Sphingosine 1-Phosphate Has Dual Functions in the Regulation of Endothelial Cell Permeability and Ca\(^{2+}\) Metabolism

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ABSTRACT

Ca\(^{2+}\) signaling plays an important role in endothelial cell (EC) functions including the regulation of barrier integrity. Recently, the endogenous lipid derivative, sphingosine-1-phosphate (S1P), has emerged as an important modulator of EC barrier function. We investigated the role of endogenously generated S1P in Ca\(^{2+}\) metabolism and barrier function in human umbilical endothelial cells (HUVECs) stimulated by thrombin, histamine, or other agonists. Barrier function was assessed by dextrans transients through HUVEC monolayers, and Ca\(^{2+}\) transients were measured using a fluoroprobe. Thrombin or histamine increased Ca\(^{2+}\) release from the endoplasmic reticulum (ER) and Ca\(^{2+}\) entry through store-operated channels (SOCs) that was accompanied by increased EC permeability. Inhibition of S1P synthesis by a specific sphingosine kinase inhibitor (SKI) decreased thrombin or histamine-induced increased permeability and decreased Ca\(^{2+}\) entry via SOC in a concentration-dependent fashion. SKI had minuscule effects on thrombin or histamine-induced Ca\(^{2+}\) release from ER. SKI also inhibited thapsigargin or ionomycin-induced Ca\(^{2+}\) entry via SOC without affecting Ca\(^{2+}\) release from the ER. In contrast to the effects of endogenously generated S1P, when S1P was administered externally, it initiated Ca\(^{2+}\) release from ER similar to thrombin and histamine while decreasing EC permeability. These observations indicate that after agonist-induced conditions, endogenously generated S1P functions as a positive modulator of Ca\(^{2+}\) entry via SOC and a mediator of increased cell permeability. In contrast, extracellular exposure to S1P has different signaling mechanisms and effects. Thus, the potential dual roles of endogenous and exogenous S1P on EC function need to be considered in pharmacological studies targeting sphingosine metabolism.

Alterations in endothelial cell (EC) barrier integrity are important determinants of organ pathology following inflammation, sepsis, or septic shock (Hallström et al., 1991). For example, increases in EC permeability is one of the underlying causative mechanisms of the adult respiratory distress syndrome, which is one of the most frequent life-threatening inflammatory mediators including cytokines, nitric oxide, activated complement proteins, or reactive oxidant species may also contribute to changes in EC Ca\(^{2+}\) signaling and associated changes in permeability and barrier function (Hasleton and Roberts, 1999).

In recent years, the endogenous lipid derivative, sphingosine-1-phosphate (S1P), has emerged as a potentially im-
portant regulator of EC barrier function (Itagaki and Hauser, 2003; McVerry and Garcia, 2004; Finigan et al., 2005; Seol et al., 2005; Zheng et al., 2006). Upon a variety of stimuli leading to protein C activation, S1P is generated from sphingosine through the action of ubiquitous sphingosine kinase (SK). The newly produced intracellular S1P may act on the Ca\(^{2+}\) signaling machinery through not yet identified intracellular receptors or by interacting with the components of Ca\(^{2+}\) transporters. Furthermore, S1P can be released from platelets or white blood cells at the sites of EC injury, thrombus formation, or neutrophil attachment, thereby targeting ECs from the extracellular space (Itagaki and Hauser, 2003). Because exogenous administration of S1P has been shown to enhance EC barrier integrity, it is believed that S1P released into the blood stream supports endothelial function during inflammatory states (McVerry and Garcia, 2004; Finigan et al., 2005; Seol et al., 2005). Despite these observations, the exact role of S1P in the regulation of Ca\(^{2+}\)mediated EC permeability has not been fully elucidated.

Therefore, in the current study, we investigated the relationship among S1P metabolism, Ca\(^{2+}\) signaling, and EC permeability utilizing two physiologically relevant mediators, thrombin and histamine. We also employed a recently developed highly specific sphingosine kinase inhibitor (SKI) to modulate intracellular S1P levels and test associated changes in cellular Ca\(^{2+}\) signaling. We hypothesized that S1P generated intracellularly by SK or administered exogenously has distinct and different effects on Ca\(^{2+}\) signaling and endothelial cell permeability.

**Materials and Methods**

**Materials.** The SKI, 2-[(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole, which was formally named SK-II, was developed and synthesized by our group as described previously (French et al., 2003) and was used as a specific sphingosine kinase inhibitor previously (Lee et al., 2004, 2005; Francy et al., 2007; Jung et al., 2007). In this article, we described SK-II as SKI for simplicity. S1P, thrombin, histamine, and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2-acetoxymethyl ester was from Molecular Probes (Eugene, OR). Ionomycin was purchased from Calbiochem (La Jolla, CA). All other chemicals and reagents were from Sigma-Aldrich.

**Cell Culture.** Human umbilical vein endothelial cells (HUVECs) were from BioWhittaker (Walkersville, MD) and were cultured to confluence at 37°C with 95% O\(_2\)/5% CO\(_2\) in endothelial cell basal media. Only cells of low passage (three to five) were used for assays. SKI was administered in the concentration range of 10 to 40 \(\mu\)M. Only cells of low passage (three to five) were used for assays. Despite these observations, the exact role of S1P in the regulation of Ca\(^{2+}\)-mediated EC permeability has not been fully elucidated.

**Results**

**SKI Prevents Thrombin- and Histamine-Induced Increase in EC Permeability.** Figure 1A shows that thrombin significantly increased clearance of 40-kDa dextran rhodamine through a confluent monolayer of ECs. SKI caused a dose-dependent attenuation of thrombin-induced increase in EC permeability. Likewise, histamine caused an increase in EC permeability, and SKI abrogated the histamine-induced effect at 30 \(\mu\)M SKI (Fig. 1B). SKI alone did not cause any change in EC permeability, indicating that endogenous S1P activity does not significantly contribute to EC permeability under nonstimulated “baseline” conditions. Because SKI was dissolved in DMSO, we tested its effect on EC permeability. We found that DMSO had no effect on EC permeability at concentrations up to 0.5%. Taken together, these results indicate that S1P has a role in G-protein-coupled agonist-
induced changes in EC permeability. We next examined the cellular mechanisms of S1P effects on EC permeability.

**Effects of SKI on Thrombin- and Histamine-Induced Increase in Intracellular Ca²⁺ Concentration.** An increase in cytosolic Ca²⁺ has been shown as the initial pivotal signal for agonist-induced increase in EC permeability (Mehta and Malik, 2006). To determine whether SKI effects on EC permeability are associated with changes in intracellular Ca²⁺ concentration, ECs were loaded with Fura-2, and intracellular Ca²⁺ concentration was measured. Figure 2 shows the effects of thrombin and histamine on intracellular Ca²⁺ transients. Consistent with earlier observations (Mehta and Malik, 2006), upon EC stimulation with thrombin (Fig. 2A) or histamine (Fig. 2B), an increase in cytosolic Ca²⁺ concentration is apparent. There are two phases of Ca²⁺ transients. The initial peak is the result of Ca²⁺ release from the endoplasmic reticulum (ER) Ca²⁺ store, which is coupled to inositol 1,4,5-triphosphate receptors. The second, more sustained response is secondary to Ca²⁺ entry through non-selective cation channels known as store-operated Ca²⁺ channels (SOCs) (Mehta and Malik, 2006). Figure 2, A and B, show that thrombin and histamine caused immediate increase in the initial phase of the Ca²⁺ transient, presumably due to stimulation of Ca²⁺ release from the intracellular ER store (Itagaki et al., 2002; Itagaki and Hauser, 2003). When extracellular Ca²⁺ (1.8 mM) was added to the bath medium, the sustained component of Ca²⁺ transient was evident. Both agents induced dose-dependent increase in both phases of Ca²⁺ transients.

To elucidate the role of S1P on agonist-induced changes in EC Ca²⁺ signaling, cells were pretreated with a specific SKI, and the effects of thrombin and histamine on Ca²⁺ transients were determined. As shown in Fig. 3A, the addition of SKI did not significantly affect the thrombin-induced initial peak of Ca²⁺ transients from the ER. In contrast, the second Ca²⁺ influx via SOC was significantly reduced by pretreatment with SKI. Likewise, SKI blocked the histamine-induced second peak of Ca²⁺ transients without change in the initial peak (Fig. 3B). Because SKI decreased agonist-induced EC permeability, these results suggested that the Ca²⁺ influx via SOC after ER store depletion might promote EC permeability.

**Effects of SKI on Thapsigargin- and Ionomycin-Induced SOC Activation in EC.** The effects of SKI on Ca²⁺ transients were also assessed after release of Ca²⁺ from stores with the Ca²⁺ pump blocker, thapsigargin (TG), which depletes Ca²⁺ from the ER as a slow leak in the absence of external Ca²⁺ (Fig. 4A). Reintroduction of Ca²⁺ to the bath solution results in Ca²⁺ influx via SOC activation. Pretreatment with SKI blocked the second phase of Ca²⁺ entry without changing the initial phase of Ca²⁺ transients (Fig. 4A). We also conducted this experiment after store depletion with a Ca²⁺ ionophore, ionomycin (to eliminate Ca²⁺ release through reactivated inositol 1,4,5-triphosphate). Ionomycin induced a more rapid and complete release of stored Ca²⁺ (compared with Fig. 3 or 4A). Readdition of external Ca²⁺ resulted in SOC-mediated Ca²⁺ entry. Preincubation of EC with SKI reduced ionomycin-induced SOC activation during reintroduction of external Ca²⁺ without significant effects on the initial Ca²⁺ transients (Fig. 4B). These results suggest...
that the effects of SKI on agonist-induced increases in EC permeability are mediated by Ca\(^{2+}\) entry via SOC but not Ca\(^{2+}\) released from ER. Our results also suggest that ER Ca\(^{2+}\) stores and SOC are not coupled to regulate EC permeability.

**Effects of SKI on Thrombin-Induced S1P Synthesis.**

We next measured thrombin-induced S1P synthesis and the effects of SKI to confirm that SKI’s effects are mediated by alterations of intracellular S1P levels through the action of SK. Figure 5 shows the effects of thrombin on S1P production and the effects of SKI. Upon thrombin receptor stimulation, S1P synthesis was significantly increased in HUVECs. Pre-treatment with SKI inhibited S1P synthesis to a value comparable with the control level.

**Effects of Extracellularly Applied S1P on EC Permeability and Ca\(^{2+}\) Signaling.**

The presented findings indicate that inhibition of intracellular S1P production supports EC barrier function by decreasing agonist-induced Ca\(^{2+}\) influx through store-operated calcium entry. These observations however may be interpreted as contradictory to previous findings indicating that exogenously administered S1P improves EC barrier function (Mehta et al., 2005). Therefore, in the last series of experiments, we tested the effects of extracellular administration of S1P on EC permeability as well as cellular Ca\(^{2+}\) transients. In accordance with previous investigations by others, exogenous S1P administration to endothelial monolayers markedly decreased basal permeability, a finding that is consistent with enhanced barrier function (Fig. 6A). Furthermore, as shown in Fig. 6B, S1P administration to EC induced Ca\(^{2+}\) transients with very similar kinetics to that reported previously (Mehta et al., 2005).

**Discussion**

This study demonstrates for the first time that S1P manifests opposing effects on EC barrier function depending on whether it is generated intracellularly or targets ECs from the extracellular space. Although the surface receptor for S1P has not been identified, independent studies demonstrated that S1P administered extracellularly initiates G-protein coupled signaling and Rac activation followed by the release of Ca\(^{2+}\) from endoplasmic reticulum stores and subsequent Ca\(^{2+}\) entry via SOC (Mehta et al., 2005). These effects of S1P were accompanied by enhanced endothelial barrier functions that involved changes in cortactin translocation and its interaction with myosin light chain kinase.
histamine, and S1P alter cell permeability through different pathways. Thus, based on these observations, the finding that exogenous or endogenous S1P has different effects on cell permeability also suggests the potential existence of different extra- and intracellular S1P receptors and the involvement of different signaling pathways mediating these S1P effects. The potential dual roles of endogenous and exogenous S1P as a critical lipid messenger molecule regulating EC function are consistent with previous observations on other cells and experimental settings as summarized in recent reviews (Young and Nahorski, 2001; Sanchez and Hla, 2004; Rosen and Goetzl, 2005; Milstien et al., 2007). It is also important to mention that although the phosphorylated form of sphingosine may remain imbedded in membranes through its hydrophobic portion, it is likely that the presence of charged phosphorylated groups on S1P prevents agonist-induced increase in the permeability of endothelial monolayers, indicating enhanced barrier function.

The relationship between Ca\(^{2+}\) signaling and EC barrier function is not yet fully elucidated. Independent investigations demonstrated that inflammatory agents including thrombin and histamine markedly increase endothelial permeability (Mehta and Malik, 2006). These agents initiate characteristic Ca\(^{2+}\) kinetics with an early transient release from the ER followed by a more sustained Ca\(^{2+}\) entry via SOC (Mehta and Malik, 2006). Thrombin-induced EC permeability requires Ca\(^{2+}\) entry from the extracellular space as well as Ca\(^{2+}\) depletion from the ER (Ahmmed and Malik, 2005; Mehta and Malik, 2006). The reduction of EC permeability involves the reduction of transient receptor potential canonical proteins such as transient receptor potential canonical 1 and 4 (TRPC1 and 4) (Tiruppathi et al., 2001, 2002; Ahmmed and Malik, 2005). It was also suggested that increases in EC Ca\(^{2+}\) result in the phosphorylation of myosin light chain through Rho kinase, which consequently increases EC permeability (Ahmmed and Malik, 2005). These data strongly suggest that regulation of Ca\(^{2+}\) influx is a key process in maintaining EC permeability.

Interestingly, extracellular application of S1P initiates a Ca\(^{2+}\) response that is similar to that caused by thrombin or histamine, albeit the SOC phase is less pronounced. These observations are consistent with earlier reports (Mehta et al., 2005). However, despite the similar Ca\(^{2+}\) responses after thrombin (or histamine) and extracellular S1P administration, these agents have opposing effects on EC barrier function. This discrepancy between Ca\(^{2+}\) responses and the parallel changes in cell permeability indicate that thrombin, histamine, and S1P alter cell permeability through different signaling pathways. Thus, based on these observations, the finding that exogenous or endogenous S1P has different effects on cell permeability also suggests the potential existence of different extra- and intracellular S1P receptors and the involvement of different signaling pathways mediating these S1P effects. The potential dual roles of endogenous and exogenous S1P as a critical lipid messenger molecule regulating EC function are consistent with previous observations on other cells and experimental settings as summarized in recent reviews (Young and Nahorski, 2001; Sanchez and Hla, 2004; Rosen and Goetzl, 2005; Milstien et al., 2007).

Our observation that the inhibition of endogenous S1P synthesis down-regulated store-operated Ca\(^{2+}\) entry initiated by all of the investigated Ca\(^{2+}\)-mobilizing agonists has a particular importance. These agents initiate Ca\(^{2+}\) mobilization through different mechanisms; thrombin and histamine acting through their distinct surface receptors and TG directly targeting the ER, causing slow Ca\(^{2+}\) release, whereas ionomycin is a general Ca\(^{2+}\) ionophore. Thus, the fact that after administration of all of the employed agonists SKI decreased SOC similarly, whereas it minimally affected Ca\(^{2+}\) release from the ER, suggests that intracellular S1P modulates SOC at downstream targets of signaling cascades. Because sphingosine is a membrane-associated lipid, its phosphorylation by SKI may alter its assembly within membrane rafts with consequent changes in the interaction between S1P and membrane-associated proteins in the vicinity. Thus, we propose that endogenously generated S1P may modulate Ca\(^{2+}\) entry via SOC by direct interactions with components of the transport protein complex. It is evident that the elucidation of the specific mechanisms responsible for the observed effects of endogenous S1P needs further investigations. In summary, our observations indicate an important role of endogenously generated S1P in the modulation of agonist-induced Ca\(^{2+}\) signaling and accompanied changes in
EC permeability. Endogenous S1P functions as a positive modulator of Ca\(^{2+}\) uptake via SOCE, resulting in increased cell permeability upon a variety of agonist-induced conditions including thrombin and histamine. These observations also suggest that S1P targeting ECs from the extracellular space exerts its effects through different receptor and signaling mechanisms than those of S1P generated intracellularly. The potential dual roles of endogenous and exogenous S1P need to be considered in pharmacological interventions aiming to prevent EC dysfunction under pathological conditions.

References


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