Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells

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Exogenous sphingosine 1-phosphate (S1P) increased cytosolic Ca^{2+} concentration, stimulated thymidine incorporation (DNA synthesis) and inhibited cell migration in rat aortic smooth-muscle cells (AoSMCs). Although exogenous sphingosine, a substrate of sphingosine kinase or a precursor of S1P, markedly induced the intracellular accumulation of S1P, the lipid failed to mimic the S1P-induced actions. In contrast, dihydrosphingosine 1-phosphate (DHS1P), an S1P receptor agonist, duplicated these S1P actions even though DHS1P was approx. 20–50-fold less potent than S1P. The pharmacological properties of DHS1P for the S1P receptor subtypes Edg-1, Edg-3, Edg-5 and Edg-6 were compared in Chinese hamster ovary (CHO) cells that were overexpressing the respective receptor. In these S1P-receptor-overexpressing cells, DHS1P was approx. 20–30-fold less potent than S1P for the displacement of [^{3}H]S1P binding and inositol phosphate response in Edg-5-expressing CHO cells, as was the case for AoSMCs. However, it was slightly (not more than 3-fold) less potent than S1P in cells expressing Edg-1, Edg-3 or Edg-6. Of the above-mentioned four types of S1P receptor, Edg-5 was abundantly expressed in AoSMCs, as demonstrated by Northern blotting. These results suggest that the intracellular accumulation of S1P is not necessary for the S1P-induced Ca^{2+} response, for the stimulation of DNA synthesis or for the inhibition of cell migration. Thus these S1P-induced actions might be mediated through extracellular (or cell-surface) S1P receptors in AoSMCs: Edg-5 might be a most important receptor subtype.

Key words: calcium response, dihydrosphingosine 1-phosphate, sphingosine 1-phosphate receptor, thymidine incorporation.

INTRODUCTION

It has been suggested that sphingosine 1-phosphate (S1P), one of the sphingolipid metabolites, affects a variety of cellular processes, including the proliferation, differentiation and motility of the cells [1–4]. These cellular responses elicited by S1P were first thought to be mediated through one or more intracellular targets [1], but more recently an extracellular mechanism has been supposed [5–8]. Supporting the latter mechanism, several isoforms of G-protein-coupled S1P receptors, Edg-1, Edg-3, Edg-5 and Edg-6, have been identified [3,4,9–15]. Thus S1P is a unique signalling molecule that behaves not only as an intracellular second messenger but also as an extracellular lipid mediator [1–4].

The proliferation and migration of vascular smooth-muscle cells (SMCs) are important events in the development of vascular disorders such as atherosclerosis and restenosis [16,17]. These cellular functions in vascular SMCs are regulated by a variety of cytokines and growth factors [17]. S1P has recently been suggested to be one of the regulators for these responses associated with several early signalling events, including the activation of phospholipase C and the increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in human vascular SMCs [18]. In bronchial SMCs, a receptor-mediated mechanism has been suggested for exogenous S1P-induced extracellular-signal-regulated kinase (ERK) activation and DNA synthesis [19]. In contrast, in rabbit femoral SMCs S1P is thought to activate ERK and proliferation through an intracellular mechanism [20]. In human vascular SMCs, it has not yet been explained whether the S1P-induced actions were mediated through a receptor-mediated mechanism or an intracellular mechanism [18]. Even if an extracellular receptor mechanism were important, it is difficult to determine which receptor subtype might mediate these S1P-induced actions. One approach to answering these questions is to analyse and compare the pharmacological specificities of S1P derivatives or their related compounds.

In the present study we employed sphingosine and dihydrosphingosine 1-phosphate (DHS1P) and compared their effects with those of S1P. If an intracellular mechanism is important for exogenous S1P-induced actions, then sphingosine, a precursor of S1P, should mimic them, but DHS1P, an agonist for S1P receptors [21], should not. However, our results showed just the opposite. Sphingosine failed to mimic these actions even though S1P accumulated to a large extent in the cells. However, DHS1P

Abbreviations used: AoSMCs, aortic SMCs; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CHO, Chinese hamster ovary; DHS1P, dihydrosphingosine 1-phosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HAECs, human aortic endothelial cells; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SMCs, smooth-muscle cells; ERK, extracellular-signal-regulated kinase.

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mimicked the actions. We further characterized the specificity of DHS1P as a receptor agonist and found that the S1P derivative can be used as a tool to discriminate the S1P receptor subtype involved in a given response. Our results indicate that intracellular S1P is not always necessary, at least for DNA synthesis and migration responses in rat aortic SMCs (AoSMCs), suggesting the importance of an extracellular mechanism. The pharmacological characterization of S1P receptors suggested that Edg-5 might be responsible for these responses induced by S1P. Edg-5 mRNA was abundantly expressed in AoSMCs, which supported this idea.

EXPERIMENTAL

Materials

1-Oleoyl-*sn*-glycero-3-phosphate (lysophosphatidic acid; LPA) and D-*erythro*-sphingosine were purchased from Sigma; S1P from Cayman Chemical Co.; DHS1P from Biomol Research Lab.; platelet-derived growth factor (PDGF)-BB from Pepro Tech. Ltd; and [^{3}H]sphingosine (20.0 Ci/mmol) and [^{3}H]-thymidine (20.0 Ci/mmol) from American Radiolabeled Chemicals. [^{3}H]S1P was synthesized enzymically from [^{3}H]-sphingosine by sphingosine-kinase-catalysed phosphorylation as described previously [12]. The sources of all other reagents were the same as those described previously [12,22,23].

Cell cultures

Rat thoracic AoSMCs from male Wistar rats weighing approx. 400 g were isolated by digestion with collagenase and elastase, then cultured by the procedure described previously [24]. The cells thus obtained were positive for smooth-muscle-specific α-actin. AoSMCs (passage number between 7 and 15) were cultured on 12-well plates for DNA synthesis and on 10 cm dishes for measurements of cellular S1P content, cell migration activity, S1P receptor mRNA species and $[Ca^{2+}]_i$. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) fetal bovine serum (FBS) in a humidified air/CO_2 (19:1) atmosphere. The culture dishes and plates were coated with 100 μg/ml rat tail collagen. The medium was changed to fresh DMEM containing 0.1 % BSA 24 h before experiments. Where indicated, pertussis toxin (PTX) (100 ng/ml) or its vehicle (final 2 mM urea) was added to the culture medium 24 h before experiments unless stated otherwise.

Human aortic endothelial cells (HAECs) with a passage number of 3 were purchased from Whittaker Bioproducts (Walkersville, MD, U.S.A.). The cells (passage number between 7 and 12) were cultured on collagen-coated dishes as described previously [22].

Edg-1-expressing Chinese hamster ovary (CHO) cloned cells (Edg-1 CHO/C13), which were used for the quntitative measurement of S1P, were maintained on 12-well plates in DMEM containing 5 % (v/v) FBS in a humidified air/CO_2 (19:1) atmosphere as described previously [25]. The medium was changed to fresh DMEM containing 0.1 % BSA 24 h before experiments.

In the experiments shown in Figure 6, CHO cells, that permanently expressed the respective S1P receptor were used. These cells were prepared by transfection of the pEFneo vector containing the respective receptor cDNA [12]. In vector-transfected cells, we could not evaluate the significant binding activity from a Scatchard plot. In contrast, we detected clear binding activity in all the receptor-transfected cells: the maximal bindings (pmol/mg of protein) and dissociation constants (nM) evaluated from the Scatchard plot were 4.25 and 13.2 respectively for cells transfected with Edg-1, 4.80 and 26.2 respectively for cells transfected with Edg-3, 5.69 and 20.4 respectively for cells transfected with Edg-5 (AGR16) and 12.80 and 19.1 respectively for cells transfected with Edg-6 [12,13]. Thus maximal binding was 2–3-fold higher in Edg-6-transfected cells than in other receptor-transfected cells but there was no appreciable difference in the dissociation constant. These cells were cultured on 10 cm dishes for the measurement of inositol phosphate [Ins(4,5)P_2 + Ins(1,4,5)P_3] response and on 12-well plates for S1P binding. The cells were maintained in DMEM containing 5 % (v/v) FBS in a humidified air/CO_2 (19:1) atmosphere. At 24 h before experiments, the medium was changed to inositol-free DMEM containing 20 μCi [^{3}H]inositol (in 6 ml) and 0.1 % BSA for inositol phosphate response, and to fresh DMEM containing 0.1 % BSA for S1P receptor binding experiments.

Measurement of $[Ca^{2+}]_i$

AoSMCs and HAECs were harvested from dishes by trypsin and loaded with fura 2 acetoxymethyl ester. $[Ca^{2+}]_i$ was estimated from the fluorescence of the fura-2 as described previously [12].

[^{3}H]Thymidine incorporation

AoSMCs were cultured for 20 h with the indicated agents in fresh DMEM containing 0.1 % BSA and for a further 4 h with [^{3}H]thymidine (1 μCi in 1 ml). The radioactivity in the trichloroacetic-acid-insoluble fraction was measured as described previously [22].

Migration assay

The migration of AoSMCs was quantified with a blind Boyden chamber apparatus by procedures essentially the same as those described previously [12]. In brief, the lower well of the chamber was filled with DMEM containing 0.1 % BSA (fraction V) and test agents. AoSMCs were harvested from dishes with trypsin. Cells (10^5 in 200 μl) were then loaded on the upper well. The number of cells that had migrated during 4 h to the lower surface of the membrane was determined by counting the cells at four places under microscopy at $\times$400 magnification [4 high power field ('HPF')].

RNA extraction and Northern blot analysis

Total RNA was prepared from AoSMCs, HAECs and HL-60 cells in accordance with the manufacturer's instructions for Trizol Reagent (Life Technologies). Northern blot analysis was performed as described previously [22,23,26]. HL-60 cells were used as a positive control for the expression of Edg-6 mRNA [13].

Quantitative measurement of S1P content in AoSMCs

AoSMCs were incubated for the indicated durations with appropriate concentrations of S1P or sphingosine in Hepes-buffered medium, which consisted of 20 mM Hepes, pH 7.5, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 5 mM glucose and 0.1 % BSA (fraction V). The cells were washed twice with the same Hepes-buffered medium; cellular S1P was selectively extracted by the procedure described previously [25]. The S1P content was measured by a recently established radio-receptor binding assay based on the competition of [^{3}H]S1P with S1P in the test sample on Edg-1-expressing CHO cells [25].

Measurement of [^{3}H]inositol phosphate production

For inositol phosphate response in CHO cells expressing Edg-3 or Edg-5, the [^{3}H]inositol-labelled cells were harvested from the 10 cm dishes with trypsin and incubated for 1 min with the indicated doses of S1P or DHS1P in suspension as described previously [12]. Where indicated, the results were normalized to 10^5 d.p.m. of total radioactivity incorporated into the cellular inositol lipids. The radioactivity of the 5% (w/v) trichloroacetic-acid-insoluble fraction was taken as the total radioactivity.

S1P receptor binding

For measurement of S1P receptor binding on CHO cells expressing the respective S1P receptor, the cells were incubated with 1 nM [^{3}H]S1P (approx. 15000 d.p.m. in 0.4 ml) in the presence of the appropriate quantity of S1P or DHS1P. Other experimental procedures were as described in a previous paper [12]. The specific binding of S1P to its receptor was estimated by subtracting the radioactivity in the presence of 1 μM unlabelled S1P. Usually, the specific S1P binding was 1000–1500 d.p.m. regardless of receptor subtype.

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as means ± S.E.M. for at least three separate experiments unless stated otherwise.

RESULTS

S1P increases $[Ca^{2+}]_i$, stimulates DNA synthesis and inhibits cell migration of rat AoSMCs

Consistent with the results of studies of human AoSMCs [18] was the observation that S1P induced a marked increase in $[Ca^{2+}]_i$ (Figure 1A) and inhibited cell migration (Figure 1C) in rat AoSMCs. As for migration of the cells, the inhibitory effect of S1P was clear when the migration was stimulated by PDGF. In human AoSMCs, S1P was a very weak stimulator of DNA synthesis [18]; however, this lipid clearly stimulated DNA synthesis in rat AoSMCs (Figure 1B). The DNA synthesis activity of S1P was almost identical with the PDGF-induced activity and was roughly additive to it. Thus S1P exerted stimulatory effects on $[Ca^{2+}]_i$ increase and DNA synthesis but had an inhibitory effect on the migration of the cells.

PTX is a powerful agent in evaluating the involvement of G_i/G_o-proteins and hence their coupled receptors. The S1P-induced increase in $[Ca^{2+}]_i$ was partly inhibited by PTX (Figure 1A). Under these conditions, the LPA-induced Ca^{2+} response was totally inhibited by treatment with toxin. Thus part of the Ca^{2+} response to S1P might be mediated by G_i/G_o-protein-coupled receptors. In contrast, the toxin hardly affected S1P-induced and PDGF-induced actions on DNA synthesis and cell migration. However, this does not rule out the possible involvement of G-protein-coupled receptors in S1P signalling.

Possible involvement of an extracellular mechanism in exogenous S1P-induced actions

DHS1P has been shown to stimulate S1P receptors but not intracellular putative target molecules [21]. We therefore examined the dose-dependent effects of DHS1P on these cell functions in AoSMCs and compared them with the effects of S1P. The S1P derivative was also effective in these responses, although much higher concentrations were required to obtain the maximal response (Figure 2).

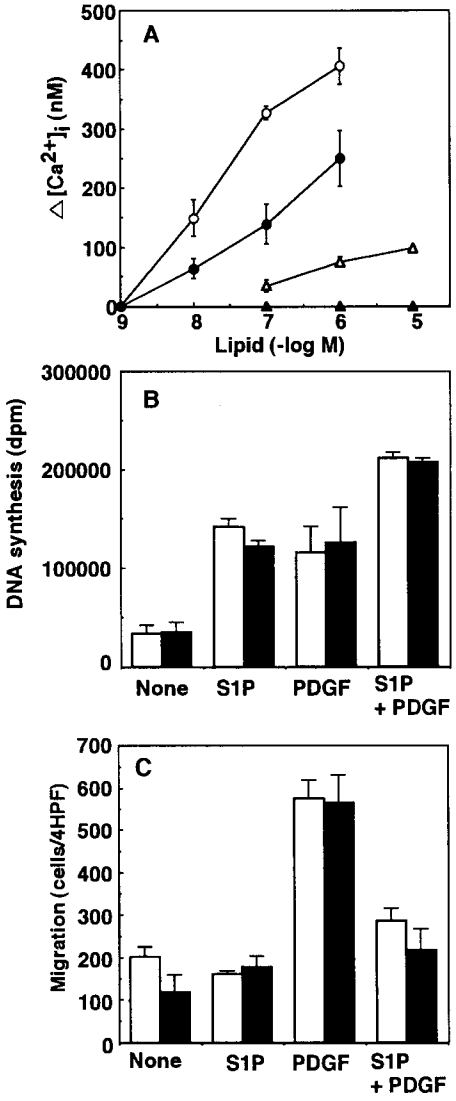

Figure 1 Effects of PTX on S1P-induced $[Ca^{2+}]_i$ increase, DNA synthesis and cell migration in AoSMCs

AoSMCs were pretreated with (●, ▲, filled columns) or without (○, △, open columns) PTX. (**A**) Cells were incubated with the indicated concentrations of S1P (○, ●) or LPA (△, ▲) to monitor $[Ca^{2+}]_i$. The increment (peak value at approx. 15 s minus the basal value) induced by S1P or LPA was plotted. (**B**) Cells were incubated with S1P (1 μM) and/or PDGF (10 ng/ml) to measure [^{3}H]thymidine incorporation. Results are expressed as radioactivities (d.p.m. per well) incorporated into the DNA fraction during 4 h. (**C**) The lower well of the chamber was filled with medium containing S1P (1 μM) and/or PDGF (30 ng/ml); the number of migrated cells was determined.

S1P is synthesized from sphingosine by sphingosine kinase inside the cells. In fact, treatment of the cells with sphingosine induced a intracellular accumulation of S1P moderately (approx. 5-fold) at 1 μM and enormously (200–300-fold) at 10 μM, at least during the 4 h incubation period (Figures 3A and 3B), the time assigned for cell migration activity in the present study. Nevertheless, sphingosine, even at these high concentrations, failed to induce these functional responses (Figure 2). In contrast, treatment with S1P did not induce a significant effect on lipid accumulation at 10 nM and increased it only 2-fold at 100 nM (Figure 3B), whereas S1P induced clearly functional responses (Figure 2). In this context it should be noted that the lipid con-

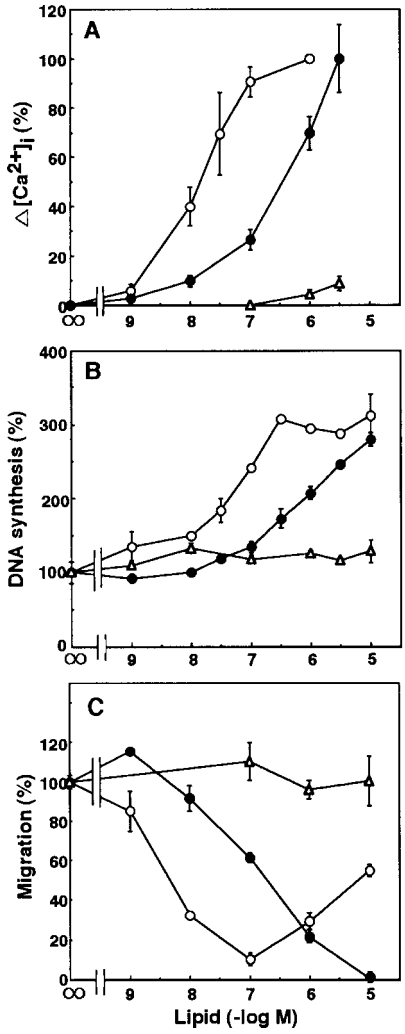

Figure 2 Dose-dependent effects of S1P, DHS1P and sphingosine on $[Ca^{2+}]_i$ increase, DNA synthesis and cell migration in AoSMCs

(**A**, **B**) AoSMCs were incubated with the indicated concentrations of S1P (○), DHS1P (●) or sphingosine (△) for Ca^{2+} response (**A**) and DNA synthesis (**B**). Results are expressed as percentages of maximal value (410 ± 30 nM) obtained at 1 μM S1P (**A**) and as percentages of basal value (19645 ± 843 d.p.m. per well) without test agents (**B**). (**C**) The lower well of the chamber was filled with the medium containing the indicated concentrations of test agents [the symbols are the same as those for (**A**) and (**B**)] with or without PDGF (30 ng/ml). The number of migrated cells was then determined. PDGF-induced cell migration is shown. Results are expressed as percentages of the PDGF-induced effects in the absence of these test agents. Other experimental conditions were essentially same as those for Figure 1.

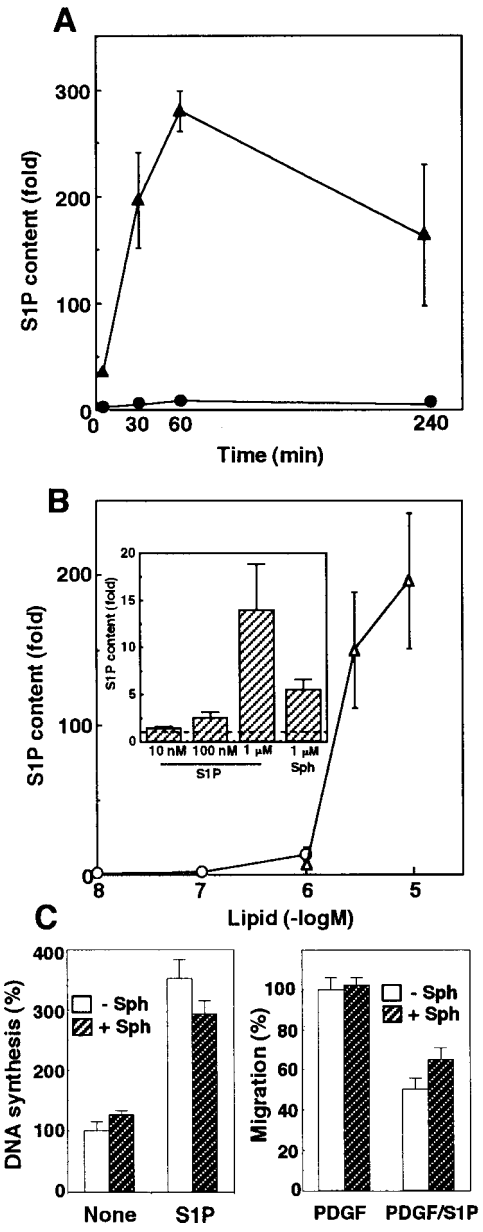

Figure 3 Change in intracellular S1P content might not be necessary for S1P-induced actions

(**A**, **B**) AoSMCs were incubated with 1 μM (●) or 10 μM (▲) sphingosine for the indicated duration (**A**) and with the indicated concentrations of S1P (○) or sphingosine (△) for 30 min, to measure S1P content (**B**). Results are expressed as fold increase compared with the value in unstimulated cells (1.79 ± 0.22 pmol/mg). (**C**) Left panel: AoSMCs were incubated with the indicated agents (1 μM S1P and/or 10 μM sphingosine) to measure DNA synthesis activity. Results are expressed as percentages of the basal value without test agents. Right panel: the lower well of the chamber was filled with medium containing PDGF (30 ng/ml) and the indicated agents (1 μM S1P and/or 10 μM sphingosine). The number of migrated cells was then determined. Results are expressed as percentages of the value obtained by PDGF alone.

tent, when S1P was used, might also have included the lipid bound to the cell surface, so, the real intracellular content of S1P might have been smaller than the estimated value.

Sphingosine is known as a potent inhibitor of protein kinase C [27]. Therefore one might assume that the intracellular S1P-induced actions were masked by the inhibitory action of sphingosine against the enzyme. However, the exogenous S1P-induced stimulation of DNA synthesis and the inhibition of cell migration were hardly affected by sphingosine (Figure 3C). These results suggest that an intracellular accumulation of S1P is not necessary for exogenous S1P-induced actions, and also that an increase in intracellular S1P concentration does not induce these functional responses in AoSMCs.

Edg-5 might be responsible for S1P-induced actions in AoSMCs

Figure 4 shows Northern blotting of S1P receptor subtypes Edg-1, Edg-3 and Edg-5 in AoSMCs. We used HAECs as a control. In AoSMCs, Edg-5 was expressed abundantly and Edg-3 moderately. As for Edg-1, a very weak band was also detected (Figure 4), but we could not detect Edg-6 mRNA (results not shown).

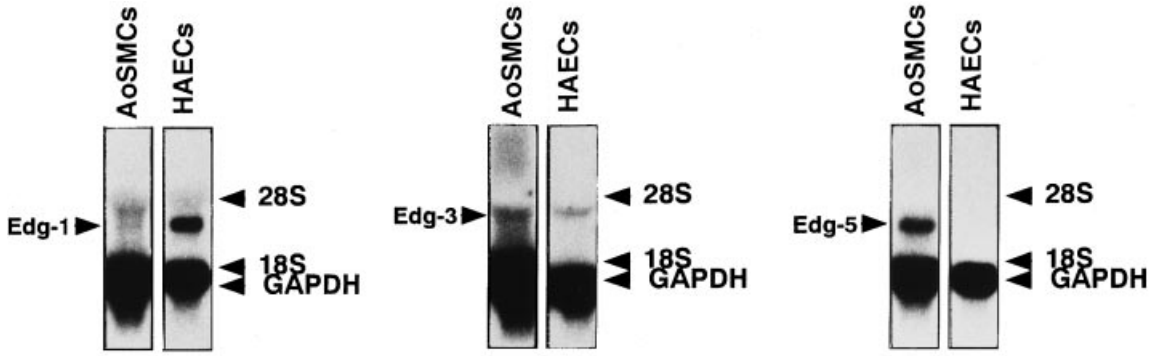

Figure 4 Northern blot analysis of S1P receptors

Total RNA species (20 μg) prepared from AoSMCs and HAECs were subjected to electrophoresis to analyse the expression of the mRNA species of Edg-1, Edg-3 and Edg-5. To compare the amount of RNA in each lane, the membrane filters were also hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In AoSMCs, as shown in Figure 2, the EC_{50} values for the S1P-induced actions varied from approx. 5 to 50 nM and those for DHS1P-induced actions varied from 100 nM to 1 μM depending on the given responses. However, the DHS1P-to-S1P EC_{50} ratios for the respective responses were not very different from each other and gave similar values of 20–50 (Figures 2 and 5B). In HAECs, S1P also increased $[Ca^{2+}]_i$ (Figure 5A) and stimulated DNA synthesis [22]. As for cell migration, S1P was a stimulatory signal rather than an inhibitory signal [22]. In this cell type, DHS1P was only slightly less potent than S1P in stimulating DNA synthesis and cell migration; the DHS1P-to-S1P EC_{50} ratio was 2.5 for DNA synthesis and 1.3 for cell migration (Figure 5B) [22]. Consistent with this was the finding that the potency of DHS1P to increase $[Ca^{2+}]_i$ was approx. 5-fold less than that of S1P in HAECs (Figure 5). Thus both S1P and DHS1P seem to be full agonists for both cell types, but their potencies to induce functional responses are different between the cell types. One plausible explanation for this difference is that the receptor subtypes involved in the induction of these responses are different between cell types. Edg-1 and Edg-3 are expressed in HAECs (Figure 4); both receptors seem to be involved in S1P-induced DNA synthesis and cell migration [22]. In contrast, in AoSMCs, Edg-5 mRNA was expressed as a major band, as shown in Figure 4.

To verify this possibility we compared the binding properties of DHS1P and S1P for the respective S1P receptor subtype overexpressed in CHO cells. As shown in Figure 6(A), the binding affinity of DHS1P and S1P was estimated as the ability to displace [^{3}H]S1P binding (1 nM). The apparent affinity of S1P was almost identical regardless of the receptor subtype, as demonstrated by the similar IC_{50} values of 10–30 nM in the respective receptor-expressing cells. However, the IC_{50} of DHS1P was dependent on the receptor species, being approx. 200 nM in the Edg-5-expressing cells but approx. 30 nM in other S1P receptor-subtype-expressing cells. Thus the apparent binding affinity of DHS1P was approx. 20-fold less than that of S1P in the Edg-5-expressing cells, whereas DHS1P was at most 3-fold less potent than S1P in cells expressing Edg-1, Edg-3 and Edg-6 (Figure 6C).

To confirm that the difference in the binding affinity of DHS1P for Edg-5 was reflected in a difference in agonist activity, we measured the inositol phosphate response to the lipids (Figure 6B). As a control we also examined the inositol phosphate response in Edg-3-expressing cells. The EC_{50} of DHS1P in the stimulation of inositol phosphate production was approx. 30-fold that of S1P in Edg-5-expressing cells. However, the S1P derivative was only 3-fold less potent than S1P in Edg-3-expressing cells (Figures 6B and 6C).

DISCUSSION

There are at least two mechanisms of action for exogenous S1P: an intracellular mechanism and an extracellular (or intercellular) mechanism. Several reports have suggested the participation of an intracellular mechanism in the stimulation of DNA synthesis [1,21,28] and the inhibition of cell migration [29], although the intracellular primary target or targets for S1P have not yet been identified. However, our results suggest that S1P-induced Ca^{2+} mobilization, stimulation of DNA synthesis and inhibition of cell migration might be mediated by the extracellular mechanism through S1P receptors in rat AoSMCs. Of the S1P receptor subtypes, Edg-5 seems to be responsible for these S1P-induced actions.

It has been widely recognized that PDGF induces S1P accumulation inside the cells and that intracellular S1P mediates some of the PDGF-induced actions, including Ca^{2+} mobilization and the stimulation of DNA synthesis [1,21,28]. The proposal of an intracellular role for S1P in the actions of PDGF was based largely on experiments with inhibitors of sphingosine kinase such as dimethylsphingosine and dihydrosphingosine [1,21,28]. However, it should be noted that these inhibitors are not specific for sphingosine kinase, so the inhibition of PDGF-induced actions by these inhibitors was not always due to the inhibition of sphingosine kinase [30]. Furthermore, in the migration of rat AoSMCs, S1P induced the opposite action to that induced by PDGF: the lipid inhibited the PDGF-induced stimulation of cell migration. Thus, even if PDGF induced an increase in intracellular S1P level, it is unlikely that intracellular S1P mediates the PDGF-induced migration of rat AoSMCs. In the present study we have shown that sphingosine, a substrate of sphingosine kinase or a precursor of S1P, failed to induce the stimulation of DNA synthesis, the inhibition of migration or the mobilization of Ca^{2+}, even though the lipid clearly induced the intracellular accumulation of S1P. In contrast, extracellular S1P induced these responses without a detectable accumulation of S1P in the cells. These results suggest that an accumulation of S1P inside the cells never induces these functional responses and also

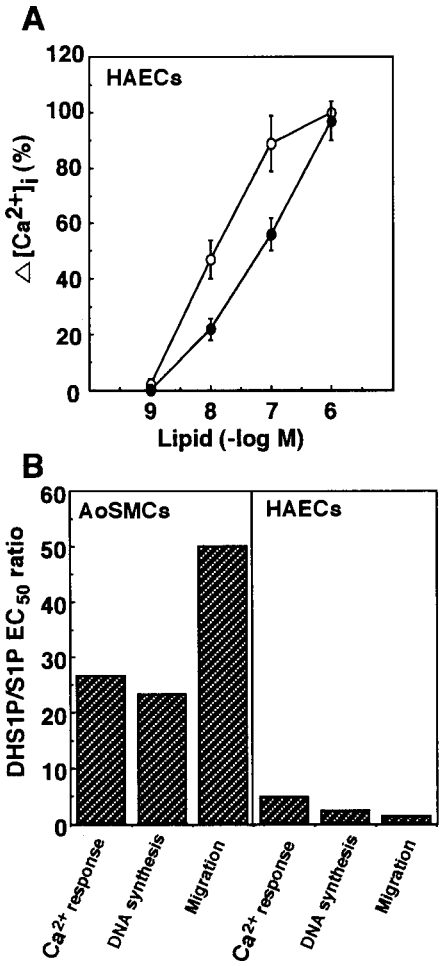

Figure 5 Comparison of the potency of DHS1P and S1P in inducing functional responses in AoSMCs and HAECs

(**A**) HAECs were incubated with the indicated concentrations of S1P (○) or DHS1P (●) to monitor $[Ca^{2+}]_i$. The increment (peak value at approx. 15 s minus the basal value) induced by these agents was plotted. (**B**) The ratio of EC_{50} of DHS1P to EC_{50} of S1P in inducing the indicated responses was calculated. For example, the DHS1P-to-S1P EC_{50} ratio for Ca^{2+} response in AoSMCs was estimated by dividing the EC_{50} of DHS1P (331 nM) by the EC_{50} of S1P (12.6 nM). Data were derived from Figure 2 for all responses in AoSMCs and from (**A**) for Ca^{2+} response and also from published results (Figure 1 of [22]) for DNA synthesis and migration in HAECs.

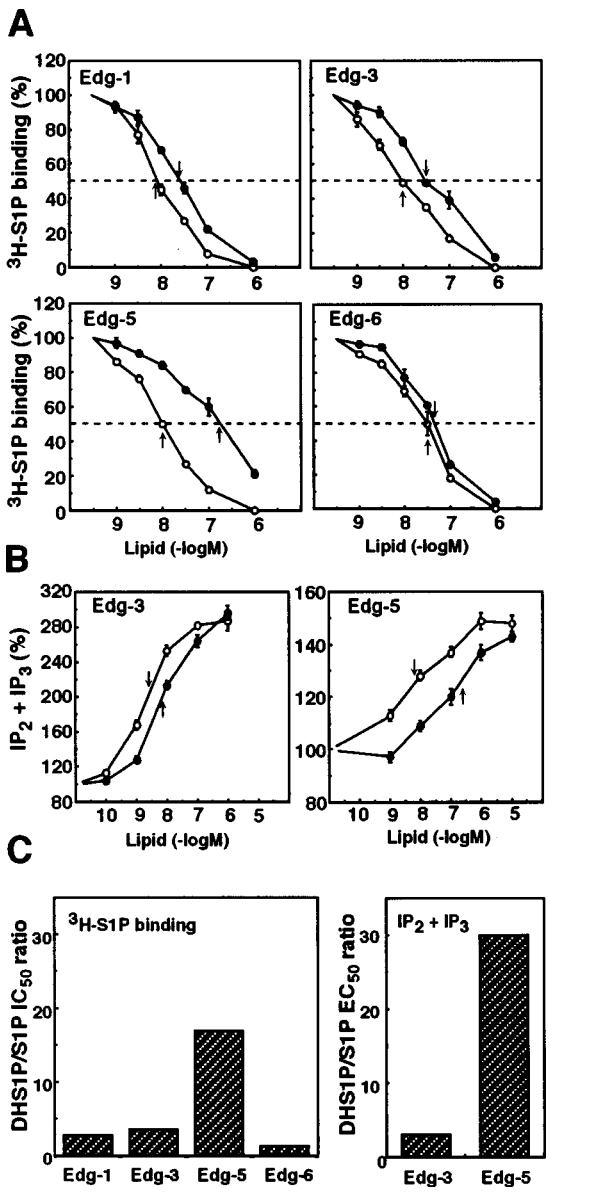

Figure 6 Pharmacological specificity of DHS1P and S1P for the respective S1P receptor subtype

(**A**) CHO cells overexpressing the indicated S1P receptor subtype were incubated with 1 nM [^{3}H]S1P in the presence of the indicated concentrations of S1P (○) or DHS1P (●). Results were expressed as percentages of the values obtained without test agents. The arrow indicates IC_{50}. (**B**) CHO cells expressing Edg-3 or Edg-5 were incubated with the indicated concentrations of S1P (○) or DHS1P (●) to measure the production of inositol phosphate [Ins(4,5)P_2 + Ins(1,4,5)P_3]. Results are expressed as percentages of basal activities without test agents. Normalized basal values were 829 ± 36 d.p.m. for Edg-3-transfected cells and 879 ± 21 d.p.m. for Edg-5-transfected cells. The arrow indicates EC_{50}. (**C**) The ratio of IC_{50} of DHS1P to IC_{50} of S1P in displacing [^{3}H]S1P binding in the cells expressing the respective S1P receptor subtype (left panel) and the ratio of EC_{50} of DHS1P to EC_{50} of S1P in stimulating inositol phosphate production (right panel) was calculated. Results were derived from (**A**) for the left panel and from (**B**) for the right panel.

that the intracellular accumulation of S1P is not necessary for S1P-induced actions in AoSMCs. Furthermore, the pharmacological specificity of S1P and DHS1P for these responses matches closely the characteristics of Edg-5 on the basis of the S1P receptor binding and inositol phosphate response in CHO cells overexpressing the respective receptor subtype. In fact, Edg-5 mRNA is abundantly expressed in AoSMCs.

The EC_{50} of the S1P-induced responses varied; there was at most one order magnitude of difference between them (Figure 2). However, this might simply reflect a difference in the coupling efficiency of the respective signalling pathway rather than an involvement of different S1P receptor subtypes. Nevertheless, the present study does not rule out the possible involvement of Edg-3 and other receptor subtypes to a small extent in the S1P-induced actions. In fact, Edg-3 mRNA and, to a smaller extent, Edg-1 mRNA are expressed in addition to Edg-5 mRNA in AoSMCs. Furthermore, during preparation of this manuscript, Edg-8 was listed as a fifth S1P receptor that couples to PTX-sensitive G_i/G_o proteins [31]. This novel S1P receptor showed a similar affinity for both DHS1P and S1P [31]. We do not know

whether Edg-8 is expressed in rat AoSMCs. However, it is unlikely that Edg-8 is a major S1P receptor subtype responsible for the S1P-induced regulation of DNA synthesis and cell migration in AoSMCs, because of the difference in its pharmacological properties. In AoSMCs, these responses to S1P were hardly affected by PTX treatment (Figure 1); moreover, DHS1P was approx. 20–50-fold less potent than S1P (Figure 2). Experiments with more specific tools, such as subtype-specific antagonists (not available at present) and anti-sense genes or anti-sense oligonucleotides might be helpful for further characterization of receptors involved in the S1P-induced functional responses in AoSMCs. In any event, the extracellular mechanism, probably through Edg-5, might be important for the S1P-induced stimulation of DNA synthesis, the inhibition of migration and the mobilization of Ca^{2+} in rat AoSMCs. Such receptor-mediated actions of S1P have recently been demonstrated for DNA synthesis and cell survival responses in cells overexpressing Edg-3 or Edg-5 [32].

In previous studies [21,28] DHS1P was used as an agent to discriminate the extracellular S1P action from the intracellular one. This was based on the observation that the S1P derivative interacted with the respective S1P receptors in S1P binding experiments and mimicked the S1P receptor-mediated actions, but failed to mimic the cell survival activity. Thus the authors concluded that the cell survival activity of S1P might be mediated by the intracellular mechanism [21,28]. However, in these experiments the dose-dependent effect was not examined. As shown in the present study, DHS1P was approx. 20–30-fold less potent than S1P in Edg-5-expressing cells, so if the cells expressed Edg-5 predominantly, one would miss the receptor-mediated DHS1P effect. In fact the DHS1P effect was marginal at the same concentration at which S1P half-maximally activated inositol phosphate production (Figure 6B). Thus careful examination was necessary to determine whether the given S1P action could be mediated by an intracellular or extracellular mechanism on the basis of the DHS1P effects. However, the discrepancy in the apparent affinity between S1P and DHS1P might be useful in discriminating between S1P receptor subtypes involved in the responses observed.

The physiological and pathophysiological roles of S1P and its receptor system in vascular SMCs have not yet been elucidated. The stimulation of DNA synthesis in rat AoSMCs suggests that S1P might be atherogenic for the vascular system. In contrast, the finding that S1P inhibits the migration of AoSMCs suggests that S1P is an anti-atherogenic signalling molecule rather than an atherogenic one. However, it should be noted that S1P is a very weak stimulator of DNA synthesis in human AoSMCs, even though S1P potently inhibits PDGF-induced cell migration [18]. Furthermore, even in rat AoSMCs, a much higher concentration of S1P is required for the stimulation of DNA synthesis than that for the inhibition of cell migration; the EC_{50} for S1P-induced DNA synthesis (approx. 50 nM) was roughly one order of magnitude higher than the EC_{50} for S1P-induced inhibition of cell migration (approx. 3 nM) (Figure 2). Thus, at least at lower concentrations, S1P seems to behave as an anti-atherogenic molecule. Recent studies suggest that S1P or its related lipid adsorbs on low-density lipoprotein (LDL) and high-density lipoprotein [33,34]. It is well known that LDL, especially oxidized LDL, is one of the important factors involved in such vascular diseases as atherosclerosis [16,17]. These results suggest that S1P receptors might also be stimulated when LDL or oxidized LDL acts on vascular cells such as SMCs and endothelial cells, although it remains unclear whether the S1P receptors mediate some of the atherogenic reactions of lipoproteins or function as negative regulators against atherogenic lipids in lipoproteins. The elucidation of the roles of S1P-induced actions and their mechanisms in vascular cells, including SMCs, might facilitate an understanding of the cellular mechanism by which LDL or oxidized LDL initiates atherosclerotic lesion. Such studies might also be important for the exploitation of antagonists and agonists for S1P receptors as drugs for atherosclerosis and other cardiovascular diseases.

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