Transforming growth factor-β1 downregulates caveolin-1 expression and enhances sphingosine 1-phosphate signaling in cultured vascular endothelial cells

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Cytokines that belong to transforming growth factor-β (TGF-β) superfamily regulate numerous essential functions of mammalian cells. In cardiovascular systems, for example, they play indispensable roles in angiogenesis during development. Vascular endothelial cells express multiple functional receptor systems specific to TGF-β superfamily cytokines that, when activated, lead to diverse cellular responses (reviewed in Ref. 23). In addition to aforementioned developmental angiogenesis, these cytokines are supposed to play significant roles in the pathogenesis of adult cardiovascular diseases including atherosclerosis (reviewed in Ref. 36). It is therefore key to more fully understand mechanisms whereby TGF-β superfamily cytokines regulate functions of cardiovascular cell types, including those of endothelial cells. Specifically, at least some of the vascular responses evoked by these cytokines appear to occur in concert with those elicited by other receptor stimuli, including polypeptide growth factors and lipid mediators (18). Thus it is important to expand our understandings of whether or not and how TGF-β superfamily cytokines exhibit cross-talks with other receptor pathways within the cardiovascular system. Sphingosine 1-phosphate (S1P) is a serum-borne naturally occurring sphingolipid mediator that binds to and activates a family of G protein-coupled receptors termed S1P receptors (reviewed in Ref. 9). In relation to TGF-β superfamily cytokines, it is interesting that signals evoked by receptor stimulation both with S1P as well as with TGF-β are indispensable in angiogenic responses of vascular endothelial cells (7, 26). Although S1P per se is able to modulate effector molecules of TGF-β superfamily cytokines termed Smad proteins in kidney mesangial cells (39), it has remained to be elucidated whether or not these two key pathways exhibit cross-talks of receptor signal transduction in endothelial cells. S1P induces robust migratory responses of cultured vascular endothelial cells by way of a signaling pathway comprising AMP-activated protein kinase (AMPK), a small G-protein Rac1, and kinase Akt (24). However, effects of TGF-β on these aspects of S1P responses have remained less well appreciated.

In vascular endothelium, plasmalemmal caveolae, which are specialized flask-shaped microstructures, are enriched in a wide array of receptor signaling proteins and thereby play pivotal roles in mediating signaling cues from cell surface receptors to their downstream effector molecules (for review see Ref. 32). Caveolins are structural proteins of caveolae membrane microstructures, of which caveolin-1 isoform binds to endothelial caveolae-associated signaling proteins and regulates their functions (4). Because at least some of the receptor molecules for both S1P and TGF-β are associated with caveolae and caveolin-1 (13, 33), we developed a hypothesis that TGF-β superfamily cytokines regulate expression levels of caveolin-1 and modulate the magnitudes of endothelial responses to S1P.

In the present study, we provide evidence that TGF-β1, a well-characterized member of TGF-β superfamily cytokines, downregulates expression levels of caveolin-1 at both levels of protein and mRNA. We further show that caveolin-1 down-
regulation by TGF-β1 is associated with enhanced endothelial responses to subsequent stimulation with S1P.

**EXPERIMENTAL PROCEDURES**

**Reagents.** Fetal bovine serum (FBS) was from HyClone (Logan, CT). All other cell culture reagents and media were from Life Technologies (Rockville, MD). S1P was from BioMol (Plymouth Meeting, PA). Human recombinant TGF-β1 was from RELIATech (Braunschweig, Germany). Anti-phospho-Akt antibody (Ser473), anti-Akt polyclonal antibody, anti-phospho-AMPK antibody (Thr172), anti-AMPK antibody, and anti-phospho-Smad1/Smad5 antibody (Ser463/465) were from Cell Signaling Technologies (Beverly, MA). Anti-phospho-Smad2/Smad3 antibody (Ser423/425) and anti-β-actin polyclonal antibody were from Santa Cruz (Santa Cruz, CA). Anti-caveolin-1 and anti-eNOS monoclonal antibodies were from BD Biosciences (San Jose, CA). Anti-S1P1 antibody was raised against native NH2-terminal fragment peptide of rat EDG-1 (S1P1) (29) and is a gift from Roger A. Sambadi (San Diego State University). SuperBlock reagents, SuperSignal substrates for chemiluminescence detection, and secondary antibodies conjugated with horseradish peroxidase (HRP) were from Pierce (Rockford, IL). Rac1 activity assay kit was from Cytoskeleton (South Acoma, CO). RNeasy minicolumns were from Qiagen (Valencia, CA). SuperScript RNase H− reverse transcriptase (RTase) was from Invitrogen (Carlsbad, CA). Tag DNA polymerase was from Promega (Madison, WI). Small interfering RNA (siRNA) was from PROLIGO (Boulder, CO). CytoSelect 24-well Cell Migration Assay kits (8 μm, Colorimetric Format) were from Cell Biolabs (San Diego, CA). cDNA encoding full-length human S1P1 receptor epitope-tagged with FLAG peptide (FLAG/S1P1) subcloned into pcDNA3 (Invitrogen) was originally provided by Timothy Hla (University of Connecticut). cDNA encoding full-length canine caveolin-1 epitope-tagged with c-myc (c-myc/cav-1) subcloned into pcDNA3 was originally provided by Michael P. Lisanti (Thomas Jefferson University). These plasmid cDNAs had been previously characterized (13). FuGENE 6 was from Roche Applied Sciences (Mannheim, Germany). All other materials, including SB-431542 and goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) were from Pierce (Rockford, IL). Rac1 activity assay kit was from Cytoskeleton (South Acoma, CO). RNeasy minicolumns were from Qiagen (Valencia, CA). SuperScript RNase H− reverse transcriptase (RTase) was from Invitrogen (Carlsbad, CA). Tag DNA polymerase was from Promega (Madison, WI). Small interfering RNA (siRNA) was from PROLIGO (Boulder, CO).

**Cell culture.** Bovine aortic endothelial cells (BAEC) obtained from Cell Systems (Kirkland, WA) were split at a ratio of 1:4 and were maintained in culture using medium A from Cell Systems (Kirkland, WA) and were then subjected to experiments. They were transfected the next day with cDNA encoding FLAG/S1P1 (3 μg) using FuGENE 6 as described (14) 16 h before transfection with siRNA.

**Drug treatment.** TGF-β1 was resolved into PBS containing fatty acid-free BSA (0.4%, wt/vol) at a concentration of 10 μg/ml and was stored at −20°C. SB-431542 was resolved in DMSO and was kept at −20°C under nitrogen. Other drug treatments were performed exactly as described previously (14, 15). The final concentration of solvents including DMSO did not exceed 0.1% (vol/vol) in any experiment.

**Immunoblot analyses.** Immunoblot analyses were performed as described previously (14, 15).

**Immunostaining analyses.** For cell-imaging studies, BAEC were plated on gelatin-coated coverslips. They were washed with PBS on ice and were fixed with 1% paraformaldehyde in PBS (wt/vol) for 10 min at ambient temperature, followed by permeabilization with acetone-ethanol (1:2 in vol/vol) for 5 min at −20°C. The fixed cells were incubated for 1 h at room temperature in 10% goat serum and then incubated with monoclonal anti-caveolin-1 antibody at 1:150 dilution for 1 h at room temperature. The cells were then washed three times with PBS and were incubated with goat anti-mouse IgG antibody conjugated with FITC at 1:200 dilution for 1 h at room temperature. After being washed with PBS for another three times, they were mounted on a glass slide and were observed using a confocal laser microscope (Radiance 2100/ Rainbow, Bio-Rad, Hercules, CA) (28).

**Rac1 activity assay.** Rac1 activity in BAEC was assessed essentially as described (6) using a commercially available kit. Briefly, BAEC in 100-mm dishes were harvested on ice into a lysis buffer (provided). Then cell lysates that bear equal quantities of cellular proteins were subjected to a pull-down assay using beads conjugated with the glutathione S-transferase-tagged p21-binding domain of p21 activated kinase 1 (PAK-PBD) that specifically recognizes GTP-bound (activated) form of Rac1. Bound Rac1 proteins were eluted using Lamelli’s sample buffer and were subjected to immunoblot analyses as above, using an antibody directed to Rac1 (supplied).

**Semiquantitative RT-PCR analyses.** RNA isolation and RT-PCR assays were performed as described previously (14, 15). The primers used were 5′-AGC CCA ACA AGG CTA TG-3′ (sense) and 5′-GAT GCC ATC GAA ACT GTG TG-3′ (antisense) for caveolin-1 (38), 5′-GCT TGA GAC CCT CAG TCA GG-3′ (sense) and 5′-GCT CTC CAG TCT TGA GCT GG-3′ (antisense) for eNOS (31), 5′-AAG ACC TGT GAC CTC TTC-3′ (sense) and 5′-ATG AAC CTT TTA GGA GCT TCA A-3′ (antisense) for S1P1 (14), and 5′-ACC ACA GTG GCT GTC AC-3′ (sense) and 5′-TCC ACC CTG TGT CTG TA-3′ (antisense) for GAPDH (14, 15), respectively. We optimized the assay conditions and verified that increasing amounts of a starting mRNA sample yield increasing amounts of RT-PCR product under these conditions in each primer pair.

**Transfection with siRNA/plasmid cDNA.** BAEC were split at a ratio of 1:4 in medium A and were plated on gelatin-coated 6-well plates. They were transected the next day with 1 nM of siRNA using LipofectAMINE 2000 and OptiMEM as described previously (15). Sequence of siRNA directed to bovine caveolin-1 and that of corresponding control siRNA were exactly as described (6). After the transfection, cells were incubated in medium A for 24 h, serum-starved overnight using medium B, and then subjected to experiments. In some experiments, BAEC in a 100-mm dish had been transfected with cDNA encoding FLAG/S1P1 (3 μg) using FuGENE 6 as described (14) 16 h before transfection with siRNA.

**COS-7 cells were split at a ratio of 1:8 in six-well plates using medium A. They were transfected the next day with cDNA encoding FLAG/S1P1 (250 ng) and c-myc/cav-1 (or empty vector: 60 ng) using FuGENE 6. Twenty-four hours later they were serum starved using medium B for 16 h and were then subjected to experiments.

**Cell migration assay.** The migration assay used the CytoSelect 24-well cell migration assay kit essentially as described (27). BAEC were trypsinized and subjected to cell counting assay using a particle counter (14). Harvested cells were suspended in medium B, and 1 × 105 cells were seeded into an gelatin-coated upper chamber insert at a volume of 300 μl. Inserts bearing BAEC were placed into a 24-well plate containing 500 μl of medium B with or without S1P (500 nM) and were incubated at 37°C for 6 h (30). They were then washed with PBS, and nonmigrated cells were gently removed using cotton swabs. The inserts were placed in cell staining solution for 10 min and shaken with 200 μl of extraction solution. The degrees of BAEC migration across the insert membranes were quantified by measuring the optical density at 560 nm.

**Isolation of caveolae-enriched fractions.** Caveolae-enriched fractions were separated by using ultracentrifugation with a discontinuous sucrose gradient system essentially as previously described (6, 13). Briefly, BAEC from two 100-mm dishes were scraped together into 2 ml of “carbonate buffer” containing 500 mM sodium carbonate (pH 11), 25 mM 2-(N-morpholino)ethanesulfonic acid and 150 mM NaCl, and the cells were homogenized and sonicated. After an aliquot of
whole cell lysate had been saved, the resulting cell suspension was brought to 45% sucrose (wt/vol) by adding 2 ml of carbonate buffer containing 90% sucrose and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the 45% sucrose bed by adding 4 ml each of 35% and 5% sucrose solutions prepared in carbonate buffer. After centrifugation using a RPS40T rotor (Hitachi, Tokyo, Japan), 12 ml fractions were collected starting at the top of each gradient. An equal volume of each fraction was analyzed by SDS-PAGE and immunoblotting.

Electron microscopic observation. Cells that had been plated in 60-mm dishes were subjected to either treatment with TGF-β1 (5.0 ng/ml for 24 h or vehicle) or transfection with siRNA directed to caveolin-1 (or control) as described above. Cells were then washed with cold PBS twice, fixed with 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) for 30 min, postfixed in 2% OsO4 (wt/vol) for 2 h at 4°C, and stained with 0.5% uranyl acetate (wt/vol). Samples were then dehydrated with ethanol, infiltrated with propylene oxide, and embedded in Epon. Sections were examined with a JEM-100 CX electron microscope (JEOL, Akishima, Tokyo, Japan).

Approvals for usage of recombinant DNA. The study procedures using recombinant DNA had been approved by the Recombinant DNA Usage Committees at Kagawa University.

Fig. 1. Effects of transforming growth factor (TGF)-β1 on endothelial signaling protein abundance in bovine aortic endothelial cells (BAEC). Shown are the results of protein immunoblot assays analyzed in cell lysates derived from BAEC treated with TGF-β1. BAEC were treated with TGF-β1 (5.0 ng/ml) for the times indicated, and equal quantities of cellular protein were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analyses probed with antibodies directed to caveolin-1 (Cav-1); endothelial nitric oxide synthase (eNOS); sphingosine 1-phosphate receptor (S1P1); or β-actin (actin), as indicated. Top: representation of six independent experiments that produced equivalent results. Bottom: results of densitometric analyses from pooled data, plotting the fold increase of the degree of expression levels of caveolin-1 and eNOS at the TGF-β1 treatment duration indicated, relative to the signals obtained in the absence of TGF-β1. Each data point represents the mean ± SE derived from 6 independent experiments. *P < 0.05 vs. cells not treated with TGF-β1.

Fig. 2. Characterization of TGF-β1-elicted decreases in caveolin-1 protein abundance in BAEC. A: results of protein immunoblot analyses in which BAEC were treated with various TGF-β1 concentrations for 24 h. Equal quantities of cellular protein were analyzed in immunoblots probed with antibodies as indicated. Top: representative data of four independent experiments that yielded equivalent results. Bottom: results of densitometric analyses from pooled data, plotting the fold increase of the degree of expression levels of Cav-1 at the TGF-β1-concentration indicated, relative to the signals obtained in the absence of TGF-β1. Each data point represents the mean ± SE derived from 4 independent experiments. *P < 0.05 vs. cells not treated with TGF-β1. B: typical photomicrographs of BAEC processed for immunostaining as described in the text. Left: representative image of Cav-1-immunolabeled BAEC studied under basal conditions. Right: representative image following treatment of endothelial cells with 5.0 ng/ml TGF-β1 for 24 h and followed by Cav-1 immunostaining. Both images are at ×250 magnifications and are photographed with identical brightness and contrast settings. Three independent cultures were examined.
Other methods. Protein determinations were made with the Bio-Rad Protein Assay Kit (Hercules, CA). LDH activity of culture medium was measured using a CytoTox-ONE Homogenous Membrane Integrity Assay kit (Promega), using DMEM and cell lysates extracted with a lysis buffer containing Triton-X (0.1% vol/vol) as negative and positive controls, respectively. All experiments were performed at least three times. Mean values for individual experiments are expressed as means ± SE. Statistical differences were analyzed by ANOVA followed by Scheffe’s F test using STAT VIEW II (Abacus Concepts). A P value <0.05 was considered statistically significant.

RESULTS

We first explored whether or not TGF-β1 modulates expression levels of caveolin-1 protein in BAEC, an archetypal model of cultured vascular endothelial cells. Cells had been treated with 5.0 ng/ml concentration of TGF-β1 up to 48 h and were
then lysed and subjected to immunoblot analyses. Figure 1 indicates that abundance of caveolin-1 markedly decreased when cells were treated with TGF-β1 for durations longer than 12 h. Abundance of endothelial nitric oxide synthase (eNOS) protein was reciprocally increased, in accordance with earlier findings (17). Expression levels of several other endothelial signaling proteins were also examined. Over the TGF-β1 treatment up to 48 h, immunoreactive signals corresponding to actin (Fig. 1), kinase Akt (Fig. 5), kinase AMPK (Fig. 5), as well as receptors S1P₁ (Fig. 1), did not change. Dose-response experiments were performed using increasing concentrations of TGF-β1 for 24 h. Figure 2A demonstrates that caveolin-1 protein expression started declining at 1.0 ng/ml. We also performed immunostaining of otherwise identically cultured BAEC in the presence and absence of TGF-β1 (5.0 ng/ml for 24 h). Figure 2B demonstrates that immunoreactive signals corresponding to caveolin-1 protein were significantly less in TGF-β1-treated cells than in those with vehicle. We sought to determine whether or not TGF-β1-induced attenuation of caveolin-1 protein expression levels took place at a level of mRNA. We performed semiquantitative RT-PCR analyses using RNA samples derived from TGF-β1-treated BAEC. Figure 3A indicates that TGF-β1 decreased expression levels of caveolin-1 transcripts, concomitantly with augmented levels of transcripts that encode eNOS, yielding similar patterns with changes in immunoblot analyses observed at a level of protein (Fig. 1). Figure 3B shows that TGF-β1 downregulated caveolin-1 transcript expression levels in a dose-dependent fashion. Under these conditions of TGF-β1 treatment, total protein recovery, cell numbers, and LDH release into culture media did not change (data not shown). In vascular endothelial cells, TGF-β1 superfamily modulates receptor molecules termed activin receptor-like kinases ALK-5 and ALK-1 (7). We made use of SB-431542, which is a specific inhibitor of ALK-5 (21). Figure 4 shows that this agent completely counteracted TGF-β1-induced decreases in caveolin-1 abundance and increases in eNOS abundance. Together, these data indicate that TGF-β1 led to attenuated expression of caveolin-1 in BAEC at both levels of protein and mRNA, mediated by ALK-5 receptor subtype.

We then sought to explore the consequences of caveolin-1 downregulation induced by TGF-β1. S1P is an angiogenic lipid growth factor that activates a signaling cascade consisting of AMPK, Rac1, and Akt in vascular endothelial cells (24). Because receptor signals evoked by S1P are modulated by caveolae and caveolin-1 (6, 13, 14, 24), we developed a hypothesis that caveolin-1 downregulation by TGF-β1 leads to an alteration of the magnitudes of S1P responses. For this, we first assessed the degrees of protein kinase responses to S1P with or without pretreatment with TGF-β1. We performed immunoblot analyses in lysates prepared from TGF-β1-pretreated BAEC that had been subsequently exposed to S1P, using phospho-specific antibodies as probes. Figure 5, A and B, shows the results of Western blot analysis using antibodies specific to phosphorylated (activated) forms of Akt and AMPK. BAEC were first incubated with TGF-β1 (5 ng/ml for 24 h) or its vehicle and then treated with S1P (500 nM for up to 10 min). S1P induced phosphorylation of these proteins in BAEC that had not been pretreated with TGF-β1 (also see Refs. 11 and 24). When BAEC had been first preincubated with TGF-β1 for 24 h, the degrees of subsequent phosphorylation of Akt and AMPK induced by S1P were augmented (Fig. 5, A and B). A dose-response study was performed at a level of Akt phosphorylation using S1P for 5 min. We chose to perform a dose-response study at a level of Akt because the amplitude of activation elicited by S1P was higher than those observed in AMPK or in Rac1. Figure 5C shows that TGF-β1 induced promotion of Akt response to S1P at multiple concentrations. We then examined whether or not TGF-β1 augments the magnitudes of Rac1 activation. Figure 5D indicates that S1P-evoked Rac1 activation was enhanced in TGF-β1-pretreated cells compared with vehicle control. Whereas TGF-β1 per se

Fig. 4. Pharmacological characterization of TGF-β1-elicited decreases in caveolin-1 protein abundance in BAEC. Shown are the results of protein immunoblot analyses in which effects of SB-431542, an inhibitor of ALK-5, on TGF-β1-mediated downregulation of endothelial Cav-1 protein abundance were examined. BAEC were treated with SB-431542 (1.0 μM for 30 min) or vehicle and then incubated with TGF-β1 (5.0 ng/ml for 24 h) or vehicle, as indicated. Equal quantities of cellular protein were examined in immunoblot analyses probed with antibodies specific to Cav-1, eNOS, and β-actin (actin), respectively. Left: representative data of four independent experiments that yielded identical results. Right: results of densitometric analyses from pooled data plotting the fold increase of the degree of expression levels of Cav-1 (top) or eNOS (bottom) relative to the signals obtained in the absence of TGF-β1 and SB-431542. Each data point represents the mean ± SE derived from 4 independent experiments.
Fig. 5. Effects of TGF-β1 on S1P-elicited signaling responses in BAEC. A: results of a protein immunoblot assay analyzed in cell lysates derived from BAEC treated with TGF-β1 followed by S1P. BAEC had been incubated with TGF-β1 (5.0 ng/ml for 24 h) or vehicle, and then they were treated with S1P (500 nM). After addition of S1P, cells were harvested at the times indicated, and equal quantities of cell lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies directed to phospho-Akt (Ser473) and Cav-1. Equal loading of samples was confirmed by reprobing the immunoblots with an antibody against (total) Akt. Top: results from a representative data from an experiment that was independently repeated four times with similar results. Bottom: results of densitometric analyses from pooled data, plotting the fold increase of the degree of phosphorylation levels of Akt at the S1P treatment conditions as indicated, relative to the signals obtained in the absence of TGF-β1 and S1P. Each data point of the graphs represents the mean ± SE derived from 4 independent experiments. Open and closed circles represent values obtained with and without pretreatment with TGF-β1, respectively. *P < 0.05 vs. S1P (–). †P < 0.05 vs. TGF-β1 (–). B: cellular proteins were probed with antibodies directed to a phosphorylated form of AMP-activated protein kinase (AMPK) (Thr172) and total AMPK instead of Akt. C: results of S1P dose-response study are shown. BAEC had been incubated with TGF-β1 (5.0 ng/ml for 24 h) or vehicle, and then they were treated with S1P (indicated concentrations for 5 min). D: results of Rac1 activity assay. BAEC had been incubated with TGF-β1 (5.0 ng/ml for 24 h) or vehicle, and then they were treated with S1P (100 nM for 4 min). After agonist stimulation, Rac1 activity in the cell lysates was measured by pulldown of Rac1-GTP. Precipitated Rac1 was quantified in immunoblots probed with a monoclonal antibody directed against Rac1. Aliquots of total cell lysates were subjected to immunoblots in separate gels using antibodies specific to Rac1 and caveolin-1, as indicated. n = 3.
appeared to increase Rac1 activity slightly, the difference did not reach statistical significance. We studied whether or not cells pretreated with TGF-β1 exert higher degree of migratory responses to S1P using a modified Boyden chamber assay. S1P alone induced robust migration of BAEC (Fig. 6). TGF-β1 by itself did not markedly alter the degree of cell migration. However, when BAEC had been treated with TGF-β1 before S1P, the degrees of endothelial migration were further augmented by ~77% (Fig. 6). Thus pretreatment with TGF-β1 of BAEC-induced enhanced responses to subsequent S1P treatment at the levels of both signal transduction and cell migration.

We examined whether or not TGF-β1-induced enhancement of protein kinase response to S1P was attributable to decreases in caveolin-1 protein. We utilized a genetic knockdown approach of caveolin-1 protein by means of transfection with siRNA. This siRNA directed to bovine caveolin-1 sequence led to >90% reduction of caveolin-1 abundance compared with control siRNA (Fig. 7; see also Ref. 6). Genetic knockdown of caveolin-1 protein induced remarkable enhancement of responses to S1P at a level of Akt and of AMPK (Fig. 7, A and B). It also led to promotion of S1P-induced migratory responses (Fig. 7C). These results demonstrate that transfection with siRNA directed to caveolin-1 mimicked the enhanced responses to S1P in TGF-β1-pretreated BAEC. We then examined whether or not reduction of caveolin-1 protein alters subcellular localization pattern of S1P1 receptors. We double transfected BAEC with cDNA encoding FLAG/S1P1 and with caveolin-1-specific siRNA, and then we subjected them to a subcellular fractionation procedure to isolate caveolae-enriched fractions, because endogenous S1P1 can no longer be detected with currently available antibodies after this operation in immunoblot. Figure 7D shows that the distribution of FLAG/S1P1 protein did not change despite quantitative reduction of caveolin-1 after siRNA transfection. We sought to gain further insights if gain-of-function of caveolin-1 protein attenuates the magnitudes of S1P responses. To this end, we exploited COS-7 cells with transient transfection strategy with plasmid cDNAs due to much lower overexpression efficiency in BAEC. Figure 8 shows that S1P treatment of COS-7 cells leads to marked Akt responses in the presence of transfected S1P1 receptors, as previously reported (20). Our results also indicate that coexpression of c-myc-tagged exogenous caveolin-1 protein led to markedly attenuated phosphorylation responses of kinase Akt compared with cells transfected with empty vector. Thus gain-of-function of caveolin-1 protein decreases the amplitudes of signaling responses elicited by S1P. To explore whether reduction of caveolin-1 protein alters morphology of caveolae microstructures, we performed an electron microscopic experiment in which cells were either treated with TGF-β1 or transfected with siRNA directed to caveolin-1. Results indicate that the number of peripheral membrane invaginations (likely reflecting that of caveolae microstructures) decreases by ~40% in cells transfected with siRNA specific to caveolin-1, but not in those treated with TGF-β1, each compared with appropriate control cells (Fig. 9).

DISCUSSION

The present study demonstrates that TGF-β1 attenuates caveolin-1 expression at both levels of protein and mRNA in BAEC. Downregulation of caveolin-1 occurs at physiologically relevant concentration of the cytokine (1.0–5.0 ng/ml) (23). Our results indicate that expression levels of eNOS are reciprocally upregulated, in line with an earlier study (17), thereby serving as a positive control response of our cell culture system. TGF-β1 may induce growth retardation in BAEC when cultured in the presence of 10% FBS (37), whereas our study used 1% serum. Under the present culture conditions, cell numbers, total protein recovery, and LDH release into culture medium do not change over various TGF-β1 treatment protocols (data not shown), and a pharmacological inhibitor of ALK-5 receptor subtype SB-431542 is capable of counteracting downregulation of caveolin-1. Thus TGF-β1 appears to attenuate caveolin-1 expression by regulating its specific signaling machineries rather than by simply exerting cellular toxicity or by introducing growth retardation. Cytokines that belong to TGF-β superfamily regulate expression levels of diverse genes in cardiovascular cell types (reviewed in Ref. 36). Our results indicate that caveolin-1 of vascular endothelial cells can be now added to this list of cardiovascular genes whose expression levels are modulated by TGF-β1. It should be also noted that caveolin-1 expression levels are downregulated by several other endothelial stimuli, including vascular endothelial growth factor (VEGF), a polypeptide growth factor (25), as well as clinically relevant cholesterol-lowering drugs termed statins (3).

TGF-β1 elicits its cellular responses by binding to and activating a large heteromeric receptor complex that comprises several receptor subunits and accessory molecules. Among the receptor complex molecules, type I TGF-β receptor is a serine-threonine protein kinase that phosphorylates its substrates termed Smad proteins that then translocate into nucleus and modulate gene transcription, thereby plays key roles in mediating TGF-β1 receptor signal transduction (23). Previous studies have established that endothelial cells express two distinct type I TGF-β receptor subtypes, i.e., ALK-5 and ALK-1 (23). Present results suggest that ALK-5 subtype plays a major role
for TGF-β1 downregulation of caveolin-1 expression, because SB-431542, a specific inhibitor of ALK-5 subtype, counteracts decreases in caveolin-1 protein expression evoked by TGF-β1. Note that this inhibitor has been widely used at similar concentrations as the present study to selectively inhibit ALK-5 kinase activity in various cell types (8, 16, 21). Since caveolin-1 steady-state abundance decreases at both levels of protein and mRNA when treated with TGF-β1, it is likely that the cytokine attenuates transcription levels of caveolin-1 gene. Whereas 5′-flanking region of caveolin-1 gene was characterized in several cellular systems (2, 19), to our knowledge there has been no prior report that explored the effects of TGF-β superfamily cytokines on caveolin-1 gene promoter activity in vascular endothelial cells. Alternatively, it is also possible that TGF-β1 promotes degradation of caveolin-1 transcripts. Thus molecular mechanisms whereby TGF-β1 downregulates caveolin-1 mRNA/protein expression levels by way of ALK-5 remain to be elucidated in greater detail at this point.

Caveolae are flask-shaped invaginations of plasma membrane that represent specialized signaling compartments; these
transduction (activation of kinases AMPK and Akt as well as that of a small G protein Rac1) and cell migration. We interpret these data that TGF-β1 enhances S1P responses by decreasing caveolin-1 expression, because genetic knockdown of caveolin-1 protein by means of transient transfection with siRNA mimics these effects of TGF-β1. We note that the present study and earlier reports by others (6, 24) exhibit good agreement in terms of promotion of S1P responses in BAEC transfected with siRNA specific to caveolin-1. Conversely, overexpression of caveolin-1 protein decreases S1P-induced Akt responses in COS-7 cells that transiently express S1P1 receptors.

Protein subcellular localization, more specifically targeting to caveolae microdomains, represents another important factor that influences cellular signal transduction. However, the present study and earlier observations by others (6) clearly indicate that reduction of caveolin-1 expression by means of siRNA transfection does not largely alter targeting to caveolae-enriched fractions of many S1P-related signaling proteins, including S1P1 receptors, Rac1, Akt, as well as eNOS, determined with a well-established detergent-free subcellular fractionation procedure. Expression of caveolin-1 protein is supposed to contribute to the acquisition of unique morphological and functional properties of caveolae microstructures (32). Our electron microscopic studies indicate that genetic knockdown of caveolin-1 by means of siRNA transfection, but not treatment with TGF-β1, appears to decrease the number of membrane invaginations, likely reflecting that of caveolae-like microstructures. We speculate that siRNA directed to caveolin-1, which may indeed decrease the number of caveolae-like structures, does not significantly alter subcellular localization of endothelial proteins. Nonetheless, the cytokine enhances S1P responses in BAEC, mimicked by genetic knockdown of caveolin-1. We therefore propose that it is the abundance of caveolin-1, rather than the disruption of caveolae structure/function, that plays a key role to determine the amplitudes of S1P signaling in these systems. However, it is known that caveolae may undergo dynamic alterations into and from other related membrane microstructures. We speculate that siRNA directed to caveolin-1, rather than the disruption of caveolae structure/function, that plays a key role to determine the amplitudes of S1P signaling in these systems. However, it is known that caveolae may undergo dynamic alterations into and from other related membrane microstructures. We speculate that siRNA directed to caveolin-1, rather than the disruption of caveolae structure/function, that plays a key role to determine the amplitudes of S1P signaling in these systems. However, it is known that caveolae may undergo dynamic alterations into and from other related membrane microstructures.
distribution profiles of receptor components for TGF-β1 and for S1P lead to differential responses of Smad proteins to S1P in mesangial cells and in BAEC. Thus we speculate that TGF-β1 and S1P may exert cross-talks of their signaling with each other by regulating activities of Smad proteins and/or expression levels of caveolins, likely in a cell type-dependent manner. It is also noteworthy that in cardiac fibroblasts TGF-β1 activates sphingosine kinase, an enzyme that produces S1P (5), and that sphingosine kinase augments TGF-β1-induced collagen gene regulation in skin fibroblasts (35). Like S1P pathways, TGF-β1 receptor signal transduction machineries are associated with caveoleae microstructures; caveolin-1 and caveoleae may thereby modulate the degrees of tissue matrix deposition regulated by TGF-β1 (reviewed in Ref. 1). For example, at least some of TGF-β receptor components are targeted to caveoleae and interact with caveolin-1 in heterologous expression systems (33) as well as in human microvascular endothelial cells (34). Downregulation of caveolin-1 expression by interleukin-4 leads to activation of TGF-β1 signaling in mouse lung fibroblasts (22). When taken together with these earlier observations, our results provide another example that TGF-β1 and caveolae/caveolins display multiple layers of interaction in various cell types.

In vascular endothelial cells, several extracellular stimuli are known to modulate expression levels of S1P1 receptor subtype and thereby regulate the degrees of endothelial responses to S1P. Such factors include VEGF (12), hydrogen peroxide (14), as well as statins (15). Although TGF-β1 does not alter the expression levels of S1P1 receptor protein/mRNA under these conditions, it does augment the magnitudes of BAEC responses to S1P by downregulating caveolin-1 expression. Thus our results indicate that TGF-β1 represents another example of endothelial stimulus that influences the degrees of S1P-elicted cellular responses. Additionally, to our knowledge cytokine-evoked downregulation of caveolin-1 is a novel mode of endothelial regulation that modifies the amplitudes of S1P
responses. Many endothelial signaling pathways other than those of S1P are also associated with and modulated by caveolae/scaffolding-1 protein (reviewed in Ref. 32). It will be therefore important to expand our understandings of whether or not TGF-β1, which reduces caveolin-1 protein abundance, alters the magnitudes of endothelial responses to a stimulus other than S1P.

In conclusion, we have demonstrated that physiologically relevant concentrations of a pluripotent cytokine TGF-β1 attenuate the expression levels of caveolin-1, a structural protein of plasmalemmal caveola, in cultured vascular endothelial cells. Cells pretreated with TGF-β1 exhibit augmented responses to a lipid growth factor S1P at both levels of signal transduction and cell migration, which are mimicked by genetic knockdown of caveolin-1 protein. We propose that TGF-β1 downregulation of caveolin-1 identifies a novel point of cross-talk at which cytokines and lipid growth factors influence their receptor-dependent signaling pathways with each other.

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