JAK-STAT Signaling Mediates Gangliosides-induced Inflammatory Responses in Brain Microglial Cells*

Received for publication, April 22, 2002, and in revised form, July 19, 2002 Published, JBC Papers in Press, August 20, 2002, DOI 10.1074/jbc.M203885200

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Neuronal cell membranes are particularly rich in gangliosides, which play important roles in brain physiology and pathology. Previously, we reported that gangliosides could act as microglial activators and are thus likely to participate in many neuronal diseases. In the present study we provide evidence that JAK-STAT inflammatory signaling mediates gangliosides-stimulated microglial activation. Both in rat primary microglia and murine BV2 microglial cells, gangliosides stimulated nuclear factor binding to GAS/ISRE elements, which are known to be STAT-binding sites. Consistent with this, gangliosides rapidly activated JAK1 and JAK2 and induced phosphorylation of STAT1 and STAT3. In addition, gangliosides increased transcription of the inflammation-associated genes inducible nitric-oxide synthase, ICAM-1, and MCP-1, which are reported to contain STAT-binding elements in their promoter regions. AG490, a JAK inhibitor, reduced induction of these genes, nuclear factor binding activity, and activation of STAT1 and -3 in gangliosides-treated microglia. AG490 also inhibited gangliosides-induced release of nitric oxide, an inflammation hallmark. Furthermore, AG490 markedly reduced activation of ERK1/2 MAPK, indicating that ERKs act downstream of JAK-STAT signaling during microglial activation. However, AG490 did not affect activation of p38 MAPK. We also report that the sialic acid residues present on gangliosides may be one of the essential components in activation of JAK-STAT signaling. The present study indicates that JAK-STAT signaling is an early event in gangliosides-induced brain inflammatory responses.

Gangliosides are sialic acid-containing glycosphingolipids that are constituents of mammalian cell membranes. Gangliosides are particularly abundant in neuronal cell membranes and participate in various cellular events of the nervous system (1, 2). The major types of gangliosides in the brain are GM1, GD1a, GD1b, GT1b, and GQ1b, which differ in their profiles of sialic acid residues and carbohydrate moieties. Several lines of evidence point to the importance of the brain-derived gangliosides in immune responses and the pathogenesis of brain disease. There are reports that brain injury can cause release of gangliosides from damaged neuronal cells into the extracellular space, which may lead to pathophysiological conditions (3–5). Gangliosides have also been reported to interact with Aβ, suggesting they play a role in Aβ toxicity as well as in the deposition of Aβ into senile plaques associated with Alzheimer’s disease (6–9). In addition, gangliosides regulate the production of various inflammatory mediators, such as cytokines and inducible nitric-oxide synthase (iNOS) (10, 11). Despite the evidence of a role for gangliosides in brain pathology, there appears to be little known about how gangliosides act.

Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathways have been reported to be involved not only in the immune response of numerous cytokines but also in the actions of primarily non-immune mediators such as growth factors and hormones. Specific subtypes of JAK and STAT molecules are activated by different signals, resulting in specificity of response (12, 13). The binding of ligand to its receptor induces assembly of an active receptor complex and consequent phosphorylation of the receptor-associated JAKs (JAK1, JAK2, JAK3, and TYK2). Phosphorylated JAKs lead to the activation of neighboring JAKs, receptor subunits, and several other substrates. Phosphorylation of JAKs provides the docking sites for STATs, which in turn become phosphorylated on tyrosine and serine residues; the phosphorylation of both amino acid species is required for full STAT activity. Phosphorylated STATs are released from the receptor complex and form dimers. These dimers translocate to the nucleus where they directly bind to the promoter region of specific target genes, thus regulating transcription of these genes, many of which are involved in immune responses (13–16).

Microglia are the major immune effector cells in the brain, and microglial activation is an early event in central nervous system inflammation (17, 18). Previously, we demonstrated that gangliosides could activate microglia, inducing release of inflammatory mediators such as tumor necrosis factor-α and nitric oxide (NO) (10, 19). Here we show that the molecular mechanisms underlying this gangliosides-induced activation of microglia include triggering of the JAK-STAT signaling pathway.

EXPERIMENTAL PROCEDURES

Reagents—Bovine brain gangliosides mixture, GM1 and GD1a, was purchased from Matreya (Pleasant Gap, PA). Asialoganglioside GM1 was from Sigma. Rat IFN-γ, α-cyano-(3,4-dihydroxy)-N-benzylcin-
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Phytoextraction of Gangliosides

- Phytoextraction of gangliosides from brain-derived tissues.
- Solubilization of gangliosides using aqueous solutions.

Characterization of Gangliosides

- Identification of gangliosides using HPLC and MS.
- Quantification of gangliosides using UV-visible spectroscopy.

In vitro Studies

- Antibody-mediated assay for ganglioside recognition.
- Functional study of gangliosides on microglial cells.

Expression of iNOS

- Detection of iNOS mRNA using RT-qPCR.
- Western blot analysis of iNOS protein.

Cell Culture

- Primary microglia from Sprague-Dawley rat brains.
- Culture conditions: DMEM with 10% FBS.

Gangliosides Treatment

- Treatment of microglial cells with gangliosides.
- Assessment of iNOS expression and NO production.

Signal Transduction Pathways

- Activation of JAK-STAT pathways by gangliosides.
- Involvement of STAT1 and STAT3 in gangliosides-mediated activation.

Results

- Gangliosides induce transcription of iNOS and nuclear factor binding.
- Activation of JAK-STAT pathways in microglial cells.

Conclusions

- Gangliosides trigger JAK-STATs during microglial activation, suggesting a role in neuroinflammation.

FIG. 1. Gangliosides stimulate iNOS transcription and nuclear factor binding to GAS/ISRE elements. A, transcript level of iNOS and nuclear factor binding to GAS/ISRE element in gangliosides-treated primary microglia. B, time course analysis of nuclear factor binding activity in BV2 microglial cells. C, gel shift assay using anti-STAT1 and anti-STAT3 in BV2 cells. Determination of NO Release—Media nitrite concentration was measured as an indicator of NO release.

Westbrook Blot Analysis—Cells were washed twice with cold phosphate-buffered saline and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na3VO4, and 1 mM NaF) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mM EDTA). The lysates were then centrifuged at 10,000 × g at 4°C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies and peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) and then visualized using an enhanced chemiluminescence system (Sigma).
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Fig. 2. Gangliosides induce the phosphorylation of STAT1 and STAT3 in microglial cells. Rat primary microglial cells (A) and mouse BV2 microglial cells (B) were serum-starved for 12 h and then stimulated with 50 µg/ml Gmix for the indicated times. Cell lysates were separated by 10% SDS-PAGE and Western blots were probed with anti-pSTAT1 (Tyr-701), anti-pSTAT1 (Ser-727), or pSTAT3 (Tyr-705). The membrane was then stripped and analyzed with anti-STAT1 antibody to determine loading. Data are representative of four independent experiments.

Fig. 3. Gangliosides stimulate phosphorylation of JAK1 and JAK2 in rat primary microglial cells. A, phosphorylated levels of JAK1 and JAK2 were serum-starved for 12 h and then stimulated with 50 µg/ml Gmix for 5 min. The phosphorylation of JAK1 and JAK2 was determined by Western blot analysis using antibodies specific for phospho-JAK1 or -2. B, inhibition of gangliosides-induced phosphorylation by AG490. Cells were pretreated with 10 µM AG490 for 1 h and then stimulated with 50 µg/ml Gmix for 2 min. Western blots were probed with anti-pSTAT1 (Tyr-701) and pSTAT3 (Tyr-705). The membrane was subsequently stripped and probed with anti-STAT1 and STAT3 antibodies. C, inhibition of nuclear factor binding to GAS/ISRE oligonucleotides by AG490. Cells were pretreated with 10 µM AG490 for 1 h and then stimulated with 50 µg/ml Gmix for 5 min. Nuclear extracts were prepared, and binding activity to GAS/ISRE oligonucleotides was determined by EMSA. *, inset is a short-exposed autograph of the upper band (dotted box).
activation was accompanied by induction of NO release. Thus, we tested whether gangliosides induced NO release via JAK-STAT signaling. First, we examined the effect of JAK inhibition on gangliosides-induced transcription of iNOS in rat primary microglial cells. RT-PCR analysis showed that the inhibitor AG490 reduced mRNA levels of iNOS (Fig. 5). Second, we investigated the effect of AG490 on NO release. In these studies, the ERK inhibitor, PD98059, was also used since we have shown previously (10) that it reduced gangliosides-induced NO release. In the presence of AG490, microglial cells were treated with 50 μg/ml gangliosides for 48 h, and the amount of NO produced was determined by measuring the amount of nitrite converted from NO in the media. AG490 significantly reduced gangliosides-enhanced NO release, as did PD98059 (Fig. 5). Compared with cells treated with gangliosides alone, NO release was reduced to 38.6 ± 4.3 and 25.2 ± 14% in cells co-treated with PD98059 and AG490, respectively. These results are consistent with the results shown in Figs. 3 and 4. The findings indicate that JAK-STAT signaling is required for NO release and provide evidence of the critical functional involvement of JAK-STAT signaling in gangliosides-induced microglial activation.

**ERK Activity Appears to be Regulated by JAK Activation**—There are several reports (32, 33) showing that the transcriptional activity of STATs is regulated through mitogen-activated protein kinases (MAPKs). MAPKs are considered as common intracellular signaling molecules involved in microglial activation. Previous reports by others and us (10, 34) showed that gangliosides induced activation of MAPKs in microglia. In the present study, we used pharmacological inhibitors to examine possible cross-talk between the JAK-STAT and MAPKs signaling pathways. When primary rat microglial cells were pretreated for 2 h with the JAK inhibitor AG490, gangliosides-induced activation of ERK1/2 was significantly reduced compared with controls with no AG490 (Fig. 6). In contrast, no significant suppression of p38 was observed under this condition. However, in the presence of PD98059, an ERK inhibitor, not only ERK but also p38 activation was completely inhibited. These results indicate that gangliosides-stimulated JAK activation leads to activation of ERK in microglial cells. These pharmacological studies also indicate that gangliosides-stimulated activation of p38 may not be due to activation of ERK by JAK.

**Sialic Acid Residues Are Important for Gangliosides-induced Phosphorylation of STAT**—The major types of gangliosides in brain are GM1, GD1a, GD1b, GT1b, and GQ1b. These gangliosides differ with respect to the number and position of sialic acid residues attached to the carbohydrates (35). The approximate percentages of each ganglioside present in the brain gangliosides mixture used in the current study are 18% GM1, 55% GD1a, 15% GD1b, 10% GT1b, and 2% others. To address whether the structural diversity of gangliosides affected activation of STAT, we compared the effect of GM1, which has one molecule of sialic acid, with GD1a, which has two molecules of sialic acid, on phosphorylation of STAT1. Primary microglial cells were treated with GM1 or GD1a for 2 min, and levels of phosphorylated STAT1 were determined by Western blot analysis using antibodies against Tyr-701-STAT1. The data in Fig.
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JAK-STAT signaling has been reported to be closely involved in inflammation. Although STAT proteins were discovered during the course of analysis of interferon signaling, recent studies (40–42) have revealed that STAT signaling can account for various cellular responses to a number of cytokines, growth factors, and hormones. IL-4 and IL-13 stimulate enhanced expression of major histocompatibility complex class II, CD23, IL-4Ra chain, and Ig class switching to IgE and IgG via activation of STAT6 (43, 44). The common cytokine receptor γ-chain, which is shared by receptors for IL-2, -4, -7, -9, and -15, associates with JAK3, thus resulting in STAT-dependent immune responses (45, 46). Prolactin, erythropoietin, and growth hormone are all known to activate JAK2 (47).

The functional association between cytokine signaling and JAK-STAT signaling prompted us to examine the involvement of STATs in gangliosides-induced inflammatory responses. Consistent with a connection between gangliosides and STAT activity, we found that gangliosides treatment of brain microglial cells increased the binding activity of a nuclear factor to a consensus GAS/ISRE element (Fig. 1). It has been reported that STAT1 and -3 are major STAT types that bind GAS and ISRE elements, and that they function to regulate the transcription of numerous genes (21, 24). By using supershift antibodies for STAT1 and STAT3, we determined that they are constituents of nuclear factor binding complex to the GAS/ISRE element in gangliosides-stimulated microglial cells (Fig. 1C). At the present time, we cannot clearly identify the individual bands since all the three bands were reduced by addition of not only anti-STAT1 but also anti-STAT3. However, our EMSA data and Western blotting data convincingly proved the involvement of STAT1 and STAT3 in gangliosides-induced inflammatory responses. Next, we investigated whether gangliosides could induce the phosphorylation of these particular STATs. As expected, phosphorylation of STAT1 and -3 was induced within 1 min and then rapidly decreased to basal levels (Fig. 2). The kinetics of these phosphorylation events were consistent with the timing of binding activity to GAS/ISRE elements. These results show that STAT1 and -3 may directly mediate gangliosides-induced microglial activation. NF-κB is also reported to be involved in gangliosides-induced microglial activation, but it is considered to be a pathway common to a range of microglial activators (10). Thus, it may be that early activation of STATs by gangliosides is a specific mechanism underlying gangliosides-induced microglial activation.

Phosphorylation of JAKs leads to their activation, and activated JAKs phosphorylate and hence activate STATs. We examined the effect of gangliosides on JAK1 and JAK2 to determine the cause of STAT1 and STAT3 phosphorylation. We found that both JAK1 and JAK2 were phosphorylated within 1 min of the addition of gangliosides (Fig. 3). Furthermore, AG490, a JAK inhibitor, diminished both gangliosides-enhanced phosphorylation of STATs and nuclear factor binding activity. These data provide strong evidence to indicate that gangliosides activate STATs through activation of JAKs.

In general, inflammatory stimuli induce release of mediators such as cytokines, chemokines and cell adhesion molecules (49). Regulation of mediator release is important for controlling inflammation. We investigated the transcription of inflammation-associated genes that contain functional GAS elements in their promoters. RT-PCR analysis showed that gangliosides enhanced the transcript level of ICAM-1 and MCP-1 within 3 h, whereas pretreatment with AG490 inhibited this increase (Fig. 4). These results provide further evidence for involvement of JAK-STAT inflammatory signaling in gangliosides-induced microglial activation. Moreover, AG490 significantly reduced gangliosides-induced NO release, indicating that NO production

**DISCUSSION**

Increasing evidence indicates that gangliosides act not only as mediators for cellular interactions but also as modulators of signal transduction in a variety of cellular events. These functions appear to occur simultaneously and influence each other (38). Identification of the precise mechanisms underlying how gangliosides regulate cellular responses has been the subject of many investigations, but it appears there is still much that is unknown (11, 34, 39). In this study, we reveal that gangliosides directly induce the activation of JAK-STAT signaling, a key pathway in inflammation, which leads to the expression of several inflammation-associated genes.

**FIG. 6.** Activation of ERK1/2 follows JAK-STAT activation in gangliosides-treated primary microglial cells. Primary microglial cells were pretreated with AG490 or PD98059 for 1 h and then treated with 50 μg/ml Gmix for 30 min. Cell lysates were separated by 10% SDS-PAGE and Western blots probed with anti-phospho-ERK and anti-phospho p38, respectively. The membrane was then stripped and probed with anti-ERK antibody. At least four experiments were independently performed, and representative data are shown in this figure.

**Fig. 7A** show both GM1 and GD1a stimulated phosphorylation of STAT1 within 2 min. The level of STAT1 phosphorylation stimulated by either GM1 or GD1a was similar to that caused by the gangliosides mixture, suggesting that the number of sialic acid residues per ganglioside molecule has little effect on the phosphorylation of STAT1 in microglial cells (Fig. 7A). Because sialic acid residues are characteristic of gangliosides, we examined whether sialic acid residues were important for gangliosides-stimulated STAT phosphorylation. Gangliosides were preincubated with either 550 or 1000 units/ml A. ureafaciens neuraminidase, which is known to release sialic acid attached to an internal galactose in any gangliosides including GM1 (36, 37). Primary microglia cells were stimulated with gangliosides or neuraminidase-treated gangliosides (desialylated gangliosides) for 2 min, and levels of phosphorylated STAT1 were determined by Western blot analysis. The data presented in Fig. 7B show a dose-dependent inhibitory effect of neuraminidase treatment on phosphorylation of STAT1, indicating that sialic acid residues are required for stimulation of JAK-STAT signaling. To rule out the possibility that these reductions are due to contaminating sialic acid or neuraminidase, we compared the effect of GM1 and asialo-GM1(Sigma) on phosphorylation of STAT and transcription of STAT-responsive genes. Consistent with Fig. 7, not only the phosphorylation of STAT1 and STAT3 but also the transcriptions of iNOS, MCP-1, and ICAM-1 were not induced in asialo-GM1-treated primary microglial cells (Fig. 8). Taken together, these results suggest that the presence of sialic acid residues is important for gangliosides-stimulated JAK-STAT signaling, although the number of sialic residues per ganglioside molecule may not influence phosphorylation.
was partly dependent on JAK-STAT signaling (Fig. 5). Taken together, these results suggest that inflammatory mediators including MCP-1, ICAM-1, and NO may be induced in response to gangliosides through JAK-STAT signaling.

Next we examined other signaling events that may be associated with JAK-STAT activation in gangliosides-treated microglial cells. Having previously observed that ERKs and p38 MAPKs were activated by gangliosides, we investigated whether gangliosides-stimulated JAK-STAT signaling was linked to activation of ERKs and p38 MAPKs. By using pharmacological inhibitors, we found that gangliosides-stimulated activation of JAK resulted in phosphorylation of ERK1/2 (but not p38) (Fig. 6), suggesting cross-talk between JAK and ERK pathways. Interestingly, inhibition of ERK by PD98059 resulted in complete inhibition of p38 activation, indicating cross-talk between different MAP kinase pathways. Because AG490 only partially reduced the phosphorylation of ERK, but PD98059 completely inhibited the activation of ERKs and p38, it is likely that activation of p38 is downstream of ERK activation and that any connection occurs via signaling molecules other than JAK-STATs. The activation profile of signaling molecules seems to depend on stimulators and/or cell types. Furthermore, upstream and downstream signaling molecules may be specifically affected by cross-talk and convergence in a particular environment. For example, Gouni-Berthold et al. (34) recently reported that gangliosides had no effect on phosphorylation of p38 in vascular smooth muscle cells, and that platelet-derived growth factor-BB-induced phosphorylation of p38 was not influenced by PD98059. In contrast, both MAPKs are activated by lipopolysaccharides and gangliosides in primary glial cells (10, 50). In view of the fact that gangliosides-induced activation of JAK-STAT signaling occurs more rapidly than activation of ERKs, and that inhibition of JAK activation reduced ERKs activation, it appears gangliosides-stimulated JAK-STAT signaling regulates ERKs activation in microglial cells. In contrast, p38 activation does not seem to be linked to JAK-STAT signaling, even though it too plays a role in microglial activation.

Gangliosides are amphipathic molecules that belong to a class of anionic glycosphingolipids. They contain sialic acid residues (N-acetylneuraminic acids), linked to the sugar residues of a ceramide oligosaccharide. It has been reported (34, 51) that different types of gangliosides have distinct roles in several cell types. To gain insight into the mechanism underlying JAK-STAT activation by gangliosides, we investigate whether GM1 and GD1a, the major types of gangliosides mixture used in these studies (18 and 55%, respectively), had different effects on STAT activation. Despite containing different numbers of sialic acid residues per molecule, GM1 containing one and GD1a containing two, both gangliosides activated STAT1 to a similar extent, which was similar to the activation caused by the gangliosides mixture. Although the different number of sialic acid residues on a ganglioside appeared to have no influence on STAT phosphorylation, the presence of sialic acid on a ganglioside was critical, since removal of sialic acid by neuraminidase resulted in reduced STAT1 phosphorylation (Fig. 7). These results are further confirmed by experiments to compare activities of GM1 and asialo-GM1. Unlike GM1, asialo-GM1 did not induce phosphorylation of STAT or expression of STAT-responsive genes including iNOS (Fig. 8). Thus, it appears sialic acid residues of gangliosides are important for STAT activation, and one residue per molecule is sufficient. However, we do not exclude the possibility that other moieties of gangliosides also importantly affect JAK-STAT signaling in microglial cells.

It is intriguing, but unresolved, as to how gangliosides stimulate phosphorylation of JAKs. One possibility is that gangliosides act by binding to specific cell surface receptors. However, although galectin-1 and -3 are reported to bind GM1, the specific receptors of gangliosides have not been clearly elucidated (51). Alternatively, gangliosides may act by modulating other signaling molecules. For example, there are reports (52–55) that incorporation of gangliosides into plasma membranes regulates CD4, growth factor receptors, and phospholipase C. It may be that the gangliosides-enriched membrane domains, assigned as lipid rafts, regulate intermolecular associations in the plasma membranes. Many transmembrane receptors are reported to be constitutively or inducibly localized within lipid rafts (36, 56–58). It has been reported that the Src family kinases Rho, FAK, Lyn, and Lck are associated with ganglioside-enriched lipid rafts (56, 39, 59, 60). Interestingly, a recent report (28) showed that JAK1 and JAK2 are exclusively local-
induced microglial inflammation, and such knowledge will assist in the better understanding of the pathogenesis of brain disease.

Acknowledgment—We thank Dr. E. J. Choi (Korea University, Seoul, Korea) for providing BV2 cells.

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**Fig. 8. Asialo-GM1 does not activate the JAK-STAT signaling in primary microglial cells.** A, effect of GM1 and asialo-GM1 on phosphorylation of STAT. Primary microglial cells were treated with 20 μg/ml of GM1 or asialo-GM1 for 2 min. Cell lysates were subjected to Western blot analysis, and levels of phosphorylated STAT were determined using anti-pSTAT1 and anti-pSTAT3. Con, control. B, effect of GM1 and asialo-GM1 on STAT-responsive transcription. Total RNA was isolated and analyzed for levels of iNOS, MCP-1, and ICAM-1 mRNA using an RT-PCR-based assay.
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