Mol. Cells *29*, 105-111, February 28, 2010
DOI/10.1007/s10059-010-0042-y

Minireview

Molecules and Cells

Sphingolipids and the Orchestration of Endothelium-Derived Vasoactive Factors: When Endothelial Function Demands Greasing

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Vasomotor tone is regulated by a complex interplay of a variety of extrinsic neurohumoral and intrinsic factors. It is the endothelium that has a major influence on smooth muscle cell tone via the release of intrinsic vasoactive factors and is therefore an important regulator of vasomotor tone. Sphingolipids are an emerging class of lipid mediators with important physiological properties. In the last two decades it has not only become increasingly clear that sphingolipid signaling plays a pivotal role in immune function, but also its role in the vascular system is now becoming more recognized. In this mini-review we will highlight the possible cross-talk between sphingolipids and intrinsic vasoactive factors released by the endothelium. Via this cross-talk sphingolipids can orchestrate vasomotor tone and may therefore also be involved in the pathophysiology of disease states associated with endothelial dysfunction.

Sphingolipids

Over the last decades, much attention has been given to signal transduction by so called 'bioactive lipids'. Insight has been gained in many formerly known signaling pathways, which are now recognized to utilize lipids as signaling mediators. An important group of bioactive lipids are the sphingolipids, firstly described by Thudichem in a 'Treatise on the chemical constitution of the brain' in 1884. Full appreciation of the signaling complexity of these sphingolipids gained excessively only recently. Now, it is generally accepted that sphingolipids are participating in regulation of, amongst others, cellular growth, differentiation and migration (for review see Hannun and Obeid, 2008). Virtually all eukaryotic cells contain sphingolipids, with sphingomyelin in general being the most abundant species. Sphingomyelin is an ubiquitous membrane (sphingo)phospholipid that may serve as a substrate for sphingomyelinases (SMase) to generate ceramide. Three different SMase isoforms have been described in eukaryotic cells that differ in optimal pH, namely acid, neutral and alkaline SMase (Goni and Alonso, 2002). Especially acid and neutral SMase have been implicated in cardiovascular signaling (Pavoine and Pecker, 2009) and reside in endosomal/lysosomal compartments and the ER/golgi/ plasma membrane respectively. Several factors can activate SMase, of which TNF-α is the most extensively described factor (Hannun and Obeid, 2008). SMase activity leads to the removal of the phosphorylcholine head group from sphingomyelin generating ceramide. Ceramide is implicated in many biological processes and targets multiple proteins such as ceramide-activated protein kinases and phosphatases, phospholipase A_2, specific PKC isotypes (Hannun and Obeid, 2008). Ceramide can be further transformed by phosphorylation via ceramide kinase into ceramide-1-phosphate (C1P). C1P generation has been associated with activation of PI3K/Akt and cytosolic phospholipase A_2, and inhibition of protein phosphatase 1 and 2A and acid SMase (Chalfant and Spiegel, 2005). Next to C1P formation, ceramide can be transformed via glucosylcera-mide synthase (at the cytosolic membrane of the golgi) or galactosylceramide synthase (at the luminal membrane of the ER) into glucosylceramide and galactosylceramide respectively (van Meer and Holthuis, 2000). Further glycosylation steps form a plethora of complex sphingolipids, which are implicated in many biological processes (for review see Merrill et al., 2007). Enzymatic deacylation of ceramide by ceramidase will lead to the generation of sphingosine which is, like ceramide, also implicated in induction of apoptosis. Downstream sphingosine targets are, amongst others, phospholipase D, PKC and cell cycle regulators (Merrill et al., 1997). Subsequently, sphingosine can be phos-phorylated by sphingosine kinase (SK) 1 and 2 to yield sphingosine-1-phosphate (S1P). SK1 is activated by several factors, such as growth factors, cytokines, angiotensin II and acetylcoline, and S1P can target five G-protein coupled S1P receptors ($S1P_{1-5}$), of which mainly $S1P_{1-3}$ are expressed in the cardiovascular system (Alewijnse and Peters, 2008; Brinkmann and Baumruker, 2006; Mulders et al., 2006; Waeber et al., 2004). Besides S1P receptor activation, SK activity has been shown to induce receptor-independent effects (Meyer Zu Heringdorf, 2004), although the existence of an intracellular target for S1P still needs to be determined. S1P receptor activation has been shown to induce migration, barrier function alteration regulation and proliferation of many cell types including vascular cells (for review see Peters and Alewijnse, 2007). Thus S1P, in contrast to its precursor ceramide and sphingosine, has anti-apoptotic effects. Therefore, this balanced system is also referred to as the ceramide/S1P rheostat (Edsall et al., 1998).

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Received January 5, 2010; accepted January 8, 2010; published online January 29, 2010

Keywords: EDCF, EDRF, endothelium, sphingolipids, vascular tone

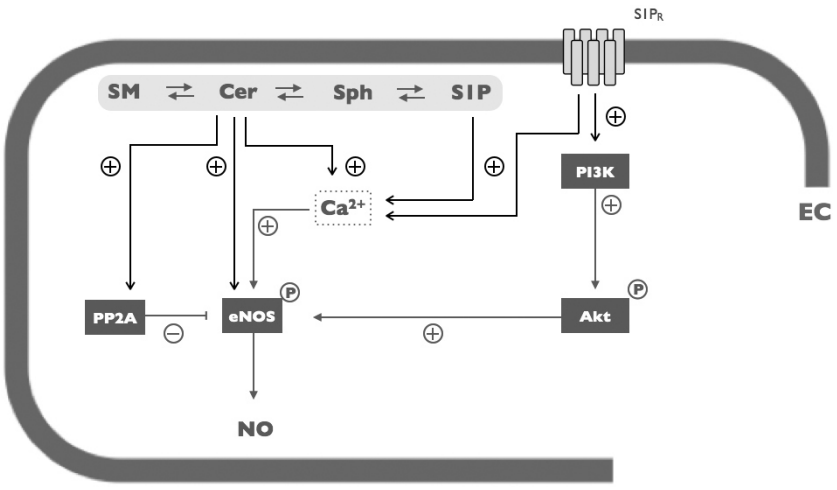

Fig. 1. Schematic representation of the modulation of eNOS activity by sphingolipids. See main text for details. EC, endothelial cell; SM, sphingomyelin; Cer, ceramide; Sph, sphingosine; S1P, sphingosine-1-phosphate; $S1P_R$, S1P receptor; PP2A, protein phosphatase 2A; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PI3K, phosphoinositide 3 kinase; Akt, protein kinase B.

The vascular system

Blood vessels are composed of three distinct layers; the endothelium (intima), a smooth muscle cell layer (media) and a layer consisting of connective tissue (adventitia). The endothelial cells comprising the endothelium are the primary contact points of the vessels with circulating blood and orchestrate a plethora of vascular functions including vascular tone regulation (Vanhoutte, 2003). The underlying smooth muscle cell layer is responsible for the execution of vasomotor tone changes, but also supports the vascular integrity. Besides extrinsic neurohumoral factors, vascular tone is maintained by a delicately balanced release of endothelium-derived relaxing factors (i.e. nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor) and contractile factors (i.e. thromboxane A_2 and endothelin-1) (Vanhoutte, 2003).

In this minireview we will concisely address a possible interplay of sphingolipids with these endothelium-derived vasoactive substances. It is not the purpose to give an in depth review of the available data, but to highlight some examples indicating that sphingolipids may play an orchestrating role in endothelium-dependent regulation of vasomotor tone.

Nitric oxide

Nitric oxide (NO), either produced by endothelial or smooth muscle cells, diffuses homogenously and non-directed upon production and can readily pass cellular membranes independent of cellular transport mechanisms (Ignarro, 2002). NO activates soluble guanylyl cyclase in the smooth muscle cell, which leads to cGMP formation and ultimately to dephosphorylation of myosin light chain and vascular relaxation. The production of the endothelial NO is mediated by endothelial nitric oxide synthase (eNOS), which resides in inactivated form at caveolae (Maniatis et al., 2006). Caveolae are flask-like invaginations of the plasma membranes of the endothelial and other cells. These specialized membrane microdomains are enriched in cholesterol, sphingolipids (sphingomyelin and glycosphingolipids) and phosphatidylinositol (Hope and Pike, 1996; Liu and Schnitzer, 1999; Liu et al., 1997) and harbor many other molecules (for review see Anderson, 1998) such as the structural component caveolin. This composition forms a rigid surface grouping several proteins like nSMase, G-protein coupled receptors including S1P receptors, and second messenger proteins like adenylyl cyclase and eNOS (Czarny et al., 2003; Igarashi and Michel, 2000; Rizzo et al., 1998). eNOS activity is influenced by Ca^{2+}-calmodulin interaction and post-translational modification of which phosphorylation by the PI3K/Akt pathway is well described (Maniatis, Brovkovych et al., 2006). Caveolin-1 is able to bind the calmodulin binding-domain of eNOS, thereby inhibiting Ca^{2+}-induced NO production. Sphingolipids have been implicated in regulation of eNOS activity, both under healthy and pathological conditions. Sphingosine-1-phosphate signaling is generally accepted to activate eNOS via $S1P_1$ and $S1P_3$ receptor signaling (Igarashi and Michel, 2000; Nofer et al., 2004). This activation is either by PI3K activation resulting in eNOS phosphorylation, or elevation of intracellular Ca^{2+} concentrations granting Ca^{2+}-calmodulin interaction with eNOS (for overview see Fig. 1). S1P has also been shown to mediate cytosolic calcium elevation receptor-independently (Meyer Zu Heringdorf, 2004), however the precise mechanism behind this remains elusive.

The role of ceramide in eNOS regulation is more complex as both activation as well as inhibition of eNOS by ceramide has been reported. Ceramide can activate eNOS via mostly calcium-independent mechanisms. Inhibition can be established via ceramide-activated protein phosphatase 2A activation that dephosphorylates eNOS and thus reduces NOS activity (Igarashi et al., 1999a; Smith et al., 2006; Xiao-Yun et al., 2009) (see Fig. 1). Shear stress-induced NO production in BAECs appeared to be mediated by ceramide produced by nSMase activity due to putative mechanosensing properties (Czarny and Schnitzer, 2004). Neutral SMase activity generated, and exogenously applied ceramide in these cells, activated the PI3K/Akt pathway but whether this was due to ceramide itself or further metabolization of ceramide to S1P was not addressed.

Next to the effects of ceramide on NO bioavailability, lactosylceramide which is a glycosylated form of ceramide, inhibited eNOS mRNA expression in HCAECs (Bismuth et al., 2009), thereby affecting NO bioavailability in a negative manner. Since eNOS activity is highly dependent on its cellular localization, sphingolipid metabolism may also affect eNOS activity by alterations in membrane lipid composition and affect membrane microdomains. Thus sphingolipids can, via multiple mechanisms fine-tune or orchestrate eNOS activity in the endothelium.

Endothelium-derived hyperpolarizing factor

Although NO-mediated vasodilation is a major feature of large conduit arteries and depends on cGMP formation, smooth muscle cell membrane hyperpolarisation appeared also to be part of the NO-induced relaxation (Feletou and Vanhoutte, 2007).

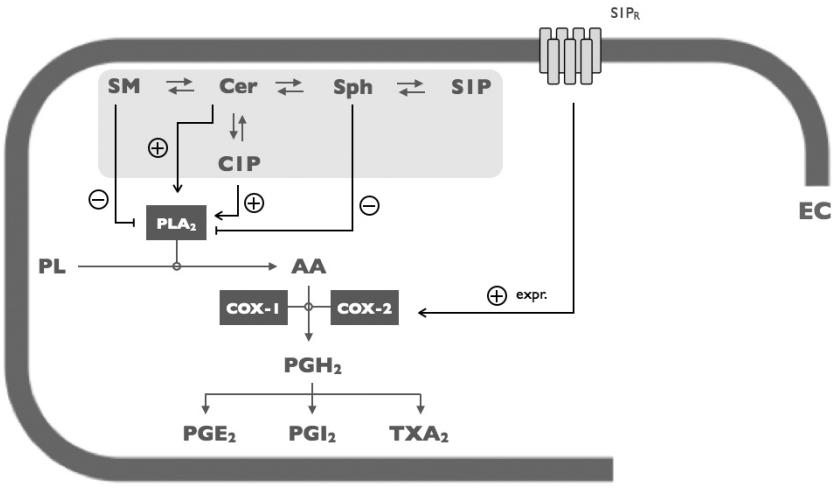

Fig. 2. Schematic representation of the cross-talk between the eicosanoid system and sphingolipids. See main text for details. EC, endothelial cell; SM, sphingomyelin; Cer, ceramide; Sph, sphingosine; S1P, sphingosine-1-phosphate; $S1P_R$, S1P receptor; C1P, ceramide-1-phosphate; PLA_2, phopholipase A_2; PL, phospholipid; AA, arachidonic acid; COX, cyclooxygenase; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; TXA_2, thromboxane A_2.

Since many relaxating substances, released from the endothelium, mediate smooth muscle cell hyperpolarisation, this process is regarded as an additional major pathway for relaxation induced by substances collectively termed endothelium-derived hyperpolarizing factors (EDHF). Candidate EDHFs are several arachidonic acid metabolites, radicals and peptides and mainly involve direct and indirect influence on potassium channel and consequently calcium channel 'open probability' (Feletou and Vanhoutte, 2007). The role of sphingolipids in EDHF signaling has been studied only sparsely. Research from our laboratory showed that in rat mesenteric arteries, inhibition of sphingosine kinase resulted in augmented EDHF-mediated relaxation after M_3 receptor stimulation, suggesting an inhibitory role of S1P on EDHF signaling (Mulders et al., 2009). Furthermore, there are indications that potassium channel activity, which is involved in EDHF signaling, is under direct influence of the surrounding membrane composition. Although small and intermediate conductance K^+ channels are highly expressed in endothelial cells (Crane et al., 2003), large conductance Ca^{2+} activated K^+ channels (BK_{Ca}) which are involved in EDHF signaling (Hilgers et al., 2006), are highly expressed on the vascular smooth muscle cells (Dimitropoulou et al., 2001). In an artificial membrane setup, BK_{Ca} open-probability was prolonged when situated in increasingly thicker membrane domains, rich in sphingomyelin (Yuan et al., 2007) as found in e.g. caveolar structures. This finding was further substantiated by Kim et al., which indicated stimulation of BK_{Ca} activity after S1P addition in HUVECs, which was S1P receptor - and calcium-independent (Kim et al., 2006). However, it is important to note the low expression level, if present at all under physiological conditions, of BK_{Ca} in endothelial cells (Sandow and Grayson, 2009). Whether small and intermediate K_{Ca} channels are affected by lateral migration to or from specific membrane micro domains similarly to BK_{Ca}, thus possibly altering EDHF signaling, remains to be determined.

Prostanoids

Prostanoids are well known endothelium-derived vasoactive compounds. While prostacyclin (PGI_2, a product of prostacyclin synthase) has mainly dilatory actions in the vasculature, prostaglandin E_2 (PGE_2, produced by prostaglandin synthases) and thromboxane A_2 (TXA_2, produced by thromboxane synthase) are potent vasoconstrictors. These endothelium-derived prostanoids, act on different receptors on the smooth muscle cells of the vessel: PGI_2 stimulates G_s-coupled IP receptors, TXA_2 G_q-coupled TP receptors and PGE_2 stimulates EP receptors that couple to different G-proteins. The precursor for all these prostanoids is PGH_2 synthesized from arachidonic acid by cyclooxygenases. The main source of arachidonic acid in its turn is the breakdown of membrane phospholipids by phospholipase A_2 (PLA_2). Endothelial cells express different isoforms of PLA_2 including cytosolic PLA_2 ($cPLA_2$), secreted PLA_2 ($sPLA_2$) and calcium-independent PLA_2 ($iPLA_2$). Several sphingolipids have been show to modulate arachidonic acid metabolism and thereby they can, at least in theory, orchestrate endothelium-dependent vasomotion. Both, ceramide and ceramide-1-phosphate have been reported to activate $cPLA_2$ (Huwiler et al., 2001; Nakamura et al., 2006; Pettus et al., 2004; Sato et al., 1999; Stahelin et al., 2007). It was shown that ceramide modulates $cPLA_2$ activity by a direct interaction with the CalB (calcium-dependent phospholipid binding) domain of the enzyme, which facilitates membrane docking (Huwiler et al., 2001). In a similar fashion, C1P has been suggested to increase the membrane association of $cPLA_2$ *in vitro* via a site in the cationic beta-groove of the C2 domain (Stahelin et al., 2007).

Sphingomyelin has inhibitory actions on both $cPLA_2$ and $sPLA_2$ (Koumanov et al., 1997; Nakamura et al., 2009; Singh et al., 2007). Sphingomyelin most likely decreases PLA_2 activity by inhibiting its binding to membrane-associated phosphatidylinositol bisphosphate, thereby promoting membrane dissociation of $cPLA_2$ (Nakamura et al., 2009).

Thus activation of sphingomyelinase will lead to an activation of $cPLA_2$ firstly because of a decrease in inhibitory sphingomyelin, and secondly by the generation of of the PLA_2 activator ceramide. Further metabolization of ceramide to sphingosine will again lower $cPLA_2$ activity because of the inhibiting activity of sphingosine on $cPLA_2$ (Nakamura et al., 2004) (for overview see Fig. 2). Arachidonic acid that is released by PLA_2 activity has been shown in several cellular systems to stimulate sphingomyelinase activity (Chan et al., 1998; Jayadev et al., 1994; Robinson et al., 1997; Visnjic et al., 1997), thus providing a positive feedback loop.

Sphingolipids also interact with the eicosanoid system at a transcriptional level. For instance, ceramide may increase $cPLA_2$ expression (Hayakawa et al., 1996) and S1P is an inducer of COX-2 expression (Pettus et al., 2003; 2005). The latter is mediated most likely via a S1P receptor, $G\alpha_{12}$, and NF-κB-dependent mechanism (Ki et al., 2007; Nodai et al., 2007). Therefore, also this sphingomyelin breakdown system may act as a positive feedback loop; S1P increases expression of COX-2 whereas its

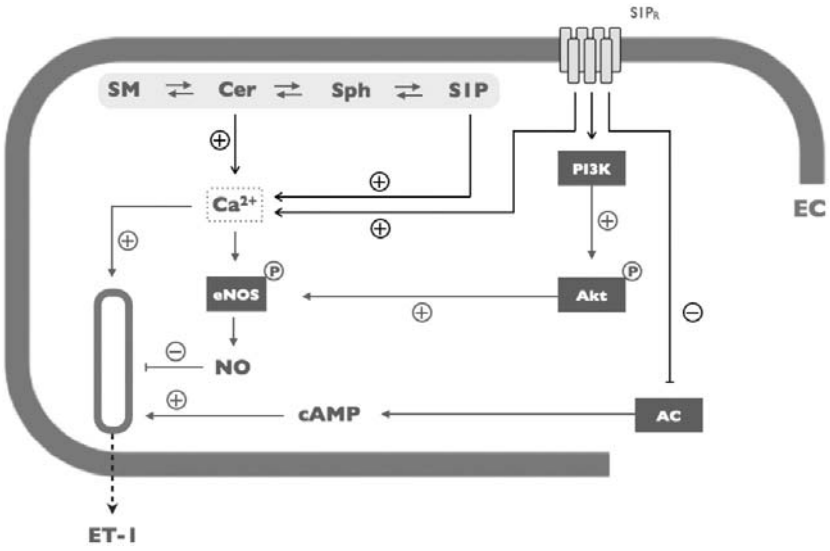

Fig. 3. Schematic representation of the possible regulation of Weibel-Palade body exocytosis by sphingolipids. EC, endothelial cell; SM, sphingomyelin; Cer, ceramide; Sph, sphingosine; S1P, sphingosine-1phosphate; $S1P_R$, S1P receptor; AC, adenylyl cyclase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PI3K, phosphoinositide 3 kinase; Akt, protein kinase B.

precursor ceramide and ceramide-1-phosphate increase substrate delivery via activation of $cPLA_2$ (Pettus et al., 2005).

To what extend the aforementioned effects of sphingolipids on arachidonic acid metabolism take place in the endothelium, still needs to be determined. As indicated, there are several ways by which, at least in theory, sphingolipids may orchestrate the production of dilatory and contractile prostanoids.

Endothelin-1

Endothelin-1 is a potent vasoconstrictor peptide released by the endothelium and is synthesized from a precursor (big-endothelin-1) by the activity of endothelin converting enzyme. Endothelin-1 signals through activation of two specific G-protein-coupled receptors termed ET_A and ET_B. These receptors are expressed in a variety of tissues. In the vasculature, smooth muscle cells express both ET_A and ET_B receptors, whereas the endothelium mainly expresses ET_B receptors [only rat and human brain endothelial cells are suggested to express ET_A receptors (Dehouck et al., 1997)]. The contractile responses to endothelin-1 are mediated via the ET_A receptors on the smooth muscle cells. Only very limited information is available on how sphingolipids are involved in, or can modulate, endothelin-1 signalling. ET_B receptor stimulation in neuronal tissue has been shown to increase ceramide levels via sphingomyelin and glycosphingolipid metabolism (Catalan et al., 1996). ET_A receptor-mediated activation of sphingosine kinase is reported to be involved in myometrial contraction (Leiber et al., 2007). In the bovine brain microvasculature, endothelin-1 is reported to increase ceramide levels via an ET_A dependent mechanism, but the functional implication of this pathway is not established yet (Collado et al., 2003). Information on interactions between the endothelin and sphingolipid systems in the endothelium are lacking so far.

However, sphingolipids can to a certain extent regulate the release of endothelin-1 from endothelial cells. Endothelial cells contain unique organelles first described in 1964 by Weibel and Palade as “rod-shaped cytoplasmic components, which consist of a bundle of fine tubules” (Weibel and Palade, 1964). These Weibel-Palade bodies (WPB) contain a variety of factors that are involved in coagulation (von Willebrand Factor, factor XIIIA and Tissue Plasminogen Factor), inflammation (interleukin-8, eotaxin, p-selectin) and also vasomotion since these structures also contain endothelin-1, endothelin converting enzyme and calcitonin gene-related peptide. The release of endothelin-1 from the endothelium is accomplished through both constitutive and regulated pathways, in which the latter is achieved by a rapid release from WPBs. These WPBs can release their content by exocytosis upon agonist stimulation. Several agonists are known to induce exocytosis of WPBs by stimulating G_q- or G_s-coupled receptors, inducing a rise in intracellular Ca^{2+} or cAMP respectively. For instance, thrombin and histamine enhance exocytosis by stimulating G_q-coupled receptors, whereas adrenaline and vasopressin achieve this by stimulating G_s-coupled receptors. Also growth factor receptors such as the vascular endothelial growth factor (VEGF) receptor 2 can stimulate WPB exocytosis by Ca^{2+} and cAMP-dependent responses (Goligorsky et al., 2009; Rondaij et al., 2006). S1P receptors can increase intracellular calcium via both G_i- and G_q-dependent mechanisms. In addition, due to their G_i-coupling they also will lower cAMP levels and because of these properties S1P receptor stimulation could potentially affect WPB exocytosis. Indeed, stimulation of human aortic endothelial cells with S1P triggers WPB exocytosis in a concentration dependent manner (Matsushita et al., 2004). Interestingly, this response proved to be pertussis toxin sensitive indicating G_i-dependent responses. The fact that a phospholipase C inhibitor inhibited, and calcium-free medium prevented S1P-stimulated exocytosis of WPBs, suggests that S1P via stimulation of G_i-coupled receptors most likely via the β/γ subunits of the G_i-protein, activates phospholipase C and subsequent calcium increases. As described before, $S1P_1$ and $S1P_3$ receptors can activate the PI3K/Akt pathway in endothelial cells that, via phosphorylation of eNOS, increases NO production. In the report by Matsushita, PI3K inhibition potentiated WPB exocytosis which was associated with decreased eNOS phosphorylation and concomitant lower NO production. The same group had shown before that NO inhibits exocytosis via S-nitrosylation of N-ethylmaleimide-sensitive factor, a protein that plays a role in membrane fusion (Matsushita et al., 2003). Because of the aforementioned, it was concluded that S1P has opposing effects on WPB exocytosis; by stimulating calcium signalling exocytosis is triggered, while eNOS activation by S1P has the opposing effect, possibly as a sort of negative feedback loop. In addition, it remains unanswered which receptor subtype mediates the observed responses since all three S1P receptors normally expressed in endothelial cells are G_i coupled and both

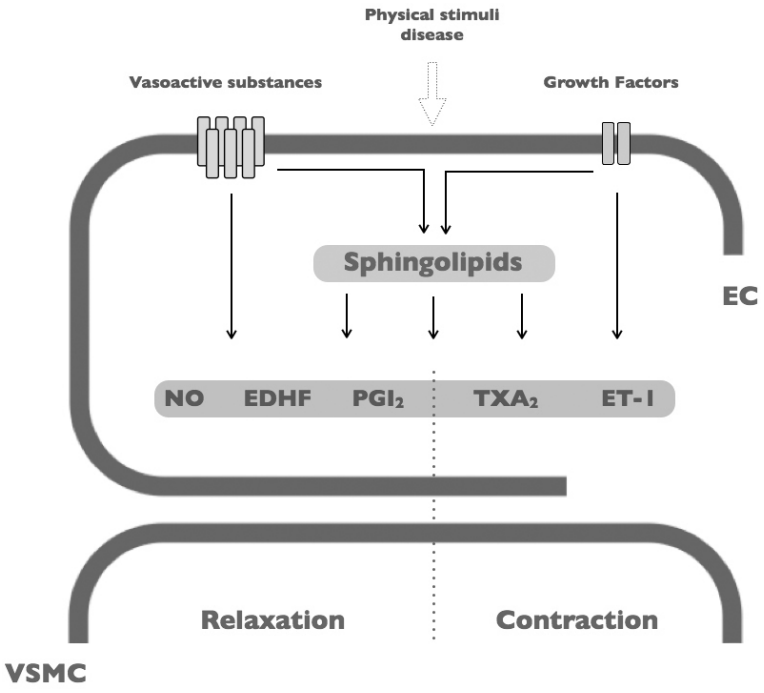

Fig. 4. Schematic representation of how sphingolipid metabolism may influence vasomotor tone by modulating the release of vasoactive substances from the endothelium. Alterations in this system may contribute in the etiology and/or pathophysiology of endothelial dysfunction. EC, endothelial cell; VSMC, vascular smooth muscle cell; NO, nitric oxide, EDHF, endothelium-derived hyperpolarizing factor; PGI_2, prostacyclin; TXA_2, thromboxane A_2; ET-1, endothelin-1.

$S1P_1$ and $S1P_3$ receptors are known to activate the PI3K/Akt pathway (Peters and Alewijnse, 2007).

As discussed before, also ceramide can induce calcium signalling and modulate eNOS activity in endothelial cells. The same group that reported the effects of S1P on WPB exocytosis showed that also exogenously applied as well as endogenously generated ceramide increases exocytosis (Bhatia et al., 2004). In this report the authors show that, in contrast to the S1P-mediated effects, exocytosis induced by ceramide is inhibited by intracellular calcium chelation and is not affected by extracellular calcium depletion. Therefore it was suggested that ceramide by releasing calcium from intracellular stores induces WPB exocytosis. Inhibition of eNOS increased ceramide-induced exocytosis, while exogenous NO had an inhibitory action. In analogy with S1P it could be possible that also ceramide has dual actions on WPB exocytosis by activating eNOS via calcium increases and/or directly in a calcium-independent manner as has been demonstrated before (Igarashi et al., 1999b), however, this was not addressed in this study. Although the effects of ceramide are not consistently reported by the same group (Bhatia et al., 2004; Matsushita et al., 2004), the available data suggest that WPB exocytosis, and the concomitant release of endothelial cell mediators, is a process that can be tightly regulated by a delicate balance of ceramide and S1P. WPB exocytosis is also triggered by several physical stimuli such as hypoxia and radiation (Hallahan and Virudachalam, 1999; Pinsky et al., 1996). Since ceramide levels are known to increase during hypoxia and radiation it is tempting to speculate that ceramide may mediate the increases in exocytosis of WPBs.

The data discussed above implicate that sphingolipids and the enzymes involved in sphingolipid metabolism, are important regulators of endothelial function with respect to exocytosis of coagulation, pro-inflammatory and vasoactive substances (for overview see Fig. 3). Especially under circumstances of endothelial dysfunction (decreased NO bioavailability), this may explain the pro-inflammatory and pro contractile effects of sphingosine kinase activation.

CONCLUSION/ FUTURE PERSPECTIVES

Previous paragraphs clearly indicate that sphingolipids may interfere with endothelial mediators in multiple ways. They potentially can influence the release of dilatory (NO, PGI_2 and EDHF) and contractile (TXA_2 and endothelin-1) mediators released from the endothelium, and it is therefore becoming increasingly clear that sphingolipids are regulators of vascular tone. Several extrinsic and intrinsic factors (e.g. growth factor, vasoactive substances, shear stress etc.) initiate sphingolipid signalling by activating sphingolipid-metabolizing enzymes, and in this way influence vasomotor function (see Fig. 4). However, many intriguing questions remain unanswered. For instance, the distribution of cell surface and intracellular sphingolipid pools has not been addressed in relation to endothelial function. Several pathways described above have been studied using exogenously added sphingolipids. Importantly, exogenous addition of sphingolipids to cell cultures or tissue could obviously evoke effects that are not derived from the initial sphingolipid but due to metabolic products. For endothelial cells like HUVECS, sphingolipid-associated enzymes have been found to be exported and present extracellularly (Ancellin et al., 2002), contributing to the importance of monitoring overall changes in sphingolipid species when describing sphingolipid-induced effects. Furthermore, since endothelial cells are equipped with specialized membrane compartments such as caveolae and Weibel-Palade bodies that are build up with sphingolipids, it is not unlikely that alterations in sphingolipid composition per se will affect endothelial function. The fact that both, sphingolipids themselves as well as sphingolipid-metabolizing enzymes are highly compartmentalized, forms an additional dimension of regulation has to be addressed in the coming years.

In addition, there is still information lacking about the pathophysiological role of sphingolipids in disease states associated with endothelial dysfunction, such as hypertension, diabetes and atherosclerosis. How do these disease states affect (endothelial) sphingolipid levels? Is decreased NO-bioavailability associated

with alterations in sphingolipid biology? Answers to these questions will most likely also answer the question whether the sphingolipid system is an attractive target to restore endothelial function in these disease states.

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