

Agonist-modulated Targeting of the EDG-1 Receptor to Plasmalemmal Caveolae

eNOS ACTIVATION BY SPHINGOSINE 1-PHOSPHATE AND THE ROLE OF CAVEOLIN-1 IN SPHINGOLIPID SIGNAL TRANSDUCTION*

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Plasmalemmal caveolae are membrane microdomains that are specifically enriched in sphingolipids and contain a wide array of signaling proteins, including the endothelial isoform of nitric-oxide synthase (eNOS). EDG-1 is a G protein-coupled receptor for sphingosine 1-phosphate (S1P) that is expressed in endothelial cells and has been implicated in diverse vascular signal transduction pathways. We analyzed the subcellular distribution of EDG-1 in COS-7 cells transiently transfected with cDNA constructs encoding epitope-tagged EDG-1. Subcellular fractionation of cell lysates resolved by ultracentrifugation in discontinuous sucrose gradients revealed that ~55% of the EDG-1 protein was recovered in fractions enriched in caveolin-1, a resident protein of caveolae. Co-immunoprecipitation experiments showed that EDG-1 could be specifically precipitated by antibodies directed against caveolin-1 and vice versa. The targeting of EDG-1 to caveolae-enriched fractions was markedly increased (from 51 ± 11% to 93 ± 14%) by treatment of transfected cells with S1P (5 μM, 60 min). In co-transfection experiments expressing EDG-1 and eNOS cDNAs in COS-7 cells, we found that S1P treatment significantly and specifically increased nitric-oxide synthase activity, with an EC₅₀ of 30 nM S1P. Overexpression of transfected caveolin-1 cDNA together with EDG-1 and eNOS markedly diminished S1P-mediated eNOS activation; caveolin overexpression also attenuated agonist-induced phosphorylation of EDG-1 receptor by >90%. These results suggest that the interaction of the EDG-1 receptor with caveolin may serve to inhibit signaling through the S1P pathway, even as the targeting of EDG-1 to caveolae facilitates the interactions of this receptor with ligands and effectors that are also targeted to caveolae. The agonist-modulated targeting of EDG-1 to caveolae and its dynamic inhibitory interactions with caveolin identify new points for regulation of sphingolipid-dependent signaling in the vascular wall.

Plasmalemmal caveolae have been characterized as membrane invaginations that can serve as microdomains for the sequestration of a wide array of signaling proteins, including receptors and their downstream modulators and effectors, including the endothelial isoform of nitric-oxide synthase (1). The transmembrane protein caveolin is the key scaffolding protein in caveolae. Caveolin directly interacts with several signaling proteins and may serve a broad role in modulating receptor-activated signaling pathways in caveolae. In addition to serving as sites for targeting of specific proteins, plasmalemmal caveolae also have a distinctive lipid composition; caveolae are relatively depleted of phospholipids and are enriched in cholesterol and sphingolipids (2). The specific lipid composition of caveolae serves to create a "liquid-ordered phase" within these small membrane structures (3) that may thereby facilitate the physical proximity and interactions of the various signal transducing proteins targeted to caveolae. In addition to this essential structural role, sphingolipids have become increasingly appreciated as serving key roles in signal transduction in a variety of cellular pathways.

Sphingolipids are acylated derivatives of sphingosine, the core structure of this class of lipids. As was described previously for phospholipid-derived compounds, it has been observed more recently that sphingolipids and their metabolites appear to be active in a variety of signaling pathways in mammalian cells (reviewed in Ref. 4). One biologically active sphingolipid is sphingosine 1-phosphate (S1P),¹ which has been implicated in intercellular as well as intracellular signaling (reviewed in Ref. 5). S1P may elicit biological responses as diverse as cellular proliferation (6), hypertrophy (7), differentiation (8), migration (9), and inhibition of apoptosis (10). Previous studies have shown that responses to S1P are mediated, at least in some cells, by the binding of S1P to the EDG-1 receptor (11, 12). The EDG-1 receptor (endothelial differentiation gene-1) was originally cloned as an orphan gene that is induced upon the stimulation of human umbilical vein endothelial cells with phorbol esters (8). The EDG receptor family is now known to comprise at least seven independent subtypes (13–15) that are activated either by S1P or lysophosphatidic acid (13). The EDG-1 receptor belongs to the superfamily of G protein-coupled receptors (16); receptor activation by S1P can lead to the activation of numerous downstream effectors, including the mobilization of intracellular calcium and the inhibition of adenylate cyclase (17). Recent studies have suggested

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¹ The abbreviations used are: S1P, sphingosine 1-phosphate; NOS, nitric-oxide synthase; eNOS, endothelial isoform of NOS; FLAG/EDG-1, EDG-1 receptor epitope-tagged with FLAG peptide; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor.

that S1P-mediated activation of EDG-1 receptor leads to morphological alterations in cultured vascular endothelial cells *in vitro* and induces vessel structure formation *in vivo* (12), thus implicating S1P/EDG-1 in signal transduction pathways leading to angiogenesis. However, the subcellular targeting of EDG-1, the proximal interactions of EDG-1 with other membrane-associated signaling proteins, and the relationship of EDG-1 to nitric oxide signaling pathways all remain less well understood.

It seemed plausible to us that the targeting of S1P precursors in caveolae might provide a mechanism for the activation of the EDG-1 receptor if it were similarly localized in this organelle. Moreover, the biological responses elicited by S1P share some features with pathways involved in nitric oxide signaling, an observation made more compelling by the fact that the endothelial isoform of nitric-oxide synthase (eNOS) is targeted to plasmalemmal caveolae (18). In the present study, we provide evidence demonstrating that the EDG-1 receptor is targeted to caveolae and explore the implications of this discovery for the involvement of eNOS and caveolin in sphingolipid-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was from Hyclone (Logan, CT); all other cell culture reagents, media, and LipofectAMINE were from Life Technologies, Inc. S1P was from BioMol (Plymouth Meeting, PA). S1P was solubilized in methanol following the supplier's instruction and stored at -20°C ; the same volume of methanol was used as a vehicle-control for S1P, and the final concentration of methanol did not exceed 0.4% (v/v) in any experiment. [^3H]-L-Arginine was from Amersham Pharmacia Biotech. Protein determinations were made with the Bio-Rad protein assay kit. Anti-eNOS monoclonal and anti-caveolin polyclonal antibodies were from Transduction Laboratories (Lexington, KY). Super Signal substrate for chemiluminescence detection, nonimmune IgG and secondary antibodies conjugated with horseradish peroxidase were from Pierce. [^{32}P]Orthophosphate was from ICN (Costa Mesa, CA). All other reagents, including anti-FLAG monoclonal antibody, were from Sigma.

Plasmid Construction—cDNA encoding full-length human EDG-1 receptor epitope-tagged with FLAG peptide (FLAG/EDG-1; described in Ref. 16) was provided by Timothy Hla (University of Connecticut) and was subcloned into pcDNA3 (Invitrogen) at *Hind*III and *Xba*I sites. cDNA encoding full-length canine caveolin-1 epitope-tagged with c-Myc (19) was provided by Michael P. Lisanti (Albert Einstein Medical College) and cloned into pcDNA3 at *Hind*III and *Bam*HI sites. eNOS cDNA subcloned into pBK-CMV was described previously (20).

Cell Culture and Plasmid Transfection—COS-7 cells were maintained in culture as described previously (21). The day before transfection, cells were split at a ratio of 1:8 in Dulbecco's modified Eagle's medium containing 10% (v/v) charcoal-treated fetal bovine serum (11). For most experiments, cells in a 100-mm culture plate were transfected with 3 μg of plasmid DNA encoding FLAG/EDG-1, using LipofectAMINE according to the manufacturer's protocols. In co-transfection experiments analyzed for quantitation of intracellular eNOS activity, cells in a 60-mm plate were co-transfected with cDNAs encoding FLAG/EDG-1 (2 μg), eNOS (0.03 μg), and/or caveolin-1 (0.04 μg). In co-transfection experiments studying phosphorylation of EDG-1 receptor, cells in a given well of a 6-well plate were co-transfected with plasmid DNAs encoding FLAG/EDG-1 (0.5 μg) and caveolin-1 (0.03 μg). Each (co)-transfection condition was optimized through preliminary experiments using varying amounts of DNA for the different combinations of plasmid DNAs and experimental conditions. When cells were co-transfected with multiple plasmid DNAs, "empty" vector (no cDNA insert) was used to normalize the total amount of transfected DNAs among the groups. Approximately 30 h after transfection, culture medium was switched to Dulbecco's modified Eagle's medium containing 0.5% (v/v) charcoal-treated fetal bovine serum, and incubation proceeded for 16 h prior to the experiments.

Isolation of Caveolae-enriched Fractions—Caveolae-enriched fractions were separated by using ultracentrifugation with a discontinuous sucrose gradient system as described previously (22). Briefly, COS-7 cells from 2–100-mm dishes were scraped together into 2 ml of carbonate buffer containing 500 mM sodium carbonate (pH 11), 25 mM MES and 150 mM NaCl, and the cells were homogenized (40 strokes in a

Dounce homogenizer) and sonicated (3 \times 20 s bursts in a Branson Sonifier 450). The resulting cell suspension was brought to 45% sucrose (w/v) 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 as described (22), 12 \times 1 ml fractions were collected starting at the top of each gradient. An equal volume of each fraction was analyzed by SDS-PAGE and immunoblotting and probed for FLAG epitope or caveolin with corresponding antibodies, as described previously (23).

Co-immunoprecipitation of FLAG/EDG-1 with Caveolin-1—COS-7 cells were lysed in 500 μl of buffer containing *n*-octyl β -glucopyranoside (OG buffer, described in Ref. 24). Cell lysates were precleared with corresponding nonimmune IgG (1 μg) incubated together with protein A- or protein G-Sepharose for 30 min at 4°C . Cleared lysates were then incubated with 1 μg of anti-FLAG antibody or anti-caveolin antibody for 1 h at 4°C . Nonimmune mouse IgG1 (for anti-FLAG antibody) or rabbit IgG (for anti-caveolin antibody) were used as a negative control, respectively. Cell lysates were then incubated with protein G-Sepharose (for anti-FLAG antibody) or protein A-Sepharose (for anti-caveolin antibody) for 1 h at 4°C . Protein A or G beads were then extensively washed with OG buffer. Proteins were eluted from beads, resolved by SDS-PAGE, and subjected to Western blot analysis as described previously (23).

Quantitation of Intracellular NO Generation—eNOS enzyme activity was quantified as the formation of [^3H]-citrulline from [^3H]-L-arginine as described previously, with minor modifications (25). Briefly, transfected COS-7 cells in a 60-mm dish were incubated in 2 ml of buffer containing 25 mM HEPES, 109 mM NaCl, 5.4 mM KCl, 0.9 mM CaCl_2 , 1 mM MgSO_4 , and 25 mM glucose (pH 7.3) for 1 h at 37°C . eNOS activity was assayed by adding a mixture of unlabeled L-arginine (10 μM), [^3H]-L-arginine (10 $\mu\text{Ci/ml}$) and S1P (5 μM) or vehicle to the culture (each treatment was performed in duplicate cultures, and then each was assayed in duplicate). Following incubation at 37°C for 10 min, cells were washed with ice-cold phosphate-buffered saline and scraped into 2 ml of solution containing 20 mM sodium acetate, 2 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA (pH 5.5) followed by sonication. An aliquot was withdrawn for determination of the total protein content and total cellular ^3H incorporation, and the remaining sample was applied to Dowex 50WX8–400 column to separate [^3H]-citrulline. The flow-through fraction was analyzed by liquid scintillation counting; [^3H]-citrulline formation in COS-7 cells was expressed as fmol [^3H]-citrulline produced per mg of cellular protein/min. In parallel with the experiments above, another individual set of culture plates corresponding to each co-transfection protocol was analyzed to verify the expression levels of co-expressed FLAG/EDG-1, eNOS, and caveolin-1 proteins by Western blot analysis using anti-eNOS, anti-FLAG, or anti-caveolin antibodies.

Phosphorylation of EDG-1 Receptor by S1P—Transfected COS-7 cells in 6-well dishes were washed twice and incubated in phosphate-free Dulbecco's modified Eagle's medium containing 20 $\mu\text{Ci/ml}$ of [^{32}P]phosphate for 4 h at 37°C and then stimulated with S1P for 5 min. Cells were then washed with ice-cold phosphate-buffered saline and scraped into 500 μl of buffer containing 50 mM Tris-HCl (pH 7.4), 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_2VO_4 , 1 mM NaF, and a mixture of protease inhibitors as described (26). An aliquot of cell lysate was processed for Western blot analysis. The lysate was immunoprecipitated with anti-FLAG antibody and analyzed by autoradiography after proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane.

Other Methods—Densitometric analyses of Western blots and autoradiograms were performed using a ChemiImager 4000 (Alpha-Innotech). All experiments were performed at least three times. Mean values for individual experiments are expressed as the means \pm S.E. Statistical differences were analyzed by analysis of variance followed by Scheffe's F test using STAT VIEW II (Abacus Concepts). A *p* value less than 0.05 was considered statistically significant.

RESULTS

Targeting of EDG-1 to Plasmalemmal Caveolae and Association with Caveolin-1—Cell lysates from COS-7 cells transiently transfected with cDNA encoding FLAG/EDG-1 were analyzed by ultracentrifugation using a discontinuous sucrose gradient system that was previously shown to resolve caveolae-enriched subcellular fractions (22, 27). As shown in Fig. 1,

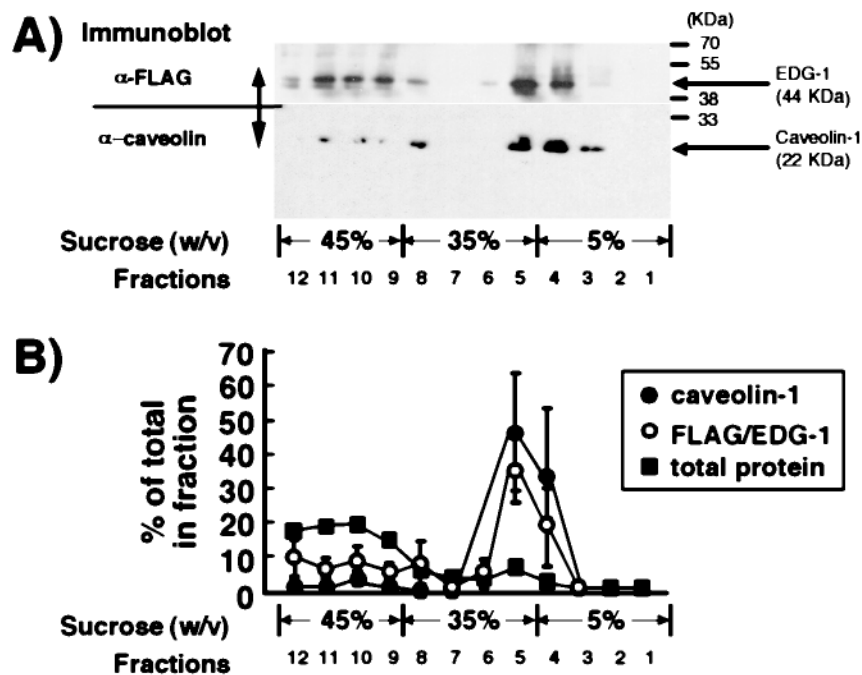


FIG. 1. Targeting of EDG-1 receptor to caveolae-enriched fractions. Shown are the results of subcellular fractionation of COS-7 cells transiently transfected with the FLAG/EDG-1 cDNA. *A* shows the results of SDS-PAGE and immunoblot analysis from a representative subcellular fractionation experiment. Sonicated cell lysates from FLAG/EDG-1-transfected COS-7 cells were separated using a discontinuous sucrose gradient system as described under "Experimental Procedures." An equal volume from each fraction was separated by SDS-PAGE, followed by immunoblot analysis using antibodies directed against FLAG or caveolin, as indicated. This figure is representative of three independent experiments. *B* shows graphic presentation of the subcellular distribution of caveolin, EDG-1, and total protein, pooled from three independent experiments. For each fraction from the sucrose gradient, the total protein content was determined (shown as closed squares), as well as the intensity of SDS-PAGE/immunoblots probed with antibodies against caveolin-1 (closed circles) or FLAG/EDG-1 (open circles) assessed by densitometry. For each parameter, the data are presented as the fraction of the total signal present within each individual fraction and show the means \pm S.E. derived from three independent preparations.

almost all of the endogenously expressed caveolin-1 in COS-7 cells is recovered in the interface between the 5 and 35% sucrose solutions (fractions 4 and 5, corresponding to previously characterized "light vesicle" or caveolae-enriched fractions). By contrast, the vast majority of total cellular protein is distributed in the denser sucrose fractions (fractions 8–12); taken together, these results indicate that this ultracentrifugation method (22) resolves caveolin-enriched fractions in transfected COS-7 cells. Importantly, when the same gradient fractions were analyzed in Western blots probed for FLAG/EDG-1, a significant fraction (\sim 55%) of the total EDG-1 (with the expected molecular mass of \sim 44 kDa) is recovered in fractions 4 and 5, demonstrating the presence of the EDG-1 receptor in caveolin-enriched fractions.

As shown in Fig. 2, antibodies directed against caveolin co-immunoprecipitate FLAG/EDG-1, and, conversely, anti-FLAG antibodies co-immunoprecipitate caveolin. The specificity of these associations was explored in co-immunoprecipitation experiments that were identically configured but used nonimmune IgG, which yield no co-immunoprecipitation whatsoever (Fig. 2). The combination of subcellular fractionation and co-immunoprecipitation data provide complementary lines of evidence indicating that the EDG-1 receptor is targeted to caveolae.

Agonist-modulated Targeting of EDG-1 to Caveolae—To test whether treatment of cells with S1P alters the targeting of EDG-1 receptor to caveolae, we treated COS-7 cells transfected with FLAG/EDG-1 cDNA with S1P for varying times, then harvested the cells, and analyzed subcellular fractions as described above. We used S1P at a concentration of 5 μ M, because serum S1P concentration may reach micromolar range upon platelet activation (28, 29). As shown in Fig. 3, although the majority of EDG-1 can be recovered in the caveolae-enriched

fraction prior to S1P treatment, a substantive fraction of EDG-1 is present in the noncaveolar fractions (fractions 8–12). Within 20 min after the addition of S1P (5 μ M), the proportion of EDG-1 in caveolae-enriched fractions begins to increase, with a concomitant decrease in the fraction of EDG-1 in the noncaveolar fractions; by 60 min almost all of the EDG-1 is recovered in the caveolae-enriched fractions. The overall recovery of EDG-1 protein (determined by immunoblot analyses of unfractionated cell lysates; data not shown) does not change following S1P treatment (30), thereby indicating that EDG-1 undergoes a translocation from noncaveolar fractions to caveolae-enriched fractions following addition of agonist. By contrast, the subcellular distribution of endogenous caveolin-1 analyzed in these same transfected COS-7 cells shows no change following S1P treatment: \sim 90% of the total caveolin-1 is recovered in the caveolae-enriched fractions at all time points following the addition of S1P (Fig. 3).

Activation of eNOS by S1P/EDG-1—We next explored whether S1P activates eNOS by performing co-transfection experiments with cDNA constructs encoding eNOS and EDG-1. eNOS activity was quantitated by measuring the formation of L-citrulline, which is produced by the NOS-catalyzed oxidation of L-arginine to yield NO plus L-citrulline; the latter is an easily measured co-product (31). COS-7 cells transiently expressing FLAG/EDG-1 and/or eNOS cDNAs were incubated with [3 H]-L-arginine and treated with S1P or vehicle; cells were harvested, and lysates were analyzed for [3 H]-L-citrulline formation as described above. As shown in Fig. 4A, there is no detectable [3 H]-L-citrulline formation in cells not transfected with eNOS cDNA. When cells are transfected with plasmid cDNA encoding eNOS alone, there is a small increase in basal eNOS, which is seen either in the presence or absence of added S1P. However, when cells are co-transfected with plasmids cDNAs encoding

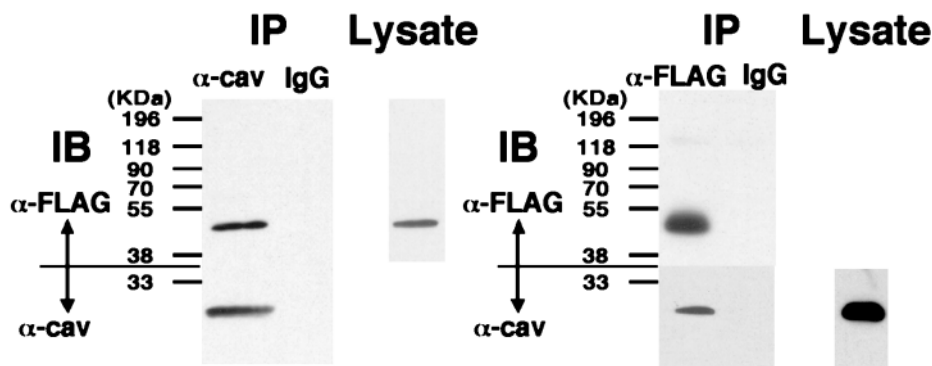


FIG. 2. Co-immunoprecipitation of EDG-1 with caveolin-1. Shown are the results of immunoblots prepared from lysates of COS-7 cells transfected with plasmid DNA encoding FLAG/EDG-1 and immunoprecipitated with antibodies as shown. After removing an aliquot of the total lysate for analysis, solubilized cell lysates were immunoprecipitated (IP) using antibodies directed against FLAG or caveolin or nonimmune IgG as indicated. After immunoprecipitation, the samples were processed for SDS-PAGE and immunoblot analysis (IB) using antibodies as shown. Shown are the results of a representative experiment performed three times with identical results.

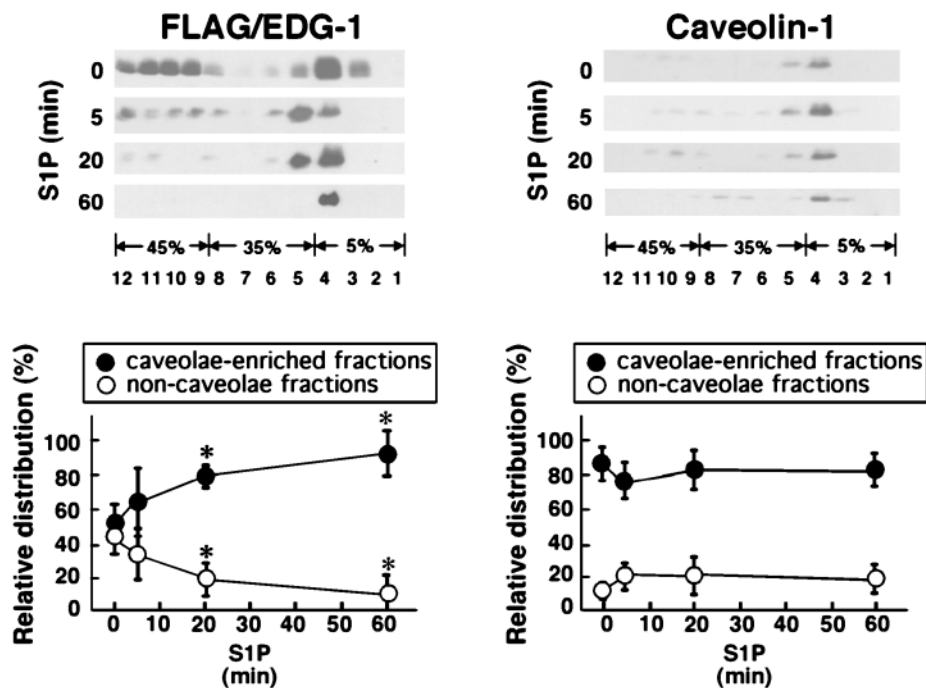


FIG. 3. Translocation of EDG-1 induced by sphingosine-1-phosphate. Shown are the results of subcellular fractionation of EDG-1-transfected COS-7 cells that were treated with sphingosine 1-phosphate ($5 \mu\text{M}$). Following S1P treatment for varying times as indicated, the cells were harvested and processed for analysis by subcellular fractionation using ultracentrifugation in a discontinuous sucrose gradient as described under "Experimental Procedures." An equal volume of each sucrose gradient fraction was separated by SDS-PAGE and analyzed by immunoblot using antibodies directed against FLAG epitope or caveolin, as shown. Presented in the upper panels are results from a representative experiment analyzing the subcellular distribution of FLAG/EDG-1 (left) or caveolin-1 (right) at various time points following S1P treatment. The lower panels show the results of densitometric analysis, plotting the relative distribution of FLAG/EDG-1 (left) or caveolin (right) in caveolae-enriched membranes (pooled from fractions 4–5; closed circles) or in noncaveolae fractions (pooled from fractions 8–12; open circles). Each data point in the lower panels represents the mean \pm S.E. derived from at least four independent preparations. An asterisk indicates $p < 0.05$ compared with value at $t = 0$, analyzed by analysis of variance.

FLAG/EDG-1 as well as eNOS, treatment with S1P ($5 \mu\text{M}$) leads to a marked increase in eNOS activity. We studied both FLAG epitope-tagged as well as untagged EDG-1 plasmid cDNAs and found an equivalent level of S1P-dependent eNOS activation mediated by the tagged and untagged constructs (data not shown). We performed dose response experiments with S1P (Fig. 4B), measuring eNOS activity in cells co-transfected with FLAG/EDG-1 and eNOS. The EC_{50} for S1P activation of eNOS in this experimental system is $\sim 30 \text{ nM}$, which is in good agreement with previous studies of S1P responses mediated by the EDG-1 receptor (11, 13). There is no change in the incorporation of [^3H]arginine into the cells under any of these experimental conditions (data not shown). These results indicate that S1P activates eNOS in COS-7 cells only when these

cells are co-transfected with cDNAs encoding both eNOS as well as the EDG-1 receptor, with an EC_{50} value in a physiologic range.

Inhibition of EDG-1 Signaling by Caveolin—We explored the functional consequences of the interaction between EDG-1 with caveolin by co-transfecting plasmid cDNA encoding FLAG/EDG-1 along with plasmids encoding eNOS and/or caveolin-1. When cells are co-transfected with constructs encoding caveolin-1, a new protein band is seen, appearing slightly larger than the endogenous caveolin band because of the Myc epitope tag on the recombinant protein (Fig. 5, lower panel). The subcellular distribution of the transfected Myc epitope-tagged caveolin-1 is identical to that of endogenous caveolin-1 (data not shown). It appears that caveolin cDNA transfection of COS-7

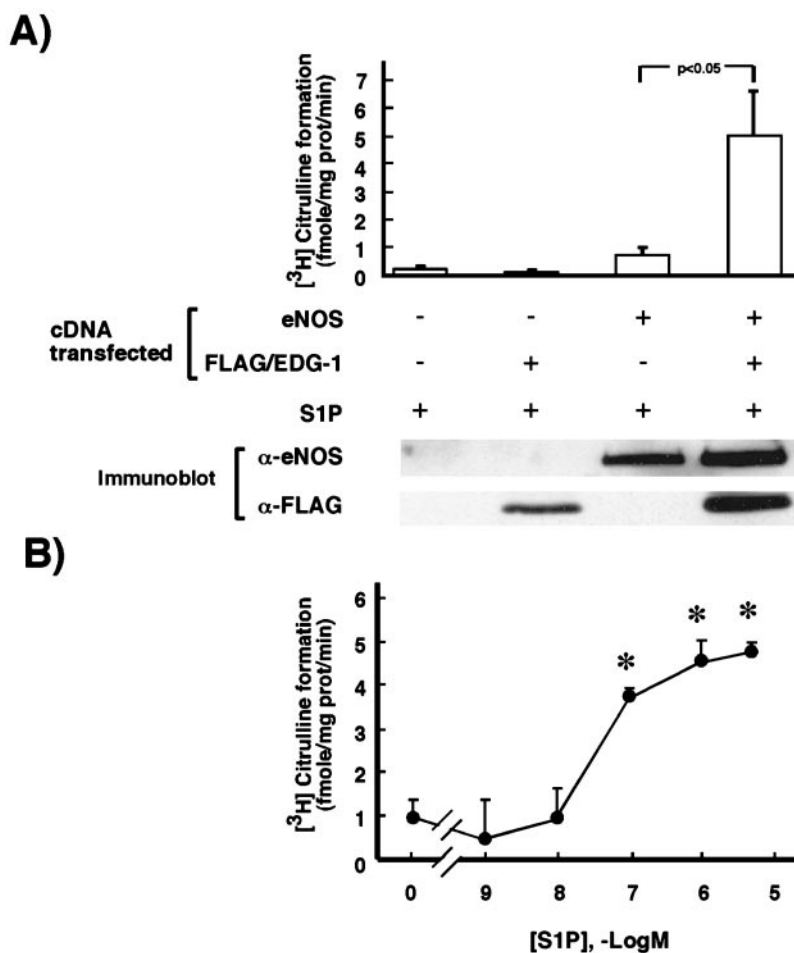


FIG. 4. Activation of eNOS by S1P via EDG-1 receptor. Shown are the results of eNOS activity assays performed in transiently transfected COS-7 cells. *A* shows the results of eNOS activation assays in the cells co-transfected with FLAG/EDG-1 and/or eNOS as indicated. The transfected cells were labeled with [³H]L-arginine and exposed to S1P (5 μM) for 10 min, then harvested, and analyzed for [³H]L-citrulline content as described in the text. Each data point in the *upper panel* represents the mean ± S.E. of [³H]L-citrulline production derived from four independent cell preparations, each performed in duplicate. To verify equal production of EDG-1 and eNOS protein under these different conditions, cells identically transfected and processed in parallel were analyzed by SDS-PAGE and immunoblot; shown *below* the corresponding data point for [³H]L-citrulline production are the lanes from these immunoblot analyses using the antibodies as shown. The immunoblot data are shown for a representative experiment that was repeated four times with equivalent results. *B* shows the results of a S1P dose response experiment measuring activation of eNOS in COS-7 cells co-transfected with cDNAs constructs encoding FLAG/EDG-1 and eNOS. Cells were treated with increasing concentrations of S1P (as indicated) or vehicle, and eNOS activity was assayed by determining formation of [³H]L-citrulline from [³H]L-arginine, as described above. Each data point represents the mean ± S.E., derived from three independent cell preparations, each performed in duplicate. *, *p* < 0.05 versus vehicle treatment.

cells increases the amount of caveolin-1 protein expression by approximately 2–3-fold overall (Fig. 5), but this figure may be an underestimate of the degree of overexpression in a given cell, because only a fraction of the cells are successfully transfected with exogenous plasmids. Moreover, the apparent increase in caveolin expression following cDNA transfection cannot be rigorously quantitated because of the potential differences in caveolin immunoreactivity that are secondary to species differences between the exogenous (canine) caveolin expressed by the cDNA and the endogenous (simian) caveolin present in COS-7 cells. We next analyzed eNOS activity in these co-transfection experiments, quantitated by measuring [³H]L-citrulline formation from [³H]L-arginine. As shown in Fig. 5 (*upper panel*), eNOS activity is not detected in sham-transfected cells. Transfection of eNOS cDNA leads to a small increase in [³H]L-citrulline formation that is not affected by caveolin-1 overexpression. In cells expressing both EDG-1 and eNOS, treatment with S1P markedly increases eNOS activity (as seen also in Fig. 4). However, overexpression of exogenous caveolin-1 in addition to EDG-1 and eNOS abrogates the aug-

mentation of NO production elicited by S1P (Fig. 5). These results indicate that overexpression of caveolin-1 has an inhibitory effect on the activation of eNOS elicited by S1P/EDG-1. Interpretation of this observation is confounded by the fact that caveolin itself directly inhibits eNOS activation (32). It is therefore difficult to discriminate the direct effects of caveolin on eNOS activity from any inhibitory effect of caveolin on EDG-1 itself. We therefore explored whether caveolin might regulate an independent agonist-dependent EDG-1-mediated response, namely the agonist-induced phosphorylation of the EDG-1 receptor (33).

As shown in Fig. 6, when COS-7 cells transfected with EDG-1 cDNA are treated with S1P, there is a striking increase in EDG-1 phosphorylation relative to vehicle-treated cells (5.0 ± 1.5-fold increase, *p* < 0.01). However, the co-transfection of caveolin-1 markedly attenuates the S1P-induced increase in EDG-1 phosphorylation. Expression of EDG-1 is unaffected by co-transfection with caveolin-1 (Fig. 6). Taken together, these results indicate that caveolin-1 overexpression inhibits agonist-induced EDG-1 receptor phosphorylation.

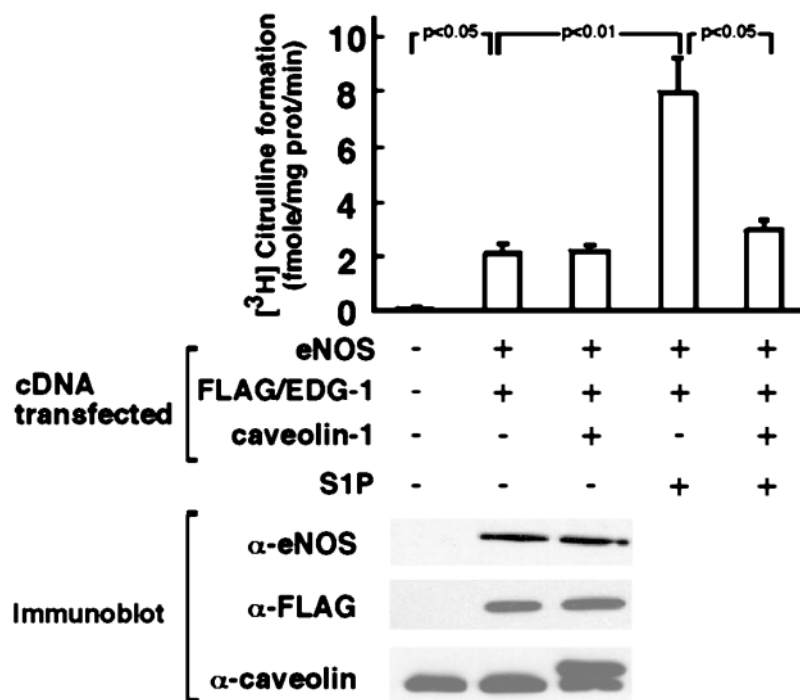


FIG. 5. **Inhibitory effects of caveolin-1 on eNOS activation by S1P/EDG-1.** Shown are the results of eNOS activity assays performed in COS-7 cells that have been transiently transfected with plasmid DNA encoding FLAG/EDG-1, eNOS, and/or caveolin-1, as indicated. Because of the presence of a c-Myc epitope tag, the exogenously overexpressed caveolin-1 has higher molecular weight compared with the endogenous caveolin-1 in COS-7 cells. The transfected cells were labeled with [^3H]L-arginine and exposed to S1P ($5\ \mu\text{M}$) or vehicle for 10 min, then harvested, and analyzed for [^3H]L-citrulline production. Each data point in the upper panel represents the mean \pm S.E. derived from four independent cell preparations, each performed in duplicate. To verify equal production of EDG-1 and eNOS protein under these different conditions, cells identically transfected and processed in parallel were analyzed by SDS-PAGE and immunoblot; shown below are the corresponding data point for [^3H]L-citrulline production are the lanes from these immunoblot analyses using the antibodies as shown. The immunoblot data are shown for a representative experiment that was repeated four times with equivalent results.

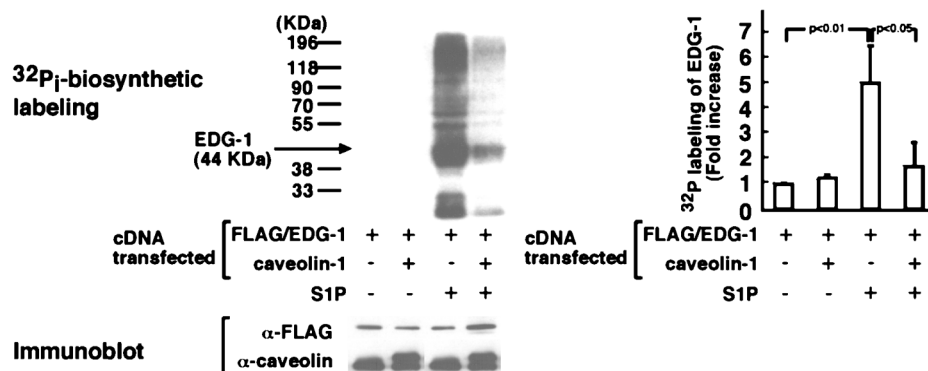


FIG. 6. **Inhibitory effects of caveolin-1 on phosphorylation of EDG-1 receptor elicited by S1P.** Shown are the results of assays for *in situ* phosphorylation of the EDG-1 receptor expressed in COS-7 cells. Cells were transiently transfected with plasmids encoding FLAG/EDG-1 and caveolin-1, as indicated. The transfected cells were labeled with [^{32}P]orthophosphate for 4 h and treated with S1P ($5\ \mu\text{M}$) for 5 min, then harvested, lysed, and immunoprecipitated with the anti-FLAG antibody; eluted proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by autoradiography and by immunoblot. The left panels show the results of a representative experiment that was repeated four times with equivalent results. The upper left panel shows the results of autoradiography for [^{32}P]-labeled and immunoprecipitated EDG-1 under different conditions as shown, and the lower left panels show the results of corresponding Western blot analyses of cell lysates in the same experiment, probed for FLAG/EDG-1 or caveolin-1 (as indicated). Shown in the right panel are the results of pooled densitometric data obtained from four independent preparations quantitating [^{32}P]-labeled FLAG/EDG-1 by autoradiography under the different conditions as shown.

DISCUSSION

These studies present several lines of experimentation indicating that the EDG-1 receptor is targeted to plasmalemmal caveolae. Subcellular fractionation experiments (Fig. 1) reveal that a significant fraction of EDG-1 is targeted to plasmalemmal caveolae in transfected COS-7 cells, and co-immunoprecipitation experiments (Fig. 2) indicate that caveolin and EDG-1 proteins associate with one another (of course, these data do not establish a *direct* protein-protein interaction between caveolin and EDG-1). We also found that treatment of FLAG/EDG-1-transfected COS-7 cells with S1P specifically al-

ters the subcellular distribution of the EDG-1 receptor (Fig. 3); the receptor appears to undergo translocation from noncaveolae to caveolae-enriched fractions after prolonged treatment with S1P, whereas the distribution of caveolin is unaffected by S1P treatment. Interestingly, Liu *et al.* (30) have previously observed that green fluorescent protein-tagged EDG-1 receptor can undergo translocation from sites in the plasma membrane to unidentified intracellular locales. The lack of availability of specific EDG-1 antibodies has hampered studies of the trafficking of endogenously expressed EDG-1 receptor in native cells. The present studies have identified plasmalemmal caveolae as

the key intracellular site for the localization of recombinant EDG-1 and establish noteworthy parallels between EDG-1 and some other G protein-coupled receptors that similarly undergo agonist-modulated translocation from noncaveolar membranes to caveolae-enriched fractions (27, 34, 35). These parallels suggest the presence of common cellular machinery that transport specific G protein-coupled receptors to caveolae after prolonged stimulation with their cognate ligands. Furthermore, the fact that the FLAG-tagged EDG-1 receptor undergoes agonist-dependent translocation from noncaveolar membranes to caveolae-enriched fractions indicates that the fundamental regulatory features of the native receptor are retained in the epitope-tagged construct (11, 16).

Specific caveolae-targeted sphingolipids, such as ceramide, may be converted to S1P by the actions of ceramidase and sphingosine kinase (13). Ceramide has recently been found to activate eNOS in cultured endothelial cells (36), and it is intriguing to speculate that the targeting of EDG-1 to caveolae serves to facilitate the physical proximity of the EDG-1 receptor to the sphingolipid precursors of S1P present in caveolae. It must be noted, however, that the concentration of S1P in most cells is quite low, possibly reflecting the limited expression of its key synthetic enzyme, sphingosine kinase. Blood platelets represent a notable exception; platelets release substantial amounts of S1P and have a robust sphingosine kinase activity (13), and it is possible that the expression of EDG-1 in endothelial cells may serve principally to transduce extracellular signals from platelet-derived S1P. We found an EC₅₀ of 30 nM for S1P activation of eNOS (Fig. 4B); this figure is within the range of S1P concentrations found in human plasma, making it possible that S1P plays a role in the physiological regulation of NO synthesis. It remains to be determined whether endogenous S1P plays an autocrine role as a ligand for the EDG-1 receptor, although endothelial cell activation may be accompanied by an increase in intracellular S1P levels (10, 37). Thus, despite the proximity to sphingolipids afforded by the caveolar targeting of EDG-1, it is possible that the localization of EDG-1 in caveolae serves principally to sequester the receptor along with other signaling proteins similarly targeted to caveolae.

These studies have identified a possible role for EDG-1 in modulating the activity of eNOS, a key signaling protein targeted to caveolae in endothelial cells and cardiac myocytes (23). The localization of eNOS in caveolae is essential for the activation of the enzyme by cell surface receptors (38). Receptors that activate eNOS have been localized in caveolae, including the m2 muscarinic cholinergic receptor (27) and the B2 bradykinin receptor (35). Like these other G protein-coupled receptors, EDG-1 is known to mobilize intracellular calcium (17, 29), a key regulatory component of eNOS activation (39, 40). The signal transduction pathways modulated by eNOS bear some striking parallels with the cellular responses elicited by S1P and EDG-1. For example, both eNOS and EDG-1 have been individually implicated as key modulators of angiogenesis, yet the role of nitric oxide synthesis in S1P-activated angiogenesis is not well understood. eNOS can be activated by polypeptide growth factors such as vascular endothelial growth factor (VEGF), leading to angiogenesis (41, 42); in similar fashion, S1P, in a synergistic fashion with VEGF, markedly stimulates angiogenesis (12). We speculate that eNOS stimulated by the S1P-activated EDG-1 receptor may modulate angiogenesis pathways in vascular endothelial cells. It should be noted that caveolin-1 also may regulate signaling pathways implicated in angiogenesis. For example, angiogenic polypeptide growth factors down-regulate the expression of caveolin-1 protein in an endothelial cell line (43), leading to the hypothesis that caveolin tonically suppresses angiogenesis. Moreover, receptors for

VEGF and related growth factors are markedly enriched in caveolae (reviewed in Ref. 44); present data showing the targeting of EDG-1 to caveolae and inhibitory association with caveolin-1 may indicate a more general mechanism for the coordinated regulation of angiogenesis pathways by independently derived ligands. Whereas caveolae may thereby serve as sites to sequester these and other vascular signaling pathways, the inhibitory effects of caveolin may serve to tonically attenuate the activation of these pathways until their activation by agonist ligands.

As indicated in Fig. 5, overexpression of caveolin-1 markedly attenuates S1P/EDG-1 mediated eNOS activation, but not the basal activity, in the co-transfected COS-7 cells. This inhibition of S1P/EDG-1 activation of eNOS by caveolin is intriguing but difficult to interpret. The eNOS enzyme activity is inhibited by caveolin *in vitro* (32), and the possible inhibitory effects of caveolin overexpression on EDG-1 cannot be distinguished from the known direct inhibitory effects of caveolin on eNOS. By contrast, the inhibition by caveolin of ligand-induced phosphorylation of the EDG-1 receptor more strongly implicates a direct effect of caveolin on S1P-dependent signaling pathways that is independent of NO. The molecular locus for this inhibitory effect of caveolin on EDG-1 receptor phosphorylation remains to be defined. Although the specific protein kinases involved in agonist-dependent phosphorylation of the EDG-1 receptor have not yet been identified, it has been proposed that kinase pathways similar to those modulating agonist-dependent phosphorylation of other G protein-coupled receptors may be involved. If such is the case, the fact that caveolin overexpression so very dramatically influences ligand-induced EDG-1 receptor phosphorylation may indicate a role for caveolin-modulated pathways in the control of S1P signaling.

In conclusion, we have presented evidence that the sphingolipid receptor EDG-1 is targeted to plasmalemmal caveolae, where the receptor can be immunoprecipitated in a complex with caveolin. The targeting of EDG-1 to caveolae appears to facilitate its agonist-dependent activation of eNOS, identifying a potential locus for interactions between these two signaling pathways in the control of angiogenesis and other vascular responses. Caveolin appears to inhibit signaling pathways activated by S1P, suggesting that caveolin may attenuate sphingolipid-dependent signaling even as caveolar targeting may enhance the interactions of signaling molecules expressed in caveolae. The agonist-modulated targeting of EDG-1 to caveolae and its dynamic inhibitory interactions with caveolin identify new points for regulation of sphingolipid-dependent signaling in the vascular wall.

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REFERENCES

1. Shaul, P. W., and Anderson, R. G. W. (1998) *Am. J. Physiol.* **275**, L843–L851
2. Brown, D. A., and Rose, J. K. (1992) *Cell* **68**, 533–544
3. Brown, D. A., and London, E. (1997) *Biochem. Biophys. Res. Commun.* **240**, 1–7
4. Mathias, S., Pena, L. A., and Kolesnick, R. N. (1998) *Biochem. J.* **335**, 465–480
5. Hla, T., Lee, M. J., Ancellin, N., Liu, C. H., Thangada, S., Thompson, B. D., and Kluk, M. (1999) *Biochem. Pharmacol.* **58**, 201–207
6. Olivera, A., and Spiegel, S. (1993) *Nature* **365**, 557–560
7. Sekiguchi, K., Yokoyama, T., Kurabayashi, M., Okajima, F., and Nagai, R. (1999) *Circ. Res.* **100**, 1000–1008
8. Hla, T., and Maciag, T. (1990) *J. Biol. Chem.* **265**, 9308–9313
9. Wang, F., Van Brocklyn, J. R., Hobson, J. P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., and Spiegel, S. (1999) *J. Biol. Chem.* **274**, 35343–35350
10. Xia, P., Wang, L., Gamble, J. R., and Vadas, M. A. (1999) *J. Biol. Chem.* **274**, 34499–34505
11. Lee, M., J., Van Brocklyn, J., R., Thangada, S., Liu, C., H., Hand, A., R., Menzeleev, R., Spiegel, S., and Hla, T. (1998) *Science* **279**, 1552–1555
12. Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999) *Cell* **99**, 301–312
13. Goetzl, E. J., and An, S. (1998) *FASEB J.* **12**, 1589–1598
14. Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H.,

- Okajima, F., and Ohta, H. (2000) *Biochem. Biophys. Res. Commun.* **268**, 583–589
15. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) *J. Biol. Chem.* **274**, 27776–27785
 16. Lee, M. J., Evans, M., and Hla, T. (1996) *J. Biol. Chem.* **271**, 11272–11279
 17. Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and Takuwa, Y. (1998) *J. Biol. Chem.* **273**, 27104–27110
 18. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. W., and Michel, T. (1996) *J. Biol. Chem.* **271**, 6518–6522
 19. Scherer, P. E., Tang, Z., Chun, M., Sargiacomo, M., Lodish, H. F., and Lisanti, M. P. (1995) *J. Biol. Chem.* **270**, 16395–16401
 20. Robinson, L. J., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11776–11780
 21. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6348–6352
 22. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 9690–9697
 23. Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) *J. Biol. Chem.* **271**, 22810–22814
 24. Feron, O., Michel, J. B., Sase, K., and Michel, T. (1998) *Biochemistry* **37**, 193–200
 25. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M., and Shaul, P. W. (1999) *J. Clin. Invest.* **103**, 401–406
 26. Busconi, L., and Michel, T. (1993) *J. Biol. Chem.* **268**, 8410–8413
 27. Feron, O., Smith, T. W., Michel, T., and Kelly, R. A. (1997) *J. Biol. Chem.* **272**, 17744–17748
 28. Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997) *J. Biochem. (Tokyo)* **121**, 969–973
 29. Lee, H., Goetzl, E. J., and An, S. (2000) *Am. J. Physiol.* **278**, C612–C618
 30. Liu, C. H., Thangada, S., Lee, M. J., Van Brocklyn, J. R., Spiegel, S., and Hla, T. (1999) *Mol. Biol. Cell* **10**, 1179–1190
 31. Bredt, D. S., and Schmidt, H. H. W. (1996) *Methods in Nitric Oxide Research*, pp. 249–255, John Wiley & Sons, New York
 32. Michel, J. B., Feron, O., Sase, K., Prabhakar, P., and Michel, T. (1997) *J. Biol. Chem.* **272**, 25907–25912
 33. Lee, M. J., Thangada, S., Liu, C. H., Thompson, B. D., and Hla, T. (1998) *J. Biol. Chem.* **273**, 22105–22112
 34. Ishizaka, N., Griendling, K. K., Lassegue, B., and Alexander, R. W. (1998) *Hypertension* **32**, 459–466
 35. de Weerd, W. F. C., and Leeb-Lundberg, L. M. F. (1997) *J. Biol. Chem.* **272**, 17858–17866
 36. Igarashi, J., Thatte, H. S., Prabhakar, P., Golan, D. E., and Michel, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12583–12588
 37. Xia, P., Gamble, J. R., Rye, K. A., Wang, L., Hii, C. S., Cockerill, P., Khew-Goodall, Y., Bert, A. G., Barter, P. J., and Vadas, M. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14196–14201
 38. Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998) *J. Biol. Chem.* **273**, 3125–3128
 39. Marletta, M. A. (1994) *Cell* **78**, 927–930
 40. Nathan, C., and Xie, Q. W. (1994) *Cell* **78**, 915–918
 41. Ku, D. D., Zaleski, J. K., Liu, S., and Brock, T. A. (1993) *Am. J. Physiol.* **265**, H586–H592
 42. He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B., and Caldwell, R. B. (1999) *J. Biol. Chem.* **274**, 25130–25135
 43. Liu, J., Razani, B., Tang, S., Terman, B. I., Ware, J. A., and Lisanti, M. P. (1999) *J. Biol. Chem.* **274**, 15781–15785
 44. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273**, 5419–5422