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Curcumin Suppresses Janus Kinase-STAT Inflammatory Signaling through Activation of Src Homology 2 Domain-Containing Tyrosine Phosphatase 2 in Brain Microglia[1]

Hee Young Kim, Eun Jung Park, Eun-hye Joe, and Ilo Jou[2]

Curcumin has been strongly implicated as an anti-inflammatory agent, but the precise mechanisms of its action are largely unknown. In this study, we show that the inhibitory action of curcumin on Janus kinase (JAK)-STAT signaling can contribute to its anti-inflammatory activity in the brain. In both rat primary microglia and murine BV2 microglial cells, curcumin effectively suppressed the ganglioside-, LPS-, or IFN-γ-stimulated induction of cyclooxygenase-2 and inducible NO synthase, important enzymes that mediate inflammatory processes. These anti-inflammatory effects appear to be due, at least in part, to the suppression of the JAK-STAT inflammatory signaling cascade. Curcumin markedly inhibited the phosphorylation of STAT1 and 3 as well as JAK1 and 2 in microglia activated with gangliosides, LPS, or IFN-γ. Curcumin consistently suppressed not only NF binding to IFN-γ-activated sequence/IFN-stimulated regulatory element, but also the expression of inflammation-associated genes, including ICAM-1 and monocyte chemoattractant protein 1, whose promoters contain STAT-binding elements. We further show that activation of Src homology 2 domain-containing protein tyrosine phosphatases (SHP)-2, a negative regulator of JAK activity, is likely to be one of the mechanisms underlying the curcumin-mediated inhibition of JAK-STAT signaling. Treatment of microglial cells with curcumin led to an increase in phosphorylation and 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. ***The Journal of Immunology*****, 2003, 171: 6072–6079.**

Microglial activation occurs rapidly after brain injury (1, 2). Activated microglia change morphologically from resting ramified to amoeboid phagocytic cells, and proliferate and migrate to injured sites. Upon activation, microglia release various proinflammatory cytokines and inflammatory mediators such as NOs and PGs that have protective functions (3, 4). However, when overactivated, microglia-induced inflammatory responses may exacerbate neuronal damage (5–8). Activated microglia are observed in various human neurological diseases, including AIDS dementia complex, multiple sclerosis, and Alzheimer's disease (9–11). Thus it may be functionally important to tightly regulate the degree of microglial activation after injury or in chronic disease.

Janus kinase (JAK)[3]-STAT signaling, originally identified as the signaling pathway for IFNs, mediates the immune responses of various cytokines as well as the actions of many growth factors and hormones, and thus participates in inflammation (12–14). Specific subtypes of JAK and STAT molecules mediate different signals, resulting in specificity of responses (15, 16). 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 tyrosine kinase 2. Phosphorylated JAKs lead to the activation of neighboring JAKs, receptor subunits, and several other substrates and provide the docking sites for STATs, 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 (17–19).

Cytokine-derived immune signaling is strictly regulated with respect to both magnitude and duration. Inhibitory molecules including the suppressors of cytokine signaling (SOCS) family (SOCS1–7 and cytokine-induced STAT inhibitor) and protein inhibitor of activated STAT have been suggested to be part of the negative feedback regulation of JAK-STAT signaling (20–24). These proteins are reported to inactivate JAKs and block the access of STATs to receptor binding sites. SOCS inactivate JAKs, cytokine-induced STAT inhibitor binds to the sites of STAT on the activated receptor and blocks the access of STAT-receptor binding. Protein inhibitor of activated STAT has been reported to associate with tyrosine-phosphorylated STAT dimers. Protein tyrosine phosphatases (PTPases) including Src homology 2 (SH2) domain-containing PTPases (SHP)-1 and SHP-2 are other important regulators of cytokine signaling. SHP-1 and SHP-2 have two SH2 domains at the N terminus and a PTPase catalytic domain at the C terminus, which catalyze the tyrosine dephosphorylation of JAKs, receptor or other cellular proteins, and thus play critical roles in the control of cytokine signaling (14, 25–28). SHPs are autoinhibited in their resting state by an intramolecular interaction between their SH2 domains and the PTPase domain. The PTPase

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[3] Abbreviations used in this paper: JAK, Janus kinase; GAS, IFN-γ-activated sequence; ISRE, IFN-stimulated regulatory element; SOCS, suppressor of cytokine signaling; PTPase, protein tyrosine phosphatase; SH, Src homology; SHP, SH2 domain-containing PTPase; iNOS, inducible NO synthase; COX, cyclooxygenase; MCP, monocyte chemoattractant protein.

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domain is activated after its association with tyrosine-phosphorylated proteins including membrane receptors and JAKs (29). 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 (30).

Curcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-di-one) is a major component of tumeric. 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 antitumor activities (31–33). The anti-inflammatory actions of curcumin seem to be closely related to the suppression of proinflammatory cytokines and mediators of their release such as TNF-γ, IL-1β, and NOs. There are reports that curcumin inhibits cytokine-mediated NF-κB activation by blocking a signal leading to I-κB kinase activity in intestinal epithelial and mouse fibroblast cells, and also suppresses phorbol ester-induced c-Jun/AP-1 activation (34–36). However, the mechanisms underlying interactions of curcumin with these signaling pathways are poorly understood. Recently, we have shown that JAK-STAT inflammatory signaling modulates glial activation (37, 38). Thus, we examined whether curcumin inhibits the JAK-STAT pathways in activated microglia. Curcumin inhibits the phosphorylation of JAK1 and JAK2 via the increased phosphorylation of SHP-2 and its association with JAK1/2, thus attenuating inflammatory response. Our results show that curcumin acts via a novel anti-inflammatory mechanism and is also a negative regulator of the JAK-STAT pathway by the activation of SHP-2.

Materials and Methods

Reagents

Curcumin and LPS were purchased from Sigma-Aldrich (St. Louis, MO), and IFN-γ was purchased from Calbiochem (San Diego, CA). Gangliosides mixture was purchased from Matraya (Pleasant Gap, PA). Abs against STAT1, phospho-STAT1 (Tyr^{701}, Ser^{727}) and phospho-STAT3 (Tyr705) were purchased from Cell Signaling Technology (Beverley, MA). Abs against inducible NO synthase (iNOS), phosphotyrosine (4G10), JAK1, and JAK2 were purchased from Upstate Biotechnology (Lake Placid, NY), and Abs against cyclooxygenase (COX)-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-JAK1 and phospho-JAK2 Abs were purchased from Calbiochem and SHP-1 and SHP-2 Abs were purchased from BD Transduction Laboratories (Lexington, KY).

Cell culture

Primary microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague Dawley rats as previously described (37). Briefly, the cortices were triturated into single cells in MEM containing 10% FBS (HyClone Laboratories, Logan, UT) and plated in 75-cm^2 T-flasks (0.5 hemisphere/flask) for 2–3 wk. Microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes. Cells were plated in six-well plates (7×10^4 cells/well), 60-mm dishes (5×10^5 cells/dish), or 100-mm dishes (10^6 cells/well). One hour later, the cells were washed to remove unattached cells before being used in experiments. BV2 immortalized murine microglial cells were obtained from Dr. E. J. Choi (Korea University, Seoul, Korea). The BV2 cell line was grown in DMEM and supplemented with 5% FBS. Cells were serum-starved overnight before RT-PCR and Western blot analysis.

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 DTT, 0.5 mM PMSF) including 0.5% Nonidet P-40. Cells were centrifuged at $500 \times g$ for 10 min at 4°C, and the pellet (nuclear fraction) was saved. The nuclear fractions were resuspended in a buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF, were incubated on ice for 60 min with occasional gentle shaking, and were centrifuged at $12{,}000 \times g$ for 20 min. The crude nuclear proteins in the supernatant were collected and stored at −70°C for EMSAs. 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 $ZnSO_4$, 50 μg/ml poly-D-(I-C), 1 mM DTT, 0.3 mg/ml BSA, 6 mM $MgCl_2$, and γ-^{32}P-radiolabeled oligonucleotide probe (3×10^4 cpm), with or without a 20- to 50-fold excess of unlabeled probe. The dried gels were exposed to x-ray film. The following double-stranded oligonucleotide was used in these studies: IFN-γ-activated sequence/IFN-stimulated regulatory element (GAS/ISRE), 5′-AAGTACTTTCAGTT TCATATTACTCTA-3′, 27 bp (sc-2537; Santa Cruz Biotechnology). 5′-end-labeled probes were prepared with 40 μCi [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, Madison, WI) and were purified on Quick Spin Columns Sephadex G-25 (Boehringer Mannheim, Indianapolis, IN).

Western blot analysis

Cells were washed twice with cold PBS, and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (2 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mM EDTA). The lysate was centrifuged for 20 min at $12{,}000 \times g$ at 4°C and the supernatant collected. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with primary Abs and peroxidase-conjugated secondary Abs (Vector Laboratories, Burlingame, CA), and then visualized using an ECL system (Sigma-Aldrich).

RT-PCR

Total RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX) and cDNA was prepared using reverse transcriptase from avian myeloblastosis virus (Takara Shuzo, Otsu, 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 were as previously described (37).

Immunoprecipitation

Cell extracts were prepared by using modified RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM $NaVO_4$). Lysates (500 μg) were incubated with 1 μg of the appropriate Ab at 4°C overnight and precipitated with protein G-agarose beads (Upstate Biotechnology) for 2 h at 4°C. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Western blot analysis was performed with several Abs as indicated.

Results

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, we first examined the effect of curcumin on the expression of iNOS and 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 Abs against either iNOS or COX-2. All three microglial activators significantly induced the expression of iNOS and COX-2, but curcumin suppressed this induction in a dose-dependent manner (Fig. 1*A*). Although curcumin has been reported to show anti-inflammatory effects or chemopreventive effects in the range of 10−40 μM in other cells, 20 μM curcumin sometimes affected cell viability in microglial cells (39, 40). Thus, we 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 with IFN-γ or LPS (Fig. 1*B*). NOs and PGs, products of iNOS and COX-2 respectively, are mediators of microglial activation. Because curcumin strongly suppressed the induction of iNOS and COX-2 in activated microglia, we further explored the mechanism of action of curcumin in inflammatory response.

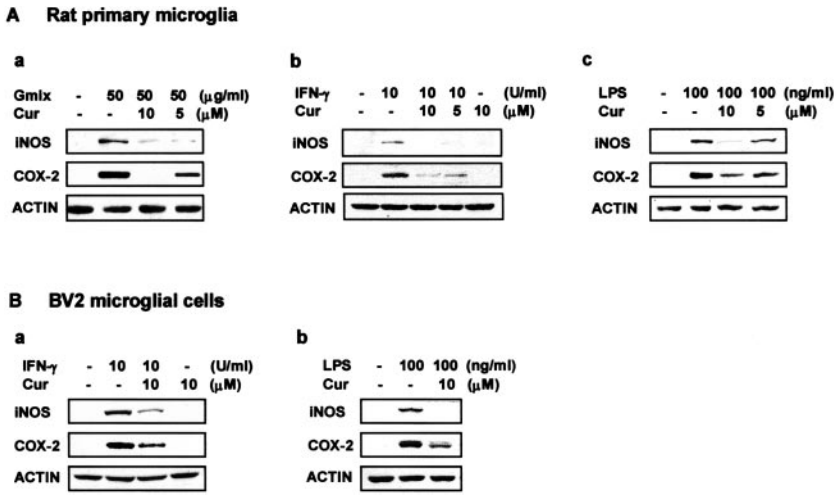

FIGURE 1. Curcumin inhibits the up-regulation of iNOS and COX-2 in activated microglia. *A*, Rat primary microglia were pretreated with curcumin (cur) for 30 min and then stimulated with brain gangliosides mixture (Gmix) (*a*), IFN-γ (*b*), or LPS (*c*) for 8 h. *B*, Murine BV2 microglial cells were stimulated with IFN-γ (*a*) or LPS (*b*) in the absence or presence of curcumin. Western blot analysis was performed with Abs against iNOS, COX-2, and actin. Data shown are representative of three independent experiments.

Curcumin suppresses the NF binding to GAS/ISRE in activated microglia

Several inflammatory mediators including iNOS and COX-2 have functional GAS elements in their promoter regions (41, 42). Because activation of GAS-NF binding has been implicated in inflammatory responses, we hypothesized that the inhibitory effects of curcumin are related to the activation of GAS-NF binding. Based on our previous report that gangliosides activate microglia through rapid activation of JAK-STAT signaling, including GAS-NF binding (37), we examined the effects of curcumin on ganglioside-induced NF binding to GAS in primary microglia. Cells were stimulated with 50 μg/ml gangliosides in the absence or presence of either 10 or 5 μM curcumin, and the nuclear protein extracts were examined by EMSA for the binding activity of a γ-^{32}P-labeled consensus GAS/ISRE oligonucleotide probe. Within 10 min, gangliosides rapidly induced NF binding to GAS/ISRE, and this increased binding activity was suppressed with inclusion of curcumin (Fig. 2*A*). The inhibitory effects of curcumin on the binding activity of GAS/ISRE were also observed in IFN-γ-stimulated BV2 cells (Fig. 2*B*). At all of the times tested, from 5 to 30 min after IFN-γ treatment, curcumin significantly inhibited NF binding. These results indicate that curcumin suppresses NF binding to GAS/ISRE sequences in activated microglia, which contribute, at least in part, to the attenuation of inflammatory response.

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. Because curcumin reduced NF binding activity to GAS/ISRE sequences, we asked whether 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 analyses were performed using phosphotyrosine STAT1 (Tyr^{701}), phosphoserine STAT1 (Ser^{727}), and actin Abs. In agreement with NF binding activity to GAS/ISRE, both phosphorylations of STAT1 by gangliosides, LPS, or IFN-γ were markedly suppressed in curcumin-treated primary microglia (Fig. 3*A*). 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. 3*B*)

Because we previously observed that gangliosides and IFN-γ activated STAT3 as well as STAT1 in microglia (37), we 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. 4*A*). Similar effects of curcumin on STAT3 were observed in BV2 microglial cells. As shown in Fig. 4*B*, the inhibitory effect of curcumin on STAT3 phosphorylation was similar to that of STAT1. Our 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.

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 ganglioside- or IFN-γ-stimulated microglial cells (37), we 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

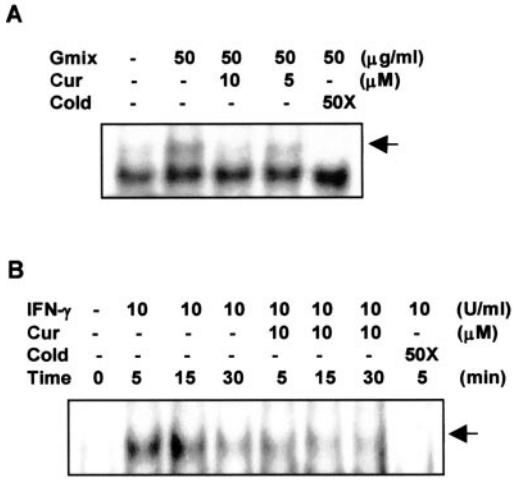

FIGURE 2. Curcumin suppresses the NF binding to GAS/ISRE in activated microglia. *A*, Rat primary microglia were pretreated with curcumin (cur) 10 or 5 μM for 30 min and stimulated with 50 μg/ml gangliosides mixture (Gmix) for 10 min. Nuclear extracts were prepared and binding activity to GAS/ISRE oligonucleotides 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 are representative of two independent experiments.

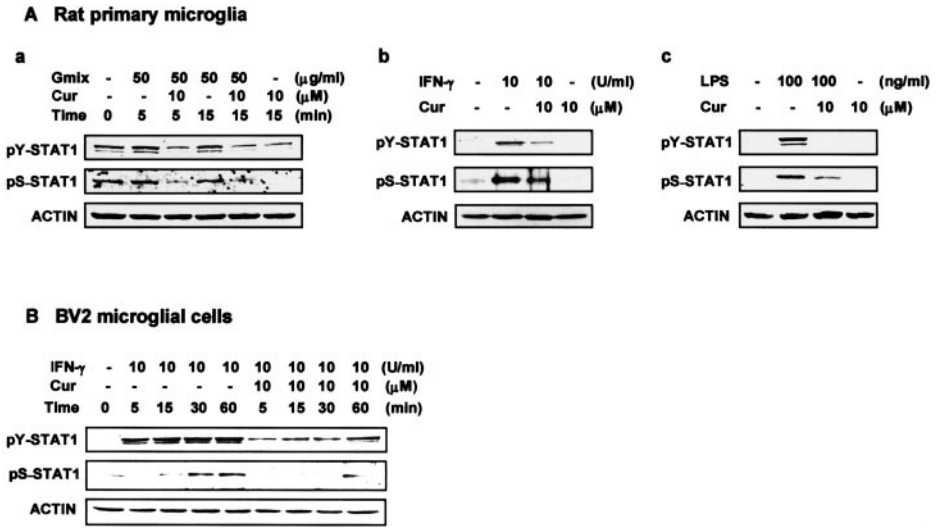

FIGURE 3. Curcumin suppresses the tyrosine and serine phosphorylation of STAT1 in activated microglia. *A*, Rat primary microglial cells were pretreated with curcumin for 30 min, and then stimulated with gangliosides mixture (Gmix) for the indicated time periods (*a*), IFN-γ for 30 min (*b*), or LPS for 180 min (*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 ~8–10% 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 Ab to determine the amount of loading. Data shown are representative of three independent experiments.

analysis was performed with Abs specific either to phospho-JAK1 or phospho-JAK2. Both gangliosides and IFN-γ rapidly induced the phosphorylation of JAK1 and JAK2, whereas addition of curcumin suppressed phosphorylation (Fig. 5*A*). Similar results were obtained in IFN-γ-activated BV2 cells (Fig. 5*B*). The inhibitory action of curcumin on JAK phosphorylation was correlated with its effect on STAT phosphorylation and GAS binding activity. Taken together, our results suggest that in activated microglia, curcumin inhibits the initiation of the JAK-STAT signaling cascade, at least from the point of JAK phosphorylation.

Curcumin suppresses STAT-responsive inflammatory gene expression

To validate the inhibitory actions of curcumin on JAK-STAT signaling in activated microglia, we 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 (MCP)-1 and ICAM-1 mRNA, and the addition of curcumin suppressed this expression (Fig. 6). Similar results were obtained in BV2 cells (data not shown).

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

Because 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 Fig. 7, we added 10 μM 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 effects of curcumin on phosphorylation of JAK1 and

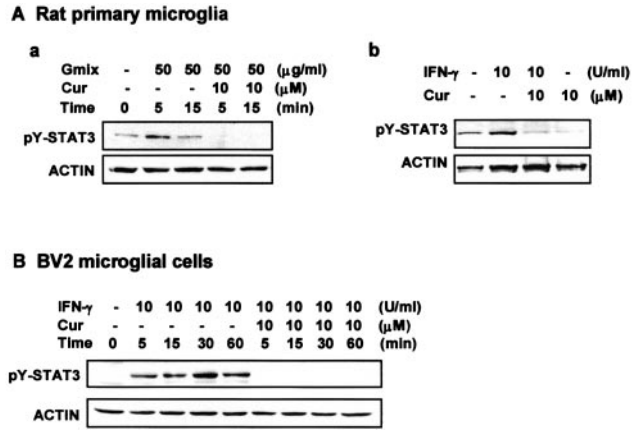

FIGURE 4. Curcumin suppresses the tyrosine phosphorylation of STAT3 in activated microglia. *A*, Rat primary microglial cells were serum-starved for 12 h. The cells were pretreated with curcumin for 30 min and stimulated with gangliosides mixture (Gmix) for the indicated time periods (*a*) or with IFN-γ 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 Abs for phospho-STAT3 and actin. Data are representative of two independent experiments.

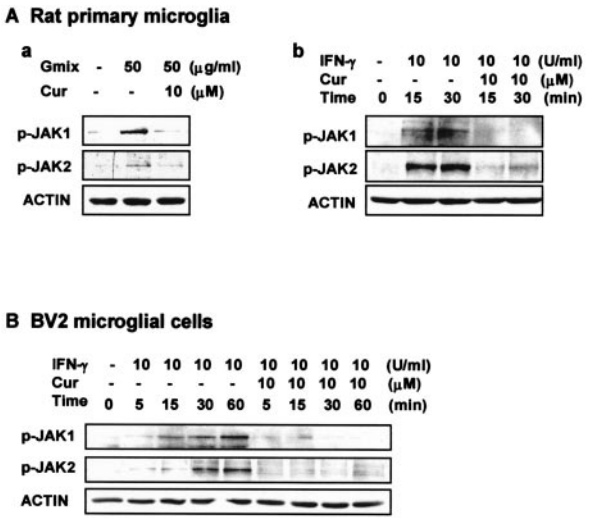

FIGURE 5. Curcumin suppresses the phosphorylation of JAK1 and JAK2 in activated microglia. *A*, Rat primary microglia were pretreated with curcumin for 30 min and then stimulated with 50 μg/ml gangliosides mixture (Gmix) for 10 min (*a*) or IFN-γ for the indicated time periods (*b*). The phosphorylation level of JAK1 and JAK2 were determined by Western blot analysis using Abs 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 Abs for phospho-JAK1 or phospho-JAK2. Data shown here are representative of three independent experiments.

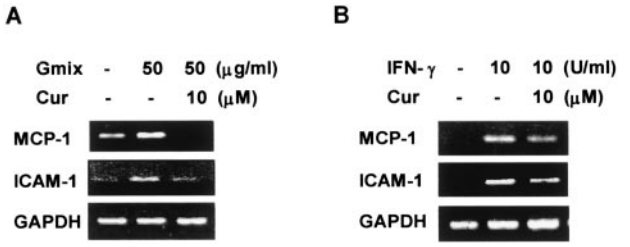

FIGURE 6. Curcumin suppresses STAT-responsive inflammatory gene expressions. Rat primary microglia were treated with 50 μg/ml gangliosides mixture (Gmix) (*A*) or 10 U/ml IFN-γ (*B*) for 3 h. Total RNA was isolated and analyzed for transcript levels of MCP-1 and ICAM-1 using an RT-PCR-based assay. The transcript of GAPDH was measured for normalization. Data shown are representative of three independent experiments.

JAK2 were observed at all the times tested. The tyrosine-phosphorylated level of STAT l 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.

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

We next examined the mechanisms underlying curcumin-mediated suppression of JAK activation. Firstly, we 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, we tested the effects of curcumin on the transcription of SOCS1 and SOCS3 in rat primary microglia and BV2 cells. However, we did not observe the induction of SOCS1 and SOCS3 transcripts in curcumin-treated microglial cells (Fig. 8). Secondly, we tested the involvement of the PTPases, 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 either at a receptor or at phosphotyrosine residue at the C terminus of SHPs itself (29–30, 43). Thus, we 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. Western blot analyses with 4G10 Ab 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, whereas changes in SHP-1 phosphorylation were smaller compared with changes in SHP-2 phosphorylation under the same experimental conditions (Fig. 9*A*). To confirm these results, we 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 Ab 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 with control cells (Fig. 9*B*), whereas 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 in cells with IFN-γ alone (Fig. 9*B*). Therefore, it is likely that curcumin rapidly alters the level of phosphorylation of SHP-2, thus inhibiting the initiation of JAK-STAT inflammatory signaling.

Discussion

In this study, we 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. For example, curcumin has been reported to reduce oxidative damage and amyloid pathology in an Alzheimer transgenic mouse (44) and to inhibit IL-12 signaling in experimental allergic encephalomyelitis (40). 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 (37, 38, 40, 44). Thus we 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. We determined the effects of curcumin on the expression of COX-2 and iNOS in activated microglia, because curcumin is reported to reduce both enzymes in several cancer cell lines, and both genes have STAT-binding sites in their promoters. As expected, curcumin suppressed the up-regulation of COX-2 and iNOS in activated microglia (Fig.

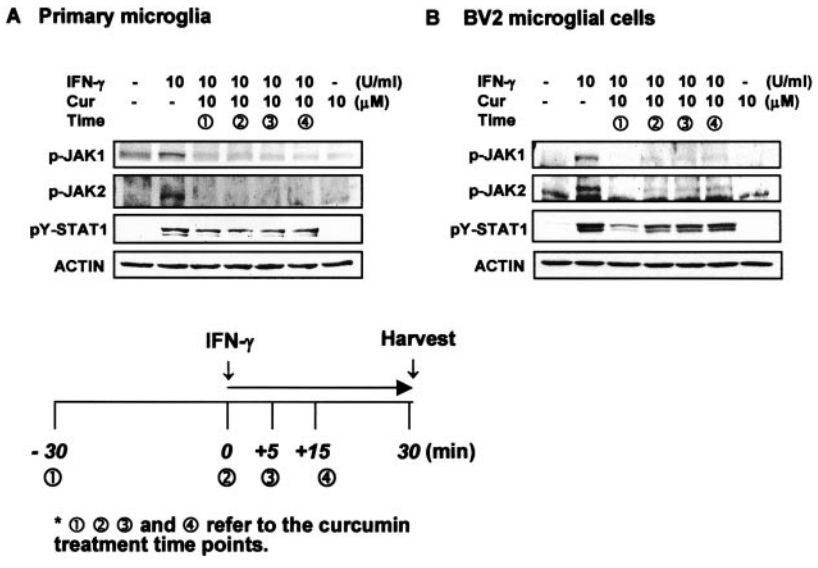

FIGURE 7. Curcumin treatments after IFN-γ stimulation also suppress the phosphorylation of JAK-STAT signaling. In primary microglia (*A*) and BV2 cell (*B*), curcumin 10 μM was treated as time schedule shown in diagram, and IFN-γ was treated for 30 min. Western blot analysis was performed with Abs for phospho-JAK1, phospho-JAK2, or phospho-STAT1 (Tyr^{701}). Curcumin inhibits the tyrosine phosphorylation of JAK1, JAK2, and STAT1 in all the case that curcumin is pretreated or after treatment. The diagram shows the time schedule of curcumin treatment.

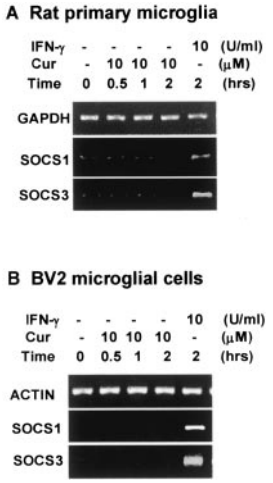

FIGURE 8. Curcumin does not induce the transcription of SOCS1 and SOCS3. Rat primary microglia (*A*) and murine BV2 cells (*B*) were treated with 10 μM curcumin for 0.5, 1, and 2 h, and with IFN-γ as a positive control, for 2 h. Total RNA was isolated and analyzed for transcript levels of SOCS1 and SOCS3 using an RT-PCR-based assay.

1), supporting our hypothesis that curcumin suppresses inflammatory responses via inhibition of JAK-STAT signaling. In subsequent experiments, we observed that curcumin reduced the phosphorylation of STAT1 and STAT3 as well as NF binding to GAS/ISRE sequences (Figs. 2–4). In addition, phosphorylations of JAK1 and JAK2, molecules upstream of STAT phosphorylations, were significantly suppressed in ganglioside-, 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 (45). In contrast, curcumin inhibited the IL-12-induced tyrosine phosphorylation of STAT3 and 4 by blocking tyrosine kinase 2 and JAK2 activation in T lymphocytes (40). In our 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, we 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 ganglioside- 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, we addressed the question of how curcumin inhibits the phosphorylation of JAKs. We considered the involvement of inhibitory proteins and PTPases. In an experiment to reveal the involvement of inhibitory regulators including SOCS1 and SOCS3, which are known to inhibit JAK phosphorylation, curcumin did not induce either (Fig. 8). So, we tested the involvement of PTPases, including SHP-1 and SHP-2, because 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 receptor tyrosine kinases 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. 9*A*). To confirm these results, we used immunoprecipitation to test the association of JAKs with SHP-2. In an agreement with its effect on phosphorylation, curcumin did increase the binding of SHP-2 with JAK1 as well as with JAK2 (Fig. 9*B*). In addition, the binding of SHP-2 to JAK1 significantly increased in microglial cells with IFN-γ plus curcumin than in cells with IFN-γ alone (Fig. 9*B*). 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 (27, 46, 47), it has also been reported to be a positive

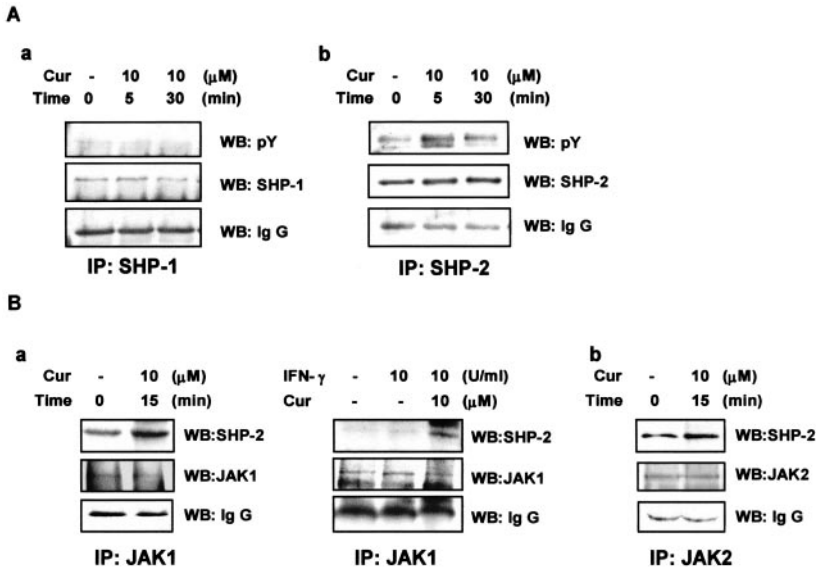

FIGURE 9. Curcumin induces the activation of SHP-2. *A*, BV2 microglial cells were treated with curcumin for the indicated time periods and immunoprecipitated with an Ab against SHP-1 (*a*) or SHP-2 (*b*). Western blot analysis was performed with 4G10 tyrosine Ab. The membrane was then stripped and analyzed 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 Abs, respectively. Data shown are representative of three independent experiments.

regulator of prolactin- or angiotensin II-induced JAK2 phosphorylation (48–50). Our 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, we recently obtained evidence that suggests the involvement of SHP-2 in curcumin-induced inhibitory responses. We 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 nonraft to lipid rafts and thus regulate T cell activation (51–54). IFN-γ receptor is also reported to be localized to membrane rafts (53), 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. We recently observed that activation of platelet-derived growth factor receptor β, another representative raft-mediated signaling molecule, also inhibited by curcumin, which further supports our assumption that curcumin could affect rafts-mediated signaling cascades (data not shown). In this regard, we are 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 mediates microglial activation and deactivation after brain injury and in chronic disease.

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