Pro-Inflammatory Role of S1P3 in Macrophages

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Abstract

Sphingosine 1-phosphate (S1P) is a specific agonist for five G protein-coupled receptors, S1P1-5 (Park and Im, 2017). In response to inflammatory stimuli, up-regulation or activation of sphingosine kinase 1 (SphK1) and increased generation of its product, S1P, have been observed in many cell types, including RAW264.7 macrophages and microglia (Xia et al., 1998; Pettus et al., 2003; Hammad et al., 2008; Jin et al., 2018). S1P caused pro-inflammatory responses; for examples, S1P increases COX-2, iNOS, PGE2 levels, interleukin-1β (IL-1β) as well as tumor necrosis factor-α (TNF-α) in several cell types, including murine peritoneal macrophages (Lee et al., 2002; Pettus et al., 2003; Hammad et al., 2008; Muller et al., 2017). On the contrary, S1P was shown to block lipopolysaccharide (LPS)-dependent stimulation of NF-κB activation and NO production, implying that S1P exerts an anti-inflammatory role (Hughes et al., 2008). The inhibitory role of S1P2 in macrophage recruitment during inflammation (Michaud et al., 2010) and the proatherogenic role of S1P3 signaling in the plaque macrophage have been reported (Skoura et al., 2011). Therefore, although anti-inflammatory function of S1P1 has been reported, specific S1P receptors involved in the pro-inflammatory function of S1P have not been fully elucidated. Especially, the function of S1P3 in macrophages has not been studied well. Therefore, we hypothesized that S1P3 induction in the inflammatory conditions and its pro-inflammatory roles. Targeting S1P3 might be a strategy for regulating inflammatory diseases.

Key Words: S1P, S1P3, Macrophage, Inflammation, Caspase 1, GPCR
MATERIALS AND METHODS

Materials
LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). TY52156 was purchased from Tocris Bioscience (cat. 5328, Bristol, UK).

Isolation and culture of mouse peritoneal macrophages
Mouse peritoneal macrophages were isolated from the peritoneal cavity of C57BL/6 mice treated with 3% thioglycollate at 4 days after treatment and cultured at 37°C in a 5% CO2 humidified incubator. Isolated macrophages were maintained in RPMI1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 μg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 18 h, and then incubated in 0.5% FBS-containing media for 24 h. Samples for RNA or protein analysis were collected at 5 h or 24 h after treatment with LPS (10 ng/ml or 100 ng/ml), respectively. TY52156 was added 1 h before LPS treatment.

Reverse Transcription-PCR
Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined by a Nanodrop ND-1000 spectrophotometer. One microgram of RNA was transcribed by using a ImProm-II Reverse Transciption System (Promega, Madison, WI, USA) in accordance with the manufacturer’s protocol. First-strand cDNA was synthesized from isolated total RNA using Trizol reagent (Invitrogen). Synthesized cDNA products and primers for each gene were used for PCR, which was conducted using Go-Taq DNA polymerase (Promega). Specific primers for IL-6 (sense 5’-CCG GAG AGG AGA CTT CAC AG-3’, antisense 5’-TGG TCT TGG TCC TTA GCC AC-3’), IL-1β (sense 5’-GGA GAA GCT GTG GCA GCT A-3’, antisense 5’-GCT GAT GTA CCA GTT GGG GA-3’) were used to amplify gene fragments. PCR was performed over 27 amplification cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s) in an Mastcycler gradient PCR machine (Eppendorf, Hamburg, Germany). Specific primers for TNF-α (sense 5’-CAA AGA AAG CCG CCT CAA AC-3’, antisense 5’TTC ACA GAG AGG GTC ACA GC-3’) were used and annealing was performed at 57°C. iNOS (sense 5’-ACC TAC CAC ACC CGA GAT GGC GTA CAG-3’, antisense 5’-GGA GGA GCT GTG GTG AAT-3’) and GAPDH (sense 5’-ACC ACC ATG GAG AAC AC-3’, antisense 5’-TTG TCT TGG TCC TTA GCC AC-3’) were used and annealing was performed at 57°C and over 30 amplification cycles. For S1P1 (sense 5’-CAC CGG CCC ATG TAC TAT-3’, antisense 5’-CCA GCC CAT ATC TAA CG-3’), S1P2 (sense 5’-CAA GAG AGT GCC CTA TGT-3’, antisense 5’-GGA GTG AGT GCC TTA TTA GC-3’), S1P3 (sense 5’-GCA GAC ATG GAC TGT CTA G3’), and GAPDH (sense 5’-TTG TCT TGG TCC TTA GCC AC-3’, antisense 5’-CA AAG TAA CTA TCT-3’), annealing was undertaken at 57°C. Aliquots (5 μl) were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Western blotting
Macrophages were harvested and resuspended in RIPA lysis buffer (GenDEPOT, Baker, Carlsbad, CA, USA). Concentrations were measured using a BCA protein assay kit (Thermo Scientific). An equal amount of protein was loaded and separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane and blocked in 5% (w/v) non-fat milk in TBST at room temperature for 1 h. Membranes were incubated overnight at 4°C with the primary antibodies against iNOS (cat. #5692, Cell Signaling Technology) and GAPDH (cat. #5174, Cell Signaling Technology) in 5% (w/v) non-fat milk in TBST. Membranes were incubated with anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature. Membranes were washed and developed with an ECL kit (Amersham, Piscataway, NJ, USA). The relative protein levels were estimated by densitometry analysis.

Fig. 1. Changes in the expression of S1P1 and SphK1 caused by LPS stimulation. (A) Mouse peritoneal macrophages were treated with vehicle or LPS 10 ng/mL for 5 h, and RT-PCR was then performed to detect the expression of the S1P1, S1P2, S1P3, and SphK1 genes. The data shown are representatives of three independent experiments. (B) Histogram of quantitated mRNA expression. Results are the mean ± standard deviation of three independent experiments. Statistically significant at **p<0.01 level vs. the vehicle-treated macrophages.
The concentration of proteins was determined using a BCA protein assay (ThermoScientific, Rockford, IL, USA). Proteins (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose paper, which was incubated with specific primary antibodies recognizing β-actin, COX-2, iNOS, IL-1β, and caspase 1, and then incubated HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA). Signals were developed using an enhanced chemiluminescence system (Pierce Biotechnology Inc., Rockford, IL, USA).

Nitrite measurement

NO production was estimated by measuring the amount of nitrite (a stable metabolite of NO) in medium using Griess reagent, as previously described (Kang et al., 2018). Cells were pretreated with different concentrations of TY52156 for 1 h and subsequently stimulated with LPS (100 ng/ml) for 48 h. Nitrite concentrations in the medium was determined using a Griess Reagent System (Promega).

ELISA

Ninety-six-well plates (NUNC) were coated with capture antibodies overnight at 4°C (eBioscience, San Diego, CA, USA, IL-1β cat 14-7012-85). The plates were washed, and then blocked with blocking buffer for 1 h at room temperature. Standard dilutions of cytokines were prepared and added to the wells with supernatants. The plates were incubated for 2 h at room temperature with shaking, and then washed five times. Biotinylated detection antibody (eBioscience, IL-1β cat 13-7112-81) was then added to the wells, which were incubated for 1 h at room temperature with shaking. The plates were then washed five times and Avidin-HRP was added for 30 min at room temperature with shaking. The plates were then washed fourteen times and incubated with substrate solution for 15 min at room temperature. Stop solution (eBioscience) was then added and absorbance was read at 450 nM.
Animals
Eight- to ten-week-old male C57BL/6 (19-22 g) mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a Laboratory Animal facility at Pusan National University (Busan, Korea), and provided food and water ad libitum. The animal protocol used in this study was reviewed and approved beforehand by the Pusan National University-Institutional Animal Care Committee (PNU-IACUC) with respect to ethics and scientific care.

Statistics
All results were expressed as mean ± standard deviation. The data were analyzed by one-way ANOVA. A p-value<0.05 was considered statistically significant.

RESULTS

Induction of S1P3 in peritoneal macrophages
Previously, LPS, a cell wall component of Gram-negative bacteria, was shown to induce the expression and activation of SphK1 in RAW264.7 macrophages. In addition, its product, S1P, has been well studied in relation with the functions of S1P1 and S1P2. We confirmed an LPS-induced increase of SphK1 expression as well as the presence of S1P3 mRNA and its simultaneous induction in mouse peritoneal macrophages after LPS treatment (Fig. 1). Because S1P3 has been poorly investigated in inflammatory responses compared to S1P1 and S1P2, we studied the function of S1P3 by using an S1P3 antagonist, TY52156 (Murakami et al., 2010).

Fig. 4. Effect of TY52156 on the protein expression of COX-2 in macrophages. Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with LPS 100 ng/mL for 24 h. Western blotting was conducted on cell lysates. The data shown in (A) are representative of three independent experiments. Relative protein levels of COX-2 versus β-actin are presented as histograms (B). Results are the means ± standard deviation of three independent experiments. Statistically significant at ***p<0.001 level vs. the vehicle-treated macrophages, and at *p<0.05 vs. the LPS-treated macrophages.

Fig. 5. Effect of TY52156 on the expression of IL-1β, IL-6, and TNF-α in macrophages. (A) Mouse peritoneal macrophages were treated with the indicated concentrations of TY52156 for 1 h, and then treated with vehicle or LPS 10 ng/mL for 5 h, and RT-PCR was performed to detect the expression of the pro-inflammatory genes, IL-1β, IL-6, and TNF-α. The data shown are representative of three independent experiments. (B-D) Histograms of quantitated mRNA expression of IL-1β, IL-6, and TNF-α. Results are the means ± standard deviation of three independent experiments. Statistically significant at ***p<0.001 level vs. the vehicle-treated macrophages, and at *p<0.05, **p<0.01 and ***p<0.001 vs. the LPS-treated macrophages.
TY52156 inhibits induction of COX-2 and iNOS in peritoneal macrophages

In the presence of 10 ng/ml LPS, expression of pro-inflammatory genes, iNOS and COX-2, was increased, and the induction of iNOS and COX-2 expressions at mRNA level was significantly inhibited by TY52156 in a concentration-dependent manner (Fig. 2).

The inhibitory effect of TY52156 on iNOS and COX-2 was studied at protein level. In the presence of 100 ng/ml LPS, western samples were made. The protein expression of iNOS was significantly suppressed by treatment with TY52156 (Fig. 3A, 3B). Also, the level of NO, the product of iNOS, was significantly decreased (Fig. 3C).

The inhibitory effect of TY52156 on COX-2 mRNA expression was also further studied at protein level. As shown in Fig. 4, TY52156 strongly inhibited the induction of COX-2 at protein level in a concentration-dependent manner (Fig. 4).

TY52156 inhibits induction of IL-1β in peritoneal macrophages

Treatment of TY52156 also inhibited LPS-induced induction of other inflammatory genes of IL-1β, IL-6 and TNF-α (Fig. 5). Because treatment of TY52156 inhibited LPS-induced induction of the IL-1β gene (Fig. 5), the protein level of IL-1β was examined by western blotting and ELISA (Fig. 6). TY52156 inhibited induction of preform and mature form IL-1β. ELISA result showed significant inhibition on IL-1β secretion in the media (Fig. 6D).

TY52156 inhibits caspase 1 expression in peritoneal macrophages

Furthermore, we evaluated the expression of caspase 1, which converts the preform IL-1β to mature IL-1β. Caspase 1 expression was induced by LPS and suppressed by treatment of TY52156 (Fig. 7).

On the basis of these findings, we hypothesized that LPS stimulation may induce not only inflammatory genes, such as, iNOS, caspase 1, and IL-1β, but also SphK1 and S1P3. Induction of SphK1 may increase production of S1P, which activates S1P3, thereby amplifying inflammatory response. In order to confirm this signaling route, we applied dimethylsphingosine (DMS), a specific inhibitor of SphK1. As shown in Fig. 8, in the presence of DMS, LPS induced less expression of caspase 1. However, treatment with CYM5541, a specific agonist of S1P3, reversed DMS-induced gene suppression (Fig. 8), implying that the induction of SphK1 and its product, S1P3, and the action of S1P on S1P3 sequentially mediated the pro-inflammatory response of LPS in macrophages (Fig. 9).

DISCUSSION

In this study, we found that LPS induced S1P3 expression in murine macrophages. Previously, expression of S1P3 was reported to be very low in macrophages (Lee et al., 2002). However, its expression was confirmed by immunofluorescence, RT-PCR and western blotting in human monocytes/macrophages and murine bone marrow-derived macrophages (Duong et al., 2004; Durafourt et al., 2011; Yang et al., 2015; Muller et al., 2017). In addition, S1P3 was induced during mac-
Upregulation of S1P3 in astrocytes was also previously reported in a mouse model of Sandhoff disease, a prototypical neuromuscular lysosomal storage disorder (Wu et al., 2008). Therefore, induction of S1P3 has been reported in many cell types.

In our study, SphK1 and S1P3 were concomitantly upregulated, but S1P1 was downregulated. Similarly, concomitant regulation of SphK1 and S1P3 was shown to play a pivotal role in mouse cardiac fibrosis and in the transdifferentiation of myoblasts into myofibroblasts (Cencetti et al., 2010; Takuwa et al., 2010). Furthermore, SphK1 and S1P3 were functionally upregulated in astrocytes under pro-inflammatory conditions (Fischer et al., 2011).

In this study, we observed a pro-inflammatory function of S1P3, which was supported by the result of a previous study that bone marrow-derived S1P3-deficient macrophages produced low MCP-1 in response to LPS stimulation (Keul et al., 2011). Previously, the functions of S1P3 have been reported in macrophage migration. S1P exerts a potent migratory action in bone marrow-derived macrophages via S1P2 and S1P3 in cholestatic liver injury (Yang et al., 2015). In vitro, S1P induces a chemotactic action in wild-type peritoneal macrophages, but not in S1P3-deficient peritoneal macrophages (Keul et al., 2011). S1P3 mediates the chemotactic effect of S1P in macrophages in vitro and in vivo, and plays a causal role in atherosclerosis by promoting inflammatory recruitment of monocyte/macrophage (Keul et al., 2011). This was also supported by a finding that S1P3, a constituent of HDL, acutely protects the heart against ischemia/reperfusion injury in vivo via an S1P3-mediated and NO-dependent pathway (Thelmieier et al., 2006).

We observed that S1P3 mediated iNOS, COX-2, IL-1β, IL-6, and TNF-α expression under LPS stimulation (Fig. 9). The pro-inflammatory action of S1P has previously been reported in several cell types, including macrophages. For examples, SphK1 activation by LPS or cytokines, including TNF-α and IL-1β, has been observed (Xia et al., 1998; Pettus et al., 2003; Billich et al., 2005; Hammad et al., 2008; Nayak et al., 2010; Snider et al., 2010). Moreover, S1P caused increases in COX-2 and PGE2 levels in several cell types (Pettus et al., 2003; Hammad et al., 2008). S1P increases the expression of iNOS under M2-polarizing conditions (Muller et al., 2017). Furthermore, S1P induces the release of IL-1β and TNF-α from murine peritoneal macrophages (Lee et al., 2002). Therefore, S1P has been reported to play crucial roles in pro-inflammatory responses. However, a specific S1P receptor for the responses has not been elucidated, and for the first time S1P3 was reported as the pro-inflammatory receptor in this study (Fig. 9). On the basis of the results of this study, S1P targeting therapeutics may be beneficial as an anti-inflammatory therapy.
S1P3, resulting in increased productions of prostaglandins (PGs), produced by SphK1 activates pro-inflammatory signaling through NF-κB.


