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The 15-Deoxy-Δ[12,14]-Prostaglandin J$_2$ Suppresses Monocyte Chemoattractant Protein-1 Expression in IFN-γ-Stimulated Astrocytes through Induction of MAPK Phosphatase-1[1] ⊘

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The 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$ Suppresses Monocyte Chemoattractant Protein-1 Expression in IFN-γ-Stimulated Astrocytes through Induction of MAPK Phosphatase-1[1]

Jee Hoon Lee, Joo Hong Woo, Sang Uk Woo, Kwang Soo Kim, Sang Myun Park, Eun-hye Joe, and Ilo Jou[2]

The 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) is a cyclopentene PG generated from PGD$_2$. It is an endogenous ligand of the peroxisome proliferator-activated receptor-γ that is primarily involved in adipocyte differentiation and lipid metabolism. Its anti-inflammatory actions have recently attracted considerable research attention, although the precise role and underlying mechanisms of these actions are largely unknown. In the present study, we focused on the inhibitory action of 15d-PGJ$_2$ on the chemokine MCP-1, which plays a key role in the initiation and progression of inflammation by recruiting inflammatory cells to lesion sites. We found that 15d-PGJ$_2$ suppressed MCP-1 transcription and protein secretion in IFN-γ-stimulated brain astrocytes. The inhibitory effects of 15d-PGJ$_2$ on MCP-1 resulted from its actions on the transcription factors, AP-1 and specificity protein-1, which play key roles in IFN-γ-induced MCP-1 expression in astrocytes. Of interest, the negative effects of 15d-PGJ$_2$ on AP-1/specificity protein-1 signaling and the resulting inhibition of MCP-1 expression were mediated by MAPK phosphatase (MKP)-1 activity, which was induced by 15d-PGJ$_2$ in a peroxisome proliferator-activated receptor-independent manner. Thus, our data demonstrate a novel anti-inflammatory mechanism of 15d-PGJ$_2$ involving MKP-1. Considering the importance of MCP-1 in inflammatory processes, our results suggest that 15d-PGJ$_2$ analogues may have therapeutic potential to attenuate inflammatory brain diseases by inducing MKP-1 expression. *The Journal of Immunology,* 2008, 181: 8642–8649.

Accumulating clinical and experimental evidence suggests that chronic inflammation contributes to the development of degenerative neuronal diseases, including multiple sclerosis, brain ischemia, and Alzheimer's disease (1–3). During inflammation, cytokines and chemokines are produced and released by astrocytes and microglia, resulting in the recruitment of resident glia and blood cells to lesion sites and the potentiation of inflammatory responses (4, 5). Among these chemokines, MCP-1 is particularly important and is primarily responsible for the initiation and progression of inflammatory responses by promoting migration and recruitment of inflammatory cells (6). Experimental evidence for this is provided by MCP-1-deficient mice, in which brain cytokine synthesis induced by LPS is reduced (7), and in MCP-1-knockout cells, which, when transplanted, exhibit little migration to lesion sites (8). In an animal model of prion disease, mice deficient in MCP-1 showed a delayed onset of inflammatory disease and an increase in survival time (9). MCP-1 overexpression is also associated with a variety of disease states, including atherosclerosis (10, 11) and ischemic stroke (12). Thus, pharmacological inhibition of MCP-1 expression could be exploited for the control of disease states in which monocyte-rich infiltrates play crucial roles.

The 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$),[3] a cyclopentene PG generated from arachidonate via PGD$_2$ (13), is an endogenous ligand of peroxisome proliferator-activated receptor (PPAR)-γ. Synthetic ligands of PPAR-γ are represented by the glitazone family of drugs (14), which are currently being used and are under development as antidiabetic drugs. In addition, growing evidence supports an anti-inflammatory role for PPAR-γ. The 15d-PGJ$_2$ can inhibit monocyte and macrophage activation and suppress the production of proinflammatory cytokines, such as IL-1β and TNF-α (15). In vivo studies also suggest that 15d-PGJ$_2$ is effective in various acute and chronic inflammation conditions, including arthritis (16), inflammatory bowel disease (17), experimental allergic encephalomyelitis (18), and cerebral ischemia (19).

PPAR-γ is a ligand-activated transcription factor that is primarily involved in lipid metabolism, but can also act as a transcriptional modulator to inhibit the transcription of proinflammatory mediators. Acting independently of PPAR, 15d-PGJ$_2$ blocks proinflammatory NF-κB signaling by directly interacting with signaling molecules (20). We have also reported that 15d-PGJ$_2$ acts in a receptor-independent manner to up-regulate the transcription of suppressor of cytokine signaling 1 and 3, resulting in suppression of JAK-STAT signals and downstream inflammatory mediator release (21).

In this study, we assessed the mechanism by which 15d-PGJ$_2$ inhibits MCP-1 expression in IFN-γ-stimulated brain astrocytes. We found that 15d-PGJ$_2$ suppresses MCP-1 transcription and protein secretion as a result of its actions on AP-1 and specificity

[3] Abbreviations used in this paper: 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGJ; ChIP, chromatin immunoprecipitation; MKP, MAPK phosphatase; PP, protein phosphatase; PPAR, peroxisome proliferator-activated receptor; siRNA, small interfering RNA; Sp, specificity protein.

FIGURE 1. The 15d-PGJ₂ reduces MCP-1 transcript levels and protein release in IFN-γ-stimulated astrocytes. *A*, Rat brain astrocytes were stimulated with 10 U/ml IFN-γ for 2 h in the absence or presence of the indicated dose of 15d-PGJ₂, and MCP-1 transcript levels were determined by RT-PCR. The graph represents the normalized intensities of MCP-1 bands against those of GAPDH bands. Data are presented as means ± SEM of three independent experiments. *B*, Cell supernatants collected 12 h after IFN-γ challenge in the absence or presence of the indicated dose of 15d-PGJ₂, and MCP-1 levels were measured using ELISA. Data are presented as means ± SEM of three independent experiments (*, $p < 0.05$; **, $p < 0.01$ vs IFN-γ group).
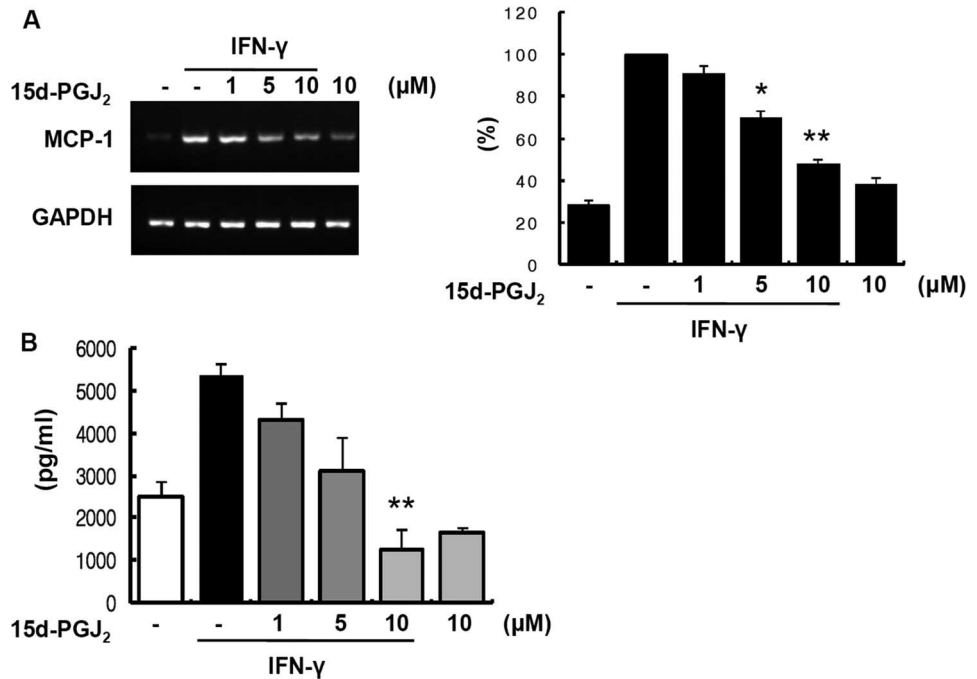
protein (Sp)-1, which play key roles in the transcriptional induction of MCP-1 by IFN-γ. Specifically, we show that 15d-PGJ₂ induced the PPAR-γ-independent expression of MAPK phosphatase (MKP)-1, which negatively regulates AP-1/Sp-1 signaling, resulting in the inhibition of MCP-1 expression. Our results demonstrate a novel anti-inflammatory mechanism of 15d-PGJ₂ involving MKP-1 that leads to the specific suppression of MCP-1 during inflammatory processes. Although further studies will be required to determine how 15d-PGJ₂ induces MKP-1 expression, our results suggest that 15d-PGJ₂ analogs have therapeutic potential to attenuate inflammatory brain diseases by inducing MKP-1 expression.

Materials and Methods

Reagents

IFN-γ was purchased from Calbiochem. Abs against phosphoserine, phospho-c-Jun, and phospho-JNK were purchased from Cell Signaling Technology. Anti-MKP-1 and anti-Sp-1 Abs were purchased from Santa Cruz Biotechnology. The 15d-PGJ₂ was purchased from BIOMOL. SP600125 and mithramycin were purchased from Sigma-Aldrich.

Cell culture

Primary microglia and astrocytes were cultured from the cerebral cortices of 1-day-old Sprague Dawley rats. Briefly, cortices were triturated into single cells in MEM containing 10% FBS (HyClone), plated onto 75-cm² T-flasks, and cultured for 2 wk. Following the removal of microglia, primary astrocytes were isolated by trypsinization.

RT-PCR analysis

Total RNA was isolated using easy-BLUE (Intron), and cDNA was prepared using Avian Myeloblastosis Virus reverse transcriptase (Promega), according to the manufacturer's instructions. PCR using 32 cycles were performed with the following primers (purchased from Bioneer): GAPDH, forward, 5′-TCCCTCAAGATTGTCAGCAA-3′ and reverse, 5′-AGATC CACAACG GATACATT-3′; MCP-1, forward, 5′-ATGCAGGTCTC TGTCACGCT-3′ and reverse, 5′-CTAGTTCTCTGTCATACTGG-3′; MKP-1, forward, 5′-AGGACACCACAAGGCAGAC-3′ and reverse, 5′-TGATGGGGCTTTGAAGGTAG-3′; and PPAR-γ, forward, 5′-TCTGAT TATGGGGTGAAACTC-3′ and reverse, 5′-TTTCTACTCTTTTTGTGG ATC-3′.

Western blot analysis

Cell lysates for Western blot analysis, prepared as previously described (22), were centrifuged for 20 min at 13,000 rpm at 4°C. Supernatant proteins were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. Membranes were incubated with primary Abs and peroxidase-conjugated secondary Abs, and bands were visualized using an ECL system (Sigma-Aldrich).

EMSA

EMSAs were performed, as previously described (22). Briefly, cells were harvested and suspended in hypotonic solution (9× packed-cell volume) with occasional gentle shaking, then centrifuged at 13,000 rpm for 20 min. The nuclear extracts thus obtained were incubated with reaction buffer and [γ-³²P]-labeled probe with or without a 20-fold excess of unlabeled probe. DNA-Protein complexes were separated on 6% polyacrylamide gels in Tris/glycine buffer, and dried gels were exposed to x-ray film. The oligonucleotide probe, 5′-CCTGACTCCACCTCTGGC-3′, specific for the AP-1/Sp-1 binding site of rat MCP-1 (positions −129 to −111), was purchased from Bioneer. For supershift experiments, protein extracts were incubated with 0.2 μg of phospho-c-Jun and Sp-1 Abs (Santa Cruz Biotechnology) for 1 h before the addition of γ-³²P-labeled probe.

ELISA

Primary microglia and astrocytes were seeded onto 6-well plates. After incubating cells with IFN-γ in the presence or absence of 15d-PGJ₂, 500 μl of cell-conditioned medium was collected and assayed using rat MCP-1 ELISA kits (BD Biosciences), according to the manufacturer's instructions.

Construction of luciferase reporter plasmids

Rat MCP-1 promoter fragments corresponding to positions −3554 to +76 (pGL3-MCP-1), −2355 to −81 (pGL3-κB-AP1-SP1), −2404 to −2062 (pGL3-κB), and −228 to +45 (pGL3-AP-1-Sp-1) were amplified by PCR using rat genomic DNA as a template with the following specific primer sets: −3554 to +76 fragment, forward, 5′-CTCAAAGGTGCTGCAG AGTTACTT-3′ and reverse, 5′-CATAGTGGTGGAGGAAGA-3′; forward, −2355 to −81 fragment, 5′-TGAGAGCTGCTTGGCTGTAA-3′ and reverse, 5′-TTGTAAGCCAGAGGGTGGAG-3′; forward, −2404 to −2062 fragment, 5′-GAGCTCAGACTATGCCTTTGT-3′ and reverse, 5′-GAGCCTGGGAGGTCACCA-3′; and −228 to +45 fragment, forward, 5′-CGCGGTTTCTCCCTTCTACT-3′ and reverse, 5′-GAGAGTTGGCT GGTTTCTGG-3′. Fragments were ligated into *Mlu*I and *Xho*I sites of the luciferase reporter plasmid, pGL3-Basic (Promega), yielding the reporter constructs. Site-directed mutagenesis was performed on the AP-1 or Sp-1
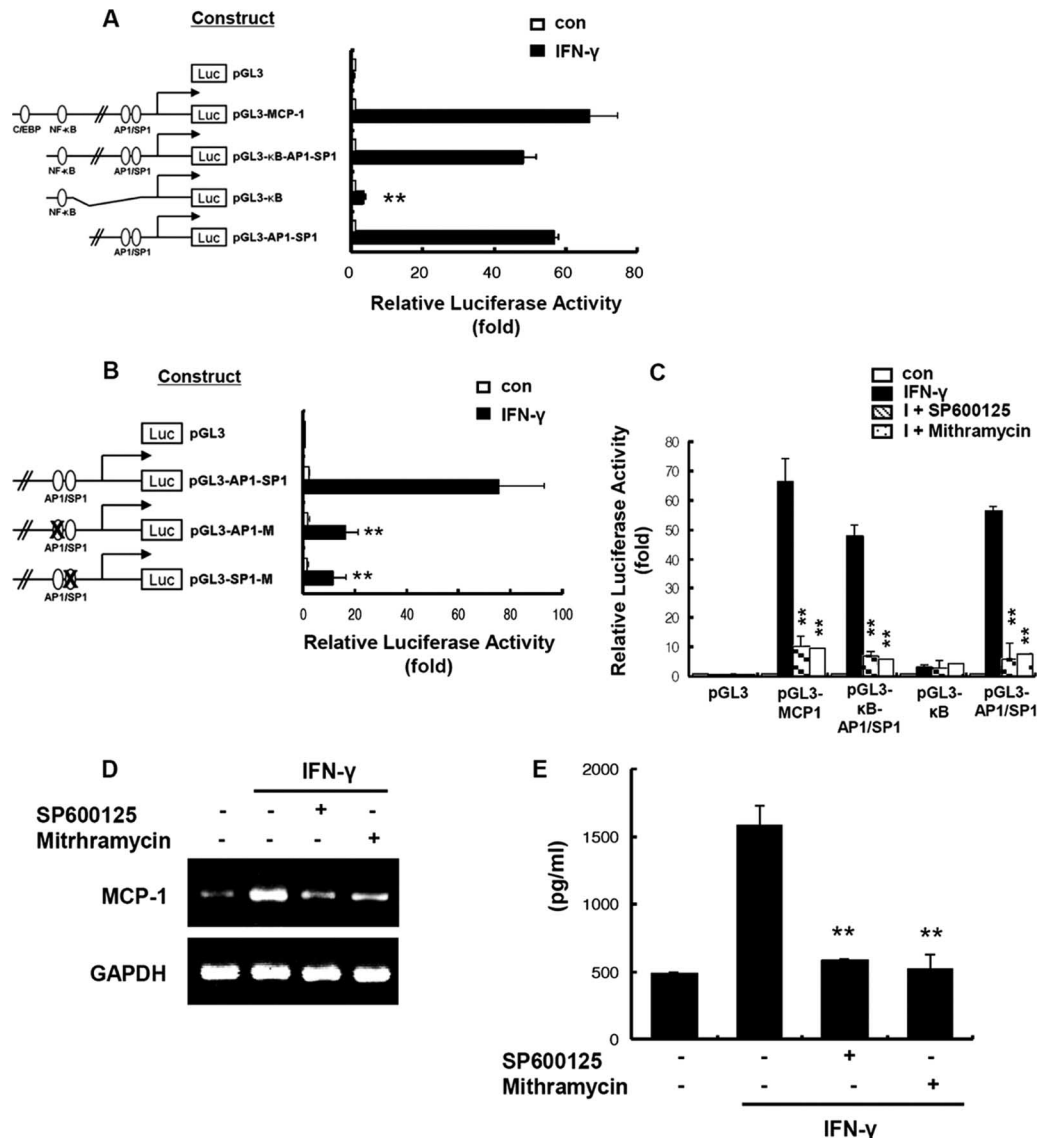
FIGURE 2. Experiments with deletion constructs and point mutations indicate the importance of AP-1 and Sp-1 in IFN-γ-stimulated MCP-1 promoter activation. *A* and *B*, Astrocytes were transiently transfected with the indicated 5′-deleted constructs of MCP-1 promoter (*A*) or AP-1- or Sp-1-mutated constructs (*B*) for 48 h. After stimulation with IFN-γ for 6 h, cells were harvested and luciferase activity was measured. Data are presented as means ± SEM for three independent experiments (**, $p < 0.01$ vs pGL3-MCP-1 group (*A*) and pGL3-AP-1/SP-1 group (*B*), respectively). *C*, Astrocytes were transfected with 5′-deleted constructs of the MCP-1 promoter used in *A* for 48 h and treated with SP600125 (JNK inhibitor) or mithramycin (Sp-1 inhibitor). The activity of MCP-1 promoter was determined by measuring luciferase activity. Data are presented as means ± SEM of three independent experiments. *D* and *E*, Astrocytes were stimulated with IFN-γ in the presence of SP60012 or mithramycin for 2 h. Total RNA was isolated and used in RT-PCR assays (*D*), and the cell supernatant was used for ELISA (*E*). Data are presented as means ± SEM of three independent experiments (**, $p < 0.01$ vs IFN-γ group).

binding sites in the proximal promoter region using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. The mutant AP-1 and Sp-1 constructs, pGL3-AP1-M and pGL3-SP1-M, were generated from the oligonucleotides 5′-CCTGACga CACCCTC-3′ and 5′-TCCACCagCTGGCT-3′, respectively, and used to examine site-specific mutations in AP-1 and SP-1 binding sites.

Transfection and luciferase assay

Transient transfections were performed in duplicate on 35-mm dishes using LipofectAMINE Plus reagents (Invitrogen) for 24 h. To normalize for variations in cell number and transfection efficiency, all cells were cotransfected with pCMV-β-galactosidase. Luciferase activity was measured using a luminometer (PerkinElmer Vitor (3)) and normalized to β-galactosidase activity (measured at A_{420}).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed, as described previously (23). Briefly, astrocytes were treated with IFN-γ and 15d-PGJ$_2$ before cross-linking for 10

min with 1% formaldehyde. Cross-linked adducts were resuspended and sonicated, yielding DNA fragments of 500–600 bp. Supernatants were collected and immunoprecipitated with c-Jun and Sp-1 Abs overnight at 4°C; protein-bound, immunoprecipitated DNA was recovered by phenol/chloroform extraction. Following extraction and precipitation, DNA was amplified by PCR using the primer pair, forward, 5′-TTCCACTCTC CATCGCTCAT-3′ and reverse, 5′-TCTGCATTTCTAGCGGCTCT-3′, spanning the promoter region of MCP-1 (position −155 to −73) containing AP-1/Sp-1 elements.

Synthesis and transfection of small interfering RNA (siRNA)

siRNA duplex oligonucleotides targeting MKP-1 (5′-CCAATTGTCCTA ACCACTT-3′) and PPAR-γ (5′-CGAAGAACCAUCCGAUUGAUU-3′) were chemically synthesized by Samchully Pharm. Confluent astrocyte and microglia cultures were transfected with siRNA oligonucleotides using Oligofectamine (Invitrogen), according to the manufacturer's instructions. All assays were performed at least 48 h after RNA transfection.

Phosphatase assay

Cell extracts were prepared by immunoprecipitation, as previously described (21). Briefly, lysates (300 μg) were incubated with anti-MKP-1 Ab (1 μg) at 4°C overnight, and precipitated with protein G-agarose beads (Upstate Biotechnology) for 2 h at 4°C. Phosphatase activities were measured in two ways. First, to measure MKP-1 activity, immunoprecipitated proteins were incubated with the substrate, *p*-nitrophenylphosphate (Sigma-Aldrich), for 4 h at 37°C and then analyzed spectrophotometrically at 405 nm. Second, to specifically measure p-JNK-linked MKP-1 activity, immunoprecipitated proteins were incubated with lysates from IFN-γ-stimulated astrocytes (as substrates). Bead-protein conjugates were then boiled, and the resulting elutes were analyzed by Western blotting using Abs against MKP-1 or p-JNK.

Statistical analysis

Differences among groups were determined using one-way ANOVA test. A *p* value of 0.05 was considered to be of significance. Values are presented as mean ± SEM.

Results

The 15d-PGJ$_2$-induced decrease in MCP-1 transcription and protein secretion in IFN-γ-activated brain astrocytes

To examine the inhibitory effects of 15d-PGJ$_2$ on MCP-1 expression, we stimulated cultured astrocytes with IFN-γ (10 U/ml) in the presence of the indicated doses of 15d-PGJ$_2$, and then measured MCP-1 transcripts and protein secretion into the medium using RT-PCR and ELISA, respectively. Treatment with 15d-PGJ$_2$ (1–10 μM) induced a dose-dependent reduction in MCP-1 transcript levels and protein release into the medium (Fig. 1). Similar inhibitory effects were observed with 15d-PGJ$_2$ pretreatment (data not shown). These results suggest that the inhibitory effect of 15d-PGJ$_2$ on MCP-1 expression is primarily mediated at the transcriptional level. Because 10 μM 15d-PGJ$_2$ produced a maximal inhibitory effect without causing cell death, this concentration was used in subsequent experiments.

Crucial role of AP-1 and Sp-1 signals in MCP-1 transcriptional induction

To determine the mechanisms involved in the transcriptional regulation of MCP-1 by 15d-PGJ$_2$, we first performed a deletion analysis using variably deleted rat MCP-1 promoter/luciferase reporter gene constructs (*Materials and Methods*), each of which contained the indicated transcription factor-binding sequences. Rat primary astrocytes were transfected with the MCP-1 promoter/luciferase reporter gene constructs, and luciferase activity was measured (Fig. 2A). The 3632-bp 5'-flanking region (−3554 to +76), containing C/EBP, NF-κB, AP-1, and Sp-1 sites, exhibited substantial luciferase activity in astrocytes following IFN-γ stimulation. pGL3-κB-AP1-Sp1 (−2355 to −81), a C/EBP-deleted construct, displayed a comparable response to IFN-γ. In contrast, astrocytes transfected with pGL3-κB (−2404 to −2062) containing NF-κB sites only showed no response to IFN-γ treatment, whereas cells transfected with pGL3-AP1-Sp1 (−228 to +45), containing both AP-1 and Sp-1 sites, exhibited a luciferase response comparable to that of the full-length promoter.

The essential roles of AP-1 and Sp-1 were further investigated by mutation analyses. Two reporter gene constructs, one containing a mutation at the AP-1 (pGL3-AP1-M) site and one mutated at the Sp-1 (pGL3-Sp1-M) site, exhibited a marked decrease in IFN-γ responsiveness (Fig. 2B). Moreover, inhibition of AP-1 or Sp-1 markedly inhibited IFN-γ-induced promoter activity (Fig. 2C), and decreased MCP-1 transcript levels (Fig. 2D) and protein release (Fig. 2E). SP600125 (10 μM) or mithramycin (1 μM) are used as pharmacological inhibitors of AP-1 and SP-1, respectively (24–26). These results confirmed the importance of AP-1 and Sp-1 in the transcriptional induction of MCP-1.
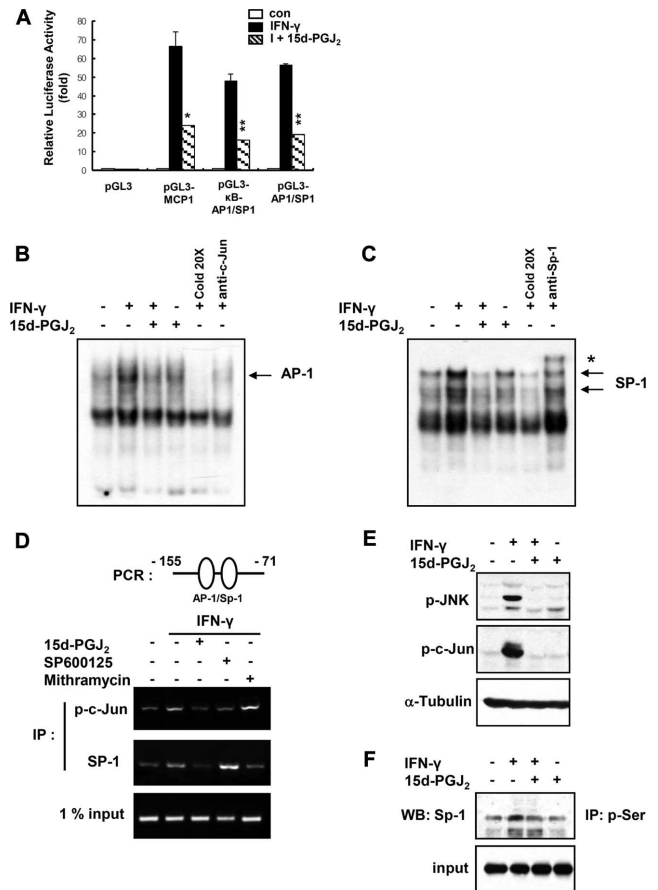

FIGURE 3. The 15d-PGJ$_2$-induced MCP-1 suppression is dependent on inhibition of JNK-AP-1 and Sp-1 signals. *A*, Astrocytes were transfected with 5'-deleted constructs of the MCP-1 promoter used in Fig. 2A for 48 h and treated with or without IFN-γ and 15d-PGJ$_2$. Activity of the MCP-1 promoter was determined by measuring luciferase activity. Data are presented as means ± SEM for three independent experiments (∗, *p* < 0.05, and ∗∗, *p* < 0.01 vs IFN-γ group). *B* and *C*, Astrocytes were stimulated with IFN-γ in the absence or presence of the 15d-PGJ$_2$, and NF-binding activities of AP-1 (*B*) and Sp-1 (*C*) were measured by EMSA using oligonucleotide probes specific for AP-1 of rat MCP-1 (−129 to −111). The arrows represent specific DNA-protein complexes, and asterisks indicate supershifted bands. *D*, Astrocytes were treated with 15d-PGJ$_2$, SP600125, or mithramycin and prepared for ChIP assays (see *Materials and Methods* for details). The input indicates control PCR and shows the amount of MCP-1 promoter DNA present in each sample before ChIP. *E* and *F*, Astrocytes were stimulated with IFN-γ for 2 h in the absence or presence of 15d-PGJ$_2$, and JNK and c-Jun activities were measured by Western blot analysis using phospho-JNK and phospho-c-Jun Abs (*E*). Cell lysates were immunoprecipitated with phosphoserine Abs, and then phosphorylation of Sp-1 was measured by Western blot analysis using an anti-Sp-1 Ab (*F*).

Molecular mechanisms of 15d-PGJ$_2$ action involving the inhibition of JNK/AP-1/SP-1

Next, we examined whether 15d-PGJ$_2$ could reduce IFN-γ-induced luciferase activity in cells transfected with one of the four MCP-1 promoter deletion constructs. The luciferase activity of astrocytes transfected with constructs containing AP-1/Sp-1 sites was reduced by treatment with 15d-PGJ$_2$ (Fig. 3A). To further evaluate the effect of 15d-PGJ$_2$, we performed EMSA using oligonucleotide probes specific for the AP-1 and Sp-1 binding sites of rat MCP-1, and found that 15d-PGJ$_2$ effectively inhibited the binding activity of transcription factors at these sites. A supershift assay using Abs against AP-1 and SP-1 confirmed that the protein complexes bound to these probes were, in fact, AP-1 and Sp-1
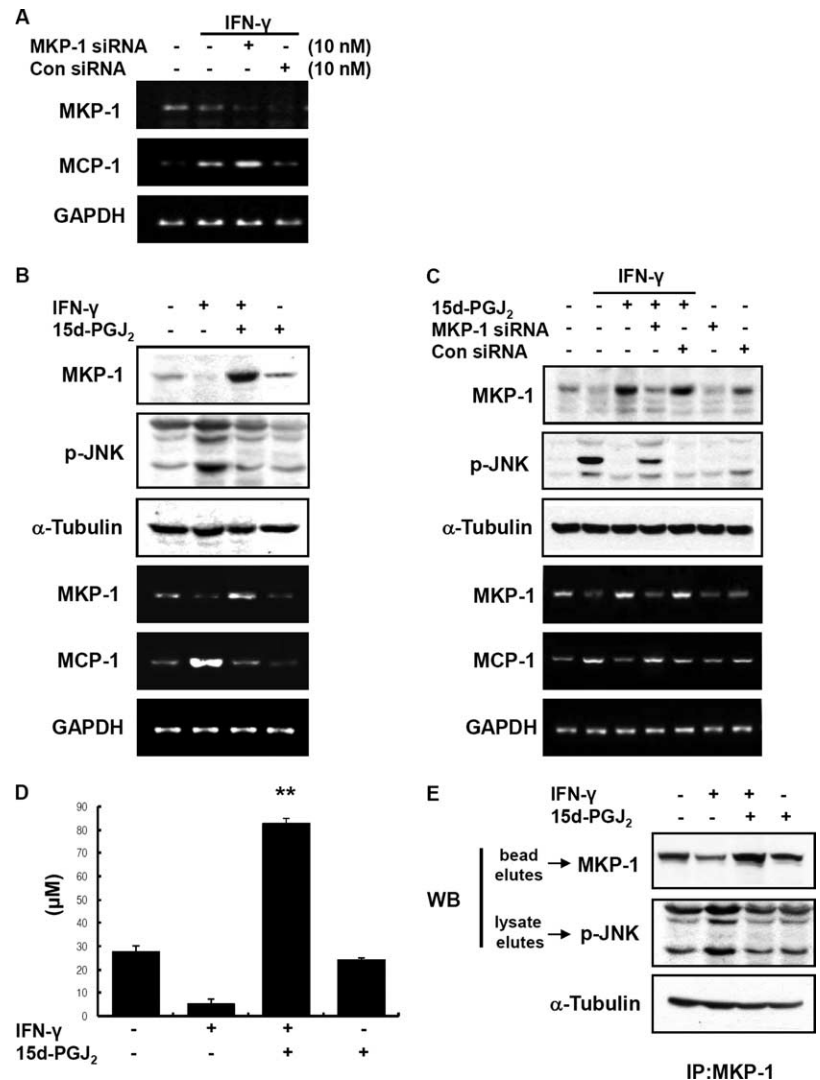
FIGURE 4. The 15d-PGJ$_2$ suppresses MCP-1 expression by inducing MKP-1 expression and phosphatase activity. *A*, Rat astrocytes were transfected with a MKP-1-specific siRNA duplex or a nonsilencing control siRNA using Oligofectamine. At 48 h after transfection, cells were stimulated with IFN-γ for 2 h. MKP-1 and MCP-1 transcript levels were determined by RT-PCR analysis. *B*, 15d-PGJ$_2$-treated astrocytes were stimulated with IFN-γ for 2 h and then analyzed by Western blotting and RT-PCR. *C*, Cells were transfected with a MKP-1-specific siRNA duplex or a nonsilencing control siRNA using Oligofectamine. At 48 h after transfection, cells were stimulated with IFN-γ for 2 h in the presence or absence of 15d-PGJ$_2$, and then analyzed by Western blotting and RT-PCR. *D* and *E*, Cell lysates immunoprecipitated with an anti-MKP-1 Ab were incubated with *p*-nitrophenylphosphate for 4 h and then analyzed spectrophotometrically at 405 nm (*D*), or incubated with lysates from IFN-γ-activated astrocytes, followed by Western blot analysis of elute (*E*). Data are presented as means ± SEM of three independent experiments (∗∗, $p < 0.01$ vs IFN-γ group).

(Fig. 3, *B* and *C*). These results were further confirmed in ChIP assays using c-Jun and Sp-1 Abs. The amount of AP-1/Sp-1 promoter DNA present in the immunoprecipitated chromatin fractions was analyzed by PCR using primer pairs derived from the AP-1/Sp-1 binding sites (−155 to −73) of rat MCP-1. As shown in Fig. 3*D*, 15d-PGJ$_2$ reduced the binding of both phospho-c-*jun* and Sp-1 at the promoter, whereas SP600125 and mithramycin selectively reduced the binding of p-c-Jun and Sp-1, respectively (Fig. 3*D*).

Because AP-1 is comprised of JNK-phosphorylated c-Jun homo- or heterodimers (27), we examined whether 15d-PGJ$_2$ acted at the level of JNK phosphorylation. JNK and c-Jun phosphorylation in response to IFN-γ stimulation was evident within 2 h, an effect that was markedly suppressed by 15d-PGJ$_2$ (Fig. 3*E*). Because Sp-1 is similarly activated by a phosphorylation event, we examined whether 15d-PGJ$_2$ also regulates Sp-1 activity at this level. Because Sp-1 is activated via phosphorylation at serine residues, astrocyte extracts were immunoprecipitated with a phosphoserine Ab and Sp-1 serine phosphorylation was analyzed by Western blot analysis. Consistent with the AP-1 results, IFN-γ-induced phosphorylation of Sp-1 was markedly suppressed by 15d-PGJ$_2$ (Fig. 3*F*).

MKP-1 as a mediator of 15d-PGJ$_2$-induced suppression of JNK/AP-1/MCP-1

Based on the above results, we first focused on the phosphorylation of JNK, which is principally regulated by MKP-1 (28). In MKP-1

knockdown experiments, we found that the IFN-γ-induced increase in MCP-1 transcription was further enhanced in astrocytes expressing MKP-1-specific siRNA (Fig. 4*A*). Therefore, we examined whether 15d-PGJ$_2$ regulated the transcription and/or activity of MKP-1, and consequently, JNK phosphorylation and MCP-1 expression in IFN-γ-stimulated astrocytes. Western blot analyses and RT-PCR showed that MKP-1 transcription and protein expression were induced within 2 h in the presence of 15d-PGJ$_2$. Increased MKP-1 expression was significantly correlated with a decrease in JNK phosphorylation and suppression of MCP-1 expression (Fig. 4*B*). These effects of 15d-PGJ$_2$ on JNK phosphorylation and MCP-1 expression were reversed by siRNA-mediated MKP-1 knockdown, indicating that MKP-1 most likely mediates these effects (Fig. 4*C*).

Additionally, MKP-1 phosphatase activity was markedly increased with 15d-PGJ$_2$ treatment (Fig. 4*D*). These results were confirmed in a more specific phosphatase assay using lysates containing excess phospho-JNK as a MKP-1 substrate instead of *p*-nitrophenylphosphate (see *Materials and Methods* for details) (Fig. 4*E*). Taken together, these results clearly indicate that 15d-PGJ$_2$ regulates MKP-1 transcript levels and enzymatic activity.

PPAR-γ independence of 15d-PGJ$_2$ inhibitory actions

Some studies have shown that 15d-PGJ$_2$ regulates inflammatory responses in a PPAR-γ-independent manner (21, 29, 30). To determine whether the actions of 15d-PGJ$_2$ on MKP-1 are receptor
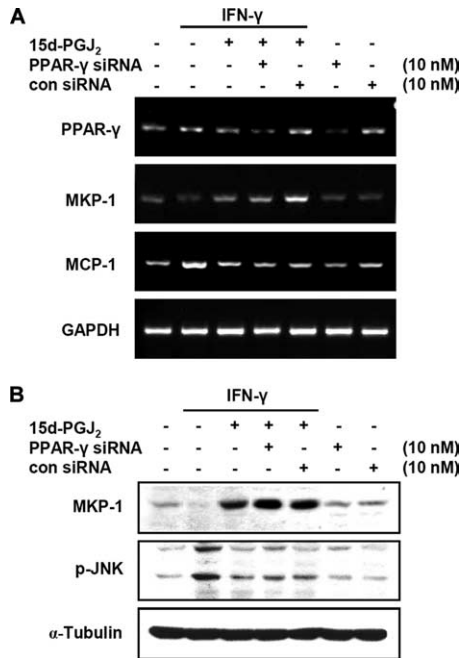
FIGURE 5. PPAR-γ is not required for the anti-inflammatory actions of 15d-PGJ$_2$. A and B, Rat astrocytes were transfected with a PPAR-γ-specific siRNA duplex or a nonsilencing control siRNA using Oligofectamine. At 48 h after transfection, cells were stimulated with IFN-γ for 2 h in the presence or absence of 15d-PGJ$_2$. MKP-1, MCP-1, and PPAR-γ transcript levels were determined by RT-PCR analysis (A), and MKP-1 protein levels and JNK phosphorylation were analyzed by Western blotting (B).

mediated, we used a siRNA-mediated PPAR-γ silencing approach. Astrocytes transfected with PPAR-γ-specific or control siRNA for 72 h were stimulated with IFN-γ in the presence or absence of 15d-PGJ$_2$ for 2 h, then analyzed by Western blotting and RT-PCR. As shown in Fig. 5, 15d-PGJ$_2$-induced MKP-1 expression was not reversed by siRNA-mediated PPAR knockdown. MCP-1 expression levels were similarly unaffected, suggesting that the action of 15d-PGJ$_2$ occurred in receptor-independent manner (Fig. 5).

The inhibitory effects of 15d-PGJ$_2$ in brain microglia

Next, we tested whether the 15d-PGJ$_2$ inhibitory effects and pathways elucidated in astrocytes were applicable to microglia, another major immune effector cell in the CNS. Cultured microglia were stimulated with IFN-γ (10 U/ml) in the absence or presence of 15d-PGJ$_2$, and MCP-1 transcripts and protein secretion into the medium were measured using RT-PCR and ELISA, respectively. Consistent with the results obtained from astrocytes, 15d-PGJ$_2$ reduced the level of MCP-1 transcripts and suppressed MCP-1 protein release (Fig. 6, A and B). These effects were attenuated by specific inhibitors of JNK and Sp-1 (Fig. 6, C and D). Moreover, 15d-PGJ$_2$ also inhibited the phosphorylation of JNK/c-*jun* and Sp-1 (Fig. 6, E and F) in a PPAR-γ-independent manner (Fig. 6G). Collectively, our results indicate that the MKP-1-mediated inhibitory effects of 15d-PGJ$_2$ on MCP-1 are common to both brain microglia and astrocytes.

Discussion

The anti-inflammatory effects of 15d-PGJ$_2$ have attracted considerable recent research interest; however, the underlying mechanisms remain largely unknown. In the present study, we showed that the anti-inflammatory actions of 15d-PGJ$_2$ on MCP-1 result from its effects on MKP-1 expression and activity. In particular, this is the first report to show that the anti-inflammatory effect of

15d-PGJ$_2$ is due, in part, to its actions on the protein phosphatase, MKP-1, effects that are exerted in PPAR-γ receptor-independent manner.

We showed that 15d-PGJ$_2$ inhibited MCP-1 transcriptional and protein release. The MCP-1 promoter contains binding sites for transcription factors, including STATs, NF-κB, and AP-1/Sp-1, that play key roles in its transcriptional regulation. Our experiments using 5′-deleted promoters revealed that MCP-1 expression was strongly dependent on the AP-1/Sp-1 signal. Although several reports have highlighted the role of NF-κB in MCP-1 induction (31–34), critical roles for AP-1/Sp-1 have also been reported. In endothelial cells, AP-1 plays a key role in the molecular mechanisms underlying induction of MCP-1 by vascular endothelial growth factor (35). Inhibition of AP-1 abrogates MCP-1 expression by delaying proteosomal degradation of c-Jun in kidney mesangial cells (36). In addition, Sp-1 binding is vital for promoter assembly and activation of the MCP-1 gene by TNF-α in fibroblasts (37). Similarly, CpG DNA acting on TLR9 activates CD34$^+$ cells to express IL-8 through MAPK-dependent and NF-κB-independent pathways (38). AP-1 additionally acts as a key transcription factor in IFN-γ-induced expression of matrix metalloproteinase-3 (MMP-3), and PPAR activators differentially regulate expression through mediation of AP-1 signaling (22).

The transcription factor c-Jun is downstream of JNK. Various stimuli trigger JNK phosphorylation, leading to the activation of c-Jun/AP-1. JNK activation is followed by induction of MKP-1, which in turn down-regulates JNK activity. MKP-1, a dual specificity (Ser/Thr or Thr/Tyr) protein phosphatase, is responsible for inactivation of JNK/p38 (28). Knockout (39) or ectopic expression (40) of MKP-1 dramatically augments or inhibits TNF-α and IL-6 production in innate immune cells, implying that MKP-1 controls MAPK-dependent inflammation during the innate immune response. However, the precise roles of MKP-1 in inflammatory processes remain to be established. Our results show that MKP-1 expression and phosphatase activity, potentiated by 15d-PGJ$_2$, suppress the release of MCP-1 in IFN-γ-activated brain glial cells. The endocannabinoid, anandamide, triggers MKP-1 expression in microglial cells, thus protecting neurons during CNS inflammation (41). In our experiments, 15d-PGJ$_2$ alone did not significantly induce MKP-1 expression or phosphatase activity, but potentiated that induced by IFN-γ, implying posttranscriptional rather than transcriptional regulation. Moreover, MKP-1 induction by 15d-PGJ$_2$ is more prominent at the protein than the mRNA level. MKP-1 stability or degradation could be involved. Although these mechanisms have not been demonstrated, a previous report that dexamethasone enhanced the stability of MKP-1 by reducing MKP-1 ubiquitination (42, 43) supports this possibility. Ongoing experiments in our laboratory are designed to determine whether this is the case for 15d-PGJ$_2$.

Sp-1 acts as a key regulator of MCP-1 gene expression, along with AP-1. However, the mechanisms by which PPAR activators modulate expression or activity are currently unclear. In addition to MKP-1, active phosphorylated Sp-1 is regulated by the Ser/Thr protein phosphatases, protein phosphatase (PP) 1 or PP2A, via a negative feedback loop (44). Our data demonstrated a crucial role for Sp-1 in MCP-1 transcription and the resultant effects of 15d-PGJ$_2$, but the precise mechanism by which 15d-PGJ$_2$ suppresses Sp-1 phosphorylation remains to be resolved. We cannot rule out the involvement of PP1 or PP2A upstream of Sp-1.

Whether 15d-PGJ$_2$ is truly a PPAR-γ ligand is a matter of some controversy. In this study, suppression of MCP-1 by 15d-PGJ$_2$ occurred in a receptor-independent manner. We performed the same experiments with the synthetic PPAR-γ ligands, ciglitazone and rosiglitazone. Although they did reduce the levels of MCP-1
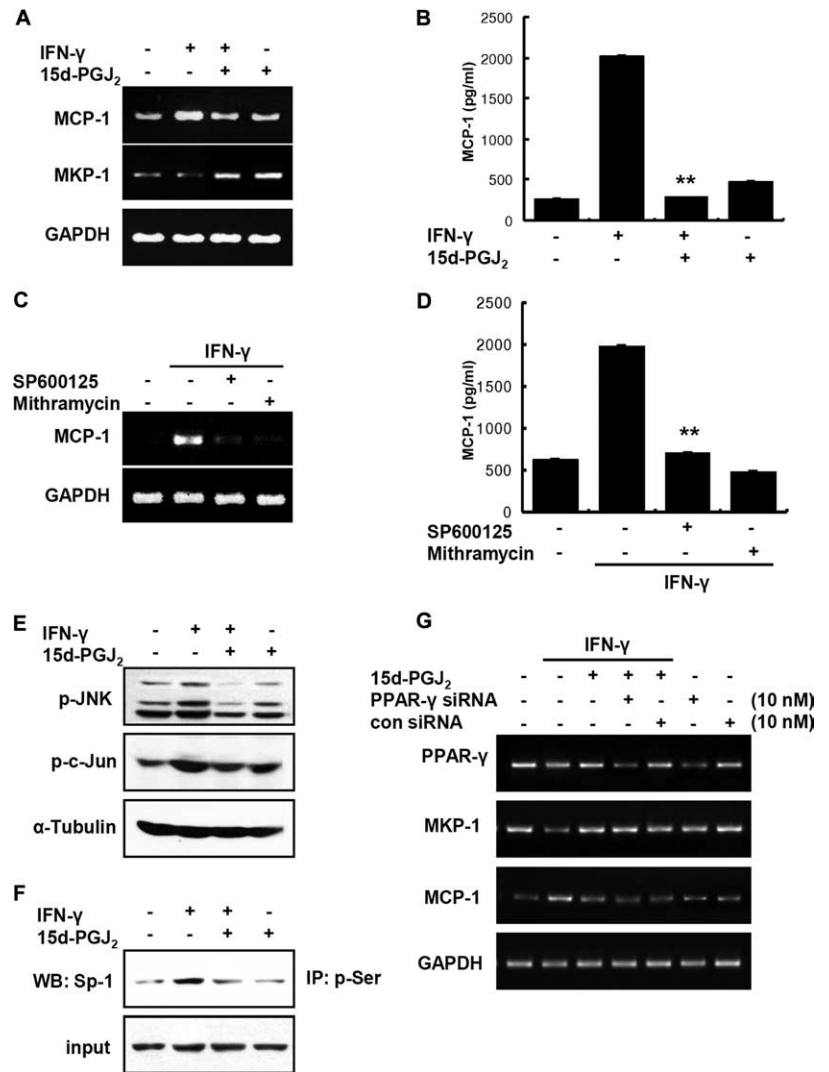
FIGURE 6. The 15d-PGJ₂ suppresses MCP-1 expression by inducing MKP-1 expression in brain microglia. *A* and *B*, Primary microglia were stimulated with 10 U/ml IFN-γ for 2 or 12 h in the absence or presence of 15d-PGJ₂, and MCP-1 transcript levels and protein release were examined by RT-PCR (*A*) and ELISA (*B*). Data are presented as means ± SEM of two independent experiments. *C* and *D*, Microglia were stimulated with IFN-γ in the presence of SP60012 or mithramycin for 2 h for RT-PCR (*C*), or 12 h for ELISA (*D*) (∗∗, $p < 0.01$ vs IFN-γ group). *E* and *F*, 15d-PGJ₂-treated microglia were stimulated with IFN-γ for 2 h. JNK and c-Jun phosphorylation levels were determined by Western blot analysis (*E*), and Sp-1 phosphorylation level was analyzed using an immunoprecipitation method (*F*). *G*, Rat microglia were transfected with a PPAR-γ-specific siRNA duplex or a nonsilencing control siRNA using Oligofectamine. At 48 h after transfection, cells were stimulated with IFN-γ for 2 h in the presence or absence of 15d-PGJ₂. MKP-1, MCP-1, and PPAR-γ transcript levels were determined by RT-PCR analysis.

transcripts and protein, they did not induce MKP-1 expression. Moreover, the inhibitory actions of these glitazones on MCP-1 were PPAR-γ dependent (data not shown), suggesting that these agents act through a distinct mechanism.

Inflammation and innate immune responses occur ubiquitously throughout the body, and reflect a complex series of interactions between diverse inflammatory cells and environment, comprised of cytokines, chemokines, and other mediators. Thus, controlling inflammation for therapeutic purposes requires a strategic approach. In this respect, targets for next-generation anti-inflammatory therapy might be developed in a target-specific manner. In this study, we provide important insights into the regulation of MCP-1 by 15d-PGJ₂ via mediation of MKP-1, demonstrating a dynamic interplay between 15d-PGJ₂ and MKP-1 in the regulation of MCP-1 expression. Despite the therapeutic potential of glitazones as anti-inflammatory drugs, the diverse mechanism-dependent adverse actions of these agents, ranging from metabolic disruption to cell death, limit their therapeutic usefulness. One clue to solving this problem may be provided by our results, which demonstrate a novel anti-MCP-1 action by 15d-PGJ₂. Importantly, this action occurred in a PPAR receptor-independent manner through a mechanism distinct from that of glitazone drugs. Moreover, recent reports revealing novel anti-inflammatory mechanisms of cannabinoids acting as PPAR ligands suggest complex interplay between 15d-PGJ₂, PPARs, and cannabinoids (45, 46). Taken to-

gether, our data shed a possibility to potentiate anti-inflammatory capacity, minimizing adverse reactions in brain inflammation.

Disclosures

The authors have no financial conflict of interest.

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