High density lipoproteins (HDL) are major plasma carriers of sphingosine 1-phosphate (S1P). Here we show that HDL increases endothelial barrier integrity as measured by electric cell substrate impedance sensing. S1P was implicated as the mediator in this process through findings showing that pertussis toxin, an inhibitor of G<sub>i</sub>-coupled S1P receptors, as well as antagonists of the S1P receptor, S1P<sub>1</sub>, inhibited barrier enhancement by HDL. Additional findings show that HDL stimulates endothelial cell activation of Erk1/2 and Akt, signaling pathway intermediates that have been implicated in S1P-dependent endothelial barrier activity. HDL was also found to promote endothelial cell motility, a process that may also relate to endothelial barrier function in the context of a vascular injury response. The effects of HDL on endothelial cell Erk1/2 and Akt activation and motility were suppressed by pertussis toxin and S1P<sub>1</sub> antagonists. However, both HDL-induced barrier enhancement and HDL-induced motility showed a greater dependence on Akt activation as compared with Erk1/2 activation. Together, the findings indicate that HDL has endothelial barrier promoting activities, which are attributable to its S1P component and signaling through the S1P1/Akt pathway.

Sphingosine 1-phosphate (S1P)<sup>2</sup> is a plasma-borne lysosphinogolipid that has been shown to regulate endothelial barrier integrity (1). For example, treatment of cultured endothelial cells with S1P associated with the carrier albumin acts to increase endothelial barrier activity as indicated by increased transendothelial electrical resistance (2, 3). Moreover, S1P administration greatly reduces lung capillary leakage induced in mice by lipopolysaccharide treatment (4). Mechanistically, S1P acts to enhance tight junction formation in neighboring endothelial cells by influencing subcellular distributions of tight junctional components including ZO-1 and claudin-5 (5). In addition, S1P induces endothelial cortical actin assembly (1) and relocation of endothelial cell junctional adhesion molecules including platelet endothelial cell adhesion molecule and vascular endothelial-cadherin to cell-cell junctional areas (5).

In plasma, S1P is found associated with multiple lipoproteins including low density lipoproteins, very low density lipoproteins, and high density lipoproteins (HDL). However, the bulk of the lipoprotein particle-associated S1P (54%) is bound to HDL (6). A number of recent studies point to the S1P cargo of HDL as being a mediator of many of the cardiovascular effects of HDL including the ability to promote vasodilation, vasoconstriction, and angiogenesis, protect against ischemia/reperfusion injury, and inhibit/reverse atherosclerosis (7). One important cardiovascular-related effect of S1P that has not yet been attributed to HDL is regulation of endothelial barrier activity, a major physiological function of the endothelium. Here we investigate the role of HDL as a regulator of endothelial barrier integrity processes known to be dependent on S1P signaling.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Erythro-S1P was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Stock solutions of S1P (100 μM) were prepared by dissolving S1P into Dulbecco’s phosphate-buffered saline (DPBS) containing 4 mg/ml fatty acid-free bovine serum albumin (BSA; Sigma). S1P levels in the stock solution were confirmed by the Lipidomics Core Facility at the Medical University of South Carolina, Charleston, SC (Department of Biochemistry) using liquid chromatography/mass spectrometry (LC-MS-MS). Pertussis toxin (PTX) was purchased from Sigma. The S1P1 antagonist 857390 and the S1P1/S1P3 antagonist VPC23019 were purchased from Avant Polar Lipids and reconstituted at 1 mM in acidified DMSO, 4 mg/ml BSA. The Akt inhibitor, LY294002, and Erk inhibitor, PD98059, were purchased from R&D Systems, Inc. (Minneapolis, MN).

**HDL Preparation**—Blood for HDL isolation was collected from healthy volunteers in 0.4 mM EDTA after 12 h of fasting. The donors were normolipemic volunteers, not receiving prescription medication for any acute or chronic condition and without family history of coronary artery disease, peripheral vascular disease, or stroke. None of the volunteers was receiving antioxidant therapy. Low density lipoprotein was isolated from pooled plasma (typically from 4 to 5 donors) after density adjustment (1.019 < d < 1.063 g/ml) with potassium bromide (KBr), by preparative ultracentrifugation at 50,000 rpm (about 17,000 g) for 18 h. The supernatant was fractionated into low density lipoprotein, very low density lipoprotein, and HDL fractions separated by rate zonal ultracentrifugation. The lipoprotein particles were isolated from the eritrocilemic supernatant by ultracentrifugation and dialyzed against phosphate-buffered saline (PBS) containing 0.1% BSA. The pooled HDL was further purified by agarose gel electrophoresis to remove low density lipoprotein and very low density lipoprotein. The purified HDL was concentrated by ultrafiltration and dialyzed against phosphate-buffered saline containing 0.1% BSA.

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360,000 × g) for 17 h, 5 °C, on a Beckman Optima™ XL-100K ultracentrifuge, using a type 70 Ti rotor. HDL was then isolated after density adjustment (1.063 < ρ < 1.21 g/ml) with KBr at 68,000 rpm (500,000 × g) for 22 h using the same rotor. Isolated HDL was washed by ultracentrifugation (SW 41 Ti rotor, 40,000 rpm, 280,000 × g, 36 h), dialyzed against a 0.15 M sodium chloride solution containing 0.3 mM EDTA, pH 8.0. Prior to use in cell culture, the HDL was dialyzed against DPBS containing 0.3 mM EDTA. Protein levels in HDL preparations were measured by the Lowry method. To determine S1P levels in the purified HDL preparations, aliquots were diluted 1:50 in DPBS and subjected to LC-MS-MS described by Bielawski et al. (8). These analyses showed that the average S1P level was 381.87 ± 32.22 pmol/mg of HDL protein (n = 4).

Cells—Human umbilical vein endothelial cells (HUVEC) (Cascade Biologics, Inc., Portland, OR) were maintained on Corning tissue culture dishes (Corning Inc., Corning, NY) in a humidified atmosphere of 5% CO2, 95% air in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD), which contains 2% fetal bovine serum. Cells were used between passages 3 and 7.

Electrical Cell Substrate Impedance Sensing Assay—Electric cell substrate impedance sensing (ECIS) is a non-invasive means of measuring electrical resistance across cultured cell monolayers in real time (9). In ECIS, cells are grown on the surface of gold-film electrodes. From readings of the electrical impedance (a measure of opposition to a sinusoidal alternating current) of the cell-covered electrode performed with low amplitude-sensing voltages, alterations in cell-cell contacts can be deduced. Changes of impedance are attributed to changes of the resistance in the paracellular pathway and the resistance between the ventral cell surface and the electrode (a: a = R(p/

FIGURE 1. HDL enhances transendothelial electrical resistance. A minimal TEER plateau was reached within ~24 h of replacing the culture medium of confluent endothelial cells with serum-free medium. The monolayers were then incubated with varying concentrations S1P (A) or HDL (B). In B, the concentration of S1P in the HDL tested ranged from 5 to 403 nm. The TEER tracings shown in panels A and B each represent mean data from three independent experiments each with two replicates per condition. As a control, monolayers were treated with 40 μg/ml BSA, a concentration corresponding to the amount of BSA carrier used for the highest concentration of S1P tested. Impedance values were normalized by dividing each value by the level of impedance measured just prior to the addition of effectors.

FIGURE 2. HDL-enhanced transendothelial electrical resistance is inhibited by pertussis toxin and S1P1 antagonists. Confluent endothelial monolayers were grown under serum-free conditions until a minimal TEER plateau had been reached. The cells were then incubated with S1P or HDL in the presence or absence of PTX (A and B) or S1P1 antagonists (C and D). In A and B, S1P was used at 833 nm, HDL was used at 1000 μg/ml (containing 400 nm S1P), and PTX was used at 1 μg/ml. In C and D, S1P was used at 250 nm, HDL was used at 621 μg/ml (containing 250 nm S1P), the S1P1 antagonist 8S7390 was used at 10 μm, and the S1P1/S1P3 antagonist VPC23019 was used at 10 μm. As controls, monolayers were treated with BSA-containing serum-free medium (SFM) plus or minus vehicle buffer. Each of the TEER tracings shown is an average of two replicate wells and representative of three independent experiments. Impedance values were normalized by dividing each value by the level of impedance measured just prior to the addition of effectors.
where $R$ is the cell radius, $\rho$ is the resistivity of the culture medium, and $h$ is the gap between the cells and the electrode) (10, 11). Disruption in the barrier integrity of an epithelial monolayer results in increased ion flow across the cell layer leading to decreased electrical resistance. ECIS has been used to monitor transendothelial electrical resistance (TEER) as an index of endothelial cell barrier function (1, 5).

Herein, TEER was measured using an ECIS Model 1600 instrument (Applied Biophysics, Troy, NY). HUVEC in EGM-2 medium were seeded into the wells of ECIS 8W10E electrode arrays at a density of $1 \times 10^5$ cells/well. The arrays were pre-coated with human plasma fibronectin (Invitrogen) at 100 $\mu$g/ml in 0.15 M NaCl, 0.01 M Tris, pH 8.0. The cells were cultured in EGM-2 medium, and impedance was measured every 5 min at 15 kHz frequency. For every 48 h of culture, 50% volume of medium was replaced with fresh EGM-2. When the electrical resistance reached a maximal plateau (3 days), the medium was replaced with serum-free endothelial basal medium (EBM; Lonza) containing 1% penicillin-streptomycin-glutamine (Invitrogen). Electrical resistance was monitored until a minimal plateau was reached (~24 h). Effectors (i.e. S1P-albumin, HDL, or albumin in DPBS) were introduced into the culture medium by removing a volume corresponding to that of the effector to be added. The maximum volume of each effector added did not exceed $1/3$ of the 400-$\mu$l volume of conditioned culture medium in each well. For experiments evaluating the effects of PTX on TEER, PTX (2 $\mu$g/ml in 50% glycerol with 50 mM Tris, 10 mM glycine, and 0.5 M NaCl, pH 7.5) was added to EBM during the final 12 h of serum starvation. Controls received PTX buffer alone. S1P or HDL was then added to the medium.

For experiments evaluating the effects of S1P1 antagonists on TEER, antagonist stocks (1 mM in 5% acidified DMSO, 4 mg/ml BSA) were diluted 1:100 into the conditioned EBM at the same time that S1P or HDL was added. For experiments evaluating the effects of Akt and Erk inhibition on TEER, inhibitor stocks (25 $\mu$M LY294002 in DMSO or 50 $\mu$M PD98059 in DMSO) were diluted to 50 nM into the conditioned EBM 1 h prior to the addition of S1P or HDL.

**Bio-Plex Phosphoprotein Detection Assay**—HUVEC were seeded into 6-well dishes (Corning) at $1 \times 10^6$ cells/well and grown for 18 h in EGM-2. The medium was then replaced with EBM containing 0.1% fetal bovine serum, and the cells were grown for 24 h. The medium was then supplemented with a range of S1P concentrations (37 nM-1 $\mu$m) or a range of HDL concentrations (37 $\mu$g–1 mg of protein/ml, corresponding to 18–493 nM S1P). For experiments evaluating the effects of PTX, PTX (100 ng/ml) or the PTX buffer was added to EBM during the final 12 h of serum starvation. For experiments evaluating the effects of S1P1 antagonists 857390 and the S1P1/S1P3 antagonist VPC23019, the antagonists (1 mM in 5% acidified DMSO, 4 mg/ml BSA) were diluted 1:100 into the conditioned EBM during the final 15 min of serum starvation.
S1P or HDL was then added to the medium and allowed to incubate for 3 min. The cells were then extracted with Bio-Plex cell lysis buffer (Bio-Rad), and protein concentration was determined by the Bio-Rad DC protein assay (Bio-Rad). Levels of phospho-Erk1/2 and phospho-Akt in the extracts were determined by multiplex bead assay using kits (Bio-Rad) and a Bio-Plex-200 instrument (Bio-Rad).

**HUVEC Migration Assay**—ECIS was also used to evaluate the effects of S1P and HDL on HUVEC motility. HUVEC were seeded onto 8W1E electrode arrays at $1 \times 10^5$ cells/well in EGM-2 medium. The cells were grown until electrical resistance reached a maximal plateau. Each cell-covered electrode was subjected to a pulse of 4 V for 20 s. This brief elevated field effectively killed the cells on the electrode as indicated by a drop in impedance. Electrical resistance was then monitored for the subsequent 10-h period during which, under control conditions, electrical resistance recovers as cells migrate and fill in the void. During this period, the EBM medium was supple-
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RESULTS

HDL Augments TEER—As shown in Fig. 1A, S1P induces a dose-dependent increase in TEER. The TEER response to S1P displays a bimodal distribution over a 5-h period. Each of the two modes spans 2 h. For all of the S1P doses tested, the height of the second peak was always less than that of the first. HDL treatment also produces a dose-dependent increase in TEER (Fig. 1B). Like S1P, HDL also produced a bimodal TEER response. By contrast, the effect of HDL on the second TEER peak was sustained longer at the higher HDL doses than observed with any S1P dose tested.

HDL-induced Enhancement of TEER Is Pertussis Toxin-sensitive—G_i protein-coupled S1P receptor signaling in endothelial cells is inhibited by PTX (12). As shown in Fig. 2A, the TEER response of endothelial cell monolayers to S1P treatments was inhibited by PTX. Similarly, the TEER response to HDL was completely abrogated by PTX (Fig. 2B), suggesting the requirement for G_i-coupled S1P receptors.

S1P Receptor Antagonists Inhibit HDL-induced Enhancement of TEER—The S1P receptor, S1P1, has been shown to mediate S1P-stimulated augmentation of endothelial barrier activity (1). We therefore tested the effect of S1P1 receptor antagonists on the barrier enhancement response of endothelial cell monolayers to HDL. Both the S1P1 antagonist, 857390, and the S1P1/S1P3 antagonist, VPC23019, inhibited the TEER response to HDL as well as the TEER response to S1P (Fig. 2, C and D).

HDL Stimulates Erk1/2 and Akt Activation in Endothelial Cells—S1P signaling in endothelial cells involves Erk1/2 and Akt activation (13). Multiplex microbead suspension array analysis was performed to evaluate the effect of HDL on the activation of Erk1/2 and Akt signaling pathway intermediates, which have been implicated in endothelial barrier function (5, 14, 15). As shown in Fig. 3A, S1P elicits a transient increase in Erk1/2 phosphorylation with peak phosphorylation detectable within 3–5 min of treatment. The response to S1P was dose-dependent, although a plateau was not reached using the highest dosage of S1P tested (Fig. 3B). Similarly, HDL produced a transient increase in Erk1/2 phosphorylation with peak phosphorylation detectable within 3–5 min of treatment (Fig. 3C). The activation response to HDL was also dose-dependent, reaching a maximal plateau at 333 µg/ml (based on LC-MS-MS analysis, this amount of HDL contained 135 nM S1P) (Fig. 3D). Similar effects of HDL and S1P on the activation of Akt were also observed (Fig. 3, E–H). Furthermore, the findings indicate that when equimolar amounts of S1P were carried either on HDL or on albumin, they elicit a similar magnitude of Erk and Akt activation (Fig. 3, compare B with D and compare F with H).

Activation of Erk1/2 and Akt by HDL Is Blocked by Pertussis Toxin and an S1P1 Antagonist—Previous studies have shown that S1P-mediated activation of Erk and Akt is PTX-sensitive (16, 17) and mediated by the S1P receptor, S1P1 (18, 19). We performed multiplex microbead suspension array analysis to evaluate the effect of PTX on HDL-induced activation of Erk1/2 and Akt in endothelial cells. The activation of Akt and Erk1/2 by either HDL or S1P was inhibited by PTX (Fig. 4, A and B).
suggesting the requirement for G\(_i\)-coupled S1P receptors. The S1P receptor, S1P1, was implicated in this process by the finding that the S1P1 antagonist, 857390, and the S1P1/S1P3 antagonist, VPC23019, inhibited HDL-induced and S1P-induced activation of Erk1/2 and Akt (Fig. 4, C and D).

Inhibition of Akt Blocks HDL-induced Enhancement of TEER—In light of the finding that HDL stimulates activation of both Erk and Akt, we next evaluated the effects of antagonists of activation of these signaling pathway intermediates on endothelial barrier function. Inhibition of both S1P-induced and HDL-induced TEER was observed using the Akt inhibitor LY294002 (Fig. 5, A and B). By contrast, partial inhibition of both S1P-induced and HDL-induced TEER was observed using the Erk inhibitor PD98059 (Fig. 5, C and D). Control multiplex microbead suspension array analyses showed that LY294002 blocked 100% of Akt phosphorylation and PD98059 blocked ~70% of Erk1/2 phosphorylation in response to either S1P or HDL stimulation in HUVEC (data not shown).

**HDL Promotes Endothelial Cell Motility via S1P1/Akt Signaling**—Endothelial cell motility has been shown to be regulated by the S1P1/Akt/Rac small GTPase pathway (18, 20). An

induced endothelial cell motility. As a result, it was found that inhibition of Akt abrogated HDL-induced motility (Fig. 7B). Similarly, inhibition of Akt also abrogated motility induced by S1P carried on albumin (Fig. 7A). By contrast, inhibition of Erk1/2 activation produced partial inhibition of the motility induced by either HDL or S1P (Fig. 7, C and D). Together, these findings indicate that HDL is capable of enhancing endothelial cell motility via S1P signaling primarily through the S1P1/Akt pathway. This signaling may play an important role in the process by which an injured endothelium is repaired.

**DISCUSSION**

Here we explored the effects of HDL on endothelial permeability. A number of studies have established that HDL administration can reduce organ injury and mortality in animal models of shock and ischemia/reperfusion (21–23). Mechanistically, such beneficial effects have been attributed to a variety of HDL properties including the ability of HDL to bind and inactivate lipopolysaccharide, to inhibit cytokine-induced expression of endothelial cell adhesion molecules, and to prevent neutrophil adhesion and transmigration through the
endothelium (23–26). These latter activities involve effects on the semipermeable barrier function of the endothelium. Alteration in endothelial barrier function is a factor underlying postischemic edema, the recruitment and migration of monocytes, as well as the introduction of triglyceride-rich lipoprotein particles into the intima of the blood vessel (27). The findings presented in the present study establish that HDL can act to promote endothelial barrier integrity as evidenced by its ability to increase TEER in the ECIS assay.

This study also provides insights into the mechanisms underlying HDL-mediated endothelial barrier enhancement. It is shown that the elevated TEER response to HDL is PTX-sensitive, thus implicating G_i-coupled S1P receptor signaling. This inference was substantiated by the finding that HDL enhancement of TEER is blocked by S1P1 antagonists. These results are consistent with findings presented here and elsewhere showing that S1P activation of endothelial cells by S1P carried on albumin leads to redistribution of tight junction-associated proteins to cell-cell junctions via the S1P1/G(i)/Akt/Rac pathway (5).

It was also observed that both HDL and S1P (carried on albumin) induced bimodal TEER responses over a range of doses. Considering that S1P receptors are G-coupled receptors, these findings may relate to the process by which many G protein-coupled receptors when exposed to agonist become refractory to further stimulation by the same agonist (29). This process, known as agonist-induced desensitization, can be mediated by receptor internalization, down-regulation, and G protein uncoupling. Importantly, S1P has been shown to induce internalization and recycling of the endothelial cell S1P receptor, S1P1 (30). Thus, agonist-induced internalization and recycling of S1P receptors may underlie the observed bimodal TEER response to HDL-S1P.

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