# Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions

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The concentration of sphingosine 1-phosphate (S1P) in plasma or serum is much higher than the half-maximal concentration of the sphingolipid needed to stimulate its receptors. Nevertheless, the inositol phosphate response to plasma or serum mediated by Edg-3, one of the S1P receptors, which was overexpressed in Chinese hamster ovary cells, was much smaller than the response expected from the total amount of S1P in these samples. The inositol phosphate response to exogenous S1P was markedly attenuated in the presence of charcoal-treated low-S1P serum. The inhibitory effect was lost by boiling but not by dialysis of the serum. The inhibitory action of the serum was specific to S1P and was associated with the trapping of exogenous S1P; the inositol phosphate response to $P_2$-purinergic agonists was somewhat enhanced by the charcoal-treated serum. Among the components of plasma or serum, lipoproteins such as low-density and high-density lipoproteins showed a stronger activity for trapping S1P than lipoprotein-deficient serum. Consistent with this observation, we detected a 15–100-fold higher amount of S1P per unit amount of protein in lipoproteins than in the lipoprotein-deficient serum. Thus even though the protein content of the lipoprotein fraction contributes to only 4% of the total protein content of plasma or serum, more than 60% of S1P is distributed in this fraction. These results suggest that the tight binding of S1P to the components of serum or plasma, including lipoproteins, may interfere with the S1P binding to its receptors and thereby attenuate the lipid-receptor-mediated actions in the cells.

Key words: Edg-3, high-density lipoprotein, low-density lipoprotein, sphingosine 1-phosphate binding.

## INTRODUCTION

Sphingosine 1-phosphate (S1P), one of the sphingolipid metabolites, has recently been suggested to be a unique signalling molecule with respect to its mode of action: it behaves as an intracellular second messenger of some kinds of cytokines and also as an extracellular autocrine or paracrine mediator [1,2]. Thus S1P stimulates a variety of intracellular signalling events including activation of phospholipase C [3–6], increase in cytosolic $Ca^{2+}$ concentration [3–10], regulation of adenylate cyclase [3,5–7,10], activation of the mitogen-activated protein kinase cascade [10–13] and activation of the Rho cascade [14,15].

Several G-protein-coupled receptors specific for S1P whose mRNAs are expressed in a variety of cells and tissues have recently been identified [16–27]. Furthermore, S1P has been shown to be present in plasma or serum [28–30]. These results support the physiological role of S1P as an extracellular mediator. Five subtypes of S1P receptors (Edg-1, -3, -5, -6 and -8) have been reported so far; the dissociation constants ($K_d$) of S1P for these receptors are similar, all within the range 2–30 nM [19,24,26,31,32]. However, the concentration of S1P in plasma is about 200 nM [29]. If this were the case *in vivo*, S1P receptors would be almost saturated with the ligand and an increase in plasma S1P levels could not be expected to cause an S1P-receptor-mediated effect. Since S1P is released from activated platelets [33], plasma samples might contain S1P derived from platelets activated artificially during withdrawal or centrifugation of blood. In such cases, the plasma S1P concentration *in vivo* might be lower than the $K_d$ value of S1P for the lipid receptors. Alternatively, the effective or active concentration of S1P in plasma might be much lower than the concentration estimated from the total amount of plasma S1P. For example, in plasma or serum, there might be unidentified components that interfere with S1P to stimulate receptors. S1P is lipophilic and easily bound to proteins such as albumin; therefore, it is reasonable to speculate that plasma proteins might reduce the freedom of the lipid to interact with its receptor.

In the present study, we found that serum or plasma inhibited the S1P receptor Edg-3-mediated inositol phosphate response in receptor-transfected Chinese hamster ovary (CHO) cells. This inhibitory effect of serum or plasma was associated with their trapping of S1P. Among components in serum or plasma, lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) exhibited potent ability to bind exogenous S1P. Indeed, we detected a considerably higher amount of S1P in LDL and HDL compared with the lipoprotein-deficient serum (LPDS) or lipoprotein-deficient plasma (LPDP). Thus lipoproteins seem to interfere with the S1P-induced actions by trapping S1P in their particles.

## EXPERIMENTAL

### Materials

S1P was purchased from Cayman Chemical Co.; fatty acid-free BSA was from Calbiochem-Novabiochem; *myo*-[2-$^3$H]inositol

Abbreviations used: S1P, sphingosine 1-phosphate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDS, lipoprotein-deficient serum; LPDP, lipoprotein-deficient plasma; CHO, Chinese hamster ovary; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium.

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(23.0 Ci/mmol) and [$^3$H]sphingosine (20.0 Ci/mmol) were from American Radiolabelled Chemicals; plasma lipoproteins including LDL and HDL were from Biomedical Tech. (Stoughton, MA, U.S.A.); and LPDS and fetal bovine serum (FBS) were from Sigma (St. Louis, MO, U.S.A.). In most experiments, unless otherwise specified, we used lipoproteins and LPDS that were obtained commercially. For the experiments shown in Figure 5, however (see below), we prepared lipoproteins and LPDP by density gradient centrifugation; very low-density lipoprotein (density $< 1.019$ g/ml), LDL (1.019–1.063 g/ml), HDL (1.063–1.21 g/ml) and LPDP ($> 1.21$ g/ml) were separated from freshly isolated human plasma by sequential ultracentrifugation as described previously [34]. Human serum or plasma was collected from normal healthy volunteers. For preparation of charcoal-treated serum, 10 ml of FBS, 10 ml of LPDS or 5 ml of human serum was treated with 1 g of activated charcoal powder (Sigma) and then serum was recovered by centrifugation and filtration (0.45 $\mu$m). For the preparation of charcoal-treated plasma lipoproteins (LDL and HDL), the lipoproteins (2.5 mg of protein/ml) were treated with 100 mg of BSA-pretreated charcoal, which was prepared by mixing with 1 % BSA and subsequent washing with PBS. For preparation of dialysed serum, serum samples were dialysed for 24 h against ice-cold PBS. [$^3$H]S1P was synthesized enzymically from [$^3$H]sphingosine by sphingosine kinase-catalysed phosphorylation as described previously [24]. By this method, we could obtain [$^3$H]S1P with the same specific activity as the labelled sphingosine. The sources of all other reagents were the same as described previously [4–6,13,21,24].

### Cell cultures

CHO cells, which had been transfected with the pEFneo empty vector or a pEFneo S1P-receptor (Edg-3 or Edg-1)-expression vector as described previously [24], were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) FBS in a humidified air/$CO_2$ (19:1) atmosphere. For measurement of S1P content, Edg-1-expressing CHO cloned cells (Edg-1 CHO/C13) were maintained on 12 multi-plates. Edg-3-expressing bulk CHO cells were maintained on 10 cm dishes for measurement of the inositol phosphate response, by which we evaluated an effective or active S1P content. Before the experiments (24 h), the medium was changed to fresh DMEM (without serum) containing 0.1 % (w/v) BSA. In the case of the inositol phosphate response, the medium was changed to the inositol-free DMEM containing 20 $\mu$Ci of [$^3$H]inositol (in 6 ml) and 0.1 % (w/v) BSA.

### Extraction of S1P and its quantitative measurement

S1P in plasma or serum and plasma lipoproteins were extracted selectively by the procedure described by Yatomi et al. [28,29]. In brief, S1P in plasma or serum (0.5 ml) or plasma lipoproteins (around 1 mg in 0.5 ml) were first extracted into the aqueous phase and separated from other lipids under alkaline conditions. They were then re-extracted into the chloroform phase under acidic conditions. The chloroform was evaporated, and the dried samples were sonicated with a Tris-buffered medium consisting of 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 15 mM NaF and 0.4 % (w/v) BSA. Overall recovery of S1P by this procedure was $24 \pm 2$ % [30]. The S1P content was then measured by a recently established radioreceptor assay based on competition of [$^3$H]S1P with S1P in a test sample on Edg-1-expressing CHO cells [30]. The procedure is essentially identical with that for the measurement of S1P-receptor binding [24]. In brief, Edg-1-expressing CHO cells were washed twice with ice-cold Tris-buffered medium, and then the cells were incubated in the same medium containing 1 nM [$^3$H]S1P (about 18000 d.p.m./plate) with or without test agents in a final volume of 0.4 ml. The plates were kept on ice for 30 min, and the cells were washed twice with the same ice-cold medium to remove the unbounded ligand. The cells were solubilized with a cell-solubilizing solution composed of 0.1 % SDS, 0.4 % NaOH and 2 % $Na_2CO_3$, and the radioactivity was counted.

### Measurement of [$^3$H]inositol phosphate production

The [$^3$H]inositol-labelled cells were harvested from the 10 cm dishes with trypsin (0.05 % in PBS containing 0.53 mM EDTA) and washed by sedimentation (250 ***g*** for 5 min) and resuspension in the Hepes-buffered medium. The Hepes-buffered medium 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 % (w/v) BSA. The washing procedure was repeated, and the cells were finally resuspended in the same medium. Unless otherwise specified, the cells (about $5 \times 10^6$ cells) were preincubated for 5 min with 10 mM LiCl in polypropylene vials (20 ml) in a final volume of 1.8 ml. The test agents (0.2 ml) were then added to the medium, and the cells were incubated further at 37 °C for 1 min. The cell suspension (0.5 ml) in triplicate was transferred to tubes containing 1 ml of chloroform/methanol/HCl (100:100:1). $^3$H-Labelled inositol bisphosphate and inositol trisphosphate were separated as described previously [24]. In order to normalize the effects of plasma and serum samples in vector-transfected or Edg-3-transfected cells, data were first normalized to $10^5$ d.p.m. of the total radioactivity incorporated into the cellular inositol lipids in each experiment and then the results were expressed as percentages of the maximal activity obtained at 1 $\mu$M S1P in Edg-3-transfected cells. The radioactivity of the trichloroacetic acid (5 %)-insoluble fraction was measured as the total radioactivity.

### [$^3$H]S1P binding to serum or its components

Serum or lipoproteins were mixed with 1 nM [$^3$H]S1P in Tris/HCl buffer (pH 7.4) containing 100 mM NaCl at room temperature, and then the mixture (0.5 ml) was applied to a Sephadex G-50 column (1 ml). The serum or lipoprotein samples were then eluted with the same buffer (1 ml) and the radioactivity of the total eluate (1.5 ml) was counted. Under these conditions, the protein-unbound [$^3$H]S1P is trapped to the column material.

### Data presentation

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

## RESULTS

### Existence of a very high amount of S1P in serum or plasma

In Figure 1, S1P was extracted from serum or plasma samples, and then its content was measured by a new quantitative method based on the radioreceptor assay [30]. Consistent with the previous results reported by Yatomi et al. [29], we detected a high amount of S1P even in human plasma. The lipid content was significantly higher in serum compared with plasma, possibly due to the release of S1P from activated platelets [33]. In biological samples, there might be ligands other than S1P, such as sphingosylphosphorylcholine, to interact with S1P receptors, resulting in the displacement of [$^3$H]S1P binding. This might lead to the

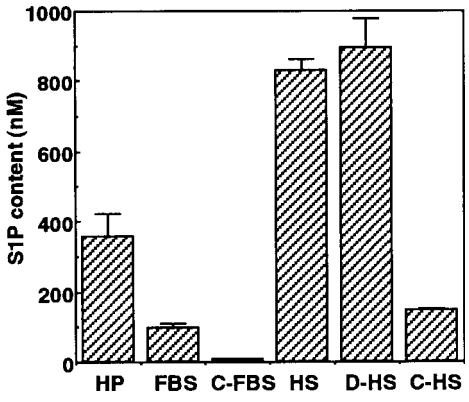


**Figure 1 S1P content in serum or plasma**

S1P was extracted from 0.5 ml samples including human plasma (HP), FBS, charcoal-treated FBS (C-FBS), human serum (HS), dialysed human serum (D-HS) and charcoal-treated human serum (C-HS), and then the lipid content was measured based on the radioreceptor assay described in the Experimental section. Data are means ± S.E.M. from three to five different samples.

overestimation of S1P content in the samples. In order to confirm that the value determined in Figure 1 reflected the actual S1P content, S1P in the samples was further purified by TLC (silica gel 60 with a solvent system of butanol/acetic acid/water, 3:1:1) and then its content was measured. We could not detect any significant difference in the values between before and after the lipid purification of serum samples (results not shown). This suggests that S1P is the only ligand to stimulate Edg-1 or S1P receptors, at least in normal human serum samples.

We also found the S1P content in FBS to be lower than in human serum. Dialysis of the serum hardly affected the S1P content, suggesting that S1P may be bound to the serum components with high molecular mass, such as albumin, reflecting its lipophilic nature. The S1P bound to the serum components was reduced to 10–20% of initial levels by charcoal treatment.

### Activity of S1P in serum or plasma to stimulate S1P receptors is apparently much lower than the activity expected from the total S1P content

In order to evaluate the activity of S1P in serum or plasma samples to stimulate its receptors, we measured inositol phosphate production in CHO cells overexpressing Edg-3 [24]. As shown in Figure 2(A), S1P increased inositol phosphate production in a dose-dependent manner, with an $EC_{50}$ value of about 2 nM. In vector-transfected cells, both S1P and serum samples exerted only a small effect. On the other hand, in the Edg-3-transfected cells, serum or plasma clearly increased the production in a dose-dependent manner (Figure 2B). Thus this assay system is useful for the detection of the S1P-receptor (Edg-3)-stimulating activity of serum or plasma.

The S1P-receptor agonist activity based on the inositol phosphate response in Edg-3-transfected cells is here tentatively termed active S1P, because, apart from S1P, no other S1P-receptor agonist has been detected in serum or plasma, as mentioned above. Based on the S1P dose–response curve (Figure 2A), we evaluated the active S1P content in serum or plasma and compared it with the total S1P content, which was estimated using the radioreceptor assay (Figure 1). Results are summarized in Figure 2(C). In all cases, on increasing the amount of serum or plasma in the assay medium the ratio of active S1P content to

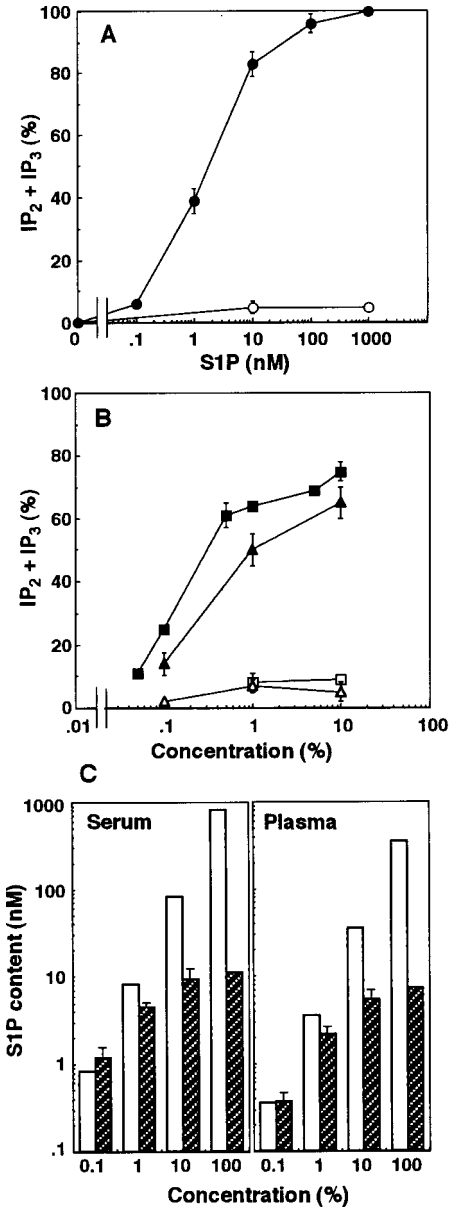


**Figure 2 Apparent activity of S1P in serum or plasma is less than the activity estimated from the total amount of S1P**

In (**A**) and (**B**), Edg-3-transfected (closed symbols) and vector-transfected (open symbols) cells were used. The cells were incubated for 1 min with the indicated concentrations of S1P (**A**), human plasma (△, ▲ in **B**) or human serum (□, ■ in **B**) in the presence of 0.1% BSA, and then inositol phosphate production was measured. Results were expressed as percentages of the maximal response attained by 1 $\mu$M S1P in Edg-3-expressing cells. Data are means ± S.E.M. of three separate experiments. $IP_2$ and $IP_3$, inositol bisphosphate and inositol trisphosphate, respectively. In (**C**), total S1P content and active S1P content (plotted on a logarithmic scale) are compared at the indicated concentrations of human serum (left-hand panel) or human plasma (right-hand panel). Total S1P content (open bars) in serum or plasma samples was derived from Figure 1. Active S1P content (hatched bars) was estimated based on the activity to stimulate inositol phosphate production shown in (**A**) and (**B**). The maximal active content of S1P at 100% serum or plasma was estimated from an Eadie–Scatchard plot. See the text for more details.

total lipid content became clearly lower (note that S1P content is shown on a logarithmic scale). For example, the active S1P content and total lipid content were 9.47 and 82.9 nM in 10% human serum but 4.52 and 8.29 nM in 1% human serum,

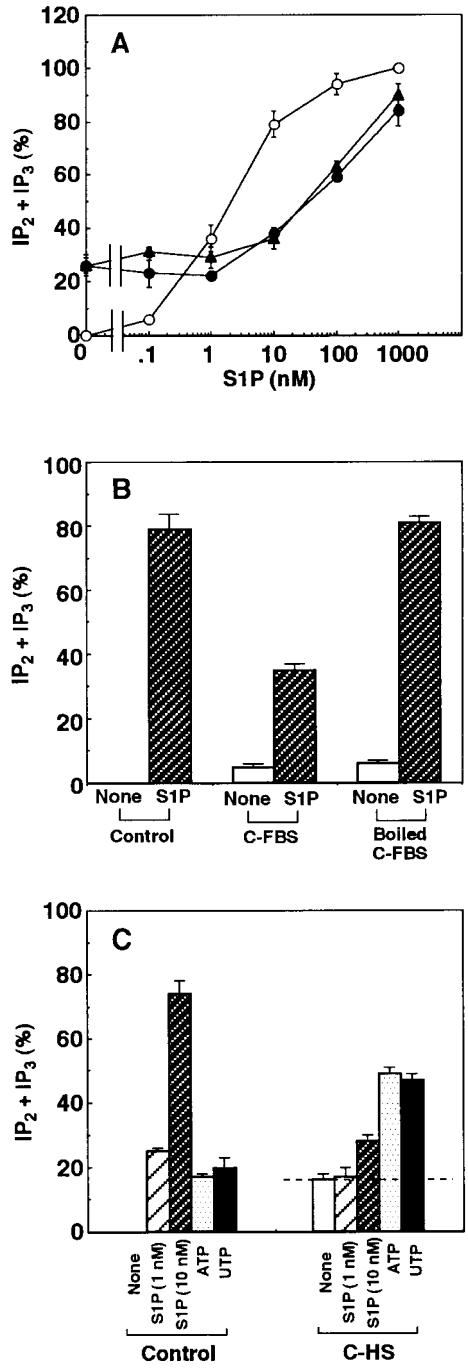


**Figure 3 Serum components specifically inhibit S1P-receptor-mediated action**

Inositol phosphate response in Edg-3-expressing cells was measured. In (**A**), cells were incubated with the indicated concentrations of S1P in the absence (○) or presence of 5 mg of protein/ml (≈ 8%, v/v) charcoal-treated human serum (●) or dialysed charcoal-treated human serum (▲). In (**B**), cells were incubated with (hatched bars) or without (open bars) 10 nM S1P in the absence (Control) or presence of 2.8 mg of protein/ml (≈ 8%, v/v) charcoal-treated FBS (C-FBS) or 2 min boiled charcoal-treated FBS (Boiled C-FBS). In (**C**), the cells were incubated without (None) or with the indicated concentrations of S1P, ATP (100 $\mu$M) or UTP (100 $\mu$M) in the presence (C-HS) or absence (Control) of 5 mg of protein/ml charcoal-treated human serum. In all experiments, 0.1% BSA was included in the incubation medium. All the results are expressed as percentages of the maximal response attained by 1 $\mu$M S1P in Edg-3-expressing cells. Data are means ± S.E.M. of three or four separate experiments. $IP_2$ and $IP_3$, inositol bisphosphate and inositol trisphosphate, respectively.

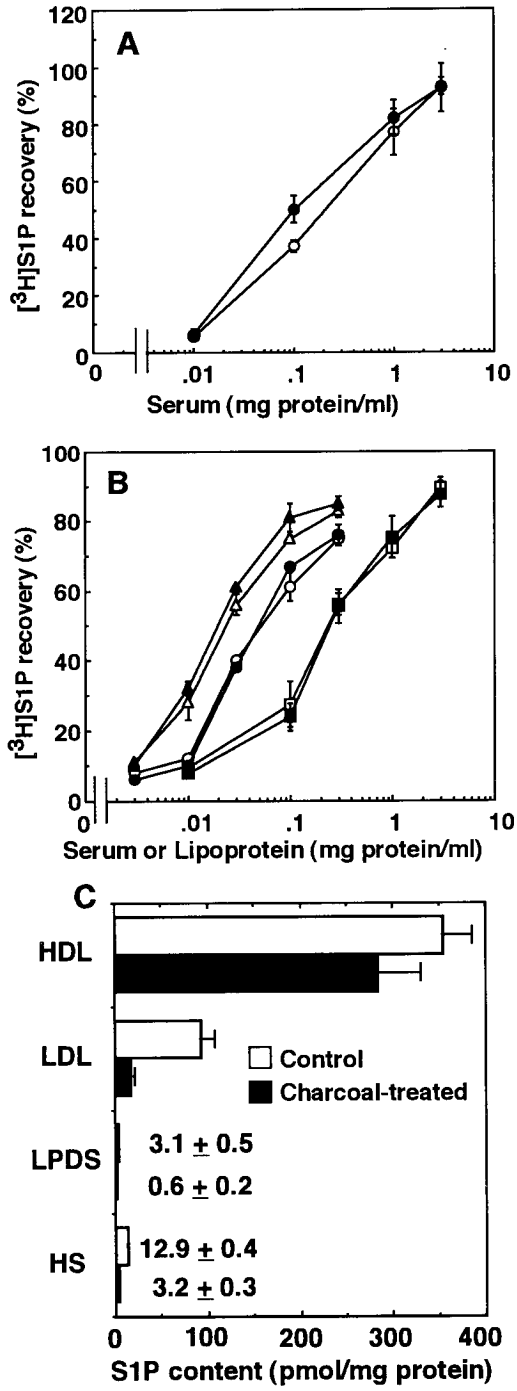


**Figure 4 The ability of serum components to trap S1P and their lipid content**

In (**A**), the indicated concentrations of human serum (○) or charcoal-treated human serum (●) (1 mg of protein/ml corresponds to 1.6%, v/v) were mixed with 1 nM $[^3H]$S1P, and then applied to a Sephadex G-50 column. The $[^3H]$S1P recovered (1.5 ml of eluate) through the column is expressed as a percentage of the total $[^3H]$S1P applied to the column. Data are means ± S.E.M. of three separate experiments. In (**B**), the experimental protocol was essentially the same as for (**A**), except that the indicated concentrations of LDL (○), charcoal-treated LDL (●), HDL (△), charcoal-treated HDL (▲), LPDS (□) or charcoal-treated LPDS (■) were used instead of human serum. Data are means ± S.E.M. of three separate experiments. In (**C**), S1P content of the indicated lipoproteins or serum samples was measured by the radioreceptor assay as described in the Experimental section. Results are expressed as S1P contents (pmol) in the indicated components (1 mg of protein). Data are means ± S.E.M. of three separate experiments.

respectively. From the Eadie–Scatchard plot, we evaluated the maximal values possibly reflecting the active S1P contents in 100 % serum or plasma as 11.2 nM for human serum and 7.3 nM for human plasma. For these samples, the total S1P contents were measured as 829 and 357 nM, respectively.

### Serum components specifically suppress the S1P-induced actions

The foregoing results suggest that S1P in plasma or serum cannot exert full activity to stimulate the lipid receptors. In order to characterize the serum effect further, we prepared low-S1P serum by treatment with activated charcoal. By this procedure, S1P content was decreased to about 10 % of the initial content for FBS and to about 20 % of the initial content for human serum (Figure 1).

Figure 3(A) shows the inositol phosphate response to S1P in the presence or absence of charcoal-treated human serum. Since the depletion of endogenous S1P was not complete, the charcoal-treated serum itself induced a small effect on the inositol phosphate response. However, in the presence of the serum, the exogenous S1P-induced response was suppressed markedly. Thus more than 10 nM S1P was necessary to induce a significant effect in the presence of the serum, whereas 0.1 nM was effective to achieve significant induction of the response in the absence of the serum. The inhibitory effect of the serum on the S1P-induced response was hardly affected by dialysis (Figure 3A), suggesting that the inhibitory substance is bound to the serum component with high molecular mass or is itself a high-molecular-mass substance. The inhibitory effect on the S1P-induced action was also detected by FBS. Thus the charcoal-treated FBS significantly inhibited the S1P-induced inositol phosphate response (Figure 3B). The inhibitory action was reversed by boiling the serum (Figure 3B), suggesting that the inhibitory substance may be heat-labile.

In Figure 3(C), we examined the effect of the human serum on the $P_2$-purinergic agonist-induced action. While the S1P action was suppressed markedly, ATP- or UTP-induced actions were somewhat enhanced by charcoal-treated human serum. Thus the inhibitory serum effect appears to be specific to S1P.

### Serum or plasma components, including lipoproteins, can trap exogenous S1P

As already shown, S1P appears to bind to high-molecular-mass components of serum (Figure 1). The ability of serum components to trap exogenous S1P was evaluated by measuring the amount of [$^3$H]S1P recovery through a Sephadex G-50 gel-filtration column; the $EC_{50}$ values of serum samples were about 0.1 mg protein/ml regardless of the treatment with charcoal (Figure 4A). Thus serum components interact with S1P and thereby may interfere with the lipid binding to its receptors and the subsequent actions on the cells.

We postulated lipoproteins as candidate components to trap S1P, because various lipids have been shown to be concentrated in their particles [35–37]. As expected, HDL and LDL contained a very high amount of S1P per unit amount of protein compared with serum or LPDS (Figure 4C). These samples were treated with the activated charcoal for further experiments. Although HDL was treated with charcoal similarly to LDL, the S1P content was not changed significantly. This suggests tighter interaction of S1P with HDL than with LDL (Figure 4C). We examined whether these serum and lipoprotein samples actually possess the ability to trap S1P. As expected from the S1P content in the native condition (without charcoal treatment; Figure 4C), the serum or lipoprotein samples, regardless of the charcoal treatment trapped S1P in the ranking order of HDL, LDL and then LPDS (Figure 4B).

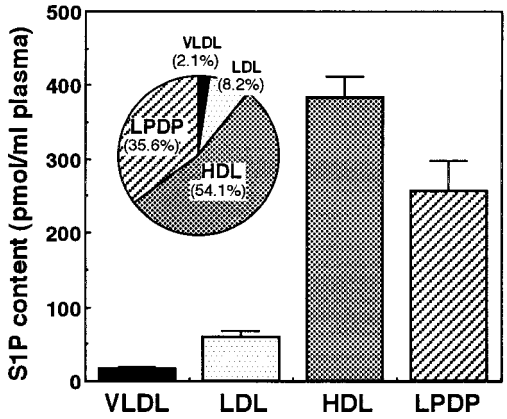


**Figure 5 Distribution of S1P among lipoproteins or LPDP fraction in human plasma**

The indicated lipoproteins and LPDP were separated by density-gradient centrifugation as described in the Experimental section. The protein content (mg/ml of plasma) in each fraction was 0.41 ± 0.09 for very low-density lipoprotein (VLDL), 1.09 ± 0.05 for LDL, 1.86 ± 0.11 for HDL and 76 ± 5 for LPDP. S1P content in each fraction was measured by the radioreceptor assay. Results are expressed as S1P content (pmol) in plasma (1 ml). Inset: the percentage distribution of S1P in each fraction in plasma is shown as a pie chart. Data are means ± S.E.M. of four different samples.

In order to evaluate the relative distribution of S1P among these lipoproteins and the lipoprotein-deficient fraction (LPDP) in plasma, these lipoproteins and LPDP were separated from the same source of plasma sample by density gradient centrifugation (Figure 5). Although the protein content in total lipoproteins (3.4 ± 0.2 mg of protein/ml of plasma) is much smaller than the protein (mainly albumin) content in LPDP (76 ± 5 mg of protein/ml of plasma), more than 60 % of total S1P is bound to the lipoproteins.

## DISCUSSION

Although cell-surface S1P receptors have been identified, the role of S1P as an extracellular signalling molecule has not always been recognized satisfactorily. One question about the extracellular mediator is that resting plasma S1P concentration estimated from total lipid content is much higher than the $K_d$ value of S1P for its receptors, regardless of subtype. In the present study, we have shown that plasma or serum S1P is not fully active to stimulate the lipid receptors. Thus the effective or active concentration of S1P in plasma or serum appears to be close to or lower than the $K_d$ value under physiological conditions (Figure 2C). Plasma components, including lipoproteins such as LDL and HDL, may trap S1P and thereby decrease the activity of S1P to stimulate its cell-surface receptors.

An addition of charcoal-treated serum to the incubation medium suppressed the S1P-induced inositol phosphate response but not the one induced by the $P_2$-purinergic receptor agonist in Edg-3-expressing CHO cells (Figure 3). Thus serum specifically suppresses S1P-induced actions. The serum components inhibiting S1P actions seem to be charcoal-unadsorbable, heat-labile and high-molecular-mass substances. One of the possibilities is that serum components might antagonize the S1P binding at the receptor level. However, pretreatment of the cells with serum followed by rapid washing resulted in no detectable effect on the subsequent [$^3$H]S1P binding, whereas similar pretreatment

with unlabelled S1P markedly inhibited subsequent ligand binding (results not shown). This result does not support the antagonistic nature of the serum components on S1P receptors.

An alternative possibility is that serum components trap S1P and thereby inhibit its binding to receptors. S1P is lipophilic and has been shown to bind to proteins such as albumin. Actually, serum S1P did not permeate through a dialysis membrane (Figure 1). Among serum components, lipoproteins such as LDL and HDL showed a higher activity in trapping exogenous S1P per unit amount of protein than LPDS. In fact, we observed a considerable amount of S1P in LDL and HDL per unit amount of protein. The existence of S1P-like lipids in these lipoproteins has recently been suggested by another group besides ours [37], although, in that study, the authors failed to specify the S1P-like lipids and quantify the amount of S1P in these lipoproteins. To our knowledge, the present study is the first indication of the quantitative measurement of S1P in these lipoproteins and its distribution among several components in plasma. In addition to lipoproteins, we should not neglect albumin in LPDS or LPDP as components to trap S1P, because the protein content in these fraction, of which a major protein component is albumin, is much higher (about 24 times) than the total protein content in the lipoprotein fraction per unit volume of serum or plasma. Actually, one-third of total S1P is distributed in the LPDP fraction. Thus under physiological conditions, not only lipoproteins but also albumin may be important components to trap S1P.

The interaction of S1P with these components in serum or plasma appears to reduce the apparent or active concentration of S1P to a level close to the $K_d$ value of the receptors (Figure 2C). Thus, *in vivo*, an increase or decrease in total plasma S1P content may lead to a change in the active S1P concentration around the $K_d$ value and thereby allow regulation of S1P-receptor activity. A change in lipoprotein content might be another possible mechanism for regulating the concentration of active S1P. Thus an increase in plasma lipoproteins may decrease the active S1P concentration without a change in the total S1P content and vice versa. Such situations might occur under physiological and pathological conditions. For example, a fasted/fed cycle may alter the plasma level of lipids and lipoproteins. Disorders such as diabetes and hyper- and hypothyroidism may cause a change in lipid and lipoprotein metabolisms in circulation. Thus in addition to a quantitative measurement of the total S1P content in plasma or serum, measurement of the effective or active S1P concentration is also important for understanding the role of the extracellular S1P *in vivo*. The novel radioreceptor assay of S1P after lipid extraction shown in Figure 1 is simple and sensitive enough ($>3$ nM S1P) for the former purpose. On the other hand, the inositol phosphate assay without the extraction procedure of S1P in Edg-3-expressing cells (and vector-transfected cells as a control) is sensitive enough ($>0.1$ nM S1P) and suitable for the latter measurement of active S1P concentration.

The finding that plasma lipoproteins behave as S1P carriers is interesting in relation to vascular disorder. It is well known that LDL, especially oxidized LDL, is an important factor involved in vascular diseases such as atherosclerosis. Recent studies showed that S1P regulates various functions of cells involved in vascular remodelling, including endothelial cells, smooth-muscle cells and blood cells such as lymphocytes, monocytes and platelets [9,10,38,39]. In these cells, LDL or oxidized LDL has also been reported to affect these cell functions [40,41]. These results suggest that S1P-receptor-mediated actions may be involved in part in the LDL- or oxidized LDL-induced actions. At the present stage of investigation, however, the role of S1P in LDL or oxidized LDL particles has not yet been defined. Such studies may be important for understanding the mechanism by which oxidized LDL initiates atherosclerotic lesion.

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