15d-PGJ₂ and Rosiglitazone Suppress Janus Kinase-STAT Inflammatory Signaling through Induction of Suppressor of Cytokine Signaling 1 (SOCS1) and SOCS3 in Glia

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Peroxisome proliferator-activated receptor (PPAR)-γ agonists are now emerging as therapeutic drugs for various inflammatory diseases. However, their molecular mechanism of action remains to be elucidated. Here we report a novel mechanism that underlies the PPAR-γ agonist-mediated suppression of brain inflammation. We show that 15-deoxy-Δ12,14-prostaglandin J₂ (15d-PGJ₂) and rosiglitazone reduce the phosphorylation of STAT1 and STAT3 as well as Janus kinase 1 (JAK1) and JAK2 in activated astrocytes and microglia. The PPAR-γ agonist-mediated reduction in phosphorylation leads to the suppression of JAK-STAT-dependent inflammatory responses. The effects of 15d-PGJ₂ and rosiglitazone are not mediated by activation of PPAR-γ. 15d-PGJ₂ and rosiglitazone rapidly induce the transcription of suppressor of cytokine signaling (SOCS) 1 and 3, which in turn inhibit JAK activity in activated glial cells. In addition, Src homology 2 domain-containing protein phosphatase 2 (SHP2), another negative regulator of JAK activity, is also involved in their anti-inflammatory action. Our data suggest that 15d-PGJ₂ and rosiglitazone suppress the initiation of JAK-STAT inflammatory signaling independently of PPAR-γ, thus attenuating brain inflammation.

Astrocytes and microglia are cells with immune functions in the central nervous system. They are rapidly activated in response to pathological stimuli and lead to various inflammatory processes. Upon activation, astrocytes and microglia change their immunophenotype as well as the expression pattern of inflammatory mediators including cytokines, chemokines, and neurotoxic substances at the transcriptional level (1, 2). Activated glial cells can exert both beneficial and harmful actions on brain. Although transient activation is beneficial for defense processes, either chronic activation or overactivation may lead to or exacerbate neuronal diseases such as Alzheimer’s disease, ischemia, and human immunodeficiency virus encephalitis (3). Inflammatory mediators are involved in many effects of activated glial cells. Whereas cytokines such as interleukin-10 reduce glial cytotoxicity (4), other cytokines such as interferon-γ (IFN-γ) cause activation of glial cells (5), eventually leading to neuronal injury via inflammatory cascade.

The JAK-STAT (Janus kinase-signal transducers and activators of transcription) cascade is an essential inflammatory signaling pathway that mediates immune responses. Specific subtypes of JAK and STAT are activated by different signals and transduce signals from cell surface receptors to different subsets of target genes, many of which are involved in immune responses (6, 7). To prevent detrimental effects, the intensity and duration of JAK-STAT activation are tightly regulated. Recently, a family of proteins called suppressors of cytokine signaling (SOCS) has been isolated (8–10). Generally, SOCS are present in cells at very low levels but are rapidly transcribed after exposure of cells to stimulus. SOCS can negatively regulate the response of immune cells either by inhibiting JAK activity or by competing with signaling molecules for binding to the phosphorylated receptor (11). For example, SOCS1 and SOCS3 bind to the JAKs and inhibit their tyrosine kinase activity (12). The ability of SOCS to suppress cytokine signaling in vivo has been confirmed by animal models including knock-out mice (13, 14). SOCS family is now considered as important regulators of normal immune physiology and immune disease (15).

Peroxisome proliferator-activated receptor (PPAR)-γ is a ligand-dependent nuclear receptor whose ligands include several prostanoids including 15-deoxy-Δ12,14-prostaglandin J₂ (15d-PGJ₂) (16) and antidiabetic thiazolidinediones such as rosiglitazone (17). Although PPAR-γ was originally shown to play a key role in adipocyte differentiation and lipid metabolism (18), some PPAR-γ ligands have recently been reported to exert anti-inflammatory actions by reducing inflammation-associated molecules such as cytokines and nitric oxides (19–24). However, the precise mechanisms of action underlying the anti-inflammatory effects of PPAR-γ agonists are poorly understood. Here we provide new insights into the anti-inflammatory actions of 15d-PGJ₂ and rosiglitazone based on their effects on JAK-STAT inflammatory signaling. We found that there is a PPAR-γ-independent link between the anti-inflammatory action of 15d-PGJ₂ and rosiglitazone and the induction of SOCS1 and SOCS3. Our findings suggest not only a novel molecular explanation for the therapeutic efficacy of PPAR-γ agonists but also a new potential therapeutic intervention for inflammatory diseases.

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EXPERIMENTAL PROCEDURES

Reagents—Lipopolysaccharide was purchased from Sigma, and IFN-γ was from Calbiochem. 15d-PGJ2 was purchased from Bioworld (St. Louis, MO). Rosiglitazone was a gift from Dr. K. S. Park (Seoul National University, Seoul, Korea). Antibodies against STAT1, Tyr-701-phosphorylated STAT1, and Tyr-705-phosphorylated STAT3 were from Cell Signaling Technology (Beverly, MA). Antibodies against phosphorylated JAK1 and JAK2 were from Affinity Bioreagents (Denver, CO).

Cell Culture—Primary astrocytes and microglia were cultured from the cerebral cortices of 1-day-old Sprague-Dawley rats. The cortices were triturated into single cells in minimal essential medium (Invitrogen) containing 10% fetal bovine serum and incubated for 2–3 weeks. Microglia were detached from the flasks, and the cells remaining in the flask were harvested with 0.1% trypsin to prepare pure astrocytes. BV2 murine microglia cells were obtained from Dr. E. J. Choi and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum.

Reverse Transcription (RT)-PCR Analysis—Total RNA was isolated using RNAzol B (TEL-TEST, Inc., Friendswood, TX), and cDNA was prepared using AMV reverse transcriptase (Promega, Madison, WI). Total RNA was analyzed for the messenger levels of indicated genes. Primary astrocytes were treated with 15d-PGJ2 (10 μM) and treated with interferon regulatory factor-1 and actin antibody. PPAR-γ agonists also suppress the events downstream of JAK-STAT activation. a, primary astrocytes were transfected with 8-GAS luciferase reporter plasmid and pCMV-β-galactosidase, and the cells were treated with combinations of 15d-PGJ2 (10 μM), rosiglitazone (20 μM), and/or IFN-γ (10 units ml−1) for 18 h. The cell extract was assayed for luciferase activity and β-galactosidase activity. b, primary astrocytes were pretreated with 15d-PGJ2 (10 μM), and then stimulated with IFN-γ (10 units ml−1) for 2 h. Western blot analysis was performed with interferon regulatory factor-1 and actin antibody. MCF, monocyte chemotactic protein-1; IRF, interferon regulatory factor-1; Con, control; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 15d, 15d-PGJ2; Ro, rosiglitazone.

RESULTS

To better define the mechanism of action by which PPAR-γ agonists suppress inflammation, we examined the effects of 15d-PGJ2 and rosiglitazone on JAK-STAT inflammatory signaling in rat brain. Pretreatment of 15d-PGJ2 or rosiglitazone for 2 h markedly suppressed the IFN-γ-stimulated phosphorylation of STAT1 and STAT3 in rat primary astrocytes (Fig. 1c). At all of the time points tested from 2 min to 2 h after IFN-γ treatment, phosphorylation of STAT1 and STAT3 was strongly reduced by treatment with either of the PPAR-γ agonists (data...
Western blot analysis was performed with pSTAT1, pSTAT3, or total 15d-PGJ2 and rosiglitazone can inhibit the activation of 15d-PGJ2 and rosiglitazone. Similar effects were observed in primary microglia. PPAR-agonists influence JAK-STAT inflammatory signaling. To address this question, we examined whether PPAR-γ mediates the inhibitory action of its agonists on JAK-STAT signaling. First, we examined the y-interferon-activated sequence (GAS) promoter activity in primary astrocytes, because activated STAT dimers bind to GAS elements. Transient transfection analysis showed that PPAR-γ agonists significantly reduced the IFN-γ responsiveness of the GAS promoter (Fig. 2a). We then examined the mRNA level of genes whose promoters have binding sites for STATs and act as mediators of inflammation, namely monocyte chemoattractant protein-1 (27) and interferon-inducible protein-10 (28). IFN-γ rapidly induced the transcription of both genes, but this induction was inhibited by pretreatment with 15d-PGJ2 or rosiglitazone (Fig. 2b). Similar patterns of transcriptional repression were observed for pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Fig. 2c). We further tested the effects of PPAR-γ agonists at the level of protein expression. As expected, PPAR-γ agonists suppressed the IFN-γ-stimulated induction of interferon regulatory factor-1 protein, which is a regulator of host defense and has binding sites for STAT in its promoter (Fig. 2d) (29). These observations convincingly demonstrate that PPAR-γ agonists can exert inhibitory actions on JAK-STAT inflammatory signaling, thus regulating brain inflammation.

An important question raised by the above findings is how PPAR-γ agonists influence JAK-STAT signaling. To address this question, we examined whether PPAR-γ mediates the inhibitory action of its agonists on JAK-STAT signaling. First, we examined the existence of PPAR-γ in rat primary astrocytes and microglia. Even in unstimulated cells, PPAR-γ transcripts could be detected (Fig. 3a and data not shown). We then compared the inhibitory effects of PPAR-γ agonists on JAK-STAT signaling between cells transiently transfected with PPAR-γ and vector. Interestingly, we did not observe any differences of GAS-luciferase activity in cells with PPAR-γ agonists compared with cells with vector (Fig. 3b). Different concentrations either of PPAR-γ agonists or of drugs also did not affect the GAS activity (data not shown). In contrast, a significant activation of PPAR-γ-responsive element (PPRE)-luciferase was observed in the same experimental conditions (Fig. 3b). Thus, overexpression of PPAR-γ did not lessen GAS activity but enhanced PPRE activity, indicating that PPAR-γ agonists act independently of PPAR-γ activation in the JAK-STAT signaling pathway. Overexpression of PPAR-γ consistently failed to improve the inhibitory effects of PPAR-γ agonists on JAK-STAT phosphorylation. In cells transiently transfected with vector, PPAR-γ, or PPAR-γ, S112A (a PPAR-γ active mutant) (30), PPAR-γ agonists exerted equivalent effects on the phosphorylation of protein.
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**Fig. 6.** Effects of 15d-PGJ2 and rosiglitazone on expression of SOCS1 and SOCS3. a–c, primary astrocytes (a and c) and BV2 (b) cells were treated with IFN-γ (10 units ml⁻¹), 15d-PGJ2 (15d) (10 μM), or rosiglitazone (20 μM) as indicated. RT-PCR analysis was performed using SOCS1 primers (a and b) and SOCS3 primers (c), and the products were analyzed on ethidium bromide-stained agarose gel. d, primary astrocytes were transiently transfected with pSV or PPAR-γS112A, and the cells were treated with IFN-γ or 15d-PGJ2 for 2 h. The transcript level of SOCS1 was detected by RT-PCR. e, primary astrocytes were transiently transfected with pEF, pEF-SOCS1, or pEF-SOCS3, and the cells were treated with IFN-γ (10 units ml⁻¹) for 5 min. Western blots were probed with pSTAT1, pSTAT3, or STAT1 antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 7.** Phosphorylation of SHP2 by 15d-PGJ2 in rat primary astrocytes. Primary astrocytes were treated with 15d-PGJ2 (10 μM) at indicated times, and the cell extracts were immunoprecipitated with SHP2 antibody. Western blot analysis was performed with 4G10 tyrosine antibody. The membrane was then stripped and analyzed with SHP2 antibody. WB, Western Blot.

**Fig. 8.** Induction of SOCS1 and SOCS3 by 15d-PGJ2 and rosiglitazone are independent on activation of STATs. Primary astrocytes were treated with 15d-PGJ2 (10 μM), rosiglitazone (20 μM), or IFN-γ (10 units ml⁻¹) for 1 h. Western blots were probed with pSTAT1, pSTAT3, or STAT1 antibody.

STATs (Fig. 3c). To further confirm this effect, we examined the effects of PPAR-γ agonists in murine BV2 microglial cells that are reported to lack PPAR-γ or express it at very low levels (31). We could not detect the PPAR-γ transcripts in BV2 cells, but we did observe an inhibitory effect of PPAR-γ agonists on STAT phosphorylation (Fig. 4a). Furthermore, PPAR-γ agonists reduced the transcription of not only IFN-γ-responsive genes but also inflammation-associated genes in BV2 cells (Fig. 4, b and c). Thus, we conclude that the inhibitory actions of 15d-PGJ2 and rosiglitazone on JAK-STAT signaling occur independently of PPAR-γ activation in brain.

We next focused our attention on how JAK activity might be inhibited independently of PPAR-γ in cells pretreated with 15d-PGJ2 and rosiglitazone. We considered the possibility that SOCS1 and SOCS3 could be involved in these events because they have been identified as negative regulators of JAK activation and as key regulators in the immune system (15). Interestingly, we found that 15d-PGJ2 and rosiglitazone rapidly induced the transcription of SOCS1 as well as SOCS3 in primary astrocytes and BV2 cells. Within 1–2 h after treatment of either 15d-PGJ2 or rosiglitazone, the transcript levels of SOCS1 and SOCS3 were significantly elevated in a dose-dependent fashion (Figs. 5 and 6). In agreement with JAK-STAT signaling events, overexpression of PPAR-γS112A failed to increase the ability of PPAR-γ agonists to induce SOCS1 and SOCS3 (Fig. 6d), indicating that elevation of SOCS transcripts occurs independently of PPAR-γ. To confirm the functional role of SOCS in brain inflammatory responses, we tested whether overexpression of SOCS1 or SOCS3 attenuated the phosphorylation of STATs in primary astrocytes. Levels of phosphorylated STAT1 and STAT3 were considerably attenuated in primary astrocytes transiently transfected with either SOCS1 or SOCS3 compared with vector (Fig. 6c). These results suggest that SOCS1 and SOCS3 may mediate the anti-inflammatory action of 15d-PGJ2 and rosiglitazone.

In subsequent experiments, we unexpectedly found that the phosphorylations of JAKs and STATs were also suppressed at 5 min after simultaneous exposure of cells to IFN-γ and either 15d-PGJ2 or rosiglitazone, implying that 15d-PGJ2 or rosiglitazone does not act only via the induction of SOCS1 and SOCS3 on JAK-STAT inflammatory signaling. Thus, we further examined the mechanism explaining the rapid inhibition of JAK-STAT activation following treatment with 15d-PGJ2 or rosiglitazone. Because SHP has been known to be an important negative regulator of JAK-STAT signaling, we investigated the involvement of SHP in anti-inflammatory action of 15d-PGJ2 and rosiglitazone. Interestingly, we observed that SHP2 was phosphorylated within 5 min after treatment of 15d-PGJ2 (Fig. 7). The activity of SHP2 has been reported to correlate with its phosphorylation, and the phosphorylated SHP2 can catalyze the tyrosine phosphorylation of JAKs, receptor, or other cellular proteins (32). In this regard, the activation of SHP2 may be part of a mechanism involved in inhibitory action of 15d-PGJ2 and rosiglitazone.

**DISCUSSION**

Some PPAR-γ agonists have been reported to effectively suppress inflammation and are thus promising potential therapeutic agents for chronic inflammatory diseases such as Alzheimer’s disease (33). Recent in vivo studies provide evidence for the therapeutic potential of PPAR-γ agonists in various inflammatory diseases (22, 23, 34). However, the mechanisms underlying the role of PPAR-γ agonists in ameliorating inflammation are poorly understood. Most of the information on the mechanism of action of PPAR-γ agonists is based on studies of NFκB activity. PPAR-γ agonists have been shown to inhibit multiple steps in the NFκB signaling pathway through covalent modi-
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