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Thrombin induces suppressor of cytokine signaling 3 expression in brain microglia via protein kinase Cδ activation

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Abstract

Microglia (brain macrophages) are activated upon brain damage. In this study, we demonstrated that thrombin, a pro-inflammatory stimulator of microglia, induced expression of suppressors of cytokine signaling (SOCS) in microglia. RT-PCR analysis and Northern blot analysis showed that thrombin induced SOCS3 mRNA expression. Further experiments indicated SOCS3 expression was not affected by cycloheximide, indicating thrombin directly stimulated SOCS3 transcript expression without de novo protein synthesis. We investigated whether PKCδ played a role in thrombin-stimulated SOCS3 expression. We found that thrombin activated PKCδ, and the specific inhibitor of PKCδ, rottlerin, significantly suppressed thrombin-stimulated SOCS3 expression. In thrombin-pretreated cells, microglial activation-induced by another inflammatory stimulator, lipopolysaccharide, was attenuated compared to that in non-pretreated cells. These results suggest thrombin induce not only proinflammatory mediators but also negative feedback regulators of inflammation, SOCS, which prevent prolonged inflammatory reactions in microglia.

Keywords: Thrombin; SOCS; PKC; Microglia

Microglia are major immune effector cells in the central nervous system. Inflammatory activation of microglia is important as a host defense mechanism. However, inflammatory mediators released from activated microglia can aggravate brain damage, resulting in the enhanced onset, and progression of brain diseases [1–3]. Thus, it is important to understand how inflammation intensity and duration are regulated.

Suppressors of cytokine signaling (SOCS) family members are negative feedback regulators of JAK/STAT pathways. The SOCS family consists of at least eight members, namely cytokine-induced SH2 protein (CIS) and SOCS1-7. SOCS proteins contain a central SH2 domain, a conserved C-terminus referred to as the SOCS box, and a unique N-terminus [4]. SOCS proteins are induced through JAK/STAT pathways by many cytokines and growth factors [5]. SOCS proteins inhibit JAK activity or inhibit binding of STATs to the cytoplasmic domains of receptors [6], and thus inhibit JAK/STAT signaling. SOCS, particularly SOCS1 and SOCS3, participate in the resolution of interferon-γ (IFN-γ)-induced inflammation. SOCS1 and SOCS3 reduce IFN-γ-induced expression of ICAM-1, HLA-DR, IFN-γ-inducible protein-10 (IP-10), and MCP-1 [7]. Stronger STAT3 activation and more severe colitis were detected in SOCS3 mutant transgenic mice compared to wild-type mice [8].

Thrombin is a well-known protease involved in blood coagulation and wound healing. Recently, many studies have described pro-inflammatory activities of thrombin. Thrombin acts as a chemotactic agent for inflammatory cells such as monocytes, macrophages, and neutrophils, and stimulates secretion of cytokines from those cells [9–11]. The central nervous system (CNS) can be exposed to thrombin when the blood–brain barrier breaks down due to trauma, stroke, or chronic neurodegenerative diseases such as Alzheimer's disease and

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cerebral–vascular dementia [12,13]. It has been reported that thrombin also induces brain inflammation by activating microglia [14].

In this study, we found that thrombin-induced SOCS expression through activation of PKCδ. Therefore, although thrombin is a pro-inflammatory stimulator, thrombin also appears to possess auto-regulatory activity that controls the duration and extent of inflammatory responses in microglia.

Materials and methods

Reagents. Thrombin, phorbol-12-myristate-13-acetate (PMA), bisindolylmaleimide (BIM), lipopolysaccharide (LPS), and minimal essential medium (MEM) were purchased from Sigma (St. Louise, MO). Rottlerin was from Biomol (Plymouth meeting, PA). IL-13 and interferon γ (IFN-γ) were from Peprotech (Rocky Mountain, NJ). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Hyclone (Logan, UT). RNAzol B was from TEL-TEST (Friendswood, TX). AMV reverse transcriptase was from Promega (Madison, WI). Oligonucleotide primers were purchased from Bioneer (Korea). Antibodies against the phosphorylated form of STAT1, STAT3, STAT5, STAT6, JAK1, JAK2, ERK, p38, JNK, and PKCδ were from Cell Signaling Technology (Beverly, MA). Antibody against PKCδ was from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated secondary antibodies were from Zymed (South San Francisco, CA).

Preparation microglia. Microglia were cultured from the cerebral cortices of 1- to 3-day-old Sprague–Dawley rats as previously described [15]. Briefly, the cortices were triturated into single cells in minimal essential medium (MEM) containing 10% fetal bovine serum and plated in 75 cm^2 T-flasks (0.5 hemisphere/flask) for 2 weeks. Microglia were then detached from flasks by mild shaking and filtered through a nylon mesh to remove astrocytes and cell aggregates. Cells were seeded in plates or dishes, washed 1 h later to remove unattached cells, and attached cells were used in experiments.

BV2 murine microglial cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum.

Reverse transcription and polymerase chain reaction. Total RNA was extracted using RNAzol B and cDNA was prepared using reverse transcriptase according to the manufacturer's instructions. The sequences of PCR primers were as follows: for SOCS3, (F) 5′-ACCAGCGCCACTTCTTCACG-3′, (R) 5′-GTGGAGCATCATACTGATCC-3′; for iNOS, (F) 5′-TCACTGGGACAGCACAGAAT-3′, (R) 5′-TGTGTCTGCAGATGTGCTGA-3′; and for β-actin, (F) 5′-CATGTTTGAGACCTTCAACACCCC-3′, (R) 5′-GCCATCTCCTGCTCGAAGTCTAG-3′. PCR products were separated by electrophoresis in 1.5% agarose gels and detected under UV light (Bio-Rad, Hercules, CA).

Northern blot analysis. Total RNA was extracted using RNAzol B, separated by electrophoresis in 1% agarose–formaldehyde gels, transferred to nylon membranes, and cross-linked to membranes using UV light. The membranes were hybridized with a SOCS3 probe which consisted of purified insert fragments of the expression vector pEF-SOCS3 (provided by Shong M.H., Chungnam National University school of Medicine, Korea) and radiolabeled using random priming. Level of 18S RNA was used as an internal loading control.

Western blot analysis. Cells were lysed in 50 mM Tris–HCl, pH 7.4, containing 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors. Proteins transferred to nitrocellulose membranes were visualized using primary antibodies, peroxidase-conjugated secondary antibodies, and the ECL system. The intensities of the bands detected in Western blot were measured using Image Gauge (Fuji photo film, Japan).

Determination of NO. The amount of nitrite produced from the conversion of NO was measured by mixing the culture medium (50 μl) with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, and 2.5% H_3PO_4). The optical density at 540 nm was then determined.

Results

Thrombin induces SOCS mRNA expression in microglia

Previously, we have reported that thrombin induces pro-inflammatory mediators in microglia [14]. Here, we investigated whether thrombin also stimulates expression of negative regulators of inflammation. We found that in primary cultured rat brain microglia, 20 U/ml thrombin increased SOCS1 and SOCS3 mRNA expression (Fig. 1A). SOCS3 mRNA expression increased within 30 min of thrombin addition, peaked at 3 h, and levels remained elevated for up to 6 h. Increase of SOCS1 mRNA was first detected at 3 h after thrombin addition and was at higher levels after 6 h.

The remaining studies focused on SOCS3 expression given it was more responsive to thrombin compared to SOCS1 expression. Using RT-PCR assays, we investigated dose-dependent SOCS3 expression in BV2 murine microglial cells treated with 0–50 U/ml thrombin. SOCS3 expression was detected in cells treated with 5 U/ml thrombin and showed a maximum plateau between 20 and 50 U/ml (Fig. 1B). Northern blot analysis also showed SOCS3 mRNA expression increased in response to 20 U/ml thrombin (Fig. 1C). We investigated the

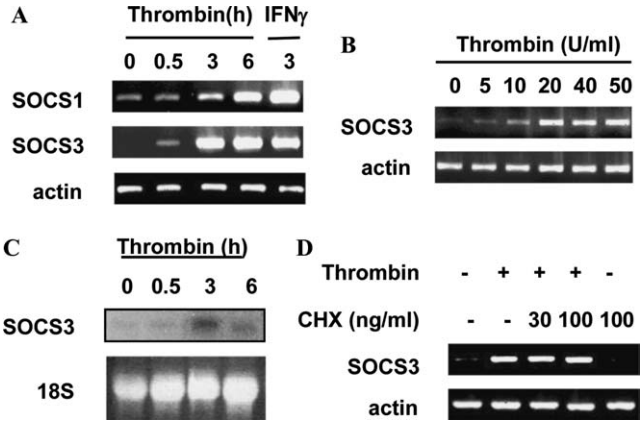

Fig. 1. Thrombin induces SOCS expression in microglia. (A) Primary cultured rat brain microglia were treated with 20 U/ml thrombin for the indicated times, and the indicated products were identified using RT-PCR. (B) BV2 microglia were incubated with indicated dose of thrombin for 3 h and SOCS3 expression was detected using RT-PCR. (C) BV2 microglia were treated with 20 U/ml thrombin for the indicated times, and the indicated products were detected using Northern blotting. (D) BV2 microglia were treated with 20 U/ml thrombin in the absence or presence of the indicated amount of cycloheximide for 3 h. The indicated products were detected using RT-PCR.

effect of cycloheximide on SOCS3 mRNA expression to determine whether thrombin directly induced SOCS3 without de novo protein synthesis. SOCS3 mRNA expression was not affected by 30–100 ng/ml cycloheximide (Fig. 1D). At this concentration, cycloheximide inhibited thrombin-induced iNOS protein expression (data not shown). These results suggest that thrombin directly induces SOCS3 without de novo protein synthesis.

Thrombin-induced SOCS3 expression involves PKCδ

SOCS family proteins are induced by cytokines, growth factors, and several immunomodulators via JAK/STAT pathways [5]. Thus, we examined activation of JAKs and STATs in thrombin-treated cells by analyzing tyrosine phosphorylation of these proteins in Western blot assays. Unexpectedly, we could not detect activation of JAK1, JAK2, STAT1, STAT3, STAT5 or STAT6 in cells treated for up to 60 min with thrombin, while activation of JAK1, JAK2, STAT1, STAT3, and STAT5 was detected in IFN-γ-treated cells, and activation of JAK1 and STAT6 was observed in IL-13-treated cells (Fig. 2). These results suggest JAK/STAT pathways may not be the main signaling pathways to mediate thrombin-induced SOCS3 expression.

Since PKC is an important signaling molecule in mediating thrombin action in microglia [14], we examined whether PKC activity was associated with thrombin-stimulated SOCS3 expression. We found that the general PKC inhibitor bisindolylmaleimide (BIM, 2 and 5 μM) dose-dependently inhibited thrombin-induced SOCS3 expression (Fig. 3A). Thrombin-induced SOCS3 expression was also significantly suppressed when PKC was down-regulated by long-term (18 h) phorbol-12-myristate-13-acetate (PMA, 0.1 and 1 μM) treatment (Fig. 3B). We examined which PKC isotypes regulated

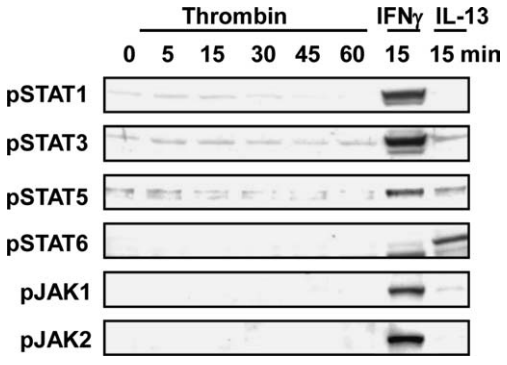

Fig. 2. Thrombin does not activate JAK/STAT pathways in microglia. BV2 microglia were treated with 20 U/ml thrombin for the indicated times. Cell lysates were separated using 8% SDS–PAGE and Western blot analysis was performed using antibodies against tyrosine phosphorylated (p)-STAT1, STAT3, STAT5, STAT6, JAK1, and JAK2. As positive controls, cells were treated with 10 U/ml interferon-γ or 20 ng/ml IL-13 for 15 min.

Fig. 3. PKC mediates thrombin-induced SOCS3 expression. (A) BV2 microglia were pretreated with the indicated amount of bisindolylmaleimide (BIM) for 30 min, then treated with 20 U/ml thrombin for 3 h, and the indicated products were identified using RT-PCR. (B) Cells were pretreated with phorbol-12-myristate-13-acetate (PMA) for 18 h, then treated with 20 U/ml thrombin for 3 h, and the indicated products were identified using RT-PCR.

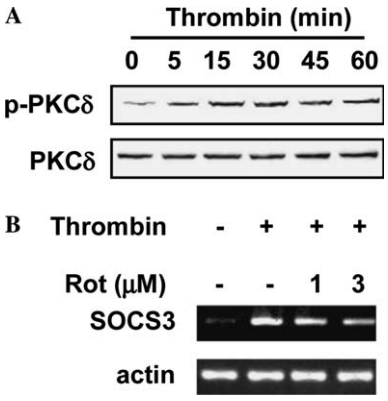

Fig. 4. PKCδ mediates thrombin-induced SOCS3 expression. (A) BV2 microglia were treated with 20 U/ml thrombin for the indicated times, and phosphorylated PKCδ (p-PKCδ) was detected using Western blotting. (B) BV2 microglia were treated with 20 U/ml thrombin in the presence of the indicated amounts of rottlerin (Rot) for 3 h. The indicated products were identified using RT-PCR.

SOCS3 expression in thrombin-treated cells. In a previous study we found that long-term treatment of cells with PMA down-regulated PKC isotypes α, β, δ, and ε in BV2 cells [16]. Furthermore, thrombin has been reported to activate PKCδ. Therefore, we examined the involvement of PKCδ in thrombin-induced SOCS3 expression. As expected, thrombin activated PKCδ in microglia, which was determined by analyzing the activation-dependent phosphorylation of the threonine 505 residue in the activation loop of PKCδ [17–22]. Thrombin increased phosphorylation of PKCδ within 15 min and the phosphorylated state remained for up to 60 min (Fig. 4A). We found that addition of the PKCδ-specific inhibitor, rottlerin (Rot, 1 and 3 μM) decreased thrombin-induced SOCS3 expression (Fig. 4B). These results suggest that PKCδ mediates thrombin-induced SOCS3 expression.

Pretreatment with thrombin reduced LPS-induced NO release and activation of MAP kinase

Since thrombin-induced SOCS1 and SOCS3 mRNA expression in microglia, we examined whether microglial

inflammatory activities could be attenuated in cells pretreated with thrombin. We investigated iNOS mRNA expression and MAPK activation induced by lipopolysaccharide (LPS) in BV2 cells pretreated with thrombin. Cells were pretreated with 20 U/ml thrombin for 1–6 h and then treated with 100 ng/ml LPS for 3 h. We found that iNOS mRNA expression was lower in these cells compared to cells treated with LPS alone (i.e., no thrombin pre-treatment) or compared to cells simultaneously co-treated with thrombin and LPS (co) (Fig. 5A, upper panel). We found that the longer the thrombin pretreatment, the more powerful was the inhibition.

In an agreement with iNOS mRNA expression, LPS-induced NO production was reduced in cells pretreated with thrombin. No production was determined by measuring nitrite formed from NO in the media. In response to 100 ng/ml LPS, cells non-pretreated with thrombin produced 10.63 ± 0.90 μM nitrite. However, cells pretreated with thrombin produced only 5.94 ± 0.48 μM nitrite in the same condition (Fig. 5A, lower panel). Cells untreated or treated with only thrombin produced 2.99 ± 0.47 or 2.91 ± 0.19 μM (Fig. 5A, lower panel).

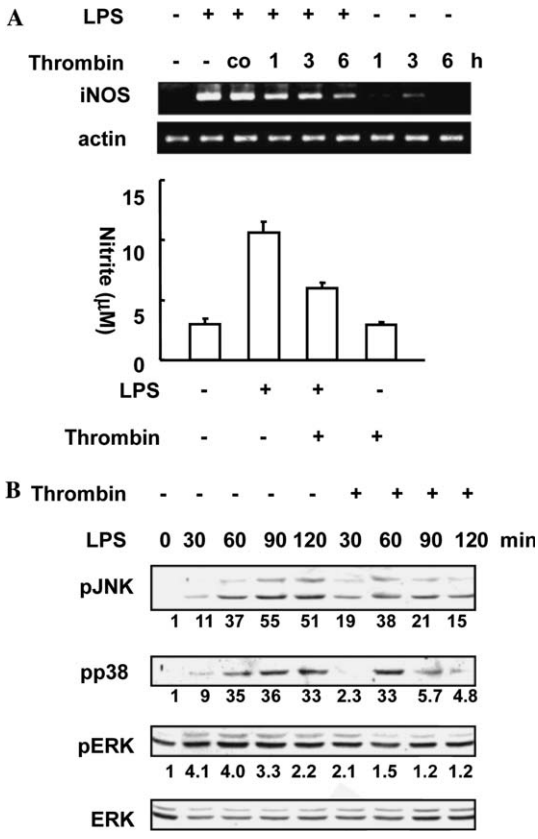

Fig. 5. Thrombin pretreatment suppresses LPS-induced iNOS mRNA expression/NO release and activation of MAP kinase. (A, upper panel) BV2 microglia were treated with 100 ng/ml LPS alone or together with 20 U/ml thrombin (co) for 3 h, or pretreated with 20 U/ml thrombin for the indicted times and subsequently stimulated with 100 ng/ml LPS for 3 h. iNOS mRNA expression was determined using RT-PCR. (A, lower panel) BV2 cells were pretreated with 20 U/ml thrombin for 6 h and then treated with 100 ng/ml LPS for 48 h. The amount of nitrite formed from NO was measured as described in "Materials and methods." (B) BV2 cells were treated with LPS alone for the indicated times, or pretreated with 20 U/ml thrombin for 6 h and then treated with LPS for the indicated times. Phosphorylated MAP kinases (pJNK, pp38, and pERK) were detected using Western blotting. The Arabic figures under each blot represent the normalized intensities of the bands of phosphorylated kinases against those of unphosphorylated ERK bands using Image Gauge program.

Next, we investigated the effect of thrombin pretreatment on the intensity and duration of phosphorylation of ERK, JNK, and p38 MAP kinase induced by LPS since these kinases mediate LPS-induced microglial NO production [23]. LPS-induced phosphorylation of JNK and p38 reached to the peak at 60–90 min with similar intensities in cells either non-treated or pretreated with thrombin. However, phosphorylation of JNK and p38 rapidly reduced in cells pretreated with thrombin while phosphorylation of them was sustained for up to 120 min in cells non-treated with thrombin. On the contrary, ERK activation was just slightly attenuated in cells pretreated with thrombin through the whole experimental times from 30 to 120 min (Fig. 5B). These results suggest that via expression of negative regulators of inflammation such as SOCS3, thrombin can regulate the duration and extent of microglial activation by attenuating the action of continuously applied inflammatory stimulators.

Discussion

Despite the essential roles of inflammation in host defense mechanisms, bio-organisms must regulate the intensity and duration of inflammation to minimize its harmful side effects. The data from this study revealed that thrombin, an important inflammatory stimulator [14], can stimulate expression of SOCS. That thrombin can stimulate expression of both pro- and anti-inflammatory mediators suggests by which thrombin can control the duration and extent of inflammation.

SOCS expression is known to be mediated via the JAK/STAT pathways [24,25]. In vascular smooth muscle cells it has been reported that thrombin activates JAK/STAT pathways [26]. In contrast, here we found that in microglia, thrombin appeared to have little effect on JAK/STAT activation, as determined using antibodies against phosphorylated forms of JAK/STATs. Others have also reported JAK/STAT-independent SOCS expression. IL-10-induced SOCS3 expression independently of STATs [27], and CpG DNA-induced expression of SOCS1 and SOCS3 via ERK- and p38 MAP kinase-dependent pathways in J774 macrophages [28].

PKC has been reported to be an important downstream signaling mediator for thrombin [14]. We found

that inhibition of PKC significantly reduced thrombin-stimulated SOCS3 expression. It appeared PKCδ in particular was the isoform that mediated SOCS3 expression in thrombin-treated cells. Thrombin-induced PKCδ phosphorylation, indicating activation [17–22], and rottlerin, a specific inhibitor of PKCδ, reduced thrombin-stimulated SOCS3 expression. We also examined whether mitogen-activated protein kinases (MAPKs) were involved in thrombin-induced SOCS3 expression. We found neither ERK pathway (PD98059) nor p38 MAP kinase (SB203580) inhibitors affected thrombin-induced SOCS3 expression, while both inhibitors reduced thrombin-induced iNOS expression (data not shown). These results suggest that thrombin-induced SOCS3 and iNOS expression are separately regulated in microglia.

It was important to determine whether SOCS expression affected the duration and extent of inflammation in our system. We found that thrombin pretreatment significantly reduced LPS-induced iNOS expression/NO release and activation of MAP kinase in microglia. It has been reported that SOCS regulates a diverse range of signaling pathways in addition to JAK/STAT pathways. SOCS1 inhibits NF-κB transcription activity induced by LPS [29]. In SOCS1-deficient pancreatic β cells, TNF-α-induced activation of p38 MAP kinase and expression of iNOS are enhanced [30]. Since NF-κB and MAP kinase are important mediators of LPS-induced iNOS expression in microglia [23,31], thrombin pretreatment may suppress LPS-induced iNOS expression by reducing activation of NF-κB and MAP kinase through expression of SOCS. We also examined whether IFN-γ-induced STAT activation was reduced in microglial cells pretreated with thrombin since SOCS3 inhibits IFN-γ-induced gene expression by suppressing tyrosine phosphorylation of STAT1 and STAT3 [7,32]. Unexpectedly, we found that thrombin pretreatment did not alter tyrosine phosphorylation of STAT1, 3, 5, nor MCP-1 mRNA expression in IFN-γ-treated microglia (data not shown). It has been suggested that PKCδ is critical for STAT-mediated transcription [33,34]. In thyroid cells, PKC inhibition reduced TSH-induced STAT3 tyrosine phosphorylation [35]. Thus, thrombin might influence IFN-γ-induced STAT activation both positively and negatively by activating PKC and inducing SOCS, respectively.

In summary, the present study suggests that thrombin not only induces inflammatory mediators but also induces negative regulators of inflammation, such as SOCS. This could be a mechanism to prevent sustained inflammation even in the continuous presence of inflammatory stimulators. Therefore, inflammatory cells, such as microglia, may possess auto-regulatory mechanisms to prevent severe inflammation that causes surrounding tissue damage.

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