

## REVIEW

# Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites

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**1** Upon various stimuli, cells metabolize sphingomyelin from the cellular plasma membrane to form sphingosylphosphorylcholine (SPC) or ceramide. The latter can be further metabolized to sphingosine and then sphingosine-1-phosphate (S1P). Apart from local formation, S1P and SPC are major constituents of blood plasma.

**2** All four sphingomyelin metabolites (SMM) can act upon intracellular targets, and at least S1P and probably also SPC can additionally act upon G-protein-coupled receptors. While the molecular identity of the SPC receptors remains unclear, several subtypes of S1P receptors have been cloned and their distribution in cardiovascular tissues is described.

**3** In the heart SMM can alter intracellular Ca<sup>2+</sup> release, particularly *via* the ryanodine receptor, and conductance of various ion channels in the plasma membrane, particularly I<sub>K(Ach)</sub>. While the various SMM differ somewhat in their effects, the above alterations of ion homeostasis result in reduced cardiac function in most cases, and ceramide and/or sphingosine may be the mediators of the negative inotropic effects of tumour necrosis factor.

**4** In the vasculature, SMM mainly act as acute vasoconstrictors in most vessels, but ceramide can be a vasodilator. SMM-induced vasoconstriction involves mobilization of Ca<sup>2+</sup> from intracellular stores, influx of extracellular Ca<sup>2+</sup> *via* L-type channels and activation of a rho-kinase.

**5** Extended exposure to SMM, particularly S1P, promotes several stages of the angiogenic process like endothelial cell activation, migration, proliferation, tube formation and vascular maturation.

**6** We propose that SMM are an important class of endogenous modulators of cardiovascular function.

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**Keywords:** Sphingosine-1-phosphate; sphingosylphosphorylcholine; sphingosine; ceramide; heart; vasoconstriction; angiogenesis

**Abbreviations:** ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NOS, NO synthase; PI3K, phosphatidylinositol 3'-kinase; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; SMM, sphingomyelin metabolites; SPC, sphingosylphosphorylcholine; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor

## Introduction

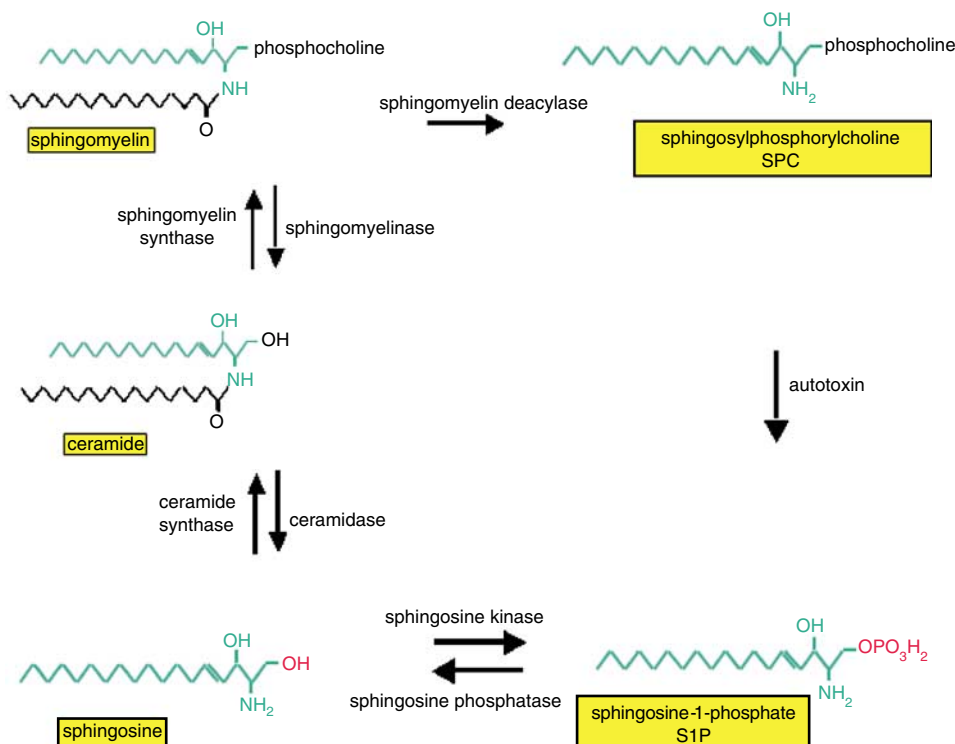
The biologically active sphingomyelin metabolites (SMM) sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC), sphingosine and ceramide are important lipid mediators which are generated upon cell activation from membrane phospholipids as part of the sphingomyelin cycle (Hannun *et al.*, 2001; Kolesnick, 2002). Various stressful stimuli activate sphingomyelinase, which catalyses the hydrolysis of sphingomyelin to ceramide, which can then be metabolized by ceramidase to sphingosine (Figure 1). Phosphorylation of sphingosine by sphingosine kinase, a ubiquitous enzyme in the cytosol and endoplasmic reticulum, finally yields S1P (Figure 1). Momentarily, less is known about the formation and degradation of SPC under physiological conditions (Meyer zu Heringdorf *et al.*, 2002). Under pathological

conditions, SPC was shown to be formed from sphingomyelin by the enzyme sphingomyelin deacylase (Higuchi *et al.*, 2000). Interestingly, it has been shown recently that SPC can be hydrolysed into S1P by autotoxin, an ectoenzyme that belongs to the nucleotide and pyrophosphatase and phosphodiesterase family (Clair *et al.*, 2003).

S1P is present in plasma and serum in high nanomolar concentrations. Recently, SPC was also detected in plasma and serum at concentrations similar to that of S1P (Liliom *et al.*, 2001). Both, S1P and SPC in plasma and serum are associated with lipoproteins, especially the high-density lipoprotein (HDL) (Okajima, 2002). The major source for S1P in plasma and serum are platelets. High concentrations of S1P are stored in platelets as these lack the enzyme S1P lyase, an enzyme that catalyses the degradation of S1P to hexadecanal and ethanolamine-phosphate (Yatomi *et al.*, 1997). The stored S1P is released upon activation of the platelets.

Many of the actions of S1P are shared by the structurally related SPC, and these two lipids may thus share common

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**Figure 1** Sphingomyelin metabolism.

mechanisms and sites of action (Meyer zu Heringdorf *et al.*, 2002). With regard to cellular signalling, S1P is most thoroughly investigated, whereas the exact targets of SPC remain largely unknown. S1P has unique properties as it can act both as an intracellular messenger and also as an intercellular messenger (Pyne & Pyne, 2000; Payne *et al.*, 2002; Spiegel & Milstien, 2003). To date, the intracellular effects of S1P are not that well understood, as the putative intracellular target(s) that mediates these effects has not been identified yet. However, evidence is increasing that some of the S1P effects cannot be explained by intracellular generated S1P acting extracellularly (Spiegel & Milstien, 2003).

The extracellular effects of S1P are much easier to investigate as these are mediated *via* its interaction with G-protein-coupled receptors. To date, a family of five structurally related receptors with high affinity for S1P has been identified. Recently, Uhlenbrock *et al.* (2002) reported the identification of three additional members of the S1P receptor family, gpr3, gpr6 and gpr12. Very recently, S1P was also found to be a low-affinity agonist for the GPR63 receptor (Niedernberg *et al.*, 2003), and the existence of even more S1P receptors cannot be excluded, particularly since not all S1P effects which apparently are receptor-mediated can be explained based on the presently identified receptor subtypes. It has been speculated that specific G-protein-coupled receptors also exist for SPC (Xu *et al.*, 2000), but recent evidence could not confirm the cloned protein to be an SPC receptor (Bektas *et al.*, 2003). However, SPC can act as a low-affinity agonist at S1P receptors (Pyne & Pyne, 2000; Spiegel & Milstien, 2000). Ceramide, one of the precursors of S1P, is also an important signalling molecule in pathways that are mediated by diverse stress stimuli. The mechanisms how

ceramide functions in the stress signal cascade still need to be elucidated.

Studying the functional effects and signal transduction mechanisms of SMM is rather complex as the various SMM can be converted into each other. The functional effect found for a particular SMM may, in some cases, not be the effect of the actual compound itself, but of one of its metabolites. For example, the functional effects found for sphingosine are most likely explained by its conversion either to ceramide or to S1P. Thus, it has been reported that the administered sphingosine is rapidly converted into S1P by sphingosine kinase upon intracoronary administration in rat hearts perfused in Langendorff mode (Cavalli *et al.*, 2002).

### Signalling characteristics of SMM and its receptors

The first S1P receptor identified was the S1P<sub>1</sub> receptor. The receptor was initially named endothelium differentiation gene (Edg) 1 receptor, because the corresponding gene is abundantly expressed in differentiating endothelial cells (Hla & Maciag, 1990b). According to the official IUPHAR nomenclature, Edg receptors were renamed as S1P receptors (Chun *et al.*, 2002). Rapidly after cloning of the S1P<sub>1</sub> receptor the S1P<sub>2</sub> (formerly Edg-5), S1P<sub>3</sub> (formerly Edg-3), S1P<sub>4</sub> (formerly Edg-6) and S1P<sub>5</sub> (formerly Edg-8) receptors have been cloned (Pyne & Pyne, 2000).

The S1P receptors, being part of the G-protein-coupled receptor family, transduce their signals *via* heterotrimeric G-proteins. As S1P receptor subtypes show subtype-specific G-protein coupling, the set of intracellular signalling pathways

activated by a particular SIP receptor is unique (for a review, see Takuwa *et al.*, 2001). The signal transduction mechanism of SIP receptors will only be discussed here shortly, as this has been the subject of several recent comprehensive reviews (Takuwa *et al.*, 2001; English *et al.*, 2002; Spiegel & Milstien, 2002).

The SIP<sub>1</sub> receptor is coupled exclusively *via* G<sub>i/o</sub>, leading to an inhibition of adenylyl cyclase and activation of Ras and extracellular signal-regulated kinases (ERKs) of the mitogen-activated protein kinase (MAPK) family. Stimulation of SIP<sub>1</sub> receptors also results in activation of phospholipase C, probably through the  $\beta\gamma$  subunit of G<sub>i/o</sub>. Like the SIP<sub>1</sub> receptor, the SIP<sub>2</sub> and the SIP<sub>3</sub> receptors are also coupled to Ras/ERK *via* G<sub>i/o</sub>. These receptors are, like the SIP<sub>1</sub> receptor, also found to activate phospholipase C and Ca<sup>2+</sup> mobilization. In contrast to the SIP<sub>1</sub> receptors, phospholipase C activation and Ca<sup>2+</sup> mobilization by these receptors is *via* both, pertussis toxin (PTX)-insensitive and PTX-sensitive G-proteins, most likely G<sub>q/11</sub> and G<sub>i/o</sub>, respectively. In addition, the SIP<sub>2</sub> and SIP<sub>3</sub> receptors are coupled to stimulation of Rho *via* G<sub>12/13</sub>. For the SIP<sub>2</sub> receptor, the most efficient coupling is *via* G<sub>q/11</sub> to phospholipase C and *via* G<sub>12/13</sub> to Rho. The SIP<sub>2</sub> receptor is also coupled to p38 MAPK and c-Jun N-terminal protein kinase *via* a PTX-insensitive G-protein (Gonda *et al.*, 1999).

Signal transduction pathways of the SIP<sub>4</sub> and SIP<sub>5</sub> receptor are not that well defined yet. SIP<sub>4</sub> is reported to couple to G<sub>i/o</sub>, leading to stimulation of phospholipase C and ERK in a PTX-sensitive manner. The SIP<sub>5</sub> receptor was shown to couple to G<sub>i</sub> and G<sub>12</sub> and to inhibit cAMP production in a PTX-sensitive manner. This receptor, however, did not activate ERK, but rather inhibited the serum-induced ERK activation. The mechanism by which SIP<sub>5</sub> mediates this effect is not understood yet (Takuwa, 2002).

The signal transduction pathways mediated by the recently identified gpr3, gpr6, gpr12 and GPR63 have not been extensively studied to date. In transiently transfected HEK-293 cells, the gpr3, gpr6 and gpr12 receptors were found to constitutively activate adenylyl cyclase (Uhlenbrock *et al.*, 2002). As PTX affected the basal and SIP-induced cAMP levels in HEK-293 cells transiently transfected with the gpr3, gpr6 or gpr12 receptor, the authors concluded that these receptors also constitutively couple to G<sub>i</sub>. The authors, however, also showed that untransfected HEK-293 cells already express a wide variety of other SIP receptor subtypes. Thus, the effect of PTX found could also originate from uncoupling of background SIP receptors as all SIP receptor subtypes are known to couple to G<sub>i</sub>-proteins. This explanation could be excluded in case PTX would not affect the basal cAMP production in mock-transfected cells, but unfortunately this has not been tested within the above study. Strong evidence for constitutive G<sub>i</sub> coupling of the gpr3, gpr6 and gpr12 receptors, for example, by reconstitution experiments in Sf9 cells, is thus still lacking.

SIP is a low-affinity agonist for the GPR63 receptor. The receptor can thus not really be classified as a SIP receptor, as the existence of another endogenous ligand with higher affinity cannot be excluded. The GPR63 receptor is shown to couple to G<sub>q</sub> and G<sub>i</sub> proteins (Niedernberg *et al.*, 2003).

As described above, besides its low affinity for SIP receptors, the target for SPC is not known. It is speculated that SPC also acts *via* a G-protein-coupled receptor based

upon findings, such as stereo-selectivity and PTX-sensitivity, of some of its effects, but the receptor mediating this has not been identified yet (Meyer zu Heringdorf *et al.*, 2002). Although the signal transduction mechanisms for ceramide are also not that clear, it is known that this lipid does not act *via* G-protein-coupled receptors. A number of enzymes can be activated by ceramide, including both protein kinases, such as protein kinase C  $\zeta$  and c-Jun N-terminal protein kinases, and protein phosphatases such as protein phosphatase 1 (Ruvolo, 2003). The mechanisms by which ceramide directly activates these enzymes still need to be elucidated.

Various studies in the heart or in cardiac myocytes have indicated that SMM can also regulate the activity of various ion channels, including L-type Ca<sup>2+</sup> channels, K<sup>+</sup> channels and Na<sup>+</sup> channels. The effects of SMM on ion channels are rather complex and will be discussed in more detail in the section describing the cardiac effects of SMM.

### Localization of SIP receptor subtypes in the cardiovascular system

To date the SIP receptor subtypes that are found to play a functional role in the cardiovascular system are the SIP<sub>1</sub>, SIP<sub>2</sub> and SIP<sub>3</sub> receptor. A summary of the expression of SIP receptor subtypes at the mRNA and protein level in various cell types of the cardiovascular system is presented in Table 1. Tissue expression analysis of SIP receptor subtypes in mice indicates that the heart and lung have the highest overall expression of SIP<sub>1</sub>, SIP<sub>2</sub> and SIP<sub>3</sub> mRNA transcripts (Zhang *et al.*, 1999; Ishii *et al.*, 2001). Expression of the SIP<sub>4</sub> receptor is mainly limited to lymphoid and haematopoietic tissues as well as the lung (Graler *et al.*, 1998), and transcripts for this receptor were not detected in the heart. Overall expression of the SIP<sub>5</sub> receptor is also limited and is highest in the brain, particularly in the midbrain, hindbrain and spinal cord (Im *et al.*, 2000).

Cardiac expression of SIP receptors has been studied in mice, rats and humans. The murine heart expresses transcripts for the SIP<sub>1</sub>, SIP<sub>2</sub> and SIP<sub>3</sub> but not the SIP<sub>4</sub> and SIP<sub>5</sub> receptor (Ishii *et al.*, 2001). While SIP<sub>1</sub>, SIP<sub>2</sub> and SIP<sub>3</sub> receptor mRNA expression also dominates in rat heart (Nakajima *et al.*, 2000; Liliom *et al.*, 2001), RT-PCR analysis additionally detected the presence of transcripts for the SIP<sub>5</sub> (but not SIP<sub>4</sub>) receptor in rat atrial and ventricular myocytes (Liliom *et al.*, 2001). When assessing these potential species differences it should be noted that the experiments in the mouse heart were performed on total RNA isolated from cardiac tissue whereas those in rats were done with RNA specifically isolated from cardiomyocytes. If overall expression of the SIP<sub>5</sub> receptor in the heart is low and restricted to certain areas, its signal might evade detection in whole tissue lysates. The localization of SIP<sub>1</sub>, SIP<sub>2</sub> and SIP<sub>3</sub> receptors within the human heart was analyzed using *in situ* hybridization and immunohistochemical techniques (Mazurais *et al.*, 2002). While expression of SIP<sub>2</sub> and SIP<sub>3</sub> receptors was lower than that of SIP<sub>1</sub> receptors, all three subtypes exhibited a similar distribution pattern. Thus, they were present in left and right ventricle, septum and atrium. Himmel *et al.* (2000) had also detected transcripts for these three subtypes of SIP receptors in human atrial cardiomyocytes.

**Table 1** Detection of S1P receptor subtypes in cardiovascular tissue at the mRNA and protein level

Receptor	S1P <sub>1</sub>		S1P <sub>2</sub>		S1P <sub>3</sub>		S1P <sub>4</sub>		S1P <sub>5</sub>	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Ventricular cardiomyocytes	+++ <sup>a</sup> , ++ <sup>b</sup>	+++ <sup>a</sup>	+ <sup>a</sup> , ++ <sup>b</sup>	+ <sup>a</sup>	++ <sup>a,b</sup>	+++ <sup>a</sup>	— <sup>b</sup>			++ <sup>b</sup>
Septal cardiomyocytes	+++ <sup>a</sup>	+++ <sup>a</sup>	+ <sup>a</sup>	+ <sup>a</sup>	+++ <sup>a</sup>	+++ <sup>a</sup>	— <sup>b</sup>			++ <sup>b</sup>
Atrial cardiomyocytes	+++ <sup>a</sup> , ++ <sup>b,c</sup>	+++ <sup>a</sup>	+ <sup>a</sup> , ++ <sup>b,c</sup>	+ <sup>a</sup>	++ <sup>a,b,c</sup>	+++ <sup>a</sup>	— <sup>b</sup>			++ <sup>b</sup>
Coronary artery endothelial cells	+++ <sup>a</sup>	+++ <sup>a</sup>	— <sup>a</sup>		— <sup>a</sup>					
Coronary artery smooth muscle cells	— <sup>a</sup>	— <sup>a</sup> , + <sup>d</sup>	— <sup>a</sup>	— <sup>a</sup>	+++ <sup>a</sup>	+++ <sup>a</sup>				
Aortic endothelial cells	— <sup>a</sup> , +++ <sup>e,f</sup>		— <sup>a,c</sup> , + <sup>f</sup>		— <sup>a</sup> , +++ <sup>e</sup>					
Aortic smooth muscle cells	— <sup>a,g</sup> , + <sup>h,i,k,l</sup>	— <sup>a</sup> , + <sup>d</sup>	+ <sup>a</sup> , +++ <sup>g,h,i</sup>	+ <sup>a</sup> , ++ <sup>d</sup>	+++ <sup>a,h,i</sup> , ++ <sup>g</sup>	+++ <sup>a</sup> , ++ <sup>d</sup>	— <sup>g,j</sup>			— <sup>g,j</sup>
Umbilical vein endothelial cells	+++ <sup>f,g</sup>		— <sup>g</sup> , + <sup>f</sup>		++ <sup>f</sup>		— <sup>g</sup>			— <sup>g</sup>

+++ : high expression; ++ : intermediate expression; + : low expression; — : not detected.

<sup>a</sup>Mazurais *et al.* (2002). <sup>b</sup>Liliom *et al.* (2001). <sup>c</sup>Himmel *et al.* (2000). <sup>d</sup>Coussin *et al.* (2002). <sup>e</sup>Kimura *et al.* (2000). <sup>f</sup>Wang *et al.* (1999). <sup>g</sup>Ryu *et al.* (2002). <sup>h</sup>Tamama *et al.* (2001). <sup>i</sup>Hobson *et al.* (2001). <sup>j</sup>Kluk *et al.* (2001). <sup>k</sup>Allende & Proia (2002). <sup>l</sup>Liu *et al.* (2000).

The S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> receptor are also expressed in human aorta as indicated by Northern and Western blot analysis (Mazurais *et al.*, 2002). The S1P<sub>1</sub> transcript is strongly and equally expressed in the aorta as in different compartments of the adult human heart. The S1P<sub>2</sub> receptor is also equally expressed in the aorta and in the different regions of the human heart although the abundance of this transcript is generally lower than that of the S1P<sub>1</sub> transcript. In line with these findings the S1P<sub>2</sub> receptor protein was also equally present in the aorta and the human heart (Mazurais *et al.*, 2002). The mRNA for the S1P<sub>3</sub> receptor, in contrast, was more abundant in the aorta than in the human heart regions. In agreement with these results the protein expression of this receptor was also higher in the aorta than in the heart (Mazurais *et al.*, 2002). RT-PCR detection studies in rat showed, besides the transcripts for the S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> receptor, also transcripts for the S1P<sub>5</sub> receptor (Salomone *et al.*, 2003).

More precise studies on the localization of S1P receptors in aorta were carried out using immunohistochemical and other techniques in aortic smooth muscle and endothelial cells. The data on the expression profile of S1P receptors in aortic smooth muscle cells are not conclusive as some groups report the expression of S1P<sub>1</sub> (Coussin *et al.*, 2000; Liu *et al.*, 2000; Kluk & Hla, 2001; Tamama *et al.*, 2001), whereas others did not detect its expression in these cells (Mazurais *et al.*, 2002; Ryu *et al.*, 2002). The expression profile in a particular cell type might also reflect particular ages of the animals at the time of the smooth muscle cell isolation, as indicated by a study of Kluk & Hla (2001), who showed a higher S1P<sub>1</sub> expression level in vascular smooth muscle cells isolated from rat pups than in adult medial smooth muscle cells.

In general, it can be concluded from all reports that the S1P<sub>2</sub> receptor and the S1P<sub>3</sub> receptor are the receptors that are expressed most abundantly in aortic vascular smooth muscle cells (Table 1). The S1P<sub>1</sub> receptor is probably also expressed in these cells, but at much lower levels than the other two subtypes. In aortic endothelial cells the expression pattern of S1P receptors is completely different. In these cells, the S1P<sub>1</sub> receptor is the most prominently expressed receptor (Wang *et al.*, 1999; Kimura *et al.*, 2000). Expression of the S1P<sub>2</sub> receptor in these cells is generally reported to be much lower than that of the S1P<sub>1</sub> receptor (Wang *et al.*, 1999), and in some studies the receptor is even undetectable (Kimura *et al.*, 2000; Mazurais *et al.*, 2002). Aortic endothelial cells in addition also express low levels of S1P<sub>3</sub> receptors (Wang *et al.*, 1999; Kimura *et al.*, 2000).

Interestingly, the expression pattern of S1P receptors in the aorta does not necessarily reflect the expression pattern found in other vessels since, in contrast to many other blood vessels, aorta does not contract in response to S1P (see below). The expression profile of the different S1P receptor subtypes may thus vary among different arteries. RT-PCR studies in rat, using RNA isolated from different arteries, however, did not indicate any difference in expression profile between different arteries (Salomone *et al.*, 2003). Basilar, carotid, femoral, coronary arteries and aorta all contained mRNA for the S1P<sub>1</sub>, S1P<sub>2</sub>, S1P<sub>3</sub> and S1P<sub>5</sub> receptors (Salomone *et al.*, 2003). In contrast to these results, no S1P<sub>2</sub> receptor expression was detected in human cardiac vessels, although not as extensively studied as the aorta, whereas this receptor was highly expressed in aortic smooth muscle cells of different species (Coussin *et al.*, 2000; Liu *et al.*, 2000; Hobson *et al.*, 2001; Kluk & Hla, 2001; Tamama *et al.*, 2001). Human coronary

smooth muscle cells were only shown to express S1P<sub>3</sub> receptors (Mazurais *et al.*, 2002). In line with the findings in aortic endothelial cells, cardiac endothelial cells also express high levels of the S1P<sub>1</sub> receptor (Mazurais *et al.*, 2002). Transcripts for the S1P<sub>2</sub> and S1P<sub>3</sub> receptor were not detected in these cells (Mazurais *et al.*, 2002).

High expression levels of S1P<sub>1</sub> receptors seem to be a general characteristic of endothelial cells. Besides aortic and cardiac endothelial cells, human umbilical vein endothelial cells also predominantly express the S1P<sub>1</sub> receptor (Wang *et al.*, 1999; Ryu *et al.*, 2002). Expression of the S1P<sub>2</sub> receptor in endothelial cells is generally low, if present at all. Interestingly, in smooth muscle cells the S1P expression profile seems to be just the opposite (see above).

As discussed earlier, a few years ago, three potential additional members of the S1P receptor family, *gpr3*, *gpr6* and *gpr12*, have been identified (Uhlenbrock *et al.*, 2002). Information about the precise distribution of these receptors in the cardiovascular system is lacking. However, it was shown by Uhlenbrock *et al.* (2003) using RT-PCR analysis that most primary human endothelial cells and most vascular smooth muscle cells are positive for *gpr3*, *gpr6* and *gpr12* transcripts.

## Acute effects of sphingolipids in the cardiovascular system

### Cardiac effects

SMM effects on cardiac function have been determined at the levels of cell signalling, isolated tissue function and *in vivo*. Not unexpectedly, ceramide, sphingosine, S1P and SPC have at least partly yielded different effects at each of these levels. Therefore, we will discuss the levels of cardiac SMM effects in this order with separate descriptions for each of the SMM.

Several studies have addressed SMM effects on cardiac function at the signal transduction level. Although inhibition of adenylyl cyclase is a prototypical signal transduction mechanism of SMM receptors (see above), such inhibition has not consistently been found in the heart. Sphingosine reduced basal and isoprenaline-stimulated cAMP levels in neonatal rat cardiomyocytes, but failed to inhibit forskolin-stimulated values (Benediktsdottir *et al.*, 2002). Studies in membrane preparations from adult rat right atrium or left ventricle (Sugiyama *et al.*, 2000a) or dog right atrium or right ventricle (Sugiyama *et al.*, 2000b) did not detect inhibition of adenylyl cyclase by S1P. Although inhibition of adenylyl cyclase can be difficult to measure and hence false-negative results cannot be excluded in the absence of a positive control, the above data certainly do not support the idea that SMM primarily affect cardiac function by modulating intracellular cAMP levels.

Numerous studies have indicated that SMM can alter intracellular ion concentrations in the heart by regulating the activity of various ion channels in the membranes of the sarcoplasmic reticulum and/or in the cell membrane. However, the net effect may differ among the SMM. Thus, sphingosine was consistently found to reduce free intracellular Ca<sup>2+</sup> concentrations in neonatal rat (McDonough *et al.*, 1994), adult rat (McDonough *et al.*, 1994; Amadou *et al.*, 2002), guinea-pig (Sugishita *et al.*, 1999) and feline cardiomyocytes (Oral *et al.*, 1997). Moreover, sphingosine appears to be the mediator of tumour necrosis factor (TNF)- $\alpha$ -induced lowering

of cardiac Ca<sup>2+</sup> concentrations (Oral *et al.*, 1997; Sugishita *et al.*, 1999; Amadou *et al.*, 2002). Other SMM have not been systematically studied in this regard, but it has been reported that the sphingosine precursor ceramide and the sphingosine metabolite S1P can increase free intracellular Ca<sup>2+</sup> concentrations in cardiomyocytes from adult (Liu & Kennedy, 2003; Relling *et al.*, 2003) and neonatal rats (Nakajima *et al.*, 2000).

The observed SMM-induced alterations in free intracellular Ca<sup>2+</sup> concentration could result from alterations in intracellular Ca<sup>2+</sup> handling and/or from alterations in the influx of extracellular Ca<sup>2+</sup>. With regard to the former, the ryanodine receptor is an important mediator of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum. Caffeine and doxorubicin enhance high-affinity [<sup>3</sup>H]ryanodine binding to isolated sarcoplasmic reticulum membranes from the canine heart and promote Ca<sup>2+</sup> release from this organelle; presumably this involves ryanodine receptors. Both effects were concentration-dependently inhibited by sphingosine (Dettbarn *et al.*, 1994). Similar observations were also made in sarcoplasmic reticulum from rabbit heart (Webster *et al.*, 1994). These observations are well in line with the consistently reported lowering of cardiac Ca<sup>2+</sup> concentration upon sphingosine administration. With regard to SPC effects on the ryanodine receptor, conflicting data have been reported. Thus, it was reported that SPC can strongly induce Ca<sup>2+</sup> release from the sarcoplasmic reticulum of the canine heart *via* the ryanodine receptor, possibly due to an SPC-induced increase in the Ca<sup>2+</sup>-sensitivity of the ryanodine receptor (Betto *et al.*, 1997). Interestingly, sphingosine was found to inhibit this SPC effect (Betto *et al.*, 1997). On the other hand, studies with incorporation of rabbit sarcoplasmic reticulum membranes into lipid bilayers have found that SPC reduces the open probability of ryanodine receptor following cytoplasmic but not luminal application (Uehara *et al.*, 1999; Yasukochi *et al.*, 2003). Whether these are true species differences, remains to be determined. Moreover, it remains unclear how these alterations in ryanodine receptor activity relate to overall SPC-induced changes in free intracellular Ca<sup>2+</sup> concentrations. Whether ceramide or S1P affects the cardiac ryanodine receptor has not yet been reported.

Other studies have looked at SMM effects upon ion channel activity in the plasma membrane. Sphingosine-induced inhibition of L-type Ca<sup>2+</sup> channels have been reported in cardiomyocytes from neonatal rats (McDonough *et al.*, 1994), adult rats (McDonough *et al.*, 1994; Yasui & Palade, 1996) and cats (Friedrichs *et al.*, 2002). The latter was accompanied by decreases of action potential duration and a lower action potential height (Friedrichs *et al.*, 2002). A reduced L-type Ca<sup>2+</sup> channel activity was also reported in rat cardiomyocytes for SPC (Yasui & Palade, 1996) and ceramide (Liu & Kennedy, 2003). In rabbit isolated sinoatrial node cells, S1P did not affect the basal activity of L-type Ca<sup>2+</sup> channels but reversed the effects of the  $\beta$ -adrenergic agonist isoprenaline (Guo *et al.*, 1999). Thus, it appears that all SMM exert some inhibitory effect upon cardiac L-type Ca<sup>2+</sup> channels. Moreover, sphingosine, SPC and S1P were reported to inhibit rat cardiac Na<sup>+</sup> channels (Yasui & Palade, 1996; MacDonell *et al.*, 1998). Interestingly, the S1P effects on the latter channel were not PTX-sensitive (MacDonell *et al.*, 1998).

Most studies have investigated SMM effects on cardiac K<sup>+</sup> channels. They have consistently found that S1P activates

$I_{K(ACh)}$  in guinea-pig atrial myocytes (Bünemann *et al.*, 1995; 1996; van Koppen *et al.*, 1996; Himmel *et al.*, 2000; Liliom *et al.*, 2001). A similar S1P-induced activation of  $I_{K(ACh)}$  was seen in freshly isolated myocytes from mouse and human atrium (Himmel *et al.*, 2000) and in rabbit isolated sinoatrial node cells (Guo *et al.*, 1999). The S1P effects in the guinea-pig atrium were inhibited by pre-treatment with PTX (Bünemann *et al.*, 1995) or with suramin (Himmel *et al.*, 2000). SPC was found to mimic the S1P effects in guinea-pig atrial myocytes, and the two SMM caused cross-desensitization of each other (Bünemann *et al.*, 1996; Liliom *et al.*, 2001). Since these studies observed no cross-desensitization with acetylcholine acting on muscarinic receptors, it was proposed that S1P and SPC may act on the same receptor. It was further proposed that S1P and SPC account for the plasma-induced activation of  $I_{K(ACh)}$  in guinea-pig atrial myocytes (Liliom *et al.*, 2001). On the other hand, in contrast to S1P, SPC did not activate  $I_{K(ACh)}$  in freshly isolated myocytes from mouse and human atrium (Himmel *et al.*, 2000). In rabbit isolated sinoatrial node cells, S1P did not alter basal activity of the  $I_f$  channel but reversed the isoprenaline effects (Guo *et al.*, 1999). In rat ventricular myocardium, S1P did not significantly affect the inward rectifier  $K^+$  channel (MacDonell *et al.*, 1998).

Taken together, sphingosine inhibits both  $Ca^{2+}$  release from the sarcoplasmic reticulum *via* ryanodine receptor channels and  $Ca^{2+}$  influx *via* L-type channels, and hence lowers free intracellular  $Ca^{2+}$  concentrations; its effects upon  $Na^+$  and  $K^+$  channels have not been established (Table 2). S1P inhibits  $I_{K(ACh)}$  and, perhaps,  $I_f$   $K^+$  channels,  $Na^+$  channels and L-type  $Ca^{2+}$  channels, but its effect upon ryanodine receptors is unknown. SPC mimics S1P effects upon  $I_{K(ACh)}$  in guinea-pigs (possibly even acting *via* the same receptor) but not in mice or humans; SPC possibly shares inhibitory S1P effects upon  $Na^+$  channels and L-type  $Ca^{2+}$  channels, but whether it stimulates or inhibits ryanodine receptor channels remains controversial. Too few data are available to determine ceramide effects on cardiac ion homeostasis. Based upon these actions, it would be expected that all SMM primarily inhibit frequency and force of myocardial contraction, albeit perhaps using somewhat different mechanisms.

SMM effects on cardiac rhythm have been determined using cultured cardiomyocytes, isolated sinoatrial node cells, isolated hearts and *in vivo* studies. These studies support the notion

that SMM lower heart rate under most but not all conditions. Thus, experiments at the level of the isolated pacemaker have found S1P-induced increases of sinoatrial rate in dogs (Sugiyama *et al.*, 2000b) but reductions of basal rate and reversal of the effects of the  $\beta$ -adrenergic agonist isoprenaline in rabbits (Guo *et al.*, 1999). Sphingosine reduced the beating rate of neonatal rat isolated cardiomyocytes (Benediktsdottir *et al.*, 2002) and of skinned myocytes from rabbit heart (Webster *et al.*, 1994). SPC reduced the rate of isolated guinea-pig hearts (Liliom *et al.*, 2001). The systemic administration of S1P reduced heart rate in rats (Sugiyama *et al.*, 2000a; Benediktsdottir *et al.*, 2002), but in some studies this effect was seen only at high doses (Friedrichs *et al.*, 2002) or not at all (Bischoff *et al.*, 2000b; 2001a). In one study in which S1P-induced reductions of heart rate had been observed, this was not affected by changes of atrio-ventricular or intra-ventricular conduction (Sugiyama *et al.*, 2000a). Two recent studies in mice also described S1P-induced lowering of heart rate (Forrest *et al.*, 2004; Sanna *et al.*, 2004). Most interestingly, this effect was abolished in S1P<sub>3</sub> receptor knockout mice (Sanna *et al.*, 2004), and the ability of several S1P receptor agonists to lower heart rate in wild-type mice correlated better with their potency at S1P<sub>3</sub> than at S1P<sub>1</sub> receptors (Forrest *et al.*, 2004). Taken together, it appears that SMM, particularly S1P, lower heart rate under most conditions, but these effects may not be of sufficient magnitude to be detectable in all cases, particularly *in vivo*.

The above signalling studies would also predict SMM to cause negative inotropic effects. This has been tested at the level of isolated cardiomyocytes, isolated myocardial strips, isolated hearts and *in vivo*. Sphingosine was shown to inhibit contractions of isolated cardiomyocytes from both cats (Oral *et al.*, 1997) and guinea-pigs (Sugishita *et al.*, 1999). In either case, sphingosine not only mimicked the negative inotropic effects of TNF- $\alpha$  but mediated them. Sphingosine also mimicked the negative inotropic effects of TNF- $\alpha$  in rat right ventricular trabeculae, and this occurred by impairing the economy of chemo-mechanical energy transduction (Hofmann *et al.*, 2003). In line with these *in vitro* observations, sphingosine caused negative inotropy upon systemic administration in rats *in vivo* (Sugiyama *et al.*, 2000b). Moderate negative inotropic effects have also been reported for S1P in isolated dog heart (Sugiyama *et al.*, 2000b), but these were largely attributed to its prominent contractile effect on the coronary vessels. Studies on inotropic effects of ceramide are less consistent. While it was found that ceramide increased contraction of cardiomyocytes and hearts isolated from adult rats (Liu & Kennedy, 2003; Relling *et al.*, 2003), another study detected negative inotropic effects of ceramide upon i.v. administration in rats (Friedrichs *et al.*, 2002). Thus, the majority of studies support the notion that SMM, particularly sphingosine, will reduce cardiac contractility.

The negative chronotropic and inotropic effects of SMM make them a logical candidate to mediate impaired cardiac function under pathophysiological conditions, particularly in the context of hypoxia and ischaemia. It is well established that cardiac hypoxia and ischaemia are associated with release of TNF- $\alpha$ . Hypoxia and ischaemia also lead to the formation and release of ceramide, for example, in neonatal rat cardiomyocytes (Bielawska *et al.*, 1997), and of sphingosine, for example, in adult rat isolated cardiomyocytes (Cavalli *et al.*, 2002), in Langendorff-perfused rabbit heart (Cavalli *et al.*, 2002) and in

**Table 2** Sphingolipid effect on cardiac ion homeostasis

	<i>Ceramide</i>	<i>Sphingosine</i>	<i>S1P</i>	<i>SPC</i>
$Ca^{2+}$ concentration	↑	↓	↑	ND
Ryanodine receptor	ND	↓	↓	↓
L-type $Ca^{2+}$ channel	↓	↓	↓	↓
$Na^+$ channel	ND	ND	↓	↓
$I_{K(ACh)}$ channel	ND	ND	↑	→ <sup>a</sup>
$I_f$ channel	ND	ND	↓	ND
Inward rectifier $K^+$ channel	ND	ND	→	ND

Arrows indicate increased, decreased or unchanged concentration or activity. Note that only sphingosine effect in general and S1P effects on  $I_{K(ACh)}$  channels have been confirmed by multiple investigators and/or in multiple studies. ND: not determined.

<sup>a</sup>No effect in mouse and human; but increase in guinea-pig. For details and references, see text.

dog heart *in vivo* (Thielmann *et al.*, 2002). This is further supported by the detection of hypoxia-induced activation of neutral sphingomyelinase in cardiac myocytes (Hernandez *et al.*, 2000). Since a TNF receptor-blocking antibody prevented hypoxia-induced sphingosine release in adult rat cardiomyocytes, sphingosine formation occurs secondary to TNF receptor activation (Cavalli *et al.*, 2002). Accordingly, the ceramidase inhibitor *N*-oleoylethanolamine prevented microembolization-induced sphingosine formation but not TNF release in dog heart (Thielmann *et al.*, 2002). Hypoxia/ischaemia-induced ceramide and sphingosine formation appear to primarily have negative effects on cardiac function. Thus, ceramide and sphingosine can induce apoptosis in neonatal cardiomyocytes (Krown *et al.*, 1996; Bielawska *et al.*, 1997). Moreover, the increase in ceramide content upon ischaemia in myocytes *in vitro* and in adult heart *in vivo* are accompanied by apoptosis (Bielawska *et al.*, 1997). Additional evidence for a deleterious role, particularly of sphingosine, comes from findings that the ceramidase inhibitor *N*-oleoylethanolamine prevented microembolization-induced contractile dysfunction in anaesthetized dogs (Thielmann *et al.*, 2002) and also improved survival after acute myocardial infarction in rats (Friedrichs *et al.*, 2002). Since cardiac tissue can rapidly and effectively convert sphingosine to S1P (Cavalli *et al.*, 2002), it was important to determine whether these adverse effects are indeed mediated by ceramide and sphingosine or by its metabolite S1P. In studies with neonatal rat cardiomyocytes, S1P protected against hypoxia-induced cell death, whereas dimethylsphingosine, an inhibitor of sphingosine kinase, enhanced cell death; moreover, ganglioside GM-1, an activator of sphingosine kinase, prevented the adverse effects of dimethylsphingosine (Karlner *et al.*, 2001). A similar protection against ischaemia-induced cardiac damage was found for S1P and the sphingosine kinase activator ganglioside GM-1 in mice (Jin *et al.*, 2002). Interestingly, this protection was absent in the hearts of protein kinase C- $\epsilon$  knockout mice, indicating a mediator role of this kinase (Jin *et al.*, 2002). Taken together, these data demonstrate that ceramide and sphingosine are formed during cardiac hypoxia/ischaemia, contribute to the myocardial damage under these conditions and mediate at least some of the adverse effects of TNF- $\alpha$  in this regard. In contrast, conversion of ceramide and sphingosine to S1P will counteract these effects and is cardioprotective. Therefore, we propose that manipulation of the relative balance between ceramide and sphingosine on the one hand and S1P on the other hand may be a possible target for the treatment of myocardial ischaemia.

### Vascular effects

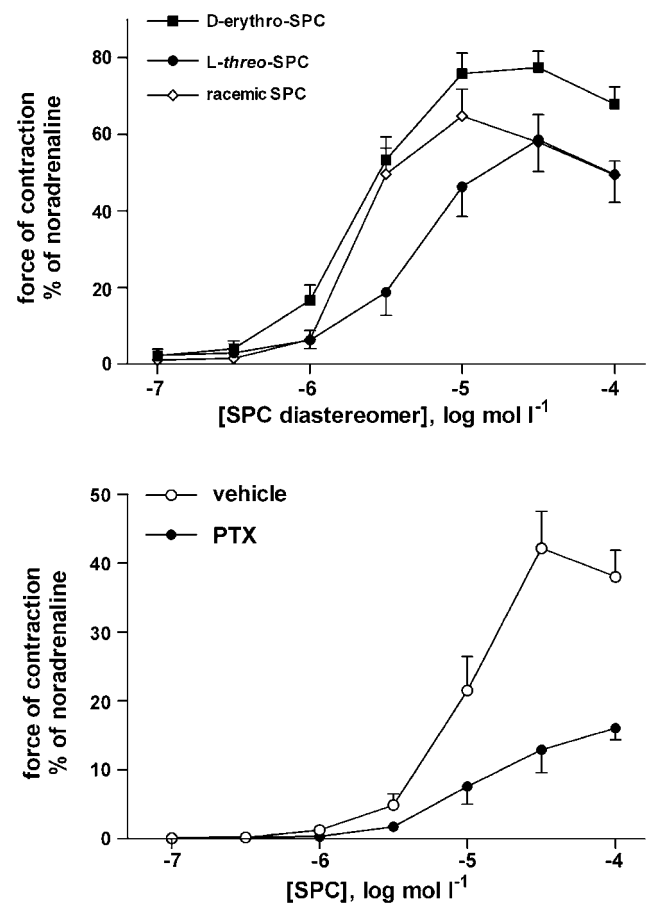
While cardiac SMM effects had originally been studied at the level of cell signalling and extended only later to that of isolated tissue function, studies on vascular SMM effects were pioneered by experiments on the contraction of isolated blood vessels. While the effects of ceramide on vascular tone remain controversial, S1P and the other SMM cause vasoconstriction in most blood vessels (for exceptions see below, for summary see Table 3). Rat mesenteric microvessels and intra-renal microvessels (inter-lobar arteries) were the first isolated vascular preparations in which S1P-induced vasoconstriction was reported (Bischoff *et al.*, 2000a) (Figure 2); these effects were confirmed in subsequent studies by the same group

**Table 3** Sphingolipid effects on the tone of isolated blood vessels

	<i>Ceramide</i>	<i>Sphingosine</i>	<i>S1P</i>	<i>SPC</i>
Aorta	↓	ND	→	ND
Carotid artery	ND	ND	→	ND
Femoral artery	ND	ND	→	ND
Mesenteric arteries	↓	↑	↑ <sup>a</sup> ↓	↑
Intra-renal arteries	ND	↑	↑ <sup>a</sup>	↑
Basilar artery	ND	ND	↑ <sup>a</sup>	↑
Cerebral artery	ND	ND	↑ <sup>a</sup>	↑
Coronary artery	↑	↑	↑ <sup>a</sup>	↑ ↓

Arrows indicate increased, decreased or unchanged vascular tone. While several findings were shown for multiple species (rat, mouse, dog, cow, pig), not all effects have been tested in more than one species. ND: not determined.

<sup>a</sup>Confirmed *in vivo* studies. For details and references, see text.



**Figure 2** Effects of diastereomers of SPC (upper panel) and effect of PTX in SPC-induced contraction (lower panel) of rat isolated mesenteric microvessels vasculature *in vitro*. Adapted from Bischoff *et al.* (2000a).

(Bischoff *et al.*, 2001a; Altmann *et al.*, 2003d; Hedemann *et al.*, 2004). S1P can also constrict many other rat blood vessels including basilar artery (Salomone *et al.*, 2003), cerebral artery (Coussin *et al.*, 2002; Salomone *et al.*, 2003), and coronary arteries (Salomone *et al.*, 2003), but apparently causes little if any contraction of aorta (Coussin *et al.*, 2002), carotid and femoral arteries (Salomone *et al.*, 2003). This pattern would

suggest that S1P is more important for the contractile function of resistance than of conduit vessels. In line with this proposal, we have recently observed that agonist-induced force of contraction strongly decreases with vessel diameter for the  $\alpha$ -adrenergic agonist noradrenaline, but only little if any for S1P (unpublished observations). In this regard, it appears to be interesting that maximum S1P-induced contraction was consistently only approximately half of maximum noradrenaline-induced,  $\alpha_{1A}$ -adrenoceptor-mediated effects in rat mesenteric and renal vessels. Nevertheless, S1P can elicit at least some additional vasoconstriction when added to maximally effective concentrations of an  $\alpha$ -adrenergic agonist (Czyborra *et al.*, 2002).

S1P-induced vasoconstriction was also found in rats *in vivo* following local or systemic injection, for example, for mesenteric and renal blood flow which were dose-dependently and transiently reduced in anaesthetized rats (Bischoff *et al.*, 2000b; 2001a, b; Wecking *et al.*, 2001). Upon direct injection into the renal artery, the effects of S1P on renal blood flow were stronger than upon systemic administration (Bischoff *et al.*, 2000b). Continuous infusions of S1P also lowered mesenteric and renal blood flow in anaesthetized rats (Bischoff *et al.*, 2001b). Comparison of the time courses of S1P-induced blood flow reductions upon bolus injection vs continuous infusion indicates that both pharmacokinetic effects and tachyphylaxis may contribute to the transient nature of *in vivo* vasoconstriction by this agonist. S1P has also been shown to reduce rat cerebral blood flow upon intra-arterial infusion (Salomone *et al.*, 2003), and S1P administration into cerebrospinal fluid was reported to cause a long-lasting reduction of the diameter of the basilar artery in dogs (Tosaka *et al.*, 2001).

Additional studies have demonstrated that S1P is not the only SMM which can elicit vascular smooth muscle cell contraction and that SMM-induced vasoconstriction is not limited to rats. The initial study reporting S1P-induced contraction of rat mesenteric and intra-renal vessels found that SPC, sphingosine and glucopsychosine also induced a concentration-dependent contraction in both vessels (Bischoff *et al.*, 2000a). SPC-induced contraction was also found in murine isolated mesenteric vessels (Hedemann *et al.*, 2004), bovine isolated middle cerebral artery (Shirao *et al.*, 2002), canine basilar artery (Tosaka *et al.*, 2001) and porcine coronary artery smooth muscle cells (Todoroki-Ikeda *et al.*, 2000). Sphingosine and sphingomyelinase were found to contract porcine coronary arteries (Murohara *et al.*, 1996). One group of investigators described ceramide-induced contraction and inhibition of endothelium-dependent dilation of bovine isolated small coronary arteries (Li *et al.*, 1999; Zhang *et al.*, 2001), but ceramide was found to cause vasodilatation in all other preparations (see below). S1P-induced vasoconstriction was also found in dogs *in situ* following intra-coronary administration (Sugiyama *et al.*, 2000b), in murine isolated mesenteric vessels (Hedemann *et al.*, 2004) and in human coronary smooth muscle cells (Ohmori *et al.*, 2003). Nevertheless, as compared to S1P, SPC, sphingosine and glucopsychosine caused much less if any alterations of mesenteric and renal blood flow upon *in vivo* injection (Bischoff *et al.*, 2000b; 2001a) or infusion (Bischoff *et al.*, 2001b; Wecking *et al.*, 2001). Thus, several SMM, most importantly S1P and SPC, can contract vascular smooth muscle cells in various vascular beds of several species both *in vitro* and *in vivo*. In line with this

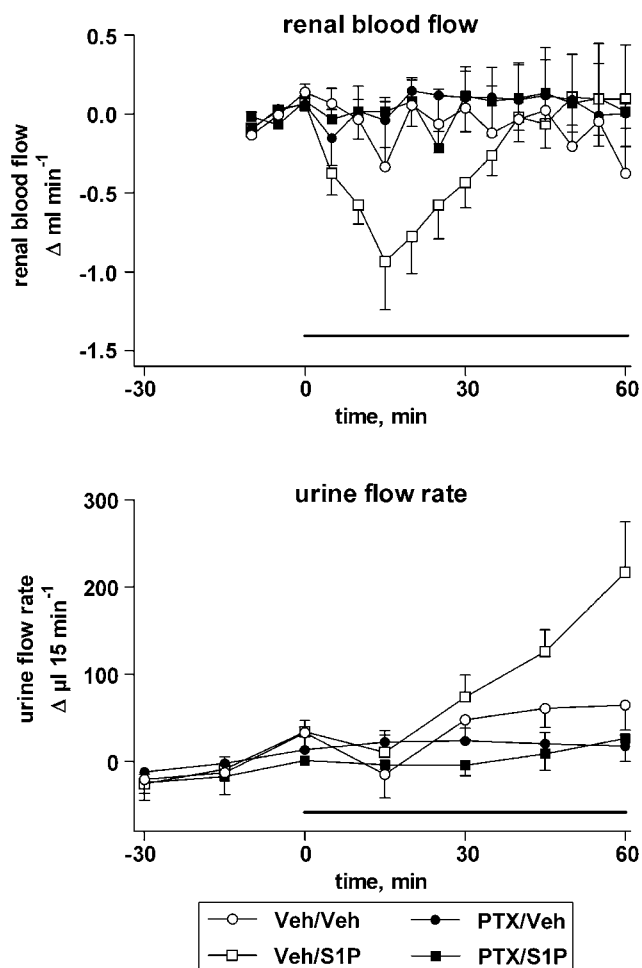
addition of the enzyme, sphingomyelinase can also cause vasoconstriction (Murohara *et al.*, 1996). Moreover, studies with forced expression of sphingosine kinase or a dominant-negative mutant thereof in hamster resistance arteries suggest that SMM may also play a crucial role in the development of basal tone and myogenic responses of blood vessels (Bolz *et al.*, 2003a).

Nevertheless, most studies failed to detect any major alterations of blood pressure upon injection or infusion of S1P or other SMM (Bischoff *et al.*, 2000b; 2001a, b; Wecking *et al.*, 2001; Forrest *et al.*, 2004). This could be explained if many vascular beds were largely insensitive to SMM-induced vasoconstriction, but this is unlikely in light of the sensitivity of many vascular beds *in vitro*. A more likely explanation for the lack of blood pressure alteration would be concomitant cardio-depressant effects, that is, a reduced cardiac output. While these have not been determined by direct *in vivo* studies, many of the above *in vitro* studies make them likely to occur. Finally, the lack of blood pressure effect in anaesthetized animals could relate to experimental circumstances because SMM-induced hypertension was prominent in conscious rats and mice (Forrest *et al.*, 2004).

Based upon the original debate whether SMM effects occur *via* intracellular sites and/or specific SMM receptors in the cell membrane, several investigators have studied the involvement of receptors and, more recently, specific SMM receptor subtypes in vasoconstriction. One characteristic of receptor- vs non-receptor-mediated SMM effects is stereo-selectivity of SPC responses (Meyer zu Heringdorf *et al.*, 1997). In this regard, SPC-induced contraction of rat isolated mesenteric microvessels was stereo-selective with *D-erythro*-SPC being more potent than *L-threo*-SPC (Bischoff *et al.*, 2000a) (Figure 2). Another characteristic of receptor-mediated SMM effects is sensitivity towards inhibition by PTX (Meyer zu Heringdorf *et al.*, 1997). Pre-treatment with PTX markedly inhibited SMM-induced contraction of rat mesenteric microvessels *in vitro* (Bischoff *et al.*, 2000a) (Figure 2) and of S1P-induced reductions of rat mesenteric and renal blood flow *in vivo* (Bischoff *et al.*, 2000b; 2001b) (Figure 3). While S1P-induced contraction of rat cerebral arteries was largely resistant to PTX treatment (Salomone *et al.*, 2003), this does not exclude receptor involvement.

Due to the lack of widely available, specific SMM-receptor antagonists with good selectivity, the following approaches have been used to identify the subtypes involved in SMM-induced vasoconstriction: agonist order of potencies, the nonspecific but moderately subtype-selective antagonist suramin, antisense approaches and receptor knockout mice. The initial study reporting S1P-induced contraction of rat mesenteric and intra-renal vessels found that several SMM induced a concentration-dependent contraction with a rank order of potency of SPC > S1P > sphingosine  $\geq$  psychosine  $\geq$  glucopsychosine; in this regard, all five SMM acted in the micromolar range and the latter three were considerably less efficacious than SPC and S1P (Bischoff *et al.*, 2000a). Two observations are noteworthy in this context: Firstly, in this as in most other studies, micromolar SMM concentrations were required to elicit vasoconstriction. This contrasts the nanomolar affinity of the SMM for their cloned receptors but fits reported plasma concentrations of S1P and SPC (see above). Secondly, in both rat preparations (Bischoff *et al.*, 2000a) and in mouse vessels (Hedemann *et al.*, 2004), S1P and SPC were active in a similar





**Figure 3** S1P infusion reduces renal blood flow (upper panel) and increases urine flow rate (lower panel), effects of PTX. The solid line indicates the period of S1P infusion. Adapted from Bischoff *et al.* (2001b).

concentration range. While a similar situation had also been found for the activation of  $I_{K(ACh)}$  in guinea-pig atria (see above), none of the cloned SMM receptors has similar affinity for both SMM (see above), indicating that they either act on a common site distinct from all known SMM receptors or *via* multiple concomitantly expressed receptors.

It had been reported that rat cerebral artery is much more responsive to S1P than aorta, and, based upon the finding that cerebral artery expresses considerably more S1P<sub>2</sub> and S1P<sub>3</sub> receptors but similar amounts of S1P<sub>1</sub> receptors as aorta, it had been proposed that one of the former mediates contraction of this vessel (Coussin *et al.*, 2002). A more detailed study regarding the receptor subtype(s) mediating S1P-induced contraction was presented for a closely related vessel, that is, rat basilar artery, using the antagonist suramin and antisense constructs (Salomone *et al.*, 2003). Suramin has been proposed to be an antagonist at SMM receptors with some selectivity for the S1P<sub>3</sub> relative to the S1P<sub>1</sub> and S1P<sub>2</sub> subtypes (Ancellin & Hla, 1999). In rat basilar artery, suramin produced some inhibition of S1P-induced contraction, and a similar degree of inhibition was observed with adenoviral transfer of S1P<sub>3</sub> receptor antisense (Salomone *et al.*, 2003). In contrast, S1P<sub>2</sub> receptor antisense did not cause inhibition, and suramin did not inhibit responses to serotonin in that study. Moreover, the

authors also reported antagonism of S1P-induced reductions of rat cerebral blood flow *in vivo* by suramin (Salomone *et al.*, 2003). Based upon these data, it appears that S1P<sub>3</sub> receptors contribute to contraction of rat basilar artery, but additional contributions of other subtypes cannot be excluded. In another study, suramin also caused inhibition of S1P-induced contraction of rat mesenteric microvessels; this inhibition was stronger than that of SPC-induced contraction in the same preparation and that caused by S1P in murine mesenteric vessels of similar size (Hedemann *et al.*, 2004). *In vivo* studies have detected blood pressure elevations by SMM receptor agonists in wild-type but not in S1P<sub>3</sub> receptor knockout mice (Forrest *et al.*, 2004). While all of these findings point towards an involvement of S1P<sub>3</sub> receptors, S1P-induced contraction of human coronary smooth muscle cells was inhibited by the S1P<sub>2</sub>-selective antagonist JTE-013 (Ohmori *et al.*, 2003). Thus, considerable additional efforts will be required to establish which subtype(s) of SMM receptors is/are important for vasoconstriction.

Key mechanisms to elicit vascular smooth muscle contractions are an elevation of free intracellular Ca<sup>2+</sup> concentrations, through mobilization from intracellular stores and/or through influx of extracellular Ca<sup>2+</sup>, and a sensitization of the contractile filaments towards ambient Ca<sup>2+</sup> concentrations, frequently involving a rho-kinase. Both mechanisms have been implicated in SMM-induced vasoconstriction. S1P- and/or SPC-induced, PTX-sensitive Ca<sup>2+</sup> elevations have directly been demonstrated in rat (Bischoff *et al.*, 2000a; Coussin *et al.*, 2002; 2003) and porcine vascular smooth muscle cells (Chin & Chueh, 1998; 2000; Nakao *et al.*, 2002), but apparently were absent in bovine cerebral artery smooth muscle (Shirao *et al.*, 2002). Moreover, forced overexpression of a sphingosine kinase in hamster resistance vessels elevated basal intracellular Ca<sup>2+</sup> concentrations (Bolz *et al.*, 2003a).

A role for a mobilization of Ca<sup>2+</sup> from intracellular stores was indicated by several findings: Firstly, SPC can activate a phospholipase C in porcine vascular smooth muscle cells (Chin & Chueh, 1998). Secondly, 2-aminoethoxydiphenylborate, an inhibitor of inositol-trisphosphate receptors, reduced S1P-induced Ca<sup>2+</sup> mobilization in rat cerebral arteries (Coussin *et al.*, 2003). Thirdly, pre-treatment with thapsigargin, an inhibitor of sarcoplasmic calcium-ATPase, ryanodine or the phospholipase C inhibitor, U 73,122 (but not its negative control, U 73,343) almost completely prevented S1P- and SPC-induced Ca<sup>2+</sup> elevation in rat basilar artery (Coussin *et al.*, 2002) and porcine aorta smooth muscle cells (Chin & Chueh, 1998), respectively. Finally, U 73,122 but not U 73,343 markedly inhibited SPC-induced contraction of rat mesenteric microvessels (Altmann *et al.*, 2003d). In contrast, SK&F 96,365, an inhibitor of store-operated Ca<sup>2+</sup>-channels, or the phospholipase D inhibitor butan-1-ol relative to its negative control butan-2-ol did not affect SPC-induced contraction of mesenteric microvessels (Altmann *et al.*, 2003d). These data demonstrate that mobilization from an intracellular store contributes to SMM-induced Ca<sup>2+</sup> elevations in vascular smooth muscle cells; this appears to occur largely *via* inositol-trisphosphate- and perhaps also ryanodine-sensitive stores, whereas store-operated Ca<sup>2+</sup>-channels or phospholipase D do not appear to play a major role.

A contribution of mobilization from intracellular stores does not necessarily exclude additional contributions from the influx of extracellular Ca<sup>2+</sup>, but the available data are

contradictory. Removal of extracellular  $\text{Ca}^{2+}$  and/or blockade of L-type  $\text{Ca}^{2+}$ -channels was reported to have little if any effects on SIP- or SPC-induced  $\text{Ca}^{2+}$  elevation in vascular smooth muscle cells from rat cerebral artery (Coussin *et al.*, 2002) or porcine aorta (Chin & Chueh, 1998), respectively. In contrast to the above, other investigators reported nifedipine to block at least part of the SIP-induced  $\text{Ca}^{2+}$  increase in rat cerebral artery (Coussin *et al.*, 2003). Similarly, removal of extracellular  $\text{Ca}^{2+}$  markedly attenuated SIP- and SPC-induced contraction of rat mesenteric microvessels (Bischoff *et al.*, 2000a; 2001a). L-type  $\text{Ca}^{2+}$ -channel inhibitors significantly inhibited SIP-induced contraction of rat intra-renal microvessels (Bischoff *et al.*, 2001a) and, to a lesser extent, SPC-induced contraction of mesenteric microvessels (Bischoff *et al.*, 2000a). Nifedipine also dose-dependently attenuated SIP-induced reductions of renal blood flow in anaesthetized rats *in vivo* (Bischoff *et al.*, 2001a). Direct electrophysiological proof of SMM-induced activation of L-type  $\text{Ca}^{2+}$ -channels was obtained in smooth muscle cells from rat cerebral arteries, where it was found to occur secondary to inhibition of voltage-gated  $\text{K}^{+}$ -channels (Coussin *et al.*, 2003). Thus, the role of extracellular  $\text{Ca}^{2+}$  and specifically of L-type, dihydropyridine-sensitive  $\text{Ca}^{2+}$ -channels may depend upon the blood vessel under investigation. In the only vessel where ceramide was reported to cause vasoconstriction, that is, bovine small coronary arteries, this was accompanied by an inhibition of the activity of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$ -channels (Li *et al.*, 1999).

Several protein kinases may be involved in coupling the above primary signals to smooth muscle contraction. Controversial results have been obtained with regard to protein kinase C. Thus, protein kinase C was reportedly not involved in SPC-induced  $\text{Ca}^{2+}$  sensitization in bovine cerebral artery (Shirao *et al.*, 2002), but on the other hand found to mediate SIP-induced inhibition of voltage-gated  $\text{K}^{+}$ -channels and hence activation of L-type  $\text{Ca}^{2+}$ -channels in rat cerebral artery smooth muscle (Coussin *et al.*, 2003). Sphingosine-induced contraction of porcine isolated coronary arteries was insensitive to the protein kinase C inhibitor staurosporine (Murohara *et al.*, 1996). One study in rat mesenteric microvessels looked at the role of phosphatidylinositol-3'-kinase (PI3K) in SPC-induced vasoconstriction; it reported ambiguous results with the kinase inhibitor LY 294,002 and, to a smaller extent, its negative control LY 303,511 causing inhibition of the SPC effect, but wortmannin being ineffective as an inhibitor (Altmann *et al.*, 2003d). Within the same study, the tyrosine kinase inhibitors genistein (relative to its negative control daidzein) and the src-type tyrosine kinase inhibitor PP2 (relative to its negative control PP3) also produced little inhibition (Altmann *et al.*, 2003d). In contrast, the src-kinase inhibitors PP1 (but not PP3) and EPA inhibited SIP-induced  $\text{Ca}^{2+}$  elevation, fyn (but not c-src) translocation, rho-kinase translocation and contraction in porcine coronary artery (Nakao *et al.*, 2002). Another group of studies has determined a possible role of ERKs in SMM-induced vasoconstriction. SPC- and SIP-induced activation of such kinases was described in cultured porcine aortic smooth muscle (Chin & Chueh, 1998) and rat aorta and cerebral artery (Coussin *et al.*, 2002), respectively. Although the activation was of similar magnitude in the latter two, SIP induced contraction only in cerebral artery but not in aorta, calling into doubt the role of this kinase in smooth muscle contraction. Studies with pharmacological inhibitors have yielded equivocal results,

since an inhibitor of ERK activation, PD 98,059, attenuated SPC-induced contraction of rat mesenteric microvessels, whereas a chemically distinct inhibitor, U 126, or its inactive analogue, U 124, did not share the effect (Altmann *et al.*, 2003d). Thus, the overall data indicate an SMM-induced activation of such kinases, but make it doubtful that they are important for the control of vasoconstriction.

As stated above, vasoconstriction can not only be brought about by elevations of intracellular  $\text{Ca}^{2+}$  but also by a  $\text{Ca}^{2+}$  sensitization of the contractile filaments, which frequently involves a rho-kinase. Indeed, SIP and SPC were found to cause  $\text{Ca}^{2+}$  sensitization in hamster skeletal muscle microvessels (Bolz *et al.*, 2003b) and in bovine cerebral artery (Shirao *et al.*, 2002). Moreover, SIP was found to activate rho A in rat cerebral artery (Coussin *et al.*, 2002). Accordingly, inhibition of the rho A/rho kinase pathway inhibited SMM-induced contraction of bovine cerebral artery (Shirao *et al.*, 2002), dog basilar artery (Tosaka *et al.*, 2001), rat cerebral artery (Coussin *et al.*, 2002), rat mesenteric microvessels (Altmann *et al.*, 2003d), hamster skeletal muscle microvessels (Bolz *et al.*, 2003b), porcine coronary artery (Nakao *et al.*, 2002) and human coronary smooth muscle cells (Ohmori *et al.*, 2003). Such inhibition was achieved using the rho-kinase inhibitors Y 27,632 (Tosaka *et al.*, 2001; Coussin *et al.*, 2002; Nakao *et al.*, 2002; Shirao *et al.*, 2002; Altmann *et al.*, 2003d; Ohmori *et al.*, 2003; Salomone *et al.*, 2003) and fasudil (Altmann *et al.*, 2003d), C3 exotoxin (Bolz *et al.*, 2003b; Ohmori *et al.*, 2003; Salomone *et al.*, 2003), or dominant-negative mutants of rho kinase (Shirao *et al.*, 2002).

Taken together, it appears that SMM-induced vasoconstriction is mediated by a combination of  $\text{Ca}^{2+}$  mobilization from inositol-trisphosphate-sensitive stores,  $\text{Ca}^{2+}$  influx through L-type channels and  $\text{Ca}^{2+}$  sensitization of contractile filaments via a rho-kinase pathway. In this regard, it is interesting to note that SPC-induced and  $\alpha_1$ -adrenoceptor-mediated vasoconstriction in rat mesenteric microvessels, despite occurring via  $\text{G}_i$  and  $\text{G}_q$ , respectively, were not only well correlated in their magnitude but also exhibited an almost identical profile of sensitivity towards signal transduction inhibitors (Altmann *et al.*, 2003d).

Although endothelial cells can express SMM receptors (see above), the role of the endothelium in SMM-induced vasoconstriction remains unclear. Thus, neither endothelial denudation nor the NO synthase (NOS) inhibitor  $N^G$ -nitro-L-arginine attenuated SPC-induced contraction of rat isolated mesenteric microvessels (Bischoff *et al.*, 2000a). While one study reported a minor attenuation of SPC-induced vasoconstriction in the presence of the cyclooxygenase inhibitor indomethacin, whether or not the NOS inhibitor  $N^G$ -nitro-L-arginine was present (Bischoff *et al.*, 2000a), another study by the same group did not confirm this effect of indomethacin (Altmann *et al.*, 2003d). Accordingly, these authors also reported that indomethacin failed to inhibit SIP-induced reductions of mesenteric or renal blood flow in anaesthetized rats *in vivo* (Wecking *et al.*, 2001). On the other hand, it was found that sphingosine-induced contraction of porcine coronary arteries was endothelium-dependent and partly indomethacin-sensitive, but insensitive to NOS inhibition (Murohara *et al.*, 1996). Thus, the question of an endothelial involvement in SMM-induced vasoconstriction needs to be studied in additional vascular beds to get a more comprehensive view.

S1P can activate endothelial NOS in bovine endothelial cells (Mogami *et al.*, 1999; Tanimoto *et al.*, 2002). Accordingly, it has also been found that SPC can cause relaxation of bovine coronary arteries in an NO-dependent manner (Mogami *et al.*, 1999). Moreover, S1P can transiently relax pressurized mesenteric vessels from rats and wild-type mice, but not those from knockout mice lacking the endothelial NOS (Dantas *et al.*, 2003). Within the latter study, S1P-induced vasodilatation was abrogated by removal of the endothelium or an NOS inhibitor, but not by the cyclooxygenase inhibitor indomethacin or the K<sup>+</sup>-channel inhibitor 4-aminopyridine. Moreover, it was inhibited by PTX, the PI3K inhibitor wortmannin and the intracellular Ca<sup>2+</sup>-chelator BAPTA. Nevertheless, vasoconstriction appears to be the most frequent vascular response to SMM administration (see above).

The role of ceramides on vascular tone has remained controversial. In bovine small coronary arteries, ceramide was found to cause vasoconstriction (Li *et al.*, 1999) and to inhibit endothelium-dependent vasodilatation (Zhang *et al.*, 2001). The former may involve activation of Ca<sup>2+</sup>-activated K<sup>+</sup>-channels, while the latter may involve attenuated NO function due to increased O<sub>2</sub><sup>-</sup> formation. In contrast, ceramide-induced vasodilatation was demonstrated in rat isolated thoracic aorta (Johns *et al.*, 1997), a finding later confirmed by the same (Johns *et al.*, 1998) and other authors (Zheng *et al.*, 1999). Ceramide-induced vasodilatation was also seen in rat mesenteric microvessels (Czyborra *et al.*, 2002). Particularly in the latter preparation, ceramide-induced relaxation was concentration-dependent and inhibited specific structure-activity relationships. However, relaxation of mesenteric microvessels was only transient, which may involve desensitization of the ceramide response as well as metabolism of the ceramides to inactive metabolites (Czyborra *et al.*, 2002). Two findings suggest that ceramide-induced vasodilatation may be an endogenously active process. Firstly, activation of sphingomyelinase, which generates endogenous ceramides, imitates the vasodilating effect of ceramide in rat aorta (Johns *et al.*, 1997). Secondly, the ceramidase inhibitor (1*S*,2*R*)-*D*-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol imitated the vasodilating effect of ceramide in rat mesenteric microvessels in a concentration where it hastened the vasodilatation by exogenous ceramide (Czyborra *et al.*, 2002). Whether the above discrepancies reflect heterogeneity in vascular ceramide responses between vascular beds and/or between species remains to be determined. Moreover, it appears possible that this reflects a methodological difference since studies reporting ceramide-induced vasodilatation have used vessel strips whereas those reporting vasoconstriction or inhibition of vasodilatation have used isolated perfused vessels.

Whether ceramide-induced vasodilatation primarily occurs at the level of the vascular smooth muscle cells or, at least partly, is endothelium-dependent has remained controversial. Thus, ceramide-induced relaxation of rat aorta (Johns *et al.*, 1998) and bovine coronary arteries (Zhang *et al.*, 2001) was reported to be at least partly endothelium-dependent, whereas that of rat mesenteric microvessels was not (Czyborra *et al.*, 2002). In line with the latter study, it was shown that ceramide can attenuate elevations of intracellular Ca<sup>2+</sup> concentrations induced by vasoconstricting agents such as phenylephrine in isolated smooth muscle cells (Zheng *et al.*, 1999). Czyborra *et al.* (2002) have reported extensive studies into the molecular mechanism of ceramide-induced vasodilatation in rat mesen-

teric microvessels, that is, in a preparation where vasodilatation appears to occur endothelium-independently. Inhibitors of cyclic nucleotide-dependent signalling pathways such as the protein kinase A inhibitors H7 and H89 or the guanylyl cyclase inhibitor ODQ had little effect on vasodilatation, the latter observation being in line with the lack of effect of endothelium removal in this preparation. Similarly, inhibitors of various types of K<sup>+</sup>-channels, including tetraethylammonium, apamine, glibenclamide, iberiotoxin and charybdotoxin, also caused small, if any, attenuation of ceramide-induced vasodilatation. However, a combination of ODQ and charybdotoxin almost completely abolished it. Thus, both guanylyl cyclase and charybdotoxin-sensitive K<sup>+</sup>-channels may be involved in ceramide-induced vasodilatation, but only combined inhibition of both reveals this effect, possibly because they can largely compensate for each other when only one of them is inhibited. In the absence of proposals for receptor-mediated ceramide effects, it appears to be determined what the molecular ceramide targets are that activate these signalling mechanisms.

While several studies have investigated cardiac SMM responses under pathophysiological conditions, only few have addressed such possible alterations in blood vessels. One study has looked at alterations of mesenteric microvessel vasoconstriction responses to S1P and SPC and vasodilatation responses to ceramide in spontaneously hypertensive relative to normotensive Wistar-Kyoto rats (Altmann *et al.*, 2003b). Numerous previous studies have established that vascular responsiveness to various contractile stimuli is enhanced in this model of genetic hypertension, whereas that to relaxing stimuli is attenuated in most cases. In confirmation of such findings, that study reported an enhanced vasoconstriction to the  $\alpha_1$ -adrenoceptor agonists noradrenaline and methoxamine, whereas vasodilatation in response to the  $\beta$ -adrenoceptor agonist isoprenaline and to nitroprusside-sodium was attenuated. In contrast, vasoconstriction responses to both S1P and SPC were not only not enhanced but rather significantly attenuated in hypertension. Similarly, the attenuation of vasodilating ceramide responses in hypertension was less pronounced than that of isoprenaline or nitroprusside-sodium. Thus, it was proposed that the regulation of vascular SMM responses in hypertension may differ from that of most established vasoactive agents, possibly related to specific effects upon expression of SMM receptors. In a second study, the same group has determined the effects of ageing on rat vascular responses to S1P and SPC (Altmann *et al.*, 2003a) or ceramide (Altmann *et al.*, 2003c). The authors have used mesenteric microvessels from 3 vs 23 months old male Wistar rats. Both age groups had similar blood pressure, but older rats had slightly lower heart rate and markedly larger internal vessel diameters. Nevertheless, vasoconstriction responses to noradrenaline and methoxamine or to S1P exhibited little, if any, difference between age groups. In contrast, vessels from aged rats exhibited an approximately 50% greater wall tension in response to SPC. Vasodilatation in response to isoprenaline, nitroprusside-sodium and ceramide was not altered in vessels from aged rats.

### Renal effects

The kidney is the organ with the greatest impact on long-term blood pressure control (Cowley, 1992). Moreover, enhancements and reductions of renal blood flow generally tend to

enhance and reduce diuresis, respectively, and SMM, particularly, SIP, have been shown to cause renal vasoconstriction *in vitro* and *in vivo* (see above). Therefore, some studies have investigated the possible effects of SMM on renal function. Based on the pronounced SIP effects on renovascular tone *in vitro* (Bischoff *et al.*, 2000a; 2001a) and *in vivo* (Bischoff *et al.*, 2000b; 2001a), it had been expected that SMM might inhibit diuresis and natriuresis. Surprisingly, however, intravenous infusion of SIP did not reduce but rather enhanced diuresis (Figure 3), natriuresis, calciuresis and, to a lesser extent, kaliuresis (Bischoff *et al.*, 2001b). This occurred under conditions where the SIP infusion dose-dependently but transiently reduced renal blood flow and did not cause major alterations of mean arterial pressure or glomerular filtration rate, as assessed by endogenous creatinine clearance. Moreover, the SIP effects upon tubular function occurred and abated slower than those on renovascular function. This overall pattern is typical for agents which act in distal nephron segments. Diuretic effects of SIP were confirmed by the authors in a second study (Wecking *et al.*, 2001).

Interestingly, other SMM such as SPC, sphingosine and glucosylsphingosine lacked the renovascular effects of SIP, but shared its diuretic effects (Bischoff *et al.*, 2001b). Actually, diuretic effects by the former three were larger and exhibited a clearer dose-dependency than those elicited by SIP, indicating that the renovascular effects of SIP might at least partly counteract its effect on the renal tubules. The finding that the effects of both SIP and SPC were abolished by PTX pre-treatment of the animals suggests that at least these two SMM exerted their effects in a receptor-mediated manner (Bischoff *et al.*, 2001b). Apart from the fact that none of the cloned SMM receptors is activated by SIP and SPC in a similar concentration range (see above), another finding suggests that both SMM exerted their tubular effects *via* distinct receptors. Thus, pre-treatment with the cyclooxygenase inhibitor indomethacin enhanced the diuretic and natriuretic effects of SIP, but abolished those of SPC (Wecking *et al.*, 2001). Thus, it appears that SMM can affect renal function indirectly *via* receptors located on the renal vasculature, and directly *via* one or more receptors located on the renal tubules. However, it should also be noted that the diuretic and natriuretic effects of SIP and SPC are of limited magnitude and hence, for example, the diuretic effects of SPC did not reach statistical significance under all experimental conditions (Bai *et al.*, 2002).

Thus, it appears that SMM can affect renal function *via* at least two different mechanisms. One of them is a receptor located on the renal vasculature, which is preferentially activated by SIP and mediates reductions of renal blood flow and possibly indirectly inhibits diuresis. The other is more likely to be located on tubular cells, activated by several SMM, and directly enhances diuresis. The latter may be a potential target for treatment of renal dysfunction, but considerable additional studies are required.

## Long-term effects of SMM in the cardiovascular system

### *SMM in vascular development*

The formation of new blood vessels is essential for a variety of physiological processes like embryogenesis and the female

reproduction system, as well as pathophysiological processes such as wound healing and neovascularization of ischaemic tissue. In the developing embryo, vasculogenesis and angiogenesis are the mechanisms responsible for the development of the blood vessels. Vasculogenesis resembles the process in which haemangioblasts differentiate to blood cells, and ultimately form a primitive vascular plexus *de novo*. Although also derived from the blood-forming bone marrow, vasculogenic stem cells contribute only little to the regular vascular repair mechanisms in adult life. Angiogenesis refers to the formation of capillaries from pre-existing vessels in the embryo and adult organism. While pathologic angiogenesis includes the role of post-natal neovascularization in the pathogenesis of arthritis, diabetic retinopathy, tumour growth and metastasis, therapeutic angiogenesis, either endogenously or in response to administered growth factors, involves the development of collateral blood vessels in tissue ischaemia. Major progress in understanding the underlying mechanisms regulating blood vessel growth has offered novel therapeutic options in the treatment of a variety of disease states. Since the identification of SIP<sub>1</sub> as a G-protein-coupled receptor homologue that is induced during the *in vitro* differentiation of human endothelial cells (Hla & Maciag, 1990a) and the discovery that SIP is its endogenous ligand (Lee *et al.*, 1998), it has become clear that SMM play a role in vascular development. Numerous investigations to date have shown that SMM may be involved in several steps of vascular development. Here we will discuss the involvement of SIP in several aspects of angiogenesis: endothelial cell activation, extracellular matrix degradation, proliferation and migration, tube formation and vessel maturation.

### *SIP, VEGF crosstalk and endothelial cell activation*

Vascular endothelial growth factor (VEGF) is most likely one of the most important growth-promoting factors involved in angiogenesis. VEGF transcription is stimulated by hypoxia, which induces the generation and binding of hypoxia-inducible factor-1 $\alpha$  to the hypoxia responsive element in the VEGF promoter region. VEGF binds to its specific receptor tyrosine kinase (VEGFR) and thereby promotes and modulates several aspects in the angiogenic process. The VEGFR family is composed of VEGFR-1 (Flt-1), VEGFR-2 (Flk-1 or KDR) and VEGFR-3 (Flt-4). The VEGFR-2 is the main receptor *via* which VEGF exerts its effects, such as vascular permeability, endothelial cell proliferation, migration and tube formation (for a review, see Carmeliet, 2003).

VEGF is known to stimulate PI3K- and Akt-dependent phosphorylation of eNOS, resulting in activation of eNOS and increased NO production (Fulton *et al.*, 1999). Activation of eNOS is an essential step in the action of VEGF, since it has been shown that eNOS inhibition blocks VEGF-induced endothelial cell migration, proliferation and tube formation *in vitro*, as well as VEGF-induced angiogenesis *in vivo* (Murohara *et al.*, 1998; Lee *et al.*, 1999c; Rikitake *et al.*, 2002).

Recent studies revealed that SIP binding to its SIP<sub>1</sub> receptor also leads to eNOS activation and NO synthesis (Igarashi *et al.*, 2001; Kwon *et al.*, 2001; Morales-Ruiz *et al.*, 2001; Rikitake *et al.*, 2002). SIP treatment of bovine aortic endothelial cells leads to an Akt-mediated eNOS phosphorylation at Ser<sup>1179</sup> and

concomitant NO production (Igarashi *et al.*, 2001). This S1P-mediated eNOS activation was PTX sensitive, indicating that these events are mediated by surface receptors rather than *via* intracellular mechanisms (Igarashi *et al.*, 2001). Inhibition of  $G_i$  signaling with PTX leads to a decrease in S1P-mediated endothelial cell chemotaxis, whereas NOS inhibition by means of  $N^G$ -nitro-L-arginine methyl ester was ineffective in this respect. In contrast, NOS inhibition effectively blocked the chemotactic actions of VEGF (Morales-Ruiz *et al.*, 2001). In a later study, Rikitake *et al.* (2002) have shown that inhibition of PI3K, Akt or eNOS significantly decreases endothelial cell migration and tube formation, whereas  $N^G$ -nitro-L-arginine methyl ester, but not the PI3K inhibitor LY294002 failed to inhibit endothelial cell viability and proliferation induced by S1P. However, another study suggests that NO is involved in S1P-mediated prevention of serum-deprived apoptosis of human umbilical vein endothelial cells (Kwon *et al.*, 2001). These results show that, in analogy with VEGF, S1P leads to activation of Akt and eNOS. The resulting production of NO may be involved in different aspects of the angiogenic action of these two agonists.

The relation between S1P and VEGF is probably even more complex, since several groups have shown a rather complicated receptor crosstalk (receptor transactivation and upregulation) between these two compounds.

In a recent report by Shu *et al.* (2002), evidence was presented that sphingosine kinase mediates VEGF-induced activation of Ras and MAPK. Thus, VEGF activated sphingosine kinase, and the VEGF-induced activation of ERK1/2 type MAPK was prevented by the sphingosine kinase inhibitor dimethylsphingosine. It was proposed by the authors that sphingosine may increase the activity of Ras GTPase-activating proteins and thereby decrease Ras activity, and that S1P can block the ability of sphingosine to activate Ras GTPase-activating proteins. Thus VEGF-induced sphingosine kinase activity will result in the conversion of sphingosine to S1P and a subsequent displacement of sphingosine from Ras GTPase-activating proteins will increase the levels of activated Ras-GTP and hence activated Raf (Shu *et al.*, 2002). In another recent report by Endo *et al.* (2002), it was shown that S1P induces membrane ruffling (a renowned characteristic of migratory cells) of human umbilical vein endothelial cells *via* the vascular endothelial growth factor receptor (VEGFR-2), Src family tyrosine kinase(s) and the CrkII adaptor protein (which is known to be involved in cellular migration and the induction of membrane ruffling). S1P-induced CrkII phosphorylation was blocked by PTX and overexpression of the carboxyl terminus of  $\beta$ -adrenergic receptor kinase, indicating that the  $\beta\gamma$ -subunit of  $G_i$  was required for the phosphorylation. In addition, the authors showed that S1P-induced CrkII phosphorylation was also abolished by inhibitors of VEGF receptor or Src family tyrosine kinase. Interestingly, it has been shown that the previously discussed S1P-induced eNOS activation involving  $G_i$  protein, PI3K and Akt is dependent on VEGFR-2 transactivation (Tanimoto *et al.*, 2002).

In addition to these complex signalling interactions, it has recently been shown that VEGF can induce S1P<sub>1</sub> expression and concomitant enhanced intracellular signalling responses to subsequent S1P treatment in endothelial cells (Igarashi *et al.*, 2003).

### Regulation of matrix degradation

The breakdown of the extracellular matrix (ECM) by proteinases is an essential step in the angiogenic process, since it creates an environment to support endothelial cell migration and proliferation. Furthermore, breakdown of the matrix will liberate additional growth factors that are normally bound to heparan sulphates in the extracellular matrix. Matrix degradation is mainly achieved by proteolytic activity of membrane-type metalloproteinases. By making use of three-dimensional collagen and fibrin matrices, it was shown that S1P potently stimulated human endothelial cell invasion (Bayless & Davis, 2003). The S1P-induced invasion response was PTX sensitive and completely dependent on integrins and matrix metalloproteinases. In another study, treatment of bovine aortic endothelial cells overexpressing membrane type 1 matrix metalloproteinase with antisense oligonucleotides directed against S1P<sub>1</sub> and S1P<sub>3</sub> significantly inhibited membrane type 1 matrix metalloproteinase-dependent cell migration and morphogenic differentiation (Langlois *et al.*, 2004).

### Endothelial cell proliferation and migration

Subsequent to matrix degradation, endothelial cell proliferation and migration are critical steps in the angiogenic process and in both of these processes SMM do play a regulatory role. Since VEGF is a potent mitogen for endothelial cells and S1P and VEGF share many signalling pathways as discussed previously, it is not surprising that also S1P induces endothelial cell proliferation (Lee *et al.*, 1999b; Wang *et al.*, 1999; Kimura *et al.*, 2000). The S1P-induced proliferation was PTX-sensitive, indicating a receptor-dependent mechanism (Lee *et al.*, 1999b; Kimura *et al.*, 2000). In addition, Kimura *et al.* (2000) showed that the S1P-induced <sup>3</sup>H-thymidine incorporation into human aortic endothelial cells was blocked by the selective MAPK kinase inhibitor PD98059 but not by the MAPK p38 inhibitor SB203580.

Although S1P inhibits cell migration in most cell types, it is now clear that it stimulates migration in endothelial cells (Lee *et al.*, 1999b; Wang *et al.*, 1999; English *et al.*, 2000; Kimura *et al.*, 2000; Panetti *et al.*, 2000; Liu *et al.*, 2001; Morales-Ruiz *et al.*, 2001; Paik *et al.*, 2001). Also, the migratory effects of S1P seem to be mediated *via*  $G_i$ -coupled cell surface receptors since these effects could be blocked by PTX in all the before-mentioned studies. S1P-induced human umbilical vein endothelial cell migration is potently inhibited by antisense phosphothioate oligonucleotides against S1P<sub>1</sub> as well as S1P<sub>3</sub> receptors (Liu *et al.*, 2001; Paik *et al.*, 2001). Interestingly, both S1P<sub>1</sub> and S1P<sub>3</sub> receptors were required for Rho activation. In addition, C3 exotoxin blocked the S1P-induced migration process, suggesting that the Rho pathway is critical for S1P-induced cell migration (Paik *et al.*, 2001). Also, in the study by Liu *et al.* (2001), various strategies to interrupt Rho family signalling, including C(3) exotoxin, dominant/negative Rho, or the addition of Y27632 (a cell-permeable Rho-kinase inhibitor), significantly attenuated S1P- but not VEGF-induced migration. Also, a study by Morales-Ruiz *et al.* (2001) showed that the  $G_i$ -coupled S1P<sub>1</sub> receptor is critical for S1P-induced endothelial cell migration. As discussed previously, this study also showed that inhibition of NO synthesis diminished VEGF-induced migration, but not that induced by S1P. Exposure of endothelial cells to S1P leads to a PTX-sensitive

activation of both ERK and p38 (Lee *et al.*, 1999b; Kimura *et al.*, 2000). However, conflicting results have been reported regarding their respective roles in S1P-induced endothelial cell migration. For instance, in the report by Panetti *et al.* (2000), MAPK kinase inhibition with PD98059 caused a loss of phosphorylation of ERK, but resulted in only a minimal decrease in migration. This is in accordance with the findings of Lee *et al.* (1999b), who showed that S1P-induced migration was not affected by inhibition of ERK with U0126 and p38 MAPK with SB203580. Also, Kimura *et al.* (2000) have reported that MAPK kinase inhibition with PD98059 was ineffective in this respect. However, in this same study, SB203580 could inhibit S1P-induced endothelial cell migration, as has been shown by Liu *et al.* (2001). In a later report of the same group similar results were reported (Kimura *et al.*, 2003). Thus, although both ERK and p38 are activated upon S1P stimulation, there is most likely only a role for p38 MAPK in S1P-induced migration.

### Tube formation

Subsequent to migration and proliferation, the new outgrowth of endothelial cells needs to reorganize into a patent three-dimensionally tubular structure. Among many others, S1P might be a factor that has a regulatory role in this process. S1P induces tube formation in umbilical vein endothelial cells on Matrigel (a matrix-rich product prepared from Engelbreth-Holm-Swarm tumour cells whose primary component is laminin) (Lee *et al.*, 1999b) and bovine aortic endothelial cells grown on collagen gel (Wang *et al.*, 1999). This S1P-induced tube formation was PTX-sensitive and was markedly inhibited by the MAPK kinase inhibitor U0126 in contrast to S1P-induced migration. Similar results were later reported by Miura *et al.* (2003), who demonstrated that a Ras/Raf1-dependent ERK activation, mediated by PTX-sensitive G-protein-coupled receptors, mediates S1P-induced human coronary endothelial cell tube formation. Also, the PI3K, Akt, eNOS pathway may play a role in S1P-induced tube formation, since inhibition of either of these three components inhibited tube formation on Matrigel (Rikitake *et al.*, 2002). In a recent paper, Bayless & Davis (2003) have shown that S1P-induced endothelial cell invasion and tube formation in collagen and fibrin three-dimensional matrices require metalloproteinases. Future research is necessary to elucidate the exact role of S1P and other factors in the complex regulation of tube formation.

### Vascular maturation

The now newly formed tube has to be stabilized by recruiting mural cells (pericytes and vascular smooth muscle cells) and the production of an extracellular matrix. Besides platelet-derived growth factor B, angiopoietin 1 and transforming growth factor- $\beta$ , S1P is a key player in this process. First evidence for the role of S1P and its S1P<sub>1</sub> receptor in vascular maturation came from a study by Liu *et al.* (2000), in which they globally disrupted the S1P<sub>1</sub> gene in mice.

While this did not appear to affect vasculogenesis or angiogenesis, it led to embryonic haemorrhage and thereby to intrauterine death between E12.5 and E14.5. This was linked to incomplete vessel maturation, which was due to a defect in the recruitment of mural cells to the vessel wall. Later studies from these investigators using conditional endothe-

lium-specific and smooth muscle-specific S1P<sub>1</sub> receptor knockout animals demonstrated that the above effects were attributable to an endothelial S1P<sub>1</sub> receptor (Allende *et al.*, 2003). Although disruption of the platelet-derived growth factor B or its receptor  $\beta$  gene leads to a similar phenotype as disruption of the S1P<sub>1</sub> receptor gene (Leveen *et al.*, 1994; Soriano, 1994; Lindahl *et al.*, 1997) and although platelet-derived growth factor can stimulate S1P formation by smooth muscle cells (Olivera & Spiegel, 1993; Hobson *et al.*, 2001), the involvement of an endothelial S1P<sub>1</sub> receptor questions the proposed role of S1P in platelet-derived growth factor-induced vascular maturation.

Although there is a very clear important role for S1P in vessel maturation, further research is warranted to elucidate the mechanisms involved.

### In vivo angiogenesis

Owing to all the aforementioned characteristics of S1P, being involved in almost every aspect of the angiogenic process, one would expect S1P to be a potent *in vivo* angiogenic factor. Indeed, S1P proved to be very potent in promoting angiogenesis in the developing chick embryo chorioallantoic membrane (English *et al.*, 2001). However, in the matrigel plug implant model, S1P significantly potentiated the basic fibroblast growth factor and VEGF-induced angiogenesis, but S1P alone proved ineffective. Similar results were obtained in the mouse cornea micro pocket assay, where also S1P alone was not very potent but the angiogenic responses to basic fibroblast growth factor were markedly potentiated by the addition of S1P (English *et al.*, 2000). Basic fibroblast growth factor and S1P treatment increased the number of neovessels with well-developed adherens junctions and basement membrane-like structures (Lee *et al.*, 1999a).

### Other SMM in angiogenesis

Most studies concerning the role of SMM in angiogenesis have focused on the role of S1P in this process, as discussed in previous paragraphs. Only a few studies have investigated the role of other SMM such as SPC and sphingosine. Both SPC and sphingosine induce chemotactic migration of human and bovine endothelial cells, as potent as VEGF (Boguslawski *et al.*, 2000). In addition, both compounds induced *in vitro* tube formation on matrigel. Interestingly, in the same study, the authors showed that the response to sphingosine and SPC was associated with a downregulation of S1P<sub>1</sub> receptor mRNA. In analogy with S1P, SPC at low concentrations stimulates vascular smooth muscle cell migration, whereas higher concentrations (1  $\mu$ M or higher) have inhibitory effects (Boguslawski *et al.*, 2002).

It is unclear whether the effects of SPC and sphingosine are direct effects of these compounds or that they are mediated through the active conversion to either S1P or ceramide. As stated earlier, it is probably the actual balance between S1P and ceramide that determines cell fate, since most of the actions of S1P can be counteracted by ceramide. Ceramide has been shown to be growth inhibitory to endothelial and vascular smooth muscle cells and may induce apoptosis in these cell types (Lopez-Marure *et al.*, 2000; Johns *et al.*, 2001; Erdreich-Epstein *et al.*, 2002; Radin, 2003). For this reason, ceramide or its analogues are potential candidates to inhibit

tumour angiogenesis (Hayakawa *et al.*, 2002) or to function as anticancer agents in general, since ceramide is also involved in radiation or drug-induced apoptosis of tumour cells (Radin, 2003).

Increased knowledge of the mechanisms by which growth factors are involved in the angiogenic process may provide therapeutic targets to influence wanted or unwanted blood vessel formation. Although SIP is at least theoretically an interesting candidate for therapeutic angiogenesis because it is involved in so many aspects of angiogenesis, we have to realize that, because of the complexity of the angiogenic process, simply adding growth factors to an ischaemic area will not be sufficient to achieve therapeutic angiogenesis. However, the development of specific agonists or antagonists for the different SIP receptors may provide tools to influence at least endothelial or smooth muscle cell proliferation and migration, which also play important roles in other (patho)physiological processes.

### Chronic effects upon cardiac function

Two studies have assessed the growth effects of SMM using neonatal rat isolated cardiomyocytes as the model system. Both studies report SPC to induce hypertrophy in this model, whereas the effects of SIP were prominent in one study (Robert *et al.*, 2001) but not the other (Sekiguchi *et al.*, 1999). Moreover, dihydro-SIP was found to enhance hypertrophy in the neonatal rat cardiomyocytes (Robert *et al.*, 2001), whereas sphingosine lacked such effects (Sekiguchi *et al.*, 1999). The latter finding is in good agreement with reports that both ceramide (Bielawska *et al.*, 1997) and sphingosine (Krown *et al.*, 1996) can induce apoptosis in these cells. When SMM induce neonatal rat cardiomyocyte hypertrophy, this appears to involve activation of various protein kinases including ERK, p38, jun-N-terminal kinase, Akt and p<sup>70S6K</sup> (Sekiguchi *et al.*, 1999; Robert *et al.*, 2001).

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### Perspective

Based upon the above, it is evident that SMM can act on all major cells of the cardiovascular system. Their ubiquitous formation and their presence in plasma at high concentrations make them an interesting potential physiological regulator of cardiovascular function acutely and chronically. In our opinion, three main questions should be addressed by future research in this area. Firstly, the vast majority of studies until now has focused on cells and tissues from healthy animals, whereas, with the exception of cardiac ischaemia, only few studies have addressed SMM responses in pathophysiological settings. Such studies will be necessary to determine their role as a valid drug target in cardiovascular pharmacology. Secondly, it is one of the most fascinating aspects of SMM that several SMM, particularly SIP and SPC, can act potentially act as intra- and inter-cellular messengers. This is a biologically fairly unique role. Thus, identification of SMM targets, including their receptor subtypes, will be a major challenge. While considerable progress has been made in the identification of SIP receptor subtypes, molecular targets for other SMM, particularly the identity of possible SPC receptors, remain to be identified. Finally, it is obvious from the above that ceramide frequently has effects opposite to those of SIP and SPC, with sphingosine sometimes mimicking one and sometimes the other. This applies to the question of apoptosis/cell growth, vasodilatation/vasoconstriction and ischaemic heart damage. Since the various SMM can largely be metabolized to each other and since only few enzymes are required in this process, it appears that regulation of the activity of these enzymes could play a key role in cardiovascular pharmacology. Beyond this, opposing effects of such closely related messenger molecules is also a fairly unique situation in biology and hence its further study should also yield fundamental insight into homeostasis.

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