iNOS as measured by both mRNA and protein (Fig. 1B and data not shown). However, we did not observe any noticeable effects of MOG, a representative myelin-derived protein, on NO release and iNOS expression in primary microglia (Fig. 1B). Taken together, these results suggest that sulfatide alone can trigger activation of microglia, contributing to pathological inflammatory conditions in the brain.

Sulfatide stimulates production of inflammatory cytokines and chemokines, as well as NF binding to NF- κB and AP-1 binding elements

To further address the pathological action of sulfatide in the brain immune system, we assayed whether sulfatide could influence the activity of representative inflammation-associated transcription factors such as NF- κ B and AP-1, which bind to the promoter regions of various inflammation-related genes. After rat primary microglia were treated with 5–20 μ M sulfatide for 30 min, nuclear extracts were prepared and then analyzed by the EMSA using

 γ -³²P-labeled consensus NF- κ B or AP-1 oligonucleotide probes. Sulfatide markedly stimulated NF binding to both NF- κ B and AP-1 binding sites (Fig. 1*C*). The specificity of the shifted bands was confirmed by competition assay using excess amounts of unlabeled oligonucleotides. These results strongly indicate that sulfatide may stimulate inflammation-associated transcriptional events in brain immune cells.

Consequently, we examined the expression of several inflammatory cytokines and chemokines with functional NF- κ B or AP-1 binding elements. Rat primary microglial cells were stimulated with brain-derived sulfatide or synthetic sulfatide for the indicated times, and total RNA was extracted for RT-PCR analysis. As shown in Fig. 2, addition of sulfatide rapidly increased the mRNA levels of TNF- α , IL-6, IL-12p40, and IL-1 β in a dose-dependent manner. Additionally, the transcript levels of inflammatory chemokines such as ICAM-1, IP-10, IL-8, and MCP-1 were induced upon exposure to sulfatide, but CCR1 and CX3CR1 transcripts remained unchanged (Fig. 2 and data not shown). Using flow cytometric analysis and ELISA, we further demonstrated that expression of ICAM-1 and secretion of TNF- α , IL-6, and IL-12 were significantly increased in sulfatide-treated microglia (Fig. 2).

These findings clearly show that sulfatide can rapidly stimulate the transcriptional activation of inflammatory cytokines and chemokines.

MAPKs mediate sulfatide-induced microglial activation

We next investigated whether MAPKs, which are representative inflammation-associated signaling molecules, could be involved in sulfatide-induced glial activation. Rat primary microglia were treated with 10 μ M brain-derived sulfatide for 5–30 min, and cell lysates were analyzed by Western blot using Abs directed against phospho-p38, phospho-ERK, total ERK, and tubulin. After exposure to sulfatide, phosphorylation of ERK and p38 were significantly increased within 5 min (Fig. 3A). To confirm these results, we tested the importance of ERK and p38 in sulfatide-induced activation of inflammatory responses using pharmacological inhibitors. Pretreatment with SB203580, a p38 inhibitor, or PD98059, an ERK inhibitor, markedly suppressed the sulfatide-induced expression of inflammatory mediators including MCP-1 and IL-8 (Fig. 3B). Moreover, sulfatide-induced release of TNF- α was significantly reduced in the presence of SB203580 or PD98059 (Fig. 3C and data not shown). These results indicate that ERK and p38 are closely involved in the sulfatide-induced activation of glia.

Additionally, we observed strong phosphorylation of JNK and activation of its downstream signaling molecules including c-jun and c-fos, the transactivation component of the heterodimeric transcription factor AP-1 (26) (Fig. 4A). Treatment with sulfatide resulted not only in phosphorylation of JNK, but also in phosphorylation of c-jun and rapid induction of c-fos. Moreover, pretreatment with SP600125, a JNK inhibitor, significantly reduced not only sulfatide-induced gene expression of MCP-1 and TNF- α , but also TNF- α release by rat primary microglia (Fig. 4, *B* and *C*). Taken together, these observations convincingly demonstrate that MAPKs such as p38, ERK, and JNK contribute to sulfatide-stimulated activation of microglia.

Sulfatide-dependent inflammatory responses are not attributable to endotoxin contamination during sulfatide preparation

To examine whether sulfatide-induced inflammatory responses could be attributable to endotoxin contamination during the preparation of sulfatide, we carefully tested all sulfatide preparations using the chromogenic Limulus amebocyte lysate assay (WinKQCL, BioWhittaker). The average endotoxin levels for brain-derived sulfatide and synthetic sulfatide used in this study were less than $0.0081\pm\ 0.0042$ and $0.0079\pm\ 0.0039$ EU/ml, respectively, which were not sufficient to induce endotoxin-dependent inflammatory events.

To further rule out the possibility of our results being misinterpreted as due to endotoxin contamination of sulfatide, we also examined the effect of polymyxin B, a well-known pharmacological LPS scavenger, on sulfatide-stimulated signaling events. As shown in Fig. 5, sulfatide-induced phosphorylation of ERK and JNK was not affected by pretreatment with polymyxin B, while LPS-induced phosphorylation was significantly reduced (Fig. 5A). Moreover, we did not observe any effects of polymyxin B on sulfatide-induced production of inflammatory cytokines in all experiments by ELISA and RT-PCR analysis (Fig. 5, B and C).

To additionally address the concern that our reagents may contain endotoxin, we examined the expression of TLR4, a representative receptor for LPS. As shown in Fig. 5D, the TLR4 transcript remained unchanged in sulfatide-changed microglia treated with sulfatide, where inflammatory mediators including IL-8 and ICAM-1 were induced. FACS analysis also showed that cell surface expression of TLR4 was not altered by sulfatide in microglia; however, LPS stimulation resulted in a considerable reduction

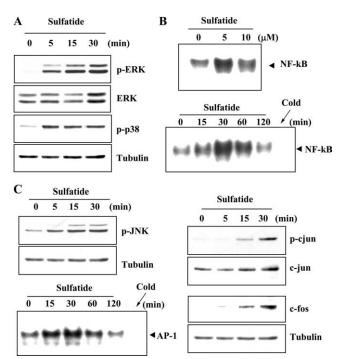


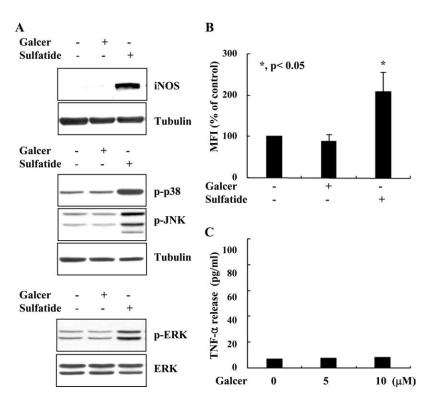
FIGURE 6. Primary astrocytes are also activated in response to sulfatide. A, Primary astrocytes were treated with 10 μM sulfatide for the indicated times. The levels of phosphorylated ERK and phosphorylated p38 were detected by Western blot analysis using phospho-ERK and phospho-p38 Abs, and the membranes were then reprobed with total ERK Ab or α-tubulin Ab for loading control. B, Primary astrocytes were stimulated with sulfatide, and binding activities to the NF-κB-specific oligonucleotide were determined by EMSA. C, Cells were treated with sulfatide for the indicated times. Western blot analysis was performed using anti-phospho-JNK (p-JNK), anti-phospho-c-jun (p-cjun), and anti-c-fos Abs. Nuclear extracts were also prepared and analyzed using a γ- 32 P-labled AP-1 consensus binding element oligonucleotide probe. Data are representative of four independent experiments.

(Fig. 5*D*). These results suggest that the sulfatide-induced inflammatory events shown in this study did not occur as a result of endotoxin activity via TLR4. Additionally, sulfatide did not influence phosphorylation of STAT1 at all time points tested, whereas LPS facilitated STAT1 phosphorylation within 2 h (Fig. 5*E*). Moreover, sulfatide had no effect on the expression of SOCS3, an inducible inhibitory molecule of STAT signaling that was significantly enhanced by exposure to LPS (Fig. 5*E*). Collectively, these findings strongly support that sulfatide itself, and not the presence of contaminating endotoxin, activates inflammatory responses in the brain.

Sulfatide also activates primary astrocytes

Astrocytes, another glial cell type vital to brain immune responses, express various cell surface receptors and produce inflammatory mediators such as cytokines. To better define the pathological action of sulfatide on brain inflammatory responses, we investigated whether sulfatide could also activate astrocytes. Rat primary astrocytes were treated with 10 μM sulfatide for the indicated times, and activation of MAPKs, NF-κB, and AP-1 were analyzed by Western blot analysis and EMSA. Consistent with the results in microglia, both ERK and p38 were significantly phosphorylated in astrocytes upon exposure to sulfatide (Fig. 6A). NF binding activities to NF-κB elements were also rapidly increased in astrocytes (Fig. 6B). Furthermore, we observed phosphorylation of JNK, activation of its downstream signaling molecules, including c-jun and c-fos, and an increase in NF binding to AP-1 binding regions

FIGURE 7. Sulfate is necessary for sulfatide-stimulated inflammatory responses in primary glial cells. A, Primary microglia were treated with 10 µM galactocerebroside or 10 µM sulfatide for 18 h. Western blot analysis was performed using anti-iNOS Ab, followed by anti-tubulin Ab. To determine the levels of phosphop38, phospho-JNK, and phosopho-ERK, primary microglial cells were treated with galactocerebroside or sulfatide for 15 min. Data are representative of three independent experiments. B, Primary microglia were treated with 10 µM galactocerebroside or 10 µM sulfatide for 18 h, and ICAM-1 surface expression levels, as well as TNF- α release, were analyzed by flow cytometric analysis using FITC-conjugated ICAM-1 Ab (B) and ELISA using TNF- α Ab (C), respectively. The results shown represent the means ± SD of triplicate wells and are representative of three individual experiments. *, p < 0.05 when compared with the mocktreated control.



in rat primary astrocytes (Fig. 6*C*). Additionally, sulfatide treatment resulted in induction of inflammatory mediators, including ICAM-1, TNF- α , and IL-6, in astrocytes (data not shown). These

results indicate that sulfatide can activate astrocytes as well as microglia, further demonstrating that sulfatide may play important roles in brain inflammatory responses and diseases.

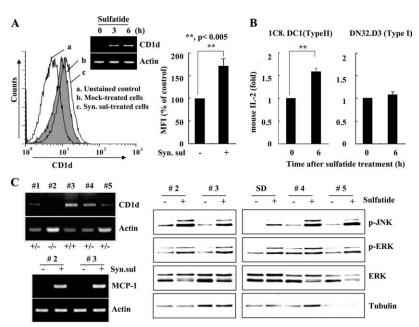
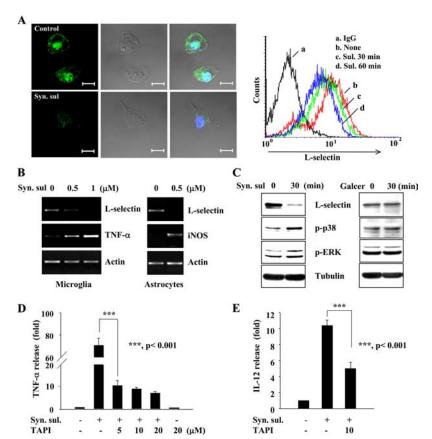


FIGURE 8. Sulfatide also stimulates phosphorylation of MAPKs in glial cells from CD1d-deficient mice. A, Rat primary microglial cells were mock treated or treated with synthetic sulfatide (1 μ M) for 18 h. Cell surface expression of CD1d was then analyzed by flow cytometry using anti-CD1d mAb. The MFI was analyzed using CellQuest software (BD Biosciences), and the change in MFI of cells incubated with CD1d Ab was calculated for sulfatide-treated cells and mock-treated cells after subtraction of the MFI obtained with isotype control Ab. The MFI values are means \pm SD of three independent experiments. **, p < 0.005 when compared with control samples. After microglial cells were treated with 10 μ M sulfatide for 3 h, transcription of CD1d was also analyzed by RT-PCR. B, Rat primary microglial cells were treated with 10 μ M sulfatide for 6 h, after which the cells were cultured with DN32.D3 cells or 1C8.DC1 cells for 15 h. ELISA was then used to determine the amount of IL-2 in the supernatant. The results shown represent the means \pm SD of triplicate wells and are representative of three individual experiments. **, p < 0.005 when compared with the mock-treated control. C, Primary glial cells either from 1-day-old SD rats, CD1d^{-/-} mice (-/-), heterozygous littermates (+/-), and wild-type littermates (+/+) were treated with 10 μ M sulfatide for 15 min. Western blot analysis was then performed using phospho-JNK, phospho-ERK, and ERK Abs. RT-PCR analysis was performed for cells treated with 1 μ M synthetic sulfatide for 3 h. The data shown are representative of at least three independent experiments.

FIGURE 9. L-selectin expression is modulated upon exposure to sulfatide. A, Primary microglia were treated with 10 µM sulfatide for 30 min. The cells were then stained with L-selectin Ab (green) and DAPI (blue) and observed by confocal microscopy (Zeiss). Scale bar, 10 μm. To assay the cell surface expression level of Lselectin, FACS analysis was performed with FITC-conjugated L-selectin Ab. Black, IgG; red, nontreated control; green, sulfatide 30 min; blue, sulfatide 1 h. B, Primary microglia and astrocytes were treated with synthetic sulfatide for 1 h, and the mRNA expression of L-selectin, TNF- α , iNOS, or actin was determined by RT-PCR. C, Primary astrocytes were treated with sulfatide or galactocerebroside for 30 min, and Western blot analysis was performed using Abs against L-selectin, phospho-p38, and phospho-ERK. The membranes were reprobed with α -tubulin Ab for loading control. D and E, Primary microglia were pretreated with TAPI for 30 min and then treated with 1 μ M synthetic sulfatide for 18 h. The media was collected and assayed for levels of TNF- α and IL-12 protein by ELISA. Data are representative of at least four independent experiments.



Nonsulfated galactocerebroside does not activate microglia and astrocytes

Sulfatide is formed by the transfer of a sulfate molecule to the third carbon of the galactocerebroside sugar ring (2). We thus examined whether nonsulfated galactocerebroside could also activate glia. Rat primary microglia were treated with 10 µM galactocerebroside or 10 μ M sulfatide, and the activities of inflammatory mediators, including iNOS and MAPKs, were examined. At all time points and concentrations tested, nonsulfated galactocerebroside did not enhance the expression of iNOS and phosphorylation of ERK, p38, and JNK (Fig. 7A and data not shown). Moreover, galactocerebroside had no effect on ICAM-1 surface expression or TNF- α release (Fig. 7, B and C). Similar results were obtained in rat primary astrocytes (data not shown). These results demonstrate that the effects of sulfatide on glial activation are not due to nonspecific effects of lipid components, and they suggest that there are significant differences in the activity of sulfatide compared with galactocerebroside on the brain immune system. Additionally, these results suggest that the sulfate residue may be necessary for sulfatide-induced activation of glia.

Sulfatide also triggers activation of MAPKs in CD1d-deficient mice

CD1d, an MHC class I-like molecule, presents lipid Ags to NKT cells. Sulfatide has been shown to bind to CD1d and activate type II NKT cells (3, 27, 28). Thus, we considered the possibility that CD1d could be involved in sulfatide-triggered inflammatory responses in the brain. We first examined the effects of sulfatide on the surface expression level of CD1d in primary microglia, since CD1d expression is modulated in an inflamed environment. Compared with the mock-treated control, the surface level of CD1d was significantly up-regulated in rat primary microglia by exposure to sulfatide (Fig. 8A). Additionally, the transcript level of CD1d was also rapidly enhanced in microglial cells treated with sulfatide

(Fig. 8A). We then examined whether sulfatide-treated microglial cells could indeed stimulate the activation of NKT cells. After rat primary microglial cells were mock treated or treated with 10 μ M sulfatide for the indicated times, the cells were cocultured with IC8.DC1 cells, a type II NKT hybridoma, or DN32.D3 cells, a type I NKT hybridoma, for 15 h, and the extent of NKT cell activation in response to Ag presentation was determined using an ELISA kit for IL-2. As expected, IL-2 levels in the supernatant were meaningfully increased by coculture with IC8.DC1 cells, but not by coculture with DN32.D3 cells (Fig. 8B). These results demonstrate that sulfatide-treated microglial cells act as APCs and actually stimulate type II NKT cells to produce IL-2.

The above results led us to investigate whether CD1d could contribute to sulfatide-triggered inflammatory responses as well as NKT cell activation in glia. For this, we cultured primary glial cells from CD1d^{-/-} knockout, heterozygous, and wild-type offspring obtained from heterozygous-by-heterozygous matings. The cells were treated with sulfatide for 30 min, followed by Western blot analysis using phospho-JNK, phospho-ERK, ERK, and tubulin Abs. Unexpectedly, sulfatide significantly stimulated the phosphorylation of MAPKs in glial cells from CD1d^{-/-} mice, and the phosphorylation levels were similar to those in wild-type mice (Fig. 8C). Additionally, there was no significant difference in the sulfatide-dependent increase of MCP-1 transcript between glial cells from CD1d-deficient and wild-type mice. Based on these findings, CD1d is not likely to directly influence sulfatide-triggered activation of MAPKs in glia, although its expression is induced in response to sulfatide.

L-selectin may be closely involved in sulfatide-triggered inflammatory responses in glia

Specific carbohydrate modification, including sulfation, has been defined as critical for selectin binding, and the interaction of L-selectin

with its ligands has been shown to generate transmembrane signals (29). Thus, we examined whether L-selectin could contribute to sulfatide-stimulated inflammatory signaling in the brain immune system. Since activation of L-selectin usually results in rapid down-regulation of L-selectin within minutes (30, 31), we tested the expression level of L-selectin in rat primary microglial cells treated with sulfatide. Interestingly, we found that L-selectin expression was rapidly down-regulated at the cell surface after exposure to sulfatide (Fig. 9A). Moreover, RT-PCR and Western blot analysis showed that L-selectin mRNA and protein were also reduced in sulfatide-treated microglia and astrocytes (Fig. 9, B and C).

To confirm the role of L-selectin in sulfatide-induced inflammatory signaling in glia, we tested the effect of TAPI, a pharmacological inhibitor of L-selectin (32), on sulfatide-dependent production of inflammatory cytokines in rat primary microglia. Sulfatide-stimulated production of TNF- α and IL-12 was dose-dependently reduced in the presence of TAPI, suggesting that these effects are L-selectin-dependent responses (Fig. 9, D and E). Taken together, these results suggest that L-selectin may function as an upstream sensor for sulfatide-induced inflammatory responses in the brain immune system.

Discussion

Lipids are emerging as key targets of disease-associated immune responses (3). Increasing reports have suggested the contribution of lipids to a range of pathophysiological conditions, and it has been generally accepted that lipids such as phospholipids and glycolipids may play a critical pathogenic role in numerous inflammation-associated diseases (12, 14). However, very little is understood about the influence of lipids on pathogenesis and their action mechanisms. In this paper, we provide an interesting finding that sulfatide, a major component of myelin, can activate brain-resident immune cells, thus contributing to pathological conditions in the brain.

Myelin is a highly organized multilamellar membrane structure that facilitates impulses via saltatory conduction in vertebrate nervous systems. Myelin differs from other plasma membranes in its composition. It contains abundant galactolipids, and about onethird of the myelin lipids comprise galactocerebrosides and their sulfated derivative, sulfatide. Recent reports have suggested that some of the myelin-specific lipids may be key contributors to the pathogenic mechanism of several CNS diseases. Individuals with MS or Parkinson's disease are reported to have different myelin lipid compositions and elevated levels of anti-sulfatide Ab in biological fluids such as serum and CSF compared with healthy individuals (14). Additionally, recent reports suggest that sulfatide might be a target autoantigen in demyelinating autoimmune diseases such as MS, and transfer of sulfatide-specific Ab caused more severe EAE (3, 14). These reports led us to question whether abnormally released sulfatide from disrupted myelin could act as a glial activator, as well as a target autoantigen during CNS inflammation, thus contributing to the pathogenesis of CNS diseases.

Interestingly, we found that both brain-derived sulfatide and synthetic sulfatide could trigger morphological changes in microglia and induce inflammation-associated molecules including NO and cytokines. Additionally, sulfatide treatment resulted in the activation of intracellular signaling pathways including MAPKs and representative inflammation-associated transcription factors. However, nonsulfated galactocerebroside, another major component of myelin, and MOG, a myelin-derived protein, had no effect on these inflammatory events in glia, suggesting the specific action of sulfatide as a glial activator. These observations suggest that sulfatide alone may cause and exacerbate inflammatory conditions in demyelinating brain. There are several reports to support the pathological actions of sulfatide in the brain. Detrimental effects of sul-

fatide in the nervous system have been observed in metachromatic leukodystrophy. Metachromatic leukodystrophy patients, characterized by accumulation of sulfatide, suffer from a progressive loss of myelin and show various neurological syndromes (6). Recently, Eckhardt and colleagues showed that increasing sulfatide synthesis in a mouse model of metachromatic leukodystrophy causes demyelination and neurological syndromes (33, 34). Based on these reports and our current observations, it may be possible that sulfatide-stimulated inflammatory responses in these patients and mice contribute to the disease phenotypes.

CD1d is a nonpolymorphic MHC class I-like molecule expressed by APCs, such as macrophages, and presents lipid Ags to NKT cells. CD1d-reactive NKT cells produce Th1 and Th2 cytokines and have regulatory functions in innate and adaptive immune responses (28, 35). Sulfatide has been reported as a self-glycolipid ligand recognized by CD1d, which stimulates type II NKT cells. Additionally, several reports have shown that sulfatide-reactive T cells are increased several fold in the CNS during EAE, and that CD1d is significantly up-regulated not only at sites of inflammation sites, but also in EAE (3, 36). Thus, we questioned whether CD1d could contribute to the sulfatide-stimulated activation of glia, known as CNS-resident APCs. Since little is known about the expression and function of CD1d in the CNS, we first examined CD1d expression and functional activity in primary glia. Compared with the mock-treated control, CD1d expression was significantly enhanced by exposure to sulfatide in primary glia. Moreover, sulfatide-treated microglia stimulated type II NKT hybridoma cells, but not type I NKT cells, to produce IL-2, providing evidence that microglia can actually present myelin lipids to T cells (Fig. 8B). To our knowledge, this is the first study to demonstrate that microglia can stimulate NKT cells to produce IL-2. However, sulfatide-stimulated activation of MAPKs was also observed in CD1d-deficient mice, and the phosphorylated levels of MAPKs were similar to those of wild-type littermates. Collectively, these results suggest that CD1d is not likely to mediate our above findings for sulfatide-triggered inflammatory responses in glia.

How then might sulfatide trigger activation of brain-resident immune cells? To address this, we considered the possibility that L-selectin (CD62L), a lectin-like glycoprotein, plays a role in sulfatide-induced activation of glia since it has been found to bind and interact with sulfatide in leukocytes (37). L-selectin is constitutively expressed in almost all leukocytes, including T lymphocytes and macrophages, and it functions in modulating immune responses as a signal transducing receptor, as well as guiding lymphocytes to inflamed sites. Activation of L-selectin usually results in its rapid degradation within minutes (30, 31, 38). For example, inflammatory agents such as PMA and LPS can cause rapid downregulation of L-selectin, which inhibits the migration of activated immune cells to inflamed sites and overactivation of intracellular signaling cascades. Thus, we examined the involvement of L-selectin in sulfatide-stimulated activation of glia. As shown in Fig. 9, L-selectin was expressed at the cell surface of microglia and astrocytes in normal conditions, but was significantly reduced in sulfatide-treated cells. Moreover, expression of L-selectin was also rapidly modulated by exposure to sulfatide at both the transcript and protein levels (Fig. 9, B and C). The contribution of L-selectin to sulfatide-triggered events was further demonstrated by examining the effects of L-selectin inhibitors. In microglia and astrocytes, L-selectin inhibitors dramatically reduced the sulfatide-dependent increase in TNF- α and IL-12 (Fig. 9, D and E). These results imply that L-selectin is regulated in response to sulfatide in the brain and may be an upstream sensor for sulfatide-induced inflammatory events.

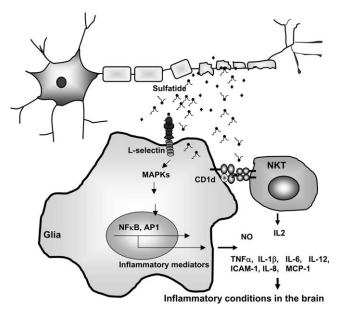


FIGURE 10. Schematic diagram depicting possible mechanisms for sulfatide-triggered inflammation in the brain. Sulfatides are released at sites of injured and/or inflamed brain lesions accompanying myelin destruction, which can activate brain-resident immune cells, thus contributing to pathological conditions in the brain.

Recently, we observed that some of the sulfatide-triggered inflammatory responses are affected by disruption of lipid raft formation, but not by L-selectin inhibitors such as TAPI, inositol, and chymotrypsin (our unpublished data). These observations raised the possibility that another mechanism besides the L-selectin-dependent mechanism might also contribute to sulfatide-stimulated activation of inflammatory responses in the brain. Lipid raft formation has recently been shown to be important for the dynamic association of multiprotein receptor complexes involved in immune responses (39, 40). Thus, we are currently investigating lipid raft formation-associated signaling events to ascertain the molecular mechanism underlying sulfatide-dependent activation of inflammatory responses in the brain.

The extracellular space (ECS) in the CNS forms the microenvironment of neurons and glia. The composition and size of the ECS change dynamically in response to pathological conditions such as ischemia, tumor, and demyelination. The changes of ECS under pathological states seem to exacerbate the pathogenesis of several diseases (41, 42). Over several years, we have investigated the pathological action of abnormally released cellular components into the ECS, such as gangliosides in immune regulation and inflammation-associated diseases of the CNS (43-45). In the present paper we propose that abnormally released sulfatide from demyelinated brain regions into the ECS may act as an endogenous inflammatory agent as well as an autoantigen in the brain immune system (Fig. 10). Our data provide an interesting clue regarding the pathogenic action of sulfatide in injured brain regions accompanying myelin destruction. Further study of sulfatide-dependent pathological signaling events and the role of glial cells should contribute to a better understanding of several CNS diseases and provide interesting insights into potential therapeutic interventions for CNS diseases accompanying demyelination and altered sulfatide levels.

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Disclosures

The authors have no financial conflicts of interest.

References

- Coetzee, T., K. Suzuki, and B. Popko. 1998. New perspectives on the function of myelin galactolipids. *Trends Neurosci.* 21: 126–130.
- Honke, K., Y. Hirahara, J. Dupree, K. Suzuki, B. Popko, K. Fukushima, J. Fukushima, T. Nagasawa, N. Yoshida, Y. Wada, and N. Taniguchi. 2002. Paranodal junction formation and spermatogenesis require sulfoglycolipids. *Proc. Natl. Acad. Sci. USA* 99: 4227–4232.
- Halder, R. C., A. Jahng, I. Maricic, and V. Kumar. 2007. Mini review: immune response to myelin-derived sulfatide and CNS-demyelination. *Neurochem. Res.* 32: 257–262.
- Vos, J. P., M. Lopes-Cardozo, and B. M. Gadella. 1994. Metabolic and functional aspects of sulfogalactolipids. *Biochim. Biophys. Acta* 1211: 125–149.
- Coetzee, T., N. Fujita, J. Dupree, R. Shi, A. Blight, K. Suzuki, K. Suzuki, and B. Popko. 1996. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. *Cell* 86: 200–210
- von Figura, K., V. Gieselman, and J. Jaeken. 2001. Metachromatic leukodystrophy. In *The Metabolic and Molecular Basis of Inherited Disease*. 8th ed. McGraw-Hill, New York, pp. 3695–3724.
- Han, X., H. Cheng, J. D. Fryer, A. M. Fagan, and D. M. Holtzman. 2003. Novel role for apolipoprotein E in the central nervous system: modulation of sulfatide content. J. Biol. Chem. 278: 8043

 –8051.
- Han, X., A. M. Fagan, H. Cheng, J. C. Morris, C. Xiong, and D. M. Holtzman. 2003. Cerebrospinal fluid sulfatide is decreased in subjects with incipient dementia. Ann. Neurol. 54: 115–119.
- Ilyas, A. A., Z. W. Chen, and S. D. Cook. 2003. Antibodies to sulfatide in cerebrospinal fluid of patients with multiple sclerosis. *J. Neuroimmunol.* 139: 76–80.
- Aruffo, A., W. Kolanus, G. Walz, P. Fredman, and B. Seed. 1991. CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell 67: 35–44.
- Nygren, C., H. von Holst, K. Ericson, and P. Fredman. 2001. Patients with primary brain tumours have elevated serum titres of antibodies to the myelin glycolipid sulphatide. Eur. Neurol. 45: 38–42.
- Han, X., M. H. D., D. W. McKeel, Jr., J. Kelley, and J. C. Morris. 2002. Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *J. Neurochem.* 82: 809–818.
- Cheng, H., J. Xu, D. W. McKeel, Jr., and X. Han. 2003. Specificity and potential mechanism of sulfatide deficiency in Alzheimer's disease: an electrospray ionization mass spectrometric study. Cell. Mol. Biol. 49: 809–818.
- Kanter, J. L., S. Narayana, P. P. Ho, I. Catz, K. G. Warren, R. A. Sobel, L. Steinman, and W. H. Robinson. 2006. Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat. Med.* 12: 138–143.
- Kreutzberg, G. W. 1996. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 19: 312–318.
- Platten, M., A. Kretz, U. Naumann, S. Aulwurm, K. Egashira, S. Isenmann, and M. Weller. 2003. Monocyte chemoattractant protein-1 increases microglial infiltration and aggressiveness of gliomas. *Ann. Neurol.* 54: 388–392.
- Nimmerjahn, A., F. Kirchhoff, and F. Helmchen. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308: 1314–1318.
- Ridet, J. L., S. K. Malhotra, A. Privat, and F. H. Gage. 1997. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci*. 20: 570–577.
- Streit, W. J., S. A. Walter, and N. A. Pennell. 1999. Reactive microgliosis. Prog. Neurobiol. 57: 563–581.
- Guo, B., E. Y. Chang, and G. Cheng. 2008. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. *J. Clin. Invest.* 118: 1680–1690.
- Prinz, M., H. Schmidt, A. Mildner, K. P. Knobeloch, U. K. Hanisch, J. Raasch, D. Merkler, C. Detje, I. Gutcher, J. Mages, et al. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* 28: 675–686.
- Park, S. H., A. Weiss, K. Benlagha, T. Kyin, L. Teyton, and A. Bendelac. 2001. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. J. Exp. Med. 193: 893–904.
- Park, S. H., J. H. Roark, and A. Bendelac. 1998. Tissue-specific recognition of mouse CD1 molecules. *J. Immunol.* 160: 3128–3134.
- Pernber, Z., M. Molander-Melin, C. H. Berthold, E. Hansson, and P. Fredman. 2002. Expression of the myelin and oligodendrocyte progenitor marker sulfatide in neurons and astrocytes of adult rat brain. *J. Neurosci. Res.* 69: 86–93.
- Kroncke, K. D., K. Fehsel, and V. Kolb-Bachofen. 1998. Inducible nitric oxide synthase in human diseases. Clin. Exp. Immunol. 113: 147–156.
- Hess, J., P. Angel, and M. Schorpp-Kistner. 2004. AP-1 subunits: quarrel and harmony among siblings. J. Cell Sci. 117: 5965–5973.
- Zajonc, D. M., I. Maricic, D. Wu, R. Halder, K. Roy, C. H. Wong, V. Kumar, and I. A. Wilson. 2005. Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity. *J. Exp. Med.* 202: 1517–1526.

 Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. Annu. Rev. Immunol. 25: 297–336.

- Gomez-Gaviro, M., M. Dominguez-Luis, J. Canchado, J. Calafat, H. Janssen, E. Lara-Pezzi, A. Fourie, A. Tugores, A. Valenzuela-Fernandez, F. Mollinedo, et al. 2007. Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on Lselectin shedding. *J. Immunol.* 178: 8053–8063.
- Feehan, C., K. Darlak, J. Kahn, B. Walcheck, A. F. Spatola, and T. K. Kishimoto. 1996. Shedding of the lymphocyte L-selectin adhesion molecule is inhibited by a hydroxamic acid-based protease inhibitor: identification with an L-selectinalkaline phosphatase reporter. J. Biol. Chem. 271: 7019–7024.
- Walcheck, B., J. Kahn, J. M. Fisher, B. B. Wang, R. S. Fisk, D. G. Payan, C. Feehan, R. Betageri, K. Darlak, A. F. Spatola, and T. K. Kishimoto. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature* 380: 720–723.
- Bennett, T. A., B. S. Edwards, L. A. Sklar, and S. Rogelj. 2000. Sulfhydryl regulation of L-selectin shedding: phenylarsine oxide promotes activation-independent L-selectin shedding from leukocytes. *J. Immunol.* 164: 4120–4129.
- Eckhardt, M., K. K. Hedayati, J. Pitsch, R. Lullmann-Rauch, H. Beck, S. N. Fewou, and V. Gieselmann. 2007. Sulfatide storage in neurons causes hyperexcitability and axonal degeneration in a mouse model of metachromatic leukodystrophy. *J. Neurosci.* 27: 9009–9021.
- 34. Ramakrishnan, H., K. K. Hedayati, R. Lullmann-Rauch, C. Wessig, S. N. Fewou, H. Maier, H. H. Goebel, V. Gieselmann, and M. Eckhardt. 2007. Increasing sulfatide synthesis in myelin-forming cells of arylsulfatase A-deficient mice causes demyelination and neurological symptoms reminiscent of human meta-chromatic leukodystrophy. J. Neurosci. 27: 9482–9490.

- Parekh, V. V., M. T. Wilson, D. Olivares-Villagomez, A. K. Singh, L. Wu, C. R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces longterm natural killer T cell anergy in mice. J. Clin. Invest. 115: 2572–2583.
- Jahng, A., I. Maricic, C. Aguilera, S. Cardell, R. C. Halder, and V. Kumar. 2004.
 Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. J. Exp. Med. 199: 947–957.
- Duchesneau, P., E. Gallagher, B. Walcheck, and T. K. Waddell. 2007. Up-regulation of leukocyte CXCR4 expression by sulfatide: an L-selectin-dependent pathway on CD4⁺ T cells. *Eur. J. Immunol.* 37: 2949–2960.
- Lee, D., J. B. Schultz, P. A. Knauf, and M. R. King. 2007. Mechanical shedding of L-selectin from the neutrophil surface during rolling on sialyl Lewis x under flow. J. Biol. Chem. 282: 4812–4820.
- Dustin, M. L. 2002. Membrane domains and the immunological synapse: keeping T cells resting and ready. J. Clin. Invest. 109: 155–160.
- Rajendran, L., and K. Simons. 2005. Lipid rafts and membrane dynamics. J. Cell Sci. 118: 1099–1102.
- Sykova, E. 2001. Glial diffusion barriers during aging and pathological states. Prog. Brain Res. 132: 339–363.
- 42. Nicholson, C. 2005. Factors governing diffusing molecular signals in brain extracellular space. *J. Neural Transm.* 112: 29–44.
- Kim, O. S., E. J. Park, E. H. Joe, and I. Jou. 2002. JAK-STAT signaling mediates gangliosides-induced inflammatory responses in brain microglial cells. *J. Biol. Chem.* 277: 40594–40601.
- 44. Yang, M. S., E. J. Park, S. Sohn, H. J. Kwon, W. H. Shin, H. K. Pyo, B. Jin, K. S. Choi, I. Jou, and E. H. Joe. 2002. Interleukin-13 and -4 induce death of activated microglia. *Glia* 38: 273–280.
- Jou, I., J. H. Lee, S. Y. Park, H. J. Yoon, E. H. Joe, and E. J. Park. 2006. Gangliosides trigger inflammatory responses via TLR4 in brain glia. Am. J. Pathol. 168: 1619–1630.