The Regulation of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 2 (SHP-2) in Brain Microglia
The Regulation of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 2 (SHP-2) in Brain Microglia

By

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Department of Medical Sciences
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김희영의 의학 박사학위 논문을 인준함.

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- ABSTRACT -

The Regulation of Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase 2 (SHP-2) in Brain Microglia

SHP-2, a member of protein tyrosine phosphatases (PTPs), plays a role on the regulation of several signaling pathway such as NF-κB, MAP kinase, JAK-STAT, and PI3 kinase. However, the roles of SHP-2 in brain microglia activation are largely unknown. Here, I determined the regulation of SHP-2 on JAK-STAT signaling pathway in brain microglia. This study firstly shows that curcumin has inhibitory effects on JAK-STAT signaling, which is mediated by SHP-2 in brain microglia. Curcumin has been strongly implicated as an anti-inflammatory agent, but the precise mechanisms of its action are largely unknown. I firstly determined whether curcumin suppressed the gangliosides-, LPS-, and IFN-γ-induced microglial activation. In both rat primary microglia and murine BV2 microglial cells, curcumin effectively suppressed the gangliosides-, LPS-, or IFN-γ-stimulated induction of COX-2 and iNOS, important enzymes that mediate inflammatory processes. Also, curcumin consistently suppressed nuclear factor binding to GAS/ISRE sequences which regulate iNOS and COX-2 expressions. Curcumin markedly inhibited the phosphorylation of STAT1/3 as well as JAK1/2 in microglia activated with gangliosides, LPS, or IFN-γ. Curcumin inhibits the expression of inflammation-associated genes, including ICAM-1 and MCP-1, whose promoters
contain STAT-binding elements. To test the inhibitory mechanism of curcumin on JAK-STAT pathway, I firstly tested the expression of SOCS1/3 by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). As a result, their expressions were not induced by curcumin. Also, I examined the phosphorylation of SHP-2 by immunoprecipitation in microglia. Treatment of microglial cells with curcumin induced phosphorylation of SHP-2. I further showed that curcumin led to an increase in association with JAK1/2 of SHP-2, which inhibit the initiation of JAK-STAT inflammatory signaling in activated microglia. Taken together, these data suggest curcumin suppresses JAK-STAT signaling via activation of SHP-2, thus attenuating inflammatory response of brain microglial cells. Secondly, this study also shows that SHP-2 is regulated by lipid rafts in gangliosides-activated microglia. It was reported that, in cultured rat brain microglia, gangliosides induce rapid and transient activation of the JAK-STAT pathway. I hypothesized that raft-mediated SHP-2 activation is involved in transient JAK-STAT signaling by gangliosides. To test this hypothesis, I first used Western blot analysis to show that SHP-2 is rapidly phosphorylated by gangliosides. This was inhibited by pretreatment with the lipid raft disrupter, filipin and was restored following filipin removal. Immunostaining using antibodies directed against p-SHP-2 and flotillin-1 revealed gangliosides-induced clustering and polarization of p-SHP-2 in membrane rafts. Raft-associated regulation of SHP-2 was further demonstrated in fractionation experiments using detergent and detergent-free sucrose gradient ultracentrifugation. Rapid SHP-2 recruitment to detergent-insoluble raft fractions by gangliosides was inhibited by
filipin, further indicating the involvement of rafts. Immunoprecipitation experiments confirmed that SHP-2 rapidly binds to JAK2 in response to gangliosides and this binding could be inhibited by filipin and restored upon filipin removal. This study therefore showed that transient activation of the JAK-STAT pathway by gangliosides is accomplished by SHP-2 in a raft-dependent manner in brain microglia. In conclusion, raft-mediated regulation of SHP-2 is suggested to be an essential component of inflammatory mechanism of brain microglia.

**Key words**: SHP-2, JAK-STAT, Curcumin, Gangliosides, Lipid raft, Microglia, Brain inflammation
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<tbody>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma Interferon Activated Site</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-Stimulated Regulatory Element</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signaling</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein Tyrosine Phosphatase</td>
</tr>
<tr>
<td>SHPs</td>
<td>Src Homology 2 Domain-Containing PTPases</td>
</tr>
<tr>
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<td>inducible NO Synthase</td>
</tr>
<tr>
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<td>Cyclooxygenase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemoattractant Protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>Gmix</td>
<td>Gangliosides Mixture</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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I. INTRODUCTION

A. Brain inflammation; microglial activation

1. Characterization of brain microglia

Microglia constitute approximately 5~20% of the total glial cell population and are known as the brain resident macrophage cells. Microglia have usually a ramified morphology, which are referred as resting states. However, when brain injuries occur by infection, stroke, and neurodegeneration, they are rapidly activated with the conversion into a round and amoeboid morphology, proliferate, and migrate to injured sites (Streit, 1996; Vilhardt, 2005). As microglia are over-activated, microglia-induced inflammatory responses may exacerbate neuronal damage. Activated microglia are observed in various human neurological diseases, including AIDS dementia complex, multiple sclerosis and Alzheimer’s disease (McRae et al., 1997; Gonzalez-Scarano and Baltuch, 1999; Aldskogius, 2001; McGeer and McGeer, 2004). Thus it may be functionally important to tightly regulate the degree of microglial activation after injury or in chronic disease. Therefore, the regulation of microglial activation could be a good therapeutic target to combat neurodegenerative disease. In this regards, the mechanisms involved in microglia activation and how to suppress it have been vigorously studied.
2. Brain microglial activation at *in vitro* and *in vivo* system

In diverse pathological conditions of brain, microglial activation is induced by inflammatory mediators such as cytokine, neuronal death, and abnormal protein aggregation. As microglial stimulator, lipopolysaccharide (LPS), interferon-γ, beta-amyloid (Aβ), and gangliosides have been used. LPS, as a cell-wall component of gram-negative bacteria, has commonly utilized for induction of CNS inflammatory response (Rock et al., 2004). LPS increases iNOS expression and NO release, and also induces the expression of interleukin-13 and TNF-α in microglia (Pyo et al., 1999; Shin et al., 2004). Interferon-γ also induces microglial activation by producing proinflammatory cytokines and prostaglandins (Possel et al., 2000; Jensen et al., 2000; Han et al., 2003). Aβ is produced in the brain of Alzheimer’s disease and induces a phagocytic response, the expression of cytokines and chemokines, and generation of reactive oxygen species in microglia (Bonaiuto et al., 1997; Pyo et al., 1998; Kopec and Carroll, 1998; Meda et al., 1999). Gangliosides, as membrane components, are especially distributed on neuronal cells and released by death or injury of many brain cells. Gangliosides are sialic acid-containing glycosphingolipid and are classified into GM1, GD1a, GD1b and GT1b according number and sites of sialic acid residues attaching to the carbohydrates. It has been reported that gangliosides activate cultured rat brain microglia through mitogen-activated protein (MAP) kinases, NF-κB (Pyo et al., 1999), and JAK-STAT pathway (Kim et al., 2002). Up to date, the mechanisms of microglial activation by several stimulators have been studied but remains to be clarified.
B. Inflammatory signaling pathways involved in microglial stimulation

Microglia activation is regulated by many signaling pathway such as NF-κB, MAP kinases, and AP-1, and so on. For example, PMA and IFN-γ synergistically enhance NO production through NF-κB and ERK (Han et al., 2003) and IL-12 induces iNOS expression by NF-κB activation in microglia (Pahan et al., 2001). Also, LPS activates MAP kinases such as ERK, JNK, and P38 in microglia (Pyo et al., 1998). Recently, JAK-STAT pathway has been reported to be involved in LPS-, IFN-γ-, gangliosides-activated microglia. This is said that JAK-STAT pathway and its regulatory mechanisms are very important in brain inflammatory response.

1. JAK-STAT signaling pathway in brain microglia

JAK-STAT signaling, originally identified as the signaling pathway for interferons (IFNs), mediates the immune responses of several cytokines as well as the actions of many growth factors and hormones, and thus participates in inflammation (Hackett et al., 1997; Rane and Reddy, 2000; Dell’Albani et al., 2001). Specific subtypes of JAK and STAT molecules mediate different signals, resulting in specificity of responses (Schindler, 1999; Cull et al., 2003). The binding of a ligand to its receptor induces assembly of an active receptor complex and subsequent phosphorylation of the receptor-associated JAKs (JAK1, JAK2, JAK3, and TYK2). Phosphorylated JAKs lead to the activation of neighboring JAKs, receptor subunits and several other substrates, and provide the docking sites for STATs (STAT1-6), which in turn become phosphorylated. Phosphorylated STATs are released from the
receptor complex and form homo- or heterodimers. These dimers translocate to the
nucleus where they directly bind to the promoter region of specific target genes, thus
regulating transcription of inflammation-associated genes (Wen et al., 1995; Park and

2. Negative regulation of JAK-STAT signaling

2-1. SOCS family and PIAS

Activated JAK-STAT pathway should be regulated negatively. Inhibitory
molecules including the suppressors of cytokine signaling (SOCS) family (SOCS1-7
and CIS) and protein inhibitor of activated STAT (PIAS) have been suggested to be
part of the negative feedback regulation of JAK-STAT signaling (Naka et al., 1997;
Starr et al., 1997; Endo et al., 1997; Starr and Hilton, 1999; Krebs and Hilton, 2001).
SOCS proteins include a src homology (SH) 2 domain and a SOCS box in C-
terminal region, and N-terminal region variable in length, of which SOCS1-3 are
short compared to SOCS4-7. SOCS1, 3, and CIS are regulated by STATs, which bind
to their STAT binding element-containing promoter regions and induce their
expression. However, interestingly, the SOCS1 and 3 expression have been reported
to be induced by peroxisome proliferators-activated receptor (PPAR)-γ agonist in a
JAK-STAT independent manner (Park et al., 2003). STAT-induced SOCS proteins act
in negative feedback loop. They are reported to inactivate JAKs and block the access
of STATs to receptor binding sites. SOCS1 binds to the JAKs and inhibits catalytic
activity. SOCS3 binds to JAK-proximal sites on cytokine receptor and inhibits JAK activity. CIS binds to the sites of STAT on the activated receptor and blocks the access of STAT-receptor binding. PIAS has been reported to be associated with tyrosine phosphorylated STAT-dimers, inducing the inactivation of STATs (Krebs and Hilton, 2001).

2-2. Src homology 2 domain-containing protein tyrosine phosphatases (SHPs)

Protein tyrosine phosphatases (PTPs) including src homology 2 (SH2) domain-containing PTPases 1 and 2 (SHP-1 and 2) are also important regulators of JAK-STAT pathway. SHPs have two SH2 domains at the amino terminus and a PTPase catalytic domain at the carboxyl terminus, which catalyze the tyrosine dephosphorylation of JAKs, receptor or other cellular proteins, and thus play critical roles in the control of cytokine signaling (Jiao et al., 1996; You et al., 1999; Qu, 2000; Qu, 2002; Lehmann et al., 2003; Neel et al., 2003). SHPs are auto-inhibited in their resting state by an intramolecular interaction between their SH2 domains and the PTPase domain. The PTPase domain is activated after its association with tyrosine phosphorylated proteins including membrane receptors and JAKs (Hof et al., 1998). SHPs are activated by phosphorylation of two tail tyrosine residues. These phosphorylations serve an adaptor function in the possible recruitment of other molecules or in itself stimulate its phosphatase activity (Lu et al., 2001; Neel et al., 2003). SHP-1 binds to and dephosphorylate JAK2 in hematopoietic cells (Jiao et al., 1996; Starr and Hilton, 1999). The experiments using SHP-1 null mutant mice show
IL-4/13-activated JAK-STAT pathway is negatively regulated by SHP-1 (Haque et al., 1998). Also, it is reported that cisplatin suppresses JAK-STAT signaling through modulation of SHP-1 in cancer cells (Song et al., 2004). SHP-2 negatively or positively regulates many cellular functions such as growth, proliferation, differentiation, apoptosis, and inflammation. SHP-2 has been reported to positively regulate the MAP kinase signaling pathway initiated by either interleukin-2 or various growth factors (Cai et al., 2002; Arnaud et al., 2004) and to negatively regulate JAK-STAT signaling pathways (You et al., 1999; Stofega et al., 2000). The positive regulation of MAP kinase is achieved through dephosphorylation of the RasGap-binding site on PDGFR, association with other adaptor molecules, or inactivation of inhibitory molecules, all of which eventually lead to Ras-Raf-MAP kinase activation (Neel et al., 2003). SHP-2 exerts its negative regulatory effects via direct dephosphorylation of tyrosine-phosphorylated signaling molecules such as JAKs and STATs, which must occur through direct targeting of SHP-2 to tyrosine-phosphorylated, or otherwise modified signaling molecules (Stofega et al., 2000).

C. Characterizations of lipid rafts

Lipid rafts, which are detergent-resistant, liquid-ordered membrane domains, are enriched for cholesterol, glycosphingolipids, and phospholipids with relatively long and saturated acyl chains. They are characterized by insolubility in non-ionic detergents such as Triton X-100 and Brij 58 at 4°C and separated in low density fractions during sucrose gradient ultracentrifugation because of its light buoyant
density. The size of raft domains is known to be range between 30 and 250 nm, indicating the model that lipid rafts coalesce upon stimulation (Mayor and Rao, 2004).

1. Functions of lipid rafts

Lipid rafts are reported to serve as platforms for several cellular functions, including vesicular trafficking and signal transduction (Draber and Draberova, 2002; Lai, 2003; Manes et al., 2003; Harder, 2004; Lucero and Robbins, 2004). Raft domains are present in several types of signaling molecules, including receptor tyrosine kinases, the Src family of non-receptor tyrosine kinases (SFKs), and G proteins, as well as structural proteins such as members of the caveolin and flotillin families. Upon stimulation, lipid rafts are implicated in the regulation of numerous cellular events, including signal transduction, endocytosis or exocytosis, transport of vesicles, antigen presentation, and viral entry/infection (Draber and Draberova, 2002; Helms and Zurzolo, 2004). Especially, initial T cell signaling related to molecules such as lck, CD45, CD8, and CD4, is regulated through lipid raft, termed “TCR raft” or TCR signalosome” (He et al., 2005).

2. SHP-2 regulation by lipid rafts

It is necessary for SHP-2 to be targeted to the tyrosine-phosphorylated molecules and SHP-2 has been reported to directly interacts with growth hormone receptor as well as the gp130 receptor (Stofega et al., 2000; Bode et al., 2003), which
exist in raft microdomains. Also, SHP-2 also regulates Rho activity through its recruitment to lipid rafts (Lacalle et al., 2002). Therefore, lipid raft-mediated regulation of SHP-2 is an essential component of many cellular signaling pathways.

D. Function and mechanism of curcumin

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-di-one] is a major component of turmeric. It has been used as an Indian medicine for centuries, and is currently commonly used as a spice for flavor and to impart a yellow color. Curcumin has recently received much attention for its anti-inflammatory, antioxidant and anti-tumor activities (Huang et al., 1991; Rao et al., 1995; Oetari et al., 1996). The anti-inflammatory actions of curcumin seem to be closely related to the suppression of pro-inflammatory cytokines and mediators of their release such as TNF-α, IL-1β, and NOs. There are reports that curcumin inhibits cytokine-mediated NF-κB activation by blocking a signal leading to IKK activity in intestinal epithelial cells and mouse fibroblast cells, and also suppresses phorbol ester-induced c-Jun/AP-1 activation (Huang et al., 1991; Singh and Aggarwal, 1995; Jobin et al., 1999). However, the mechanisms underlying curcumin’s interactions with these signaling pathways are poorly understood.
E. Objects of Studies

In this study, I tested anti-inflammatory action of curcumin and SHP-2 in microglia activated by gangliosides.

- Firstly to investigate whether curcumin inhibits the JAK-STAT pathway in activated microglia, I performed as follows.
  
  (1) I tested whether curcumin inhibits the phosphorylation of JAK1/2 and STAT1/3 in rat primary microglia and BV2 microglial cells.
  
  (2) I tested whether the suppression of JAK-STAT signaling by curcumin is accomplished through activation of SHP-2.

- Secondly to investigate that gangliosides induce the activation of SHP-2 in a raft-dependent manner, I tested as follows

  (1) I tested whether gangliosides induce the phosphorylation of SHP-2.
  
  (2) I tested whether lipid raft is involved in gangliosides-induced SHP-2 activation.
  
  (3) I confirmed whether SHP-2 is recruited to lipid raft by gangliosides.
  
  (4) I tested whether SHP-2 activation by gangliosides is associated with JAK2 in a raft-dependent manner.
II. MATERIALS AND METHODS

A. Reagents

Curcumin and lipopolysaccharide (LPS, from salmonella enteritidis) were purchased from Sigma Chemicals (St. Louis, MO, USA), and IFN-γ was purchased from Calbiochem (San Diego, CA, USA). Gangliosides mixture (Gmix) was purchased from Matraya (Pleasant Gap, PA, USA). Antibodies against STAT1, phospho-STAT1 (Y701, Y727), phospho-STAT3 (Y705), and Phospho-SHP-2 (Y580) antibody were purchased from Cell Signaling Technology (Beverley, MA, USA). Antibodies against iNOS, phospho-tyrosine (4G10), JAK1 and JAK2 were purchased from Upstate (Lake Placid, NY, USA), and antibodies against COX-2 and actin were purchased from Santa Cruz Technology (Santa cruz, CA, USA). Phospho-JAK1 and phospho-JAK2 antibodies were purchased from Calbiochem and Upstate respectively. SHP-1, SHP-2, and Flotillin-1 antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Cholera toxin B (GM1, horseradish peroxidase [HRP]-conjugated) and Filipin complex from Streptomyces filipinensis was obtained Sigma Chemicals (St. Louis, MO, USA).

B. Cell culture

Primary microglia were cultured from the cerebral cortices of 1- to 3-day old Sprague-Dawley rats as previously described (Kim et al., 2002; Kim et al., 2003). Briefly, the cortices were tritutated into single cells in minimal essential medium
eagle (Sigma Chemicals, St. Louis, MO, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA) and plated in 75-cm² 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 60 mm dishes (8×10⁵ cells/dish) or 100 mm dishes (2×10⁶ cells/dish). One hour later, the cells were washed to remove unattached cells before being used in experiments. BV2 immortalized murine microglial cells were obtained from Dr. E. J. Choi (Korea University, Republic of Korea). The BV2 cell line was grown in Dulbecco’s Modified Eagle Medium with 5% fetal bovine serum. The cells all were serum-starved overnight, before being used for experiments.

C. Electrophoretic mobility shift assays (EMSA)

Cells were harvested and suspended in 9× 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, 0.5 mM phenylmethylsulfonyl fluoride) including 0.5% Nonidet P-40. Cells were centrifuged at 500 ×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 dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, were incubated on ice for 60 min with occasional gentle shaking, and were centrifuged at 12,000 ×g for 20 min. The crude nuclear proteins in the supernatant were collected and stored at −70°C for electrophoretic mobility shift assays (EMSA). EMSA was performed for 30 min.
on ice in a volume of 20 µl, containing 1~2 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 d (I-C), 1 mM dithiothreitol, 0.3 mg/ml bovine serum albumin, 6 mM MgCl₂ and γ³²P radiolabeled oligonucleotide probe (3×10⁴ cpm), with or without a 20- to 50-fold excess of unlabelled probe. The dried gels were exposed to X-ray film. The following double-stranded oligonucleotide was used in these studies: GAS/ISRE, 5’-AAGTACTTTCAGTTTCA TA TTACTCTA-3’, 27 bp (Santa Cruz Biotechnology, Inc., sc-2537). 5’-end-labeled probes were prepared with 40 µCi of γ³²P ATP using T4 polynucleotide kinase (Promega, WI, USA) and were purified on Quick Spin Columns Sephadex G-25 (Boehringer Mannheim).

D. Western blot analysis

Cells were washed twice with cold phosphate-buffered saline (PBS), and then lysed and scraped in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM Na₃VO₄) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, 10 µg/ml pepstatin, 1 µg/ml Aprotinin and 2 mM EDTA). The lysate was centrifuged for 20 min at 12000 g at 4°C and the supernatant collected. Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membrane (Schleicher & Schuell BioScience, Dassel, Germany). The membrane was blotted with 5% skim milk in tris buffered saline (TBS) for 1 h, incubated with adequate primary antibodies at 4°C overnight, and after washing with TBS containing 1% Triton X-100 incubated with
peroxidase-conjugated anti-mouse, anti-goat, and anti-rabbit secondary antibodies for 1 h (Vector Lab., Burlingame, CA or Zymed, USA). These membranes were visualized using an enhanced chemiluminescence (ECL) system (Sigma).

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

Cells were prepared in 6 well plates and treated with proper drugs. Total RNA was extracted using RNAzol B (TEL-TEST, Friendswood, TX) and cDNA was prepared using reverse transcriptase from Avian Myeloblastosis Virus (TaKaRa, Japan), according to the manufacturer’s instructions. PCR was performed with 30 cycles of sequential reactions: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). The sequences of PCR primers show at table 1.

F. Immunoprecipitation

Cell extracts were prepared by using modified RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM NaVO₄). 500 µg lysates were incubated with 1 ~ 2 µg of the appropriate antibody at 4°C overnight and precipitated with protein G-agarose beads (Upstate) for 2 h at 4°C. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Germany). Then western blot analysis was performed with several antibodies as indicated.
Table 1. Primer Sequences for PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5’-TCCCTCAAGATTGTAGGACGACAAAA-3’&lt;br&gt;R: 5’-AGATCCACAACGGATACATT-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>F: 5’-CATGTTTTAGACCTCTCAACAACCCC-3’&lt;br&gt;R: 5’-GCCATCTCCTGCTCAGAAGTCTAG-3’</td>
</tr>
<tr>
<td>SOCS1</td>
<td>F: 5’-CACTCACTCCGACACCTTC-3’&lt;br&gt;R: 5’-AGCAGCTCGAAAAGGAGGAGCTAG-3’</td>
</tr>
<tr>
<td>SOCS3</td>
<td>F: 5’-ACCAGCGCCACTTCTTCACG-3’&lt;br&gt;R: 5’-GTGGAGCATCATACTGATCC-3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>F: 5’-ATGCAGGTCTCTGTCACGCCT-3’&lt;br&gt;R: 5’-CTAGTCTCCTGTCACTGAGGAG-3’</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>F: 5’-CTGGAGAGACAAACAGCAGAC-3’&lt;br&gt;R: 5’-AAGGCCGAGACAGCACAAAGAAGC-3’</td>
</tr>
</tbody>
</table>
G. Isolation of detergent insoluble fraction

Cells were washed twice with ice-cold PBS and lysed with Hepes buffer (10 mM sodium Hepes [pH 7.5], 150 mM NaCl, 5 mM EDTA and 0.5 mM PMSF) containing 0.5% Triton X-100. Then, the cells were incubated for 30 min and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were used as soluble fraction (S). The pellet were washed with 1 ml of cold Hepes buffer without a detergent, solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM Na3VO4), and centrifuged for 30 min at 4°C. These supernatants were used as insoluble fraction (I). Each fraction was analysed by western blotting.

H. Detergent-free, discontinuous sucrose gradient ultracentrifugation

To isolate the low-density fraction as raft regions, discontinuous sucrose gradient ultracentrifugation was used (Song et al., 1996). The cells were washed twice with ice-cold PBS and scraped into 0.5 M sodium carbonate (pH 11.0). And then, homogenization was carried out using a loose-fitting Dounce homogenizer (about 40 strokes). The homogenate was then adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A 5-35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose; 4 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) and centrifuged at 38,000 rpm for 20
h in an SW41 rotor (Beckman Instruments, Palo Alto, CA).
From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 12 fractions and precipitated with 10% Trichloroacetic acid (TCA, Aldrich, USA). Gradient fractions were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. After transfer, nitrocellulose membranes were blotted and incubated with anti-SHP-2.

I. Immunostaining and confocal microscopy
To determine p-SHP-2 and flotillin-1 localization, cells cultured on poly-D-lysine coated coverslips, were washed twice with ice-cold PBS and fixed with 100% methanol at –20°C. The fixed cells were washed with PBST (PBS containing 0.1% Triton X-100) and blotted with 10% serum for 30 min at room temperature. And then, the primary antibodies were incubated overnight at 4°C, fluorescein or rhodamine-conjugated secondary antibodies were incubated for 2 h, and the cells were mounted. The mounted cells were observed with the confocal microscopy (Zeiss, Germany).
III. RESULTS

A. Curcumin suppresses JAK-STAT inflammatory signaling through activation of SHP-2 in brain microglia

1. Curcumin inhibits the up-regulation of iNOS and COX-2 in activated microglia

To investigate the anti-inflammatory actions of curcumin and its mechanism of action in brain, I first examined the effect of curcumin on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, key enzymes in inflammatory processes in activated microglia. Rat primary microglia were stimulated with 50 µg/ml gangliosides, 100 ng/ml LPS, or 10 U/ml IFN-γ for 8 h in the absence or presence of curcumin. Cell extracts were assayed by western blot with antibodies against either iNOS or COX-2. All the three-microglial activators significantly induced the expression of iNOS and COX-2, but curcumin suppressed this induction in a dose-dependent manner (Fig. 1A). Although curcumin has been reported to show anti-inflammatory effects or chemo-preventive effects in the range of 10 to 40 µM in other cells, 20 µM of curcumin sometimes affected cell viability in microglial cells (McGeer et al., 1988; Natarajan and Bright, 2002). Thus, I used 5 or 10 µM curcumin for subsequent experiments. Similar anti-inflammatory effects of curcumin were observed in murine BV2 microglial cells. The inhibitory actions of curcumin on the induction of iNOS or COX-2 were apparent in BV2 cells stimulated
A Rat primary microglia

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Fig. 1. Curcumin inhibits the up-regulation of iNOS and COX-2 in activated microglia. A, Rat primary microglia were pretreated with curcumin (cur) for 30 min and then stimulated with brain gangliosides mixture (Gmix) (a), IFN-γ (b), or LPS (c) for 8 h. B, Murine BV2 microglial cells were stimulated with IFN-γ (a) or LPS (b) in the absence or presence of curcumin. Western blot analysis was performed with antibodies against iNOS, COX-2, and actin. Data shown here are representative of three independent experiments.
with IFN-\(\gamma\) or LPS (Fig. 1B). Nitric oxides and prostaglandins, products of iNOS and COX-2 respectively, are mediators of microglial activation. Since curcumin strongly suppressed the induction of iNOS and COX-2 in activated microglia, I further explored the mechanism of action of curcumin in inflammatory response.

2. Curcumin suppresses the nuclear factor binding to GAS/ISRE in activated microglia

Several inflammatory mediators including iNOS and COX-2 have functional GAS (gamma-interferon activated sequence) elements in their promoter regions (Eberhardt et al., 1996; Lukiw et al., 1998). Because activation of GAS-nuclear factor binding has been implicated in inflammatory responses, I hypothesized that the inhibitory effects of curcumin are related to the activation of GAS-nuclear factor binding. Based on our previous report that gangliosides activate microglia through rapid activation of JAK-STAT signaling, including GAS-nuclear factor binding (Kim et al., 2002), I examined the effects of curcumin on gangliosides-induced nuclear factor binding to GAS in primary microglia. Cells were stimulated with 50 \(\mu\)g/ml gangliosides in the absence or presence of either 10 \(\mu\)M or 5 \(\mu\)M curcumin, and the nuclear protein extracts were examined by EMSA for the binding activity of a \(\gamma^{32}\)P labeled consensus GAS/ISRE oligonucleotides probe. Within 10 minutes, gangliosides rapidly induced nuclear factor binding to GAS/ISRE, and this increased binding activity was suppressed with inclusion of curcumin (Fig. 2A). The inhibitory effects of curcumin on the binding activity of GAS/ISRE were also observed in IFN-
Fig. 2. Curcumin suppresses the nuclear factor binding to GAS/ISRE in activated microglia. A, Rat primary microglia were pretreated with curcumin (cur) 10 μM or 5 μM for 30 min and stimulated with 50 μg/ml Gmix for 10 min. Nuclear extracts were prepared and binding activity to GAS/ISRE oligonucleotides determined by EMSA. B, BV2 cells were pretreated with curcumin 10 μM for 30 min and stimulated with 10 U/ml IFN-γ for the indicated time periods. Data shown here are representative of three independent experiments.
γ-stimulated BV2 cells (Fig. 2B). At all of the times tested, from 5 min to 30 min after IFN-γ treatment, curcumin significantly inhibited nuclear factor binding. These results indicate that curcumin suppresses nuclear factor binding to GAS/ISRE sequences in activated microglia, which contribute, at least in part, to the attenuation of inflammatory response.

3. Curcumin suppresses phosphorylation of STAT1 and STAT3 in activated microglia

Phosphorylated STAT dimers are the major transcription factors that bind to GAS/ISRE sequences and that lead to inflammatory responses. Since curcumin reduced nuclear factor binding activity to GAS/ISRE sequences, I asked if curcumin suppressed the phosphorylation of STATs. Primary microglial cells were stimulated with 50 µg/ml gangliosides, 100 ng/ml LPS or 10 U/ml IFN-γ in the absence or presence of 10 µM curcumin, and western blot analysis were performed using phospho-tyrosine STAT1 (Tyr701), phospho-serine STAT1 (Ser727), and actin antibodies. In agreement with nuclear factor binding activity to GAS/ISRE, both phosphorylations of STAT1 by gangliosides, LPS, or IFN-γ were markedly suppressed in curcumin-treated primary microglia (Fig. 3A). Similar responses were observed in BV2 cells. The level of phosphorylation of STAT1 was significantly reduced at all of the time points tested, from 5 min after treatment with IFN-γ in BV2 microglial cells (Fig. 3B).
Fig. 3. Curcumin suppresses the tyrosine and serine phosphorylation of STAT1 in activated microglia. A, Rat primary microglial cells were pretreated with curcumin for 30 min, and then stimulated with Gmix for the indicated time periods (a), IFN-γ for 30 min (b), or LPS for 3 h (c). B, BV2 cells were pretreated with curcumin for 30 min and treated with IFN-γ for the indicated time periods. Cell lysates were separated by 10% SDS-PAGE and the western blots were probed with anti-phospho-STAT1 (Y701) and anti-phospho-STAT1 (S727). The membranes were then stripped and analyzed with actin antibody to determine the amount of loading. Data shown here are representative of three independent experiments.
Since we previously observed that gangliosides and IFN-γ activated STAT3 as well as STAT1 in microglia (Kim et al., 2002), I tested whether curcumin also suppressed the activation of STAT3. As expected, curcumin inhibited the phosphorylation of STAT3 in stimulated primary microglia with either gangliosides or IFN-γ (Fig. 4A). Similar effects of curcumin on STAT3 were observed in BV2 microglial cells. As shown in figure 4B, the inhibitory effect of curcumin on STAT3 phosphorylation was similar to that of STAT1. These results indicate that curcumin reduced the activation of STAT1 and 3, implying that STAT-dependent inflammatory signaling may be related to the anti-inflammatory activity of curcumin.

4. Phosphorylation of JAK1 and JAK2 are inhibited by curcumin

Phosphorylation of STATs depends on the activation of JAKs. As STAT1 and 3 are recruited and activated by phosphorylation of JAK1 and JAK2 in gangliosides- or IFN-γ-stimulated microglial cells, I examined whether the inhibitory effects of curcumin on the activation of STATs are due to the suppression of JAK activity. Primary microglia were stimulated with gangliosides or IFN-γ in the absence or presence of curcumin, and western blot analysis was performed with antibodies specific either to phospho-JAK1 or phospho-JAK2. Both gangliosides and IFN-γ rapidly induced the phosphorylation of JAK1 and 2, while addition of curcumin suppressed phosphorylation (Fig. 5A). Similar results were obtained in IFN-γ-activated BV2 cells (Fig. 5B). The inhibitory action of curcumin on JAK phosphorylation was correlated with its effect on STAT phosphorylation and GAS
Fig. 4. Curcumin suppresses the tyrosine phosphorylation of STAT3 in activated microglia. A, Rat primary microglial cells were serum-starved for 12 h. The cells are pretreated with curcumin for 30 min, and stimulated with Gmix for the indicated time periods (a) or IFN-γ for 30 min (b). B, BV2 cells were pretreated with curcumin for 30 min and treated with IFN-γ for the indicated time periods. Cell lysates were separated by 10% SDS-PAGE and the western blots were probed with antibodies for phospho-STAT3 and actin. Data are representative of three independent experiments.
Fig. 5. Curcumin suppresses the phosphorylation of JAK1 and JAK2 in activated microglia. A, Rat primary microglia were pretreated with curcumin for 30 min and then stimulated with 50 µg/ml Gmix for 10 min (a) or IFN-γ for the indicated time periods (b). The phosphorylation level of JAK1 and JAK2 were determined by western blot analysis using antibodies specific for phospho-JAK1 or phospho-JAK2. B, BV2 microglial cells were pretreated with curcumin and stimulated with IFN-γ for the indicated time periods. Western blot analysis was performed with antibodies for phospho-JAK1 or phospho-JAK2. Data shown here are representative of three independent experiments.
binding activity. Taken together, these results suggest that in activated microglia, curcumin inhibits the initiation of the JAK-STAT signaling cascade, at least from the point of JAK phosphorylation.

5. Curcumin suppresses STAT-responsive inflammatory gene expression

To validate the inhibitory actions of curcumin on JAK-STAT signaling in activated microglia, I examined the expression profiles of several inflammation-associated genes whose promoters have STAT binding sequences. Rat primary microglia was stimulated with 50 µg/ml gangliosides or 10 U/ml IFN-γ for 3 h, and total RNA was extracted for RT-PCR analysis. Both gangliosides and IFN-γ rapidly increased the expression of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) mRNA, and the addition of curcumin suppressed this expression (Fig. 6). Similar results were obtained in BV2 cells (data not shown).

6. The inhibitory effects of curcumin on JAK-STAT signaling is not due to the displacement of IFN-γ from receptors

Since curcumin is polyionic, it may displace IFN-γ from receptors. This possibility was tested by adding curcumin at times after treatment of IFN-γ in rat primary microglia and BV2 cell lines. As shown in figure 7, I added 10 µM of curcumin into cells at various time points, from 30 min pretreatment to 15 min after treatment of IFN-γ, and incubated for indicated times. In both cells, the suppressive
Fig. 6. Curcumin suppresses STAT-responsive inflammatory gene expressions.

Rat primary microglia were treated with 50 µg/ml Gmix (A) or 10 U/ml IFN-γ (B) for 3 h. Total RNA was isolated and analyzed for transcript levels of MCP-1 and ICAM-1 using an RT-PCR-based assay. The transcript of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was measured for normalization. Data shown here are representative of three independent experiments.
Fig. 7. Curcumin treatments after IFN-γ stimulation also suppress the phosphorylation of JAK-STAT signaling. In primary microglia (A) and BV2 cell (B), curcumin 10μM was treated as time schedule shown in diagram, and IFN-γ was treated for 30 min. Western blot analysis was performed with antibodies for phospho-JAK1, phospho-JAK2 or phospho-STAT1 (Tyr701). The diagram shows the time schedule of curcumin treatment.
effects of curcumin on phosphorylation of JAK1 and JAK2 were observed at all the times tested. The tyrosine phosphorylated level of STAT1 was also reduced at times after treatment of IFN-γ. These results indicate that the inhibitory actions of curcumin are not due to the displacement of microglial activators from their cellular receptors.

7. Curcumin induces an increase in phosphorylation and association with JAKs of SHP-2

I next examined the mechanisms underlying curcumin-mediated suppression of JAK activation. Firstly, I considered the possibility that SOCS, negative regulators of JAK, were involved in inhibitory action of curcumin. Generally, SOCS are present in cells at very low levels, but are rapidly transcribed after exposure of cells to stimulus. Thus, I tested the effects of curcumin on the transcription of SOCS1 and 3 in rat primary microglia and BV2 cells. However, I did not observe the induction of SOCS1 and SOCS3 transcripts in curcumin-treated microglial cells (Fig. 8). Secondly, I tested the involvement of the protein tyrosine phosphatases, SHP-1 and SHP-2, reported to be negative regulators of JAK activity via binding to JAKs. SHPs can be activated by binding to phosphotyrosine residue either at receptors or at the C-terminus of SHPs itself (Vogel et al., 1993; Hof et al., 1998; Lu et al., 2001). Thus, I investigated the phosphorylated levels of SHPs by immunoprecipitation methods. BV2 cells were treated with curcumin for various lengths of time, and cell extracts were immunoprecipitated with SHP-1 or SHP-2.
**Fig. 8. Curcumin does not induce the transcription of SOCS1 and 3.** Rat primary microglia (A) and murine BV2 cells (B) were treated with 10 μM curcumin for 0.5, 1, and 2 h, and with IFN-γ, as a positive control, for 2 h. Total RNA was isolated and analyzed for transcript levels of SOCS1 and SOCS3 using an RT-PCR-based assay.
Western blot analyses with 4G10 antibody were performed to determine the phosphorylation of SHPs. Interestingly, with the inclusion of curcumin, SHP-2 phosphorylation significantly increased in 5 min and lasted up to 30 min, while changes in SHP-1 phosphorylation were smaller compared to changes in SHP-2 phosphorylation under the same experimental conditions (Fig. 9A). To confirm these results, I examined the SHP-2 binding to JAKs in curcumin-treated cells. After BV2 microglial cells were treated with or without curcumin for 15 min, the protein extracts were immunoprecipitated with either JAK1 or JAK2, and western blot analysis was performed with an antibody against SHP-2. Consistent with its effect on SHP-2 phosphorylation, SHP-2 binding to either JAK1 or JAK2 increased in curcumin-treated cells compared to control cells (Fig. 9B), while SHP-1 binding was not detected (data not shown). Furthermore, the binding of SHP-2 to JAK1 significantly increased in microglial cells with IFN-γ plus curcumin than cells with IFN-γ alone (Fig. 9B). Therefore, it is likely that curcumin rapidly alters the level of phosphorylation of SHP-2, thus inhibiting the initiation of JAK-STAT inflammatory signaling.
Fig. 9. Curcumin induces the activation of SHP-2. A. BV2 microglial cells were treated with curcumin for the indicated time periods and immunoprecipitated with an antibody against SHP-1 (a) or SHP-2 (b). Western blot analysis was performed with 4G10 tyrosine antibody. The membrane was then stripped and analyzed with anti-SHP-1 or anti-SHP-2. B, BV2 cells were immunoprecipitated with either anti-JAK1 (a) or anti-JAK2 (b), and then western blot analysis was performed with SHP-2, JAK1 or JAK2 antibodies, respectively. Data shown here are representative of three independent experiments.
B. Raft-mediated Src homology 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) regulation in brain microglia

1. SHP-2 phosphorylation is rapidly induced by Gmix in rat primary microglia and BV2 murine microglial cell lines.

   Based on our previous observations that JAK-STAT activation occurs within 5-15 min of Gmix addition and then dissipates after 30 min in cultured rat brain microglia (Kim et al., 2002; Kim et al., 2003), I wanted to determine whether this transient activation was due to the suppression of JAKs by SHP-2. I first assessed SHP-2 phosphorylation using western blot analysis, since SHP-2 phosphatase activity is regulated by the phosphorylation of the C-terminal tyrosine residues, Y542 and Y580 (Lu et al., 2001). Primary microglia or BV2 murine microglial cells were stimulated with 50 µg/ml Gmix for the times indicated in Fig. 10. The cells were then harvested and cell lysates were subjected to western blotting using an antibody directed against SHP-2 phosphorylated at Y580. SHP-2 was rapidly phosphorylated by 5 min, but dissipated after 30 min in both Gmix-treated primary microglia (Fig. 10A) and BV2 microglial cells (Fig. 10B).

2. SHP-2 phosphorylation by Gmix is mediated via lipid rafts

   Since SHP-2 activation is known to be linked to its raft translocation, I measured raft involvement by pretreating cells with filipin, cholesterol-binding polyene macrolide that disrupts lipid rafts by dispersing cholesterol, a major raft
Fig. 10. Phosphorylation of SHP-2 is induced by Gmix. Rat primary microglia (A) and murine BV2 microglial cells (B) were treated with gangliosides mixture (Gmix) 50 μg/ml for the indicated times. Cells were lysed with RIPA buffer and Western blot analysis was performed following 10% SDS-PAGE. SHP-2 phosphorylation was detected using an antibody directed against phospho-SHP-2 (Y580). The membranes were then stripped, and probed with antibodies to SHP-2, as loading controls. The graphs indicate the normalized intensities of p-SHP-2 bands against those of SHP-2 bands measured by Image Gauge technology. Data are representative of three independent experiments.
component. Cells were pretreated with 5 µg/ml filipin for 15 min, washed with serum-free media, and then treated with 50 µg/ml Gmix. Filipin inhibited the phosphorylation of SHP-2 induced by Gmix in both rat primary microglia (Fig. 11A) and BV2 microglial cells (Fig. 11B). Lipid rafts are known to reform after filipin removal through reincorporation of cholesterol. Therefore, to confirm the regulation through lipid rafts, I measured SHP-2 phosphorylation after allowing sufficient time for raft reconstruction. Two hours after filipin removal, addition of 50 µg/ml Gmix restored the phosphorylation of SHP-2 in BV2 microglial cells (Fig. 11B). Therefore, it appears that Gmix-regulated SHP-2 phosphorylation is mediated by lipid rafts.

3. Clustering and polarization of p-SHP-2 in membrane rafts are induced by Gmix.

Lipid rafts are reorganized and polarized by recruitment of signaling molecules and are coalesced into larger domains. To investigate whether reconstitution of lipid rafts is induced by Gmix and whether p-SHP-2 is present in these lipid rafts, I examined the localization of flotillin-1 and p-SHP-2 using immunocytochemistry visualized with confocal microscopy. Cells treated with or without 50 µg/ml Gmix were incubated with antibodies against the raft marker flotillin-1 and p-SHP-2. The double-stained cells were then observed under a confocal microscope. Immunofluorescence patterns of flotillin-1 and p-SHP-2 were diffusely distributed in the control primary microglia (Fig. 12A, a and b) and BV2
Fig. 11. Gmix-induced SHP-2 phosphorylation is inhibited by filipin. Rat primary microglia (A) and murine BV2 microglial cells (B) were pretreated with filipin 5 µg/ml for 15 minutes for disassembly of lipid rafts, and the media was then removed and replaced with fresh serum-free media. Immediately following washing, cells were treated Gmix 50 µg/ml for 5 min. In BV2 microglial cells, reassembly of lipid rafts occurs 2 h after filipin removal, which is marked ‘+(R)’ (B). Cell lysates were subjected to Western blot analysis with antibody directed against p-SHP-2 or actin as loading control. The right graphs indicate the normalized intensities of p-SHP-2 bands against those of actin bands measured by Image Gauge technology. Data are representative of three independent experiments.
cells (Fig. 12B, a and b). Treatment with Gmix 50 µg/ml for 5 min induced clustering and polarization of flotillin-1 and p-SHP-2 in rat primary microglia (Fig. 12A, d and e) and BV2 microglial cells (Fig. 12B, d and e). The merged images revealed that flotillin-1 and p-SHP-2 are colocalized in Gmix-treated primary microglia (Fig. 12A, f) and BV2 cells (Fig. 12B, f). These results indicate that in Gmix-treated microglia, lipid rafts are reformed and p-SHP-2 is localized at lipid raft.

4. SHP-2 is recruited to the detergent-insoluble fractions by Gmix

Because lipid rafts are easily isolated by their insolubility in non-ionic detergents at 4°C, they are called detergent resistant microdomains (DRMs). While many signaling proteins are permanently associated to lipid rafts, some are only temporally associated with rafts via biochemical or biological modifications. SHP-2 is primarily present in cytosol and is recruited to target molecules in lipid rafts. Since I observed p-SHP-2 localization at lipid raft in Fig. 12, I investigated whether SHP-2 is recruited to lipid raft by Gmix using detergent-insoluble fractionation by non-ionic detergent, Triton X-100. Primary microglia or BV2 microglial cells were treated with 50 µg/ml Gmix for times indicated Fig. 13, and cell lysates were then fractionated into insoluble and soluble fractions using 0.5% Triton X-100. These separated fractions were then subjected to western blot analysis. SHP-2 appeared to be translocated to detergent-insoluble fraction at 5 min after Gmix treatment in rat primary microglia (Fig. 13A). Similarly, in BV2 microglial cells, Gmix increased the recruitment of SHP-2 to the detergent-insoluble fraction relatively quickly (Fig. 13B).
Fig. 12. Clustering and polarization of p-SHP-2 are induced by Gmix. Primary microglia from rat brain (A) or BV2 microglial cells (B) were fixed and immunostained with primary antibodies against p-SHP-2 and flotillin-1 as lipid raft marker. Rhodamine-conjugated anti-rabbit or fluorescein-conjugated anti-mouse were used as secondary antibodies. Magnification, 400x. Data from one of three typical experiments are shown. Scale bars indicate 20 μm.
Fig. 13. SHP-2 is recruited to detergent-insoluble fractions by Gmix. Rat primary microglia (A) and BV2 microglial cells (B) were treated with Gmix 50 μg/ml for the indicated times (B). Cells were lysed with buffer containing 0.5% Triton X-100 and separated into soluble and insoluble fraction as described in Materials and Methods. Each fraction was then subjected to Western blotting. Flotillin-1 was used as a lipid raft marker and as a control for insoluble fractions. The graphs represent the normalization of SHP-2 against flotilin-1 in insoluble fraction measured by Image Gauge technology. Data are representative of three independent experiments.
p-SHP-2 was hardly detected in our experimental condition probably either due to a lack of sufficient amount of cell lysates or a methodologic problems. Immunoblotting with flotillin-1 confirmed the presence of lipid rafts in the detergent-insoluble fraction. Flotilin-1 was used as loading control for detergent-insoluble fractions.

5. SHP-2 is recruited by Gmix to the low density fraction during sucrose gradient centrifugation

In addition to detergent insoluble fractions, lipid rafts are known to be present in the low-buoyant density of sucrose gradient, therefore, I determined whether SHP-2 is also targeted into low-density fraction by Gmix. Detergent-free sucrose gradient ultracentrifugation was performed as described in Materials and Methods. BV2 microglial cells were homogenized in detergent-free buffer, subjected to sucrose gradient ultracentrifugation, and divided into 12 fractions each containing 1 ml. Lipid rafts were detected by Western blot analysis with HRP-linked cholera toxin B, which binds to GM1, major component of lipid rafts. GM1 was primarily detected in fraction 4 and 5 in BV2 microglial cells, indicating that these fractions contained lipid rafts (Fig. 14A). SHP-2 was detected in the GM1-positive fractions and Gmix induced rapid association of SHP-2 to the raft region of GM1-positive fractions at 5 min (Fig. 14B). Fig. 14C shows the ratio of the density of lipid rafts-associated SHP-2 (fraction No. 4 and 5) over total SHP-2. Figs. 13 and 14 indicate that SHP-2 was practically recruited to lipid raft by Gmix.
Fig. 14. SHP-2 is recruited to the low density fraction during sucrose gradient centrifugation following Gmix incubation. BV2 microglial cells were treated with Gmix 50 µg/ml for the indicated times. Cells were homogenized and adjusted to 5-35-40% discontinuous sucrose gradient centrifugation, as described in Materials and Methods. Detection of GM1, a lipid raft marker, was achieved by dotting each fraction on nitrocellulose membrane and incubating it with cholera toxin B-HRP, which binds to GM1. GM1 was detected in fractions 4 and 5, indicating that these are raft-containing regions (A). Each fraction was then subject to Western blot analysis using anti-SHP-2 (B). Lipid raft-associated SHP-2 (fractions No. 4 and 5) versus total SHP-2 was quantitated using densitometry (C). Data are representative of three independent experiments.
6. SHP-2 translocation to detergent-insoluble fractions is suppressed by filipin and restored upon filipin removal

To confirm that the recruitment of SHP-2 is suppressed by disassembly of lipid rafts, I examined the effect of filipin on the translocation of SHP-2 to the detergent-insoluble fraction. As described in Fig. 11, cells were incubated with filipin for 15 min to allow for disruption of the lipid raft and subsequently incubated in fresh media for 2 h to allow raft reassembly. Cells were separated into insoluble and soluble fraction using Triton X-100 and analysed by western blotting. Pretreatment with filipin 5 µg/ml for 15 min suppressed the recruitment of SHP-2 by Gmix in rat primary microglia (Fig. 15A) and BV2 microglial cells (Fig. 15B). Two hours after filipin removal, Gmix-induced SHP-2 recruitment was restored, further confirming that SHP-2 targeting is regulated by lipid rafts.

7. JAK2 are recruited to detergent-insoluble fractions by Gmix

The above results indicate that SHP-2 is targeted to and phosphorylated in lipid rafts, suggesting that it is the place that SHP-2 is regulated. I therefore decided to search for an SHP-2 target molecule, JAK2, in the membrane raft using detergent-resistant fractionation. To examine whether the translocation of JAK2 to lipid rafts is induced by Gmix, Primary microglia (Fig. 16A, a) or BV2 microglial cells (Fig. 16A, b) were treated with 50 µg/ml Gmix for the times indicated in Fig. 16A. The cells were then lysed with 0.5% Triton X-100-containing HEPES buffer and centrifugation was performed to isolate the soluble and insoluble fractions. Each sample was then
**Fig. 15. SHP-2 recruitment to detergent-insoluble fractions by Gmix is inhibited by filipin.** Primary microglia from rat brain (A) or BV2 microglial cells (B) were stimulated with Gmix for 5 min in the presence or absence of filipin for 15 min, and cells were then harvested. Cell lysates were separated into insoluble and soluble fractions using Triton X-100, and subjected to western blot analysis using an SHP-2 antibody. Flotillin-1 (Flot-1) was used as a loading control for the raft-containing fractions.
subjected to western blot analysis. Gmix-induced recruitment of p-JAK and JAK2 to the insoluble fraction occurred by 5 min, but the levels of these proteins were restored to control levels after 30 min (Fig. 16B). The recruitment of JAK2 into the rafts was also inhibited by filipin in primary microglia and BV2 cells (Fig. 16B, a and b, respectively) and restored following filipin removal in primary microglia. These results indicate that, like SHP-2, p-JAK2 and JAK2 are regulated through lipid rafts.

8. SHP-2 targeting to JAK2 is regulated by lipid rafts

SHP-2, which is known to dephosphorylate the phosphotyrosine residues, binds JAKs and negatively regulates the JAK-STAT pathway. To demonstrate that SHP-2 interacts with and inhibits JAK2, I performed immunoprecipitation using an antibody directed against SHP-2. Cell extracts were immunoprecipitated with anti-SHP-2 and subjected to Western blotting with anti-JAK2. In BV2 microglial cells, Gmix increased the association of SHP-2 with JAK2 within 5 min, while control levels were restored after 30 min (Fig. 17A). Pretreatment of filipin suppressed the increased binding of SHP-2 with JAK2 by Gmix, and binding was restored following filipin removal in both rat primary microglia (Fig. 17B, a) and BV2 microglial cells (Fig. 17B, b), confirming that SHP-2 association with JAK2 is regulated by lipid rafts.
Fig. 16. p-JAK2 and JAK2 are translocated into insoluble fractions by Gmix.

Primary microglia from brain (A, a) and BV2 microglial cells (A, b) were treated with 50 µg/ml Gmix for the indicated times. Cells were then fractionated into soluble or insoluble fractions using Triton X-100, and each fraction was subjected to Western blot analysis with p-JAK2 and JAK2 antibodies. Primary microglia from rat brain (B, a) or BV2 cells (B, b) were stimulated with Gmix for 5 min in the absence of presence of filipin 5 µg/ml. Insoluble fractions were then subjected to Western blot analysis using an antibody directed against JAK2. Filipin removal [indicated with +(R)] was performed as described in Fig. 11. The graphs represent the normalization of SHP-2 against flotillin-1 in insoluble fraction measured by Image Gauge technology. Data are representative of more than three independent experiments.
Fig. 17. SHP-2 binding to JAK2 is increased by Gmix and inhibited by filipin. BV2 microglial cells were treated with Gmix 50 µg/ml for the indicated times (A). Cell lysates were immunoprecipitated with SHP-2 antibody and then, subjected to Western blot analysis with anti-JAK2 and anti-SHP-2 antibodies. To confirm that SHP-2 binding to JAK2 is regulated by lipid rafts, filipin pretreatment and removal was performed in rat primary microglia (B, a) and BV2 microglial cells (B, b) as described in Fig. 11. The graphs indicate the normalized intensities of JAK-2 bands against those of SHP-2 bands measured by Image Gauge technology. Data are representative of three independent experiments.
IV. DISCUSSION

A. Curcumin suppresses JAK-STAT inflammatory signaling through activation of SHP-2 in brain microglia

In this study, I report one mechanism underlying the anti-inflammatory actions of curcumin in brain microglial cells. Several lines of evidence in vivo and in vitro indicate that curcumin is a potent anti-inflammatory agent in various inflammatory diseases including Alzheimer’s disease (AD). For example, curcumin has been reported to reduce oxidative damage and amyloid pathology in an Alzheimer transgenic mouse (Lim et al., 2001) and to inhibit IL-12 signaling in experimental allergic encephalomyelitis (Natarajan and Bright, 2002). Until now, the anti-inflammatory actions of curcumin were attributed to the inhibition of NF-κB activation, a mediator involved in cytokine signaling and inflammation. However, curcumin appears to act at multiple steps in the signaling cascade underlying inflammation.

JAK-STAT inflammatory signaling has recently been reported to play a role in inflammatory responses of brain (Lim et al., 2001; Natarajan and Bright, 2002; Kim et al., 2002; Park et al., 2003). Thus I tested whether the anti-inflammatory effects of curcumin are related to the suppression of JAK-STAT activation in rat primary microglia and murine BV2 microglial cells. I determined the effects of curcumin on the expression of COX-2 and iNOS in activated microglia, because curcumin is reported to reduce both the activities of enzymes in several cancer cell
lines, and the expressions of genes which have STAT-binding sites in their promoters. As expected, curcumin suppressed the up-regulation of COX-2 and iNOS in activated microglia (Fig. 1) supporting our hypothesis that curcumin suppresses inflammatory responses via inhibition of JAK-STAT signaling. In subsequent experiments, I observed that curcumin reduced the phosphorylation of STAT1 and STAT3 as well as nuclear factor binding to GAS/ISRE sequences (Figs. 2-4). In addition, phosphorylations of JAK1 and JAK2, molecules upstream of STAT phosphorylations, were significantly suppressed in gangliosides-, LPS-, or IFN-γ-activated microglia (Fig. 5). Recently, two contradictory observations of the effects of curcumin on STAT signaling were reported. In chondrocytes, curcumin interfered with oncostatin M-induced tyrosine phosphorylation of STAT1, without affecting the JAKs activation (Li et al., 2001). In contrast, curcumin inhibited the IL-12-induced tyrosine phosphorylation of STAT3 and 4 by blocking TYK2 and JAK2 activation in T lymphocytes (Natarajan and Bright, 2002). In these experiments with brain microglia, curcumin inhibited the phosphorylation of STAT1 and STAT3 by suppressing JAK1 and JAK2 phosphorylation. These disparate results may be explained by cell type- and stimuli-specific effects of curcumin on the JAK-STAT pathway.

To validate the functional importance of the inhibitory activity of curcumin on the JAK-STAT inflammatory cascade in microglia, I examined the level of expression of mRNA transcripts for inflammatory mediators whose promoters have STAT-binding sequences, including MCP-1 and ICAM-1. Curcumin effectively
suppressed the expression of MCP-1 and ICAM-1 mRNA in gangliosides-, or IFN-γ-treated rat primary microglia and BV2 cells (Fig. 6 and data not shown). MCP-1, a member of the chemokine family, specifically attracts monocytes and macrophages, and ICAM-1 is a representative adhesion molecule involved in cellular migration toward inflammatory sites. Thus, the inhibitory action of curcumin on the up-regulation of MCP-1 and ICAM-1 in activated microglia supports the conclusion that curcumin suppresses inflammatory response through inhibition of JAK-STAT signaling in microglia.

Next, I addressed the question of how curcumin inhibits the phosphorylation of JAKs. I considered the involvement of inhibitory proteins and protein tyrosine phosphatases (PTPases). In an experiment to reveal the involvement of inhibitory regulators including SOCS-1 and 3, which are known to inhibit JAK phosphorylation, curcumin did not induce either (Fig. 8). So, I tested the involvement of PTPases, including SHP-1 and SHP-2, since they are known to regulate JAK activity. The phosphatase activities of SHP-1 and SHP-2 have been reported to be due primarily to their phosphorylation and interaction with tyrosine phosphorylated proteins such as RTK and JAKs. In an experiment to examine the phosphorylation of SHP-1 and SHP-2 in curcumin-treated BV2 cells using immunoprecipitation, curcumin increased the phosphorylation of SHP-2, but not that of SHP-1, in the same experimental conditions (Fig. 9A). To confirm these results, I used immunoprecipitation to test the association of JAKs with SHP-2. In an agreement with its effect on phosphorylation, curcumin increased the binding of SHP-2 with
JAK1 as well as with JAK2 (Fig. 9B). In addition, the binding of SHP-2 to JAK1 significantly increased in microglial cells with IFN-γ plus curcumin than cells with IFN-γ alone (Fig. 9B). There are conflicting reports about the roles of SHP-2 in the regulation of JAK phosphorylation. Whereas SHP-2 has been suggested to function as a negative regulator of the growth factor- or cytokine-induced phosphorylation of JAK and STAT (Symes et al., 1997; You et al., 1999; Yu et al., 2000), it has also been reported to be a positive regulator of prolactin-, or angiotensin II-induced JAK2 phosphorylation (Ali et al., 1996; David et al., 1996; Marrero et al., 1998). These results clearly showed that curcumin increased the phosphorylation of SHP-2 and its association with JAK1 and JAK2, and thus inhibited the phosphorylation of JAK1 and JAK2 in activated microglia. Moreover, I recently obtained evidence that suggests the involvement of SHP-2 in curcumin-induced inhibitory responses. I observed marked differences in the subcellular localization of SHP-2 between cells treated with curcumin and control cells using fluorescence and confocal microscopy (data not shown). SHP-2 was distributed in a characteristic punctate fashion in curcumin-treated cells, but was diffusely distributed throughout the cytoplasm in control cells. Although additional studies are needed to clarify the significance of this difference in subcellular localization, these observations support that SHP-2 may be involved in the inhibitory action of curcumin.

Recently, detergent-insoluble specialized membrane microdomains called rafts have attracted attention for their possible roles in signal transduction. JAKs, STATs and SHPs have recently been reported to translocate from non-raft to lipid
rafts and thus regulate T cell activation (Su et al., 2001; Goebel et al., 2002; Sehgal et al., 2002; Lacalle et al., 2002). IFN-γ receptor is also reported to be localized to membrane rafts (Sehgal et al., 2002), and gangliosides are enriched in membrane raft microdomains. Curcumin is a dihydroxyphenolic compound and thus has physicochemical properties such as hydrophobicity and instability in aqueous solutions at pH > 7 that lead us to predict that it might be localized to membranes and induce membrane changes. Thus, it is possible that curcumin rapidly intercalates into plasma membrane, thus affecting many signaling molecules in raft microdomains. Changes in SHP-2 localization in curcumin-treated cells support this possibility. I recently observed that activation of PDGF receptor β, another representative raft-mediated signaling molecule, also inhibited by curcumin, which further support our assumption that curcumin could affect rafts-mediated signaling cascades (data not shown). In this regard, I am undertaking experiments to demonstrate the localization of SHP-2 with immunostaining, confocal microscopy and subcellular fractionation. Such experiments could reveal how SHP-2 may be associated with tyrosine-phosphorylated proteins, and how stimulatory and inhibitory signals are arranged within membrane raft microdomains. Curcumin and SHP-2 may thus be part of a novel mechanism that regulates microglial activation after brain injury and in chronic disease.
B. Raft-Mediated Src Homology 2 Domain-Containing Tyrosine Phosphatase 2 (SHP-2) Regulation in Microglia

In the present study, I have demonstrated that the transient activation of JAK-STAT signal in gangliosides-stimulated microglia is due to the phosphorylation and lipid raft-mediated association of SHP-2 with JAK-2, suggesting a potential mechanism for the regulation of SHP-2 in activated microglia. JAKs and STATs are traditionally considered interferon signaling molecules, but recent reports suggest that they are also involved in signaling triggered by other cytokines and growth factors and may thus mediate a variety of cellular functions (Kisseleva et al., 2002; O'Shea et al., 2002; Shuai and Liu, 2003). JAK-STAT signaling is tightly regulated by induction of inhibitory molecules such as SOCS family proteins, activation of phosphatases, and degradation through ubiquitin-proteasome pathways (Kile and Alexander, 2001; Cooney, 2002; Wormald and Hilton, 2004). The rapid and transient activation of JAK-STAT signaling by gangliosides prompted us to consider regulation by protein tyrosine phosphatases such as SHPs, particularly because we have previously shown that JAK-STAT signals are negatively regulated by SHP-2 (Kim et al., 2003; Park et al., 2003), I have shown that SHP-2 phosphorylation is rapidly induced from 5-15 min in gangliosides-activated microglia. SHPs are auto-inhibited in their resting state by an intramolecular interaction between their SH2 domains and their PTPase domain. The PTPase domain is activated following its association with tyrosine-phosphorylated proteins, including membrane receptors and JAKs (Fuhrer et al., 1995). Recently, Lu et al. (Lu et al., 2001) demonstrated that
the two C-terminal two tyrosine residues (Y542 and Y580) are also involved in SHP-2 activation by intramolecularly interacting with SH2 domains. However, the precise mechanisms through which SHP-2 comes in contact with phosphotyrosine residues and dephosphorylates its target molecules remain to be clarified. Although SHP-2 is a cytosolic protein, it is involved in the dephosphorylation of many membrane proteins. Therefore, SHP-2 must be somehow recruited to the membrane via adaptor protein or conformational changes. The necessity of membrane recruitment suggests the possible participation of lipid rafts. In this study, I showed that disruption of lipid rafts by filipin inhibits phosphorylation of SHP-2. In addition, SHP-2 is rapidly recruited to the detergent-insoluble, low-density fraction obtained during detergent-free sucrose gradient ultracentrifugation following incubation with Gmix and this recruitment is inhibited by disrupting the lipid rafts with filipin. Immunohistochemical experiments using phospho-SHP2 and flotillin-1 antibodies further revealed the induction of raft clustering by Gmix and that phospho-SHP-2 and flotillin-1 colocalized following incubation of cells with Gmix. SHP2 targeting to lipid rafts following cell attachment has been shown to influence cell adhesion and migration by regulating Rho activity (Lacalle et al., 2002). Recently, SHP-2 has been reported to be recruited to the low density membrane fractions in confluent endothelial cells via its interaction with annexin II in a cholesterol-dependent manner (Burkart et al., 2003).

SHP-2 is known to be involved in many cellular signalings in catalytic-dependent or –independent manners. This protein modulates PI3 kinase, NF-κB,
JAK-STAT, and MAP kinase in catalytic-dependent ways. Specifically, SHP-2 positively regulates ERK activation (Shi et al., 2000; Wang et al., 2004) and the PI3-kinase/Akt pathway during cell survival (Ivins et al., 2004) in fibroblasts, while it negatively regulates hematopoietic cell survival through dephosphorylation of STAT5 (Chen et al., 2004). Although SHP-2 is generally known to act in a catalytic-dependent manner, it has also been reported to have catalytic-independent actions. Yu et al. (Yu et al., 2003) reported that catalytically inactive SHP-2 does not affect PI3 kinase activation but rather reduces JAK and ERK pathway signaling induced by IL-3, although all three signaling molecules are altered in SHP-/- hematopoietic cells, indicating that SHP-2 regulates PI3 kinase in a catalytic-independent manner. Also, SHP-2 acts as an adaptor molecule downstream of the prolactin receptor and is required for recruitment of its substrate (Minoo et al., 2003). Components of the JAK-STAT signaling cascade have previously been reported to be target molecules of SHP-2. SHP-2/- mutant mice display increased interferon-induced STAT1 activation, suggesting that SHP-2 negatively regulates the JAK-STAT pathway (You et al., 1999). SHP-2 is also known to be required as an adaptor molecule for JAK2 association with its receptor (Marrero et al., 1998). In addition, SHP-2 acts as dual-specificity phosphatase during the dephosphorylation of STAT1 on tyrosine and serine residues (Wu et al., 2002), and direct associations between SHP-2 and JAKs have been reported (Fuhrer et al., 1995; Yin et al., 1997). Moreover, our previous reports showed that SHP-2 interacts with JAK and is an important inhibitory molecule on the JAK-STAT pathway (Kim et al., 2003; Park et al., 2003). In this study, I
demonstrated that SHP-2 interacts specifically with JAK2 and that this interaction is inhibited by filipin. These results reveal that the rapid phosphorylation of SHP-2 by Gmix causes the JAK-STAT signal to subside in a raft-dependent manner.

The question still remains regarding how transient activation of the JAK-STAT signal by Gmix differs from IFN-γ-stimulated sustained JAK-STAT activation. Gmix is a potent stimulator of microglia, resulting in the release of proinflammatory cytokines, nitric oxides, and adhesion molecules. However, similar activities occur in response to IFN-γ. Interestingly, we observed that Gmix could not induce the class II transactivator (CIITA) or the class II major histocompatibility complex (MHCII) molecules in rat primary microglia (Yoon and Jou, unpublished data), while IFN-γ completely induced them, thus acting as a full antigen presenting stimulator. JAK-STAT signaling is known to be important for antigen presentation. IRF-1, an IFN-γ-responsive gene regulated by JAK-STAT signaling, is also not induced by Gmix. IRF-1 expression is induced by the binding of activated STATs to promoter region, which induces the sequential expression of CIITA and MHC-II. Oncostatin M, which also induces the transient STAT1 activation, is not capable of inducing expression of IRF-1 and MHC-I, and mutated gp130 receptor that cannot bind to SHP-2 induces sustained activation of STAT1 (Mahboubi and Pober, 2002). Although further studies are still needed, the transient activation of the JAK-STAT pathway by SHP-2 activation appears to be related to the failure of IRF-1 and MHC-II induction. In particular, studies regarding antigen presentation suggest the involvement of lipid rafts in this process. The presence of MHC-II in lipid rafts has been reported in
human B cells, dendritic cells, and T cells, and MHC-II has been reported to be regulated by lipid rafts. (Anderson et al., 2000; Poloso et al., 2004; Poloso and Roche, 2004; Dolan et al., 2004). I suggest that the regulation by lipid raft may be important for the expression of the antigen presentation-associated molecules, MHC-II and CIITA that are regulated by JAK-STAT signaling as well as for activation and targeting of SHP-2.

Taken together, my results demonstrate that transient activation of the JAK-STAT pathway by Gmix is regulated by SHP-2 activation and that its association with JAK2 occurs in a raft-dependent manner, thus described a novel role for SHP-2 targeting to lipid rafts during microglial activation. Since microglial activation plays important roles in several neurodegenerative disease including Parkinson’s disease and Alzheimer’s disease, raft-mediated control mechanisms of inflammatory signaling molecules involved in microglial activation could be a useful therapeutic target to combat neurodegenerative disease.
V. CONCLUSION

In this study, I present the regulatory mechanism and the meaning of SHP-2 in brain microglial activation. Here, I firstly showed that curcumin, known as an anti-inflammatory drug, inhibited the LPS-, gangliosides-, and IFN-γ-induced JAK-STAT signaling pathway via SHP-2 activation, which was determined by the phosphorylation of SHP-2 and its association with JAK1/2. Therefore, SHP-2 is suggested to be an important regulator of the anti-inflammatory function of curcumin. Secondly, I clarified that gangliosides, one of microglial activators, activated the SHP-2 in a lipid raft-dependent manner. Gangliosides induced the SHP-2 phosphorylation and its recruitment into the detergent-insoluble fraction and the low density fraction during sucrose gradient ultracentrifugation. In gangliosides-activated microglia, SHP-2 was also associated with JAK2 in a raft-dependent manner. Therefore, SHP-2 is thought to be finely regulated since microglia is activated. In conclusion, SHP-2 is an essential negative regulator of the JAK-STAT signaling pathway in microglial activation and is suggested as a therapeutic target molecule in neurodegenerative disease.
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뇌소교세포에서 Src Homology 2 Domain-Containing Protein

Tyrosine Phosphatase 2 (SHP-2) 조절 기전에 관한 연구

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