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Original research article

Sphingosine-1-phosphate receptor-2 mediated NFκB activation contributes to tumor necrosis factor-α induced VCAM-1 and ICAM-1 expression in endothelial cells

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ABSTRACT

Sphingosine-1-phosphate (S1P) regulates a wide array of biological functions in endothelial cells. We previously showed that S1P receptor subtype 2 ($S1P_2$) is significantly up-regulated in the atherosclerotic endothelium (*J. Biol. Chem.* 283:30363, 2008). In this study, we investigated the roles of $S1P_2$-mediated signaling in the proinflammatory responses of endothelial cells. Treatment with tumor necrosis factor-α (TNFα), a proinflammatory cytokine, increased the expression of $S1P_2$ receptors in endothelial cells. TNFα treatment also enhanced sphingosine kinase 1 expression and increased S1P production. Pharmacological inhibition or knockdown of $S1P_2$ receptors completely abrogated the TNFα-induced VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular adhesion molecule 1) expression in endothelial cells. In contrast, pharmacological inhibition or knockdown of other S1P receptor subtypes had no effect on the TNFα-stimulated ICAM-1 and VCAM-1 expression. Moreover, ectopic expression of $S1P_2$ receptors increased VCAM-1 and ICAM-1 expression in endothelial cells in response to S1P stimulation. Mechanistically, we show that antagonizing $S1P_2$ signaling markedly inhibited the TNFα-stimulated NFκB activation. Utilizing the NFκB reporter luciferase assay, the S1P/$S1P_2$ signaling was shown to stimulate NFκB activation. Moreover, the S1P/$S1P_2$-stimulated VCAM-1/ICAM-1 expression was completely abolished by the pharmacological inhibitor of NFκB. Collectively, our data suggest that TNFα treatment activates autocrine S1P/$S1P_2$ signaling, which subsequently activates NFκB and leads to the proinflammatory responses in endothelial cells.

1. Introduction

Atherosclerosis, a chronic inflammatory process, is characterized by the thickening of the arterial wall and is the primary cause of cardiovascular disease and cerebrovascular accident, two of the most common causes of illness and death worldwide [1]. An important risk factor for developing atherosclerosis is vascular inflammation, which involves the complex interactions of modified lipoproteins, monocyte-derived macrophages or foam cells, T lymphocytes, endothelial cells, and smooth muscle cells [2,3].

Spingosine-1-phosphate (S1P), a serum-borne bioactive lipid mediator synthesized by sphingosine kinase (SphK), regulates an array of biological activities in various cell types [4–7]. S1P can function either as an extracellular ligand or intracellular mediator [8–10]. When functioning as an extracellular ligand, S1P-regulated biological activities are mediated by the S1P family of G protein-coupled receptors (GPCRs) [8,11,12]. There are five identified members of the S1P receptor family: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$ (previous nomenclature: EDG-1, -5, -3, -6, -8, respectively)

Abbreviations: S1P, sphingosine-1-phosphate; GPCR, G-protein coupled receptor; $S1P_{1-5}$, high affinity GPCRs for S1P; TNFα, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; SphK, sphingosine kinase; HUVEC, human umbilical vein endothelial cells; ECs, cultured endothelial cells.

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[13]. S1P receptor subtypes couple to different G_α polypeptides to regulate distinct signaling pathways [14–16]. Various S1P receptors are expressed in distinct combinations in different cell types to produce the appropriate biological effects. For example, $S1P_1$, $S1P_2$, and $S1P_3$ receptors are expressed in endothelial cells (ECs) [17,18]. The signaling pathways regulated by $S1P_1$ and $S1P_3$ are required for cellular chemotaxis, adherens junction assembly, endothelial morphogenesis, and angiogenic response [17,19]. Moreover, the balance of $S1P_1$ and $S1P_2$ signaling contributes to vascular integrity *in vivo* [20]. Disturbance of this balance, *i.e.*, the up-regulation of $S1P_2$ signaling, may have functional implications in vascular dysfunctions such as endothelial senescence and atherosclerosis [18].

It has been shown that sphingosine kinase (SphK) plays a role in inflammatory disorders, such as rheumatoid arthritis [21,22]. However, the molecular details of S1P signaling in the regulation of inflammation remain to be elucidated. Recent studies demonstrated that $S1P_2$ deficiency led to inhibition of macrophage proinflammatory responses and atherosclerosis in apoE-deficient mice [23]. Moreover, it was shown that $S1P_3$ mediates the chemotactic effect of S1P in macrophages *in vitro* and *in vivo*, and plays a causal role in atherosclerosis by causing inflammatory monocyte/macrophage recruitment and altering smooth muscle cell behavior [24]. These studies suggest that S1P signaling cascades play an important role in the development of atherosclerosis.

However, how S1P signaling contributes to atherosclerosis and vascular inflammation, particularly in the context of the endothelium, is poorly understood. We previously showed that senescence-associated endothelial dysfunctions can be attributed to the up-regulation of $S1P_2$ signaling [18]. We also found that $S1P_2$ receptors were markedly increased in atherosclerotic endothelium [18]. These results suggest that the up-regulated endothelial $S1P_2$ signaling may play a role in mediating endothelial proinflammatory responses and atherosclerosis development. Therefore, we examined roles of S1P signaling in ECs for proinflammatory cytokine-induced endothelial inflammation in this study. We observed that TNFα treatment increased the expression of $S1P_2$ receptors and production of S1P in ECs. More importantly, inhibition of endothelial $S1P_2$ signaling significantly diminished the TNFα-induced activation of NFκB and expression of proinflammation-associated adhesion molecules (*i.e.* VCAM-1, ICAM-1) in ECs. Furthermore, $S1P_2$ signaling is capable of activating NFκB directly. Also, S1P stimulation increases the expression of VCAM-1/ICAM-1 in ECs, ectopically expressing $S1P_2$ receptors. Collectively, our results suggest that $S1P_2$ signaling plays an important role in proinflammatory cytokine induced endothelial inflammation.

2. Materials and methods

2.1. Reagents

Sphingosine-1-phosphate was purchased from Biomol; JTE-013 and VPC23019 were from Avanti. BAY 11-7085 was from Calbiochem EMD. Mouse-derived $S1P_1$ monoclonal antibody (E1-49) was generated by conventional hybridoma technology using bacteria expressed $S1P_1$ as an immunogen [25,26]. E1-49 was shown to specifically react with the human $S1P_1$ receptor by immunostaining, immunoblotting, and immunoprecipitation [20,25,26]. Polyclonal anti-$S1P_2$ and anti-$S1P_3$ were from Cayman Chemical. Monoclonal Anti-human ICAM-1/CD54 antibody was purchased from R&D Systems. Polyclonal rabbit anti-VCAM-1 (H-276), phospho-IκBα p65 (Ser32) (14D4) rabbit mAb, and polyclonal rabbit anti-actin (H-300) were purchased from Santa Cruz. Rabbit polyclonal anti-phospho-IκBα p65 was purchased from Millipore. Peroxidase-conjugated sheep anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were from MP Biomedicals and Pierce Biotechnology, respectively. Alexa conjugated secondary antibodies were from Molecular Probes. C17-sphingosine, C17-sphingosine-1-phosphate and D7-sphingosine were purchased from Avanti. Other reagents, unless specified, were purchased from Sigma.

2.2. Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVECs, Clonetics, Cc-2517, passage 4–12) were cultured in M199 medium (Mediatech) supplemented with 10% fetal bovine serum (FBS, Hyclone) and heparin-stabilized endothelial cell growth factor as we previously described [18]. Chinese hamster ovary (CHO) cells were cultured in RPMI1640 (HyClone) with 10% FBS.

2.3. RT-PCR and real-time PCR

Total RNA was isolated from cultured cells and RT-PCR was performed as we described previously [27]. PCR primer pairs used were: human $S1P_1$: sense, 5′-GCACC AACCC CATCA TTTAC-3′, anti-sense, 5′-TTGTC CCCTT CGTCT TTCTG-3′; human $S1P_2$: sense, 5′-CAAGT TCCAC TCGGC AATGT-3′, anti-sense, 5′-CAGGA GGCTG AAGAC AGAGG-3′; human $S1P_3$: sense, 5′-TCAGG GAGGG CAGTA TGTTC-3′, anti-sense, 5′-GAGTA GAGGG GCAGG ATGGT-3′; human ICAM-1: sense, 5′-GGCTG GAGCT GTTTG AGAAC-3′, anti-sense, 5′-CCTCT GGCTT CGTCA GAAT C-3′; human VCAM-1: sense, 5′-GGTGC TGCAA GTCAA TGAGA-3′, anti-sense, 5′-AAGAT GGTCG TGATC CTTGG-3′; human sphingosine kinase 1 (SphK1): sense, 5′-GAGGC TACAC AGGGG CTT-3′, anti-sense, 5′-TTTGG CTGAG GCTGA AAT-3′; human SphK2: sense, 5′-GCACA GCAAC AGTGA GCA-3′, anti-sense, 5′-GAGCC TGAGT GAGTG GGA-3′; human GAPDH: sense, 5′-GAAGG TGAAG GTCGG AGTC-3′, anti-sense, 5′-GAAGA TGGTG ATGGG ATTTC-3′. For real-time PCR quantitation, 50 ng of reversely transcribed cDNAs were amplified with the ABI 7500 system (Applied Biosystems) in the presence of TaqMan DNA polymerase. The sense and antisense primers used to detect the gene expression of S1P receptor subtypes and GAPDH were purchased from Applied Biosystems. The sense and antisense primers of VCAM-1, ICAM-1, SphK1, and SphK2 were purchased from GeneScript. The qPCR was performed by using a universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

2.4. Sphingosine kinase activity assay by LC-MS/MS

HUVECs were washed with ice cold PBS and scraped in 200 μl SK buffer (20 mM Tris buffer, pH 7.4, 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 1 mM PMSF) containing 1× Protease inhibitor cocktail (Calbiochem). Subsequently, cells were disrupted by sonication (three times, 5 s each) at 4 °C using a Vibra Cell ultrasonic processor (Sonics & Materials), followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The protein concentration of the resulting supernatant was determined by a bicinchoninic acid protein assay kit (Thermo Scientific) with BSA as the standard. The enzymatic activity of sphingosine kinase (SphK) was then measured as described previously [28] with minor modifications. Briefly, 60 μg of proteins were used in the enzyme assay. The reaction was initiated by adding 5 μl of 200 μM C17-sphingosine (dissolved in 5% Triton X-100) and 5 μl of 20 mM ATP containing $MgCl_2$ (200 mM) in a final volume of 100 μl. After incubation at 37 °C for 20 min, the reaction was terminated with 10 μl of 1 M HCl, and then 100 ng of D7-sphingosine was added as an internal standard. After vigorous vortexing for 10 s, samples were stood at room temperature for 5 min, and then 223 μl of methanol were added. The mixture was vigorously vortexed for

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