Gnetin C suppresses double-stranded RNA-induced C-C motif chemokine ligand 2 (CCL2) and CCL5 production by inhibiting Toll-like receptor 3 signaling pathway

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ABSTRACT

The innate immune system is a prerequisite for biophylactic ability, but its dysregulation can cause inflammatory and autoimmune diseases. To determine a safe method of controlling inflammatory reactions in the brain, we examined the effects of gnetin C, a natural resveratrol dimer, on C-C motif chemokine ligand 2 (CCL2) and CCL5 (pro-inflammatory chemokines) production observed after treatment with polyinosinic–polycytidylic acid [poly IC; a synthetic analog of dsRNA as a Toll-like receptor 3 (TRL3) ligand, 30 μg/mL] in cultured human astrocytoma U373MG and neuroblastoma SH-SY5Y cells. The addition of gnetin C (10 μM) to the media moderately reduced the CCL2 production and markedly suppressed CCL5 production in both cells. In the TLR3–interferon (IFN)-β–phosphorylated-STAT1 (signal transducer and activator of transcription protein 1)–RIG-I (retinoic acid-inducible gene-I) pathway that mediates CCL2 and CCL5 production, gnetin C first inhibits IFN-β expression in SH-SY5Y cells and primarily inhibits STAT1 phosphorylation in U373MG cells. In any case, gnetin C attenuated the dsRNA-activated TLR3 signaling resulting in CCL2 and CCL5 production, thus, may be useful for controlling TLR3-mediated inflammation in the brain.

Innate immune responses play a key role in controlling infection of the brain by pathogens including viral nucleic acid patterns (14, 23). Viral nucleic acids are recognized by pattern recognition receptors, which include the following three major families: Toll-like receptors (TLRs), Nod-like receptors, and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). In the central nervous system (CNS), parenchymal microglia as well as astrocytes, oligodendrocytes, and endothelial cells, and even neurons express functional levels of some of these receptors (2, 18, 28). The engagement of such receptors results in the induction of specific pathways and the release of specific cytokines that play a role in resolving the injury (14). Upon infection, viral double-stranded (ds)RNA activates TLR3 and RIG-I, leading to production and secretion of chemokines, such as C-C motif chemokine ligand 2 [CCL2, also known as monocyte chemoattractant protein-1 (MCP-1)] and CCL5 [also known as regulated and normal T-cell expressed and secreted (RANTES)] (24). These chemokines enhance migration of circulating leukocytes, such as monocytes and lymphocytes, through the blood–brain barrier (BBB). These CNS-infiltrating immune cells play cytoprotective roles together with CNS-resident cells, such as microglia and astrocytes. Meanwhile, they can also contribute to
Universal SYBR® Green Supermix solution for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Oligonucleotide primers for qPCR were custom synthesized by Fasmac/Greiner Japan (Atsugi, Japan). A biocinchonic acid (BCA™) protein assay kit from Pierce™/Thermo Scientific Inc. (Waltham, MA, USA) was also used. Polyvinylidene fluoride (PVDF) membranes and the Luminata™ Crescendo Western horseradish peroxidase (HRP) substrate were from Millipore Corporation (Billerica, MA, USA). Mouse monoclonal IgG against phosphorylated signal transducer and activator of transcription protein 1 (STAT1) (pY701.4A, #sc-136229) and rabbit polyclonal IgG against STAT1 (p84/p91, E-23, #sc-346) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). An antibody against β-actin [ACTN (C4), mouse monoclonal, #ab3280] was obtained from Abcam plc (Cambridge, UK). Anti-mouse (#7076) and anti-rabbit (#7074) IgG and HRP-linked antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture. Human astrocytoma U373MG cells (ECACC No. 89081403) and human neuroblastoma SH-SY5Y cells (ECACC No. 94030304) were purchased from the European Collection of Authenticated Cell Cultures (Porton Down, UK) and cultured in DMEM (for U373MG) and DMEM/F12 (for SH-SY5Y) containing GlutaMAX™-I supplemented with 10% FBS (22, 32). U373MG and SH-SY5Y cells have properties of astrocytes (7) and catecholaminergic neurons (30), respectively. Once the cells reached 80% confluence, they were used for experimental purposes; immediately, the medium was replaced with fresh Advanced DMEM (for U373MG) or Advanced DMEM/F12 (for SH-SY5Y) containing GlutaMAX™-I supplemented with 3% FBS. In experiments with gnetin C, cells were pretreated with this biophenol for 1 h prior to the addition of poly IC. Gnetin C was dissolved in DMSO; the maximum final concentration of DMSO in the culture medium was 0.1%. Meanwhile, control cells were treated with the vehicle alone.

ELISA. Aliquots of cell culture supernatants were stored at −80°C until further use. Levels of CCL2 and CCL5 in the cell-conditioned medium were determined using ELISA kits and an iMark/Bio-Rad microplate reader.

RT-qPCR. Total RNA was extracted from cells, and
single-stranded cDNA was synthesized using the iScript™ Advanced cDNA synthesis kit for RT-qPCR (5). A CFX96™ Real-Time PCR System (Bio-Rad) was used for quantitative analyses of 18S rRNA and mRNAs of the genes listed in Table 1. Primers were used at 500 nM. Amplification was performed in SsoAdvanced™ Universal SYBR® Green Supermix solution, and the following reaction conditions were used: 95°C for 30 s; 40 cycles of 95°C for 5 s, 55°C for 10 s, and 60°C for 15 s; and an increase from 65°C to 95°C (at 0.5°C/5 s) for the melting step. Data were analyzed with CFX Manager™ Version 2.1 software.

**Western blot analysis.** Cells cultured in a 35-mm-diameter dish were washed twice with ice-cold 20 mM phosphate-buffered saline (PBS, pH 7.4). Cells were then lysed in 50 μL hypotonic cell lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, and 0.5% NP-40] containing 0.2% proteinase inhibitors, and immediately placed at −80°C until use. Thawed and vortex-mixed lysates were centrifuged at 10,000×g for 10 min at 4°C, and the supernatants were transferred to fresh tubes. After the BCA protein assay, aliquots of the solutions containing 10 μg of the protein were mixed with 6× loading buffer and boiled for 6 min. The samples were subjected to electrophoresis on 7.5% SDS-polyacrylamide gels, and proteins were transferred to PVDF membranes. The membranes were blocked by incubation in Tris-buffered saline with Tween 20 (TBS-T, pH 7.4; 50 mM Tris-HCl, 250 mM NaCl, and 0.1% Tween-20) containing 3% nonfat dry milk (blocking buffer) for 1 h at 25°C. The membranes were then incubated with a mouse anti-pSTAT1, or rabbit anti-STAT1 antibody (1:1000 in TBS-T containing 3% BSA) overnight at 4°C, followed by a third incubation with the appropriate secondary antibodies (HRP-conjugated anti-mouse, or anti-rabbit IgG, 1:10,000 in blocking buffer) for 1 h at 25°C. The immunoreactive bands were detected using an enhanced chemiluminescence (ECL) detection system. The same blot was then reprobed with an anti-β-actin antibody (1:5000).

**Densitometric quantification.** Integrated optical density of the immunoreactive band was quantified using ImageJ 1.49i software (National Institute of Health, Bethesda, MD, USA) and was normalized to the β-actin band density.

**Statistics.** Values are expressed as mean ± SD, and statistical significance was analyzed using unpaired two-tailed t-test or one-way analysis of variance. Significance was considered at a probability (P) value of <0.05.

**RESULTS**

**Gnetin C reduces poly IC-induced CCL2 and CCL5 production**

First, we examined whether gnetin C has suppressive activity against CCL2 and CCL5 production that was enhanced by dsRNA in cultured human astrocytoma U373MG cells and neuroblastoma SH-SY5Y cells (Fig. 1). The cells were pretreated with gnetin C at the indicated concentrations for 1 h and then treated with 30 μg/mL poly IC for 24 h. Control cells were treated with DMSO during the experiment. In both U373MG and SH-SY5Y cells, gnetin C suppressed poly IC-induced CCL2 and CCL5 secretion by 79% and 96%, respectively. In SH-SY5Y cells, gnetin C at 10 μM moderately lowered the CCL2 and CCL5 secretion by 35% and 53%, respectively. Because the 10-μM dose of gnetin C did not affect cell viability (19), we used this dose for the following experiments.

| Table 1 Oligonucleotide primers used for real-time quantitative PCR |
|-------------------------|------------------------|------------------------|
| cDNA        | Forward sequence (5’ to 3’) | Reverse sequence (5’ to 3’) |
| IFN-β       | CCTGTGGCAATTGAATGGGAGGC | CCAGGCACAGTGACTGCTCTTT |
| RIG-I        | GTGCAAAAGCCCTTGGCAGTG | TTGGCTTGGGATGTGGTCTACTC |
| CCL2        | AAACCTGAAGCTCGCAGTCTCG | ATTTCTGGGTGGTGGAAGTGA |
| CCL5        | CTACTCGGAGGCTTAAAGCAGGAA | GGAGGTTGAGACCGCGGAAGC |
| 18S rRNA    | ACTCAACACGGGAAAACCTCA | AACCCAGAAAATGCTCACC |
In SH-SY5Y cells, gnetin C first inhibited IFN-β expression, whereas in U373MG cells, it first inhibited STAT1 phosphorylation. Based on the aforementioned results, we performed step-by-step investigation of the suppressive effect of gnetin C (10 μM) on the poly IC (30 μg/mL)-activated TLR3 signaling pathway in U373MG and SH-SY5Y cells (Figs. 4 and 5). In SH-SY5Y cells, gnetin C first inhibited poly IC-enhanced IFN-β mRNA expression, but in U373MG cells, gnetin C did not affect this expression step (Fig. 4). In both cells, gnetin C markedly inhibited the poly IC-induced RIG-I mRNA; however, in SH-SY5Y cells, gnetin C moderately inhibited CCL2 and CCL5 production. Because IFN-β is known to activate STAT1 phosphorylation in U373MG cells (8), next, we examined whether gnetin C affects STAT1 phosphorylation (Fig. 5). Gnetin C mostly inhibited the poly IC-induced RIG-I mRNA expression. The concentration dependence of poly IC (up to 100 μg/mL)-induced IFN-β or RIG-I expression was also confirmed at the peak time (Fig. 3), and we used the 30-μg/mL dose of poly IC in the following experiments.

**Time- and concentration-dependent poly IC-induced mRNA expression**

To set our next experimental conditions, we confirmed the time courses of representative mRNA expression in poly IC-induced TLR3 signaling (Fig. 2). In U373MG cells, IFN-β, RIG-I, CCL2, and CCL5 expression reached its peak at 4, 8, 16, or 24 h, respectively; in SH-SY5Y cells, the expression reached its peak at 4 (and another 16), 16, 16, and 24 h or more, respectively. These phase-shift patterns apparently confirmed the existence of the poly IC–TLR3–IFN-β–RIG-I–CCL2/5 axis (6) not only in U373MG cells but also in SH-SY5Y cells. The concentration dependence of poly IC (up to 100 μg/mL)-induced IFN-β or RIG-I expression was also confirmed at the peak time (Fig. 3), and we used the 30-μg/mL dose of poly IC in the following experiments.
Fig. 2  Time course of IFN-β, RIG-I, CCL2, or CCL5 mRNA expression in U373MG or SH-SY5Y cells stimulated with 30 μg/mL poly IC. mRNA expression was analyzed by real-time quantitative PCR and was normalized to 18S rRNA levels (relative value). The mean ± SD of three experiments is shown. * denotes $P < 0.05$ vs. control (0 h).
astrocytoma cells (22) and in human mesangial cells (6), although findings have suggested that the poly IC-induced TLR3–NF-κB/IRF3–CCL5 pathway is active in a case of human biliary epithelial cells (21). In this study, we confirmed that the poly IC-induced TLR3–IFN-β–pSTAT1–RIG-I pathway involved in upstream of CCL2/5 production is active not only in astrocytoma cells but also in neuroblastoma cells. We found that gnetin C first inhibits IFN-β expression in neuroblastoma cells and primarily inhibits STAT1 phosphorylation in astrocytoma cells.

A high level of RIG-I expression has been observed in the poly IC-stimulated astrocytoma cells in culture and resident astrocytes in the mouse brain injected with poly IC (31). RIG-I is one of the IFN-stimulated genes (ISGs). STAT1 is a transcription factor that mediates ISG mRNA expression involved in downstream of TLR3–IFN-β signaling. We have previously shown that, in astrocytoma cells, inhibition of STAT1 phosphorylation can decrease RIG-I expression (9). In this case, gnetin C markedly inhibited poly IC-induced pSTAT1 at initial stage and also completely suppressed RIG-I and CCL2/5 expression in astrocytoma cells.

DISCUSSION

In the present study, we examined a trans-resveratrol dimer, gnetin C, as a possible means of controlling pro-inflammatory chemokine production induced by a TLR3 ligand. This is the first study to state that gnetin C suppressed CCL2 and CCL5 production in both human astrocytoma U373MG and human neuroblastoma SH-SY5Y cells treated with poly IC which mimics a viral dsRNA.

We have previously reported that poly IC activates TLR3 signaling (8, 10) and enhances production of chemokines including CCL5 via the TLR3–IFN-β–pSTAT1–RIG-I signaling pathway in human astrocytoma cells (22) and in human mesangial cells (6), although findings have suggested that the poly IC-induced TLR3–NF-κB/IRF3–CCL5 pathway is active in a case of human biliary epithelial cells (21). In this study, we confirmed that the poly IC-induced TLR3–IFN-β–pSTAT1–RIG-I pathway involved in upstream of CCL2/5 production is active not only in astrocytoma cells but also in neuroblastoma cells. We found that gnetin C first inhibits IFN-β expression in neuroblastoma cells and primarily inhibits STAT1 phosphorylation in astrocytoma cells.

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Fig. 4  Effect of gnetin C (GNC) on IFN-β, RIG-I, CCL2, or CCL5 mRNA expression in U373MG or SH-SY5Y cells. After pretreatment with 10 μM GNC for 1 h, the cells were incubated with 30 μg/mL of poly IC for 4 or 8 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC and was also used as a vehicle control [0.1% (v/v) DMSO]. mRNA expression was analyzed by real-time quantitative PCR and was normalized to 18S rRNA levels (relative value). The mean ± SD of three experiments is shown. * denotes $P < 0.05$. 
As our results indicate, the suppressive effect of gnetin C on CCL2/5 production is more efficient in astrocytoma cells than in neuroblastoma cells. Gnetin C (C_{28}H_{22}O_{6}, 454.47 g/mol) has a lipophilic property, which makes it possible to cross BBB. These suggest that gnetin C may be effective in the whole brain because astrocytes are widely distributed over there and, in particular, constitute BBB. BBB is one of the most important components of the first line of innate immunity (12, 14, 25).

Immune and inflammatory reactions mediated by TLR3 signaling are suggested to be important in host defenses against viral or “pseudoviral” infections (6, 23). Cross-talk between functional molecules induced by TLR3 activation, such as type I IFNs and pro-inflammatory chemokines or cytokines stimu-

**Fig. 5** Effect of gnetin C (GNC) on STAT1 phosphorylation in U373MG or SH-SY5Y cells. After pretreatment with 10 μM GNC for 1 h, the cells were incubated with 30 μg/mL of poly IC for 16 h. Dimethyl sulfoxide (DMSO) was used as a solvent for GNC, and also used as a vehicle control [0.1% (v/v) DMSO]. (A, B) Phosphorylated STAT1 (pSTAT1) or STAT1 in hypotonic cell lysates was detected by Western blotting (samples were separated in a 7.5% SDS-polyacrylamide gel and probed with a mouse anti-pSTAT1 monoclonal antibody or a rabbit anti-STAT1 polyclonal antibody), and β-actin was used as a loading control. Data shown are representative of three experiments. (C, D) The integrated optical density of the blot for pSTAT1 or STAT1 in (A, B) was quantified using ImageJ 1.49i and normalized to that of β-actin. The mean ± SD of three experiments is shown. * denotes P < 0.05.
Gnetin C as a CCL2/5 suppressor

Astroglial cells

Poly IC

TLR3

IFN-β

Gnetin C

STAT1

pSTAT1

RIG-I

CCL2 and CCL5

Neuronal cells

Gnetin C

CCL2 and CCL5

Fig. 6

Suppressive potentials of gnetin C on pro-inflammatory chemokine production. We focused on the double-stranded RNA-induced CC chemokine, CCL2 and CCL5, production in cultured human astrocytoma U373MG and neuroblastoma SH-SY5Y cells. Gnetin C may act against neuroinflammation by suppressing the IFN-β-pSTAT1-RIG-I pathway, which regulates the subsequent CCL2 and CCL5 production in both astroglial and neuronal cells, although its primary target signal differs (IFN-β in SH-SY5Y cells; pSTAT1 in U373MG). Abbreviations used: poly IC, polyinosinic-polycytidylic acid; TLR3, Toll-like receptor 3; IFN-β, interferon-β; STAT1, signal transducer and transcription 1; pSTAT1, phosphorylated STAT1; RIG-I, retinoic acid-inducible gene-I; CCL2, C-C motif chemokine ligand 2 (also known as monocyte chemoattractant protein-1, MCP-1); CCL5, also known as regulated on activation normal T-cell expressed and secreted (RANTES).

lated by type I IFNs, plays protective or deleterious roles during the pathogenesis of brain inflammation (12, 13). Chemokines are small, 8- to 12-kDa cytokines that have the ability to induce the chemotaxis of leukocytes and are divided into four groups based on their initial cysteine residue: XC, CC, CXC, and CX3C (24). Among them, CCL2 and CCL5 are produced by various cell types in the brain, such as neurons, astrocytes, and microglia (15, 20), and are suggested to be involved in viral and autoimmune inflammatory neurological disorders (1, 13). Previous infection with Epstein–Bar virus may contribute to the development of multiple sclerosis (17), and elevated levels of CCL2 and CCL5 are associated with the activity of this disease (3, 29).

Gnetin C as well as other melinjo-derived biophenols are free from harmful side effects (26, 27), leading to high expectations that it could be used for many neuroinflammatory and neurodegenerative dis-

cases. Although the clinical and prophylactic efficacy of gnetin C in pathologies remains to be evaluated, its appropriate use could play a role in preventing excess or prolonged inflammation after TLR3 activation in the CNS.

In conclusion, gnetin C was found to negatively regulate IFN-β expression and STAT1 phosphorylation in poly IC-treated human astroglial and neuronal cells, respectively, and finally suppressed their downstream CCL2 or CCL5 production in both cell types (Fig. 6). Gnetin C could potentially be used for controlling the progression of virus-mediated neuroinflammation. Further studies are needed to confirm these preliminary findings.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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