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Sphingosine 1-phosphate activation of ERM contributes to vascular calcification

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Running title: Sphingolipids regulate vascular calcification

Abbreviations: BGP, β-glycerophosphate; CDase, ceramidase; CKD, chronic kidney disease; ERM, ezrin-radixin-moesin; HMU-PC, 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine; NBD-C6-SM, (6-((N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yI)amino)hexanoyl)-C6-sphingomyelin; ox-LDL, oxidised-LDL; PKCα, protein kinase Cα; PP1α, protein phosphatase 1α; qPCR, quantitative RT-PCR; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; VSMC, vascular smooth muscle cells
ABSTRACT

Vascular calcification is the deposition of mineral in the artery wall by vascular smooth muscle cells (VSMC) in response to pathological stimuli. The process is similar to bone formation and is an independent risk factor for cardiovascular disease. Given that ceramide and sphingosine 1-phosphate (S1P) are involved in cardiovascular pathophysiology and biomineralisation, their role in VSMC matrix mineralisation was investigated. During phosphate-induced VSMC mineralisation, endogenous S1P levels increased accompanied by increased sphingosine kinase (SK) activity, and increased mRNA expression of SK1 and SK2. Consistent with this, mineralisation was increased by exogenous S1P but decreased by C2-ceramide. Mechanistically, exogenous S1P stimulated ezrin-radixin-moesin (ERM) phosphorylation in VSMC and ERM phosphorylation was increased concomitantly with endogenous S1P during mineralisation. Moreover, inhibition of acid sphingomyelinase and ceramidase with desipramine prevented increased S1P levels, ERM activation, and mineralisation. Finally, pharmacological inhibition of ERM phosphorylation with NSC663894 decreased mineralisation induced by phosphate and exogenous S1P. Although further studies will be needed to verify these findings in vivo, this study defines a novel role for the SK-S1P-ERM pathways in phosphate-induced VSMC matrix mineralisation, and shows that blocking these pathways with pharmacological inhibitors reduces mineralisation. These results may inform new therapeutic approaches to inhibit or delay vascular calcification.

Keywords: Arteries, acid sphingomyelinase, biomineralisation, cardiovascular disease, ceramides, ezrin, signal transduction, sphingosine kinase, smooth muscle cells, vascular biology
Vascular calcification, the deposition of bone-like material in the media and/or intima of arteries, is an independent risk factor for cardiovascular disease (1). Vascular calcification is associated with ageing and also occurs in response to injury, ionic and metabolic imbalance (2). There is now compelling evidence that vascular calcification is an active, regulated process in which vascular smooth muscle cells (VSMC) play an essential role. In response to pathological stimuli such as inflammatory cytokines or mineral imbalance, VSMC undergo osteogenic differentiation and apoptosis, releasing vesicles that initiate the deposition of a mineralised matrix (3, 4). Despite considerable research into the mechanisms and cellular processes that underlie vascular calcification there are still no effective therapies to prevent or reverse the condition (5). Therefore, further understanding of the mechanisms that contribute to development of this pathology is essential in order to identify potential therapeutic strategies and targets.

The sphingolipids, ceramide and sphingosine 1-phosphate (S1P) are important signalling molecules, regulating many cellular processes that determine cell fate (6). Within the cardiovascular system, ceramide and S1P are implicated in vascular and cardiac morphogenesis, maintenance of vascular tone, vascular permeability, inflammation, ageing and atherosclerosis (7-12). Additionally, evidence is emerging that sphingolipids are involved in vascular and valvular calcification. For instance, in VSMC in vitro ceramide mediates oxidised-LDL (ox-LDL) induced matrix mineralisation (13) and neutral sphingomyelinase, which produces ceramide from sphingomyelin, regulates matrix vesicle release and subsequent VSMC matrix mineralisation (14). Furthermore, a role for S1P in osteogenic differentiation and mineralisation of human aortic valve interstitial cells has been reported (15). However, the mechanisms by which sphingolipids regulate VSMC matrix mineralisation are still not fully understood.

A major source of ceramide within cells is from the hydrolysis of sphingomyelin by sphingomyelinases (SMase). The main classes of SMase are acid (aSMase) and neutral (nSMase), which are expressed ubiquitously and are also implicated in cell signalling (16-18). There are two forms of aSMase, secretory (s-SMase) and lysosomal (L-SMase), which are the products of a
single gene SMPD1 but undergo alternative post-translational modifications (19). L-SMase is active in lysosomes and forms part of the lysosomal sphingolipid salvage pathway (17). To date four mammalian nSMase have been identified; SMPD2 (nSMase1), SMPD3, (nSMase2), SMPD4 (nSMase3) and SMPD5 (mitochondria-associated nSMase) (20). Neutral SMases act on sphingomyelin at the plasma membrane. Activation of nSMase or L-SMase therefore results in production of ceramide in different subcellular compartments with access to different effectors. However, ceramide may not accumulate following SMase activation because it can be hydrolysed rapidly by ceramidases (CDase) to produce sphingosine, the precursor of S1P (21, 22). Whereas many metabolic routes can form ceramide, there is only one source of S1P, the phosphorylation of sphingosine by sphingosine kinases (SK1 and 2, encoded by the genes SPHK1, SPHK2 respectively). Accordingly, alterations in the activity of enzymes within this pathway may lead to accumulation of ceramide and/or S1P within the cell. Indeed, lysomotropic inhibitors such as desipramine, which induce proteolysis of L-SMase (23, 24) and acid CDase (25, 26) increase ceramide and limit S1P production due to depletion of sphingosine (25).

Ceramide and S1P have emerged as important regulators of the ezrin, radixin and moesin (ERM) family of proteins. ERM proteins are activated by phosphorylation (27). In MCF7 breast cancer cells S1P increases ERM phosphorylation (28), conversely ceramide induces ERM dephosphorylation (6). In VSMC, ERM proteins are implicated in proliferation, migration and adhesion (29, 30). Although there have been no reports of ERM involvement in VSMC mineralisation, several observations also link them to this process. For instance, ERM proteins are activated by Rho kinase and protein kinase Cα (PKCα) (27, 31) and both these kinases have been implicated in VSMC mineralisation (32-34). Ezrin also regulates Akt activity (35) and phosphate balance (36), both of which regulate VSMC mineralisation (37-39). Finally, ERM proteins promote osteogenic differentiation of mesenchymal stem cells (40). Together these studies demonstrate that ERM proteins are activated by kinases that regulate mineralisation and that they interact with proteins, which potentially regulate pathways that mediate vascular calcification.
Therefore, this study tests the hypothesis that VSMC mineralisation is regulated by sphingolipids and investigates the role of ERM proteins in this process. Accordingly, we demonstrate for the first time that ceramide inhibits VSMC mineralisation, whereas SIP is stimulatory. We also demonstrate that elevated-phosphate induces sphingosine kinase activity and increases SIP production, which stimulates matrix mineralisation via activation of ERM signalling in VSMC.
MATERIALS AND METHODS

Materials

Reagents were analytical grade and were obtained from Sigma-Aldrich (UK) unless otherwise stated. Desipramine was purchased from Sigma; NSC663894 from Merck Millipore; C2-ceramide and S1P from Enzo Life Sciences. Anti-rabbit phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (# 3149), anti-Ezrin (# 3145), anti-Radixin (# 2636) and anti-Moesin (# 3146) were from Cell Signaling Technology, anti-mouse β-actin (A1978) was from Sigma and the HRP-conjugated secondary antibodies (# 711-035-152 and 715-545-150) were from Jackson Immunoresearch.

Cell Culture

VSMCs were isolated from bovine aortic explants and cultured in high glucose Dulbecco’s modified eagles medium (DMEM) containing 10% (v/v) foetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine and 1 x non-essential amino acids (10% FBS-DMEM). For experiments, VSMCs were seeded at 2x10⁴ cells/cm² and maintained in 10% FBS-DMEM until 95% confluence (termed Day 0). At this point, VSMCs were induced to mineralise by the addition of 3 or 5 mM β-glycerophosphate (BGP) to 10% FBS-DMEM (osteogenic medium) (41). Control VSMCs were cultured in 10% FBS-DMEM alone (control medium). Medium was changed every 48 hours up to 16 days. Two batches of VSMCs isolated from different animals were used in these studies. Cells were used between passage 8 and 11.

Cell treatments

For mineralisation experiments in the presence of inhibitors (desipramine 1 and 10 µM; NSC668394 1 and 10 µM) or lipids (C2-ceramide 10 µM; S1P 0.01-10 µM), cells were treated continuously from Day 0. Where appropriate, an equivalent volume of vehicle was used as a
control for each compound: deionised H$_2$O (desipramine), DMSO (C2 ceramide, NSC668394). Medium, containing fresh inhibitors or lipids, was changed every 48 hours for up to 16 days.

**Quantification of mineralisation**

Alizarin red (40 mM, pH 4.1) was used to stain and visualize calcium-rich deposits (37). The extent of mineralisation was quantified by dye elution (42). The stages of matrix mineralisation in control cells incubated in osteogenic medium were based on absorbance values at 405 nm as follows; early mineralisation 0.09-0.25, mid mineralisation 0.25-1.25 and late mineralisation 1.25-2.5 (arbitrary units).

**Preparation of RNA and real time quantitative (RT)-PCR analysis**

Total RNA was isolated from VSMC using the RNAeasy mini kit (Qiagen); 1 µg RNA was used to produce cDNA using TaqMan reverse transcription reagents (Applied Biosystems) in an Eppendorf Master Cycler (Eppendorf). Quantitative RT-PCR (qPCR) using SYBR green was performed for $SMPD1$ (aSMase), $SPHK1$ (SK1) and $SPHK2$ (SK2) in a standard reaction mix of 12 µl containing 50 ng of cDNA, 0.83 µM forward and reverse primers and 1X SYBR green PCR master mix (Applied Biosystems) using a CFX96 Real-Time System (BioRad). Primer sequences are shown in the Supplemental Table S1. Samples were heated to 95°C for 10 minutes, followed by 40 cycles of: 95°C for 15 seconds, 60°C for 1 minute and 72°C for 15 seconds. Amplification of a single PCR product was confirmed by melt-curve analysis and PCR products were sequenced using a 3730 DNA Analyzer (Applied Biosystems) to confirm the correct target had been amplified. qPCR using TaqMan gene expression assays (Applied Biosystems) was performed for $SMPD2$ (nSMase1; GenBank™ accession number NM_001075383.2), $SMPD3$ (nSMase2; GenBank™ accession number NM_001192363.1) and $SMPD4$ (nSMase3; GenBank™ accession number NM_001205602.1). The standard reaction mix was 20 µl containing 82.5 ng of cDNA, 0.9 µM forward and reverse primers, 0.25 µM of the reporter probe and 1X TaqMan gene expression master mix (Applied Biosystems) using the same real-time system as above. Samples were heated to 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1
minute. For both assays, reactions were performed in duplicate and averaged to give one data point. Values were normalized to house-keeping genes (PPIA and RPL12) and results were calculated using the comparative Ct (threshold cycle) method (43).

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 25 mM sodium deoxycholate, 1% Triton X-100, 3.5 mM sodium dodecyl sulphate, 5% glycerol, 1 mM dithiothreitol, 50 µM sodium orthovanadate, 200 µM sodium pyrophosphate and protease inhibitors (Complete Mini-tab; Roche)) at 4°C for 20 minutes before centrifugation at 12,000xg for 15 minutes at 4°C. The protein concentration of the lysate was estimated by Bradford assay (BioRad) and adjusted to 1.2 mg/ml before addition of 5x Laemmlli sample buffer, giving a final protein concentration of 1 mg/ml. Equivalent amounts of protein (20-50 µg) were subjected to SDS-PAGE, transferred to nitrocellulose membrane, blocked in TBS/0.1% Tween containing 5% fish skin gelatin and probed with anti-rabbit; phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558), ezrin, radixin or moesin (1:1,000 in TBS/0.1% Tween containing 0.1% fish skin gelatin, overnight at 4°C) and anti-mouse β-actin (1:10,000 in TBS/0.1% Tween containing 0.1% fish skin gelatin, 30 minutes at room temperature) as loading control, followed by the appropriate HRP-conjugated secondary antibody (1:10,000 in TBS/0.1% Tween containing 0.1% fish skin gelatin). Signals were developed with chemiluminescence, imaged using a Chemidoc (BioRad) and band intensities quantified using Chemidoc software (BioRad).

Sphingomyelinase and sphingosine kinase activity assays

Cells were washed with PBS and lysed in Tris-TX-100 buffer (50 mM Tris pH 7.4, 5 mM EDTA, 0.2% Triton X-100, protease inhibitors (Complete mini-tab, Roche), 1 mM sodium orthovanadate, 200 µM sodium pyrophosphate) at 4°C for 20 minutes before centrifugation at 12,000xg for 15 minutes at 4°C. The protein concentration of the supernatant was determined by Bradford assay.
(BioRad) and adjusted to 1 mg/ml.

Neutral sphingomyelinase activity was measured in cell lysates using 6-((N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)amino)hexanoyl)-C6-Sphingomyelin (NBD-C6-SM) as described previously (10). Briefly, cell lysate (50 µg protein) was added to 100 µl of reaction mixture containing; 100 mM Tris pH 7.4, 10 mM MgCl$_2$, 0.2% Triton X-100, 10 mM dithiothreitol, 100 µM NBD-C6-SM and 100 µM phosphatidylserine. Following 30 min incubation at 37°C, reactions were terminated by the addition of 1 ml chloroform:methanol (2:1 v:v) and 200 µl dH$_2$O for phase separation. The upper aqueous phase was discarded and the lower organic phase dried under O$_2$-free N$_2$ gas and resuspended in 15 µl chloroform:methanol (2:1 v:v). Samples and NBD-C6-ceramide standard were spotted onto heat-activated silica gel 60 thin-layer chromatography plates (Merck) and developed in chloroform/methanol/10% NH$_4$OH (7/3/0.5 v/v/v). The plates were air-dried and the fluorescent lipid detected using an Alpha-Innotech Imager. NBD-C6-ceramide was identified by the co-chromatographed standard and quantified by densitometry using AlphaEaseFC software. Lysosomal-sphingomyelinase activity was measured using 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (HMU-PC) (44). Briefly, cell lysate (25 µg protein) was added to 55 µl of reaction mixture containing; 750 mM sodium acetate pH 5.2, 0.3% Triton X-100, 3 mM EDTA, 1.5 mM HMU-PC. After 1 hour at 37°C 500 µl stop buffer (0.5 M sodium bicarbonate pH 10.7, 0.25% Triton X-100) was added and fluorescence measured in a 300 µl aliquot using excitation 404 nm and emission 460 nm filters (FLx800 microplate reader (BioTek)). The levels of liberated HMU were quantified using an HMU standard curve.

Sphingosine kinase activity was measured using a commercial Sphingosine Kinase Activity Assay Kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer’s instructions. In brief, cell lysate (5 µg protein) was incubated in reaction buffer containing 100 µM sphingosine and 10 µM ATP for 20 minutes at room temperature. The reaction was stopped
by addition of 40 µl luminescence conjugated ATP detector, and kinase activity measured using an Orion L Microplate Luminometer (Titertek-Berthold).

**Measurement of sphingolipids**

For lipid analysis by mass spectrometry, VSMC were washed with PBS, lysed in RIPA buffer for 20 minutes at 4°C and protein concentration determined by Bradford assay (BioRad). Total cell lysate containing 0.5 mg protein was analysed for ceramide, dihydroceramide and sphingoid bases by tandem liquid chromatography/mass spectrometry (45). Lipid levels were normalised to cellular total protein. Lipid analysis was carried out at the Lipidomics Core Facility, Stony Brook University Medical Center, New York, USA

**Data Analysis**

The data are shown as the mean ± the standard error of the mean (SEM) as indicated in the appropriate figure legend. Statistical analyses were performed using a student t-test, or a one-way or two-way ANOVA followed by multiple comparison post-hoc test, as indicated in the figure legends. Results were deemed significant when $P < 0.05$. All statistics were calculated using GraphPad Prism 7.
RESULTS

Inhibition of the sphingolipid salvage pathway reduces VSMC matrix mineralisation

To investigate the role of the sphingolipid salvage pathway (Figure 1A) in matrix mineralisation, VSMC were cultured in osteogenic medium or osteogenic medium plus desipramine, a dual L-SMase and aCDase inhibitor (25, 26), for up to 14 days. Control cells were incubated in control medium. Deposition of a mineralised matrix was assessed and quantified by alizarin red staining at three time-points corresponding to early, mid and late mineralisation (Figure 1B). Incubation with desipramine (1 or 10 µM) in the presence of osteogenic medium significantly inhibited matrix mineralisation compared to cells incubated with osteogenic medium and vehicle (Figure 1B).

In order to determine whether desipramine may inhibit VSMC matrix mineralisation through effects on the sphingolipid salvage pathway we measured the levels of ceramide, sphingosine and S1P at early, mid and late mineralisation with and without desipramine (10 µM). When cells were incubated in osteogenic medium, a marked increase in S1P levels were detected at the late stage of mineralisation, this increase was abolished by co-incubation with desipramine (Figure 1C). Ceramide levels were similar in VSMC cultured in osteogenic medium compared to controls at the same time points (Figure 1C). However, in the presence of 10 µM desipramine there was a trend to increased ceramide throughout the mineralisation time course (Figure 1C), consistent with inhibition of the lysosomal sphingolipid salvage pathway (Figure 1A). Further analysis of the species of ceramide in VSMC demonstrated that culture in osteogenic medium did not affect the levels of any individual species throughout the time course of mineralisation compared to controls (Figure 2). However, incubation with desipramine significantly increased the levels of several individual ceramide species, C14, C22, C24:1, C24, C26:1 and C26 ceramides (Figure 2). Together these data suggest that S1P levels are increased during mineralisation of VSMC as a consequence of increased ceramide flux through the lysosomal
sphingolipid pathway, and that interfering with this pathway using desipramine limits the biosynthesis of S1P and inhibits mineralisation.

**Exogenous ceramide inhibits and S1P stimulates VSMC matrix mineralisation**

To explore whether ceramide and/or S1P regulate the deposition of a mineralised matrix by VSMC, the effect of exogenous ceramide and S1P on VSMC matrix mineralisation was studied. Incubation of VSMC with the cell permeable C2-ceramide (10 µM) in osteogenic medium decreased matrix mineralisation compared to controls incubated in osteogenic medium plus vehicle (Figure 3A). In contrast, exogenous S1P (10 µM) increased matrix mineralisation (Figure 3B). Prolonged treatment with C2-ceramide or S1P had no effect on VSMC cultured in control medium (Supplemental Figure S1A&B). These data demonstrate that S1P and C2-ceramide have opposing effects on VSMC matrix mineralisation.

**The mRNA expression of SK1 & 2 and SK activity increases during VSMC matrix mineralisation**

Ceramide and S1P are generated by sphingomyelinase-mediated hydrolysis of sphingomyelin and sphingosine phosphorylation by SKs respectively (Fig 1A). Accordingly we next determined whether changes in SK or SMase activity and/or expression correlated with the increase in S1P production during deposition of a mineralised matrix by VSMC. Total RNA was isolated from confluent, Day 0 (D0) VSMC and VSMC cultured in control or osteogenic medium at early, mid and late mineralisation and the mRNA expression levels of SKs; SPHK1 (SK1) and SPHK2 (SK2), and sphingomyelinas; SMPDI (aSMase), SMPD2 (nSMase1), SMPD3 (nSMase2), and SMPD4 (nSMase3) determined by qPCR. To investigate whether any changes observed in mRNA levels of sphingomyelinas and SKs lead to functional effects, enzyme activities of SK, L-SMase and N-SMase were also measured at the same time points.

SPHK1 (SK1) and SPHK2 (SK2) mRNA levels were increased at mid-mineralisation and SPHK2 (SK2) remained elevated at late-mineralisation in VSMC in osteogenic medium (Figure 4A). Concomitant with elevated mRNA levels, SK activity was also increased at late-
mineralisation. The increased expression and activity of the SKs occurred at the same time point at which high levels of S1P were measured (Fig 1C(iii)). There was no change in \textit{SPHK1} (SK1) and \textit{SPHK2} (SK2) mRNA levels in VSMC in control medium at any time point (Fig 4A). Similarly SK activity remained unchanged throughout prolonged culture in control medium (Fig 4A). To confirm that the changes in SK mRNAs were associated with deposition of a mineralised matrix and not a consequence of prolonged exposure to high phosphate, a separate population of VSMC that did not deposit a mineralised matrix in response to osteogenic medium was studied. There was no change in \textit{SPHK1} (SK1) or \textit{SPHK2} (SK2) mRNA up to 12 days of culture in either control or osteogenic medium in these cells (Supplemental Figure S2).

Next we investigated whether changes in SMase mRNA expression and/or activity changed throughout the mineralisation time course. \textit{SMPD1} (aSMase) mRNA levels did not change in VSMC cultured in either control or osteogenic medium (Figure 4B). The mRNA levels of \textit{SMPD2} (nSMase1) did not change during culture in control medium, but increased in VSMC in osteogenic medium at late mineralisation (Figure 4B). In contrast, \textit{SMPD4} (nSMase3) mRNA levels increased over the mineralisation time course, but there was no difference between VSMC in control and osteogenic medium (Figure 4B). \textit{SMPD3} (nSMase2) mRNA was not detected in the VSMC at any of the time points tested (not shown) even though these primers could detect \textit{SMPD3} mRNA in aortic endothelial cells (Supplemental Figure S3).

To explore whether changes in \textit{SMPD1, 2 & 4} mRNA expression led to changes in enzyme activity, L-SMase and nSMase activity were measured. Both L-SMase and nSMase activity increased between Day 0 and the early mineralisation time point and remained elevated throughout the time course in both control and osteogenic medium. Only at late mineralisation was there a difference between control and osteogenic medium when L-SMase (Fig 4C) and nSMase (Fig 4C) activity decreased in the mineralising cells.

Taken together we show that during the deposition of a mineralised matrix there is increased SK1 and SK2 mRNA expression, SK activity and increased S1P in VSMC, which is
accompanied by a decrease in both L-SMase and nSMase activity. These data are consistent with the hypothesis that endogenous sphingolipids are implicated in the regulation of matrix mineralisation.

**Mechanisms involved in S1P-induced matrix mineralisation of VSMC**

To explore the mechanisms involved in sphingolipid regulation of matrix mineralisation we investigated the adaptor proteins ezrin, radixin and moesin, which have been identified as downstream effectors of ceramide and S1P signalling (6, 28). Ceramide and S1P reciprocally regulate ERM activity by inducing dephosphorylation and phosphorylation respectively at the activation sites Thr567-ezrin, Thr564-radixin and Thr558-moesin (27). Immunoblot analysis identified ezrin and moesin in VSMC lysates, radixin was not detected (Figure 5A). The lack of detection of radixin in our VSMC may reflect poor antibody cross-reactivity with bovine radixin because radixin is expressed in rat aortic VSMC (30). Next we investigated whether ERM phosphorylation was altered during VSMC matrix mineralisation. Protein lysates were prepared from VSMC cultured in control, osteogenic or osteogenic plus desipramine medium at early, mid and late mineralisation. For all time points lysates were prepared from cells 48 hours post medium change. A pERM antibody that recognizes phosphorylation of all three proteins at the activating threonine detected a marked increase in pERM levels in mineralised VSMC compared to controls (Figure 5B). Indeed, a doublet was detected by the pERM antibody suggesting both ezrin (81kDa) and moesin (78kDa) were activated. Interestingly, the increase in ERM phosphorylation in VSMC in osteogenic medium compared to control correlated with the time point of increased S1P (Figure 1). Furthermore, desipramine, which prevents S1P production and matrix mineralisation (Figure 1), prevented the increase in ERM phosphorylation in VSMC in osteogenic medium (Figure 5B). Whether ceramide or S1P activate ERM proteins in VSMC has not been reported, and so was investigated here. Immunoblot analysis demonstrated that short term (5 and 10 minutes) stimulation of VSMC with 10 µM C2-ceramide did not alter pERM
levels (Figure 5C). In contrast, 10 µM S1P increased pERM levels at both 5 and 10 minutes (Figure 5C), demonstrating a potential for S1P to activate ERM proteins in VSMC.

These data suggest that S1P-induced activation of ERM is important for regulating VSMC matrix mineralisation.

**S1P-induced ezrin activation is necessary for VSMC matrix mineralisation**

The ezrin inhibitor NSC668394, which inhibits ezrin Thr-567 phosphorylation (46), was used to investigate whether ezrin activity is important for matrix mineralisation. Initially, to confirm that NSC668394 inhibited S1P activation of ezrin, VSMC were incubated with 1 or 10 µM NSC668394 for 16 hours before stimulation with 10 µM S1P for 10 minutes. Pre-incubation with 1 µM NSC668394 did not prevent S1P-induced ezrin phosphorylation (Figure 6A). However, at the higher concentration of 10 µM NSC668394 did inhibit the increase in phospho-ezrin in response to S1P (Fig 6A). Accordingly, the effect of 10 µM NSC668394 on VSMC matrix mineralisation was studied. These experiments demonstrated that 10 µM NSC668394 markedly inhibited mineralisation (Figure 6B). Prolonged treatment with 10 µM NSC668394 had no effect on VSMC cultured in control medium (Supplemental Figure S1C). Finally, rescue experiments were performed where VSMC were co-incubated in osteogenic medium with 10 µM NSC668394 and 10 µM S1P. Under these conditions S1P was unable to overcome the inhibitory effect of NSC668394, whereas S1P alone promoted mineralisation as reported above (Figure 6B).

These data demonstrate that inhibition of S1P-induced phosphorylation of ezrin is important for VSMC matrix mineralisation, identifying a novel pathway for regulation of vascular calcification.
DISCUSSION

Our results establish several novel findings regarding the regulation of VSMC matrix mineralisation by sphingolipids and the mechanisms by which they exert their effects. First our inhibition studies using desipramine show that VSMC matrix mineralisation is regulated, at least in part, through sphingolipids generated via the lysosomal sphingolipid pathway. Second, lipid analysis demonstrates that S1P increases during VSMC matrix mineralisation, and using qPCR and activity assays we show that increased mRNA expression and activation of SKs directly correlates with S1P production, implicating this pathway as a key regulator of matrix mineralisation. Third, we show that activation of ERM proteins is required for VSMC matrix mineralisation and that ERM activation requires elevated S1P levels. Furthermore in the presence of ezrin inhibition, S1P is no longer able to stimulate matrix mineralisation identifying ezrin as a downstream mediator of S1P-induced VSMC mineralisation. Finally, we show that in contrast to S1P, ceramide inhibits VSMC matrix mineralisation. Taken together, these results demonstrate that therapeutic manipulation of VSMC sphingolipid metabolism by inhibition of the lysosomal sphingolipid salvage pathway inhibits VSMC matrix mineralisation.

We detected an increase in cellular S1P in VSMC cultured in osteogenic medium when mineralisation was widespread. Within the cell S1P is formed by SK-mediated phosphorylation of sphingosine. During matrix mineralisation the mRNA expression of SK1 and SK2 and SK activity increased in VSMC concomitant with an increase in intracellular S1P, although only increased SK2 mRNA expression directly correlated with SK activity and elevated S1P levels, implicating this isoform in VSMC matrix mineralisation. Use of desipramine to inhibit S1P production prevented VSMC matrix mineralisation confirming the importance of sphingolipid flux through this pathway. However, we cannot rule out that decreased degradation of S1P (by either S1P lyase or S1P phosphate phosphohydrolases (21)) may also contribute to the increase in intracellular S1P levels at late mineralisation. Also, we showed that exogenous S1P increases phosphate induced VSMC matrix mineralisation. However, 10 µM S1P was required to
consistently increase the rate of phosphate-induced VSMC mineralisation, which is approximately 10-fold higher than human plasma concentrations (47, 48). Although, our studies were conducted in the presence of 10% FBS, which would bind S1P and lower the effective concentration. Additionally, we tested lower S1P concentrations and did observe a trend to increased mineralisation with 1 µM S1P indicating that the threshold for increasing mineralisation is between 1-10 µM S1P in vitro in the presence of 10% FBS. A requirement for supraphysiological concentrations of exogenous S1P to increase phosphate-induced VSMC mineralisation may reflect uptake into the cells and activation of intracellular pathways (49). We did not measure cellular S1P levels following addition of exogenous S1P, therefore a direct comparison between the increase in endogenous S1P observed in cells in osteogenic medium and the effects of exogenous S1P is not possible. However, our study does demonstrate that S1P is a major regulator of biomineralisation by VSMC, and suggests that under osteogenic conditions such as high phosphate in CKD VSMC may be a source of S1P, which could further predispose to vascular calcification.

In addition to inhibiting S1P production, desipramine treatment of VSMC in osteogenic medium increased ceramide levels throughout early, mid and late mineralisation. Desipramine induces downregulation of A-SMase and aCDase within lysosomes, which can lead to ceramide accumulation within the lysosome (23-26). Therefore, treatment of VSMC in osteogenic medium with desipramine shifted the cellular balance between ceramide and S1P, which may contribute to the inhibition of mineralisation by desipramine. These data therefore prompted us to investigate the effects of ceramide on VSMC matrix mineralisation. Cell permeable C2-ceramide decreased VSMC mineralisation in osteogenic medium, suggesting that increased ceramide in the presence of desipramine could be involved in the prevention of mineralisation observed with this inhibitor. However, there is evidence that water soluble C2-ceramide may not directly mimic the effects of hydrophobic long-chain endogenous ceramides (50). Accordingly, further studies are required to substantiate the role of ceramide in vascular calcification. Indeed, an inhibitory role for ceramide
is in direct contrast to a recent report in human femoral artery SMC where ceramide was
identified as a mediator of ox-LDL-induced matrix mineralisation (13). The reasons for these
apparently opposing results are unclear. In this latter study (13) ox-LDL was shown to rapidly
and transiently increase N-SMase activity and ceramide levels in human femoral artery SMC and
an inhibitor of nSMase2 prevented mineralisation. Activation of N-SMase will produce ceramide
outside of the lysosomal compartment giving the ceramide access to different effectors compared
to generation of ceramide by L-SMase, which remains trapped within the lysosomes. Kapustin
and coworkers (14) have reported in human coronary artery SMC that nSMase2 inhibition
prevents matrix vesicle release and reduces mineralisation in response to osteogenic medium.
However, although we could detect nSmase 1 and 3 expression in the VSMC used in our study,
we could not detect nSMase2. Therefore, differences in response to ceramide may reflect the
different N-SMases expressed. It is also noteworthy that neither of the studies in human VSMC
(13, 14) investigated whether endogenous ceramide levels changed during matrix mineralisation,
accordingly stimulation of mineralisation may have been due to conversion of ceramide to S1P.

A further novel finding of our study is that ERM phosphorylation is required for VSMC
matrix mineralisation. To our knowledge this is the first report that ERM proteins are potential
regulators of vascular calcification. Our demonstration that S1P stimulates ERM phosphorylation
in VSMC is in agreement with studies in HeLa and breast cancer cells (51, 52). Interestingly, in
HeLa cells stimulated with epidermal growth factor, SK2-derived S1P induced ERM
phosphorylation (51), which may also suggest that increased expression of SK2 during VSMC
mineralisation may be regulating ERM activity. Although it should be noted that the signalling
data was obtained following short term addition of S1P, whereas effects on mineralisation were
observed during treatment up to sixteen days. However, a time course demonstrated that ERM
phosphorylation increased at mid and late VSMC mineralisation when increased cellular S1P was
also detected. Furthermore using NSC668394, a small molecule inhibitor of ezrin threonine-567
phosphorylation (46), we showed that ezrin activity is required for VSMC mineralisation. Thus,
NSC668394 blocked S1P-induced ERM phosphorylation and VSMC matrix mineralisation. Additionally S1P was unable to overcome the inhibition of matrix mineralisation by NSC668394 demonstrating that ezrin acts downstream of S1P to regulate calcification. Similarly desipramine, which prevented the S1P increase and VSMC mineralisation, also prevented ERM phosphorylation, although it is also possible that in the presence of desipramine increased ceramide levels induced ERM dephosphorylation. Ceramide activates protein phosphatase 1α (PP1α) to dephosphorylate ERM (53). However, whether ceramide in the lysosomal compartment would have access to cytosolic PP1α is unclear. Certainly addition of cell permeable C2-ceramide to VSMC did not reduce basal ERM phosphorylation, which might suggest that during mineralisation increased S1P is the key regulator of this pathway.

In this study we used small molecule inhibitors of lysosomal sphingolipid metabolism and ezrin activity, desipramine and NSC663894 respectively. We believe that our study is the first to show that inhibition of these pathways may be clinically relevant in vascular calcification. Although further work with in vivo models of vascular calcification is required to substantiate our in vitro findings, studies in cancer have demonstrated that lysosomotropic agents such as desipramine sensitise cancer cells to chemotherapeutic agents and ezrin inhibition prevents osteosarcoma metastasis (54, 55). Taken together these and our study suggest that using small molecule inhibitors of these pathways may be of clinical relevance.

In summary, this study has demonstrated in VSMC that inhibition of S1P formation prevents, whereas exogenous S1P stimulates matrix mineralisation induced by osteogenic medium. Moreover, we have identified ERM proteins as important downstream effectors of S1P-induced VSMC matrix mineralisation in vitro. These novel findings add further evidence to the growing recognition of S1P as a regulator of vascular pathologies. Further work is now required to determine how ERM proteins regulate VSMC matrix mineralisation and to explore the potential of desipramine as an inhibitor of vascular calcification in vivo.
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Figure 1: The effect of desipramine on VSMC matrix mineralisation and sphingolipid levels. 
A: Diagram of the lysosomal sphingolipid salvage pathway showing points in the pathway inhibited by desipramine. L-SMase, lysosomal sphingomyelinase; aCDase, acid ceramidase; SK, sphingosine kinase. B: VSMC were cultured in control, osteogenic (Osteo) or osteogenic with 1 or 10 μM desipramine (Des) medium. Matrix mineralisation was detected and quantified by Alizarin Red staining. **(i):** Photomicrographs representative of early, mid and late stages of mineralisation shown by positive Alizarin staining (red), scale bar = 500µm. **(ii):** Quantification of matrix mineralisation by elution of Alizarin Red. Data are mean±SEM from 3 independent experiments, *P<0.05 for osteogenic vs osteogenic plus desipramine. **(iii):** Mass spectroscopy analysis of **(i)** ceramide, **(ii)** sphingosine and **(iii)** S1P from VSMC at Day 0 (D0) and the early, mid and late stages of mineralisation. Data are mean±SEM from 3 independent experiments, +P<0.05 for osteogenic vs osteogenic plus desipramine, * P<0.05 for control vs osteogenic, by two-way ANOVA with Dunnett’s multiple comparisons post-test.
Figure 2: The effect of desipramine on individual ceramide species during VSMC matrix mineralisation. VSMC were cultured in control, osteogenic or osteogenic with 10 µM desipramine medium. Individual ceramide species were analysed using mass spectroscopy from VSMC at Day 0 (D0) and early, mid and late stages of matrix mineralisation. The data are mean±SEM from 3 independent experiments. * P<0.05 for osteogenic vs osteogenic plus 10 µM desipramine by two-way ANOVA with Dunnett’s multiple comparisons post-test.
Figure 3: The effect of C2-ceramide and S1P on VSMC matrix mineralisation. A: VSMC were cultured in osteogenic (Osteo) or osteogenic with 10 µM C2-ceramide medium. At mid-mineralisation the cells were stained with Alizarin Red. A(i): Representative photomicrographs showing mineralisation (red), scale bar = 500µm and A(ii): quantification of matrix mineralisation by elution of Alizarin Red. Data are mean±SEM from 6 independent experiments. *P<0.05 osteogenic vs osteogenic plus C2-ceramide by paired t-test. B: VSMC were cultured in osteogenic medium with the indicated concentrations of S1P. At early-mineralisation the cells were stained with Alizarin Red. B(i): Representative photomicrographs showing mineralisation (red), scale bar = 500µm and B(ii): quantification of matrix mineralisation by elution of Alizarin Red. Data are mean±SEM from 3 independent experiments. *P<0.05 osteogenic vs osteogenic plus S1P by one-way ANOVA with Tukey’s multiple comparisons post-test.
Figure 4: The mRNA expression profile and activity of sphingosine kinases and sphingomyelinases during VSMC matrix mineralisation. VSMC were cultured in control or osteogenic (Osteo) medium. At Day 0 (D0), early, mid and late mineralisation cell lysates were prepared for activity assays or total RNA was extracted for quantification of target genes by qPCR. A: SPHK1 and SPHK2 expression, sphingosine kinase (SK) activity, B: SMPD1, SMPD2 and SMPD4 expression, C: Lysosomal sphingomyelinase (L-SMase) and neutral sphingomyelinase (nSMase) activity. mRNA levels are shown relative to the house-keeping genes PP1A and RPL12, as detailed in Methods. The data are mean±SEM from 8 independent experiments. *P<0.05 control vs Osteogenic by two-way ANOVA with Dunnett’s multiple comparisons post-test.
Figure 5: S1P dependent activation of ERM during VSMC matrix mineralisation. 

A: Cell lysates were prepared from confluent VSMC cultured in control medium for immunoblot analysis for ezrin, radixin and moesin. β-Actin was used as a loading control. An immunoblot with duplicate samples is shown. B: VSMC were cultured in control, osteogenic or osteogenic with desipramine (10 µM) medium, and cell lysates prepared for immunoblot analysis with pERM at early, mid and late matrix mineralisation. β-Actin was used as a loading control. The immunoblot is representative of 3 independent experiments. C: VSMC were stimulated with 10 µM C2-ceramide (Cer) or 10 µM S1P for 5 or 10 minutes and cell lysates prepared for immunoblot analysis for pERM. β-Actin was used as a loading control. Representative immunoblot and densitometric data of pERM corrected for loading are shown. The data are mean±SEM from 3 independent experiments. * P<0.05 for control vs 10 µM S1P by one-way ANOVA with Tukey’s multiple comparisons post-test.
**Figure 6**

**Inhibition of S1P dependent activation of ERM prevents VSMC matrix mineralisation.**

**A:** VSMC were incubated with vehicle 0.1% DMSO, 1 or 10 μM NSC668394 for 16 hours before stimulation with 10 μM S1P for 10 minutes, and cell lysates prepared for immunoblot analysis with pERM. β-Actin was used as a loading control. Representative immunoblot and densitometric data of pERM corrected for loading are shown. The data are mean±SEM from 3 independent experiments. *P*<0.05 for control vs 10 μM S1P by two-way ANOVA with Dunnett’s multiple comparisons post-test.

**B:** VSMC were cultured in osteogenic (oste) medium, osteo plus NSC668394 10 μM, osteo plus S1P 10 μM or osteo plus NSC668394 10 μM plus S1P 10 μM. At the mid-mineralisation time point cells were stained with Alizarin Red. Representative photomicrographs showing mineralisation (red), scale bar = 500μm and quantification of matrix mineralisation by elution of Alizarin Red. Data are mean±SEM from 6 independent experiments. *P*<0.05 by one-way ANOVA with Tukey’s multiple comparisons post-test.