Sphingosine-1-phosphate induces contraction of valvular interstitial cells from porcine aortic valves

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Aims

Sphingosine-1-phosphate (S1P) has emerged as a potent bioactive lipid with multiple functions in cardiovascular pathophysiology. Potential roles of S1P in heart valve diseases and expression of relevant receptors (S1P1, S1P2, or S1P3) in valve tissue and in valvular interstitial cells (VICs), the major cell population with essential functions in maintenance of valvular structure, are currently unknown.

Methods and results

Exposure to S1P (62–2000 nM) of cultured VICs from porcine aortic valves on cell culture polystyrene resulted in contraction and nodule formation. The S1P-dependent contraction was completely inhibited by blockers of S1P2, RhoA, and RhoA-associated protein kinase (ROCK). Activated RhoA was clearly increased after S1P treatment, whereas activated Rac1 was only slightly reduced. In addition, exposure to S1P induced a transient increase in cytosolic \( \text{Ca}^{2+} \). Application of channel blockers and other effectors of \( \text{Ca}^{2+} \) homeostasis showed that the S1P effect is largely caused by \( \text{Ca}^{2+} \) release from internal stores. However, resistance to blocking S1P2, different kinetics, as well as concentration dependence exclude a major role of \( \text{Ca}^{2+} \) influx in S1P-induced nodule formation. In order to verify the effects in situ, contractions of valve tissue slices were measured. The S1P-induced isometric contraction of valve leaflets was of similar force amplitude as observed with adrenaline. The effect was fully reversed by blocking S1P2.

Conclusion

The results suggest that S1P induces contraction of VICs from porcine aortic valves by signalling via S1P2, RhoA, and ROCK. In this way, S1P may contribute to regulation of tissue tension in aortic valves.

Keywords

Sphingosine-1-phosphate • Aortic valve • Valvular interstitial cell • RhoA • Valve contraction

1. Introduction

Valvular interstitial cells (VICs) constitute the most prominent cell type in heart valve leaflets. VICs are a heterogeneous group of cells of multiple origins with mostly fibroblast properties. The main physiological roles of VICs are synthesis of extracellular matrix (ECM) components during development, and maintenance of ECM composition and valve structure throughout adult life.\textsuperscript{1–3} Like fibroblasts in general, VICs can acquire an activated state (myofibroblasts) upon exposure to different stimuli including growth factors, mediators of inflammation, or mechanical stress and changes of the ECM composition.\textsuperscript{2,4} Myofibroblasts are characterized by the ability to contract and migrate due to expression of \( \alpha \)-smooth muscle actin (\( \alpha \text{SMA} \)) and other components of the contractile apparatus, and by their increased capacity to release ECM components and remodelling enzymes like matrix metalloproteinases.\textsuperscript{2} The percentage of myofibroblasts among VICs varies throughout development, with high levels during foetal maturation and very low abundance during adult life.\textsuperscript{5} An increase in activated VICs in adult valves is considered as an indicator of pathological processes.

To a major extent, isolated VICs acquire the myofibroblast phenotype, at least under the standard two-dimensional (2D) cell culture conditions. Nevertheless, cultured VICs have become a valuable tool to investigate pathophysiologically relevant mechanisms. In particular, the ability of VICs to contract spontaneously\textsuperscript{6} and to form multicellular aggregates (nodules) accumulating Ca-phosphate has been used for \textit{in vitro} studies of valve calcification mechanisms. Both ERK\textsuperscript{7} and RhoA\textsuperscript{8} pathways were found to play...
important roles in myofibroblast differentiation and induction of nodule formation.

In early reports on valve structure, VICs were recognized as a complex cellular network spanning the entire valve and comprising 30% of the valves’ volume. The observation of closely apposed adrenergic nerve endings led to the concept that VICs may contribute to tissue tone, meaning state of tension and resistance against mechanical stress. Meanwhile, the direct effects of numerous vasoactive compounds, such as norepinephrine, serotonin, endothelin, histamine, angiotensin I, and thromboxane A2, on force of contraction and stiffness of valve tissue have been demonstrated, suggesting implications of receptor stimulation for the function and structure of valve cusps. It should be noted that several drugs acting on the serotonin 5-HT1A receptor had to be withdrawn because cases of fibrotic heart valve disease have been reported.

Because of its high abundance in plasma and multiple regulatory functions in the cardiovascular system, sphingosine-1-phosphate (S1P) is another candidate for modulating VIC functions. This ubiquitous lipid mediator induces a large array of cellular responses in smooth muscle cells and fibroblasts including contraction, migration, activation, or differentiation or proliferation. S1P has major effects on cardiovascular physiology and pathology relating to vascular tone and remodelling, permeability, heart rate, and cardiac contractility and protection against cardiac ischaemia. S1P mediates its effects via G protein-coupled receptors. Five distinct receptor subtypes (S1P1–S1P5) are presently known, and cell responses depend on the profile of receptor subtypes expressed in the particular cell type. In the cardiovascular system, S1P1–S1P3 are expressed, which couple to different G proteins thereby producing different responses in various cell types.

However, nothing is known about the potential role of S1P in heart valves and about the signalling pathways involved. If receptors are expressed in VICs, effects on cell contraction and possibly differentiation have to be expected resulting in stiffening of valve tissue with long-term pathologically relevant consequences like fibrotic modifications. Therefore, VICs isolated from porcine aortic valve leaflets were used to investigate the effects of S1P on the cellular Ca²⁺ status and RhoA-dependent contraction. The S1P receptor subtypes and signalling pathways involved in the responses were analysed with subtype-selective blockers. In addition, results obtained with cultured cells were verified by measuring force of contraction in strips of valvular tissue.

2. Methods

2.1 Cell culture

Porcine hearts from 5- to 6-month-old Landrace pigs were obtained from a local abattoir. Aortic valves were excised within 60 min of harvest. Valve leaflets were washed in Hank’s buffered salt solution and thereafter in DMEM/Ham’s F12 supplemented with 2.5% antibiotic/antimycotic. In order to remove endothelial cells, the intact leaflets were incubated at 37°C for 20 min in a shaking incubator in the same medium supplemented with 500 U/mL collagenase type II and 1 U/mL dispase II. The VICs were then released by collagenase alone (4 h, 37°C) and cultured in DMEM comprising 10% FCS, penicillin/streptomycin, and 2 mM glutamine, on collagen-coated flasks by standard procedures. Cells on 24-well plates were extracted by scraping with 100 μL of the test kit’s lysis buffer. The further procedure precisely followed the supplier’s instructions. The concentrations of total RhoA and Rac1 in the protein extracts were estimated by western blot analysis.

2.2 Analysis of VIC immunophenotype

Immunocytochemistry was performed on Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL, USA) using the ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) for immunoperoxidase staining. Cells were fixed for 10 min in acetone at −20°C and dried. Quenching (0.3% H₂O₂, 10 min), blocking, incubation with primary (1 h) and secondary (30 min) antibodies, visualization with diaminobenzidine, and counterstaining with Hematoxylin Q5 were carried out as recommended by the manufacturer.

2.3 Visualization and quantification of VIC contraction

After induction of nodule formation for 24 h in DMEM comprising 2% FCS and other additives as outlined in Section 3, cells were washed with PBS and fixed in 4% phosphate-buffered paraformaldehyde. Cells were washed again in PBS before they were stained for 5 min with eosine-methylene blue solution. Nodules and other major cell aggregates were visualized as dark spots before a clear background using an electron microscope at maximum contrast (see Supplementary material online, Figure S1). Spot staining was quantified using Gel-Pro Analyzer 3.1 software (Media Cybernetics, Silver Spring, MD, USA).

2.4 Real-time PCR

Total RNA from VICs was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany), and reverse transcribed and mRNA expression of S1P1, S1P2, and S1P3 was determined by SYBR green-based real-time PCR as described. Sequences of primers are given in Supplementary material online.

2.5 RhoA and Rac1 activation assays

Active, GTP-bound RhoA and Rac1 were assessed by the G-Lisa assay (Cytoskeleton, Denver, CO, USA). Cells on 24-well plates were extracted by scraping with 100 μL of the test kit’s lysis buffer. The further procedure precisely followed the supplier’s instructions. The concentrations of total RhoA and Rac1 in the protein extracts were estimated by western blot analysis.

2.6 Detection of intracellular Ca²⁺

The release of intracellular Ca²⁺ upon treatment with S1P was determined using test kit reagents (Screen Quod Fluoro-8 No Wash Calcium Assay) from ATT Bioquest, Sunnyvale, CA, USA. VICs were plated at 5 × 10⁴ cells/cm² in black, clear bottom 96-well plates and grown to confluence before they were serum-starved overnight in 1% FCS (100 μL/well). Cells were loaded with Fluoro-8 NW ester by adding an equal volume of the test kit’s dye-loading solution and by incubations at 37°C and subsequently at room temperature, both for 30 min. Fluorescence was measured using an excitation/emission filter pair of 485/535 nm on an Infinite F200pro plate reader (Tecan, Crailsheim, Germany) at intervals of 10 s.

2.7 Western blot analysis

Cell layers in 24-well plates were scraped off into SDS-sample buffer for electrophoresis without reducing agent and glycerol. Samples were sonicated and centrifuged before the protein content was quantified using the BCA assay (Thermo Scientific, Rockford, IL, USA). Glycerol and dithiothreitol were added, and equal amounts of protein per lane were electrophoresed as reported previously.

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2.2 Analysis of VIC immunophenotype

Immunocytochemistry was performed on Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL, USA) using the ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) for immunoperoxidase staining. Cells were fixed for 10 min in acetone at −20°C and dried. Quenching (0.3% H₂O₂, 10 min), blocking, incubation with primary (1 h) and secondary (30 min) antibodies, visualization with diaminobenzidine, and counterstaining with Hematoxylin QS were carried out as recommended by the manufacturer.
2.8 Contraction of tissue strips
Aortic valves were excised from hearts of 6- to 8-month-old Landrace pigs within 60 min of harvest. Left and right coronary aortic leaflets were cut in the circumferential direction yielding strips of \( \sim 5 \times 10 \text{ mm} \) in size. Dissection was carried out in oxygenated, modified Tyrode's solution, and force of contraction of pair-wise mounted tissue strips was recorded.\(^{28}\) Samples were loaded with the pre-tension of 3 mN and allowed to relax to a stable baseline over a period of 60 min.

Tissue viability of every strip was tested by applying KCl (90 mM), which was washed out after the contractile response had reached a stable plateau. Following return to baseline, strips were pre-incubated with S1P receptor inhibitors W146 (2 \( \mu \text{M} \)), JTE-013 (2 \( \mu \text{M} \)), and BML-241 (20 \( \mu \text{M} \)), or with equivalent amounts of vehicles, over a period of 30 min. Under all conditions, the effects of S1P were evaluated by comparing the S1P group receiving cumulative S1P concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, and 5 \( \mu \text{M} \); 10 min incubation time for each concentration) vs. a control group receiving the S1P vehicle NaOH (DMSO + NaOH group) in respective concentrations.

2.9 Statistics
Differences between the two groups were analysed by Student’s \( t \)-test, whereas ANOVA with post hoc least significant difference was used to analyse multiple treatment groups. Differences were considered significant at values of \( P < 0.05 \). Log EC\(_{50} \) (M) values were estimated from fitting a Hill function with different slopes to the concentration–effect curve of S1P. When appropriate, we used an equation for two receptor populations, taken from Graph Pad Prism version 4.03 for Windows (Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1 Characterization of VICs and S1P-induced nodule formation
Initially, cultured cells from porcine aortic valves were characterized by immunocytochemistry using antibodies against established marker proteins.\(^{29} – 31 \) Most of the cells (64 – 6%) expressed \( \alpha \text{SMA} \), almost all cells (98 ± 2%) stained positive for vimentin, and 90 ± 7% expressed smooth muscle myosin. Markers for endothelial cells and macrophages were detected in less than 1% (CD31, CD34) of the cells, and the marker of muscle cells desmin was found in 6 - 2% of the cells. These data justify the classification of the cultivated cell fraction as VICs and are in accordance with other reports on this topic.\(^{29} – 31 \) As indicated by the expression of \( \alpha \text{SMA} \), at least 64% of cells were in the activated state (myofibroblasts) due to growing on the rigid surface of the culture material. Similar results have been reported with VICs of aortic valves from various species.\(^{32} \)

Upon transfer from collagen-coated surfaces to non-coated tissue culture plastic, VICs showed the tendency to contract and aggregate into nodules (see Supplementary material online, Figure S1). Spontaneous contraction began 4 – 5 days after reaching confluence. Addition of S1P to the medium at earlier time points (1 – 2 days confluent) induced a robust increase in nodule formation. To obtain nodules of sufficient size for densitometric evaluation, measurements were carried out 24 h after addition of S1P. The concentration–response curve (Figure 1) exhibited two distinct phases. A significant increase over controls was detected already at 62 nM of S1P, with no further increase between 62 and 250 nM. The second increase in nodule formation occurred at 500 nM. Therefore, two log EC\(_{50} \) (M) values (−7.5 and −6.0) were calculated by fitting a Hill function with different slopes to the concentration–effect curve. Nodule formation was increased 34.6-fold over untreated controls at 2 \( \mu \text{M} \), the highest tested concentration of S1P.

Cellular effects of S1P in the cardiovascular system are mediated via three of the five known S1P receptor subtypes, i.e. S1P1, S1P2, or S1P3.\(^{33} \) Real-time PCR showed that mRNA of all three subtypes was expressed in porcine VICs, with S1P1 being the most abundant receptor in freshly isolated cells. Expression of all subtypes increased during subculturing up to passage 3 (see Supplementary material online, Figure S2). A panel of specific inhibitors was used to identify the relevant S1P receptor subtype(s) in VICs. The S1P2 blocker, JTE-013, completely prevented the S1P-dependent nodule formation at all tested concentrations (Figure 1), whereas the S1P3 receptor antagonist, suramin, was without effect (Figure 2A). Unexpectedly, the two blockers with affinity to S1P1 (VPD-23019 and W146) increased rather than impaired S1P activity at all tested concentrations of S1P, although neither compound was active on its own in the absence of S1P (Figure 2B and C). Taken together, the results suggest that the stimulating effects of S1P on contraction and nodule formation of VICs are mediated only via S1P2 receptors.

It should be noted that the selectivity of JTE-013 has recently been questioned. Long et al.\(^{32} \) showed that JTE-013 also blocked S1P4 which is not relevant in this context, and Salomone et al.\(^{33} \) observed that JTE-013 (10 \( \mu \text{M} \)) inhibited endothelin- and U46619-induced contraction of cerebral arteries in rats and mice. Endothelin-1 was without effect in VICs, but the thromboxane analogue U46619 induced marked contraction. This response was not inhibited by JTE-013 at 2 \( \mu \text{M} \). Furthermore, JTE-013 significantly blocked S1P-induced contraction in a concentration as low as 0.2 \( \mu \text{M} \) (see Supplementary material online, Figures S3 and S4). Therefore, it is highly improbable that the off-target effects which have been observed at high concentration of JTE-013 in another system\(^{31} \) also occurred in VICs.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/93/3/490/497245/492)
3.2 S1P signalling pathways in VICs

In most responsive cell types, activation of S1P2 receptors is coupled to contractile function via RhoA signalling. Rapid RhoA activation was observed in VICs with a maximum effect at 1 min after stimulation with 2 μM S1P (Figure 3A). The level of activated RhoA markedly decreased within the next 10 min, but a significant increase over controls was still detectable after 30 min. The essential role of RhoA in VIC contraction was further demonstrated by direct inhibition using the cell penetrating form of C3 transferase (C3) from Clostridium botulinum. The S1P-induced nodule formation was reduced to 8% of controls in the presence of 2 μg/mL C3, and significant inhibition was observed with 0.2 μg/mL C3 (Figure 3B). Accordingly, the S1P-induced RhoA activation was significantly reduced in the presence of the S1P2 blocker JTE-013 (see Supplementary material online, Figure S5). In contrast, a tendency to increase RhoA activity was observed with the blockers of S1P1 (VPC-23019: 1.15-fold, P = 0.37; W146: 1.20-fold, P = 0.21). Modification of Rac1 activity constitutes another pathway of S1P signalling, especially of S1P1-mediated effects. Treatment of VICs with S1P resulted in a small but significant (28%) reduction in Rac1 activity. However, effects of receptor blockers on Rac activation were not observed.

One of the targets of RhoA is Rho-associated protein kinase (ROCK). Therefore, the highly specific inhibitors of this kinase, Y-27632 and H-1152, were tested. With 2 μM Y-27632, S1P-induced nodule formation was completely blocked, and in the presence of 0.2 μM Y-27632, nodule formation was reduced to 59%. A similar result was obtained with H-1152, but a concentration of 0.2 μM

Figure 2 Effects of S1P receptor blockers with different specificity on S1P-dependent VIC contraction. Nodule formation was tested as outlined in the legend of figure 1, but in the presence (black bars) or absence (white bars) of suramin (A), VPC-23019 (B), or W146 (C). The concentration of inhibitors was 2 μM in all cases. The data were normalized to the values measured with 2 μM S1P in the absence of receptor blockers. While the blocker with affinity to S1P3 (suramin) was without an effect, the S1P1 blockers (VPC-23019 and W146) increased VIC contraction in the presence of S1P in all tested concentrations. Means ± SD, n = 4. *P < 0.05, S1P without vs. with receptor blocker.

Figure 3 Signalling via RhoA in relation to S1P-induced VIC contraction. (A) Activated RhoA in VICs was detected at time points ranging from 1 to 30 min after addition of S1P (2 μM) by medium change (black bars). Controls received no additions or vehicle (10 mM NaOH) in matched concentrations (white bars). (B) Nodule formation by S1P (2 μM) was inhibited by C3 transferase (C3) in concentrations of 0.2 and 2.0 μg/mL. Both agents were added simultaneously by medium change (2% FCS). Data are normalized to the values in assays with 2 μM S1P without C3. Means ± SD, n = 4. *P < 0.05, **P < 0.005, S1P vs. vehicle at each time point (A), or all other treatments vs. 2 μM S1P (B).
was sufficient for complete inhibition (Figure 4). In contrast, blockers of other potential S1P2 receptor-activated pathways, as for instance MAP kinase inhibitor PD-98059 and PI3K inhibitor LY294002, were without an effect. In addition, the inhibitor of actin-dependent serum response factor activation CCG-1423 was also ineffective (Figure 4), suggesting that de novo synthesis of αSMA and other components of the contractile apparatus\(^3^4\) was not altered by exposure to S1P. Accordingly, the expression levels of αSMA and other markers of fibroblast differentiation were not changed, even when exposure was extended over several days (see Supplementary material online, Figure S6).

The S1P-induced contraction of VICs may involve increase in cytosolic Ca\(^{2+}\) concentration.\(^2^3\) To test this hypothesis, cells were loaded with the fluorescent Ca\(^{2+}\) probe Fluo-8 before receptor activation with S1P. Addition of S1P elicited a rapid, transient increase in cytosolic Ca\(^{2+}\), with a maximum at 20–30 s and a slow return to baseline within \(~2\) min. The threshold concentration was 2 nM, and the maximum response was observed at 1 \(\mu\)M (Figure 5A). Cytosolic Ca\(^{2+}\) can be elevated due to Ca\(^{2+}\) release from internal stores [mainly endoplasmic reticulum (ER)] or due to transmembrane Ca\(^{2+}\) influx into the cell. To test the contribution from internal stores, the ER was emptied by exposure to thapsigargin (5 \(\mu\)M) before stimulation with S1P (Figure 5B). The response to S1P was significantly reduced to 26.9 ± 4.0% (\(P = 0.014\)) of control. In contrast, when transmembrane Ca\(^{2+}\) influx was blocked with the rare earth Gd\(^{3+}\) or the L-type Ca\(^{2+}\) channel blocker nifedipine, the S1P-elicted Ca\(^{2+}\) increase in VICs was not impaired. Selective blockers of S1P receptors were used to identify the receptor subtypes involved.
JTE-013 (S1P2) was without a significant effect, whereas VPC-23019 (S1P1 and S1P3) and W146 (S1P1) partially reduced the S1P-induced Ca\(^{2+}\) release (Figure 5C).

### 3.3 Contraction of aortic valve tissue

To evaluate the impact of S1P and activation of the S1P2 receptor on contraction of VICs in situ, we measured contraction of strips from aortic valve leaflets. Exposure to 90 mM KCl evoked a mean increase in force of contraction of 0.5 ± 0.03 mN under control conditions without significant difference between the experimental groups. The contraction induced by S1P in the presence and absence of inhibitors and carriers is expressed in percentage of KCl-evoked contraction. Under control conditions, tissue strips relaxed over time to up to 84% of 100% basal tone (Figure 6). Increasing concentrations of S1P significantly enhanced force of contraction up to 148.5% at 5 μM compared with control. Inhibition of S1P1 using W146 reduced the S1P effect (Figure 6A). S1P-induced contraction was completely blunted in the presence of JTE-013 (Figure 6B), whereas the S1P3 blocker BML-241 was without an inhibitory effect (Figure 6C).

### 4. Discussion

Interstitial cells in heart valves express receptors for numerous vasoactive effectors that contribute to the regulation of tissue tone and flexibility in cusps and leaflets. Here, we show that the lipid mediator S1P also exerts robust effects in porcine aortic valves. The underlying mechanisms were studied in isolated VICs. These cells are not terminally differentiated and they are activated during pathological conditions in vivo and when cultured in vitro. The resulting myofibroblast phenotype possesses properties of fibroblasts and smooth muscle cells. Activated VICs are able to contract and migrate due to the expression of αSMA and other contractile proteins. Coordinated contraction (but not migration) of a large number of adherent cells at high cell density results in the formation of nodules.\(^4\) Increase in nodule formation was prominent with S1P concentrations within the physiological range (in serum of up to 900 nM).\(^3\) The S1P-induced contraction was not correlated with an increase in αSMA expression. Therefore, the effect is most probably due to activation and possibly restructuring of the pre-existing contractile apparatus.

The receptor subtypes involved and downstream effects were analysed with specific blockers and pathway inhibitors as well as by direct determination of RhoA and Rac1 activation. The S1P-dependent nodule formation was completely blunted by the selective S1P2 receptor blocker JTE-013 as well as by the ROCK inhibitors H-1152 and Y-27632. Stimulation with S1P transiently increased activated RhoA, and nodule formation was completely blocked by cell permeable C3, a specific inhibitory toxin for RhoA. These results suggest that the S1P effect was mediated via receptor subtype S1P2 and RhoA pathway, and coupling to the contractile system was due to activation of ROCK.

Two unexpected observations should be noted: (i) the biphasic concentration dependence of S1P-induced nodule formation (Figure 1) and (ii) the activation rather than inhibition of VIC contraction with receptor blockers VPC-23019 and W146 (Figure 2). Obviously, VICs express S1P receptors with opposing effects on contraction, the stimulating S1P2/RhoA/ROCK pathway and inhibitory S1P1 receptor pathway(s). Similar situations have been described in several other cell types.\(^3\) Accordingly, activation of RhoA showed...
at least a tendency to increase after blocking S1P1, whereas blocking S1P2 caused reduction in active RhoA. Furthermore, exposure to S1P resulted in a small but significant reduction in active Rac1, suggesting that the antagonism of RhoA and Rac pathways may have contributed to the net effect of S1P.

The biphasic concentration–response curve can be interpreted as based on two receptor subtype populations with different affinities or on two binding sites with different affinities within the same receptor subtype. The latter explanation appears more likely, since both components are blocked by S1P2-selective inhibitor JTE-013. However, other effects like different functional states, interaction with other receptors, or receptor internalization could have contributed to the observed biphasic concentration–response curve. These topics clearly need further experimental verification which was beyond the scope of the present communication.

S1P-induced increase in cytosolic Ca\(^{2+}\) concentration is another well-known mechanism to enhance cellular contractility. This effect may overlap with RhoA/ROCK-mediated contraction with respect to S1P concentration range and time course of effect, as has been exemplified in cultured gastric smooth muscle cells. In this system, the initial contraction is mediated by activation of S1P1 and S1P2 and Ca\(^{2+}\) release, whereas the same pathway as in VICs consisting of S1P2, RhoA, and ROCK was responsible for sustained contraction. In accordance with numerous other investigations on similar cell types like fibroblasts or smooth muscle cells, S1P exposure of VICs induced a transient increase in cytosolic-free Ca\(^{2+}\). The dynamic concentration range of S1P was mainly below the level necessary to induce nodule formation, with a significant response at a concentration as low as 2 nM. The effect was prevented by emptying ER Ca\(^{2+}\) stores with thapsigargin but was insensitive against block of Ca\(^{2+}\) influx channels with nifedipine and Gd\(^{3+}\). These observations suggest that the Ca\(^{2+}\) response to S1P in VICs of porcine origin is mostly dependent on Ca\(^{2+}\) release from the ER. In this way, the S1P effect closely resembles the response of human VICs to histamine.

The Ca\(^{2+}\) responses to S1P are elicited in the same concentration range (up to 100 nM) as the low concentration effects on nodule formation, which also peaked around 100 nM. Again, subtype-selective blockers were utilized for analysing the Ca\(^{2+}\) effects. Ca\(^{2+}\) release was not significantly affected by S1P2 blocker JTE-013 but at least partially impaired by S1P1 blockers VPC-23019 and W146. These observations are in clear contrast to S1P effects on nodule formation, which were completely inhibited by JTE-013 over the entire concentration range (up to 100 nM) as the low concentration effects on nodule formation, which also peaked around 100 nM. Therefore, Ca\(^{2+}\) release mainly mediated via S1P1 receptors may contribute to initial VIC contraction, whereas sustained contraction which is required for overnight nodule formation would depend on S1P2 activation and the RhoA/ROCK pathway, like signalling in gastric smooth muscle cells.

VICs in situ are adapted to a microenvironment defined by multiple ECM components and other biochemical factors, by interaction with other cell types like endothelial cells and neurons, and by biomechanical influences which are related to valve function. Obviously, VICs in 2D culture are exposed to a completely different environment. Growth on the rigid tissue culture surface has been shown to affect cellular responses like differentiation into myofibroblasts or osteoblast-like cells. Therefore, the in vitro S1P effects on cell contraction needed verification in situ to demonstrate their physiological relevance. In excised aortic valve tissue strips, S1P induced contractions in a concentration-dependent manner, and these contractions were of similar magnitude as observed with adrenaline. S1P was less effective than other vasoactive agents like 5-HT and endothelin, which can elicit responses in the same order of magnitude as 90 mM KCl whereas the effects of S1P were close to 25%. The S1P-dependent force generation was completely inhibited by JTE-013 at all concentrations of S1P, indicating that S1P2-dependent signalling in valve tissue has the same dominating role as in cultured VICs. However, the inhibitory effect of W146 indicates a different role of S1P1 in valve tissue-embedded cells. The myofibroblast differentiation under cell culture conditions as well as a multitude of other effects that characterize difference in in vitro 2D culture from in situ situation have to be considered in this context, but these topics are far beyond the scope of the present investigation.

The physiological relevance of the S1P-induced force generation and similar effects of other vasoactive agents are as yet largely unknown. Nevertheless, the contractile state of VICs affects valvular tissue tone and stiffness of the valvular leaflets. An in vitro model of isolated porcine aortic roots where the valves were pressurized through the aortic arch has been used for testing the direct influence of 5-HT on aortic valve competence. The rate of leakage from intact aortic roots was significantly increased in the presence of 5-HT. In addition, S1P may also play a role in long-term pathological changes. Several recent investigations have shown that, in addition to biochemical factors, physical cues of the cellular microenvironment influence VIC activation and differentiation. Exposure to elevated levels of S1P, in concert with other vasoactive agents, may cause stiffening of valve tissue to induce differentiation of a subset of susceptible cells into myofibroblasts with the consequence of fibrotic modifications.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References

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15. Bucher D, Setola V, Roth BL, Merryman WD. Serotonin receptors and heart valve disease—it was meant to be. Pharmaco Ther 2011;132:146–157.


