Sphingolipid metabolism in vascular function

Arthur CM Mulders

SPHINGOLIPID METABOLISM IN VASCULAR FUNCTION

Arthur CM Mulders

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de openbare verdediging
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op vrijdag 14 december 2007
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Sphingolipid metabolism in vascular function

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General introduction

Aim of the thesis

Historical background

Sphingolipids are a diverse family of phospholipids and glycolipids in which fatty acids are linked via amide bonds to a sphingoid base. The term 'sphingo-' was suggested in 1884 by the German biochemist and surgeon Johann L.W. Thudichum, at that time working in London. He was the first to describe sphingomyelin as a constituent of both gray and white brain matter. He named sphingomyelin after the Sphinx because of the many enigmas that surrounded the lipid he had extracted.[1] The term sphingolipid was introduced by Herbert E. Carter and colleagues in 1947.[2] Sphingolipids comprise more than 300 structurally related lipids and are found in many living organisms including yeast, plants and humans and in virtually all cell types.[3,4]

Sphingomyelin is the main precursor for an important subset of sphingolipids, which are also referred to as sphingomyelin metabolites. Sphingomyelin can be converted into ceramide, which in turn can be metabolized further into sphingosine and through subsequent phosphorylation into sphingosine-1-phosphate (S1P). All these reactions are reversible and controlled by the presence and activity of specific enzymes (see figure 1). At first, sphingolipids were thought only to play a role in the fluidity and sub domain structure of the cell membrane. After sphingomyelin was identified, it took over 100 years for the first appreciation of sphingomyelin metabolites as signalling entities. The discovery in 1986 that protein kinase C (PKC) was inhibited by sphingosine, triggered an exponentially growing interest in the research of sphingolipids as bioactive molecules.[5-7] The first identification of signalling properties for ceramide came one year later in 1987 and it became clear that sphingomyelin metabolites were involved in various cellular processes.[8] Ceramide, sphingosine and S1P are known to have various signalling capabilities [9], in which the biological effects of ceramide and sphingosine on the one, and S1P on the other hand, can be opposite. Ceramide and sphingosine are generally involved in apoptotic responses to various stress stimuli and in growth arrest [10,11], while S1P is implicated in mitogenesis, differentiation and migration.[12,13] It was suggested in 1996 that it is not the absolute amount of either sphingolipid that is decisive, in e.g. cell death or cell proliferation, but the relative balance between them. This homeostatic system is frequently referred to as the ceramide / S1P rheostat.[14]

Already in 1992 it was suggested that S1P could act as an extracellular mediator in the control of cell motility through a putative transmembrane receptor.[15] The first actual evidence for a G protein-coupled S1P receptor was given in 1995.[16] Interestingly, the responsible endothelial differentiation gene (EDG)-1 receptor had already been cloned in 1990 from

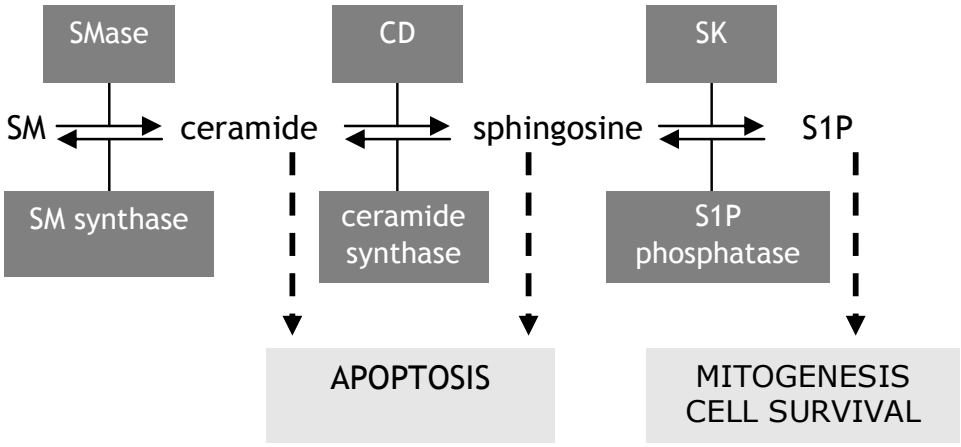

Figure 1. Ceramide / S1P rheostat. Interconvertible sphingomyelin metabolites ceramide and sphingosine can have opposite effects from S1P. The conversion of these sphingolipids is subject to regulation by indicated enzymes. SM = sphingomyelin, SMase = sphingomyelinase, CD = ceramidase, SK = sphingosine kinase.

human umbilical vein endothelial cells.[17] In 1998, it was shown that indeed S1P was the preferred ligand for the EDG-1 receptor.[18] The EDG receptors were renamed to S1P receptor isoforms $S1P_{1-5}$ in 2002 according to the International Union of Pharmacology guidelines.[19] In total, five specific S1P receptors have been identified [20], some of which are expressed in the vasculature.[21] There are several other potential S1P receptors, now still classified as "orphan" receptors.[22,23] S1P receptors are coupled to different heterotrimeric G proteins (G_q, G_i, G_{12-13}) and the small GTPases of the Rho family.[24] The importance of S1P receptors and sphingolipid signalling in embryonic development was first shown by the generation of $S1P_1$ receptor knockout mice.[25] $S1P_1$ receptor gene disruption was associated with severe vascular malformations leading to embryonic haemorrhage and thereby to intrauterine death between embryonic day 12.5 and 14. As will be discussed later on, the recent development of the immunosuppressant drug candidate FTY720 that in its phosphorylated form targets S1P receptors, stimulated sphingolipid research tremendously. In addition, the development of several other S1P receptor agonists, antagonists and enzyme inhibitors accelerates this research field.

Now, almost 125 years after the first description of sphingolipids by Thudichum, many riddles have been solved, but nevertheless, the functions of these lipids remain enigmatic. In this introduction we will review the current knowledge about sphingolipid metabolism and signalling and the biological roles of sphingolipids with a main focus on the vasculature, which is the main subject of this thesis.

Bioactive sphingomyelin metabolites

Structurally, sphingolipids are defined by the presence of a sphingoid backbone.[26] The de novo synthesis of sphingolipids primarily takes place in the endoplasmic reticulum and the Golgi apparatus [27,28], but also other sites have been described [29]. Sphingomyelin, which is a major constituent of the cell membrane, can be formed de novo, during which the by-product 1,2-diacylglycerol is formed. Upon cell stimulation, different forms of sphingomyelinases can be activated to cleave sphingomyelin and produce ceramide and phosphocholine.[30] Three groups of sphingomyelinases, acid, neutral, and alkaline, have been cloned and these groups are distinguished by their catalytic pH optimum, primary structure and localization. Interestingly, some of the sphingomyelinases have been shown to be excreted and can be active in the extracellular space.[31] In many cell types, formation of ceramide leads to cell growth arrest and apoptosis.[32,33] Ceramide formation can even be a necessary physiological intermediate step for cells to go into apoptosis.[34] This was shown for example by protection from apoptosis of cells lacking acidic sphingomyelinase activity and re-sensitization to apoptosis by exogenously added ceramide.[35-38] Moreover, genetic and pharmacological studies *in vivo* showed that radiation induces apoptosis through formation of ceramide to initiate the pathogenesis of tissue damage.[39] In parallel, oxidative stress also leads to ceramide generation in lung epithelial cells, again resulting in cellular apoptosis.[40] Several molecular targets have been identified for ceramide, e.g. protein kinase C [41], Src-like tyrosine kinases [42], phospholipase A_2 [43], Jun-N-terminal kinases [44], a ceramide-activated protein phosphatase [45], the small G-proteins Ras and Rac [46,47] and others.[11]

Ceramide can be deacylated by ceramidases and like the sphingomyelinases, ceramidases have been classified into three groups, acid, neutral, and alkaline, distinguished by their catalytic pH optimum, primary structure and localization.[31] Neutral ceramidase is most likely responsible for the regulation of stimulus-induced ceramide into sphingosine conversion. Like ceramide, increased sphingosine levels inhibit cell growth and induce apoptosis.[48,49] Although it is still not clear whether mere elevated sphingosine levels are sufficient, there is increasing evidence for specific sphingosine-dependent pathways to induce apoptosis.[50] However, for some cells the metabolism of sphingosine into ceramide is thought to be the most important pathway for apoptosis.[34] Several pathways, such as mitogen-activated protein kinase, caspases and Akt / ribosomal S6 kinase / Bad signalling cascades, have been shown to be involved in sphingosine-dependent induction of apoptosis.[51]

Sphingosine can be converted to S1P by the action of sphingosine kinase and this enzyme plays an important role in sphingolipid metabolism. While sphingomyelinase activity will alter

the total abundance of sphingomyelin metabolites, activation of sphingosine kinase shifts the balance between pro-apoptotic ceramide and sphingosine on the one and the anti-apoptotic S1P on the other hand. Two isoforms of sphingosine kinase (-1 and -2) exist that differ in their temporal and spatial distribution in mammals. During embryonic development of the mouse, sphingosine kinase-1 expression is high at embryonic day 7 and decreases thereafter, whereas sphingosine kinase-2 expression increases gradually up to embryonic day 17.[52,53] This may reflect a differential role for the two isoforms during development. In adult mouse tissues, sphingosine kinase-1 expression is highest in lung, spleen, kidney, and blood, whereas sphingosine kinase-2 is predominantly found in liver, kidney, brain and heart.[52-54] Both sphingosine kinases can be activated by a variety of external stimuli, including G protein-coupled receptors, small GTPases, tyrosine kinase receptors, pro-inflammatory cytokines, immunoglobulin receptors, Ca^{2+}, protein kinase activators, and others. Most stimuli cause a rapid, transient stimulation of sphingosine kinase activity, most likely by post-translational modification or by affecting its localization. However, some agents also induce a prolonged transcriptional upregulation after the first rapid increase in enzyme activity.[55-57] Since S1P has been shown to be important in a variety of biological processes and the development of various pathologies, tight regulation of local formation of S1P by sphingosine kinase is very important and plays a key role in S1P-mediated effects. Formation of sphingomyelin metabolites is generally, though not solely, achieved within the cellular membrane. Since sphingolipids are mostly present within the membrane, translocation of sphingosine kinase from the cytotosol to the membrane is associated with increased synthesis of S1P, although this is not necessarily due to increased enzyme activity.[58] Moreover, it has been shown that sphingosine kinase-1 and -2 can have opposing roles in the regulation proliferation and apoptosis, respectively, which is due to the cellular localization of the enzymes.[59] An important pharmacological tool to study sphingosine kinase activity is the inhibitor *N,N*-dimethylsphingosine (DMS).[60] DMS is a specific and competitive inhibitor of sphingosine kinase-1 and a non-competitive inhibitor of sphingosine kinase-2.[53] For reviews addressing structure and function of sphingosine kinases see: [61,62].

S1P, once formed, can be converted back to ceramide via S1P phosphatases and ceramide synthase activities or ultimately be irreversibly degraded by S1P lyase to ethanolamine-phosphate and hexadecenal.[63,64] Moreover, S1P can also be broken down by the less prominent intracellular lipid phosphate phosphatases.[65-67] An overview of the sphingolipid metabolism is shown in figure 2. S1P is probably the best studied sphingomyelin metabolite and has various signaling capabilities, including cell survival, cell growth, differentiation and migration and others.[13,30,63,68] Before the discovery of the S1P receptors, it was believed that S1P primarily acted as an intracellular mediator.[69,70] Several lines of evidence support an intracellular function of S1P. The membrane impenetrable dihydroS1P is an agonist at all S1P

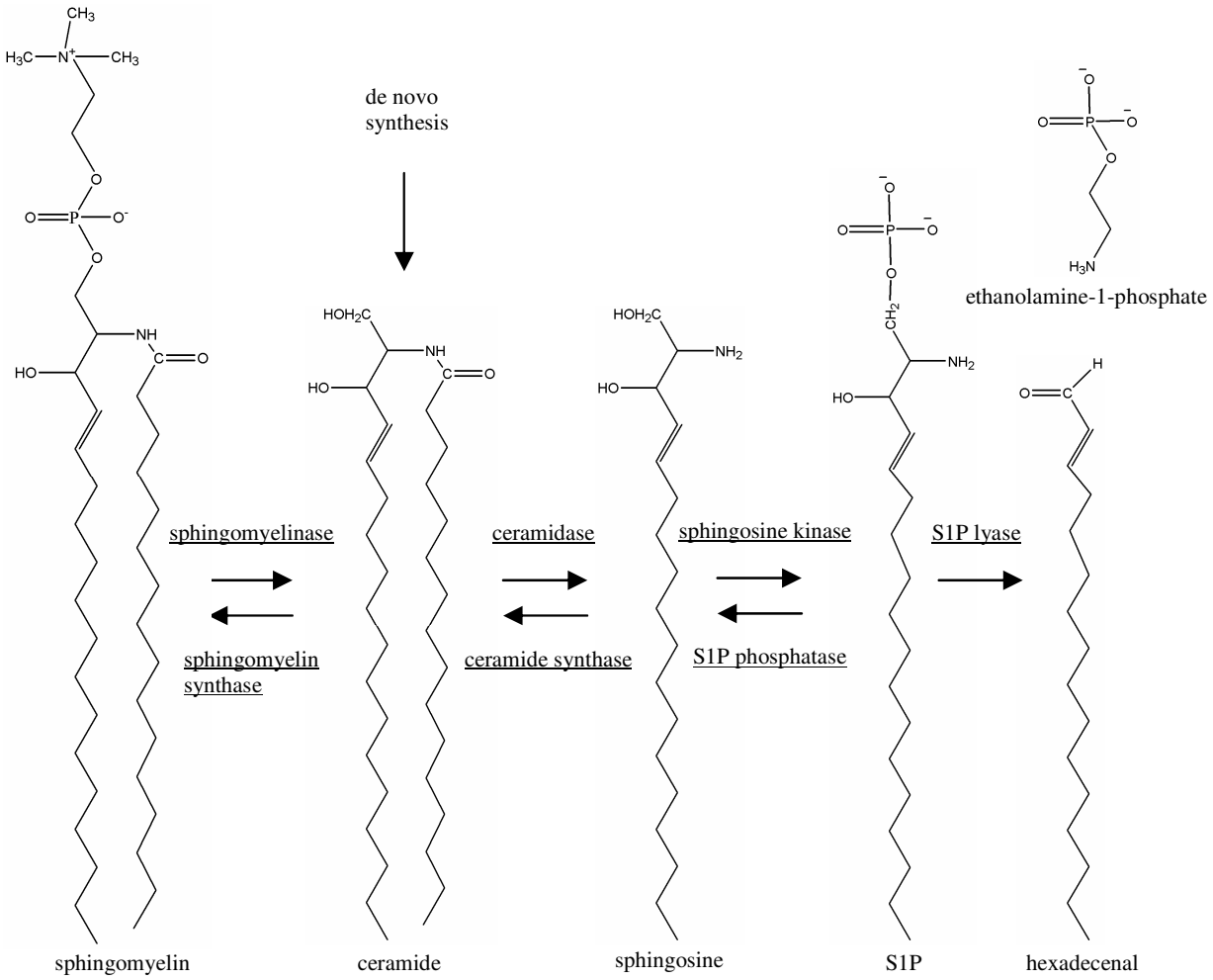

Figure 2. Chemical structures of interconvertible sphingomyelin metabolites with various signalling capabillities. Enzymes responsible for metabolism are also shown. For detailed information see main text.

receptors, yet it fails to reproduce all the effects of S1P, such as the prevention of cells to go into apoptosis.[71] In addition, several effects of S1P are only found with micromolar concentrations of S1P, which are higher than the affinity for S1P receptors. Moreover, yeast and plants lack S1P receptors, but still possess the S1P metabolizing enzymes and are responsive to S1P.[72,73] Although S1P can mobilize Ca^{2+} in cells via its interaction with its surface receptors, microinjection of S1P into cells or increasing intracellular S1P by the use of caged S1P also mobilizes Ca^{2+} in a receptor-independent manner.[74,75] The definite confirmation of the role of S1P as an intracellular second messenger awaits the identification of an intracellular target that can explain these effects.[62,76] As stated before, since ceramide and sphingosine are pro-apoptotic stimuli and S1P is a survival and mitogenic factor, the conversion of ceramide and sphingosine into S1P, and vice versa, has major impact on cellular homeostasis.[77] It was suggested that the ceramide / S1P rheostat is a critical determinant of cell fate.[14] Accordingly, it has been shown that S1P can limit ceramide-induced apoptosis, and depletion of S1P enhances ceramide-induced apoptosis.[14,78,79] One

mechanism involved may be cellular autophagy, which is the catabolic process involving degradation of proteins and organelles through the lysosomal machinery. Autophagy has been shown to act as a pro-survival or pro-apoptosis mechanism in different physiological and pathological conditions. Since ceramide and S1P have been demonstrated to trigger autophagy with opposing outcomes on cell survival it has been suggested that autophagy is key in controlling the cell fate decision made by these sphingolipids.[80]

Recent studies have shown that sphingolipid-metabolizing enzymes are also active in the extracellular space, including the outer leaflet of the plasma membrane. However, the physiological significance of extracellular sphingomyelin metabolites, beyond S1P binding to S1P receptors, is yet to be elucidated (reviewed in [31]).

Biological functions of sphingolipids

Immune system

Sphingosine kinase and S1P synthesis have been implicated in a number of (patho)physiological processes, including inflammation [81] and asthma [82]. Recent studies reveal that synthesis of sphingomyelin metabolites is critically important for initiation and maintenance of diverse aspects of immune cell activation and function.[83] It has been suggested that the ceramide / S1P rheostat determines the allergic responsiveness of mast cells.[84] The rising interest in sphingomyelin-mediated signalling in immunology started with the discovery of the immune modulator FTY720. This compound is structurally very similar to sphingosine and after phosphorylation by sphingosine kinase, FTY720 binds to and activates S1P receptor isoforms $S1P_{1,3,4,5}$, but not $S1P_2$.[85] The immunosuppressive effect of FTY720 supposedly is due to $S1P_1$ receptor internalisation in lymphocytes, after which they are no longer able to exit the lymph node, resulting in lymphopenia.[86-89] However, one of the side effects that emerged for FTY720 in renal transplantation patients as well as healthy subjects is an asymptomatic reduction in heart rate.[90-92] The pathway underlying this effect is not completely understood, but it is most likely due to stimulation of $S1P_3$ receptors in the heart.[93] Also inhibition of S1P degradation may result in immunosuppressant effects. Interestingly, a substance present in caramel food colourant with immunomodulatory properties, tetrahydroxybutylimidazole, was shown to inhibit S1P lyase activity *in vivo*.[94] Thus the sphingolipid system is an interesting target for immunesuppression.

Tumour development

Cell growth, survival, invasion, and angiogenesis are processes involved in tumour development that can be influenced by sphingomyelin metabolites.[95-97] Interestingly, the expression levels of sphingosine kinase is higher in tumour tissue than in normal tissue and inhibition of sphingosine kinase is anti-proliferative and pro-apoptotic to several tumour cell lines.[98,99] Moreover, inhibition of sphingosine kinase activity enhances the sensitivity of cancer cells to chemotherapy.[100] Therefore, it is a possibility that sphingosine kinase is an oncogene and may be an appropriate protein target for anticancer drug research.[96] Recent studies have also shown a role of S1P lyase in cancer development.[101] In line with the function of the ceramide / S1P rheostat, ceramide functions in an opposite manner of S1P as a tumour-suppressor lipid, inducing anti-proliferative and apoptotic responses in various cancer cells.[102] De novo formation of ceramide has indeed been shown to be, at least in part, important for apoptosis induced by certain anticancer drugs.[103-105] Potential therapeutic targets regarding tumour-angiogenesis mediated by local formation of sphingomyelin metabolites involve accumulation of ceramide in apoptotic mechanisms [106] and limiting S1P signalling. The latter might be achieved through pharmacological inhibition of sphingosine kinase or S1P receptor antagonists.[77]

Neurogenesis

S1P signalling has been shown to be critical for neural development, since sphingosine kinase and $S1P_1$ knockout mice displayed severly disturbed neurogenesis, including neural tube closure, that caused embryonic lethality.[107] Moreover, it has become clear that sphingolipid metabolism is essentially correlated with neuro-degeneration and neuro-transformation [108], due to effects in regulation of cellular processes in neuronal and glial cells.[109,110] Therefore, also in neurogenesis local formation of sphingomyelin metabolites represents an essential component.

Besides important regulatory functions in immune function, tumour development and neurogenesis, the different sphingomyelin metabolites are involved in several (patho)physiological processes in the vasculature that will be discussed in the next paragraphs.

Vascular effects of sphingomyelin metabolites

Blood vessels are composed of vascular smooth muscle cells and a monolayer of endothelial cells, each of which has well-defined roles. The primary function of vascular smooth muscle

tissue is contraction and relaxation of the vessel wall, in order to control the lumen diameter and thus blood flow and blood pressure. Smooth muscle cells are also essential for vascular integrity and elasticity. The endothelium covers the luminal side of the entire cardiovascular system and has been considered a distinct organ. It forms a non-thrombogenic, non-adhesive layer and contributes to the regulation of blood flow and blood pressure via communication the smooth muscle cells by releasing vasodilators, such as NO and vasoconstrictors such as endothelin-1 and thromboxanes.[111-117] NO, produced by endothelial NO synthase (eNOS), is primarily a paracrine, vasorelaxant factor for the underlying vascular smooth muscle cells.[117] Moreover, NO is also vasoprotective through maintenance of important physiological functions such as anticoagulation, leukocyte adhesion, smooth muscle proliferation, and the antioxidative capacity.[118] A variety of vasoactive substances (i.e substances that influence the diameter of a given vessel) influence specific signalling pathways in endothelial or smooth muscle cells via interaction with G protein-coupled receptors. Many of these receptors induce, amongst other effects, an increase in intracellular Ca^{2+}. In endothelial cells, elevation of intracellular Ca^{2+} levels induces activation of eNOS and thus the production of NO. In addition, phosphorylation of eNOS via activation of the PI3 kinase / Akt pathway increases eNOS activity by increasing the Ca^{2+} sensitivity of this enzyme. NO diffuses from the endothelium to the smooth muscle cells where it activates guanylyl cyclase. NO-induced increases in cGMP or increases in cAMP by a receptor-dependent activation of adenylyl cyclase, results in relaxation of the smooth muscle cell. While in larger blood vessels (so called conduit vessels) endothelium-dependent relaxation is mainly mediated by NO, in small diameter blood vessels (resistance vessels), prostaglandines and endothelium-derived hyperpolarizing factors (EDHFs) contribute to a major extend to endothelium-dependent relaxation.[119] Elevation of intracellular Ca^{2+} levels in vascular smooth muscle cells leads to constriction and the effect of a given substance on vascular tone is thus highly dependent on endothelial and smooth muscle cell receptor distribution (figure 3).

It is now generally accepted that endothelial dysfunction plays an essential role in the development of cardiovascular disease.[120-123] Endothelial dysfunction refers to impaired biological processes in endothelial cells, leading to increased adhesiveness to monocytes, increased permeability, procoagulant properties and changes in vascular tone.[124] However, in clinical and experimental settings endothelial dysfunction has been used to describe the impaired NO-mediated vasodilation.

The vascular effects of sphingolipids are diverse and are mediated through various signalling mechanisms.[125] Sphingomyelin metabolites are present in relatively high concentrations in plasma although the largest fraction of these lipids is not free, but stored in platelets, erythrocytes and lipoproteins.[126-129] However, at least for S1P, it is has been shown that they

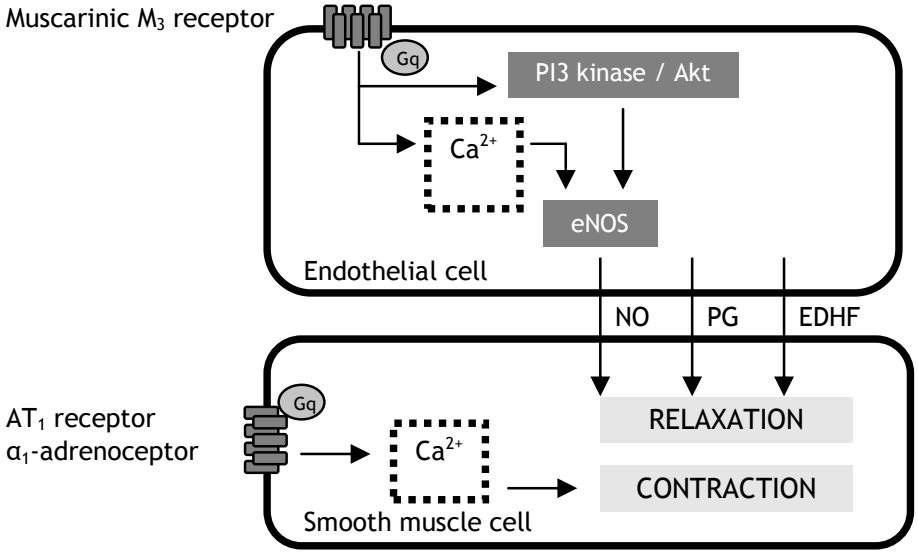

Figure 3. Agonist-induced vascular contraction and relaxation. In the endothelium, activation of for instance muscarinic receptors leads to activation of G_q, elevation of intracellular Ca^{2+} levels and activation of the PI3 kinase / Akt pathway, resulting in the activation of endothelial NO synthase (eNOS). Depending on vessel type also NO-independent relaxant factors, such as prostaglandins (PG) and endothelium-derived hyperpolarizing factors (EDHF), can be formed and released towards the vascular smooth muscle cells. In the smooth muscle cells, G_q-coupled AT_1 receptors and α_1-adrenoceptors also result in elevations of intracellular Ca^{2+} levels, resulting in contraction.

can be released from platelets upon agonist-stimulation and affect vascular tone.[130] However, not all sphingolipid-mediated effects can be completely explained by their extracellular presence. Both endothelial cells and vascular smooth muscle cells express the enzymes involved in sphingolipid metabolism and are, therefore, able to produce sphingolipids with signalling capabilities on demand.[131,132] S1P and ceramide, when applied exogenously *in vitro*, have been demonstrated to possess vasoactive properties. S1P has been shown to induce contraction [133-142] and vasorelaxation [143-145], while ceramide was also shown to induce relaxation [146-151] and contraction [152-154]. These (partly) contradictory findings may be caused by species differences, application of different methods (e.g. wire myograph versus canulated vessels), type of vascular bed, vessel function (conduit versus resistance) and possibly S1P concentration.[131] The finding that systemic S1P administration reduces regional blood flows and increases vascular resistance *in vivo* [134,136,137,142] indicates that at least S1P is more contractile in the smaller (resistance) arteries. The reports that S1P may reduce the mean arterial pressure upon systemic administration do not necessarily contradict these findings as it can be explained by a bradycardiac effect.[93,135,155] However, currently the exact role for sphingomyelin metabolites in the regulation of vascular tone in different vascular beds is unknown, especially their function as downstream signalling entities for known vasoactive compounds.

Variation in S1P receptor isoform expression between vessel types and also between cell types within the vascular wall, will partially determine the effect of S1P in a certain vascular bed. In the vasculature, mainly the S1P receptor isoforms $S1P_1$, $S1P_2$ and $S1P_3$ are expressed in both endothelium and smooth muscle.[21,156] At least at the mRNA level in vascular smooth muscle cells the $S1P_2$ receptor is most abundant, whereas the $S1P_3$ receptor is less abundant. On the other hand, in endothelial cells, relative expression of the $S1P_1$ receptor is higher, while it is lower for the $S1P_3$ receptor, but this is also depedent on the vessel type. The other isoforms $S1P_4$ and $S1P_5$ are (almost) not found at all in the cardiovascular system (for review see: [125], figure 4).

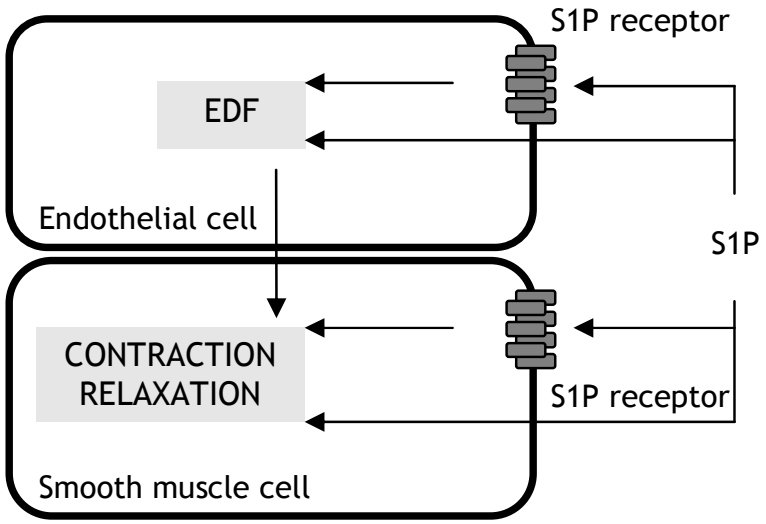

Figure 4. Overview of vascular effects of S1P. When applied *in vitro* or present in blood, S1P can signal via both receptor-dependent and –independent pathways in endothelial cells and vascular smooth smooth muscle cells. EDF = endothelium-derived factor.

Effects of sphingolipids in endothelial cells

Sphingomyelin metabolites can exert various effects in endothelial cells, in receptor-dependent and -independent pathways. The formation of the vasorelaxant factor NO in the endothelium is an important pathway for vasorelaxation that can be affected by sphingomyelin metabolites. Stimulation of the S1P receptor subtypes $S1P_1$ and $S1P_3$ by S1P has been shown to activate eNOS in bovine aortic endothelial cells through activation of protein kinase Akt, leading to phosphorylation and, therefore, increased activity of eNOS.[157] Moreover, it has been shown that this also holds true for S1P produced by the vascular endothelial cell itself.[144] Other sphingomyelin metabolites such as ceramide have also been shown to promote activation and translocation of eNOS. It was demonstrated that ceramide regulates eNOS in endothelial cells independently of the Ca^{2+}-regulated pathways.[158] Therefore, sphingomyelin metabolites can affect endothelial function when they are exogenously present or produced locally by the endothelial cell itself.

Besides effects that may influence the production of vasoactive substances in the endothelium, sphingomyelin metabolites influence the growth of endothelial cells. Angiogenesis is the formation of new blood vessels out of pre-existing ones.[159] Angiogenesis involves proliferation, migration, adhesion, and differentiation of endothelial cells, which, in a later stage, are then lined up by vascular smooth muscle cells. This is achieved through tight regulation of both pro- and anti-angiogenic factors. In adults, the vasculature is normally quiescent and angiogenesis is mainly controlled by pathological conditions, like wound healing, arthritis, psoriasis, diabetic retinopathy and cancer, with the exception of the uterus. During angiogenesis, vessels initially dilate and become leaky in response to vascular endothelial growth factor (VEGF); a specific endothelial cell growth factor. VEGF was initially identified and described as vascular permeability factor because of the strong effect on vascular permeability.[160] Interestingly, the VEGF has several interactions with the sphingolipid system. VEGF exerts pro-proliferative effects on endothelial cells during angiogensis and vasculogenesis partly by activation of sphingosine kinase.[161,162] Moreover, VEGF induces $S1P_1$ mRNA and protein expression in endothelial cells and potentiates the vascular effects mediated by S1P.[163] In the vessel wall extracellular S1P is a potent stimulator of angiogenesis [164,165], since S1P promotes endothelial cell growth, thereby promoting blood vessel formation through interaction with signalling by VEGF (see figure 5).[166] In an *ex vivo* model of angiogenesis, as well as in *in vivo* studies, a synergistic effect of S1P with other angiogenic factors such as basic fibroblast growth factor (bFGF) and stem cell factor was observed on vascular sprouting, proliferation and tube formation during vasculogenesis and neo-vascularization.[167,168] Interestingly, administration of a monoclonal S1P antibody attenuated angiogenesis *in vitro*.[169] The immunosuppressant FTY720 displays functional antagonism of vascular S1P receptors, resulting in potent inhibition of angiogenesis [170] most likely via internalisation of the $S1P_1$ receptor.[171,172] Therefore, FTY720 may also provide a novel therapeutic approach for pathologic conditions with dysregulated angiogenesis. Because under some circumstances, e.g. wound healing, the promotion of cellular growth may be beneficial, also S1P receptor agonists and activators of sphingosine kinase (e.g. monosialoganglioside GM-1) are current research topics.[173,174]

Endothelial cells are a rich and regulatable source of sphingomyelinase, which in turn allows for regulation of ceramide formation.[175] One of the upregulators of sphingomyelinase is the pro-inflammatory cytokine interleukin-1β (IL-1β).[176] Ceramide induces apoptosis in endothelial cells, as it does in many other cell types and S1P stimulates survival, proliferation and migration of human endothelial cells by activating S1P receptors.[177,178] Accordingly, S1P

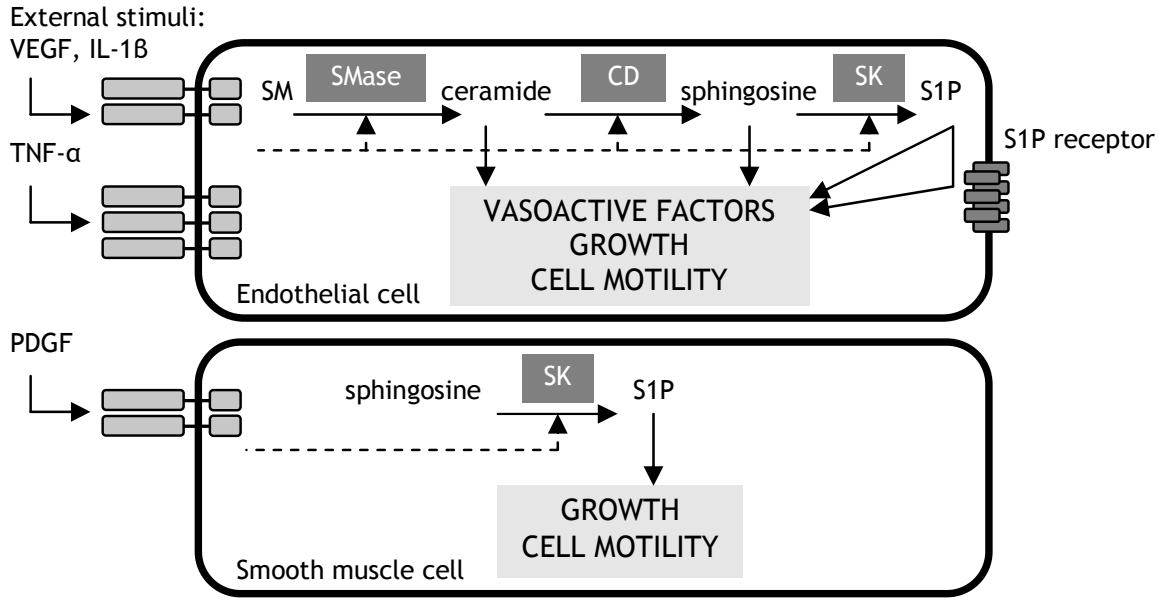

Figure 5. Local formation of sphingomyelin metabolites induced by external stimuli mediate various effects in endothelial cells and smooth muscle cells. VEGF=vascular endothelial growth factor; TNF-α=tumour necrosis factor-α; IL-1β=interleukin-1β; PDGF=platelet-derived growth factor; SM=sphingomyelin; SMase=sphingomyelinase; CD=ceramidase; SK=sphingosine kinase.

protects proliferating endothelial cells from ceramide-induced apoptosis, while this is not the case for DNA damage-induced mitotic death.[179] Ceramide formation in the endothelium has been shown to mediate apoptosis during tumour necrosis factor (TNF)-α-induced inflammation.[180,181] In accordance with these findings, cells overexpressing acid ceramidase are protected from TNF-α-induced apoptosis by increased conversion of ceramide to survival inducing S1P.[182] Moreover, De Palma et al. nicely demonstrated that eNOS activation by TNF-α, resulting in NO, was preceded by sequential activation of both neutral sphingomyelinase and sphingosine kinase and, therefore, generation of S1P [183] (see figure 5). As the vascular endothelium expresses S1P receptors, S1P formed by the endothelial cell can act as an autocrine and / or paracrine mediator of endothelial function.[125] Thus, changes in expression pattern of S1P receptors may also determine the effect of S1P synthesis. It has been demonstrated that H_2O_2, a reactive oxygen species, can upregulate $S1P_1$ receptors in bovine aortic endothelial cells and sensitize the endothelium to S1P-induced formation of the endothelium-derived vasorelaxant factor NO.[156,184] Similar to reactive oxygen species, statins (inhibitors of cholesterol synthesis) increase expression levels of $S1P_1$ receptors in vascular endothelial cells and augment eNOS responses to S1P.[185]

In vascular development the endothelium plays an essential role by interacting with vascular smooth muscle cells for cellular invasion, migration, proliferation and differentiation. As stated earlier, the endothelial $S1P_1$ receptor is required for vascular stabilization during embryonic development.[186] $S1P_1$ receptor gene disruption was associated with severe

vascular malformations leading to embryonic haemorrhage and thereby to intrauterine death between embryonic day 12.5 and 14. Interestingly, in these embryos endothelial tubes were formed and mural cells (pericytes and vascular smooth muscle cells) were recruited to the endothelial tube, however, these cells did not migrate appropriately to completely surround the endothelial cells, resulting in incomplete vascular maturation.[25] In a later study, by making use of conditional endothelium or smooth muscle cell $S1P_1$ knockout animals, it was shown that these severe vascular malformations were only caused by disruption of the endothelial $S1P_1$ receptor and not by disruption of the smooth muscle cell $S1P_1$ receptor.[187] Thus endothelial $S1P_1$ receptors are responsible for smooth muscle cell coverage and hence vascular maturation. The precise mechanism is unknown, but released soluble factors (e.g. cytokines) and / or increased expression of endothelial adhesion molecules by stimulation of the endothelial $S1P_1$ receptor may be involved.[21] The origin of the S1P acting on the S1P receptors during development of the vascular tube has not been described, but due to the absence of blood flow through those immature vessels it could be hypothesized that the required S1P is produced by the endothelial cell itself. Moreover, next to vascular to development, sphingomyelin metabolites generally represent an important component in vascular function that can elicit and control various cellular effects, either through receptor-dependent or -independent manners.

Recently, it has been shown that there is also endothelium-dependent vascular dysfunction in $S1P_2$ receptor knockout mice.[188] These mice are deaf by one month of age, exhibiting pathologies within the barrier epithelium containing the primary vasculature of the inner ear.[189] These data are in accordance with the negative regulation of endothelial morphogenesis and angiogenesis by the $S1P_2$ receptor as shown by Inoki et al.[190] Therefore, endogenously formed S1P, acting via specific S1P receptor isoforms expressed in the endothelium, can exert specific effects during vascular development.

Effects of sphingolipids in vascular smooth muscle cells

The role of sphingomyelin metabolites in vascular effects has been less well studied in vascular smooth muscle cells than in endothelial cells. However, local formation of sphingomyelin metabolites in vascular smooth muscle cells does affect vasoreactivity, as was shown by application of sphingomyelinase exogenously. As with the exogenously added sphingomyelin metabolites, both vasoconstricting [153,154,191] and vasodilating [146,192] responses have been reported. At least in some cases the net effect appears to be endothelium-independent. It has been shown by forced expression of sphingosine kinase in vascular smooth muscle cells that local formation of S1P in the smooth muscle cells of the vessel wall results in an increased vascular tone in resistance arteries and, therefore, it has been

suggested that sphingosine kinase may play an important role in the control of peripheral resistance.[193] Moreover, formation of S1P has been shown to play an important role in mediating pressure-induced, NADPH oxidase-derived reactive oxygen species formation. This in turn increases Ca^{2+} sensitivity of smooth muscle cells, leading to increased vasoconstriction.[194] Therefore, cross-talk of reactive oxygen species and the sphingolipid metabolism may also be important in mediating vascular responses.

Migration of vascular smooth muscle cells is important for development and in vascular pathologies and can be differentially regulated by activation of specific S1P receptor isoforms. While the $S1P_2$ receptor inhibits vascular smooth muscle cell migration, both the $S1P_1$ and $S1P_3$ receptor stimulate migratory responses.[156] Even the expression level of a single S1P receptor isoform, namely the $S1P_1$, has been shown to influence the migratory respons.[195] The expression pattern of S1P receptors under pathological circumstances may, therefore, affect the migratory properties of vascular smooth muscle cells.[131] Also undesirable growth of smooth muscle tissue into the lumen of a blood vessel is an important step in the development of atherosclerotic lesions. Recently, it has been shown in rat aortic vascular smooth muscle cells that S1P is a mitogenic stimulus [196,197], while ceramide induces growth arrest and apoptosis [26,198]. While the S1P receptor isoforms $S1P_{1-3}$ are expressed in the vascular smooth muscle tissue, their mitogenic signalling occurs via distinct pathways [199], of which the $S1P_1$ receptor is most effective in inducing growth via the G_i / PI3 kinase pathway.[195]

Next to sphingolipid-dependent signalling by sphingolipids present in the vicinity of the smooth mucle cells, smooth muscle proliferation can also be affected by local formation of sphingomyelin metabolites as part of growth factor signalling. Platelet-derived growth factor (PDGF) was one of the first growth factors identified to affect growth of vascular smooth muscle cells, mediating arterial wound repair.[200,201] Not much later, the first evidence was provided that PDGF can actually activate sphingosine kinase and, therefore, induce S1P formation.[202] Ceramidase and sphingomyelinase activity are also influenced by PDGF [203,204] and also S1P lyase has been identified as a downstream signalling target for PDGF [205]. In addition, PDGF has been shown to signal via PDGF-β receptor-$S1P_1$ receptor complexes [206] and these complexes can be are required for PDGF-induced mitogenic signalling.[207] It has been proposed that the differences in Ca^{2+} signalling after stimulation with PDGF during different stages of the cell cycle are due to specific modulations of the sphingolipid metabolism.[208] PDGF is also important during vasculogenesis for migration of vascular smooth muscle cells to form the vascular tube and the effects of PDGF on cell motility were found to be sphingosine kinase and $S1P_1$-dependent.[209,210] However, while several signalling

pathways have been associated with modulating the sphingolipid metabolism, the exact pathway how e.g. sphingosine kinase is activated remains to be elucidated.[211]

Besides growth factors, several other receptor systems have been shown to activate sphingolipid metabolizing enzymes in the smooth muscle. Vascular stimulation with TNF-α does not only result in activation of sphingolipid-metabolizing enzymes in the endothelium, also the production of ceramide via the activation of sphingomyelinase can be induced in smooth muscle cells.[212] The formed ceramide inhibits smooth muscle cell proliferation, as part of the inflammatory process.[181] The regulation of vascular tone by vascular smooth muscle cells is subject to endothelium-derived factors like NO, as described earlier. While the short term effect of NO in the vascular smooth muscle cell is relaxation through guanylyl cyclase activation, the long term cellular effect can be inhibition of cellular growth and apoptosis.[213] It has been shown in vascular smooth muscle cells that during NO-induced apoptosis there is an increase in ceramide synthesis, which appears to function as a mediator of apoptosis.[198,214] The importance and tight regulation of the ceramide / S1P rheostat has also been demonstrated by hyperglycemia-induced apoptosis of vascular smooth muscle cells that can be inhibited by activation of sphingosine kinase-1 and thus S1P synthesis.[215]

Hypoxia is a pathological condition in which tissue is deprived of adequate oxygen supply and can have major impact on cellular homeostasis of vascular smooth muscle cells. In order to survive, cells must cope with the new situation in which oxygen supply is limited and they do so by starting to proliferate. Interestingly, it has been shown that local formation of S1P is stimulated in hypoxia-treated vascular smooth muscle cells.[216] Recently, it has also been demonstrated that hypoxia markedly increases expression levels of sphingosine kinase-1 and -2 and even post S1P receptor signal transduction pathways may be modified.[217] Moreover, increasing levels of ceramide inhibit hypoxia-induced proliferation.[218] Therefore, hypoxia-induced vascular smooth muscle cell growth is also a cellular process that modulates sphingolipid metabolism, resulting in reduction of total intracellular ceramide level with concomitant increase in S1P formation, in order to cope with the pathological circumstances.

Conclusion

The sphingomyelin metabolites ceramide, sphingosine and S1P exert many biological effects in a variety of cell types, including endothelial and vascular smooth muscle cells. These sphingolipids are present within the extracellular space (including in blood) and can affect

vessel tone through activation of various contraction- and relaxation-inducing mechanisms in both endothelial cells and smooth muscle cells.

Sphingomyelin metabolites are not only found in blood, they can also be formed by endothelial cells and vascular smooth muscle cells by specific enzymes expressed in these cell types. After formation, they can act as both auto- and paracrine signalling entities affecting not only vascular tone, but also other processes within the vessel wall during vascular development and physiological vascular function are subject to their regulation. Moreover, variations in S1P and ceramide levels within the cellular membrane have been implicated in a number of vascular pathological conditions in which cellular apoptosis and proliferation play important roles.

Currently, it is not known whether vasoactive compounds such as angiotensin II and muscarinic receptor agonists induce the formation or degradation of sphingomyelin metabolites in order to exert their vascular effects. Moreover, it is unknown whether dysregulated cellular growth within the vessel wall during vasculopathies has consequences for the vasoactive effects of sphingomyelin metabolites. Therefore, the exact regulatory role of the sphingolipid metabolism within the vessel wall under normal and pathological circumstances remains to be elucidated. The therapeutic potential of affecting the tight regulation of production and breakdown of sphingomyelin metabolites is great, but much additional research is required to further characterize sphingomyelin metabolite-mediated molecular signalling pathways, to ultimately identify possible pharmacological interventions.

Aim of the thesis

The vasoactive properties of the different sphingomyelin metabolites have mainly been investigated by the addition of exogenous sphingolipids to either isolated vascular preparations or injecting those compounds into the blood stream. These experiments revealed that the different sphingomyelin metabolites indeed possess vasoactive properties. However, in part contradictory results have been obtained. Therefore, the exact role and mechanism of action of sphingomyelin metabolites in the different vascular beds is not precisely known. It has been well documented that most cell types can synthesise the different sphingomyelin metabolites. It is, therefore, striking to see that in the field of vascular biology, in contrast to for instance tumour biology, the role of extracellular (exogenously applied) sphingomyelin metabolites received most attention whereas the role of local formation of sphingomyelin metabolites (i.e. in the vascular wall) has been studied only

sparsely. Indeed, sphingomyelin metabolites can be found in high concentrations in serum and plasma and may act from that compartment on endothelial and smooth muscle cells. However, under normal circumstances these sphingolipids are sequestered in lipoprotein particles or stored in erythrocytes and platelets. Thus the fraction of free sphingolipids in blood may be rather low. Since also endothelial and vascular smooth muscle cells express all the enzymes involved in sphingolipid metabolism and additionally express target molecules such as S1P receptors, sphingomyelin metabolites can act as auto- or paracrine factors in the vasculature. It is unknown whether vasoactive factors make use of sphingomyelin metabolites in order to exert their vasoactive actions. We, therefore, investigated the role of local sphingolipid metabolism induced by known vasoactive compounds in vascular function.

In **chapter 2** we investigated the possible involvement of sphingolipid metabolism in a variety of contractile stimuli in isolated rat carotid arteries. These experiments revealed that angiotensin II induces S1P formation specifically in the endothelium. Here we also have elucidated the mechanism by which local sphingolipid metabolism by angiotensin II modulates vasoconstriction.

As discussed before in this chapter, the different sphingomyelin metabolites are important regulators of growth. S1P has anti-apoptotic properties and acts as a mitogen in most cell types, whereas ceramide and sphingosine, the precursors of S1P, are involved in induction of apoptosis and growth arrest. However, it is not known whether changes in growth responses, for instance as seen during vascular remodelling and intimal hyperplasia, cause alterations in sphingolipid-dependent vasoconstriction or relaxation. Since angiotensin II is a well known hypertrophic factor, we investigated the influence of growth-promoting conditions on angiotensin II-induced sphingolipid-dependent vasoconstriction in **chapter 3**. The results suggest that growth promoting conditions drastically change the role of angiotensin II-induced endothelial sphingolipid metabolism.

Because these previous experiments indicated an important role of sphingolipid metabolism in the endothelium, we additionally investigated the role of sphingolipid metabolism in a receptor system (muscarinic receptors) mainly inducing endothelium-dependent relaxation (**chapter 4**). Literature data suggests that the vasoactive properties of sphingomyelin metabolites may differ between different vascular beds. We, therefore, investigated the influence of sphingosine kinase inhibition on muscarinic receptor-mediated vasorelaxation in isolated rat aorta, carotid and mesenteric arteries.

Hypertension is associated, amongst others, with endothelial dysfunction and vascular remodelling. Concomitantly, during hypertension sphingomyelin metabolite-dependent

signalling may be altered since sphingolipids have (endothelium-dependent) vasoactive and growth regulating properties as shown in chapters 2-4. Therefore, we investigated in **chapter 5** what effect inhibition of sphingosine kinase by the inhibitor dimethylsphingosine has on angiotensin II -induced vasoconstriction and muscarinic receptor-mediated vasorelaxation in different vascular beds of spontaneous hypertensive rats.

Although there may be a role for blood-borne sphingomyelin metabolites in the vascular system, in the previous chapters we have clearly shown that the local synthesis of sphingomyelin metabolites within the vasculature contributes to vascular tone and dysregulation of the sphingolipid metabolism may be a cause or consequence of altered vascular function. In **chapter 6** we examined the downstream signalling of more vasoactive compounds, such as endothelin-1 and histamine, to assess whether local sphingomyelin metabolite formation is entwined in their signalling. It has been speculated that differential S1P receptor expression in endothelial and vascular smooth muscle cells of different vascular beds contributes to the vasoactive effects that S1P may have. Several synthetic and S1P receptor subtype specific agonists have become available recently, that make it possible to test this hypothesis. We have used a combined $S1P_1$ and $S1P_3$ agonist to investigate possible differential effects of S1P receptor stimulation in isolated rat mesenteric arteries and aorta. We also investigated the effects of local sphingolipid metabolism in regard to cell survival within the vessel wall, with special attention for the role of the endothelial sphingolipid metabolism in mediating these effects.

Also in literature, changes in local formation of sphingomyelin metabolites in endothelial cells have been shown to affect endothelial and vascular function. In **chapter 7** we highlight some recent findings on the complex interplay between the local formation of sphingomyelin metabolites and endothelial function. Focus hereby lies on functional aspects of the endothelium, that may play a role during embryogenesis and also in pathological conditions involving endothelial dysfunction such as vascular inflammation and / or chronic heart failure.

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C h a p t e r

2

Sphingosine kinase-dependent activation of endothelial NO synthase by angiotensin II

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Abstract

Objective. In addition to their role in programmed cell death, cell survival and cell growth, sphingolipid metabolites such as ceramide, sphingosine and sphingosine-1-phosphate have vasoactive properties. Besides their occurrence in blood, they can also be formed locally in the vascular wall itself in response to external stimuli. This study was performed to investigate whether vasoactive compounds modulate sphingolipid metabolism in the vascular wall and how this might contribute to the vascular responses.

Materials and methods. In isolated rat carotid arteries, we measured the contractile responses to angiotensin II in the absence or presence of the sphingosine kinase inhibitor dimethylsphingosine. Using the probe DAF-2 DA, we measured cellular NO production in the bEnd.3 endothelial cell line. For intracellular Ca^{2+} levels, we used the Ca^{2+} probe fluo-4 AM. Cell lysates were immunoblotted using the antibodies for phosphorylated eNOS and Akt.

Results. The contractile responses to angiotensin II are enhanced by dimethylsphingosine. Endothelium removal or NO synthase inhibition by n^{ω}-nitro-L-arginine results in a similar enhancement. Angiotensin II concentration-dependently induces NO production in bEnd.3 endothelial cells, that can be diminished by dimethylsphingosine. This sphingosine kinase-dependent endothelial NO synthase activation is mediated via both phosphatidylinositol 3-kinase / Akt and Ca^{2+}-dependent pathways.

Conclusion. Angiotensin II induces a sphingosine kinase-dependent activation of endothelial NO synthase, that partially counteracts the contractile responses in isolated artery preparations. This pathway may be of importance under pathological circumstances with a reduced NO bioavailability. Moreover, a disturbed sphingolipid metabolism in the vascular wall may lead to a reduced NO-bioavailability and endothelial dysfunction.

Introduction

Sphingolipids such as sphingomyelin are a major constituent of cellular plasma membranes. Various stimuli activate enzymes involved in sphingolipid metabolism. Sphingomyelinase catalyzes the hydrolysis of sphingomyelin to form ceramide.[1,2] The sequential action of ceramidase and sphingosine kinase converts ceramide to sphingosine and sphingosine-1-phosphate (S1P), and ceramide synthase and S1P phosphatase can reverse this process to form ceramide from S1P.[3,4] The sphingomyelin metabolites ceramide, sphingosine and S1P are biologically active mediators, which play important roles in cellular homeostasis. In this regard ceramide and sphingosine on the one and S1P on the other hand frequently have opposite biological effects. For example, ceramide and sphingosine are generally involved in apoptotic responses to various stress stimuli and in growth arrest[5,6], while S1P is implicated in mitogenesis, differentiation and migration.[7,8] This homeostatic system is frequently referred to as the ceramide / S1P rheostat.[9] It can be hypothesized that this rheostat also plays a role in vascular contraction and relaxation since S1P, sphingosine and ceramide are, potentially counteracting, vasoactive compounds.[10,11]

The molecular basis of ceramide effects has not been fully explored but is believed to involve stress-activated protein kinases, protein phosphatases such as protein phosphatases 1 and 2, guanylyl cyclase and charybdotoxin-sensitive K^+ channels.[11,12] The molecular basis of S1P effects has been characterized in more detail. S1P can act on specific G protein-coupled receptors, of which five subtypes have been identified until now which are termed $S1P_{1-5}$. These receptors couple to intracellular second messenger systems including intracellular Ca^{2+}, adenylyl cyclase, phospholipase C, phosphatidylinositol 3 (PI3)-kinase, protein kinase Akt, mitogen-activated protein kinases, as well as Rho- and Ras-dependent pathways.[13] The cardiovascular system primarily expresses the receptor subtypes $S1P_{1-3}$, and within the vasculature they are expressed both in vascular smooth muscle and endothelial cells.[14] S1P can cause elevation of intracellular Ca^{2+} in both cell types[10,15,16], which is likely to be the basis of contractile effects in smooth muscle, but can also cause smooth muscle relaxation via activation of endothelial NO synthase (eNOS) and subsequent production of NO.[17]

Physiologically, the vascular wall is exposed to S1P as a constituent of high density lipoproteins[18,19] or upon its release by activated platelets.[20] The experimental addition of exogenous ceramide or S1P imitates this. However, studies in several cell types and tissues demonstrate that various stimuli can elicit local ceramide and S1P formation which then act in an autocrine or paracrine manner.[1,21,22,23] Therefore, it was the aim of the present study to determine whether known vasoactive compounds may exert their vascular effects at least in

part by modulating the ceramide / S1P rheostat. For this purpose we have used the specific sphingosine kinase inhibitor dimethylsphingosine (DMS) [24] to block S1P formation. Using this approach we show that the important vasoactive modulator angiotensin II (Ang II) exerts its effects on isolated rat carotid arteries at least partly via the ceramide / S1P rheostat in the endothelium. This may be of importance in further understanding the underlying pathophysiology of various vascular diseases associated with endothelial dysfunction, such as atherosclerosis and hypertension.

Materials and Methods

Materials

Mouse brain microvascular endothelium-derived bEnd.3 cells were a kind gift from the Department of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands. Ang II was from Bachem (Bubendorf, Germany). DMS was purchased from Biomol (Plymouth Meeting, PA, USA). Dulbecco's modified Eagle's medium (D-MEM), fetal calf serum (FCS), penicillin, streptomycin and phosphate buffered saline (PBS) were from Invitrogen (Breda, The Netherlands). 4,5-Diaminofluorescein-2 diacetate (DAF-2 DA) was from Calbiochem (San Diego, CA, USA). Methacholine hydrochloride, n^{ω}-nitro-L-arginine (L-NNA), phenylephrine hydrochloride (PhE), Ca^{2+} ionophore A23187, wortmannin and bovine serum albumin (BSA) (fatty acid and endotoxin free) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Tween-20 was from Bio-Rad Laboratories (Veenendaal, The Netherlands), PD123319 (1-[(4-(dimethylamino)-3-methylphenyl)methyl]-5-(diphenylace-tyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate) from Parke Davis (Ann Arbor, MI, USA), eprosartan (4-[[2-butyl-5-(2-carboxy-3-thiophen-2-yl-prop-1-enyl)-imidazol-1-yl]methyl]benzoic acid) from Solvay (Hannover, Germany), telmisartan (2-[4-[[4-methyl-6-(1-methylbenzoimidazol-2-yl)-2-propyl-benzoimidazol-1-yl]methyl] phenyl]benzoic acid) from Boehringer Ingelheim (Ingelheim, Germany), VPC 23019 (phosphoric acid mono-[2-amino-2- (3-octyl-phenylcarbamoyl)-ethyl] ester) from Avanti (Alabaster, AL, USA) and vascular endothelial growth factor (VEGF) from Peprotech (London, U.K.). The primary antibodies against Ser^{473} phosphorylated Akt (p-Ser^{473}-Akt) and Ser^{1177} phosphorylated eNOS (p-Ser^{1177}-eNOS) were from Cell Signaling (Beverly, MA, USA).

Contraction experiments

The experiments followed a protocol approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands. Adult male Wistar rats (280 – 320 g, Charles

Rivers, Maastricht, The Netherlands) were anaesthesized by i.p. injection of 75 mg/kg pentobarbitone (O.B.G., Utrecht, The Netherlands). Heparin (500 I.U.) (Leo Pharma B.V., Weesp, The Netherlands) was administered i.p. to prevent blood coagulation. The left common carotid artery was carefully excised in a range just distal from the bifurcation until the level of the aortic arch and immediately placed in Krebs-Henseleit buffer (118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4 and 5.6 mM glucose) at room temperature, aerated with 5 % CO_2 / 95 % O_2, pH 7.4. Four segments of carotid artery were carefully prepared and two stainless steel wires with a diameter of 100 μm (Goodfellow, Huntingdon, U.K.) were inserted into the lumen of each vessel segment. The segments were then transferred into organ baths of a 4-channel wire myograph (Danish Myo Technology, Aarhus, Denmark) and subjected to a normalization procedure according to Mulvany & Halpern.[25] In short, the individual circumference was adjusted to 90 % of the value that the particular vessel would have had at a transmural pressure of 100 mmHg. Afterwards, the arteries were equilibrated for an additional 20 min and the buffer was refreshed after each period of 10 min. The preparations were contracted twice for 10 min with a depolarizing high K^+ Krebs-Henseleit solution (100 mM NaCl was replaced by 100 mM KCl) at intervals of 15 min. Subsequently, the vessels were precontracted with the α_1-adrenoceptor agonist PhE (0.3 μM). After reaching a steady level, one concentration of the endothelium-dependent vasodilator methacholine (10 μM) was added to assess the endothelial integrity. Vessels were excluded when relaxation was less than 80 %. After washing, again 100 mM KCl was added to the vessel segments to obtain the maximal contractile response. After washing and a 30 min preincubation with the sphingosine kinase inhibitor DMS (10 μM), the NOS inhibitor L-NNA (100 μM), the $S1P_1$/$S1P_3$ receptor antagonist VPC 23019 (10 μM) or appropriate vehicles, cumulative concentration response curves (CRCs) for Ang II, PhE or KCl were constructed. In some cases the endothelium was removed mechanically. Isometric force of contraction was measured continuously and data are presented in mN/mm segment length, unless stated otherwise.

Cell culture

bEnd.3 cells were maintained in D-MEM supplemented with 10 % (v/v) heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine in a humidified atmosphere of 5 % CO_2 / 95 % O_2 at 37 °C.[26] Cells were split 1:6 to 1:8 upon reaching confluence. bEnd.3 cells express high levels of eNOS, a phenomenon that is most likely caused by the polyoma virus middle T oncogene used to immortalize the primary cells.[27] These cells do not express a detectable amount of inducible NOS.[28] For NO and Ca^{2+} measurements cells were cultured in black clear-bottom 96-well plates and for immunoblot analysis in 60 mm culture dishes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Before

initiating experiments, bEnd.3 cells were grown in FCS-free culture medium supplemented with 0.1 % (w/v) BSA during 18 h for NO and Ca^{2+} determinations and 72 h for immunoblot analysis.

NO measurements

The reaction of intracellular DAF-2 and the NO oxidation product $N_2O_3^-$, results in formation of a DAF-2 triazole derivative, which is stable and highly fluorescent.[29] To measure NO production, bEnd.3 cells were washed with a Hepes-based buffer (20 mM Hepes, 133 mM NaCl, 6.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 50 μM L-arginine, 0.1 % (w/v) BSA (pH 7.4)) and incubated with 5 μM DAF-2 DA in Hepes buffer for 30 min at RT. Hereafter, cells were washed twice and preincubated for 30 min at 37 °C with buffer, L-NNA (100 μM), DMS (10 μM), VPC 23019 (10 μM), or appropriate vehicle. After this preincubation Ca^{2+} ionophore A23187 or Ang II was added to the wells at indicated concentrations. Fluorescence (excitation 485 nm; emission 538 nm) was measured at 37 °C for 70 min using a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland) and the mean increase in fluorescence was calculated per assay point.[28] NO production is expressed as fold increase in fluorescence of untreated cells. All measurements were performed in triplicate.

Verification of eNOS and Akt phosphorylation by immunoblotting

Preincubation of bEnd.3 cells with the PI3-kinase inhibitor wortmannin (200 nM) or DMS (10 μM) was done for 30 min at 37 °C in D-MEM containing 0.1 % (w/v) BSA. After stimulation of the cells with Ang II or VEGF as a positive control for 2.5 min, cells were washed with ice-cold PBS and incubated with 300 μl extraction buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton X-100, 150 mM NaCl, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 1x protease inhibitor cocktail (Pierce, Rockford, IL, USA), pH 7.5) for 10 min at 4 °C. Cells were scraped and spun down for 10 min at 15,000 g in an Eppendorf centrifuge. Protein concentrations of the supernatant were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. A standard curve was prepared using BSA, ranging from 0-2000 μg/ml in final concentration. Absorption was measured at 595 nm using a Victor 2 plate reader (Perkin Elmer, Wellesley, MA, USA). All measurements were performed in triplicate.

For SDS-PAGE followed by immunoblotting, chemicals and materials were used according to the NuPAGE® electrophoresis system from Invitrogen (Breda, The Netherlands), unless stated otherwise. In short, 10 μg of protein was subjected to SDS-PAGE, on a 4-12% Bis-Tris polyacrylamide gel combined with MOPS buffer. The molecular weight marker Magic Marker was included. After electrophoresis, proteins were transferred to PVDF membranes. Blots

were washed with PBS/T (PBS, 0.1 % (v/v) Tween-20) and incubated with blocking solution (PBS/T, 5 % (w/v) BSA) for 1 h at RT. Afterwards, the blots were incubated overnight at 4 °C with primary antibodies against p-Ser^{1177}-eNOS and p-Ser^{473}-Akt (1:2000), in PBS/T containing 1 % (w/v) BSA. Blots were washed 3x5 min with PBS/T at RT and the donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham, Buckinghamshire, U.K.) (1:10000) was incubated 1 h at RT. After washing 3x5 min, chemiluminescent detection by ECL (Roche Diagnostics, Basel, Switzerland) was performed, according to the manufacturer's instructions. As a loading control, blots were stripped for 1 h using Western Strip Buffer (Pierce, Rockford, IL, USA) and incubated with anti-α-tubulin mouse primary antibody (1:1000) and goat anti-murine secondary antibody (1:10000) (Santa Cruz, Santa Cruz, CA, USA). Bands were quantitated by densitometric analysis using ImageJ (National Institutes of Health, version 1.34n). Phosphorylation of vehicle treated cells was arbitrarily set to 100 %.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) determination

To measure $[Ca^{2+}]_i$, bEnd.3 cells were loaded with the probe fluo-4 AM (4.76 μg/ml) for 60 min at 37 °C in buffer (HBSS (Invitrogen, Breda, The Netherlands) supplemented with 20 mM Hepes and 2.5 mM probenicid (Sigma Chemical Co., St Louis, MO, USA) (pH 7.4)) supplemented with 0.1 % (w/v) BSA and 0.042 % (v/v) pluronic acid F-127 (Molecular probes, Leiden, The Netherlands). Cells were then washed twice with buffer, equilibrated for 30 min at 37 °C and incubated for 30 min with 200 μl buffer containing the antagonists/inhibitors or appropriate vehicles. Using the NOVOstar (BMG Labtechnologies, Offenburg, Germany), 22 μl of 10x concentrated agonist was applied to the cells at 37 °C, while measuring fluorescence (excitation 485 nm; emission 520 nm) every 1 sec. To determine maximum and minimal fluoresence per well, 0.5 % (v/v) Triton X-100 and 7.7 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) (Sigma Chemical Co., St Louis, MO, USA) were used, respectively. Changes in $[Ca^{2+}]_i$ were calculated in nM using basal, maximum and minimal fluorescent signal per sample and the K_d value for fluo-4 AM. All measurements were performed in triplicate.

Real-time quantitative PCR

Cells were washed twice with PBS and lysed in Trizol (Invitrogen, Breda, The Netherlands). Total RNA was isolated according to the manufacturer's protocol with minor changes, using a second chloroform extraction to remove traces of phenol in the aqueous phase, a high salt solution (0.8 M sodium citrate, 1.2 M NaCl) together with isopropanol to precipitate RNA and a second wash of the RNA pellet with 75 % (v/v) ethanol. RNA purity was verified on the Experion (Bio-Rad Laboratories, Veenendaal, The Netherlands) and the RNA concentration was determined by spectrophotometry using the Nanodrop (Isogen Life Science, IJsselstein,

The Netherlands). To eliminate genomic DNA contamination 1 μg of total RNA was treated with DNase I, Amp Grade (Invitrogen, Breda, The Netherlands). cDNA was synthesized by reverse transcription using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Veenendaal, The Netherlands) according to the manufacturer's protocol. A control for the presence of genomic DNA, in which no cDNA was synthesized, was made for each sample. The cDNA of 1 μg RNA was diluted 1:50 for use in real-time quantitative PCR.

Oligonucleotide primers were designed using the D-LUX designer software (Invitrogen, Breda, The Netherlands) based on sequences from the GenBank database (Table 1). Each primer pair was tested for selectivity, sensitivity and PCR efficiency. Constitutively expressed HPRT1 and GAPDH were used as a reference.Relative quantification of mRNA was performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands) following the thermal protocol: 95 °C for 3 min to denature, 40 cycles at 95 °C for 10 sec followed by 60 °C for 45 sec for annealing and extension. The final reaction mixture of 15 μl consisted of the diluted cDNA, 1x iQ SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands), 200 nM forward primer and 200 nM reverse primer. All the reactions were performed in 96-well plates, in duplicate. Controls for genomic DNA were included for each cDNA sample and also a negative control containing only both primers and the iQ SYBR Green Supermix. Oligonucleotide primers used for real-time quantitative PCR are shown in table 1.

Gene	Accession nr		Sequence	Amplicon size
$S1P_1$	NM_007901	forward	cgccCTCTCGGACCTATTAGCAGGcG	87
		reverse	CTGGGCAGGTGTGAGCTTGTA	
$S1P_2$	NM_010333	forward	cgtacaCTGGCTATCGTGGCTCTGTAcG	83
		reverse	CTAGCGTCTGAGGACCAGCAAC	
$S1P_3$	NM_010101	forward	cagagttATGCTGGCTGTCCTCAACTcG	100
		reverse	CTAGACAGCCGCACACCAACC	
$S1P_4$	NM_010102	forward	cggaaAATCCTCTCATCTACTCCTTCcG	100
		reverse	CTCCTGGACCTCGCAGACCTA	
$S1P_5$	NM_053190	forward	cgcgTTGCTATTACTGGATGTCGcG	101
		reverse	GGATTCAGCAGCGAGTTAGCC	
Sphk1	NM_025367	forward	cacatgaCTGTCCATACCTGGTTCATGtG	94
		reverse	CCATCAGCTCTCCATCCACAG	
Sphk2	NM_020011	forward	cgctcGTGGACATTCACAGTGAGcG	104
		reverse	GAGAGGCGTCCACGGTAGGTA	
GAPDH	NM_001001303	forward	TGAAGCAGGCATCTGAGGG	102
		reverse	CGAAGGTGGAAGAGTGGGAG	
HPRT1	NM_013556	forward	CCTAAGATGAGCGCAAGTTGAA	86
		reverse	CCACAGGACTAGAACACCTGCTAA	

Table 1. Oligonucleotide primers used for real-time quantitative PCR. Non-capital letters indicate that these nucleotides are added to form a hairpin.

Statistical analysis

All curve fitting and data analysis was done using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). All data are expressed as means ± S.E.M. for the number of experiments (n) as indicated. Data are analyzed by Student's t-test, one-way ANOVA or one sample t-test where appropriate. A *P* value < 0.05 was considered significant.

Results

Effect of sphingosine kinase inhibition on vascular contraction

In the contraction experiments, the mean normalized diameter of a total number of 82 carotid artery preparations was 1028 ± 8 μm. The maximum contraction evoked by KCl (100 mM) amounted to 4.0 ± 0.5 mN/mm segment length, and there was no significant difference in KCl-induced maximal contractile force between the compared groups. In endothelium denuded preparations KCl responses amounted to 2.6 ± 0.2 mN/mm segment length. DMS (10 μM) and VPC 23019 (10 μM) had no influence on the pretension of the preparations. Preincubation of the vessels with DMS (10 μM) had no significant effect on the potency or efficacy for KCl or PhE. However, DMS induced a leftward shift of the CRC for Ang II (pEC_{50} 9.11 ± 0.05 vs 8.57 ± 0.04 for control, n=7-8) without significantly affecting the efficacy (figure 1).

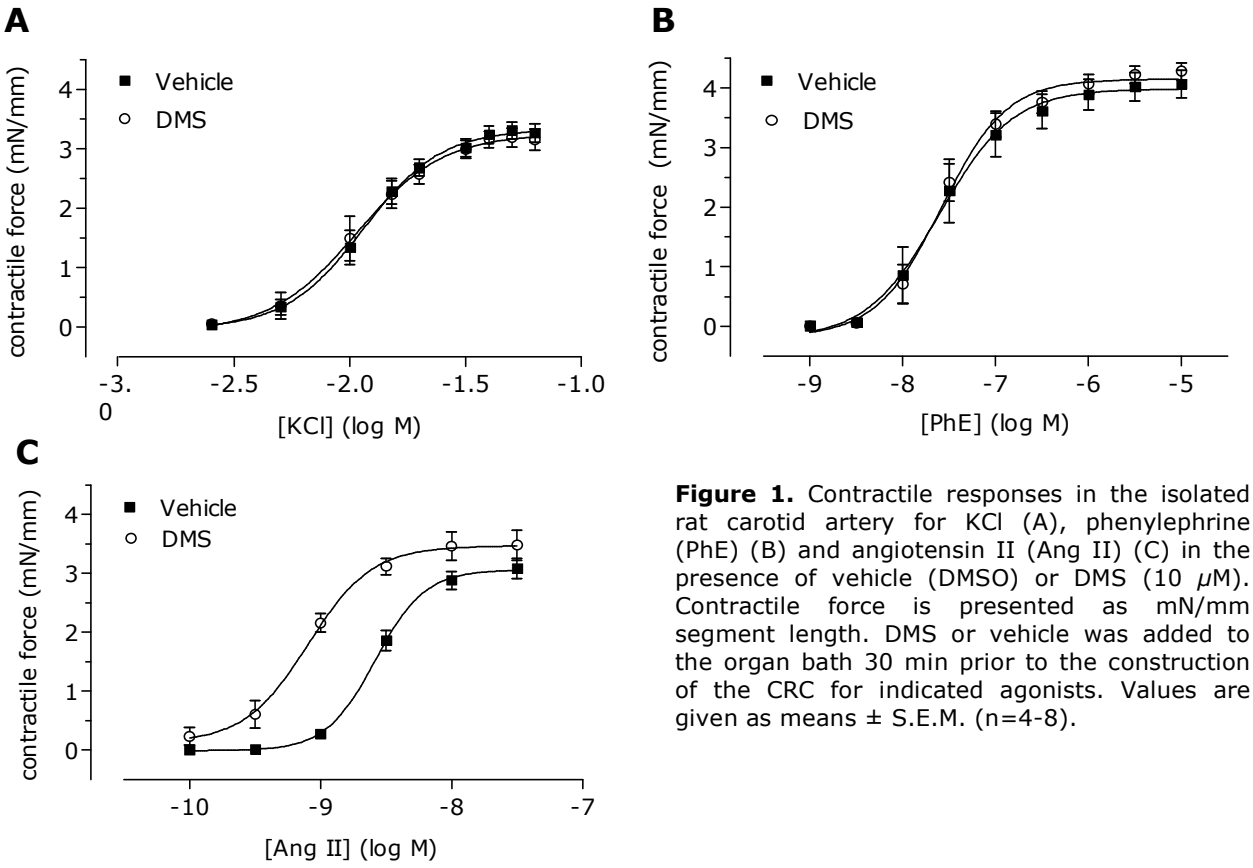

Figure 1. Contractile responses in the isolated rat carotid artery for KCl (A), phenylephrine (PhE) (B) and angiotensin II (Ang II) (C) in the presence of vehicle (DMSO) or DMS (10 μM). Contractile force is presented as mN/mm segment length. DMS or vehicle was added to the organ bath 30 min prior to the construction of the CRC for indicated agonists. Values are given as means ± S.E.M. (n=4-8).

In order to directly compare the results with and without endothelial denudation, data in figure 2A are normalized to the contractile response obtained by the 3rd 100 mM KCl. Preincubating the vessel with the NOS inhibitor L-NNA (100 μM) mimicked the effect of DMS on Ang II-induced contraction (pEC_{50} 9.17 ± 0.20) although there was a more substantial increase in in E_{max} (102.2 ± 3.7 vs 78.4 ± 1.7 % for control, n=7). More importantly, there was no additional effect of DMS when applied simultaneously with L-NNA. Removal of the endothelium resulted in a similar effect as observed for the Ang II-induced contraction in the presence of L-NNA (figure 2). Preincubation of the vessel with the $S1P_1/S1P_3$ receptor antagonist VPC 23019 (10 μM) resulted in a significant increase in E_{max} (3.20 ± 0.26 mN/mm vs 2.53 ± 0.13 mN/mm for control, n=6) and a small, although not significant, leftward shift of the curve for Ang II (figure 2). The AT_2 receptor antagonist PD123319 (10 μM) did not show any effect (data not shown).

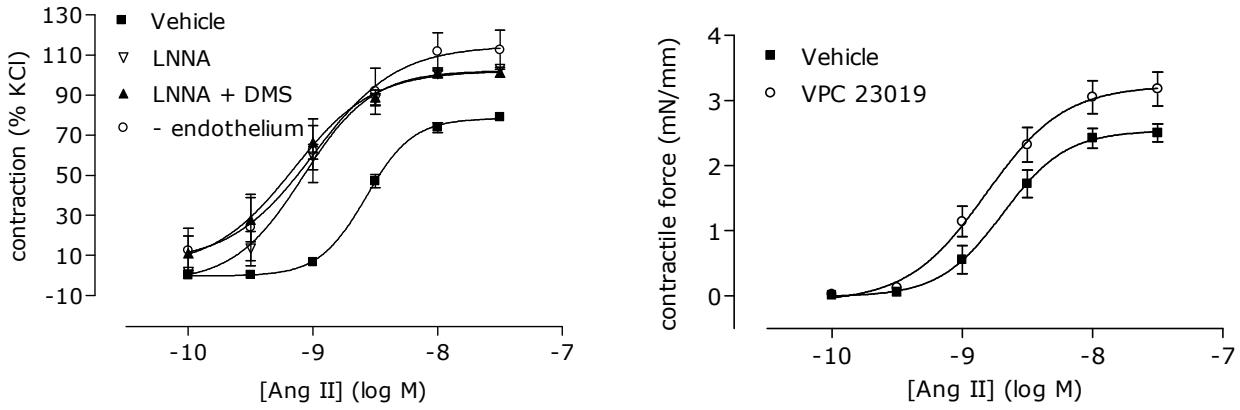

Figure 2. (left) Contractile responses for Ang II measured in the isolated rat carotid artery in the presence of L-NNA (100 μM), both L-NNA (100 μM) and DMS (10 μM), vehicle (distilled water and distilled water and DMSO, respectively) or after removal of endothelium (- endothelium). Data are normalized to the contractile response obtained by the 3rd 100 mM KCl. As a reference the control Ang II (Vehicle) curve is shown. (right) Contractile responses for Ang II measured in the isolated rat carotid artery in the presence of VPC 23019 (10 μM) or its vehicle (DMSO). Contractile force is presented as mN/mm segment length. Inhibitors or vehicles were added to the organ bath 30 minutes prior to the construction of the cumulative CRC for Ang II. Values are given as means ± S.E.M. (n=5-8).

Role of sphingosine kinase in Ang II-induced NO release *in vitro*

Ang II concentration-dependently increased NO production in the bEnd.3 cell line (figure 3). DMS and VPC 23019 had no effect on basal NO production (1.00 ± 0.10, n=10 and 0.98 ± 0.07, n=6, respectively). Preincubation of the cells with 10 μM DMS or 10 μM VPC 23019 inhibited Ang II-induced NO production to approximately basal level. 100 μM L-NNA further diminished the NO production. As a positive control, Ca^{2+} ionophore A23187 (2.5 μM) induced a NO response of approximately 2.5 fold of basal, which was not significantly influenced by DMS (figure 3). The α_1-adrenoreceptor agonist PhE did not induce NO production in bEnd.3 cells (data not shown).

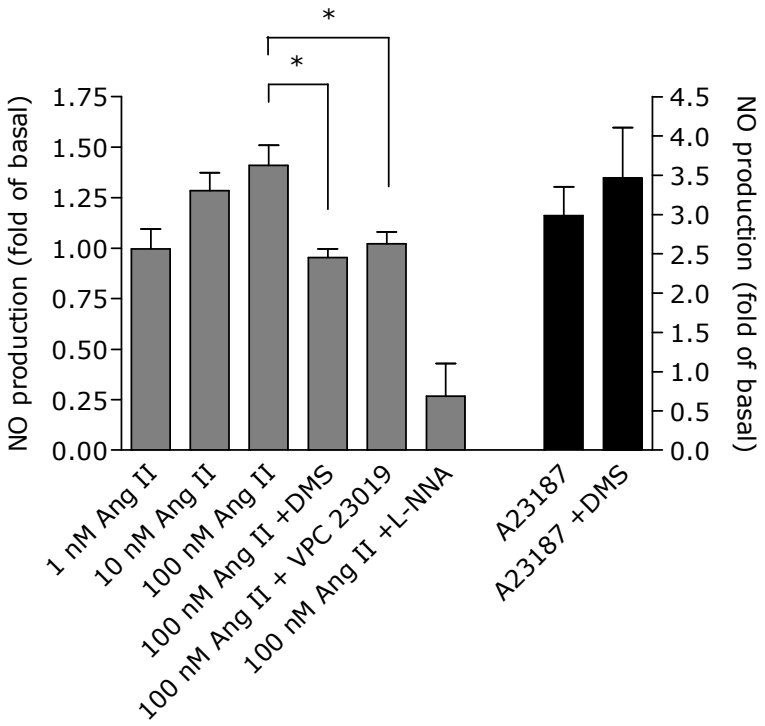

Figure 3. NO formation measured directly in bEnd.3 endothelial cells using the specific fluorescent NO probe DAF-2 DA. Cells were preincubated with DMS (10 μM), L-NNA (100 μM), VPC 23019 (10 μM), vehicle (DMSO, distilled water and DMSO, respectively) or none. Afterwards, cells were stimulated with the positive control Ca^{2+} ionophore A23187 (2.5 μM), Ang II (1, 10 and 100 nM) or vehicle (DMSO and distilled water, respectively). NO levels are calculated using the mean increase in fluorescence, measured every 2 min over a period of 70 min and are expressed as fold of basal and means ± S.E.M. (n=6-19). * P < 0.05. Note the differential right Y-axis for Ca^{2+} ionophore A23187 data.

Effects of sphingosine kinase inhibition on $[Ca^{2+}]_i$ changes

Ang II concentration-dependently increased $[Ca^{2+}]_i$ in the bEnd.3 cell line. Preincubation of the cells with 10 μM DMS prevented the Ang II-induced Ca^{2+} increase completely. The Ang II-induced Ca^{2+} release was also inhibited by the AT_1 receptor blocker telmisartan (10 nM), but not by 100 nM PD123319, an AT_2 receptor specific antagonist. Preincubation with 10 μM DMS did not influence the Ca^{2+} ionophore A23187 (2.5 μM) induced increase in $[Ca^{2+}]_i$ (figure 4).

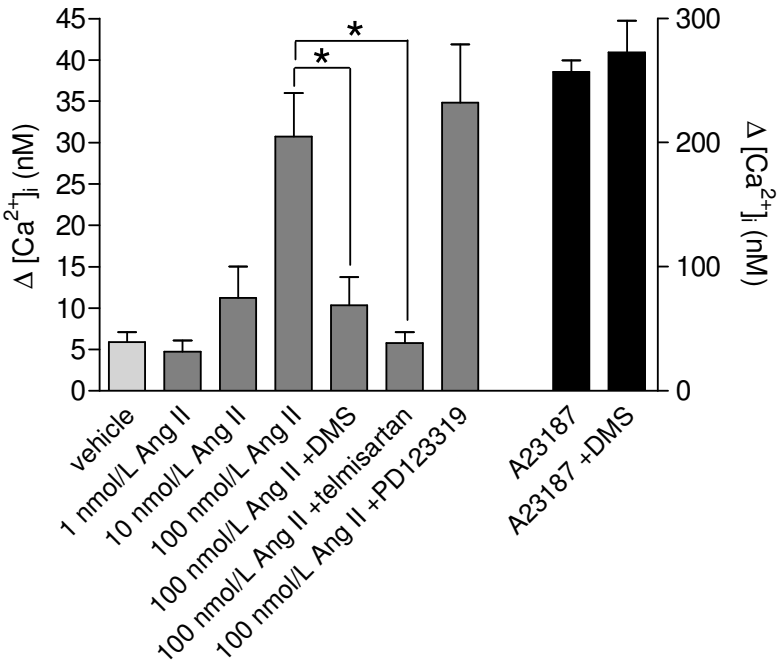

Figure 4. $[Ca^{2+}]_i$ in bEnd.3 cells. After loading with Fluo-4 AM, cells were preincubated with DMS (10 μM), the AT_1 receptor antagonist telmisartan (10 nM), the AT_2 receptor antagonist PD123319 (100 nM), vehicle (DMSO, distilled water and distilled water, respectively) or none. Cells were then stimulated with Ang II (1, 10 and 100 nM), Ca^{2+} ionophore A23187 (2.5 μM) or vehicle under constant measuring of fluorescence and changes in intracellular Ca^{2+} concentrations ($\Delta [Ca^{2+}]_i$) were calculated. Ca^{2+} levels are expressed in nM and means ± S.E.M. (n=4-9). * P < 0.05. Note the differential right Y-axis for Ca^{2+} ionophore A23187 data.

Role of Akt in Ang II-induced eNOS activation

To investigate the role of the PI3-kinase/Akt pathway in Ang II-induced sphingosine kinase activity and subsequent eNOS activation, we stimulated bEnd.3 cells with 100 nM Ang II or 20 ng/ml VEGF either in the presence or absence of 10 μM DMS or the PI3-kinase inhibitor wortmannin (200 nM). In a pilot study we investigated the time-dependency of Ang II and VEGF (as a positive control)[30]-induced phosphorylation of Akt and eNOS. This revealed that the maximal phosphorylation occurred at a timepoint of 2.5 min. Ang II (100 nM) induced Akt phosphorylation to a similar extent as VEGF, which was inhibited by DMS. DMS had no influence on basal level of Akt or eNOS phosphorylation (data not shown). Ang II (100 nM) induced eNOS phosphorylation, which was also inhibited by DMS. The PI3-kinase inhibitor wortmannin abolished both Akt and eNOS phosphorylation. As a loading control, the bands for the antibody directed against the general protein α-tubulin are shown (figure 5).

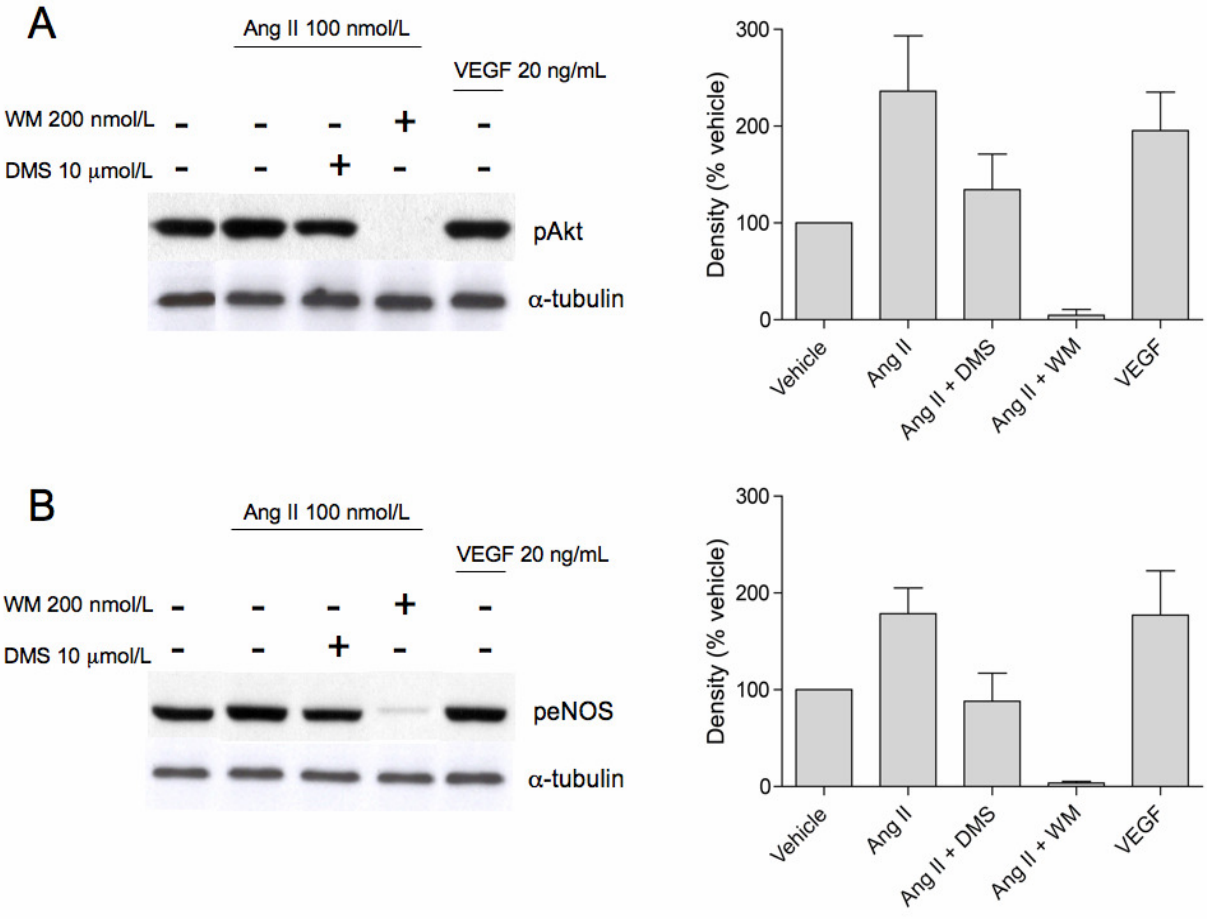

Figure 5. Ang II-mediated Akt and eNOS phosphorylation. bEnd.3 cells were stimulated with Ang II or VEGF for 2.5 min with or without preincubation with DMS, wortmannin (WM) or vehicle (DMSO for both) for 30 min. Protein extracts were analyzed for phospho-Ser^{473}-Akt (pAkt) (A) and phospho-Ser^{1177}-eNOS (peNOS) (B) by Western blotting. Loading controls for α-tubulin content are shown. All results are representative for four experiments. Densitometric analysis of blots are shown, with the phosphorylation of vehicle treated cells arbitrarily set to 100 %.

Expression of S1P receptor and sphingosine kinase subtypes

The rankorder of expression of S1P receptor subtypes in the bEnd.3 cell line, based on the raw Ct values from Real Time PCR from 3 independent experiments, was as follows: $S1P_1$ (29.2 ± 0.6) ≥ $S1P_2$ (31.5 ± 0.6) > $S1P_4$ (34.8 ± 0.5) with $S1P_3$ and $S1P_5$ not detectable. SphK2 (29.9 ± 1.0) was expressed higher than SphK1 (34.9 ± 0.5). In comparison, the Ct values for the housekeeping genes HPRT1 and GAPDH were 29.4 ± 0.7 and 21.7 ± 0.5, respectively).

Discussion

S1P, sphingosine and ceramide are interconvertible sphingolipids that have important effects on cellular homeostasis. S1P has been shown to induce cell growth and survival[7,31], whereas ceramide and sphingosine, the metabolic precursors of S1P, have been shown to induce apoptosis and growth arrest.[5,6] Accordingly, the dynamic balance between ceramide and sphingosine versus S1P, referred to as the ceramide / S1P rheostat, is thought to be an important determinant of cell fate.[9] We hypothesized that this rheostat may play a role in vascular contraction and relaxation since S1P, sphingosine and ceramide are, potentially counteracting, vasoactive compounds.[10,11] S1P and ceramide, when applied exogenously or administered *in vivo*, can have differential effects that may be dependent on type of vascular bed, species and/or method used to study vascular contraction and relaxation (e.g. *in vivo*, *ex vivo*, wire myograph, canulated vessels). It is still unknown whether physiologically relevant vasoactive factors make use of the rheostat by activating one or more of the aforementioned key enzymes in order to exert their vasoactive effects. Therefore, we have investigated the role of the rheostat in agonist-induced vascular responses by inhibition of sphingosine kinase rather than by applying sphingolipids exogenously.

Here we show that the presence of the specific competitive sphingosine kinase inhibitor DMS substantially potentiated the Ang II-induced contractile effect. In contrast, the contractile effects of the α_1-adrenoceptor agonist PhE or receptor-independent constriction by KCl were unaffected. There are early reports stating that DMS may act as a protein kinase C (PKC) inhibitor *in vitro* [32,33], however Edsall et al. [24] have shown that DMS is a specific sphingosine kinase inhibitor in cellular systems at concentrations up to 50 μM. A PKC-independent action of DMS in monocytes, at concentrations higher than 10 μM, was reported recently by Lee et al.[34] This is in concurrence with our finding that the PKC inhibitor calphostin C (100 nM) did not affect the Ang II-induced contraction (data not shown). Moreover, when DMS would be a PKC inhibitor in our system, one would, if anything, expect an opposite response (i.e. a

rightward shift of the CRC for Ang II and PhE) since PKC activation can be involved in smooth muscle cell contraction. Lastly, the fact that the CRCs for PhE and KCl are not influenced by DMS supports a specific effect on sphingosine kinase rather than a non-specific effect on PKC.

The leftward shift of the CRC for Ang II implicates that endogenous S1P, which formation is inhibited by DMS, has vasodilatory properties, or that ceramide or sphingosine (that may accumulate) have contractile properties in our system. Since NO is the major relaxing factor throughout the vasculature, we investigated whether the leftward shift of the Ang II curve by sphingosine kinase inhibition is attributable to a decrease in NOS activation. Preincubation with the NOS inhibitor L-NNA, or removal of the endothelium, indeed leads to a similar leftward shift of the CRC for Ang II. More importantly, DMS in the presence of L-NNA did not further influence the CRC for Ang II, suggesting that a decreased activation of NOS might indeed mediate the leftward shift of the Ang II CRC in the presence of DMS. This implicates that Ang II under normal circumstances induces NO production, a phenomenon that also has been shown by others.[35,36] The fact that L-NNA, in contrast to DMS, also increases the E_{max} of Ang II might be attributable to inhibition of basal NO production by L-NNA. (figure 3). NO production by Ang II has been attributed to both AT_1 and AT_2 receptor stimulation. The lack of effect of the specific AT_2 antagonist PD123319 in the present study indicates that the Ang II-induced NO production is due to AT_1 receptor stimulation, which is in accordance with findings of Boulanger et al.[37] In order to show that indeed the Ang II-induced NO production is inhibited by DMS, we measured NO formation directly in cultured vascular endothelial cells. The bEnd.3 endothelial cell line is known to express relatively high levels of eNOS and therefore is highly suitable to investigate relatively small alterations in eNOS activity.[27,28] Ang II induced a concentration-dependent increase in NO production in the bEnd.3 cell line that could be completely inhibited by DMS and L-NNA. In these experiments DMS had no influence on the NO production induced by Ca^{2+} ionophore A23187, indicating that DMS had no a-specific influences in this assay. These findings suggest that either Ang II-induced S1P production leads to activation of eNOS or that ceramide and/or sphingosine inhibit eNOS activity. The former explanation is not unlikely since it has been demonstrated before that S1P can lead to NO formation through increased eNOS activity in the endothelium, which can be mediated via both intracellular Ca^{2+} mobilization and phosphorylation of Akt and eNOS.[38,39]

To test the involvement of Ca^{2+} elevation in the Ang II-induced eNOS activation via endogenous S1P formation, we measured Ang II-induced changes in $[Ca^{2+}]_i$ in the bEnd.3 cells. $[Ca^{2+}]_i$ was modestly elevated in bEnd.3 cells after stimulation with Ang II, in a concentration dependent manner. This rise in $[Ca^{2+}]_i$ could be inhibited by DMS, whereas the changes in $[Ca^{2+}]_i$ caused by the receptor independent influx of Ca^{2+} by the Ca^{2+} ionophore A23187 were not affected by DMS, indicating that DMS has no a-specific effect in this assay.

The fact that the Ca^{2+} response for Ang II was inhibited by telmisartan, but not PD123319 demonstrates again an AT_1 receptor mediated effect.

The second major pathway leading to increased eNOS activity is via phosphorylation of Akt and eNOS. Ser^{1177} phosphorylation of eNOS by Akt (that can be activated by PI3-kinase) increases the sensitivity of eNOS for the Ca^{2+}/calmodulin complex by approximately 10-15 times and is therefore an important mechanism underlying increased NO production. Both exogenously applied S1P [17,39,40] and Ang II receptor activation [41,42] have been shown to induce Akt and eNOS phosphorylation in cultured endothelial cells. In the present study, Ang II rapidly (within 2.5 min) induced phosphorylation of Akt and eNOS that could be inhibited by DMS. Wortmannin, a specific inhibitor of PI3-kinase, also inhibited phosphorylation of Akt and eNOS induced by Ang II. Therefore it seems that sphingosine kinase activity is not only important for the mobilization of intracellular Ca^{2+}, but also the PI3-kinase/Akt pathway in the Ang II-induced activation of eNOS. The latter finding points towards a receptor-mediated phenomenon and both stimulation of $S1P_1$ and $S1P_3$ receptors have been reported to result in increased NO formation via the PI3-kinase/Akt pathway in cultured endothelial cells.[40,43] This indicates that it is most-likely S1P that increases eNOS activity via one or more types of S1P receptors expressed in the endothelium. Interestingly, a similar signalling mechanism has been shown recently for TNF-α-induced eNOS activation in endothelial cells. In this report the authors showed that silencing $S1P_1$ and/or $S1P_3$ receptors by means of siRNA, prevents eNOS activation by TNF-α.[44] To investigate whether $S1P_1$ and $S1P_3$ receptors are involved in the Ang II-induced NO production, we tested whether the novel $S1P_1$/$S1P_3$ receptor antagonist VPC 23019 also augments the contractile effects of Ang II in the rat carotid artery, as seen for DMS and L-NNA. Indeed VPC 23019, one of the few available S1P receptor antagonists, induced a significant increase in E_{max} and a small, although not significant leftward shift of the CRC for Ang II. Moreover, VPC 23019 also inhibited the Ang II-induced production of NO in the bEnd.3 cell line. These data indeed may point towards involvement of S1P receptors, but S1P receptor-independent mechanisms can not be excluded. A similar sphingosine kinase-dependent formation of NO has very recently been shown for the vasodilatory action of acetylcholine, although these effects appeared not to be mediated by S1P receptors.[45] To further investigate the role of S1P receptors, receptor subtypes or the putative intracellular targets, genetic models can be used. Using $S1P_3$ knock-out mice, it was for instance recently shown that high density lipoproteins, known to carry S1P, and the immunomodulator and S1P receptor agonist FTY720 induce an endothelium- and NO-dependent vasorelaxation via the $S1P_3$ receptor *in vitro* and *ex vivo*.[38,46]

Taken together, these data suggest that activation of the endothelial AT_1 receptor by Ang II leads to a modulation of the sphingolipid metabolism, resulting in an increased NO production. This is most likely the result of an increased sphingosine kinase activity leading to an increased production of S1P that subsequently stimulates (an) endothelial S1P receptor(s). Via activation of the PI3-kinase/Akt pathway and Ca^{2+} mobilization, eNOS activity is increased and the resulting NO formation counteracts the Ang II-induced smooth muscle cell contraction (see figure 6). This counteracting effect may be of importance under pathological circumstances with a reduced bioavailability of NO such as atherosclerosis and hypertension. Moreover, a disturbed regulation of the ceramide / S1P rheostat (e.g. reduced sphingosine kinase activity) may be another mechanism leading to reduced NO-bioavailability and endothelial dysfunction.

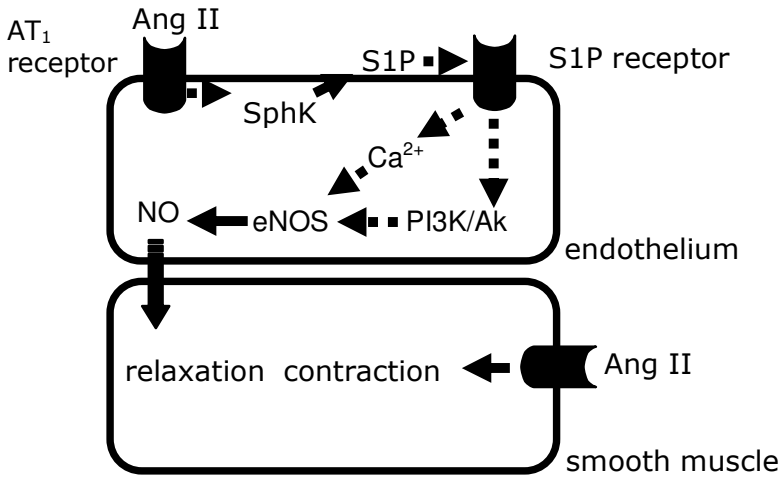

Figure 6. Overview of the suggested role of the ceramide / S1P rheostat during Ang II-induced vascular contraction. AT_1 receptor activation in the endothelial cell leads to endogenous formation of S1P via activation of sphingosine kinase (SphK). This subsequently leads to activation of eNOS involving both release of intracellular Ca^{2+} and phosphorylation of Akt and eNOS via the PI3-kinase pathway. The resulting formation of NO has a counterbalancing effect on the Ang II-induced contraction in vascular smooth muscle.

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C h a p t e r

3

Growth promoting conditions alter the role of sphingolipids in the vasoactive effects of angiotensin II

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Submitted for publication

Abstract

Introduction. We have previously shown that angiotensin II induces local (i.e. endothelial) sphingolipid metabolism resulting in an attenuation of vascular constriction by inducing NO production. Next to vasoactive properties, sphingolipids have important growth regulating properties. The present study was performed to investigate whether growth promoting conditions alter (sphingolipid-dependent) contractile effects of angiotensin II in the vasculature.

Materials and methods. The effects of the sphingosine kinase inhibitor dimethylsphingosine (DMS) on the angiotensin II-induced vasoconstriction under physiological and under growth promoting conditions (i.e. 24 h culture in the presence of 20% serum) were studied in isolated rat carotid arteries. In addition, we investigated the effects of sphingolipids on, and the role of sphingolipid metabolism in (growth factor-induced) growth of VSMCs.

Results. DMS potentiated the contractile response to angiotensin II in non-cultured preparations, whereas it attenuated this response in cultured preparations. Interestingly, this attenuation was endothelium-dependent. Sphingosine-1-phosphate concentration-dependently increased BrdU incorporation in cultured VSMCs, whereas ceramide and DMS concentration-dependently reduced BrdU incorporation and induced apoptosis in these cells. In addition, DMS concentration-dependently inhibited basic fibroblast growth factor and angiotensin II-induced VSMC proliferation.

Conclusions. VSMC growth can be modulated by exogenous and endogenous sphingolipids. Nevertheless, under both normal and growth promoting circumstances, only activation of sphingosine kinase in the endothelium by angiotensin II results in altered contractile responses, albeit with opposite effects. In vascular pathologies characterized by vessel growth, changes in endothelial sphingolipid metabolism may drastically influence angiotensin II-induced vascular contraction.

Introduction

The sphingomyelin metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) are important mediators of various cellular processes and are known modulators of vascular tone when applied to different vascular beds *in vivo* and *in vitro*.[1] Sphingomyelin metabolites which are present in blood can affect both endothelium and vascular smooth muscle cells (VSMCs). However, the majority of circulating sphingolipids are sequestered in lipoproteins or stored in platelets and erythrocytes, most likely resulting in a small fraction of free sphingolipids.[2-5] Both VSMCs and endothelial cells express the enzymes involved in sphingolipid metabolism and are thus able to synthesize the different sphingomyelin metabolites. Sphingomyelinase catalyzes the hydrolysis of sphingomyelin, an abundant phospholipid in mammalian cell membranes, to generate ceramide, which subsequently can be converted to sphingosine by ceramidase. Phosphorylation of sphingosine by sphingosine kinases yields S1P.[6,7] S1P phosphatases and ceramide synthase can reverse this process and generate ceramide from S1P.[8,9]

The different sphingomyelin metabolites frequently have opposite biological effects. S1P, in most cases, leads to cell survival, mitogenesis, differentiation and migration [10,11], while ceramide and sphingosine are generally involved in growth limiting responses (e.g. induction of apoptosis) to various stress stimuli.[12,13] It has been proposed that it is not the absolute amount, but the balance between these sphingolipids which is most important in deciding cell fate. This is referred to as the ceramide / S1P rheostat.[14]

The effect of S1P on vascular contractility is diverse, since S1P has been shown to induce contraction in several vascular beds, but vasodilation has also been described under other circumstances (reviewed in [15]). Most of the biological effects of S1P are thought to be mediated by at least 5 subtypes of G protein-coupled receptors with high affinity for S1P ($S1P_{1-5}$).[16] These receptors function via various intracellular second messenger systems, including inhibition of adenylyl cyclase, stimulation of phospholipase C, phosphatidylinositol 3-kinase / protein kinase Akt and mitogen-activated protein kinases, as well as Rho- and Ras-dependent pathways.[17] $S1P_{1-3}$ are the major S1P receptor subtypes expressed in the vasculature.[1,18]

To exert their biological effect, various stimuli (e.g. growth factors) can activate sphingolipid metabolizing enzymes resulting in the local formation of sphingomyelin metabolites.[19,20] Recently, we have shown that angiotensin II (Ang II), via stimulation of the AT_1 receptor, modulates sphingolipid metabolism in the vasculature by activating sphingosine kinase in the

endothelium. The subsequent local production of S1P leads to an activation of endothelial NO synthase, resulting in the production of NO, which partially counteracts the Ang II-mediated vascular constriction.[21] Concomitantly, the contraction inducing effects of Ang II on VSMCs are under normal circumstances not sphingosine kinase-dependent.

The vascular effects of Ang II are not limited to modulation of vessel tone. Activation of the AT_1 receptor has been linked to hypertrophy and hyperplasia of VSMCs, leading to vascular neointima formation and media thickening.[22,23] The signalling pathway of Ang II-induced VSMC proliferation is not fully understood and there are various pathways implicated to play a role (e.g. extracellular signal-regulated kinase (ERK), jun N-terminal kinase (JNK) and p38 MAP kinases).[22,24] Besides Ang II, many other growth factors (e.g. basic fibroblast growth factor (bFGF)) are implicated in VSMC growth and proliferation and several of these factors can modulate sphingolipid metabolism in order to exert their effects. Because of the dual role of sphingomyelin metabolites (having vasoactive and growth modulating properties) we investigated whether under growth promoting conditions the contractile properties of Ang II are altered due to alterations in sphingolipid metabolism and/or signalling in VSMCs and how sphingolipid metabolism affects (growth factor-induced) VSMC growth.

Here we show that VSMC growth can be modulated by exogenous and endogenous sphingolipids. However, under both normal and growth promoting circumstances, only Ang II-mediated activation of sphingosine kinase in the endothelium contributes to altered vascular responses. Interestingly, this endothelial sphingosine kinase activation results in opposite contractile responses under normal and growth promoting conditions.

Materials and Methods

Materials

Ang II was from Bachem (Bubendorf, Germany). Methacholine hydrochloride (MCh), (R)-(-)-phenylephrine hydrochloride and bovine serum albumin (BSA) (fatty acid and endotoxin free) were purchased from Sigma-Aldrich Chemical Co (St Louis, MO, USA). Dimethylsphingosine (DMS, dissolved in DMSO) was purchased from Biomol (Plymouth Meeting, PA, USA). bFGF (dissolved in 0.1 % BSA (w/v) in sterile water), Dulbecco's modified Eagle's medium (D-MEM), MEM199, fetal calf serum (FCS), penicillin, streptomycin and phosphate buffered saline (PBS) were from Invitrogen (Breda, The Netherlands). C_2-ceramide (dissolved in 0.5 % (v/v) DMSO and 6.6 % (w/v) BSA in sterile water) and S1P (dissolved in 0.4 % (w/v) BSA in sterile water) were from Avanti (Alabaster, AL, USA) and smooth muscle actin 1A4 antibody was

from Dako (Glostrup, Denmark). Unless otherwise stated, all compounds were dissolved in sterile water.

Carotid artery segment contraction experiments

The experiments followed a protocol approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands, were in line with NIH guidelines for the care and use of experimental animals and were conducted as described before.[21] Cumulative concentration response curves (CRCs) for Ang II and phenylephrine were constructed in carotid artery segments after 30 min pre-incubation with the sphingosine kinase inhibitor DMS (10 μM) or vehicle (DMSO). In some cases, carotid artery segments were cultured for 24 h in MEM199 culture medium containing 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5 % CO_2 / 95 % O_2 at 37 °C, in the absence or presence of 20 % (v/v) heat-inactivated FCS. In selected preparations the endothelium was removed (after culturing) by gently rubbing the vascular segments. Endothelium integrity was checked for all preparations as previously described.[21] Isometric force of contraction was measured continuously and data are presented as % of the 3rd KCl (100 mM) induced contraction.

Cell culture

VSMCs were obtained using a tissue outgrowth model.[25] The thoracic part of the rat aorta was cut into pieces of approximately 1 by 1 mm and cultured in MEM199 culture medium containing heat-inactivated 20 % FCS, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5 % CO_2 / 95 % O_2 at 37 °C, until cells grew out of the tissue. VSMCs were cultured in 0.1 % (w/v) gelatin / PBS coated flasks and maintained in MEM199 culture medium, containing 10 % heat-inactivated FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. VSMCs were split 1:3 to 1:4 upon reaching confluence. Using a smooth muscle actin antibody staining, the purity of the smooth muscle cell culture was confirmed. For proliferation experiments, cells were plated at 10,000 cells/well and cultured in black clear-bottom 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Before initiating experiments, VSMCs were cultured in 0.5 % FCS (v/v) culture medium for 18 h.

5-Bromo-2'-deoxyuridine (BrdU) incorporation

Proliferation of cells was quantified using a BrdU incorporation assay from Roche Diagnostics (Basel, Switzerland), according to the manufacturer's instructions. In short, cells were stimulated for 24 h in the presence of 0.5 % FCS (v/v) with S1P, ceramide, DMS, bFGF, Ang II or appropriate vehicles at the indicated concentrations. Subsequently, BrdU was added and cells were cultured for another 24 h. Cells were fixed, the DNA was denatured and an anti-

BrdU-peroxidase antibody was allowed to bind to the BrdU incorporated in the DNA. The immune complexes were detected by a chemiluminescent substrate and the luminescence was measured directly for 0.1 sec per well using a Victor 2 plate reader (Perkin Elmer, Wellesley, MA, USA). The luminescent signal of vehicle-treated cells was arbitrarily set to 100 %.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)

Apoptosis was visually detected using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, USA), according to the manufacturer's instructions. In short, cells were cultured in 8 well Lab-Tek permanox chamber slides (Nunc, Rochester, NY, USA), in 0.5 % FCS (v/v) culture medium for 24 h and stimulated with DMS, ceramide or appropriate vehicle at the indicated concentrations, in the absence or presence of 30 ng/ml bFGF, for 48 h. As a positive control, DNA fragmentation was induced by DNase I (Invitrogen, Breda, The Netherlands). Afterwards, cells were fixed, permeabilized and fluorescein-12-dUTP was allowed to incorporate at 3´-OH DNA ends using the enzyme TdT. Cells were mounted in Vectashield containing DAPI (to visualize all nuclei) (Vector Laboratories, Burlingame, CA, USA) and the fluorescein-12-dUTP-labeled DNA was visualized using an Eclipse TE2000-U fluorescence microscope (Nikon, Kawasaki, Japan).

Statistics

All curve fitting and data analysis was done using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). All data are expressed as means ± S.E.M. for the number of experiments (n) as indicated. Data are analyzed by Student's t-test or one-sample t-test where appropriate. A *P* value of less than 0.05 was considered significant.

Results

Contraction experiments

In the contraction experiments, the mean normalized diameter of a total number of 63 carotid artery preparations was 1053 ± 12 μm. The peak contraction evoked by KCl (100 mM) amounted to 3.2 ± 0.1 mN/mm segment length. DMS (10 μM) had no influence on the pretension of the preparations.

In non-cultured preparations DMS induced a leftward shift of the Ang II concentration response curve (pEC_{50} 8.8 ± 0.1 vs 8.3 ± 0.1 for vehicle, n = 4-5) without affecting the E_{max} (figure 1A). Culturing the vessels for 24 h in the presence of 20 % FCS had no significant influence on the response to Ang II, but in these preparations DMS significantly attenuated the contractile response to Ang II (E_{max} 43 ± 4, vs 71 ± 5 % for vehicle, pEC_{50} 8.2 ± 0.1 vs 8.6 ± 0.1 for vehicle, n = 5-6) (figure 1B). Interestingly, this attenuation of the Ang II-induced vasoconstriction by DMS was completely absent in endothelium-denuded preparations (E_{max} 72 ± 7 vs 68 ± 8 % for vehicle, pEC_{50} 8.4 ± 0.1 vs 8.3 ± 0.1 for vehicle, n = 6-7) (figure 1C). As a comparison, pre-incubation of the cultured vessels with DMS (10 μM) had no significant effect on the potency or efficacy for phenylephrine (figure 1D).

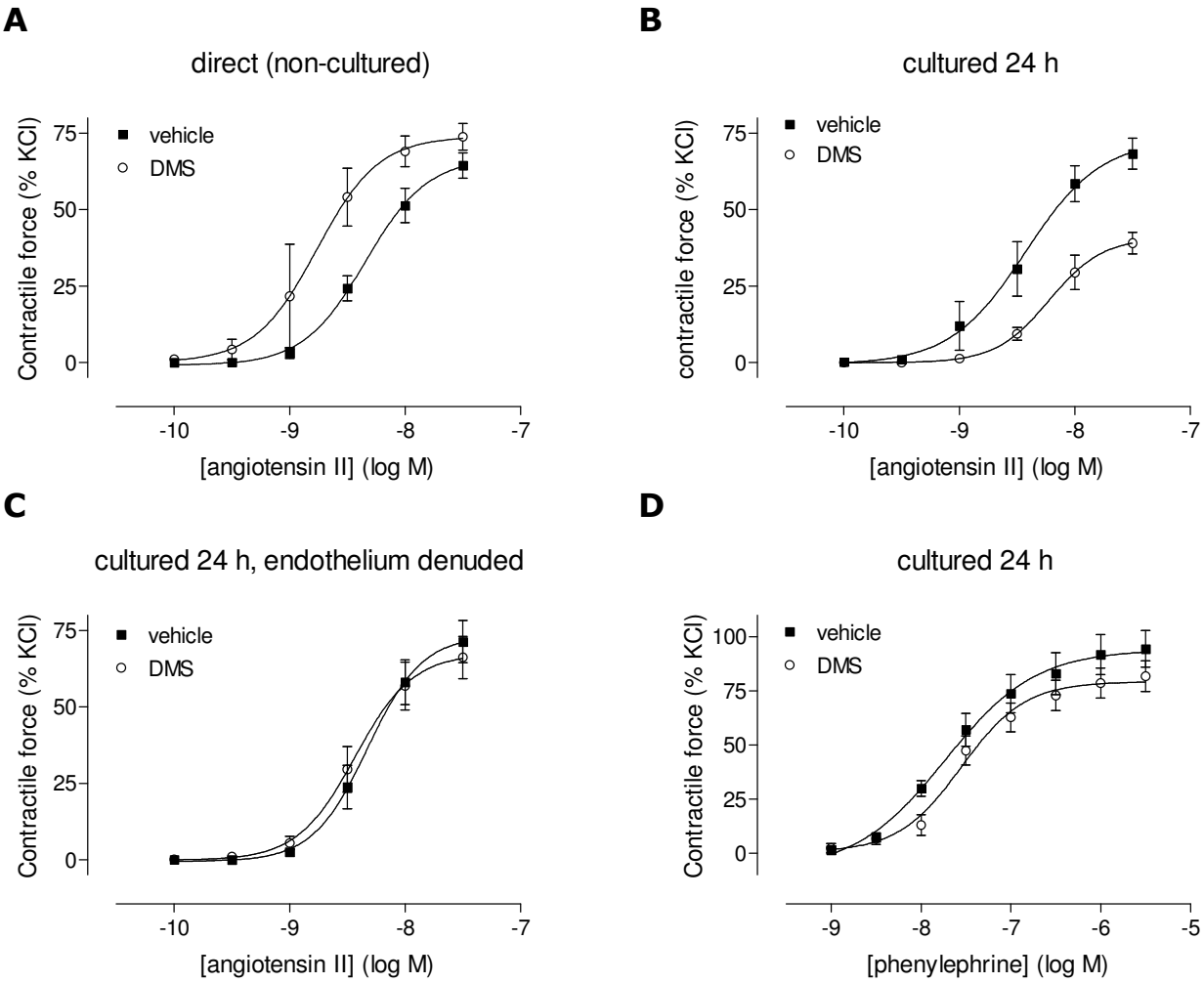

Figure 1. Influence of growth promoting conditions on the effect of DMS on vasoconstriction induced by Ang II and phenylephrine. Contractile responses to Ang II were measured in isolated rat carotid artery segments in the presence of DMS or vehicle (DMSO) in non-cultured preparations (A), after 24 h culture with 20 % FCS in the presence of a functional endothelium (B), or after 24 h culture with 20 % FCS in endothelium-denuded (after culture) preparations (C). Contractile responses to phenylephrine in 24 h cultured preparations with 20 % FCS in the presence of DMS or vehicle (DMSO) (D). DMS or vehicle was added to the organ bath 30 minutes before the construction of the concentration response curve for indicated agonists. Values are expresed as % of the 3rd KCl (100 mM)-induced constriction and given as mean ± S.E.M. (n = 4-5).

Proliferation of VSMCs

In the BrdU incorporation assay, S1P concentration-dependently induced BrdU incorporation in VSMCs in the presence of 0.5 % FCS (to 143 ± 11 % of basal at 1 μM, n = 3). Ceramide concentration-dependently decreased BrdU incorporation in these cells (53 ± 6 % of basal at 100 μM, n = 4), as did DMS (98 ± 1 % of basal at 10 μM, n = 4) (figure 2). bFGF concentration-dependently increased BrdU incorporation in VSMCs (406 ± 47 % of basal at 10 ng/ml, n = 9); ceramide and DMS inhibited the magnitude of this response without affecting the pEC_{50} of bFGF (figures 3A and 3B). Ang II also concentration-dependently increased BrdU incorporation in VSMCs, which was inhibited by DMS (figure 3C). As measured by TUNEL, ceramide (100 μM) and DMS (4 μM) induced apoptosis in VSMCs. bFGF (30 ng/ml) prevented the apoptotic effect of DMS (4 μM) (see appendix I, page 150).

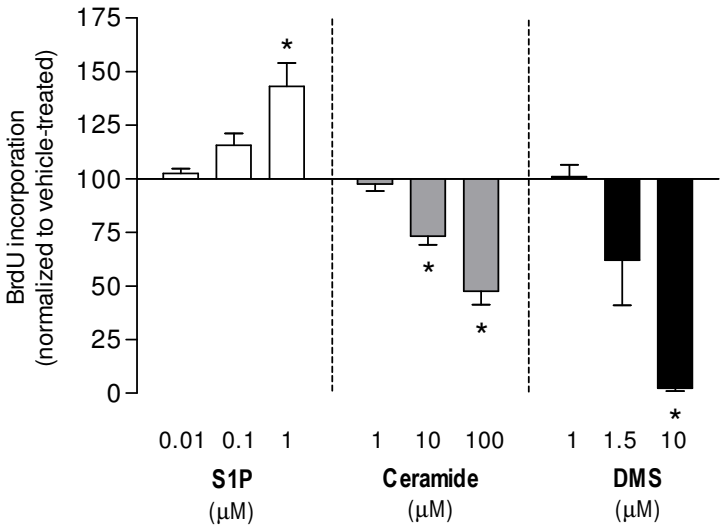

Figure 2. Effects of S1P, ceramide and DMS on DNA synthesis in VSMCs. Incorporation of BrdU was measured in the presence of 0.5 % FCS. Basal values were arbitrarily set to 100 % (258308 ± 90428, 280244 ± 37540 and 308034 ± 17270 relative luminescent units for S1P, DMS and ceramide, respectively). Data presented as % of vehicle-treated cells. Values represent mean ± S.E.M. (n = 3-4). * = $P < 0.05$ compared to vehicle in one-sample t-test.

Discussion

Sphingomyelin metabolites have various vasoactive properties and, therefore, they may be involved in the regulation of vascular tone by other vasoactive substances, as shown for instance for Ang II.[21] Ang II-mediated sphingosine kinase activation in the endothelium results in the production of the vasodilatory NO. Although sphingomyelin metabolites have also been implicated in VSMC contraction under normal circumstances, Ang II-induced VSMC contraction is not dependent on sphingolipid metabolism. Several disease states are

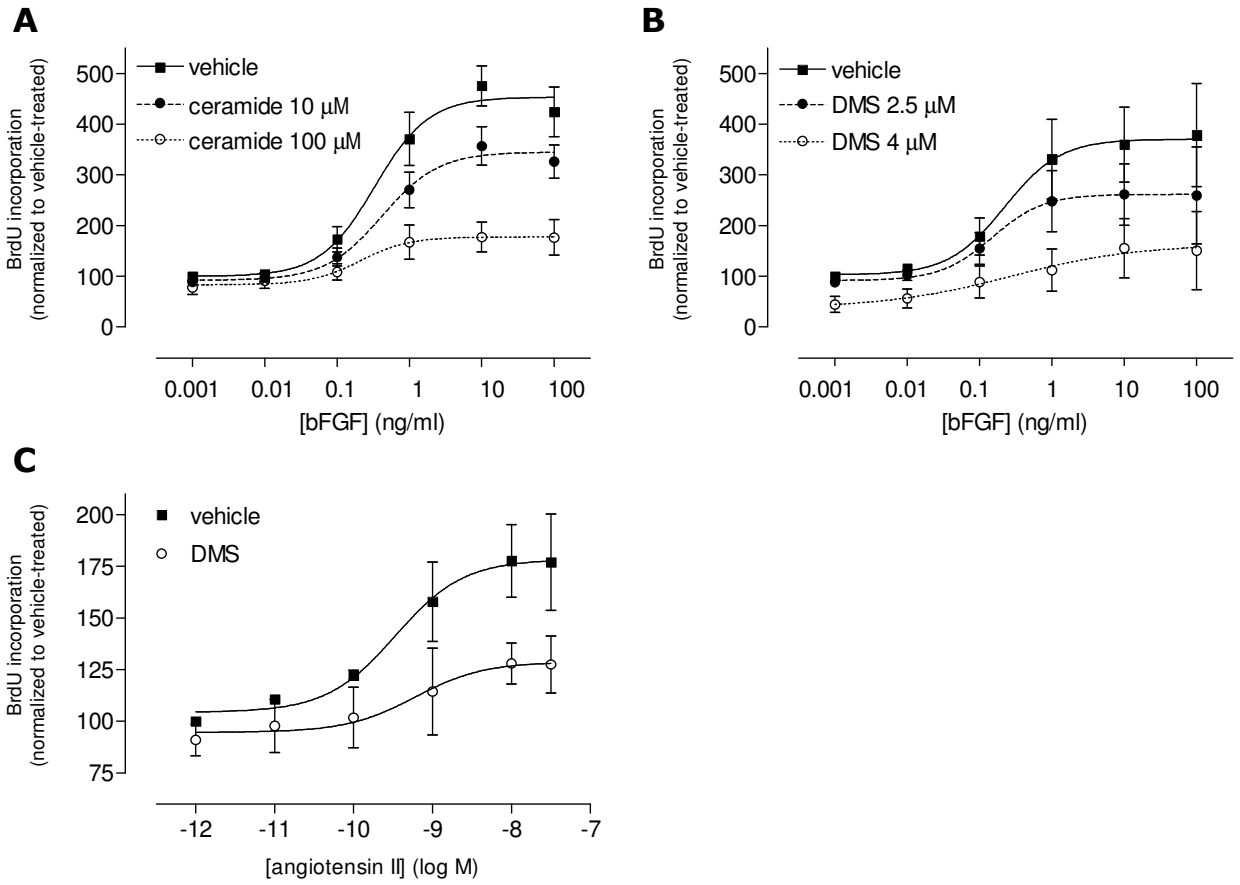

Figure 3. Influence of ceramide and DMS (and their respective vehicles) on bFGF and angiotensin II-induced DNA synthesis in VSMCs. DNA synthesis of VSMCs was measured using a BrdU incorporation assay. bFGF concentration-dependently increased proliferation of VSMCs, which could be inhibited by ceramide (A) and DMS (B) at indicated concentrations. (C) Angiotensin II concentration-dependently increased proliferation of VSMCs, which could be inhibited by DMS. Data presented as % of vehicle-treated cells. Values are given as means ± S.E.M. (n = 3-7).

characterized by an increased VSMC proliferation by systemic or locally produced growth factors. Besides regulation of vascular tone, sphingomyelin metabolites are also involved in regulation of cell growth and several growth factors have been shown to induce sphingolipid metabolism in order to exert their growth regulating properties.[26,27] Thus, stimulation of sphingolipid metabolism in VSMCs during hypertrophic conditions may also result in altered contractile responses to vasoactive factors such as Ang II because of the dual role of sphingomyelin metabolites. To test this hypothesis, we have cultured vascular segments for 24 h in culture medium supplemented with 20 % FCS and compared the contractile responses to Ang II in the presence or absence of DMS. This culturing method has been successfully applied for studying the influence of hypertrophic conditions on smooth muscle cell phenotype in rat tracheal rings.[28] Culturing for 24 h in the presence of 20 % FCS, did not affect the contractile responses to Ang II, when compared to non-cultured preparations. In non-cultured preparations, DMS induced a potentiation of the Ang II-induced contractile responses as reported previously.[21] Interestingly, this potentiation of the Ang II-mediated

contraction by DMS is inverted to an inhibition of contraction after 24 h culturing. Whereas we have shown that the leftward shift in non-cultured preparations is due to endothelial effects, the decreased maximal contractile effect of Ang II by DMS after culturing might be explained by an inhibitory action of DMS on the VSMCs. In this scenario, the Ang II-induced contraction would be partially dependent on the production of S1P in the VSMCs and indeed, several studies have shown that S1P can induce smooth muscle cell contraction.[29-32]

Surprisingly, removal of the endothelium in the vascular segments after culturing, completely prevented the inhibitory effects of DMS on the Ang II-induced contraction. This implicates that also under growth promoting conditions, only Ang II-mediated activation of sphingosine kinase in the endothelium modulates vascular constriction. However, under these circumstances a contractile factor, instead of the dilatory NO, is released from the endothelium due to local S1P generation. Another possibility may be that under growth promoting conditions, activation of endothelial Ang II receptors, gives rise to a factor that subsequently activates sphingosine kinase in the VSMC where the generation of S1P mediates contraction. It cannot be excluded that under these experimental conditions DMS-induced accumulation of ceramide and/or sphingosine gives rise to a contractile factor released from the endothelium. Although the exact mechanism of the decreased contractile responses to Ang II in these experiments remains to be elucidated, the current findings suggest that also under growth promoting conditions Ang II selectively modulates sphingolipid metabolism in the endothelium. These actions were Ang II-specific since the contractile responses to the α_1-adrenoceptor agonist phenylephrine were not substantially affected by DMS in both cultured and non-cultured preparations. The experimental growth promoting conditions for 24 h used in this study clearly induce pronounced changes in endothelial Ang II signalling, but it cannot be excluded that longer periods of growth stimulation will additionally give rise to alterations in the contractile response to Ang II in the VSMCs. To investigate this possibility a different experimental set-up or *in vivo* studies would be required.

In order to show that sphingomyelin metabolites are involved in the regulation of VSMC growth and that growth factor-induced VSMC growth indeed depends on, or is modulated by sphingolipid metabolism, we have performed proliferation experiments with isolated VSMCs. S1P has been primarily associated with growth promoting effects [10,11], while ceramide and sphingosine, the metabolic precursors of S1P, have been shown to induce apoptosis and growth arrest.[12,13] Here we show that exogenously applied S1P has modest mitogenic effects in cultured primary VSMCs, whereas ceramide inhibits proliferation of VSMCs, nicely demonstrating the ceramide / S1P rheostat principle in VSMC. The growth inhibitory effects of ceramide are most likely due to induction of apoptosis as determined in the TUNEL assay. In analogy to ceramide, also sphingosine kinase inhibition by DMS induces apoptosis (as shown

by TUNEL) and reduces BrdU incorporation in VSMCs. This indicates that under normal culturing conditions endogenous S1P generation contributes to cell survival and that removal of S1P and/or accumulation of ceramide induces apoptosis. In addition, both ceramide and DMS concentration-dependently inhibit bFGF-induced VSMC growth. Also the growth stimulatory effects of Ang II on VSMCs are inhibited by DMS. Whether these growth inhibiting effects of DMS are due to induction of apoptosis (counteracting in general a growth stimulus) or that bFGF and Ang II stimulate sphingosine kinase in these cells to exert their mitogenic effects cannot be concluded from these experiments, although the latter has been suggested for bFGF previously.[27] Overall, these results demonstrate a regulatory role for sphingomyelin metabolites in VSMC growth, which indeed may be modulated by growth factors.

Although the present study clearly demonstrates that sphingomyelin metabolites are involved in growth factor and probably also Ang II-induced VSMC growth, the direct contractile effects of Ang II in VSMCs are not sphingolipid-dependent. In contrast, the actions of Ang II on the endothelium are mediated by modulation of local sphingolipid metabolism and these actions are drastically altered under hypertrophic conditions. Therefore, in pathological states characterized by vascular hypertrophy/hyperplasia endothelial function may be affected because of altered sphingolipid metabolism.

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C h a p t e r

4

Activation of sphingosine kinase by muscarinic M_3 receptors enhances NO-mediated and inhibits EDHF-mediated vasorelaxation

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Submitted for publication

Abstract

Objective. Local formation of the sphingomyelin metabolite sphingosine-1-phosphate (S1P) within the vascular wall has been shown to modulate vascular contraction and relaxation. In this study we investigated whether sphingosine kinase, the enzyme responsible for S1P synthesis, plays a role in muscarinic receptor-mediated vascular relaxation in different vascular beds.

Materials and methods. Sphingosine kinase translocation and sphingolipid-dependent NO-production after muscarinic receptor stimulation was assessed in an endothelial cell line. Furthermore, we used the sphingosine kinase inhibitor dimethylsphingosine (DMS) to investigate the role of sphingosine kinase in the relaxant responses to the muscarinic agonist methacholine (MCh) in isolated rat aorta, carotid and mesenteric arteries.

Results. Activation of M_3-receptors in an endothelial cell line induces a fast translocation of YFP-tagged sphingosine kinase from the cytosol to the plasma membrane. Concomitant NO-production in this cell line was partially inhibited by DMS. In aorta, the relaxant responses to MCh were attenuated in the presence of DMS, whereas DMS enhanced the relaxant responses to MCh in mesenteric artery preparations. In the latter preparation, MCh-induced, endothelium-derived hyperpolarizing factor-mediated vasorelaxation was enhanced by DMS. In addition, DMS potentiated the dilatory actions of the putative endothelium-derived hyperpolarizing factor C-type natriuretic peptide.

Conclusion. We conclude that stimulation of endothelial muscarinic receptors activates sphingosine kinase and this activation results in differential effects in different vessel types. Whereas intracellularly produced S1P enhances NO-mediated vasorelaxation, it inhibits EDHF-mediated vasodilation. A disturbed regulation of sphingolipid metabolism in the vascular wall may therefore play a role in the aetiology/pathology of disease states characterized by endothelial dysfunction.

Introduction

Sphingolipids are biologically active lipids that play important roles in various cellular processes, and the sphingomyelin metabolites, sphingosine-1-phosphate (S1P), sphingosine and ceramide act as signalling molecules in virtually all cell types. Ceramide and sphingosine are generally involved in apoptotic responses to various stress stimuli [1], whereas S1P is involved in mitogenesis, cell differentiation and migration.[2] The balance between ceramide and S1P plays an important role in cell fate and is also referred to as the ceramide / S1P rheostat.[3] Sphingolipid metabolism is controlled by the action of several enzymes, e.g. sphingomyelinase converts sphingomyelin into ceramide and sphingosine kinases convert sphingosine into S1P.[4] While activation of sphingomyelinase will alter the total abundance of sphingomyelin metabolites, activation of sphingosine kinases shifts the balance between ceramide and sphingosine on the one and S1P on the other hand. Furthermore, the level of S1P is also affected by the combined activities of other enzymes including S1P phosphatases and S1P lyase.[5]

S1P has high affinity for at least 5 subtypes of G protein-coupled receptors, termed $S1P_{1-5}$ [6], and possibly several additional "orphan" receptors These receptors are coupled to different intracellular second messenger systems, including adenylyl cyclase, phospholipase C, phosphatidylinositol 3-kinase / protein kinase Akt, mitogen-activated protein kinases, as well as Rho- and Ras-dependent pathways.[7] $S1P_{1-3}$ are the major S1P receptor subtypes expressed in the cardiovascular system and are expressed in both vascular smooth muscle cells and endothelium.[8] In addition to their effects on cellular growth, ceramide, sphingosine and S1P have vasoactive properties when applied exogenously to different vascular beds *in vivo* and *in vitro*.[9] S1P has been shown to induce contraction in several vascular beds, but vasodilation has also been described under different circumstances (for review see: [9]). These partially contradicting results may be explained by the type of vascular bed, the method of measuring contraction and possibly also species differences.[10] Since sphingomyelin metabolites are found in blood, they may act on the vasculature via the bloodstream, a situation that is mimicked by the exogenous application of sphingolipids in an organ bath. However, under physiological circumstances circulating sphingolipids are sequestered in lipoproteins or stored in platelets and erythrocytes [11-14], yielding most likely a very small free fraction of sphingolipids.

Both vascular smooth muscle cells and endothelial cells express the enzymes involved in sphingolipid metabolism and are, therefore, able to synthesize the different sphingomyelin metabolites. Since these cell types also express target molecules such as S1P receptors, S1P and other sphingomyelin metabolites can act as auto- or paracrine factors in the vasculature. Recently, we have shown that vasoactive factors such as angiotensin II can activate sphingosine kinase, thereby stimulating the synthesis of S1P in the endothelium. This local synthesis of S1P results in the activation of endothelial NO synthase and hence the production of the vasorelaxant factor NO. This NO production partially counteracts the angiotensin II-induced vasoconstriction by its action on the vascular smooth muscle cells.[15]

As the role of locally formed S1P and other sphingolipid metabolites is not completely understood, we investigated whether a receptor system that induces primarily vasorelaxation (i.e. the muscarinic M_3 receptor) also stimulates local sphingolipid metabolism. It has been well documented that the relative contribution of NO to muscarinic receptor-mediated vasorelaxation varies greatly between larger conduit vessels and smaller resistance vessels.[16] Therefore, we investigated how inhibition of sphingosine kinase (by means of the sphingosine kinase inhibitor dimethylsphingosine (DMS) [17]), modulates the vasodilatory action of muscarinic receptor stimulation in isolated rat aorta, carotid artery and mesenteric artery.

Materials and methods

Materials

Mouse brain microvascular endothelium-derived bEnd.3 cells were a kind gift from the Department of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands. DMS was purchased from Biomol (Plymouth Meeting, PA, USA). Dulbecco's modified Eagle's medium (D-MEM), fetal calf serum (FCS), penicillin, streptomycin, L-glutamin and phosphate buffered saline (PBS) were from Invitrogen (Breda, The Netherlands). Methacholine chloride (MCh), U46,619, (R)-(-)-phenylephrine hydrochloride (PhE), 9,11-dideoxy-11$^{\alpha}$,9$^{\alpha}$-epoxymethanoprostaglandin $F_{2\alpha}$), Ca^{2+} ionophore A23187 (4-benzoxazolecarboxylic acid), carbamylcholine chloride (carbachol), sodium nitroprusside (SNP) and bovine serum albumin (BSA) (fatty acid free, Cohn fraction V) were purchased from Sigma Chemical Co. (St Louis, MO, USA). C-type natriuretic peptide (CNP) was from Bachem (Weil am Rhein, Germany). N_{ω}-Nitro-$_L$-arginine (L-NNA) and N_{ω}-nitro-$_L$-arginine methyl ester hydrochloride (L-NAME) were from ICN Biomed (Aurora, OH, USA), indomethacin was from MSD (Whitehouse Station, NJ, USA), and S1P was from Avanti Polar Lipids (Alabaster, AL, USA).

Cell culture

bEnd.3 cells were maintained in D-MEM supplemented with 10 % (v/v) heat inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine in a humidified atmosphere of 5 % CO_2 / 95 % O_2 at 37 °C.[18] Cells were split 1:6 to 1:8 upon reaching confluence. For translocation experiments, cells were cultured in 35-mm dishes. For NO measurements, cells were cultured in black clear-bottom 96-well plates. Before initiating experiments, bEnd.3 cells were grown in FCS-free culture medium supplemented with 0.1 % (w/v) BSA during 18 h for NO determinations and 24 h for translocation experiments.

Translocation of YFP-tagged sphingosine kinase-1a

bEnd.3 cells were transfected with Effectene (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Localization imaging of YFP-tagged sphingosine kinase-1a (generated from mouse sphingosine kinase-1a that was generously provided by Dr. Spiegel, Richmond, Virginia, USA, using the EYFP-C1 vector from Clontech (Mountain View, CA, USA)) was monitored using an inverted confocal laser scanning microscope (Zeiss LSM510) and a 63 x / 1.4 Plan Apochromat oil immersion objective (Carl Zeiss MicroImaging, Inc., Oberkochen, Germany). YFP was excited with the 488 nm line of an argon laser and emission was recorded with a 505 nm long pass filter. Fluorescence images were collected from an area of 60 x 60 µm at a spatial resolution of 1024 x 1024 pixel.

NO measurements

NO measurements in bEnd.3 endothelial cells were performed as described before.[15] After loading with the fluorescent NO probe the cells were pre-incubated for 30 min at 37 °C with buffer, L-NNA (100 μM), DMS (10 μM) or appropriate vehicle. After this pre-incubation the Ca^{2+} ionophore A23187 (2.5 μM) or MCh (5 μM) was added to the wells at indicated concentrations. NO production was measured fluorometrically and is expressed as fold increase in fluorescence of untreated cells.

Relaxation experiments

The experiments followed a protocol approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands, in accordance with EU regulation on the care and use of laboratory animals. Adult male Wistar rats (280 – 320 g; Charles River, Maastricht, The Netherlands) were anaesthetized by injection of 75 mg/kg pentobarbitone (i.p.) (O.B.G., Utrecht, The Netherlands). 500 I.U. heparin (Leo Pharma B.V., Weesp, The Netherlands) was administered i.p. to prevent blood coagulation. The thoracic aorta, the left common carotid artery (in a range just distal from the bifurcation until the level of the aortic arch) or the 2nd branch mesenteric artery were carefully excised and immediately placed in Krebs-Henseleit

buffer (118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.1 mM KH_2PO_4 and 5.6 mM glucose) at room temperature, aerated with 5 % CO_2 / 95 % O_2 (pH = 7.4). Four segments were carefully prepared of the carotid and mesenteric artery, and two stainless steel wires (Goodfellow, Huntingdon, United Kingdom) with a diameter of 100 μm for carotid and 40 μm for mesenteric artery were inserted into the lumen of each vessel segment. These were then transferred into organ baths of a 4-channel wire myograph (Danish Myo Technology, Aarhus, Denmark) and subjected to a normalization procedure according to Mulvany & Halpern.[19] The individual circumference was adjusted to 90 % of the value that the particular vessel would have had at a transmural pressure of 100 mmHg. Hereafter, the arteries were equilibrated for an additional 20 min and the buffer was refreshed after each period of 10 min. For aortas, 6 rings of approximately 5 mm in length were prepared and mounted between two triangular stainless steel hooks in water-jacketed (37 °C) organ baths of 5 ml capacity, containing Krebs-Henseleit buffer, aerated with 5 % CO_2 / 95 % O_2 (pH = 7.4). The isometric tension was recorded on a MacLab/8e data acquisition system (ADInstruments, Bella Vista, Australia) via isometric force transducers. The resting tension was adjusted to 10 mN throughout, and the aorta segments were allowed to equilibrate for at least 60 min. At 30 min intervals the medium was exchanged against fresh buffer. After equilibration, all preparations were contracted twice for 5 min with a depolarizing high KCl Krebs-Henseleit solution (NaCl was replaced on an equimolar base by KCl, 120 mM for mesenteric artery, 100 mM for carotid artery and 40 mM for aorta) at intervals of 15 min. Endothelium integrity of all vessel segments was assessed by pre-contraction with the α_1-adrenoceptor agonist PhE (3 μM for mesenteric arteries, 0.3 μM for carotid arteries and 1 μM for aorta) and subsequent relaxation with 10 μM MCh. Vessels were excluded when relaxation was less than 80 %. After washing, again high KCl Krebs-Henseleit solution was added. After a 30 min preincubation with the sphingosine kinase inhibitor DMS (10 μM) or vehicle (DMSO) the vessels were precontracted with PhE (3 μM) or U46,619 (0.1 μM) for mesenteric artery, PhE (0.3 μM) for carotid artery and PhE (1 μM) for aorta and cumulative concentration response curves for MCh and SNP were constructed. Because the pre-constriction to phenylephrine in the presence of DMS proved less stable over time in mesenteric artery preparations, both phenylephrine and U46,619 were used as pre-constrictors in selected experiments. However, the nature of pre-constriction had no influence on the effects of DMS on the dilatory responses in these vessels.

For the study of endothelium-derived hyperpolarizing factor (EDHF)-dependent relaxation (i.e. non-NO and non-prostanoid endothelium-dependent relaxation) we followed another protocol. For these experiments, mesenteric arteries were primed by two consecutive constrictions with U46,619 (1 μM) and subsequent preconstriction for vasodilation measurements was preformed with U46,619 (0.1 μM).[20] Afterwards, cumulative

concentration response curves for MCh and CNP were constructed in the presence of L-NAME (300 μM), the cyclooxygenase inhibitor indomethacin (3 μM) and DMS (10 μM) or its vehicle (DMSO). Inhibitors were added to the organ bath 30 minutes prior to the construction of the concentration response curve for MCh or CNP. Isometric force of contraction was measured continuously and all data presented are normalized to the contractile response obtained by the pre-contraction of the vessels. Since the responses under these experimental conditions did not allow a reliable curve fit, data are presented as single concentrations.

Statistical analysis

All curve fitting and data analysis was done using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). All data are expressed as means ± S.E.M. for the number of experiments (n) as indicated. Data are analyzed by Student's t-test or one-way ANOVA where appropriate. A *P* value of less than 0.05 was considered significant.

Results

Translocation of sphingosine kinase-1

In unstimulated double transfected (muscarinic M_3 receptor and YFP-tagged sphingosine kinase-1a) bEnd.3 endothelial cells, yellow fluorescence was detected mainly in the cytoplasm. Addition of the muscarinic receptor agonist carbachol (100 μM) caused a rapid increase of plasma membrane fluorescence, and a concomitant decrease of cytosolic fluorescence, indicating translocation of the YFP-sphingosine kinase-1 construct from the cytoplasm to the membrane (see appendix II, page 151).

Role of sphingosine kinase in MCh-induced NO release *in vitro*

MCh (5 μM) increased NO production in the bEnd.3 cell line to 2.6 ± 0.2 fold of basal (n = 7). Pre-incubation of the cells with 10 μM DMS inhibited the MCh-induced NO production to 2.0 ± 0.2 ($P < 0.05$, n = 7). 100 μM L-NNA further diminished NO production due to muscarinic receptor stimulation. As a control, Ca^{2+} ionophore A23187 (2.5 μM) induced a NO response to 2.4 ± 0.5 fold of basal, which was not significantly influenced by DMS (figure 2).

Effect of sphingosine kinase inhibition on vascular relaxation

The mean normalized diameter amounted to 1005 ± 13 and 292 ± 4 μm and normalized passive forces were 15.8 ± 0.3 and 2.7 ± 0.1 mN for carotid (n = 30) and mesenteric artery (n = 66) segments, respectively. Pre-contraction was 3.2 ± 0.2 and 3.1 ± 0.1 mN/mm

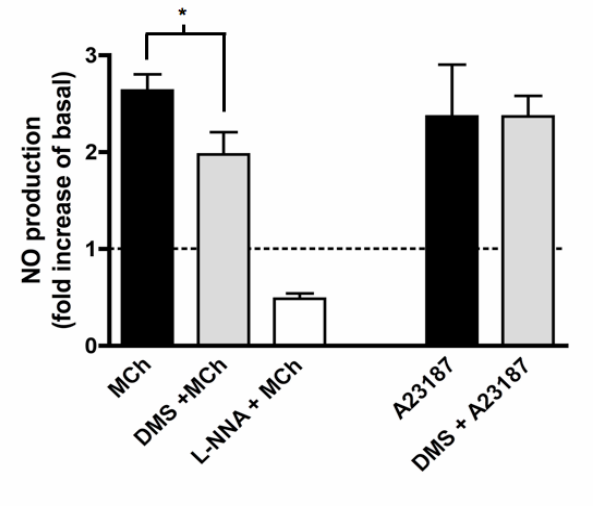

Figure 2. The effect of DMS on MCh and Ca^{2+} ionophore-induced NO production in bEnd.3 endothelial cells. Cell were loaded with the fluorescent NO probe DAF-2 DA. After pre-incubation (30 min) with DMS (10 μM), L-NNA (100 μM) or vehicle (DMSO and distilled water, respectively) cells were stimulated with the Ca^{2+} ionophore A23187 (2.5 μM), MCh (5 μM) or vehicle (DMSO and distilled water, respectively). NO production was measured fluorometrically and NO levels are expressed as fold of basal and means ± SEM. All measurements were performed in triplicate (n = 7). * = $P < 0.05$ when compared to MCh alone.

segment length for carotid and mesenteric artery, respectively and 6.5 ± 0.3 mN for aorta (n = 37). The maximum contraction evoked by the high KCl concentration was 3.6 ± 0.1 and 2.8 ± 0.1 mN/mm in carotid and mesenteric artery, respectively and 7.6 ± 0.2 mN in aorta.

DMS (10 μM) induced a significant rightward shift of the concentration response curve for MCh in aorta (pEC_{50} 6.4 ± 0.08 versus 6.9 ± 0.07 for control), and additionally lowered the E_{max} in these preparations (83 ± 2 versus 100 ± 2 % for control, n = 6, $P < 0.05$) (figure 3A). In carotid artery segments, DMS induced a small, though not significant, decrease in potency of MCh and did not change the E_{max} (figure 3B). In contrast, DMS induced a significant leftward shift of the concentration response curve for MCh in mesenteric artery (pEC_{50} 7.4 ± 0.11 versus 6.8 ± 0.08 for control, n = 5-8) without affecting the efficacy of MCh (figure 3C). DMS had no effect on either the potency or the efficacy of the SNP-induced relaxation for aorta, carotid artery or mesenteric artery (figure 4). In order to show that the observed effects of DMS are due to inhibition of S1P synthesis, we have measured the MCh-induced relaxation after pre-incubation with S1P (1 μM) in mesenteric arteries. The presence of S1P induces the opposite response of DMS in these preparations, i.e. a rightward shift of MCh concentration response curve (pEC_{50} 6.6 ± 0.08 vs 6.9 ± 0.10 for vehicle, n = 4) (figure 5).

Endothelium-dependent vasorelaxation in the mesenteric artery is known to be only partly NO-mediated, and EDHFs contribute to a major extent in this vessel type.[16] To study the effects of DMS on EDHF-dependent vasorelaxation we measured MCh-induced vasodilation in mesenteric arteries in the presence of L-NAME and indomethacine. Under these conditions MCh was still able to induce vasodilation (up to approximately 80%, figure 6A) confirming the only minor involvement of NO and prostanoids in MCh-induced vasorelaxation in this vessel type. In contrast, L-NAME alone or in combination with indomethacin, completely blocked

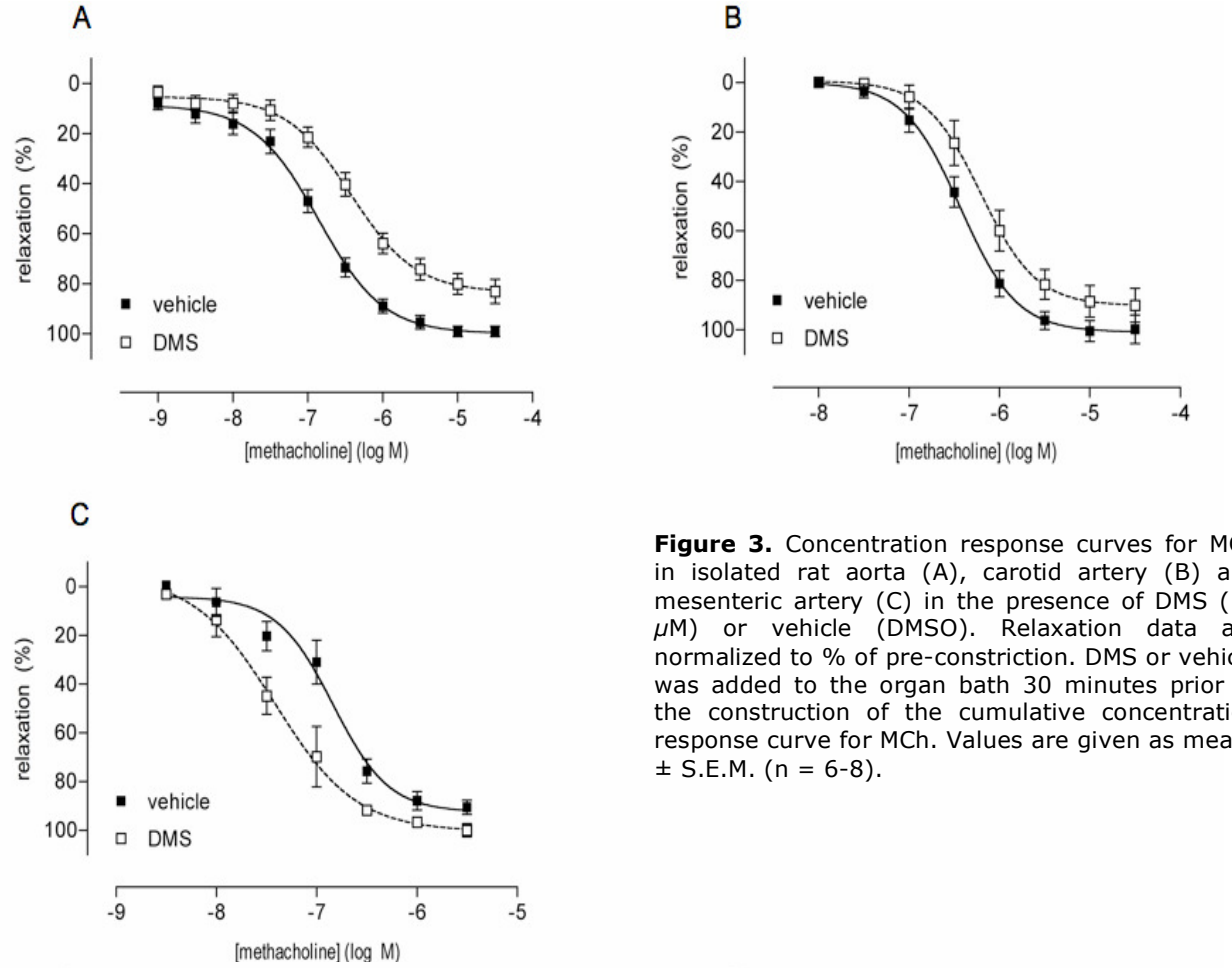

Figure 3. Concentration response curves for MCh in isolated rat aorta (A), carotid artery (B) and mesenteric artery (C) in the presence of DMS (10 μM) or vehicle (DMSO). Relaxation data are normalized to % of pre-constriction. DMS or vehicle was added to the organ bath 30 minutes prior to the construction of the cumulative concentration response curve for MCh. Values are given as means ± S.E.M. (n = 6-8).

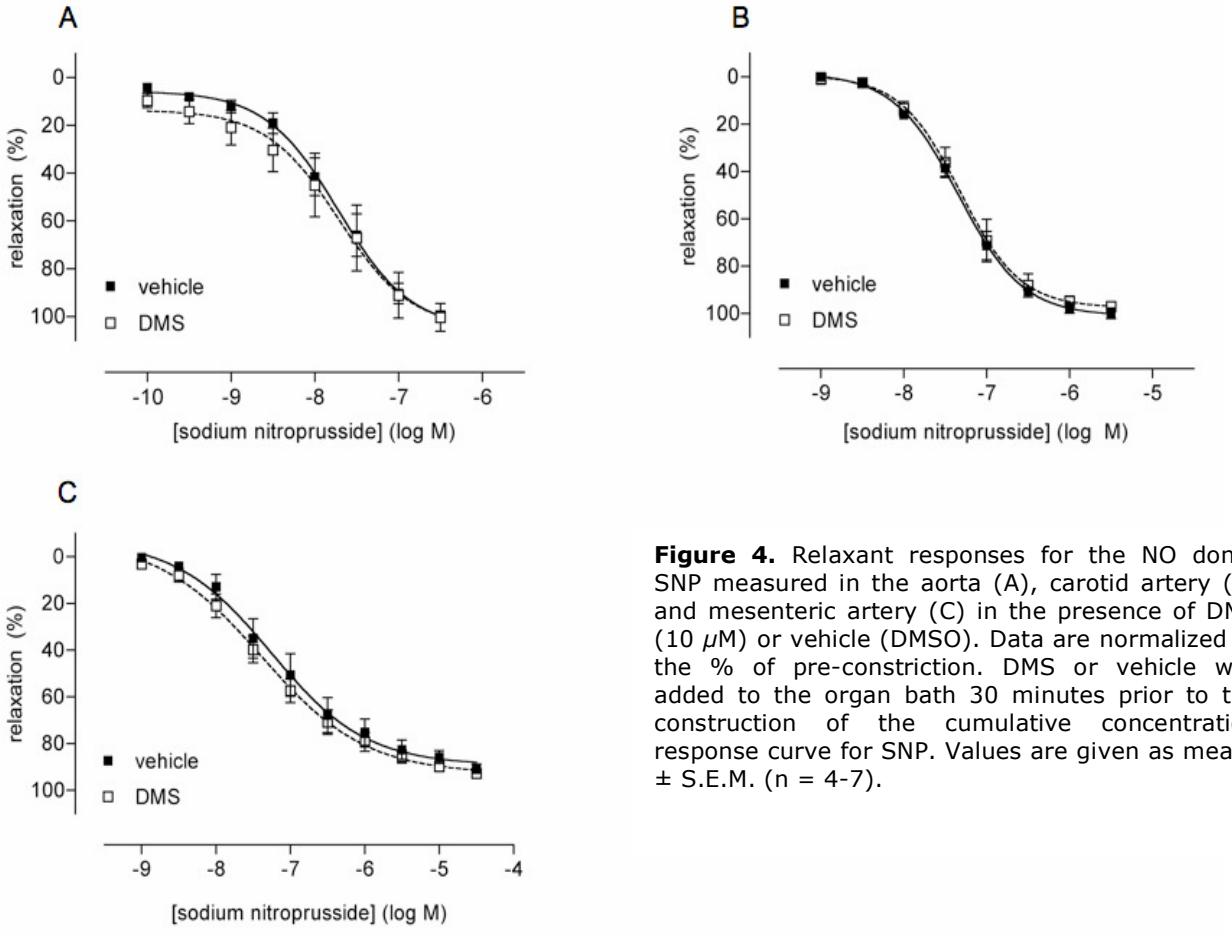

Figure 4. Relaxant responses for the NO donor SNP measured in the aorta (A), carotid artery (B) and mesenteric artery (C) in the presence of DMS (10 μM) or vehicle (DMSO). Data are normalized to the % of pre-constriction. DMS or vehicle was added to the organ bath 30 minutes prior to the construction of the cumulative concentration response curve for SNP. Values are given as means ± S.E.M. (n = 4-7).

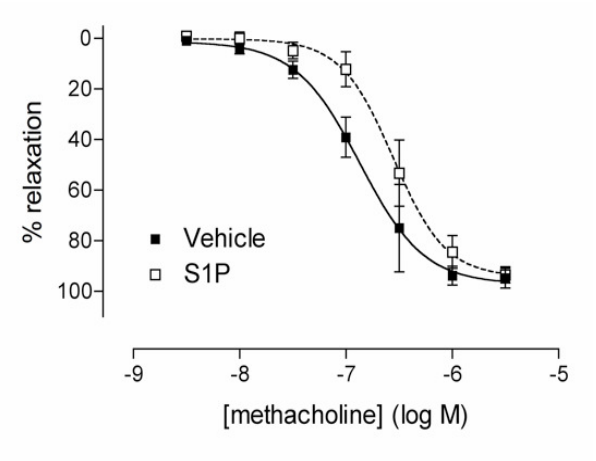

Figure 5. Relaxant responses to methacholine in the mesenteric artery in the presence or absence of S1P (1 μM). Data are normalized to the % of pre-constriction. S1P or vehicle (0.4% fatty acid free BSA in water) was added to the organ bath 30 minutes prior to the construction of the cumulative concentration response curve for MCh. Values are given as means ± S.E.M. (n = 4).

MCh-induced vasorelaxation in rat aorta (90 ± 5% for control vs -8 ± 3% in the presence of L-NAME or -5 ± 5% for L-NAME + indomethacin, n = 3-7, $P < 0.05$). In the mesenteric arteries DMS enhanced the EDHF-dependent vasodilations (figure 6A). CNP has been suggested to be a potential EDHF and, therefore, we investigated the effect of DMS on this putative EDHF. In analogy with the previous experiments, DMS also enhanced the CNP-induced vasorelaxation in mesenteric artery segments (figure 6B).

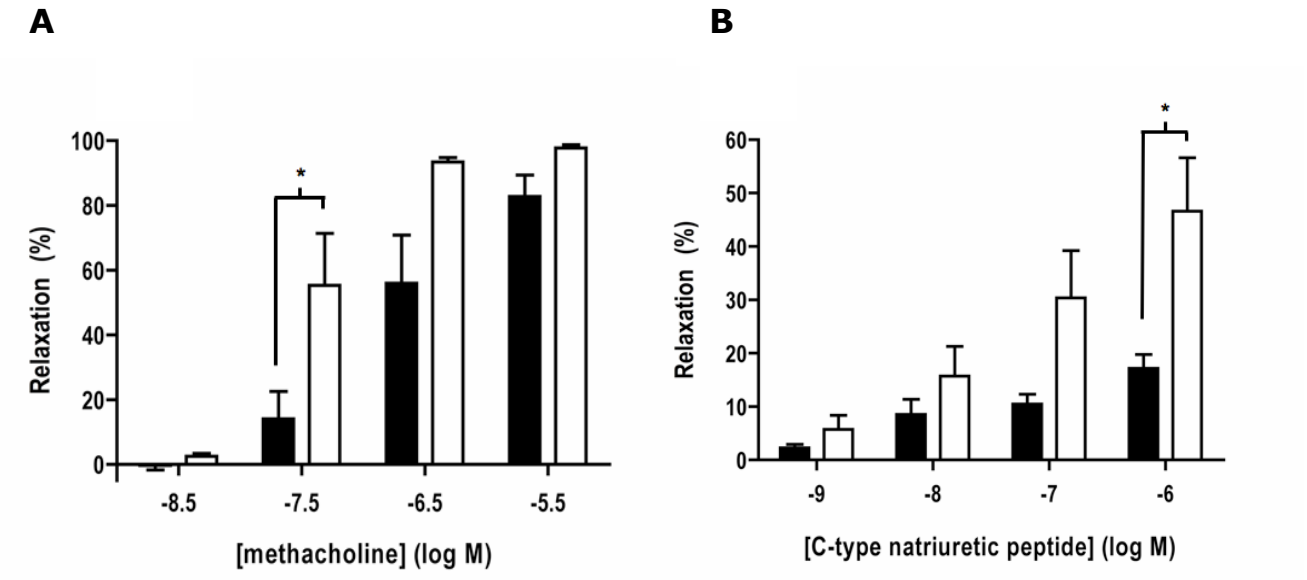

Figure 6. A) MCh-induced EDHF-dependent relaxation in rat mesenteric arteries. MCh-induced relaxation was measured in the presence of L-NAME (300 μM), indomethacin (3 μM) and DMS (10 μM)(white bars) or vehicle (DMSO)(black bars). B) CNP-induced relaxation measured in isolated rat mesenteric artery in the presence of L-NAME (300 μM), indomethacin (3 μM) and DMS (10 μM, white bars) or vehicle (DMSO, black bars). Data are normalized to % of pre-constriction. Inhibitors were added to the organ bath 30 minutes prior to the construction of the cumulative concentration response curve for MCh and CNP. Values are given as means ± S.E.M. (n = 4-7). * = $P < 0.05$ when compared to control (= vehicle).

Discussion

The interconvertible sphingolipids sphingomyelin, ceramide, sphingosine and S1P are important regulators of various cellular processes. Besides their growth regulating effects, they have also been shown to induce both contraction and relaxation in several vascular beds.[9] Since endothelial and vascular smooth muscle cells express all enzymes involved in sphingolipid metabolism and additionally express S1P receptors, S1P can be considered as an auto-, and paracrine factor in these cells.[21] Local sphingolipid metabolism plays a physiological role which can be influenced by known vasoactive compounds, as we have recently shown for angiotensin II.[15] We have now investigated the role of locally formed sphingolipid metabolites for a receptor system that induces vasodilation which is endothelium and, at least in some vascular beds, NO-dependent, the muscarinic receptor.

It has been shown previously in transfected HEK-293 cells that activation of the M_3 receptor induces sphingosine kinase-dependent Ca^{2+} signalling.[22] The translocation experiments in the present study clearly show that M_3 receptor stimulation in endothelial cells results in a rapid translocation of sphingosine kinase from the cytosol to the plasma membrane, indicating a direct coupling between M_3 receptors and sphingosine kinase. Translocation of sphingosine kinase most likely results in a concomitant increased S1P production in the plasma membrane.[23] This is also supported by our findings in isolated vessels where we have investigated endothelium-dependent responses to muscarinic receptor stimulation in the presence or absence of the specific competitive sphingosine kinase inhibitor DMS (10 μM).[17] In aorta preparations, DMS caused a rightward shift of the concentration response curve for MCh, suggesting that the locally formed S1P has a relaxant effect. This is in accordance with findings reported by Roviezzo et al. [24], who also showed a rightward shift and decrease of maximum relaxation in rat aorta to the muscarinic receptor agonist acetylcholine, using a different sphingosine kinase inhibitor, namely DL-threo-dihydrosphingosine. In addition, this provides evidence that the observed effects are indeed due to sphingosine kinase inhibition and not to non-specific effects of DMS. We have made a very similar observation for the actions of angiotensin II in the rat carotid artery, in which we demonstrated an endothelium-dependent activation of sphingosine kinase, and thus S1P production, leading to NO formation.[15] In the present study, we additionally demonstrate that DMS inhibits MCh-induced NO-production in an endothelial cell line, confirming the stimulatory action of S1P on endothelial NO production. Although Roviezzo et al. suggest S1P receptor-independent effects of the locally formed S1P, others have shown S1P receptor-dependent actions.[15,25,26] These may result in activation of eNOS, most likely via PI3 kinase and Akt/PKB-dependent pathways as described previously. In the present study, DMS had no influence on SNP-induced

vasorelaxation in neither mesenteric artery, aorta nor carotid artery preparations, indicating that sphingosine kinase indeed acts upstream of NO. Moreover, the latter is also confirmed by the inhibitory effect of DMS on MCh-induced NO production in our study.

To our surprise, DMS enhanced the MCh-induced relaxation in the mesenteric artery. This would suggest that, in contrast to the aorta, either the locally formed S1P has an inhibitory action on vasodilation, or that accumulating ceramide or sphingosine induce a relaxant effect under these experimental conditions. However, in the presence of S1P MCh-induced vasodilation is attenuated in these preparations, which suggests that the effects of DMS are caused by inhibition of S1P synthesis rather than accumulation of other sphingomyelin metabolites. The enhancement of the MCh-induced relaxation in mesenteric artery preparations by DMS may possibly be explained by an inhibition of S1P on the release or action of EDHF (i.e. a NO and prostaglandin-independent relaxant factor), which is known to play a major role in the relaxation responses of the mesenteric artery, but not of conduit vessels.[16,27-29] In the present study we show that in the presence of L-NAME and indomethacin, MCh is still able to induce vasodilation in the mesenteric artery (to approximately 80%). In contrast, the relaxant responses to MCh in the aorta were completely blocked by the same concentration L-NAME (either alone or in combination with indomethacin), which nicely reflects the differential role of NO in these preparations. The EDHF-mediated relaxation in mesenteric arteries (i.e. the relaxant response in the presence of L-NAME and indomethacin) was enhanced by DMS. Together with our finding that in the presence of S1P the MCh-induced vasodilation is attenuated in mesenteric artery, we conclude that under physiological circumstances S1P inhibits the generation or action of EDHF in these vessels.

Several factors have been proposed to function as EDHF [16], one of which is CNP [30] that acts on the smooth muscle cells via the natriuretic peptide receptors NPR-B and NPR-C. While under our experimental conditions CNP induced only a modest relaxant response, DMS substantially enhanced CNP-induced vasorelaxation in mesenteric arteries. Thus, the effects of DMS on MCh-induced relaxation in mesenteric arteries may be, at least partially, explained by an inhibitory action of S1P on the dilatory effects of CNP. While it has been suggested that the EDHF action of CNP is mainly mediated by the NPR-C receptor [30], it was shown previously that S1P potently inhibits CNP-induced NPR-B signalling in VSMCs.[31] To explain these effects of DMS in more detail, it remains to be elucidated whether CNP induces sphingosine kinase activity in the smooth muscle cells or, in the case of MCh-induced relaxation, that sphingosine kinase activity regulates endothelial CNP release.

Taken together, these data suggest that activation of muscarinic receptors in the vasculature modulates sphingolipid metabolism via activation of sphingosine kinase and thereby induces local i.e. endothelial, S1P formation. This local S1P formation enhances NO-mediated vasodilation whereas EDHF-mediated vasodilation is inhibited. Since vasorelaxation in conduit vessels is mainly mediated via NO, S1P will enhance (and accordingly, DMS will inhibit) relaxation in these arteries. S1P will have the opposite action in mesenteric arteries, since in these vessels relaxation is mainly achieved via the action of EDHF. A disturbed regulation of the ceramide / S1P rheostat (e.g. due to a reduced sphingosine kinase activity) may be important under pathological circumstances associated with endothelial dysfunction. Since especially resistance vessels are involved in the regulation of blood pressure, the inhibitory effect of S1P on vasodilatory actions in these vessels may have a negative influence on vascular tone in hypertension.

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C h a p t e r

5

Sphingolipid-dependent vascular reactivity in spontaneously hypertensive rats

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Manuscript in preparation

Abstract

Introduction. The endothelial formation of sphingosine-1-phosphate (S1P) is an important regulatory mechanism for angiotensin II (Ang II) and muscarinic receptor agonist methacholine (MCh)-induced vascular responses. Since S1P has vasoactive and growth regulating properties, we have studied the role of S1P in the actions of Ang II and MCh during hypertension, a disease state characterized by endothelial dysfunction and vascular remodeling.

Materials and methods. Aorta, carotid artery and mesenteric artery vessel segments were isolated from 33-36 weeks old Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHRs). Vascular responses to Ang II in carotid arteries and MCh in aortas, carotid and mesenteric arteries were measured using a wire myograph setup, in the presence or absence of the sphingosine kinase inhibitor dimethylsphingosine (DMS).

Results. In WKY rats, DMS enhanced the vasorelaxant effect of MCh in the mesenteric artery, but inhibited MCh-induced relaxation in the aorta. In contrast, DMS had no effect on the MCh-induced relaxation in SHR. Ang II-induced contraction in carotid arteries was facilitated by DMS in both WKY and SHR, although the effect was more pronounced in SHR. Interestingly, DMS alone induced an endothelium- and cyclooxygenase-dependent contraction in carotid artery segments of SHR, but not of those obtained from WKY rats.

Discussion. Sphingosine kinase-dependent effects of muscarinic receptor stimulation are absent in SHR, whereas the sphingolipid-dependent actions (the release of an endothelial relaxant factor) of Ang II are increased in these animals. These phenomena are most likely due to altered activation or altered constitutive activity of sphingolipid metabolizing enzymes. Moreover, the generation of sphingomyelin metabolites may induce the release of an endothelium-derived contractile factor in hypertensive rats. Alterations in sphingolipid metabolism may thus contribute to a disturbed regulation of vascular tone during hypertension.

Introduction

The interconvertible sphingomyelin metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) can exert various biological effects in most cell types including endothelial and vascular smooth muscle cells (VSMCs).[1] In general, S1P is involved in mitogenesis, cell differentiation and migration [2], while ceramide and sphingosine are involved in apoptotic responses to various stress stimuli and growth arrest.[3] S1P can activate at least five G protein-coupled receptors ($S1P_{1-5}$) of which $S1P_{1-3}$ are expressed in the vasculature.[1] Formation and degradation of sphingomyelin metabolites is achieved by several enzymes, e.g. sphingomyelinase converts sphingomyelin into ceramide, out of which ceramidase forms sphingosine, and sphingosine kinases can subsequently convert sphingosine into S1P.[4] Activation of sphingosine kinases shifts the balance between ceramide and sphingosine on the one and S1P on the other hand [5], while S1P levels are lowered by the combined activities of various enzymes including S1P phosphatases and S1P lyase.[6] Several growth factors and cytokines (e.g. platelet-derived growth factor [7], basic fibroblast growth factor [8] and tumor necrosis factor-α [9]) stimulate sphingosine kinase and other enzymes involved in sphingolipid metabolism, and, therefore, directly affect the balance between these sphingolipids for their signalling. Sphingolipid-metabolizing enzymes are expressed within the vascular wall and local formation is thought to be an important source of sphingomyelin metabolites which can act as autocrine or paracrine factors in both endothelial and VSMCs.[1,10] In addition to their effects on cellular growth, ceramide, sphingosine and S1P have vasoactive properties when applied exogenously to different vascular beds *in vivo* and *in vitro*. S1P induces contraction in several vascular beds, but also vasodilation has been described (for review see: [11]). These partially contradicting results may be explained by the type of vascular bed, the method used for measuring contraction and possibly also species differences.[12]

Recently, we have shown that angiotensin II (Ang II) induces activation of sphingosine kinase, which leads to increased activity of endothelial NO synthase via both Ca^{2+} and phosphatidylinositol 3-kinase/Akt pathways, most likely involving one or more S1P receptors. The resultant production of the vasorelaxant factor NO counteracts the contraction induced by Ang II.[13] We have also shown a differential role of S1P in vascular relaxation by methacholine (MCh) in different vascular beds in the Wistar rat. In aorta and carotid artery, local formation of S1P due to activation of the muscarinic receptor has a vasorelaxant effect because of stimulation of NO production. However, in the mesenteric artery the locally formed S1P has an opposite effect which is due to inhibition of endothelium-derived hyperpolarizing factor(s) (see chapter 4).

Hypertension is associated with endothelial dysfunction [14] and vascular remodelling.[15] In both processes sphingomyelin metabolites play an important role and, therefore, the present study was designed to investigate sphingolipid-dependent alterations in vascular responses to Ang II and MCh during hypertension. For this purpose we studied the effects of inhibition of sphingosine kinase by means of DMS [16] effects on Ang II-induced constriction in isolated carotid arteries and muscarinic receptor-induced vasorelaxation in isolated mesenteric and carotid arteries and aorta of 33-36 wks old spontaneously hypertensive rats (SHR) and WKY rats.

Materials and Methods

Chemicals

Ang II was purchased from Bachem (Bubendorf, Germany) and DMS was purchased from Biomol (Plymouth Meeting, PA, USA). MCh, the tromboxane A_2 mimetic 9,11-dideoxy-11$^{\alpha}$,9$^{\alpha}$-epoxymethanoprostaglandin $F_{2\alpha}$ (U46,619) and (R)-(-)-phenylephrine hydrochloride (PhE) were from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Indomethacin was from MSD (Whitehouse Station, NJ, USA) and N_{ω}-nitro-$_L$-arginine methyl ester hydrochloride (L-NAME) was from ICN Biomed (Aurora, OH, USA).

In vivo and *ex vivo* experiments

All animal experiments followed a protocol approved by the Animal Ethical Committee of the University of Amsterdam, The Netherlands, in accordance with EU regulation on the care and use of laboratory animals. Adult male WKY and SHR (33-36 wks) (Charles River, Maastricht, The Netherlands) were anaesthetized by injection of 75 mg/kg pentobarbitone (i.p.) (O.B.G., Utrecht, The Netherlands). For mean arterial pressure (MAP) and heart rate measurements animals were ventilated with room air at a frequency of 40 cycles/min with of volume of 200 ml/min using a Braun Melsungen (Melsungen, Germany) pump. Body temperature was kept at 37 °C by means of a thermostat-equipped table. The left carotid artery was cannulated, 50 I.U. heparin (Leo Pharma B.V., Weesp, The Netherlands) was administered to prevent blood coagulation and blood pressure was monitored continuously using a PowerLab data acquisition system (Chart 3.4, ADI instruments, Colorado Springs, CO, USA). The heart rate was derived from this signal. As expected, SHR had a higher MAP than WKY (approximately 200 vs 140 mmHg for WKY), whereas heart rates were similar for both rat strains (n = 2 each).

For contraction and relaxation experiments 500 I.U. heparin was administered i.p., and the thoracic aorta, the left common carotid artery (in a range just distal from the bifurcation until the level of the aortic arch) or the 2nd branch mesenteric artery were carefully excised and immediately placed in Krebs-Henseleit buffer (118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.1 mM KH_2PO_4 and 5.6 mM glucose) at room temperature, aerated with 5 % CO_2 / 95 % O_2 (pH = 7.4). Of the carotid artery and mesenteric artery, four segments were carefully prepared and two stainless steel wires (Goodfellow, Huntingdon, United Kingdom) with a diameter of 100 μm for carotid and 40 μm for mesenteric artery were inserted into the lumen of each vessel segment. These were then transferred into organ baths of a 4-channel wire myograph (Danish Myo Technology, Aarhus, Denmark) and subjected to a normalization procedure according to Mulvany & Halpern.[17] The individual circumference was adjusted to 90 % of the value that the particular vessel would have had at a transmural pressure of 100 mmHg. Hereafter, the arteries were equilibrated for an additional 20 min and the buffer was refreshed after each period of 10 min. For aortas, 6 rings of approximately 5 mm in length were prepared and mounted between two triangular stainless steel hooks in water-jacketed (37 °C) organ baths of 5 ml capacity, containing Krebs-Henseleit buffer, aerated with 5 % CO_2 / 95 % O_2, pH = 7.4. The isometric tension was recorded on a MacLab/8e data acquisition system (ADInstruments, Bella Vista, Australia) via isometric force transducers. The resting tension was adjusted to 10 mN throughout, and the aorta segments were allowed to equilibrate for at least 60 min. At 30 min intervals the medium was exchanged against fresh buffer. After equilibration, all preparations were contracted two times for 5 min with a depolarizing high KCl Krebs-Henseleit solution (NaCl was replaced on an equimolar base by KCl, 120 mM for mesenteric artery, 100 mM for carotid artery and aorta) at intervals of 15 min. Subsequently, the vessels were pre-contracted with U46,619 (1 μM) for mesenteric artery and (PhE) (1 μM) for carotid artery and aorta. After reaching a steady level, one concentration of the endothelium-dependent vasodilator MCh (10 μM) was added to assess the functional state of the endothelium. After washing, again a high KCl Krebs-Henseleit solution was added to the vessel segments to obtain the maximal contractile response. After a 30 min pre-incubation with the sphingosine kinase inhibitor DMS (10 μM) or vehicle (DMSO), vessels were pre-contracted with U46,619 (1 μM) for mesenteric artery and PhE (1 μM) for aorta and concentration response curves for MCh were constructed. Carotid artery segments were pre-incubated with DMS for 60 min, before either pre-contraction with PhE (1 μM) and cumulative concentration respons curves for MCh or concentration respons curves for Ang II were constructed. Isometric force of contraction was measured continuously. Contraction data are normalized to the 3rd high KCl contraction and relaxation data are normalized to the contractile response obtained by the pre-contraction of the vessels.

Statistical analysis

All curve fitting and data analysis was done using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). All data are expressed as means ± S.E.M. for the number of experiments (n) as indicated. Data are analyzed by Student's t-test. A *P* value of less than 0.05 was considered significant.

Results

Since pre-contraction in the mesenteric artery with PhE in the presence of DMS was not stable with time, we used U46,619 as a pre-constricting agonist. Previous experiments in our lab had shown that the nature of pre-constriction has no influence on DMS-induced effects. Characteristics of the aorta, carotid artery and mesenteric artery preparations used are shown in table 1. In WKY, pre-incubation with DMS (10 μM) resulted in a small leftward shift of the concentration response curve for MCh in mesenteric artery which did not reach statistical significance with the given number of experiments. In the carotid artery, there was no significant effect of DMS on the potency or efficacy of MCh. In aorta, pre-incubation with DMS (10 μM) resulted in a rightward shift of the CRC for MCh in the WKY rat. (N = 4-6, figure 1). These findings are qualitatively similar to our previously reported findings for normotensive Wistar rats (see chapter 4). In SHR we found a decreased maximal endothelium-dependent relaxation in both aorta and carotid artery, indicating endothelial dysfunction (figure 1A and 1B). A representative tracing in carotid arteries of WKY and SHR is shown in figure 3. MCh-induced relaxation of mesenteric artery and aorta preparations in SHR was less potently influenced by DMS. (N = 4-6, figure 1A and 1C). See table 2 for pEC_{50} and E_{max} values.

Mesenteric artery	**WKY**	**SHR**
N	10	11
mean normalized diameter (μm)	321 ± 9	279 ± 9
normalized passive tension (mN/mm)	0,64 ± 0,03	0,76 ± 0,03
precontraction (mN/mm)	2,7 ± 0,2	3,0 ± 0,3
Carotid artery		
N	23	45
mean normalized diameter (μm)	1073 ± 12	964 ± 11
normalized passive tension (mN/mm)	4,3 ± 0,1	3,3 ± 0,0
precontraction (mN/mm)	2,7 ± 0,2	2,0 ± 0,2
contraction 100 mM KCl (mN/mm)	3,8 ± 0,1	2,8 ± 0,1
Aorta		
N	9	10
pre-contraction (mN)	2,7 ± 0,1	4,1 ± 0,4

Table 1. Properties of vessel segments used from WKY and SHR. Values are given as means ± S.E.M.

MCh-induced relaxation		pEC_{50} vehicle	pEC_{50} DMS	E_{max} (%) vehicle	E_{max} (%) DMS
aorta	WKY	7,4 ± 0,1	6,5 ± 0,1 *	87,5 ± 5	85,3 ± 5
	SHR	7,1 ± 0,0	6,7 ± 0,1 *	49,6 ± 10	58,1 ± 6 †
carotid artery	WKY	6,6 ± 0,2	6,7 ± 0,1	80,6 ± 6	83,5 ± 6
	SHR	7,0 ± 0,1	7,1 ± 0,2	64,5 ± 8	54,8 ± 8 †
mesenteric artery	WKY	7,8 ± 0,1	8,0 ± 0,1	97,4 ± 1	97,5 ± 1
	SHR	7,9 ± 0,1	8,0 ± 0,2	97,1 ± 1	97,7 ± 1
Ang II-induced contraction					
carotid artery	WKY	8,0 ± 0,0	8,6 ± 0,2 *	12,8 ± 4	18,3 ± 3
	SHR	7,8 ± 0,1	8,4 ± 0,1 *	23,1 ± 3	45,8 ± 11 *

Table 2. pEC_{50} and E_{max} values for MCh-induced relaxation and Ang II-induced contraction in the absence and presence of DMS (10 μM) in WKY and SHRs. Values are given as means ± S.E.M. (n = 4-6). * = $P < 0.05$ compared to vehicle. † = $P < 0.05$ when compared to WKY.

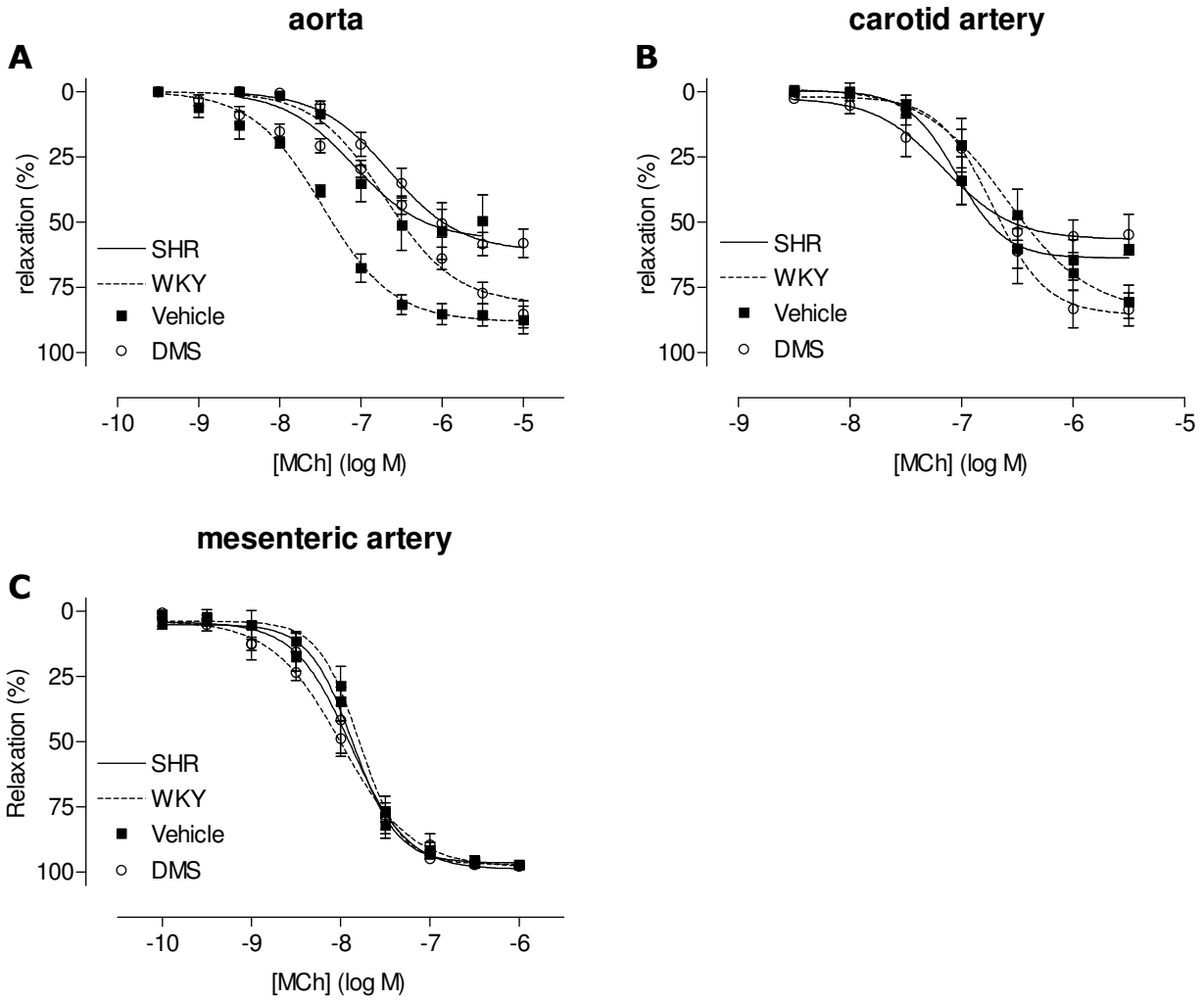

Figure 1. Concentration response curves for MCh measured in isolated (A) aorta, (B) carotid artery and (C) mesenteric artery from SHR and WKY rats in the presence of DMS (10 µM) or vehicle (DMSO). Relaxation data are normalized to % of pre-constriction. DMS or vehicle was added to the organ bath 30 minutes prior to the construction of the cumulative concentration for MCh. Values are given as means ± S.E.M. (n = 4-6).

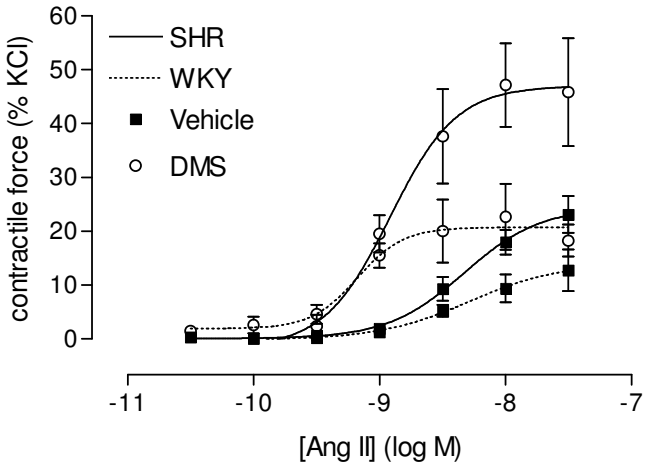

Figure 2. Contractile responses to Ang II in the isolated carotid arteries from SHR and WKY rats in the presence of DMS (10 μM) or vehicle (DMSO). Data are normalized to the contractile response obtained by the 3rd 100 mM KCl. DMS or vehicle were added to the organ bath 30 minutes prior to construction of the cumulative concentration response curve for Ang II. Values are given as means ± S.E.M. (n = 5-6).

Pre-incubation of the carotid artery segments with DMS (10 μM) induced a leftward shift of the CRC for Ang II and elevated the E_{max} in both WKY and SHR (figure 2).

Surprisingly, in the carotid artery of SHR, DMS (10 μM) induced a contractile response, which was absent in WKY. This DMS-induced vasoconstriction was transient and was mostly biphasic. Typical examples for SHR and WKY are shown in figure 3. The NO synthase inhibitor L-NAME (100 μM) enhanced the contractile effect of DMS. Moreover, the spontaneous contraction evoked by DMS was completely abolished by removal of endothelium or the administration of the cyclooxygenase inhibitor indomethacin (10 μM) (n = 5-6, figure 4). Since the DMS-induced contraction of the carotid artery in SHR was transient, the concentration response curves for Ang II were not constructed before contraction had returned to basal level.

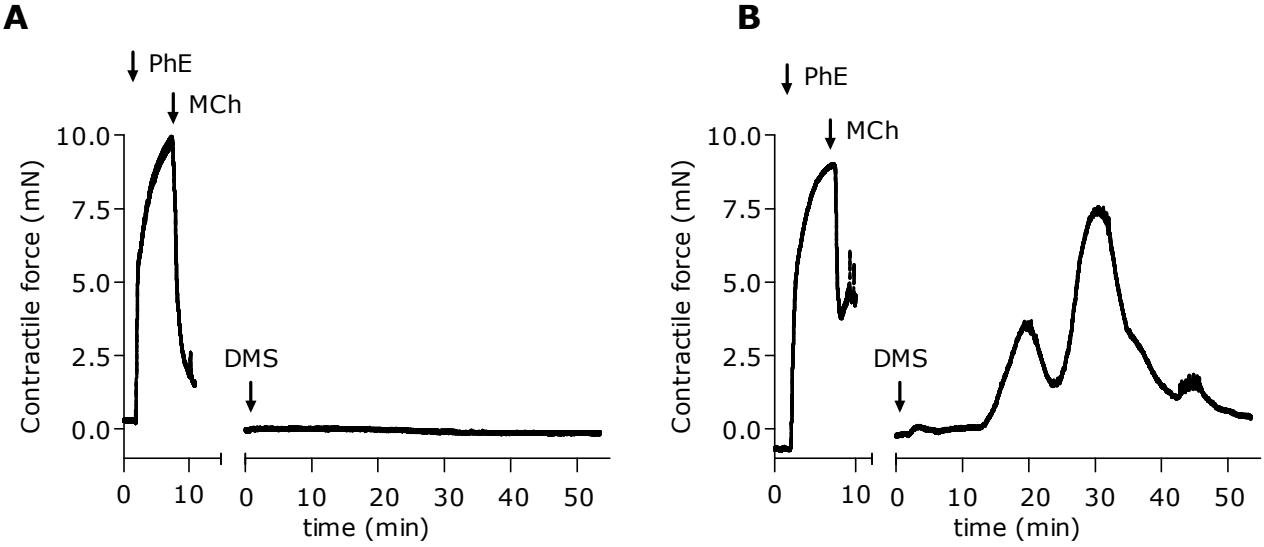

Figure 3. Typical tracings of the effect of DMS (10 μM) on basal tension of isolated rat carotid arteries from WKY rats (A) and SHRs (B). Contractile force is presented in mN. Note the impaired relaxant response to MCh (10 μM) after pre-contraction with PhE (1 μM) and a DMS-induced constriction in arteries from SHRs but not WKY rats.

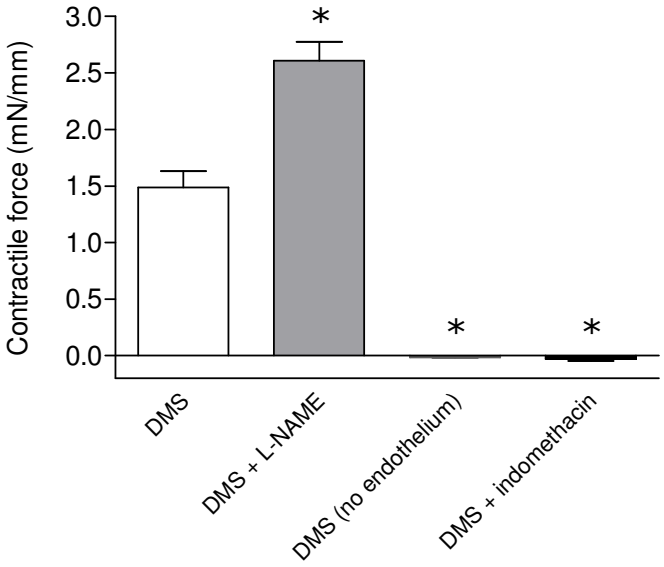

Figure 4. Contractile responses evoked by DMS (10 μM) in isolated carotid artery segments of SHR. Contractile force is presented as mN/mm segment length. Contraction was measured in the presence or absence of L-NAME (100 μM) and indomethacin (10 μM) and in endothelium-denuded preparations. Values are given as means ± S.E.M. (n = 5-6), * = $P < 0.05$ compared to DMS alone in the presence of endothelium.

Discussion

The sphingomyelin metabolites ceramide, sphingosine and S1P are important mediators of various cellular processes. Exogenous application of sphingomyelin metabolites has been shown to affect vascular contractility by inducing both contraction and relaxation.[11] We and others have recently shown that local formation of sphingomyelin metabolites is an important regulatory mechanism of vascular contraction and known vasoactive compounds such as Ang II and muscarinic agonists have been shown to induce formation and breakdown of sphingomyelin metabolites to exert their vasoactive effects.[13,18]

In the present study we investigated changes in sphingomyelin metabolite-dependent vascular responses during hypertension, a disease state that is characterized, amongst others, by endothelial dysfunction and vascular remodelling. For this reason it is not unlikely that the role of local S1P formation is altered in vessels from hypertensive subjects. We confirm that the endothelium-dependent vasorelaxation to MCh was attenuated in aorta and carotid artery preparations from SHR.[14] Interestingly, the endothelium-dependent relaxation to MCh was not reduced in mesenteric arteries of SHR when compared to WKY. Since endothelial dysfunction in hypertension has been suggested to be caused mainly by a decreased NO bioavailability, this phenomenon can partially explained by the relative minor role of NO in this vascular bed.[19,20] Endothelium-dependent relaxation in this vascular bed is mainly mediated by EDHF. Indeed, some studies suggest that EDHF might compensate for the loss of NO bioavailability in hypertensive animals [21] and also increased EDHF-mediated relaxant responses in renal arteries have been reported in SHR.[22] However, these responses

are highly dependent on age [22] and the nature of EDHF, and also attenuation of EDHF responses in hypertension have been shown.[23,24]

In the aorta of WKY rats, inhibition of sphingosine kinase by DMS resulted in a rightward shift of the CRC for MCh, which is in accordance with our previous findings in normotensive Wistar rats. The latter is most likely caused by locally synthesized S1P in response to muscarinic receptor stimulation (which is inhibited by DMS) that has a stimulatory action on endothelial NO synthase activity (see chapter 4). In addition, DMS enhances MCh-induced vasorelaxation in mesenteric arteries from normotensive animals, although this effect was much less pronounced when compared to Wistar rats and did not reach statistical significance with the given number of experiments in WKY rats. The latter may be caused because of the higher age and relative high weight of the WKY rats compared to the Wistar rats from our previous study (33-36 vs 12 weeks respectively). The enhancement of MCh-induced relaxation by DMS in normotensive rats is due to an inhibitory effect of S1P (which synthesis is inhibited by DMS) on the release or action of EDHF in these vessels (see chapter 4).

In both, aorta and mesenteric arteries derived from SHR, DMS less potently influenced MCh-induced relaxation. The decreased effect of DMS in the aorta might be partially explained by a generally decreased NO-dependent relaxation.[25] Because of the low number of experiments, the obtained results in the mesenteric artery preparations should be interpreted cautiously, but the less potent effect of DMS may be due to an altered function or identity of EDHF. Since the site of action of S1P on EDHF is not known, one can speculate that S1P may have differential effects on the different proposed EDHFs, and the nature of EDHF may be different in for instance hypertension. In this light it is noteworthy that it was recently shown that EDHF-mediated responses in mesenteric arteries facilitated by inwardly rectifying K^+ channels at muscarinic receptor stimulation are decreased in SHR compared to WKY rats.[26] It was also shown that the muscarinic receptor agonist acetylcholine evokes a fast depolarization in SHR but not in WKY rats. This depolarization is responsible for a constriction that reduces EDHF-mediated relaxation.[27] Therefore, it is indeed likely that sphingosine kinase-dependent EDHF inhibition during muscarinic receptor-mediated relaxation of the mesenteric artery is less important in SHR. However, the exact mechanism of muscarinic receptor-mediated vasorelaxation in the mesenteric artery of SHR and the role of sphingomyelin metabolizing enzymes herein, remains to be elucidated.

In both SHR and WKY rats, the potency of Ang II in carotid arteries in the presence of the sphingosine kinase inhibitor DMS was increased, which is in accordance to our earlier findings in normotensive Wistar rats.[13] However, the increase in efficacy by DMS was higher in SHR when compared to WKY. This finding suggests that in the SHR Ang II-induced S1P synthesis

induces a stronger vasorelaxant response. However, this conclusion is not in line with the observed decrease in endothelial function. Another possibility could be that under these experimental conditions the inhibition of sphingosine kinase by DMS gives rise to the synthesis of an (endothelium-derived) contractile factor (EDCF). Interestingly, when investigating the effects of DMS on Ang II-induced contraction and MCh-induced vasodilation in the carotid artery from SHR, we observed a substantial, transient and biphasic contractile response to DMS. Since this phenomenon was completely absent in WKY or Wistar rats, we investigated this DMS-induced constriction in more detail. It has been shown in animal models (SHR) and humans that in subjects with (essential) hypertension muscarinic receptor stimulation results in an endothelium-dependent vasoconstriction, instead of vasorelaxation. Although the exact mechanisms are unknown, these endothelium-dependent contractions involve the release of cyclooxygenase-derived contractile eicosanoids that subsequently stimulate thromboxane receptors on vascular smooth muscle cells.[28-31] Indeed, also under our experimental conditions application of MCh to non-pre-constricted arteries of SHR but not WKY induced vasoconstriction (data not shown). It is tempting to speculate that DMS in these arteries induces the release of a similar EDCF. Endothelial denudation indeed completely prevented the DMS-induced constriction, indicating an endothelial origin of the contractile factor in the present study. Since inhibition of cyclooxygenase by means of indomethacin also completely blocked DMS-induced constriction, this endothelium-derived contractile factor is indeed an eicosanoid. Although we have not investigated how DMS induces the release of eicosanoids from the endothelium, a likely mechanism may be the generation of ceramide-1-phosphate from accumulating ceramide due to inhibition of sphingosine kinase. Ceramide-1-phosphate, produced by ceramide kinase from ceramide, has been shown to activate cytosolic phospholipase A_2 directly [32] or in a protein kinase C-dependent way.[33] Activation of cytosolic phospholipase A_2 by ceramide-1-phosphate will lead to the synthesis of arachidonic acid that is a substrate for eicosanoid synthesis by cyclooxygenase. It remains to be investigated whether ceramide-1-phosphate indeed plays a role in EDCF synthesis and what is the relation of altered sphingolipid metabolism and hypertension. It is not unlikely that expression and/or activity of sphingolipid metabolizing enzymes are altered during hypertension, since Johns et al. have shown that ceramide synthesis is impaired in vascular smooth muscle from SHR.[34] In their study, decreased ceramide synthesis and increased activity of sphingosine kinase was linked to increased VSMC proliferation in SHR. Interestingly, in the same study it was shown that treatment of VSMCs with TNF-α, a known activator of sphingosine kinase [9], had an inhibitory effect on WKY rat VSMC proliferation, but stimulated proliferation in cells from SHR.[34]

In summary, a disturbed expression and/or activity of sphingolipid metabolizing enzymes in hypertension may indeed be the basis of the observed altered responses to DMS. Moreover,

an altered (constitutive) balance in sphingomyelin metabolites may contribute to decreased vascular tone as a compensating mechanism in hypertensive subjects. Pharmacological interventions in sphingolipid metabolism may be useful tools to modulate endothelial function in hypertension.

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C h a p t e r

6

The role of locally formed sphingomyelin metabolites in vascular function.

A general discussion

Introduction

Sphingomyelin was first discovered in the brain in 1884 by Prof. Thudichum and he did not know the complexity and variety of signaling capabilities of the lipid he had discovered and the related metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) that were to be studied.[1] Now, almost 125 years and much research later, the biology of sphingolipids remains enigmatic. We know now that it is not just the presence of a certain sphingomyelin metabolite, but its spatiotemporal formation that determines its effect. Local formation of sphingomyelin metabolites is important in the central nervous system and immune system but also in tumor development. Although certain sphingomyelin metabolites are present in high concentrations in blood, both endothelial cells and vascular smooth muscle cells (VSMCs) express the enzymes involved in the sphingomyelin metabolism and, therefore, these lipids can be formed "on demand" in both cell types. In this thesis, we sought out to describe the role of local formation of sphingomyelin metabolites induced by known vasoactive compounds for vascular function under both normal and pathological circumstances. In the previous chapters we have clearly shown that local sphingolipid metabolism induced by angiotensin II (Ang II) and muscarinic receptor agonists indeed plays an important role in the vasculature. Moreover, pathological conditions, such as hypertension and/or hypertrophy of the vessel wall have major implications on the vasoactive properties of sphingolipids.

In this chapter we will discuss the findings presented in chapters 2, 3, 4 and 5 in more detail and present some additional new data that further substantiate the importance of sphingolipid signaling in the vasculature.

Materials and methods

Contraction and relaxation experiments

Contraction experiments were performed in isolated rat carotid arteries as described in chapter 2. Concentration response curves to endothelin-1 and histamine were constructed 30 min after the addition of 10 μM DMS (or its vehicle DMSO) to the organ baths. Relaxation experiments were performed in isolated rat aorta and mesenteric arteries as described in chapter 4. Non-cumulative concentration responses were measured for the $S1P_{1,3}$ agonist VPC 23153. In selected preparations the endothelium was removed by gently rubbing the vascular segment, or the NO synthase inhibitor L-NNA was added 30 min prior the addition of VPC 23153.

Cell culture

VSMCs were obtained using a tissue outgrowth model. They were isolated and cultured as described in chapter 3. bEnd.3 cells were a kind gift from the Department of Nephrology and Hypertension, University Medical Center Utrecht, The Netherlands and cultured as described in chapter 2. For caspase-3/7 activity and proliferation experiments, cells were plated at 10,000 cells/well and cultured in black clear-bottom 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Before initiating experiments, bEnd.3 cells were grown in FCS-free culture medium supplemented with 0.1 % (w/v) BSA, and VSMCs were cultured in 0.5 % (v/v) FCS culture medium, both for 18 h.

Real-time quantitative PCR

After excision, vessels were placed directly in RNAlater (Sigma Chemical Co., St Louis, MO, USA) and stored for a minimal period of 3 days at -20 °C.[2] Afterwards, vessels were cleaned from adipose and connective tissue, cut into small pieces in Trizol (Invitrogen, Breda, The Netherlands) and homogenised 2 x 30 sec on ice using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) at full speed. Further isolation of RNA and real-time quantitative PCR were performed as described before.[3] Oligonucleotide primers were designed using the D-LUX designer software (Invitrogen, Breda, The Netherlands) based on sequences from the GenBank database, except for S1P phosphatase 1 and S1P lyase 1 (table 1). Each primer pair was tested for sensitivity and PCR efficiency. Constitutively expressed P0-ribosomal protein (P0-ribo) and elongation factor-1 (EF-1) were selected as endogenous controls to correct for potential variation in RNA loading. Relative mRNA expression of aorta smooth muscle cells (SMCs) (table 2A) and aorta (table 2B) were arbitrarily set to 1 for each experiment.

Gene	Accession nr		Sequence	Amplicon size
$S1P_1$	NM_017301	forward	TTCCTGGTTCTGGCTGTGCT	137
		reverse	gactccTTGAATTTGCCAGCGGAGtC	
$S1P_2$	NM_017192	forward	CCAACGGAGGCACTGACTAAT	96
		reverse	ATGTCTAGCCCTAAACTCGAGCC	
$S1P_3$	XM_225216	forward	caccagGCATCTTCACAGCCATTCTGGtG	79
		reverse	CTGCGGCTGCTGGACTTGAC	
Sphk1	NM_133386	forward	gaaccaTCGACTGCCCGTACTTGGtTC	111
		reverse	GCACAGCTTCACACACCATCA	
Sphk2	NM_001012066	forward	CAGCTCAGCTTTCACCCATCG	89
		reverse	cacaatAGAGGCTTCTGGAGCATTGtG	
Sgpp1	XM_343081	Acquired from Superarray (Frederick, USA)		
Sgpl1	NM_173116	Acquired from Superarray (Frederick, USA)		
P0-ribo	NM_022402	forward	cacagaAGGGTCCTGGCTTTGTCTGtG	90
		reverse	CGCAAATGCAGATGGATCG	
EF-1	NM_175838	forward	GCAAGCCCATGTGTGTTGAA	96
		reverse	TGATGACACCCACAGCAACTG	

Table 1. Oligonucleotide primers used for real-time quantitative PCR for rat $S1P_{1-3}$, sphingosine kinase-1 (Sphk1) and -2 (SphK2), S1P phosphatase 1 (Sgpp1), S1P lyase 1 (Sgpl1), P0-ribo and EF-1. Non-capital letters indicate that these nucleotides are added to form a hairpin. Sgpp1 and Sgpl1 were from Superarray (Frederick, USA).

5-Bromo-2'-deoxyuridine (BrdU) incorporation

Induction of DNA synthesis was determined by means of BrdU incororation as described in chapter 3.

Caspase 3/7 assay

To measure caspase activity in cultured bEnd.3 cells and VSMCs the Apo-ONE homogeneous caspase 3/7 assay from Promega (Madison, WI, USA) was used, according to the manufacturer's instructions. In short, cells were treated with DMS at indicated concentrations or vehicle (DMSO) in the presence of Ang II (100 nM) or vehicle (sterile water) for 6 h. Afterwards, the caspase-3/7 substrate Z-DEVD-R110 (rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide)) was added in cell lysis buffer. Upon cleavage of the aspartate residue by caspase-3/7 enzymes, rhodamine 110 becomes fluorescent. Fluorescence (excitation 485 nm; emission 510 nm) was measured using a Victor 2 plate reader (Perkin Elmer, Wellesley, MA, USA). Caspase 3/7 activity induced by DMS at various concentrations (0, 4 and 6 μM) was arbitrarily set to 100 %, and data are presented as changes (%) in caspase activity induced by Ang II in the presence of the different DMS concentrations.

S1P-induced changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ measurements were performed according to Jongsma et al., using the fluorescent indicator dye fluo-4.[4] A concentration-response curve for S1P was constructed, including the vehicle (0.4 % (w/v) BSA in sterile water). All measurements were performed in triplicate.

Statistics

All curve fitting and data analysis was done using GraphPad Prism (version 4.0; GraphPad Software, San Diego, USA). All data are expressed as means ± S.E.M. for the number of experiments (n) as indicated. Data are analyzed by Student's t-test, one-way ANOVA or one-sample t-test where appropriate. A *P* value of less than 0.05 was considered significant.

Results and discussion

Previous studies investigating the vasoactive properties of sphingolipids used exogenously applied sphingolipids, more or less mimicking the presence of sphingomyelin metabolites in serum or plasma. These studies have identified the different sphingomyelin metabolites as vasoactive lipids but, since the results are inconclusive, their functional role and the nature of

their effects (i.e. vasoralaxation or vasoconstriction) remains unclear (reviewed in [5]). Moreover, as stated previously the majority of sphingolipids present in whole blood is sequestered in blood cells, stored in lipoprotein particles or bound to albumin, most likely resulting in only a very small fraction of free sphingolipids. Because of this it can be questioned whether the exogenous administration of sphingolipids is the preferred method to investigate the physiological role of sphingolipids in the vasculature. In addition, it has been shown recently that endogenously formed S1P may induce different effects on endothelial barrier function than extracellular exposure to S1P.[6] Overexpression of sphingosine kinase has been used to identify the role of locally synthesized S1P in vascular function.[7] These studies revealed that sphingosine kinase overexpression and concomitant S1P production increases resting tone and myogenic responses. However, sphingosine kinase was (only) overexpressed in the smooth muscle cells and is therefore not representative for the *in vivo* situation. For these reasons we have opted to investigate the role of locally synthesized sphingolipids by inhibiting sphingosine kinase in vascular segments. For this purpose we have used the sphingosine kinase inhibitor dimethylsphingosine (DMS). Since all our studies rely on this inhibitor, its specificity is an important issue. Although DMS has been described as a protein kinase C inhibitor, several studies have shown that DMS is a specific sphingosine kinase inhibitor in concentrations lower than 50 μM.[8-10] Moreover, we have shown that the protein kinase C inhibitor calphostin C did not mimic the effects of DMS on the Ang II-induced vasoconstriction.[3] To undisputedly show that sphingosine kinase is activated upon agonist exposure, one would like to measure S1P concentrations. Unfortunately, most conventional assays (e.g. HPLC) to measure changes in the production of S1P are not sufficiently sensitive. Nevertheless, in chapter 4 we have demonstrated by means of translocation studies a direct coupling between muscarinic receptors and sphingosine kinase. In the same chapter we have also reported on a study that showed similar effects of an alternative sphingosine kinase inhibitor on the methacholine induced relaxation in rat aorta as we show for DMS.[11] Taken together these data suggest that the effects of DMS in the vasculature are indeed due to sphingosine kinase inhibition and not to non-specific effects.

In chapter 2 we have reported that the contractile responses to Ang II are potentiated by DMS. Endothelial denudation or inhibition of NO synthase resulted in a similar potentiation of Ang II-induced constriction, suggesting a prominent physiological role of eNOS in attenuating the contractile effects of Ang II. The underlying mechanism involves both activation of the PI3 kinase/Akt pathway and elevation of endothelial intracellular Ca^{2+} concentrations by locally formed S1P, leading to eNOS activation.

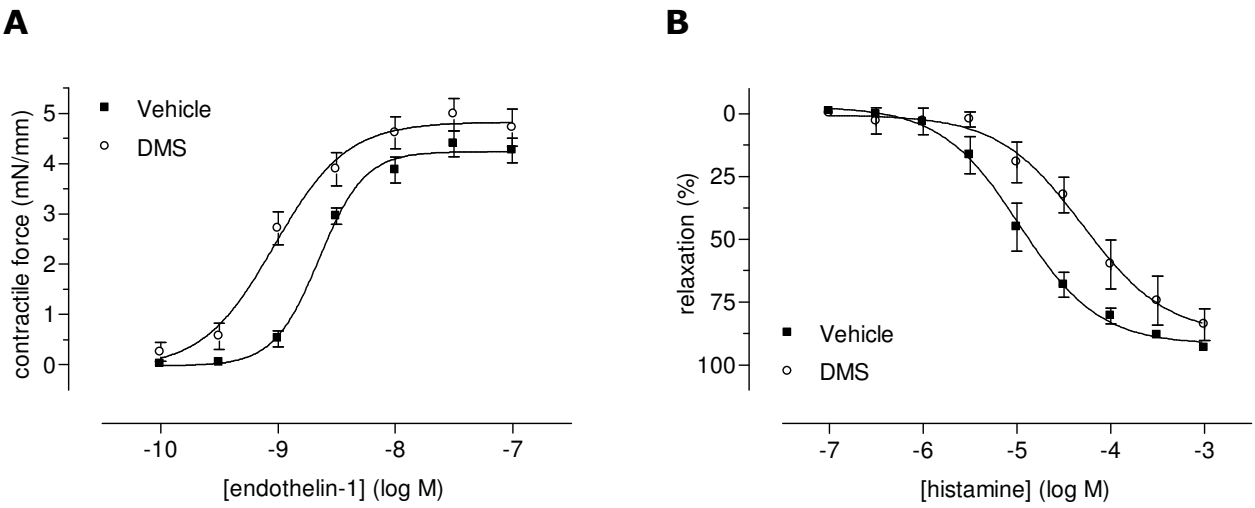


Figure 1. Contractile and relaxant responses in the isolated rat carotid artery to endothelin-1 (A) and histamine (B) in the presence or absence of DMS (10 μM). Contractile force is presented as mN/mm segment length or as % relaxation of phenylephrine (3 μM)-induced pre-constriction. DMS or vehicle was added to the organ bath 30 minutes before the construction of the concentration-response curve for indicated agonists. Values are given as mean ± SEM (n = 3-8).

Importantly, we did not find alterations in the contractile response to K^+ (receptor-independent vasoconstriction) and the α_1-adrenoceptor agonist phenylephrine. However, in addition to Ang II, the contractile properties of other constriction inducing agonists are affected by DMS. For instance, in analogy to Ang II also the concentration response curve to endothelin-1 is shifted to the left in the presence of DMS (figure 1A). It remains to be investigated whether the same molecular mechanisms are responsible for the potentiation of endothelin-1-induced vasoconstriction as we have shown for Ang II. The vasorelaxant effects of histamine in the carotid artery are attenuated by DMS (figure 1B) which indeed suggests that S1P has a relaxant effect in this vascular preparation. It would be interesting to know whether the vasodilatory response to histamine in the mesenteric artery is affected by DMS in a similar way (i.e. potentiated) as methacholine-induced relaxation as discussed chapter 4.

The surprising finding that DMS attenuates the Ang II-induced constriction in an endothelium-dependent manner in cultured preparations as we reported in chapter 3, still awaits further investigation to clarify the underlying mechanism. Nevertheless, it is likely that under growth promoting circumstances, Ang II triggers the release of an endothelium-derived contractile factor (EDCF) that is dependent on sphingolipid metabolism. It can be speculated that S1P is this EDCF or that activation of sphingosine kinase triggers the release of a contractile factor, more or less similar as the sphingolipid-dependent release of an EDCF as seen in carotid arteries from spontaneous hypertensive rats after inhibition of sphingosine kinase (chapter 5). These possibilities will be discussed in more detail later in this chapter. An alternative explanation for the DMS effects might be that Ang II in the cultured preparations

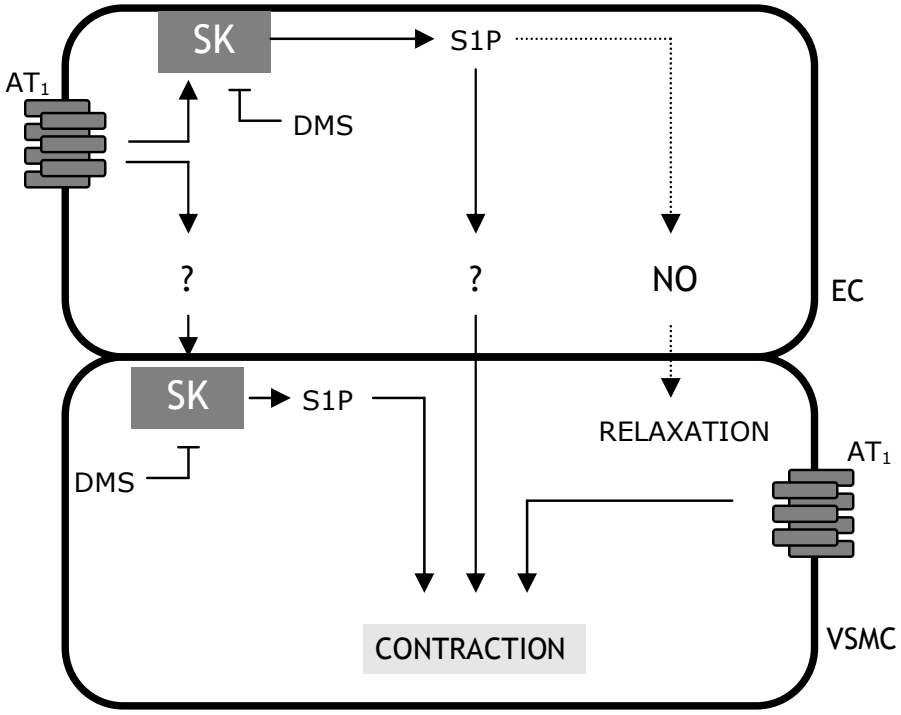


Figure 2. Schematic representation of possible mechanisms for the endothelium- and sphingosine kinase-dependent contractile effect for Ang II in cultured vessels. Either S1P acts as an endothelium-derived contractile factor (EDCF), endothelial sphingosine kinase activity leads to the release of another EDCF or the downstream signalling of an unknown EDCF is sphingosine kinase-dependent in the smooth muscle cell. (EC = endothelial cell; VSMC = vascular smooth muscle cell; SK = sphingosine kinase; AT_1 = angiotensin II type 1 receptor).

induces the release of a contractile factor that subsequently activates sphingosine kinase in the smooth muscle cells (figure 2).

It is remarkable that local sphingolipid metabolism only affects the endothelial effects of Ang II. The main acute effect of Ang II in the vasculature after all is vasconstriction by stimulating AT_1 receptors on the smooth muscle cells. As shown in chapters 2 and 3, these contractile effects are apparently not altered by sphingolipid metabolism in the smooth muscle cells. This phenomenon may also translate in differential growth regulating effects of Ang II in endothelial cells and VSMCs. In both cell types, Ang II as well as S1P, are mitogenic (figure 2 in chapter 3 and figure 3). Moreover, as depicted in figure 3, the existence of a functional ceramide / S1P rheostat can also be nicely demonstrated in endothelial cells; exogenously applied S1P induces a small mitogenic effect as measured by BrdU incorporation, whereas ceramide decreases BrdU incorporation. In addition, in analogy to VSMC, inhibition of sphingosine kinase decreases BrdU incorporation in these endothelial cells.

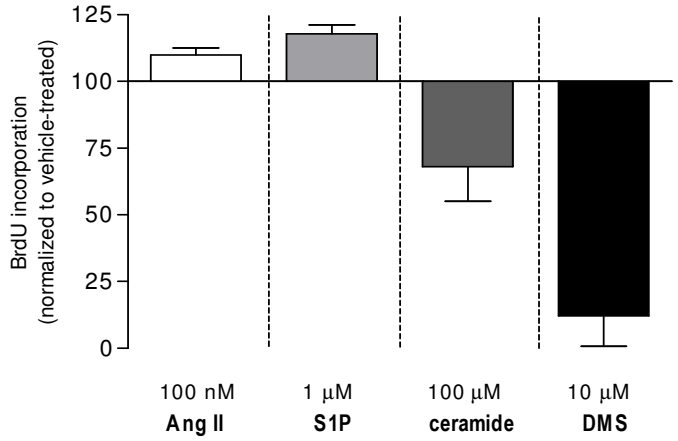


Figure 3. Effects of Ang II, S1P, ceramide and DMS on DNA synthesis in bEnd.3 endothelial cells. Incorporation of BrdU was measured in the presence of 0.1 % (w/v) bovine serum albumin. Basal values that were arbitrarily set to 100 % were 1154508 ± 158047, 1263521 ± 47220, 1209845 ± 111431, 1291542 ± 92550 relative luminescent units for Ang II, S1P, ceramide and DMS, respectively. Data presented as % of vehicle-treated cells. Values represent mean ± S.E.M. (n = 3-6).

Thus a reduced activity or expression of sphingosine kinase in endothelial cells may result in an opposite growth effect to Ang II (i.e. induction of apoptosis instead of proliferation or survival), since pro-apoptotic ceramide and sphingosine may accumulate, whereas S1P synthesis will be attenuated (see figure 4). In the case that Ang II simultaneously activates sphingomyelinase and/or ceramidase in addition to sphingosine kinase, these effects will be amplified. When indeed the Ang II effects on VSMC are sphingosine kinase-independent, a reduced expression or activity of sphingosine kinase will be without effect on the growth promoting effects of Ang II. In order to investigate this hypothesis we pre-incubated endothelial and VSMCs with DMS to reduce sphingosine kinase activity and investigated whether under these conditions Ang II reduced or increased apoptosis in these cells by measuring caspase 3/7 activity. Ang II alone (i.e. in the absence of DMS) did not induce caspase activity in endothelial or VSMCs, indicating that under normal circumstances Ang II does not induce apoptosis in these cell types, which is in accordance with our data showing that Ang II has modest mitogenic effects in both cell types. As shown previously in chapter 3, DMS induces apoptosis in the VSMCs and endothelial cells (figure 3) that is also reflected by a concomitant increase in caspase activity. In the VSMCs Ang II decreased DMS-induced caspase activity suggesting that the mitogenic effect of Ang II (partially) counteracts the DMS-induced caspase activity (figure 5). In contrast, in the endothelial cell line Ang II increased DMS-induced caspase activity. This indeed may be explained by the fact that also the growth inducing effects of Ang II in endothelial cells but not VSMCs, is (partly) dependent on sphingolipid metabolism. Accordingly, under circumstances with a reduced sphingosine kinase activity this results in a shift in the ceramide / S1P rheostat, giving rise to an altered ratio of apoptotic and mitogenic sphingomyelin metabolites (figure 4). A similar mechanism of action has been shown for TNF-α in endothelial cells under conditions with a reduced sphingosine kinase activity or expression.[12] The aforementioned phenomena may also explain the Janus-face type behaviour of Ang II in other cell types e.g. cardiomyocytes, where it can act either as a hypertrophic factor or can induce apoptosis under certain (pathological) conditions (e.g. heart failure).

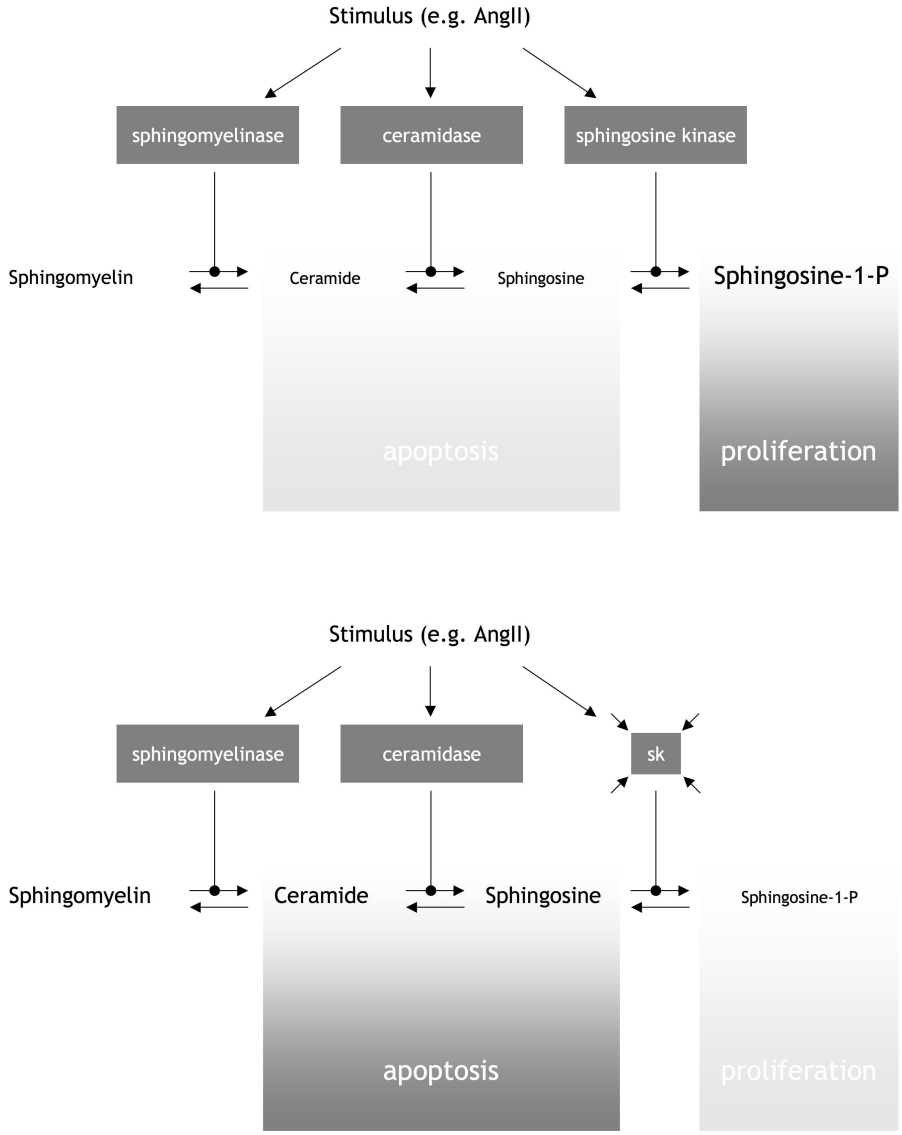
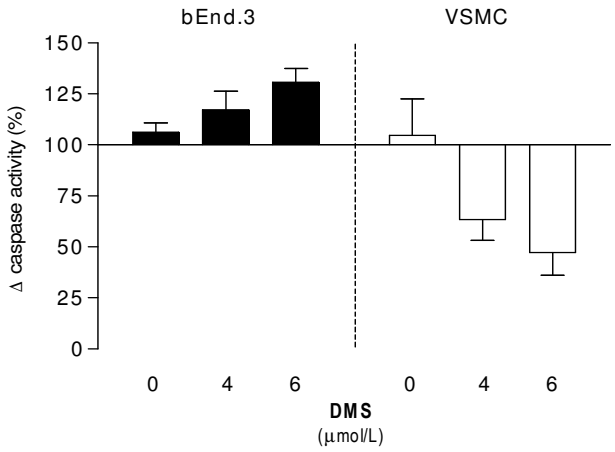


Figure 4. Several stimuli (e.g. Ang II) make use of the ceramide / S1P rheostat to exert both positive and negative effects on cellular proliferation. Under normal circumstances, activation of sphingosine kinase leads to increased S1P synthesis and a concomitant increased mitogenic response (upper panel). However, as depicted in the lower panel, reduced activity or expression of sphingosine kinase in endothelial cells may result in an opposite growth effect to Ang II since ceramide and sphingosine may accumulate, whereas S1P synthesis will be attenuated. (SK = sphingosine kinase; Sphingosine-1-P = sphingosine-1-phosphate).

Figure 5. Caspase 3/7 activity measured in bEnd.3 endothelial cells and VSMCs. Cells were incubated with DMS for 6 h in the presence of Ang II (100 nM) or vehicle. Afterwards, cells were lysed and a caspase 3/7-specific fluorescent substrate was added and fluorescence measured. Caspase 3/7 activity induced by DMS at various concentrations (0, 4 and 6 μM) was arbitrarily set to 100 %, and data are presented as changes (%) in caspase activity induced by Ang II in the presence of the different DMS concentration. Values are given as mean ± S.E.M. (n = 5-7).



The sphingolipid-dependent effects of Ang II on the endothelium of the carotid artery prompted us to investigate the role of local sphingolipid metabolism for the muscarinic M_3 receptor which vasodilatory actions are fully endothelium-dependent. We investigated the dilatory responses to the muscarinic receptor agonist methacholine not only in conduit vessels (aorta and carotid arteries) but also in isolated mesenteric arteries. As described in chapter 4, we observed some interesting differential effects of DMS on the methacholine-induced vasodilation in these three vessel types. Whereas DMS inhibits methacholine-induced vasodilation in the aorta and to a lesser extent in carotid arteries, the dilatory responses were potentiated in mesenteric artery preparations. The effects of DMS in the aorta can be explained by a similar mechanism as presented for Ang II in chapter 2. Accordingly, muscarinic receptor-mediated activation of sphingosine kinase and concomitant S1P production will, most likely via a phosphatidylinositol 3 kinase/Akt-dependent pathway, lead to an increased activity of NO synthase. Thus inhibition of sphingosine kinase will induce a rightward shift of the CRC to methacholine. More or less the opposite is true for the effects of DMS in the mesenteric artery; activation of sphingosine kinase and concomitant S1P production results in vasoconstriction or an "anti-dilatory" effect. The potentiation of the methacholine-induced relaxation in the mesenteric artery preparations by DMS may be explained by two mechanisms. Because S1P may be exported to the extracellular space [13] and because of the existence of an extracellular sphingosine kinase in endothelial cells [14,15], one possibility would be that S1P acts as an EDCF which is more pronounced in mesenteric artery preparations.[16] If S1P indeed acts as an EDCF than a possible explanation for the differential effects of DMS would be that smooth muscle cells from the mesenteric artery are more sensitive to S1P for changes in $[Ca^{2+}]_i$. Indeed, S1P has been shown to induce contraction in mesenteric artery preparations, although at very high concentrations.[17] Due to practical problems in reaching these concentrations in our organ bath setup, we were not able to confirm this. As an alternative, we measured changes in $[Ca^{2+}]_i$ in cultured smooth muscle cells from the different vascular beds, which allowed us to investigate the influence of S1P at lower and most likely more physiological concentrations. We found that S1P induced a similar concentration-dependent increase in $[Ca^{2+}]_i$ in all three types of VSMCs, so there apparently is no difference in responsiveness of these cells to S1P (figure 6). These data suggest that endothelium-derived S1P indeed may act as an EDCF, however, since all three VSMC types respond similar to S1P this does not explain the differential effects of DMS in the artery preparations.

A second explanation could be that S1P under normal circumstances inhibits the release or action of EDHF, that is known to play a major role in the relaxation responses of the mesenteric artery, but not of conduit vessels.[18,19] Indeed, in chapter 3 we have shown that DMS potentiates the actions of EDHF (i.e. the vasodilatory response in the presence of NO

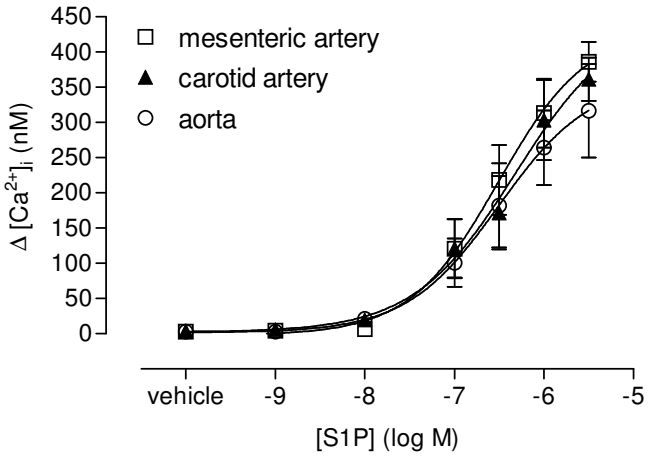


Figure 6. Intracellular Ca^{2+} measurements in cultured VSMCs from aorta, carotid and mesenteric arteries. After loading with fluo-4 AM, cells were stimulated with S1P or vehicle (0.4 % (w/v) BSA in sterile water) under constant measuring of fluorescence. With the use of Triton and EGTA, the maximal and minimal fluorescent responses were determined, and changes in intracellular Ca^{2+} concentrations (Δ $[Ca^{2+}]_i$) were calculated. Δ $[Ca^{2+}]_i$ are expressed in nM and are mean ± S.E.M. (n = 5).

synthase and cyclooxygenase inhibitors) in mesenteric arteries. Thus, in these preparations S1P may act as an inhibitor of EDHF and a likely candidate for the target of locally formed S1P is C-type natriuretic petide which vasodilatory action in mesenteric arteries is indeed potentiated by DMS.

Both, endothelial and VSMC express S1P receptors and therefore these cells may respond to locally formed S1P. The S1P receptors expressed in the vascular system (i.e. $S1P_1$, $S1P_2$ and $S1P_3$ receptors) induce a variety of signalling events in endothelial cells and VSMC that influence vascular tone (e.g. increases in $[Ca^{2+}]_i$, decreases of cAMP levels and activation of Rho kinase).[20] Therefore, the ultimate effect of S1P on a vessel or vascular bed depends, amongst many other factors, on the relative expression of S1P receptor subtypes in the endothelium and VSMCs. Accordingly, Coussin et al. have shown that differences in vascular S1P receptor expression levels can result in different contractile responses and also differences in sphingolipid metabolizing enzyme expression have been suggested to play a role in the differential effects of S1P.[21,22] Thus, the aforementioned differential effects of DMS on methacholine-induced vasorelaxation in aorta, carotid and mesenteric arteries may also be partially dependent on a differential S1P receptor and/or sphingosine kinase expression in the endothelial cells and VSMCs of these three vessel types. In order to address this possibility, we have investigated the relative expression of the S1P receptors and sphingolipid metabolizing enzymes in whole vessel preparations and cultured VSMC from the three vessel types. In the whole vessel preparations we observed a 5 to 6 fold higher expression of the $S1P_1$ and $S1P_3$ receptor subtypes in mesenteric arteries when compared to aorta (table 2A). In addition, compared to aorta, there is a modestly higher expression of sphingosine kinase in mesenteric and carotid arteries. In the VSMC isolated from the three different vessel types we did not observe substantial differences in S1P receptor or enzymes (table 2B). Although the latter finding may explain the equipotent action of S1P to increase $[Ca^{2+}]_i$ in these cells, the results are otherwise difficult to interpret. In the whole vessel preparations the ratio of

A

	Vessel type		
Gene	**Aorta**	**Carotid artery**	**Mesenteric artery**
$S1P_1$	1	1.4 ± 0.3	5.0 ± 1.4
$S1P_2$	1	1.0 ± 0.4	0.9 ± 0.4
$S1P_3$	1	0.8 ± 0.1	5.7 ± 1.9
Sphk1	1	2.3 ± 1.1	2.7 ± 1.6
Sphk2	1	0.9 ± 0.3	0.8 ± 0.4
Sgpp1	1	1.1 ± 0.2	1.3 ± 0.1
Sgpl1	1	0.7 ± 0.2	1.1 ± 0.4

B

	Cell type		
Gene	**Aorta SMC**	**Carotid artery SMC**	**Mesenteric artery SMC**
$S1P_1$	1	1.2 ± 0.1	0.3 ± 0.0
$S1P_2$	1	1.7 ± 0.1	1.6 ± 0.1
$S1P_3$	1	1.0 ± 0.0	0.8 ± 0.1
Sphk1	1	1.5 ± 0.1	0.9 ± 0.2
Sphk2	1	1.5 ± 0.1	1.5 ± 0.1
Sgpp1	1	0.7 ± 0.1	0.9 ± 0.0
Sgpl1	1	1.4 ± 0.1	1.1 ± 0.0

Table 2. (A) Real time quantitative PCR determination of relative expression levels of S1P receptors $S1P_{1-3}$, sphingosine kinase-1, -2, S1P phosphatase 1 and S1P lyase 1 in isolated rat carotid and mesenteric artery, normalized to aorta for each experiment. For aorta Ct values were 21.8 ± 1.3, 20.5 ± 1.4, 29.5 ± 1.3, 29.4 ± 0.9, 31.7 ± 1.3, 33.6 ± 0.7, 33.8 ± 0.8, 28.6 ± 0.9, 31.5 ± 1.8 and 28.5 ± 1.0 for P0-ribo, EF-1, $S1P_{1-3}$, sphingosine kinase-1, -2, S1P phosphatase 1 and S1P lyase 1, respectively (n = 3-4). B) Real time quantitative PCR determination of relative expression levels of S1P receptors $S1P_{1-3}$, sphingosine kinase-1, -2, S1P phosphatase 1 and S1P lyase 1 in cultured VSMCs from rat carotid and mesenteric artery, normalized to aorta VSMCs for each experiment. For aorta VSMCs Ct values were 18.6 ± 0.0, 17.0 ± 0.0, 31.4 ± 0.1, 25.2 ± 0.0, 25.4 ± 0.1, 27.7 ± 0.3, 26.1 ± 0.1, 26.6 ± 0.1 and 25.1 ± 0.1 for P0-ribo, EF-1, $S1P_{1-3}$, sphingosine kinase-1, -2, S1P phosphatase 1 and S1P lyase 1, respectively (n = 3). Constitutively expressed P0-ribo and EF-1 were used as a reference.

endothelium/VSMC is most likely not equal for smaller (mesenteric) and larger vessels (aorta and carotid arteries). In addition, culturing VSMC may induce changes in mRNA expression. For these reasons it is extremely difficult to explain the differential effects of DMS based on these real time PCR data. Nevertheless, the importance of differential S1P receptor expression in these three vessel types can be unmasked by making use of selective S1P receptor agonists. For instance, the $S1P_1$/$S1P_3$ agonist VPC 23153 induces a concentration-dependent vasodilation in both, isolated rat aorta and mesenteric artery. In the mesenteric artery segments VPC 23153 induces a nearly complete vasodilation that was NO- and endothelium-independent. In contrast, the same compound had only a small (approximately 30%) vasodilatory effect in the aorta that was completely abolished by the NO synthase inhibitor L-NNA or endothelial denudation (figure 7). These results suggest that also differential receptor expression can potentially contribute to different responses to for instance muscarinic agonist-induced local sphingolipid metabolism.

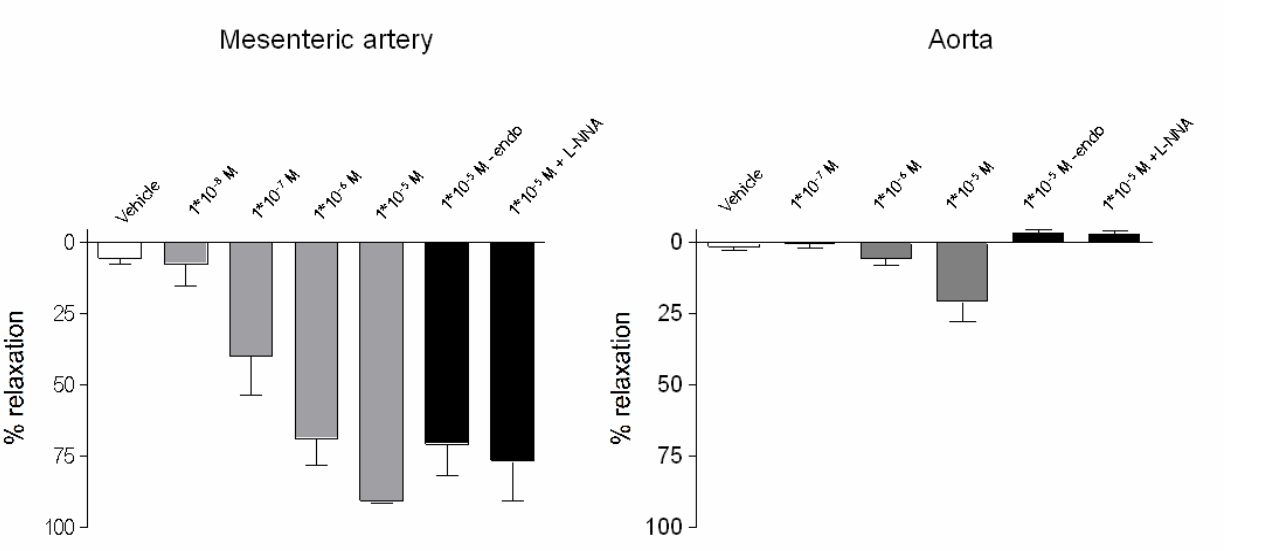


Figure 7. Concentration-dependent vascular relaxation induced by the $S1P_1/S1P_3$ agonist VPC 23153 of isolated rat mesenteric artery and aorta. In mesenteric artery segments, removal of endothelium and the presence of the NO synthase inhibitor L-NNA did not prevent VPC 23153-induced vasodilation. In contrast, the small dilatory response to VPC 23153-in rat aorta is completely abolished by L-NNA or endothelial denudation. Data are expressed as % relaxation of phenylephrine (3 μM)-induced preconstriction. Values ± SEM (n = 4-6).

One well-known risk factor for cardiovascular disease is hypertension; a disease state amongst others characterized by endothelial dysfunction and vascular remodelling. Since vascular effects for Ang II and methacholine involve endothelial formation of sphingomyelin metabolites which has been shown to be altered under hypertrophic conditions (chapters 2, 3 and 4), we hypothesized that the vasoactive properties of Ang II and methacholine are altered in vessels obtained from spontaneous hypertensive rats. DMS induced a similar leftward shift of the Ang II CRC in carotid artery segments of SHR and WKY, however, with a higher maximal effect in SHR. In the WKY rats, we observed at least qualitatively similar responses of DMS on methacholine-induced relaxation as described in chapter 4. However, when compared to WKY, the responses to DMS were less pronounced, especially in the isolated aorta of SHR. Interestingly, in carotid artery segments from SHR but not in segments from normotensive rats, DMS induced a slowly developing, transient, and mostly biphasic contraction. It has been described previously that in SHR but also in human essential hypertension, endothelium-derived contractile factors may contribute to an increased vascular tone. Since in the previous chapters we have speculated that sphingomyelin metabolites can act as, or are involved in the synthesis of EDCF, we have investigated whether the DMS-induced transient contraction could be explained by an EDCF action. As reported previously by other groups[16], methacholine induces vasoconstriction in the isolated vessel segments in the present study, confirming that EDCF responses can be detected in our vascular preparations from SHR. In literature, the EDCF in hypertension has been identified as an endothelium-derived eicosanoid which synthesis can be inhibited by cycloxygenase inhibitors or by endothelium removal.[16] In our experiments, the transient contraction by DMS

was completely absent in endothelium-denuded preparations and in preparations pre-treated with indomethacin. Accordingly, in hypertension changes in sphingolipid metabolism may give rise to endothelium-dependent vasoconstrictions. It remains to be investigated what is the relation between sphingolipids, EDCF and hypertension and whether sphingolipids are involved in the endothelium-dependent constrictions induced by acetylcholine, ATP or other neurohumoral mediators. As discussed in chapter 5, a possible explanation for the DMS effects could be that, at least in some vessel types from hypertensive animals, an altered (constitutive) balance in sphingomyelin metabolites contributes to decrease vascular tone as a compensating mechanism. Under these circumstances DMS may promote the synthesis of ceramide-1-phosphate that subsequently may activate cytoplasmic phospholipipase A2, triggering the synthesis of contractile eicosanoids. As stated previously in this chapter, the generation of endothelium-dependent eicosanoids or related compound may possibly also explain the sphingolipid-dependent nature of the Ang II-induced constriction in cultured preparations as shown in chapter 3. Further research is warranted to investigate the role of sphingolipids in endothelium-dependent contractions under certain pathological states.

General conclusion and future perspectives

Although there theoretically may be a role of blood borne sphingolipids in the vascular system, in the previous chapters we have clearly shown that the local synthesis of sphingomyelin metabolites within the vasculature can contribute to vascular tone. Moreover, altered sphingolipd metabolism as a cause or consequence of disease states may influence vascular function drastically. Since endothelial and VSMCs express all enzymes involved sphingolipid metabolism and express different targets of sphingolipids (e.g. S1P receptors), sphingolipids can act in an auto- or paracrine fashion in these cells. Several vasoactive substances such Ang II, endothelin-1, muscarinic agonists and histamine can alter sphingolipid metabolism in the vascular wall in order to exert their contractile or relaxant effects. It is striking to see the major importance of sphingolipid metabolism in the endothelium. In addition, it is interesting to observe that induction of endothelial sphingolipid metabolism by the different agonist can result in a contractile or vasodilatory action. In chapter 2 we have shown that S1P has, at least in some vessel types a vasodilatory action by increasing the synthesis of NO. The opposite action in mesenteric arteries as described in chapter 4 is most likely due to an inhibitory action of S1P on the release or action of EDHF, such as for instance C-type natriuretic peptide (see figure 8). The fact that S1P can be exported to the extracellular space by means of active transport and the existence of endothelial extracellular sphingosine kinase makes it possible that S1P itself may act as an

EDCF. However, also products that may accumulate due to sphingosine kinase inhibition such as ceramide and sphingosine, may lead to dilatory and contractile actions. For instance accumulating ceramide may be phosphorylated by ceramide kinase and the resultant ceramide-1-phosphate can subsequently activate cytoplasmic phospholipase A_2. The concomitant production of arachidonic acid by phospholipase A_2 acts as a substrate for the production of vasocative eicosanoids (e.g constriction inducing prostaglandins and thromboxanes or the vasodilatory prostaglandin prostacyclin) by cyclooxygenase (figure 8). The latter mechanism may be responsible for the transient contraction in the SHR due to inhibition of sphingosine kinase.

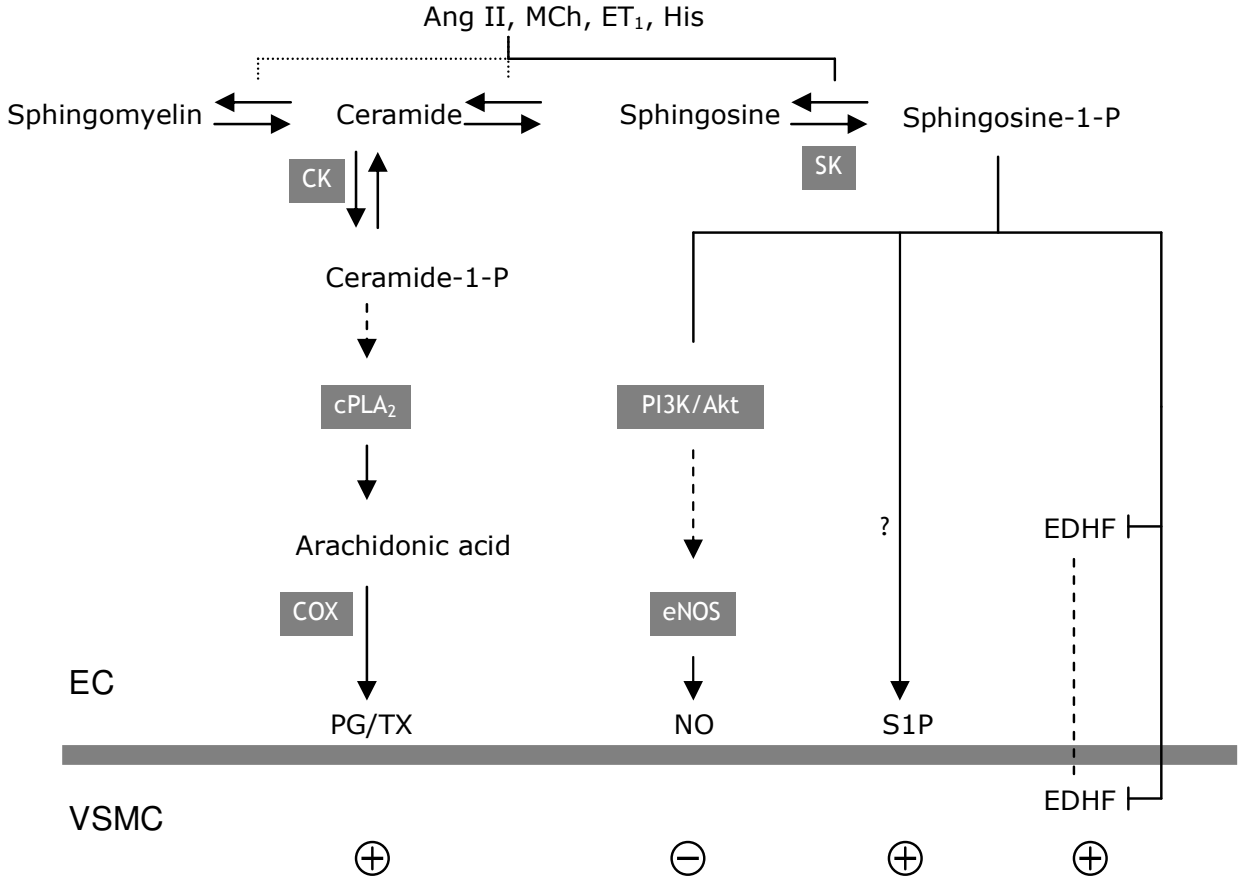


Figure 8. Overview of differential sphingomyelin metabolite-dependent signalling in the vascular wall for known vasoactive compounds (Ang II, methacholine, endothelin-1 and histamine). S1P can have a vasodilatory action by increasing the endothelial synthesis of NO as discussed in chapter 2. The contractile effect of locally formed S1P in mesenteric arteries is most likely due to an inhibitory action of S1P on the release or action of EDHF (chapter 4). In addition, extracellular S1P may act as and endothelium-derived contractile factor (chapters 3, 4 and 6). However, other sphingomyelin metabolites which accumulate due to inhibition of sphingosine kinase (such as ceramide and sphingosine) may affect vascular tone. Accumulating ceramide for instance, may be phosphorylated by ceramide kinase and the resultant ceramide-1-phosphate can subsequently activate cytoplasmic phospholipase A_2. The concomitant production of arachidonic acid by phospholipase A_2 acts as a substrate for the production of vasocative eicosanoids by cyclooxygenase (chapter 5). (EC = endothelial cell; VSMC = vascular smooth muscle cell; MCh = methacholine; ET_1 = endothelin-1; His = histamine, Sphingosine-1-P = sphingosine-1-phosphate; SK = sphingosine kinase; PI3K = phosphatidylinositol 3 kinase; Akt = protein kinase Akt; eNOS = endothelial NO synthase; CK = ceramide kinase; Ceramide-1-P = ceramide-1-phosphate; $cPLA_2$ = cytoplasmatic phospholipase A_2; COX = cyclooxygenase; PG/TX = prostaglandins and thromboxanes; EDHF = endothelium-derived hyperpolarizing factor, $\ominus$ = vasorelaxant; $\oplus$ = vasocontractile;).

It is important to realise that the ultimate effect of endothelial sphingolipid metabolism is for a major part dependent on the smooth muscle cells. For instance, hypertension or intimal hyperplasia may induce alterations in the vascular smooth muscle cells (e.g altered expression of S1P or eicosanoid receptors) in such a way that a vasodilatory action endothelial sphingolipid metabolism is inverted to a contractile action. In addition, in this thesis we have also shown that there exists a complex interplay between the growth and vasoactive effects of sphingolipids.

Local formation of sphingomyelin metabolites is an important and complex regulatory mechanism for maintaining homeostasis in cells of the vessel wall and also for controlling vascular tone. Pharmacological modulation of local sphingolipid metabolism may be of therapeutic value in cardiovascular disease states characterized by endothelial dysfunction and (smooth) muscle hypertrophy. At this time pharmacological tools to interfere with local formation of sphingomyelin metabolites are limited. The therapeutic potential of affecting the tight regulation of production and breakdown of sphingomyelin metabolites is substantial, but additional research is required to further identify and characterize possible therapeutic relevant interventions in these pathways.

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C h a p t e r

7

Sphingomyelin metabolism and endothelial cell function

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Introduction

In previous chapters we have described a central role for the vascular endothelium in mediating locally formed sphingomyelin metabolite-dependent effects. Activation of AT_1 or muscarinic receptors in the endothelium results in sphingosine kinase-mediated effects that influence vascular tone. The concomitant production of S1P has differential effects on vascular tone in different vascular beds. Moreover, we have shown that growth stimuli can drastically change the ultimate effects of endothelial sphingolipid metabolism. Accordingly, the aforementioned mechanisms may have important implications in disease states characterized by endothelial dysfunction and/or hypertrophic responses. Indeed, during hypertension we have identified alterations in sphingolipid metabolism in the endothelial cell that may contribute to a disturbed regulation of vascular tone. Also in literature, changes in local formation of sphingomyelin metabolites in endothelial cells have been shown to influence endothelial and vascular function. In this chapter we will highlight some recent findings on the complex interplay between the local formation of sphingomyelin metabolites and endothelial function.

Sphingomyelin metabolite-mediated signalling

Various stressful stimuli can activate different isoforms of sphingomyelinase, which catalyses the hydrolysis of sphingomyelin to ceramide. Ceramidase can metabolize ceramide further into sphingosine, which in turn can be phosphorylated by sphingosine kinase to yield sphingosine-1-phosphate (S1P). Other enzymes allow for a reversal of such reactions and/or can form other biologically active sphingomyelin metabolites such as sphingosylphosphorylcholine. S1P is a ligand for at least five subtypes of G protein-coupled receptors, designated $S1P_{1\text{-}5}$, which were originally described as endothelial differentiation genes. $S1P_{1\text{-}3}$ are the major receptor subtypes expressed in the cardiovascular system, in both endothelium and vascular smooth muscle cells; at least at the mRNA level $S1P_1$ appears to be the most abundantly expressed subtype in the endothelium.[1] In many cases ceramide and sphingosine on the one and S1P on the other hand have opposite effects on cellular function, e.g. by stimulating cell death and apoptosis vs. cell growth and differentiation, respectively. Accordingly, sphingomyelinases determine the amount of sphingomyelin metabolites being formed and hence can be considered as a volume regulator of sphingolipid signalling. On the other hand, sphingosine kinase has a major effect on the balance between the opposing effects of ceramide / sphingosine vs. those of S1P and hence may allow determining the direction of such signalling.

Sphingomyelin metabolites can reach endothelial cells via the blood stream. Perhaps even more importantly, they can be formed locally in the vascular wall [2-9] as endothelial cells express the enzymes involved in sphingolipid metabolism and are a regulatable source of sphingomyelin metabolites. As the endothelium also expresses receptors for some sphingomyelin metabolites such as the S1P receptor subtypes $S1P_1$ and $S1P_3$ [1], they can be considered as autocrine and/or paracrine mediators of endothelial function.

Barrier function

Classically endothelial cells were considered to mainly provide a barrier between the bloodstream and the vascular smooth muscle cells. This barrier function is based upon tight junctions between the endothelial cells. Endocytosis at the apical and subsequent exocytosis at the basolateral surface of the endothelium allows a controlled transition from the lumen to the vessel wall. Several agents can affect this endothelial barrier function via direct effects on the integrity of the tight junctions. It has now been recognized that the sphingomyelin metabolites ceramide and S1P have profound (opposite) effects on endothelial barrier function.[1] Local sphingolipid metabolism, induced for instance by activation of sphingomyelinase or sphingosine kinase, may, therefore, regulate endothelial permeability, most likely via differential actions on endothelial cell-cell junctions. Indeed, Göggel et al., have shown *in vivo* and in a perfused lung model that platelet activating factor (PAF)-induced pulmonary oedema is partly mediated by local ceramide generation.[5] In this study, it was shown that PAF increased secretory sphingomyelinase (sSM) activity and thereby elevated lung ceramide content. This effect was completely abolished in acidic sphingomyelinase deficient mice and in these animals PAF-induced lung oedema was strongly reduced when compared to wild-type animals. Therefore, it can be concluded that the local production of ceramide by the action of sphingomyelinase can increase vascular permeability leading to tissue oedema. In light of a recent study by Doehner et al. that shows increased serum activity of sSM in congestive heart failure (CHF) patients [10], it is tempting to speculate that this increase possibly contributes to heart failure-associated pulmonary oedema.

NO release

The endothelium forms and releases mediators controlling vascular smooth muscle tone, among which formation of the relaxant factor NO by endothelial NO synthase (eNOS) may be the most important. Local formation of ceramide by neutral sphingomyelinase can cause endothelium-dependent vasorelaxation through endothelial NO production.[7] This activation of eNOS has been shown not to involve cytosolic Ca^{2+} elevation, but is probably mediated by translocation of eNOS from the plasma membrane caveolae to the perinuclear region. It can not be excluded that metabolites of ceramide cause these effects since also locally formed

S1P has been shown to activate eNOS to stimulate the endothelial NO formation.[4] For example, angiotensin II can induce a sphingosine kinase-dependent activation of eNOS in the endothelium which counteracts the contractile response to angiotensin II; interestingly, both the endothelial S1P formation and the direct contraction of the smooth muscle appear to occur via the same receptor subtype, i.e. the AT_1 receptor.[8] Therefore, a disturbed sphingolipid metabolism in the vascular wall could lead to a reduced NO bioavailability and endothelial dysfunction, and contribute to the development of vascular pathologies. Such mechanisms might also contribute to the association between serum sSM activity and peripheral vasodilator capacity in e.g. CHF, which is a state of endothelial dysfunction.[10]

Vascular inflammation

Recently, an association between serum sSM activity and cytokine activation, specifically with circulating levels tumour necrosis factor-α (TNF-α) and soluble TNF-α receptor 1 has been shown.[10] Several pro-inflammatory stimuli including cytokines such as interleukin-1β, lipopolysacharides and oxidative stress can increase serum activity of sSM. Thus, the endogenous formation of sphingomyelin metabolites in endothelial cells is part of the downstream signalling of TNF-α.[4] Upon stimulation of human endothelial cells with TNF-α, the activation of eNOS was preceded by the sequential activation of neutral sphingomyelinase-2 and sphingosine kinase-1 and, therefore, the generation of S1P. Sphingolipid metabolism-dependent production of NO was linked to inhibition of expression of E-selectin and the adhesion of dendritic cells to the endothelium stimulation by TNF-α. However, high concentrations of S1P may directly induce expression of VCAM-1 and E-selectin, thus the role of S1P in adhesion is complex and not yet fully understood.

Weibel-Palade bodies are granules stored in the endothelium that contain various procoagulant and pro-inflammatory substances. One of the effects of both locally formed S1P [6] and ceramide [3] is triggering exocytocis of Weibel-Palade bodies by the endothelium. These bodies release vasoactive substances in close proximity of the endothelial cell, resulting in the initiation of vascular thrombosis and inflammation. However, S1P can also activate eNOS which forms NO and in turn inhibits exocytosis of Weibel-Palade bodies. Although these data appear contradictory for S1P, the two-faced effect of S1P allows for a tight regulation of the release of Weibel-Palade bodies by sphingomyelin metabolites upon pro-inflammatory stimulation. Since formation of atherosclerotic lesions occurs through activation of cellular events that include monocyte adhesion to the endothelium and vascular inflammation, local formation of S1P may play an important role in the pathogenesis of atherosclerotic vascular disease.

Embryonic vascular maturation

Vascular maturation during embryonic blood vessel development involves cell-to-cell communication and interactions between endothelial and vascular smooth muscle cells to form a solid new vascular structure. In conditional mutant mice with a specific deletion of $S1P_1$ from endothelial cells endothelial tubes are formed, but they are incompletely covered by smooth muscle cells. This leads to embryonic haemorrage and interuterine death.[2] This indicates that the endothelial $S1P_1$ receptor required for vascular maturation. The origin of the S1P acting on the $S1P_1$ receptor in the endothelial tube has not been investigated but due to the absence of blood flow through those vessel precursors it could be hypothesized that the required S1P is produced locally by the endothelial cell itself.

The role of sphingomyelin metabolites during blood vessel development is not limited to vascular maturation as S1P can also upregulate expression of the proteolytic enzymes matrix metalloproteinases (MMP's).[9] MMP's are involved in degradation of the extracellular matrix and play critical roles in endothelial cell migration and matrix remodelling during angiogenesis and collateral growth. Therefore, S1P formed by sequential activation of sphingomyelinase, ceramidase and sphingosine kinase may also play an important role in endothelial cell invasion during blood vessel formation by regulating the expression of MMP's.

Conclusion

Taken together, endothelial cells express various enzymes involved in the sphingolipid metabolism and can, therefore, endogenously form sphingomyelin metabolites. As the endothelium is responsive to sphingomyelin metabolites, particularly due to expression of S1P receptors, sphingomyelin metabolites appear to be auto- and paracrine regulators of endothelial function. This may play a role during embryogenesis and also in pathological conditions involving endothelial dysfunction such as vascular inflammation and/or CHF.

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Summary

Summary

Chapter 1

In chapter 1, the current knowledge regarding the role of (local formation) of sphingomyelin metabolites in the vasculature is discussed. Research in the past two decades has identified the sphingomyelin metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) as important bioactive lipids that are involved in a variety of cellular processes. For instance, these metabolites play a crucial role in cell survival and cell growth. Interestingly, S1P promotes cell survival and has mitogenic effects in several cell types while ceramide and sphingosine, the precursors of S1P, are potent inducers of apoptosis. Because sphingolipids are present in relatively high levels in blood, they potentially can exert effects on the vasculature by interacting with cells in the vascular wall and extensive research has been performed by exogenous application of sphingolipids, trying to understand the vascular role of these blood-borne sphingolipids. However, most likely the fraction of free sphingolipids in blood is small because most of these lipids are sequestered in blood cells or lipoprotein particles. Endothelial and vascular smooth muscle cells express the enzymes involved in sphingolipid metabolism and can, therefore, produce these sphingomyelin metabolites themselves. Moreover, these cells are responsive to sphingomyelin metabolites, particularly due to expression of S1P receptors and, therefore, sphingomyelin metabolites appear to be auto- and paracrine regulators of endothelial and vascular smooth muscle function.

Several factors have been associated with activation of sphingolipid metabolizing enzymes and, therefore, these factors may make use of the sphingomyelin metabolites to exert their (vascular) effects. Regulation of vascular tone, angiogenesis and endothelial barrier function are important processes in blood vessels that may be regulated or affected by locally formed sphingomyelin metabolites and, therefore, they are the main focus in this thesis. The therapeutic potential of affecting the tight regulation of sphingolipid metabolism in the vasculature is substantial, but much additional research is required to further identify and characterize possible pharmacological targets.

Chapter 2

Sphingomyelin metabolites such as ceramide, sphingosine and S1P have vasoactive properties when applied exogenously, and besides their occurrence in blood, they can also be formed locally in the vascular wall itself in response to external stimuli. Chapter 2 describes our study that was performed to investigate whether vasoactive compounds such as angiotensin II modulate sphingolipid metabolism in the vascular wall and how this might contribute to the vascular responses.

In isolated rat carotid arteries we found that the contractile response to angiotensin II was enhanced after inhibition of sphingosine kinase, by the specific sphingosine kinase inhibitor dimethylsphingosine (DMS). Removal of the endothelial cell layer or the addition of the NO synthase inhibitor L-NNA resulted in a similar enhancement. Moreover, in the presence of L-NNA, DMS had no additional effect on the angiotensin II-mediated constriction. The contractile responses to K^+ and phenylephrine were not affected by DMS. Angiotensin II concentration-dependently induced formation of the vasorelaxant factor NO in an endothelial cell line, that could be inhibited by DMS and by an AT_1 but not an AT_2 receptor blocker. Using immunoblotting for phosphorylated (and thus activated) endothelial NO synthase and phosphorylated Akt, as well as direct measurements of intracellular Ca^{2+}, we demonstrated in endothelial cells that the sphingosine kinase-dependent endothelial NO synthase activation is mediated via both phosphatidylinositol 3-kinase / Akt and Ca^{2+}-dependent pathways.

Therefore, we conclude that angiotensin II induces a sphingosine kinase-dependent activation of endothelial NO synthase via the AT_1 receptor on the endothelium, that partially counteracts the contractile responses in isolated artery preparations. This pathway may be of importance under pathological circumstances with a reduced NO bioavailability, such as hypertension and atherosclerosis. Moreover, a disturbed sphingolipid metabolism in the vascular wall may lead to a reduced NO-bioavailability and endothelial dysfunction.

Chapter 3

The study described in chapter 2 indicated that angiotensin II induces local (i.e. endothelial) formation of sphingomyelin metabolites resulting in increased NO production, subsequently attenuating vascular constriction. Under these " normal " circumstances, vascular smooth muscle-dependent effects for angiotensin II are apparently not mediated via sphingosine kinase. Several vascular pathologies are characterized by endothelial dysfunction and in more severe stages, hypertrophy and / or hyperplasia of the smooth muscle cell layer may manifest. The increased growth stimulation by various compounds in blood, including angiotensin II, leads to vascular intimal thickening. Since sphingomyelin metabolites are involved in regulation of vascular tone and have growth modulating properties, we investigated in chapter 3 whether under growth-promoting conditions the contractile properties of angiotensin II are altered due to alterations in sphingolipid metabolism and / or signalling in vascular smooth muscle cells.

We used isolated rat carotid arteries and demonstrated that under growth promoting conditions (i.e. 24 h culture of the vessel segments in the presence of 20 % serum) sphingosine kinase inhibition by DMS attenuated the angiotensin II-induced vasoconstriction, whereas under normal circumstances DMS potentiated angiotensin II-induced

vasoconstriction (as also shown in chapter 2). Surprisingly, this inhibitory effect of DMS in cultured preparations proved to be endothelium-dependent since denudation of the arteries abolished this inhibitory effect. In order to investigate the role of sphingomyelin metabolites in smooth muscle growth we examined the effects of different sphingomyelin metabolites and DMS on the growth of cultured vascular smooth muscle cells. In cultured smooth muscle cells, S1P concentration-dependently increased 5-bromo-2'-deoxyuridine (BrdU) incorporation, whereas ceramide and DMS concentration-dependently reduced BrdU incorporation and induced apoptosis in these cells, thus nicely demonstrating the existence of the ceramide / S1P rheostat in vascular smooth muscle cells. DMS concentration-dependently inhibited basic fibroblast growth factor and angiotensin II-induced vascular smooth muscle cell proliferation. Thus the growth of vascular smooth muscle tissue can be modulated by exogenous and endogenously formed sphingomyelin metabolites. However, under both normal and growth promoting circumstances, angiotensin II selectively activates sphingosine kinase in the endothelium. Whereas inhibition of this activation results in a potentiation of the angiotensin II-induced contraction under normal conditions, this is inverted into an attenuation of angiotensin II-induced contraction under growth promoting conditions. In vascular pathologies characterized by vessel growth, changes in endothelial sphingomyelin metabolism may drastically influence angiotensin II-induced vascular contraction.

Chapter 4

In the previous chapters we described the role of the formation of sphingomyelin metabolites in the vascular endothelium for angiotensin II, a peptide that primarily induces contraction in the vascular smooth muscle tissue. In chapter 4, we investigated whether sphingosine kinase plays a role in muscarinic receptor-mediated vascular relaxation, since this is primarily an endothelium-dependent effect. Because it is known that the relative contribution of NO differs per vascular bed, we have studied the role of sphingosine kinase in muscarinic receptor-mediated relaxation in several vascular beds. In an endothelial cell line we show that activation of the muscarinic M_3-receptor leads to a concomitant translocation of YFP-tagged sphingosine kinase from the cytosol to the plasma membrane. Moreover, in this cell line inhibition of sphingosine kinase by DMS attenuated muscarinic receptor-mediated NO production. In isolated rat aorta preparations, the potency of the relaxant responses to the muscarinic receptor agonist methacholine was decreased in the presence of DMS. In contrast, DMS enhanced the relaxant responses to methacholine in mesenteric artery preparations. DMS did not affect the responses to the NO donor sodium nitroprusside in the different vascular preparations, indicating that activation of sphingosine kinase is upstream of NO. In mesenteric artery preparations, methacholine-induced endothelium-derived hyperpolarizing factor-mediated vasorelaxation was enhanced by DMS. In addition, DMS also potentiated the

dilatory actions of the putative endothelium-derived hyperpolarizing factor C-type natriuretic peptide.

Therefore, we conclude that stimulation of endothelial muscarinic receptors activates sphingosine kinase and this activation results in differential effects in different vessel types. Whereas this sphingosine kinase activity promotes vasodilation in rat aorta via an NO-dependent pathway, it may inhibit relaxation in mesenteric arteries by inhibition of endothelium-derived hyperpolarizing factor-mediated vasodilation. A disturbed regulation of sphingolipid metabolism in the vascular wall may, therefore, play a role in the aetiology and / or pathology of disease states characterized by endothelial dysfunction.

Chapter 5

In chapter 2 and 4, we have shown sphingolipid-dependent vasoactive effects of angiotensin II and methacholine, respectively. Interestingly, these sphingolipid-dependent effects, including those of angiotensin II, are mainly of endothelial origin. In addition we have shown (chapter 3) that growth-promoting conditions may alter the role of local sphingolipid metabolism. Little is known about the role of local formation of sphingomyelin metabolites in hypertension, a disease state characterized by endothelial dysfunction and vascular remodelling. The study described in this chapter was designed to investigate sphingomyelin metabolite-dependent alterations in vascular reactivity to angiotensin II and methacholine in spontaneously hypertensive rats (SHRs). Aorta, carotid artery and mesenteric artery vessel segments were isolated from 33-36 weeks old SHRs and Wistar Kyoto (WKY) rats for control.
In the WKY rats we found qualitatively similar response of DMS on methacholine-induced relaxation in the three vessel types as described in chapter 4. However, in the hypertensive animals DMS had less potent effects on methacholine- induced vasorelaxation.

Angiotensin II-induced constriction was enhanced by DMS in both WKY rats and SHRs, although the enhancement of E_{max} was stronger in SHR. Interestingly, in carotid artery segments of SHR, but not those obtained from WKY, DMS induced a spontaneous transient contraction. This DMS-induced contraction was fully prevented by endothelial denudation and by the cyclooxygenase inhibitor indomethacine.

We conclude that in SHR, sphingosine kinase-dependent stimulation of endothelial NO formation in the carotid artery by angiotensin II is more pronounced, while sphingosine kinase-dependent effects of muscarinic receptor stimulation in mesenteric artery are less pronounced. These phenomena are most likely due to altered activation or altered constitutive activity of sphingolipid metabolizing enzymes. Moreover, the generation of sphingomyelin metabolites may induce the release of an endothelium-dependent contractile

factor in hypertensive rats. Alterations in sphingolipid metabolism may thus contribute to a disturbed regulation of vascular tone during hypertension.

Chapter 6

In chapter 6 we provide some additional data about the effects and consequences of local sphingolipid metabolism induced by vasoactive compounds. We show for instance that also the contractile responses to endothelin-1 and the relaxant responses to histamine are enhanced and attenuated, respectively, by DMS. In addition, we show that modulation of endothelial sphingolipid metabolism by angiotensin II has important consequences for the growth-inducing effects of angiotensin II in these cells.

Chapter 7

The previous chapters unraveled the importance of especially endothelial sphingolipid metabolism. In this thesis we mainly focused on the vasoactive aspects of local sphingolipid metabolism, however, literature data suggests that endothelial formation of sphingomyelin metabolites also influences other processes important for vascular function. This chapter highlights some recent findings in this field.

Nederlandse samenvatting

Nederlandse samenvatting

Hoofdstuk 1

In hoofdstuk 1 wordt de huidige kennis over de rol van (lokale vorming) van sfingomyeline metabolieten in het vaatstelsel besproken. Gedurende de afgelopen twee decennia heeft onderzoek de sfingomyeline metabolieten ceramide, sfingosine en sfingosine-1-fosfaat (S1P) geïdentificeerd als belangrijke bioactieve lipiden die betrokken zijn bij verschillende cellulaire processen. Deze metabolieten spelen bijvoorbeeld een cruciale rol in celoverleving en celgroei. Interessant is dat S1P celoverleving en mitogene effecten stimuleert in verschillende celtypen, terwijl ceramide en sfingosine, de voorlopers van S1P, apoptose kunnen induceren. Omdat sfingolipiden in relatief hoge concentraties in bloed aanwezig zijn, kunnen ze vasculaire effecten teweeg brengen door een interactie aan te gaan met de cellen van de vaatwand en er is veel onderzoek gedaan naar deze effecten door middel van exogene applicatie van sfingolipiden. De hoeveelheid vrije sfingolipiden in bloed is waarschijnlijk echter klein, omdat de meeste lipiden zijn opgeslagen in bloedcellen of lipoproteïne deeltjes. Endotheelcellen en vasculaire gladde spiercellen brengen de enzymen tot expressie die betrokken zijn bij het sfingolipid metabolisme en kunnen dus deze sfingomyeline metabolieten ook zelf produceren. Bovendien zijn deze cellen gevoelig voor sfingomyeline metabolieten, mede omdat ze S1P receptoren tot expressie brengen. Daarom kunnen sfingomyeline metabolieten zowel op auto- als op paracrine wijze de functie van het endotheel en glad spierweefsel in bloedvaten beïnvloeden.

Verschillende factoren zijn geassocieerd met de activatie van sfingolipid metaboliserende enzymen en zij kunnen daarom gebruik maken van sfingomyeline metabolieten tijdens het teweeg brengen van hun vasculaire effecten. De regulatie van vasculaire tonus, angiogenese en de barrièrefunctie van het endotheel zijn belangrijke processen in bloedvaten die beïnvloed kunnen worden door lokaal gevormde sfingomyeline metabolieten en daarom vormen zij de focus van dit proefschrift. De enzymen betrokken bij de regulatie van het sfingolipid metabolisme vormen mogelijk aangrijpingspunten voor nieuwe geneesmiddelen ter behandeling van verschillende soorten cardiovasculaire aandoeningen.

Hoofdstuk 2

Sfingomyeline metabolieten zoals ceramide, sfingosine en S1P hebben vasoactieve eigenschappen als ze exogeen worden toegevoegd. Naast hun aanwezigheid in bloed kunnen ze ook lokaal gevormd worden in de vaatwand in respons op externe stimuli. Hoofdstuk 2 beschrijft de studie die we hebben uitgevoerd om te onderzoeken of vasoactieve stoffen zoals

angiotensine II het sfingolipid metabolisme in de vaatwand moduleren en hoe dit een bijdrage kan leveren aan de vasculaire respons.

In geïsoleerde carotis arteriën van de rat hebben we gevonden dat de contractiele respons op angiotensine II versterkt was als sfingosine kinase was geremd door de specifieke sfingosine kinase remmer dimethylsfingosine (DMS). Verwijderen van de endotheelcellaag of het toevoegen van de NO synthase remmer L-NNA resulteerde in een vergelijkbare versterking. Belangrijker nog, in de aanwezigheid van L-NNA had DMS geen additioneel effect op de angiotensine II-geïnduceerde vasoconstrictie. De contractiele responsen door K^+ en fenylefrine werden niet beïnvloed door DMS. Angiotensine II induceerde concentratie-afhankelijk de vorming van de vasorelaxerende factor NO in een endotheelcellijn, wat kon worden geremd door DMS en een AT_1, maar niet een AT_2 receptor blokker. Door middel van detectie van gefosforyleerd (en dus geactiveerd) endotheel NO synthase en gefosforyleerd Akt, evenals het direct meten van intracellulair Ca^{2+}, hebben we laten zien dat de sfingosine kinase-afhankelijke activatie van endotheel NO synthase tot stand komt door zowel fosfatidylinositol 3-kinase / Akt-, als Ca^{2+}-afhankelijke mechanismen.

Daarom concluderen we dat angiotensine II een sfingosine kinase-afhankelijke activatie van endotheel NO synthase induceert via de AT_1 receptor op het endotheel, wat deels de contractiele respons in geïsoleerde arterie preparaten tegenwerkt. Dit mechanisme kan belangrijk zijn onder pathologische omstandigheden met een verminderde biologische beschikbaarheid van NO, zoals hypertensie en atherosclerose. Ook zou een verstoord sfingolipid metabolisme in de vaatwand kunnen leiden tot een verminderde biologische beschikbaarheid van NO en endotheel dysfunctie.

Hoofdstuk 3

De studie beschreven in hoofdstuk 2 laat zien dat angiotensine II lokale (dat wil zeggen in het endotheel) vorming van sfingomyeline metabolieten induceert, wat resulteert in een verhoogde productie van NO en vervolgens een verminderde vasculaire constrictie. Onder deze "normale" omstandigheden zijn de effecten van angiotensine II op de gladde spiercellen niet afhankelijk van sfingosine kinase. Verschillende vasculaire pathologiën worden gekarakteriseerd door endotheel dysfunctie en in een verder gevorderd stadium kunnen hypertrofie en/of hyperplasie van gladde spiercellen zich manifesteren. De verhoogde stimulatie van groei door verschillende stoffen in het bloed, waaronder angiotensine II, leidt uiteindelijk tot verdikking van de intima laag in de vaatwand. Omdat sfingomyeline metabolieten betrokken zijn bij de regulatie van de vasculaire tonus en groei modulerende eigenschappen bezitten, hebben we in hoofdstuk 3 onderzocht of de contractiele eigenschappen van angiotensine II zijn veranderd onder groei bevorderende omstandigheden

door veranderingen in sfingolipid metabolisme en/of signaal transductie in vasculaire gladde spiercellen.

We hebben hiervoor gebruik gemaakt van geïsoleerde carotis arteriën van de rat waarin we hebben laten zien dat onder groei bevorderende omstandigheden (24 uur lang kweken van de bloedvatsegmenten in de aanwezigheid van 20 % foetaal kalfsserum) remming van sfingosine kinase door DMS de angiotensine II-geïnduceerde vasoconstrictie remt. Dit in tegenstelling tot niet gekweekte preparaten waar onder normale omstandigheden DMS de angiotensine II-geïnduceerde vasoconstrictie potentieert, zoals we ook hebben laten zien in hoofdstuk 2. Opvallend was dat het remmende effect van DMS in gekweekte bloedvaten endotheel-afhankelijk bleek te zijn, omdat dit niet meer optrad in vaten waarin het endotheel was verwijderd. Om de rol van sfingomyeline metabolieten in de groei van gladde spiercellen beter te begrijpen, hebben we de effecten van verschillende sfingomyeline metabolieten en DMS op de groei van gekweekte gladde spiercellen onderzocht. In gekweekte gladde spiercellen stimuleerde S1P concentratie-afhankelijk de incorporatie van 5-bromo-2'-deoxyuridine (BrdU), terwijl ceramide en DMS concentratie afhankelijk de incorporatie van BrdU verminderden en apoptose induceerden in deze cellen. Deze tegenstelling is een mooie demonstratie van de aanwezigheid van de ceramide / S1P rheostaat in vasculaire gladde spiercellen. DMS remde concentratie-afhankelijk de groei van gladde spiercellen geïnduceerd door basische fibroblast groeifactor en angiotensine II. De groei van glad spierweefsel kan dus worden gemoduleerd door exogene en endogeen gevormde sfingomyeline metabolieten. Onder zowel normale, als groei bevorderende omstandigheden, kan angiotensine II echter selectief sfingosine kinase activeren in het endotheel. Terwijl remming van deze activatie leidt tot een potentiëring van de angiotensine II-geïnduceerde constrictie onder normale omstandigheden, wordt dit omgekeerd naar een remming van de constrictie onder groei bevorderende omstandigheden. In vasculaire pathologiën die gekarakteriseerd worden door groei, kunnen veranderingen in endotheel sfingolipid metabolisme dus een drastisch effect hebben op de angiotensine II-geïnduceerde vasculaire contractie.

Hoofdstuk 4

In de voorgaande hoofdstukken hebben we de rol van de vorming van sfingomyeline metabolieten in het endotheel beschreven voor angiotensine II, een peptide dat primair vasoconstrictie induceert in het vasculaire gladde spierweefsel. In hoofdstuk 4 hebben we onderzocht of sfingosine kinase een rol speelt bij muscarine receptor-geïnduceerde vasorelaxatie, aangezien dit primair een endotheel-afhankelijk effect is. Het is bekend dat de bijdrage van NO in deze vasorelaxatie verschilt per vaatbed en daarom hebben we de rol van sfingosine kinase-gemedieerde vasorelaxatie in verschillende vaatbedden onderzocht. In een endotheelcellijn hebben we laten zien dat activatie van de muscarinerge M_3-receptor leidde

tot een translocatie van geel fluorescent eiwit-gelabeld sfingosine kinase van het cytosol naar het plasma membraan. Daarbij gaf remming van sfingosine kinase in deze cellijn een vermindering van de muscarine receptor-geïnduceerde productie van NO.

In geïsoleerde ratten aorta preparaten was de potentie van de relaxatie respons na toevoeging van de muscarine receptor agonist methacholine verlaagd in de aanwezigheid van DMS. Dit in tegenstelling tot de responsen in geïsoleerde mesentericum arterie preparaten, waar DMS de relaxatie op methacholine versterkte. DMS beïnvloedde niet de responsen op de NO donor natrium nitroprusside in de verschillende vasculaire preparaten, wat aangeeft dat activatie van sfingosine kinase plaatsvindt voor NO productie. In mesentericum arterie preparaten werd de methacholine-geïnduceerde endotheel-afhankelijke hyperpolarizerende factor-geïnduceerde vasorelaxatie versterkt door DMS. DMS potentiëerde daarnaast ook de dilaterende acties van de mogelijk betrokken endotheel-afhankelijke hyperpolarizerende factor C-type natriuretisch peptide.

Daarom concluderen wij dat stimulatie van endotheliale muscarine receptoren leidt tot activatie van sfingosine kinase en dat deze activatie resulteert in differentiële effecten in verschillende typen vaten. Terwijl deze sfingosine kinase activiteit vasodilatie in de ratten aorta bevordert op een NO-afhankelijke manier, lijkt het dat vasodilatie in mesentericum arteriën verminderd is door remming van de endotheel-afhankelijke hyperpolarizerende factor. Omdat in deze arteriën het NO systeem geen rol van betekenis speelt zal remming van sfingosine kinase een potentierend effect hebben op de methacholine-gestimuleerde vasorelaxatie. Een verstoorde regulatie van het sfingolipid metabolisme in de vaatwand kan, daarom, een rol spelen in de etiologie en/of pathologie van ziekte processen gekarakteriseerd door endotheel dysfunctie.

Hoofdstuk 5

In hoofdstuk 2 en 4 hebben we sfingolipid-afhankelijke vasoactieve effecten van, respectievelijk, angiotensine II en methacholine laten zien. Interessant is dat deze sfingolipid-afhankelijke effecten, inclusief die van angiotensine II, voornamelijk voortkomen uit het endotheel. Daarnaast hebben we laten zien (hoofdstuk 3) dat groei-bevorderende omstandigheden de rol van lokaal sfingolipid metabolisme kunnen veranderen. Er is weinig bekend over de rol van lokale vorming van sfingomyeline metabolieten in hypertensie, een ziekte gekarakteriseerd door endotheel dysfunctie en vasculaire remodelling. De studie beschreven in dit hoofdstuk is opgezet om sfingomyeline metaboliet-afhankelijke veranderingen in vasculaire reactiviteit op angiotensine II en methacholine in spontaan hypertensieve ratten (SHR) te onderzoeken. Aorta, carotis arterie en mesentericum arterie

segmenten werden geïsoleerd van 33 tot 36 weken oude SHR en Wistar Kyoto (WKY) ratten als controle.

In de WKY ratten vonden we kwalitatief vergelijkbare responsen van DMS op methacholine-geïnduceerde relaxatie in de drie typen vaten, zoals beschreven in hoofdstuk 4. Echter, in de hypertensieve dieren had DMS minder potente effecten op methacholine-geïnduceerde vasorelaxatie. Angiotensine II-geïnduceerde constrictie werd versterkt door DMS in zowel WKY ratten, als SHR, hoewel de versterking van de E_{max} hoger was in SHR. Interessant is dat in carotis arterie segmenten van SHR, maar niet die van WKY, DMS een spontane, transiënte contractie induceerde. Deze DMS-geïnduceerde contractie werd volledig voorkomen door endotheel denudatie of door toevoeging van de cyclo-oxygenase remmer indomethacine.

We concluderen dat in SHR de sfingosine kinase-afhankelijke stimulatie van NO vorming in het endotheel van de carotis arterie door angiotensine II vergroot is, terwijl sfingosine kinase-afhankelijke effecten van muscarine receptor stimulatie in de mesentericum arterie verkleind zijn. Deze fenomenen zijn waarschijnlijk te wijten aan veranderde activatie of veranderde constitutieve activiteit van sfingolipid metaboliserende enzymen. Daarbij zou de vorming van sfingomyeline metabolieten het vrijmaken van een endotheel-afhankelijke contractiele factor in hypertensieve ratten kunnen induceren. Veranderingen in sfingolipid metabolisme zouden dus kunnen bijdragen aan een verstoorde regulatie van de vasculaire tonus tijdens hypertensie.

Hoofdstuk 6

In hoofdstuk 6 presenteren we additionele data ten aanzien van de effecten en gevolgen van lokaal sfingolipid metabolisme geïnduceerd door vasoactieve stoffen. We laten bijvoorbeeld zien dat ook de contractiele responsen op endotheline-1 en de relaxerende responsen op histamine zijn versterkt en verminderd, respectievelijk, door DMS. We laten daarnaast zien dat modulatie van sfingolipid metabolisme in het endotheel door angiotensine II belangrijke gevolgen heeft voor de groei-inducerend effecten van angiotensine II in deze cellen.

Hoofdstuk 7

De voorgaande hoofdstukken hebben het belang van sfingolipid metabolisme in vooral het endotheel ontrafeld. In dit proefschrift hebben we overwegend de focus gelegd bij de vasoactieve aspecten van lokaal sfingolipid metabolisme, maar er zijn aanwijzingen in de literatuur dat vorming van sfingomyeline metabolieten in het endotheel ook andere processen kan beïnvloeden die belangrijk zijn voor vasculaire functie. Dit hoofdstuk belicht een aantal recente bevindingen hieromtrent.

Dankwoord

Dankwoord

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Curriculum Vitae and bibliography

Curriculum Vitae

Op 25 april 1980 werd Arthur Cornelis Maria Mulders geboren te Prinsenbeek. Hij heeft 1 broer en 1 zus. Na een fantastische jeugd met de grootst denkbare speeltuin aan huis begon hij op zijn 12e met de middelbare school op het Stedelijk Gymnasium in Breda. Na het behalen van zijn diploma ging hij in september 1998 naar Nijmegen om de studie Biomedische Gezondheidswetenschappen aan de toenmalige Katholieke Universiteit Nijmegen te volgen, waarvoor hij in 2002 zijn bul behaalde. In de tussentijd was hij lid geworden van de Nijmeegse Studenten Zeilvereniging De Loefbijter, wat een groot deel van zijn sociale leven zou gaan bepalen. Hij heeft in het bestuur gezeten van De Loefbijter, heeft verschillende commissietaken vervuld en is zeilinstructeur. In 2003 begon hij aan zijn AIO-tijd aan de afdeling Farmacologie en Farmacotherapie binnen het Academisch Medisch Centrum te Amsterdam onder leiding van Prof. Dr. Martin Michel. Hij heeft zijn werk, zoals beschreven in dit proefschrift, in verschillende internationale tijdschriften gepubliceerd en gepresenteerd op verscheidene nationale en internationale conferenties. Op dit moment studeert hij Geneeskunde aan de Radboud Universiteit in Nijmegen.

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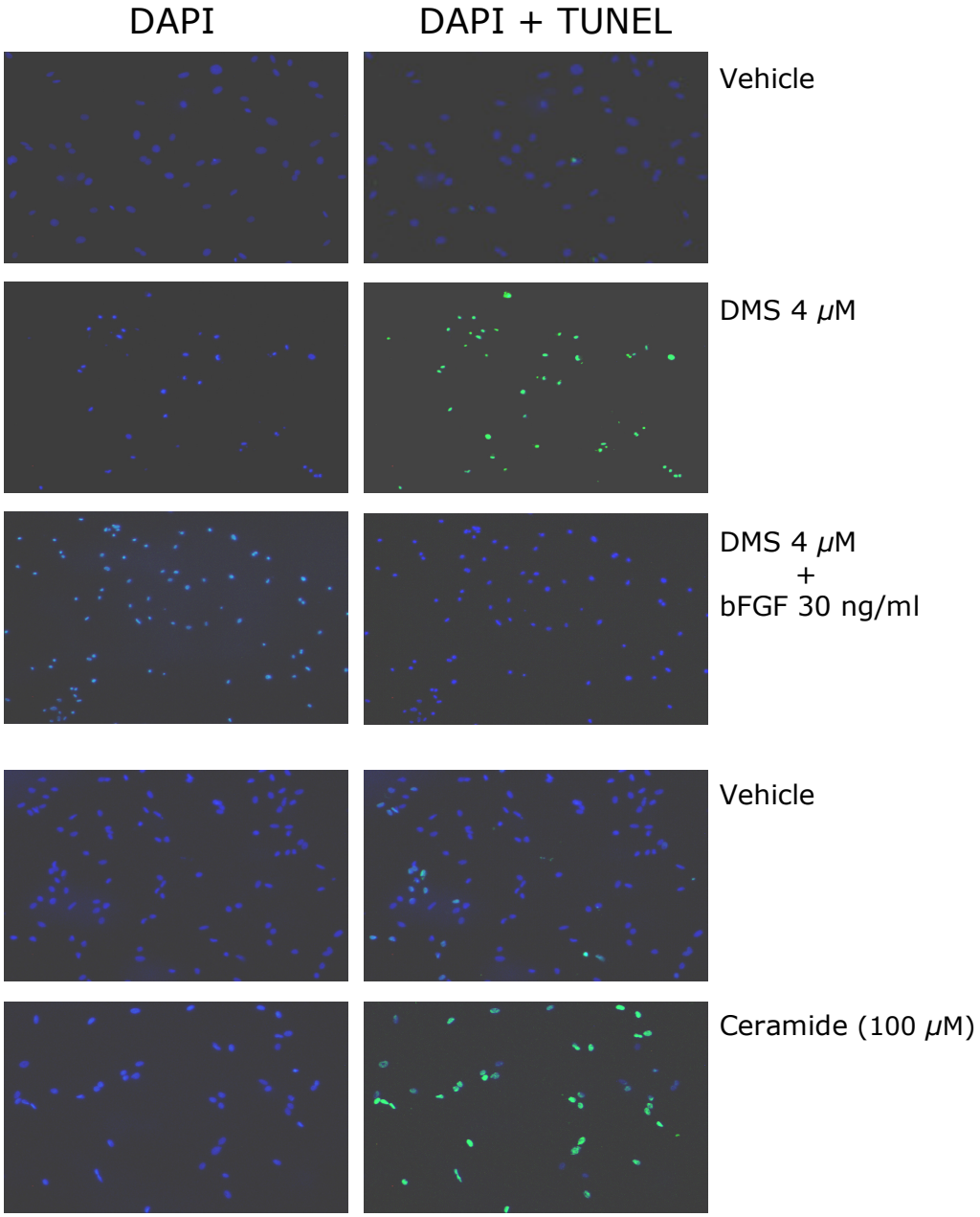
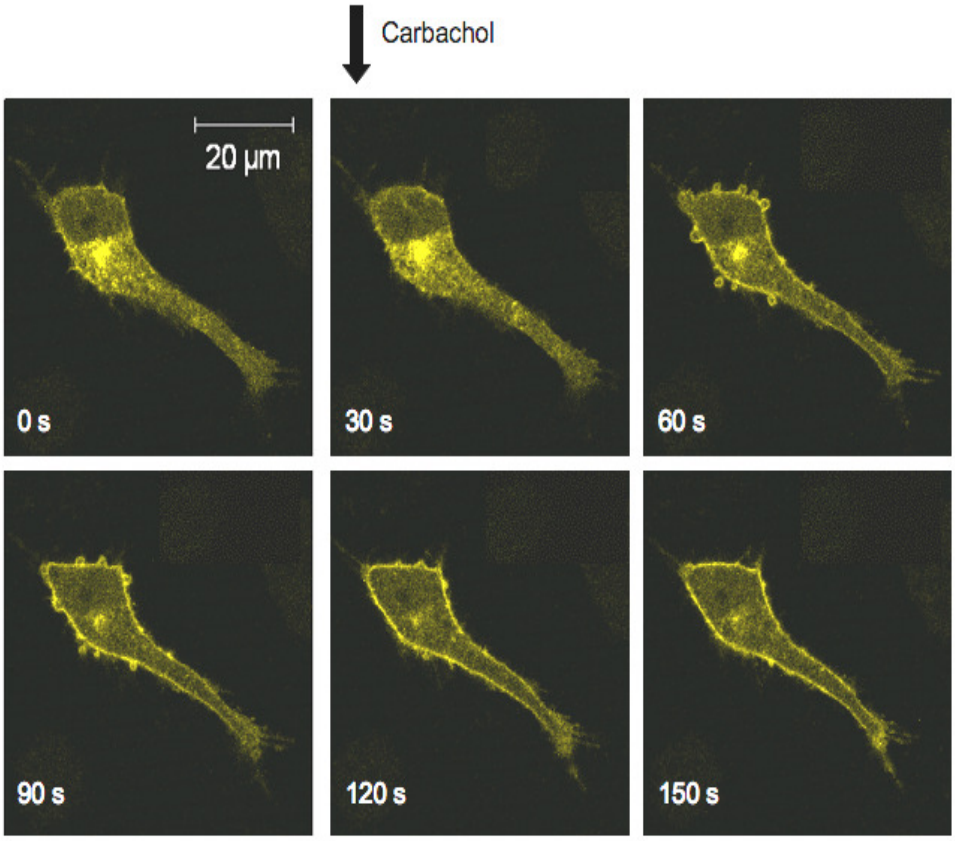
Appendices

Appendix I



Appendix I (chapter 3). Induction of apoptosis in VSMCs by ceramide and DMS. Cells cultured in chamber slides were treated for 48 h with DMS, ceramide or appropriate vehicle at the indicated concentrations. bFGF prevented DMS-induced apoptosis. Induction of apoptosis was visualized by TUNEL. Nuclei are visualized by blue stain (DAPI) and apoptotic nuclei stain green (TUNEL).

Appendix II



Appendix II (chapter 4). Translocation of sphingosine kinase-1 in bEnd.3 endothelial cells. Cells were transfected with the muscarinic M_3 receptor and YFP-tagged sphingosine kinase-1a. Upon muscarinic receptor stimulation with carbachol (100 μM) at t = 30 s, sphingosine kinase-1 translocates from the cytosol to the plasma membrane of the cell. Typical example of 3 experiments.

Stellingen

behorend bij het proefschrift:

Sphingolipid metabolism in vascular function

door Arthur C.M. Mulders.

1. Endogene vorming van sfingolipiden in het endotheel onder invloed van vasoactieve stoffen leidt tot zowel auto-, als paracrine effecten in de vaatwand (*dit proefschrift*).

2. Ook onder pathologische omstandigheden zijn de sfingosine kinase-afhankelijke effecten van angiotensine II op de vasculaire tonus waarschijnlijk voorbehouden aan het endotheel (*dit proefschrift*).

3. De met hypertensie-geassocieerde endotheel-afhankelijke vasoconstrictie zoals voor het eerst beschreven door Lüscher en Vanhoutte (Lüscher TF and Vanhoutte PM. Hypertension. 1986; 8:344-8) is gerelateerd aan een verandering van het sfingolipid metabolisme (*dit proefschrift*).

4. Er bestaat de mogelijkheid dat AT_1 blokkers niet alleen de negatieve effecten van angiotensine II tegengaan.

5. Alhoewel in plasma en serum hoge sfingosine-1-fosfaat concentraties gemeten kunnen worden, zal de hoeveelheid vrij S1P in het bloed veel lager zijn.

6. Er is maatschappelijk begrip nodig van het belang om een minimaal risico voor lief te nemen, om de steeds strengere regelgeving rondom innovatieve geneesmiddelen te nuanceren.

7. De vasculaire farmacotherapie van de toekomst ligt in het vet.

8. Promoveren is als aan de wind zeilen; altijd het compromis tussen afstand en snelheid.

9. Op de verkoop van treinkaartjes zou kansspelbelasting moeten worden geheven.

10. I am prepared to go anywhere, provided it be forward. *David Livingstone*

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