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The role of wogonin in controlling SOCS3 expression in neuronal cells

Kung-Woo Nam [a], Sungwook Chae [b], Ho-Yeon Song [c], Woongchon Mar [d], Man-Deuk Han [a,*]

[a] Department of Life Science and Biotechnology, College of Natural Science, Soonchunhyang University, Asan 336-745, Republic of Korea
[b] Herbal Quality Control Center, Korea Institute of Oriental Medicine, 488 Expo, Daejeon 305-811, Republic of Korea
[c] Department of Microbiology, College of Medicine, Soonchunhyang University, Cheonan 330-721, Republic of Korea
[d] Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

The mechanism underlying the wogonin-mediated increase in the expression of suppressor of cytokine signaling 3 (SOCS3) is unclear. Promoter deletion assay results revealed that wogonin-induced SOCS3 expression is dependent on the AP-1 consensus sequences and two STAT responsive elements (TTACAAGAA and TTCCAGGAA) in the 5′-flanking region of the SOCS3 gene in SH-SY5Y cells. Wogonin-induced SOCS3 expression was blocked by inhibitors of PI3K, Akt, Raf, p38, JNK, MEK, and STAT3, respectively. However, JAK2 inhibitors did not inhibit wogonin-induced SOCS3 expression. These results indicate that SOCS3-inducing effect of wogonin is caused by the activation of PI3K-mediated MAPK signaling pathways (Akt, ERK1/2, p38, and JNK), and the subsequent activation of AP-1 consensus sequences and STAT responsive elements in SH-SY5Y cells.

1. Introduction

Wogonin (5,7-dihydroxy-8-methoxyflavone) is one of the major constituents of *Scutellaria baicalensis* Georgi, a plant that has been used widely in traditional Oriental medicine to treat various inflammatory and neurological symptoms [1]. Wogonin protects neurons in damaged brain through the facilitation of neuronal precursor cell differentiation and also alleviates inflammatory processes by decreasing the expression of inducible cyclooxygenase-2 and monocyte chemoattractant protein-1 (MCP-1), a crucial factor for early inflammatory responses [2,3]. Moreover, the neuroprotective activity of wogonin has also been demonstrated in different ischemic animal models. Wogonin significantly reduced infarct volume after transient focal ischemia/reperfusion [4] or permanent ischemia induced in rats [5]. The neuroprotective effect of wogonin is considered to be due in part to its suppressive activity on the production of inflammatory cytokines in microglia [6]. Following the onset of cerebral ischemia, a multifaceted inflammatory reaction emerges over the course of the next few hours. Numerous inflammatory mediators are induced at the transcriptional level, including enzymes required for prostaglandin synthesis, cytokines of the tumor necrosis factor (TNF) family, and chemokines. The up-regulation of inflammatory genes is not restricted to glial cells but also occurs in neurons [7]. Experimental studies have firmly established that inflammation is closely interrelated with neuronal cell death and thereby promotes the neurological deficit.

In these inflammatory responses, NF-κB plays a central role by regulating genes encoding pro-inflammatory cytokines, chemokines, and adhesion molecules [8]. The molecular mechanisms underlying NF-κB activation have been investigated extensively and involve a sequential activation of cytoplasmic protein kinases and the ultimate nuclear translocation of the active subunit of NF-κB [9,10]. Recently, Wiejak et al. reported that flavonoids induce the expression of the suppressor of cytokine signaling (SOCS3) gene in vascular endothelial cells [11].

Cytokine signaling pathways are negatively regulated by the SOCS family of proteins [12,13]. These constitute a family of eight related members, SOCS1 through SOCS7 and cytokine-inducible Src homology 2 (SH2)-containing protein (CIS), which are characterized by the presence of a central SH2 domain, an N-terminal domain of variable length, and a C-terminal conserved domain termed the SOCS box [14]. In addition, SOCS1 and SOCS3 have a kinase inhibitory region (KIR) in their N-terminal domain. SOCS proteins are inducible by a number of cytokines and they affect signaling by inhibiting the JAK-STAT pathway in a classic negative feedback loop, using a variety of mechanisms for inhibition [15]. The SOCS3 protein plays a critical role in regulating signaling by the IL-6 family of cytokines by inhibiting STAT-3 activation [11,16]. Recent studies suggest that SOCS3 has a broader spectrum

* Corresponding author. Fax: +82 41 530 1256.
E-mail address: mdhan@sch.ac.kr (M.-D. Han).

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of action in that it can positively regulate the ERK-MAPK pathway [17], inhibit the NF-κB pathway [18], and antagonize cAMP-mediated signaling [19]. SOCS3 deficiency is embryonic lethal due to defects in placental development by an enhanced activation of the LIF signaling pathway [20]. Targeted deletion of SOCS3 in macrophages results in markedly enhanced IL-6-induced STAT-3 activation [21]. Mice deficient in SOCS3 in hematopoietic and endothelial cells demonstrate exacerbated IL-1-dependent arthritis [22]. Conversely, forced expression of SOCS3 in mouse arthritis models suppressed the induction and/or development of disease [23]. Furthermore, intracellular administration of a cell-penetrating SOCS3 suppressed the cytokine-mediated signal transduction associated with acute inflammation [24]. In spite of many previous studies, the relationship between wogonin and SOCS3 in neuronal cells has not been reported to date.

In this study, we investigated the effect of wogonin on the molecular mechanism involved in SOCS3 gene induction in neuronal cells.

2. Materials and methods

2.1. Plant materials and isolation of wogonin

Wogonin (5,7-dihydroxy-8-methoxyflavone) was isolated from *S. baicalensis* Georgi as described previously [25], with slight modifications. Samples containing 99% wogonin or more were used in all experiments unless otherwise indicated. Wogonin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution for *in vitro* study, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.2% throughout the study.

2.2. Materials and cell culture

Human SH-SY5Y neuroblastoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMED with 10% Qualified FBS at 37 °C in humidified 5% (v/v) CO_2. Primary antibodies to total anti-SOCS3, Akt1/2/3 (H-136), phospho-Akt (Ser473), anti-β-actin, anti-rabbit IgG-PE, farnesyl thiosalicylic acid, sorafenib tosylate, and wartmanin were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-NeuN antibody, SD1029, and SB230580 were obtained from EDM Millipore Co. (Billerica, MA). The anti-Iba-1 antibody and the secondary antibody anti-mouse IgG (Alexa Fluor 488) were purchased from Abcam Co. (Cambridge, UK). The anti-GFAP antibody was from BD Biosciences (Franklin Lakes, NJ). Anti-rabbit IgG conjugated with horse radish peroxidase (HRP) was obtained from GE Healthcare (Buckinghamshire, UK). Tyrphostin AG490, Cryptotanshinone, U0126, SP600125, LY294002, and Akt inhibitor were purchased from Sigma (St. Louis, MO).

2.3. Animals

Male Sprague–Dawley (SD) rats weighing 260–300 g were purchased from Samtaco Laboratories (Seoul, Korea) and maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. Rats were acclimated to their environment for a minimum of 3 days prior to use in all experiments. All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by Soonchunhyang University Institutional Animal Care & Use Committee (SCH-2012-12-01). All efforts were made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to *in vivo* techniques, if available.

2.4. In vivo ischemic model: focal transient cerebral ischemia model

Rats were initially anesthetized with 3.0% isoflurane in 70% N_2O and 30% O_2 (v/v). Focal cerebral ischemia was produced by right-sided endovascular middle cerebral artery occlusion (MCAO), as described previously [26] with slight modification. After 90 min of ischemia, sutures were released and animals were allowed to recover. Sham-operated controls were subjected to the same surgical procedures except that they did not receive MCAO.

2.5. Immunohistochemical detection of SOCS3, neurons (NeuN), astrocytes (GFAP), and microglia (Iba1)

Serial 20-μm sections were prepared using a cryostat (Leica 1850, Leica, Germany). To reduce non-specific binding, sections were first blocked with 5% normal goat serum for 1 h, and then stained with rabbit polyclonal anti-SOCS3 (1:200; Santa Cruz), or mouse monoclonal anti-NeuN (A60; 1:100; EMD Millipore) or mouse anti-GFAP (1:100; BD Biosciences), or mouse monoclonal anti-Iba1 (1:50; Abcam) antibodies at 4 °C overnight. After washing, sections were co-incubated with PE-conjugated goat anti-rabbit and Alexa 488 goat anti-mouse IgG (2 μg/ml) for 1 h at room temperature and then counterstained with Hoechst 33258 for 20 min. Fluorescence intensity and the number of immunoreactive cells were measured by confocal laser microscopy (Zeiss LSM510; Zeiss, Oberkochen, Germany).

2.6. Analysis of phospho-Akt expression by immunocytochemistry

SH-SY5Y cells (1×10^4) were cultured with or without wogonin (30 μM) in eight-well plates (Nalge Nunc Int, NY, USA) for 24 h and then gently rinsed twice in PBS. Cells were fixed by incubation in 100% (v/v) methanol for 10 min at RT, and permeabilized with 0.5% Triton X-100 and 5% fetal bovine serum in PBS for 1 h at RT. These cells were incubated with anti-phospho-Akt primary antibody (1:50) (Santa Cruz Biotechnology, TX) for 2 h at RT. Rinsed cells were treated with, anti-rabbit IgG-PE secondary antibody (1:200) (Santa Cruz Biotechnology) for 1 h at RT. After 1 h, the cells were rinsed three times for 5 min in 5% FBS containing PBS. Cells were then counter-stained with Hoechst (2 μg/ml) for 20 min at RT. Results were evaluated by confocal microscope (Zeiss LSM510; Zeiss, Oberkochen, Germany).

2.7. SOCS3 promoter constructs, permanent transfection, and luciferase assays

The 2056-bp (−2709 to −650 bp) human SOCS3 promoter was amplified from genomic DNA using the forward primer 5′-CGGGGTACCTGGTCTTGAATGTCCATGCTC-3′ and the reverse primer 5′-ACTGAAGCTTCTGGTCCCGAATCGAAGTCTC-3′ and cloned into the *Kpn*I and *Hind*III sites of the pGL4.20-puro vector. Serial deletions were synthesized by PCR using *Pfu* DNA polymerase and inserted into the *Kpn*I/*Hind*III restriction site of pGL4.20-puro vector, which contains the luciferase gene as a reporter. The SOCS3 promoter sequence was confirmed by automatic sequence analysis. The SOCS3 promoter constructs (1 μg) were permanently transfected into SH-SY5Y cells in six-well plates using the Lipofectamine Plus™ method. Puromycin-selected cells were treated with medium or wogonin for 24 h, and the luciferase activity of each sample was normalized to the total protein concentration of each well. Luciferase activity from the untreated sample was arbitrarily set at 1 for the calculation of fold-induction.

2.8. Western blotting analysis

Wogonin (30 μM)-treated SH-SY5Y cells were washed twice with PBS and then lysed for 10 min at 4 °C in a lysis buffer containing 25 mM HEPES-NaOH (pH 7.4), 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 5 mM NaF, 1 mM 4-(2-aminoethyl) benzene sulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The protein concentration was measured using the BCA Protein Assay Kit (Thermo Scientific Inc., IL, USA). The lysate was diluted 1:5 with 5× sample buffer and heated at 95 °C for 5 min prior to resolution by SDS–PAGE. Proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in TBST (25 mM Tris [pH 8], 150 mM NaCl, and 0.1% Tween 20) and then incubated at 4 °C overnight with a 1:500 dilution of rabbit anti-β-actin (NeoMarkers, Fremont, CA), 1:200 dilution of mouse anti-Akt, and phospho-Akt (Santa Cruz), or in 5% milk in TBST. Reactive proteins were visualized by HRP-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK; 1:3000 dilution in 5% milk in TBST) and chemiluminescence using Western Lightning® ECL (Amersham Biosciences).

2.9. Statistical analysis

Data were expressed as means ± SD or SEM, and analyzed for statistical significance using Student's *t*-tests or ANOVA, followed by Scheffe's test for multiple comparisons. A p value of <0.05 was considered to indicate significance.

3. Results

3.1. SOCS3 expression in rat brain

In the rat brain, neurons displayed unique SOCS3 expression. Microglia and astrocytes did not express SOCS3 in the focal ischemia-induced rat brain (Fig. 1A).

3.2. Wogonin induces human SOCS3 promoter-driven luciferase activity in SH-SY5Y cells

Neuroblastoma SH-SY5Y cells express SOCS3, and SOCS3 plays an important role in the negative regulation of cytokine-induced inflammatory gene expression. Wogonin showed a dose-dependent

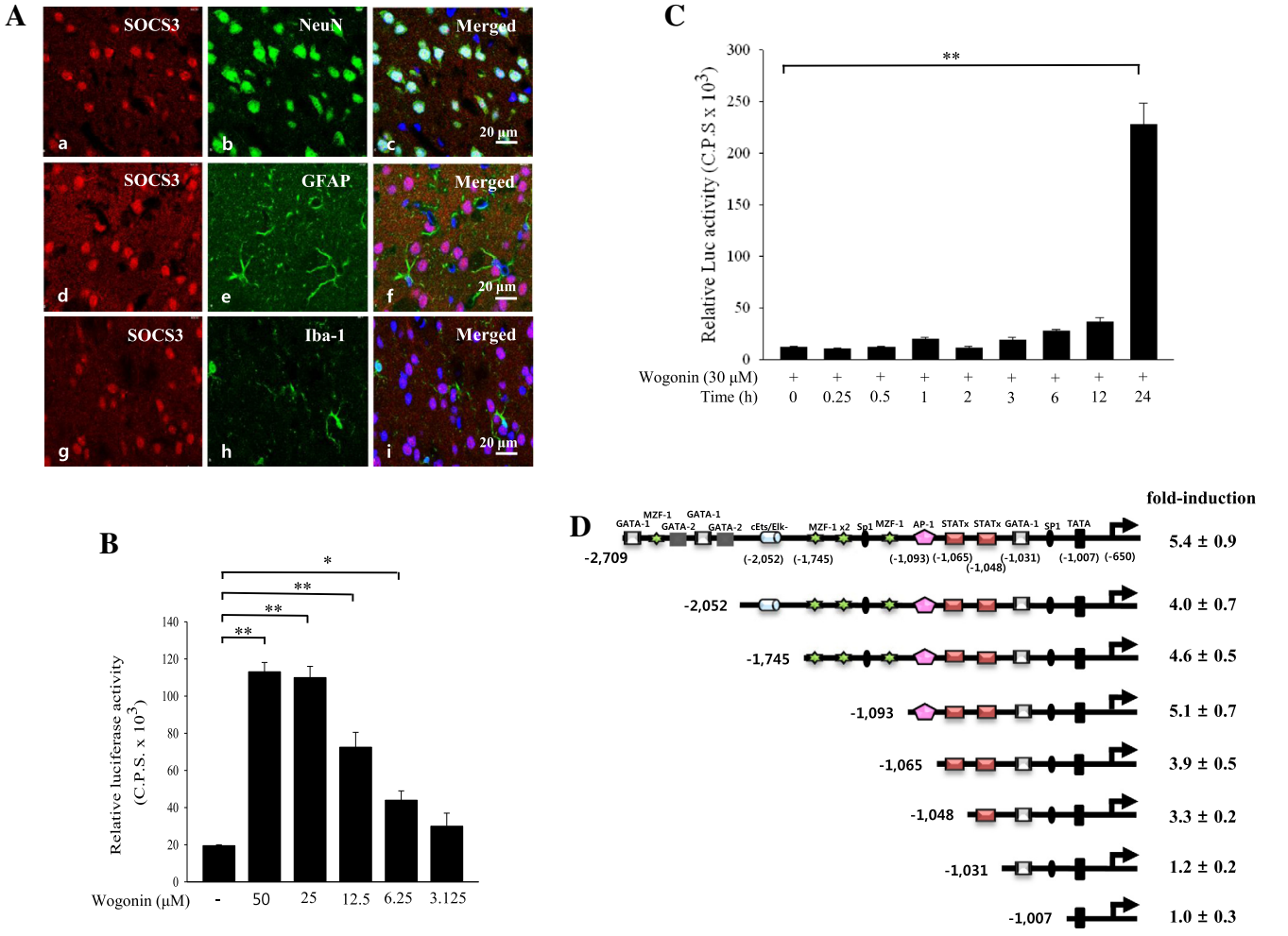

Fig. 1. Wogonin induces activation of the 2.0-kb human SOCS3 promoter in neuronal cells. (A) SOCS3 expressed only in neuronal cells in rat brain. Confocal images of SOCS3 (a, d, g: red), NeuN (b: green), GFAP (e: green), and Iba1 (h: green) immunostaining in the striatal penumbra and merged images with Hoechst-labeled nuclei (c, f, i). Neuron-specific protein (NeuN), astrocytes (GFAP), and microglia (Iba1). (B) SH-SY5Y cells were permanently transfected with 2.0-kb human SOCS3 promoter-driven luciferase, treated with wogonin (50–3.125 μM) for 24 h, and then analyzed for luciferase activity. (C) The cells were treated with wogonin (30 μM) for 0 min to 24 h, and then analyzed for luciferase activity. (D) The 2.0-kb human SOCS3 promoter deletion assay values were normalized to total protein levels and fold-induction was calculated by dividing the wogonin (30 μM) treatment values by the untreated values. Data are presented as the means ± SD of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$; data represent the means of three individual experiments.

increase in the expression of 2.0 kb SOCS3 promoter-driven luciferase activity in SH-SY5Y cells (Fig. 1B). The expression was not affected until 12 h after the addition of wogonin, and it peaked at 24 h (fivefold induction) in SH-SY5Y cells (Fig. 1C). Collectively, these results demonstrate that wogonin induces SOCS3 expression in a time-dependent manner in human neuroblastoma cells.

3.3. Wogonin-induced SOCS3 promoter activity requires AP-1- and STAT-binding elements

Within the −1.1 kb human SOCS3 promoter, three potential AP-1 sites, one G-rich region and two STAT binding elements (TTACAAGAA and TTCCAGGAA) were identified by the TFSEARCH program (Fig. 1D). To ascertain the functional roles of human SOCS3-regulatory elements, deletion constructs were generated from the 2.0-kb 5′-end of the SOCS3 promoter, as described in Section 2. The 2.0-kb wild-type human SOCS3 promoter and various deletion constructs were permanently transfected into SH-SY5Y cells, which were treated with wogonin (30 μM) for 24 h and then tested for luciferase activity. Wogonin stimulation caused a fivefold induction of human SOCS3 promoter-driven luciferase activity in SH-SY5Y cells. Deletion of the distal AP-1 element reduced the wogonin-induced SOCS3 promoter activity by 27.8% (Fig. 1D). Deletion of both AP-1- and STAT-responsive elements reduced wogonin-induced SOCS3 promoter activity by 77.8% (Fig. 1D). These data indicate that the AP-1 and STAT responsive elements are important for wogonin-induced SOCS3 promoter activity.

3.4. Jak2 receptor is independent of STAT-3 activators in wogonin-induce human SOCS3 promoter activity

It has been reported that STAT-3 activation is caused by activation of the Jaks receptor by many cytokines to induce SOCS3 gene transcription. However, whether the small molecule wogonin is recruited to Jak2-mediated SOCS3 gene transcription is unknown. To monitor transcription factor binding to the endogenous SOCS3 promoter, wogonin was incubated in the absence or presence of Jak2 inhibitors. Luciferase analysis indicated that the Jak2 inhibitors (Tyrphostin AG490 and SD1029) did not block the 2.0-kb human SOCS3 promoter-driven luciferase activity in SH-SY5Y cells (Fig. 2A). However, the STAT-3 inhibitor Cryptotanshinone blocked the SOCS3 promoter-driven luciferase activity in SH-SY5Y cells in a dose-dependent manner (Fig. 2B). These results indicate that Jak2 signal transduction is independent of the wogonin-induced SOCS3 expression. Moreover, STAT-3 plays a pivotal role in the SOCS3 up-regulation.

3.5. The PI3K, Akt, ERK1/2, JNK, and p38 MAPK pathways were involved in wogonin-induced SOCS3 gene expression

To determine the effect of wogonin on MAPK pathways, wogonin was treated to SH-SY5Y cells containing the 2.0-kb human SOCS3 promoter-driven luciferase gene. Specific pharmacological inhibitors of MEK1/2, JNK, p38, Ras, Raf, Akt, and PI3K were co-treated with wogonin (30 μM). As shown in Fig. 3C, wogonin activation was blocked by the MEK1 inhibitor U0126, by the JNK inhibitor SP600125, and by p38 MAPK inhibitor SB230580, respectively

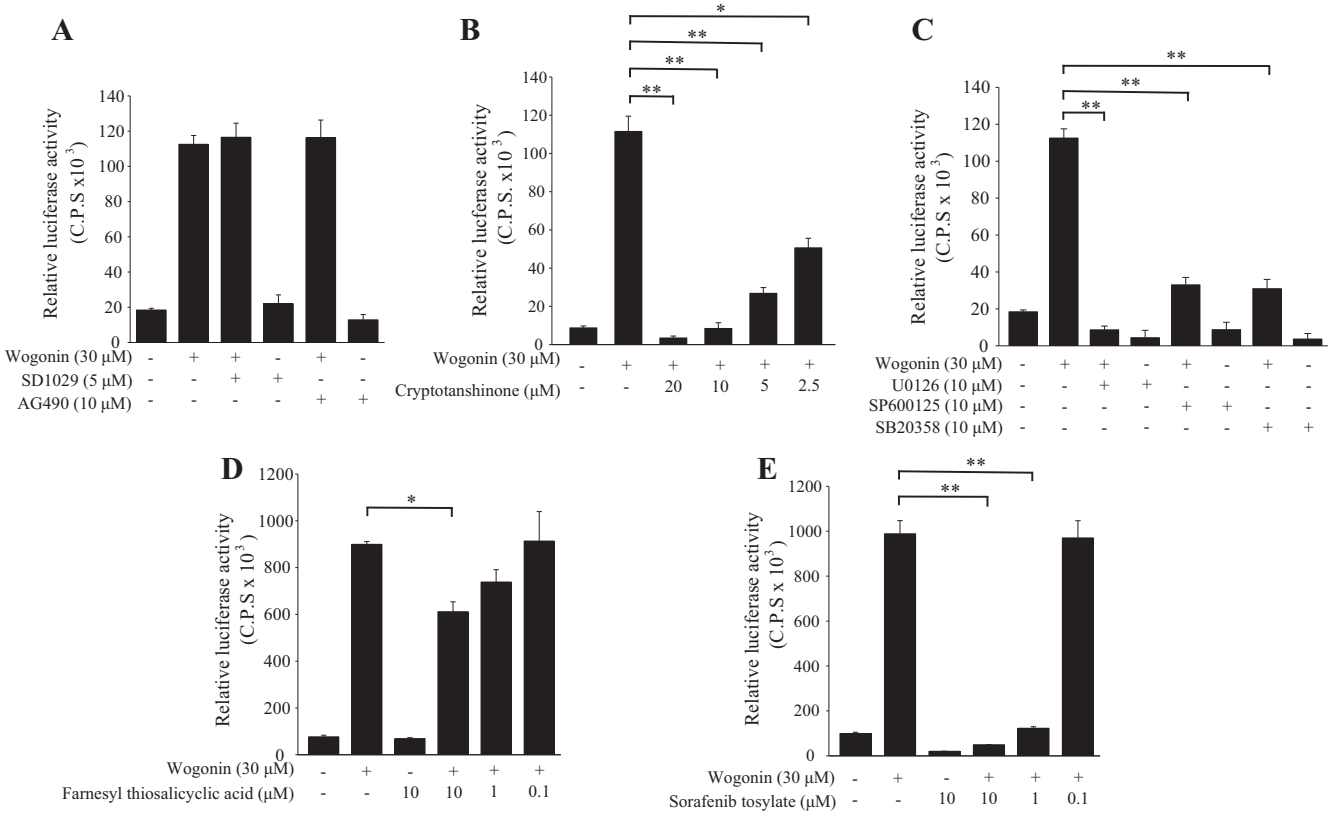

Fig. 2. Inhibition of wogonin-induced SOCS3 expression by Ras, Raf, MEK, JNK, p38, JAK, and STAT-3 inhibitors. Schematic representations of the 2.0-kb human SOCS3 promoter-driven luciferase assay in SH-SY5Y cells. (A) JAK2 inhibitors SD1029 and Tyrphostin AG490 did not inhibit wogonin (30 μM)-induced luciferase activity in SH-SY5Y cells. (B) The STAT3 inhibitor cryptotanshinone (10 μM) blocked wogonin (30 μM)-induced luciferase activity. (C) MEK inhibitor U0126 (10 μM), JNK inhibitor SP600125 (10 μM), and p38 inhibitor SB20358 (10 μM) blocked wogonin (30 μM)-induced luciferase activity, respectively. (D) Wogonin (30 μM)-stimulated luciferase activity was slightly inhibited by the Ras inhibitor farnesyl thiosalicyclic acid in a dose-dependent manner. (E) The Raf inhibitor sorafenib tosylate. $^{*}P < 0.05$, $^{**}P < 0.01$; data represent the means of three individual experiments performed in duplicate.

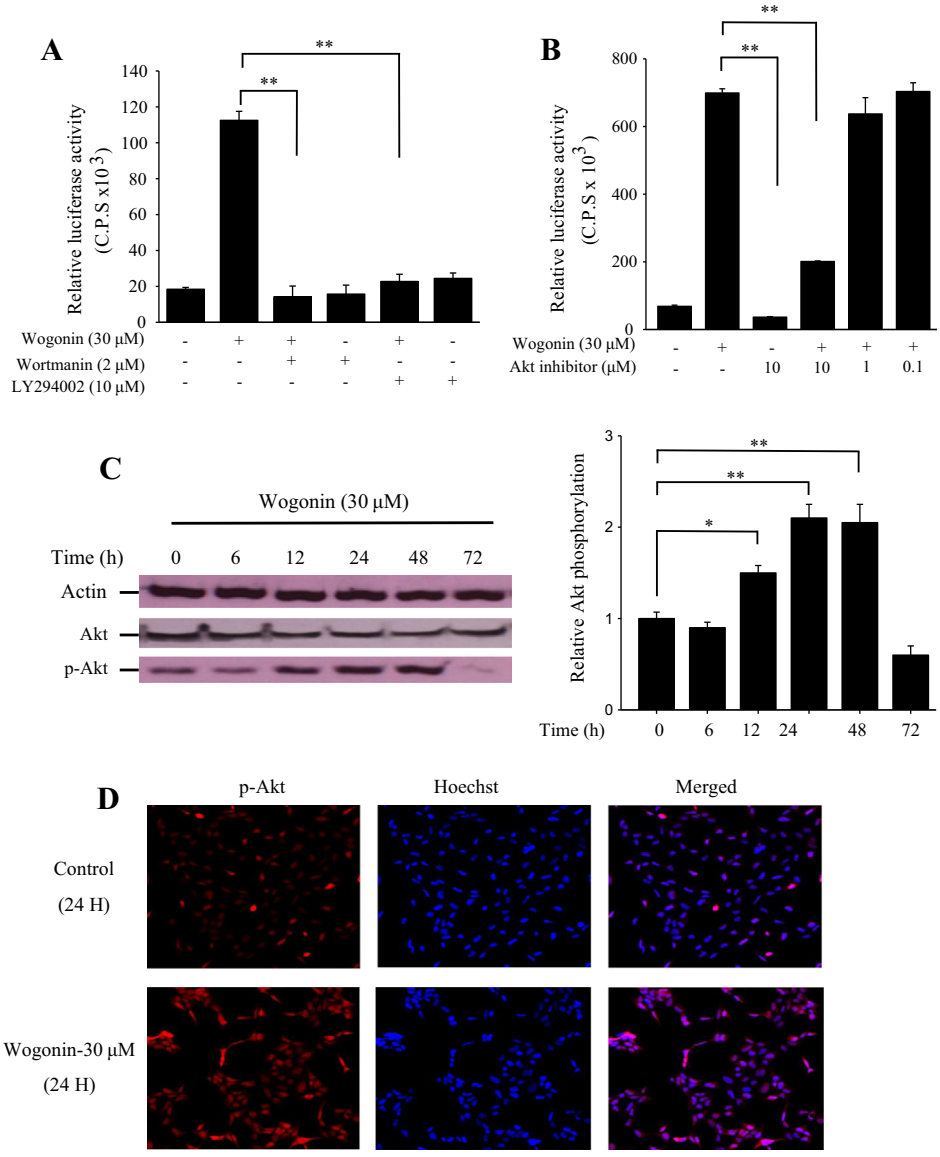

Fig. 3. Inhibition of wogonin-induced SOCS3 expression by PI3K and Akt inhibitors. (A) Schematic representations of the 2.0-kb human SOCS3 promoter-driven luciferase assay in SH-SY5Y cells. Wogonin (30 μM)-stimulated luciferase activity was blocked by the PI3K inhibitors wortmannin (2 μM) and LY-294002 (10 μM), respectively. (B) Akt inhibitor inhibited wogonin (30 μM)-induced luciferase activity dose-dependently in SH-SY5Y cells. (C) Western blot analysis of Akt and p-Akt at the indicated times in wogonin (30 μM)-treated SH-SY5Y cells. In bar graphs, the levels of Akt and p-Akt were normalized with respect to β-actin and then expressed as relative fold-changes in comparison with 0, 6, 12, 24, 48 and 72 h p-Akt of the wogonin-treatment group. (D) SH-SY5Y cells were treated with wogonin (30 μM) for 24 h, and then immunostained with phospho-Akt (p-Akt) antibody. Confocal images of p-Akt (red) and merged images with Hoechst-labeled nuclei (blue). $N = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different between the groups indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2C). Ras inhibitor farnesyl thiosalicylic acids showed modest inhibition of wogonin-induced SOCS3 expression, whereas the Raf inhibitor sorafenib tosylate blocked wogonin-induced SOCS3 expression (Fig. 2D and E). The PI3K inhibitors wortmannin and LY294002 blocked wogonin-induced SOCS3 expression (Fig. 3A). Wogonin treatment increased Akt phosphorylation from 12 h, which was and maintained at 48 h but decreased at 72 h (Fig. 3C and D); the Akt inhibitor blocked this wogonin-induced SOCS3 expression in a dose-dependent manner (Fig. 3B). These results demonstrated that the PI3K, Akt, and Raf pathways are involved in wogonin-induced SOCS3 gene expression, with a lesser contribution from the Ras pathway.

4. Discussion

The effects of SOCS3 on the JAK cytokine receptors play critical roles against inflammatory neurotoxic mediators of microglia and macrophage origin in the delayed stages following stroke SOCS3 might act either directly by hampering JAKs activation or by mediating the ubiquitination and subsequent proteasome-mediated degradation of cytokine/growth factor/hormone signal transduction [27]. It has been reported that microglia/macrophages and astrocytes express SOCS3 [28,29]; however, in the rat brain, neurons displayed unique SOCS3 expression. Microglia and astrocytes did not express SOCS3 in the focal ischemia-induced rat brain. Thus, in this study, we focused on the mechanism underlying wogonin-mediated SOCS3 induction in neuronal cells.

Using a 2.0-kb SOCS3 promoter deletion assay and diverse kinase inhibitors, we demonstrated that the SOCS3-inducing effect of wogonin is caused by the activation of PI3K-mediated MAPK signaling pathways (Akt, ERK1/2, p38, and JNK), and the subsequent activation of AP-1 consensus sequences and STAT responsive elements (TTACAAGAA and TTCCAGGAA) in the 5′-flanking region of the SOCS3 gene in SH-SY5Y cells. However, JAK2-mediated signal

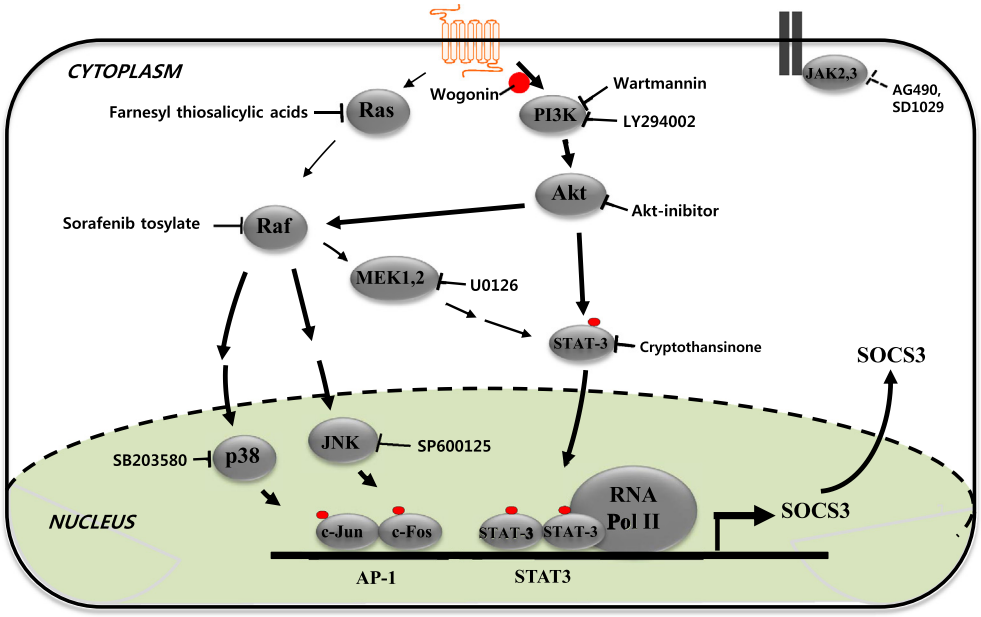

Fig. 4. Proposed model for wogonin-induced SOCS3 gene expression. Wogonin activates the PI3K mediated MAPK pathways. Akt, Raf, MEK1, JNK, and p38 lead to nuclear translocation and binding of Ap-1 and STAT responsive elements (TTACAAGAA and TTCCAGGAA) in the 5′-flanking region of the SOCS3 gene in SH-SY5Y cells. Concurrently, wogonin induces SOCS3 up-regulation. Ras is not strong enough to control SOCS3 expression. JAK2-mediated signal transduction is not related to wogonin-induced SOCS3 expression in SH-SY5Y cells.

transduction was not related to this induction by wogonin in SH-SY5Y cells. This SOCS3-inducing effect of wogonin acts at a delayed time point (12 h after stroke), and so would influence the infarct volume through the inhibition of neuroinflammatory cytokines at the delayed stages. Lee et al. reported that wogonin inhibits NO production in rat astrocytes through the suppression of iNOS induction and NF-κB activation [6]. In the current study, however, we demonstrated that wogonin increased SOCS3 expression in neuronal cells and the PI3K-mediated signaling cascades are downstream of the wogonin targets. The recruitment of transcription regulators Akt, Raf, ERK1/2, p38, and JNK are critical for the increase in SOCS3 gene expression in SH-SY5Y cells (Fig. 4). These functions and the mechanism of wogonin are different from the previously described anti-inflammatory effects of wogonin against NF-κB-mediated signal transduction.

The SOCS3 promoter contains numerous potential regulatory elements, including Sp1-, GATA-1-, STAT-binding sites (TTACAAGAA and TTCCAGGAA), and AP-1. Qin et al. [28] demonstrated that AP-1 and IFN-γ activation sequence (GAS) elements (TTCNNNGAA) are involved in LPS-induced SOCS3 transcription. Furthermore, LPS activated the MAPK-ERK1/2, JNK, and p38 pathways that, in addition to STAT3, are also involved in LPS-induced SOCS3 expression. LIF induction of SOCS3 involves both STAT-1 and STAT-3, as STAT-1 homodimers, STAT-3 homodimers, and STAT-1/STAT-3 heterodimers all bind to the proximal GAS-like element in the SOCS3 promoter [29]. Our promoter deletion analysis of the human SOCS3 promoter-driven luciferase assay suggests that the proximal AP-1- and STAT-binding elements (TTACAAGAA and TTCCAGGAA) are critical for the activation of human SOCS3 gene transcription by wogonin. We also demonstrated the involvement of PI3K as a downstream target of wogonin, which is documented by the serial results of PI3K, Akt, Ras, Raf, MEK1/2, p38, JNK, and STAT-3 inhibitors. The inhibitors blocked or inhibited SOCS3 promoter-driven luciferase activity in response to wogonin stimulation.

Taken together, these results indicate that wogonin interacts and stimulates a signal upstream from PI3K for SOCS3 gene expression in neuronal cells. The SOCS3-inducing effect of wogonin is caused by the activation of PI3K-mediated MAPK signaling pathways (Akt, ERK1/2, p38, and JNK), and the subsequent activation of AP-1 consensus sequences and STAT responsive elements.

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References

[1] S. Xing, M. Wang, Y. Peng, D. Chen, X. Li, Simulated gastrointestinal tract metabolism and pharmacological activities of water extract of *Scutellaria baicalensis* roots, J. Ethnopharmacol. 152 (2014) 183–189.
[2] J.S. Lim, M. Yoo, H.J. Kwon, H. Kim, Y.K. Kwon, Wogonin induces differentiation and neurite outgrowth of neural precursor cells, Biochem. Biophys. Res. Commun. 402 (2010) 42–47.
[3] H.Z. Piao, I.Y. Choi, J.S. Park, H.S. Kim, J.H. Cheong, K.H. Son, S.J. Jeon, K.H. Ko, W.K. Kim, Wogonin inhibits microglial cell migration via suppression of nuclear factor-kappa B activity, Int. Immunopharmacol. 8 (2008) 1658–1662.
[4] J. Cho, H.K. Lee, Wogonin inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion, Biol. Pharm. Bull. 27 (2004) 1561–1564.
[5] Y.M. Lee, P.Y. Cheng, S.Y. Chen, M.T. Chung, J.R. Sheu, Wogonin suppresses arrhythmias, inflammatory responses, and apoptosis induced by myocardial ischemia/reperfusion in rats, J. Cardiovasc. Pharmacol. 58 (2011) 133–142.
[6] H. Lee, Y.O. Kim, H. Kim, S.Y. Kim, H.S. Noh, S.S. Kang, G.J. Cho, W.S. Choi, K. Suk, Flavonoid wogonin from medicinal herb is neuroprotective by inhibiting inflammatory activation of microglia, FASEB J. 17 (2003) 1943–1944.
[7] H.K. Wang, U.J. Park, S.Y. Kim, J.H. Lee, S.U. Kim, B.J. Gwag, Y.B. Lee, Free radical production in CA1 neurons induces MIP-1{alpha} expression, microglia recruitment, and delayed neuronal death after transient forebrain ischemia, J. Neurosci. 28 (2008) 1721–1727.
[8] K.W. Nam, G.T. Oh, E.K. Seo, K.H. Kim, U. Koo, S.J. Lee, W. Mar, Nuclear factor kappaB-mediated down-regulation of adhesion molecules: possible mechanism for inhibitory activity of bigelovin against inflammatory monocytes adhesion to endothelial cells, J. Ethnopharmacol. 123 (2009) 250–256.
[9] S.M. Kim, S.H. Kim, Inhibitory effect of astragalin on expression of lipopolysaccharide-induced inflammatory mediators through NF-κB in macrophages, Arch. Pharm. Res. 34 (2011) 2101–2107.
[10] S. Gerondakis, T.S. Fulford, N.L. Messina, R.J. Grumont, NF-κB control of T cell development, Nat. Immunol. 15 (2014) 15–25.
[11] J. Wiejak, J. Dunlop, S.P. Mackay, S.J. Yarwood, Flavanoids induce expression of the suppressor of cytokine signalling 3 (SOCS3) gene and suppress IL-6-

activated signal transducer and activator of transcription 3 (STAT3) activation in vascular endothelial cells, Biochem. J. 454 (2013) 283–293.
[12] S. Xiang, J. Liu, N. Dong, J. Shi, Y. Xiao, Y. Wang, X. Hu, L. Gong, W. Wang, Suppressor of cytokine signaling 3 is a negative regulator for neointimal hyperplasia of vein graft stenosis, J. Vasc. Res. 51 (2014) (2014) 132–143.
[13] J.J. Babon, N.J. Kershaw, J.M. Murphy, L.N. Varghese, A. Laktyushin, S.N. Young, I.S. Lucet, R.S. Norton, N.A. Nicola, Suppression of cytokine signaling by SOCS3: characterization of the mode of inhibition and the basis of its specificity, Immunity 36 (2012) 239–250.
[14] E.M. Linossi, J.J. Babon, D.J. Hilton, S.E. Nicholson, Suppression of cytokine signaling: the SOCS perspective, Cytokine Growth Factor Rev. 24 (2013) 241–248.
[15] H. Qin, W.I. Yeh, P. De Sarno, A.T. Holdbrooks, Y. Liu, M.T. Muldowney, S.L. Reynolds, L.L. Yanagisawa, T.H. Fox 3rd, K. Park, L.E. Harrington, C. Raman, E.N. Benveniste, Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation, Proc. Natl. Acad. Sci. USA 109 (2012) 5004–5009.
[16] J.J. Babon, L.N. Varghese, N.A. Nicola, Inhibition of IL-6 family cytokines by SOCS3, Semin. Immunol. 26 (2014) 13–19.
[17] L. Piao, J. Park, Y. Li, S. Shin, S. Shin, G. Kong, R. Shrestha, Q. Tran, G.M. Hur, J.L. Kim, J. Park, SOCS3 and SOCS6 are required for the risperidone-mediated inhibition of insulin and leptin signaling in neuroblastoma cells, Int. J. Mol. Med. 33 (2014) 1364–1370.
[18] A.E. Karlsen, P.E. Heding, H. Frobøse, S.G. Rønn, M. Kruhøffer, T.F. Orntoft, M. Darville, D.L. Eizirik, F. Pociot, J. Nerup, T. Mandrup-Poulsen, N. Billestrup, Suppressor of cytokine signalling (SOCS)-3 protects beta cells against IL-1beta-mediated toxicity through inhibition of multiple nuclear factor-kappaB-regulated proapoptotic pathways, Diabetologia 47 (2004) 1998–2011.
[19] I. Bellezza, H. Neuwirt, C. Nemes, I.T. Cavarretta, M. Puhr, H. Steiner, A. Minelli, G. Bartsch, F. Offner, A. Hobisch, W. Doppler, Z. Culig, Suppressor of cytokine signaling-3 antagonizes cAMP effects on proliferation and apoptosis and is expressed in human prostate cancer, Am. J. Pathol. 169 (2006) 2199–2208.
[20] J.C. Marine, C. McKay, D. Wang, D.J. Topham, E. Parganas, H. Nakajima, H. Pendeville, H. Yasukawa, A. Sasaki, A. Yoshimura, J.N. Ihle, SOCS3 is essential in the regulation of fetal liver erythropoiesis, Cell 98 (1999) 617–627.
[21] K. Boyle, J.G. Zhang, S.E. Nicholson, E. Trounson, J.J. Babon, E.J. McManus, N.A. Nicola, L. Robb, Deletion of the SOCS box of suppressor of cytokine signaling 3 (SOCS3) in embryonic stem cells reveals SOCS box-dependent regulation of JAK but not STAT phosphorylation, Cell. Signal. 21 (2009) 394–404.
[22] P.K. Wong, P.J. Egan, B.A. Croker, K. O'Donnell, N.A. Sims, S. Drake, H. Kiu, E.J. McManus, W.S. Alexander, A.W. Roberts, I.P. Wicks, SOCS-3 negatively regulates innate and adaptive immune mechanisms in acute IL-1-dependent inflammatory arthritis, J. Clin. Invest. 116 (2006) 1571–1581.
[23] T. Shouda, T. Yoshida, T. Hanada, T. Wakioka, M. Oishi, K. Miyoshi, S. Komiya, K. Kosai, Y. Hanakawa, K. Hashimoto, K. Nagata, A. Yoshimura, Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis, J. Clin. Invest. 108 (2001) 1781–1788.
[24] D. Jo, D. Liu, S. Yao, R.D. Collins, J. Hawiger, Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis, Nat. Med. 11 (2005) 892–898.
[25] Y. Kimura, H. Okuda, Z. Ogita, Effects of flavonoids isolated from scutellariae radix on fibrinolytic system induced by trypsin in human umbilical vein endothelial cells, J. Nat. Prod. 60 (1997) 598–601.
[26] E.Z. Longa, P.R. Weinstein, S. Carlson, R. Cummins, Reversible middle cerebral artery occlusion without craniectomy in rats, Stroke 20 (1989) 84–91.
[27] B.J. Baker, L.N. Akhtar, E.N. Benveniste, SOCS1 and SOCS3 in the control of CNS immunity, Trends Immunol. 30 (2009) 392–400.
[28] H. Qin, K.L. Roberts, S.A. Niyongere, Y. Cong, C.O. Elson, E.N. Benveniste, Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia, J. Immunol. 179 (2007) 5966–5976.
[29] B.J. Baker, H. Qin, E.N. Benveniste, Molecular basis of oncostatin M-induced SOCS-3 expression in astrocytes, Glia 56 (2008) 1250–1262.