Transforming growth factor- β receptors localize to caveolae and regulate endothelial nitric oxide synthase in normal human endothelial cells

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Caveolae (sphingolipid- and cholesterol-rich, 100 nm flask-shaped invaginations of the cell membrane) serve as a nexus of cell signalling. In the present study caveolin-rich lipid raft domains were extracted from HUVEC (human umbilical-vein endothelial cells) using both density gradient and immunoprecipitation techniques, and demonstrated localization of the TGF- β (transforming growth factor- β) receptors T β RI and T β RII to the Cav-1 (caveolin-1)-enriched raft fractions of these normal, human endothelial cells. Immunoprecipitation demonstrated an association between T β RI and T β RII, as well as an association of the T β Rs receptors with Cav-1 and eNOS (endothelial nitric oxide synthase), suggesting a mutual co-localization to caveolae; after treatment of HUVEC with 5 ng/ml TGF- β 1 for 15 min, however, co-precipitation of eNOS with T β RI, T β RII and Cav-1 was diminished. The

loss of immunoprecipitable eNOS from Cav-1-enriched fractions was accompanied by a decrease both in phosphorylation of eNOS and in enzymatic activity (conversion of arginine into citrulline). No change in the localization of eNOS to morphologically distinct caveolae could be detected by electron microscopy after treatment of HUVEC with TGF- β 1 for 20 min. The results of these investigations provide evidence that T β RI interacts with eNOS in the caveolae of normal, human endothelial cells and has a regulatory function on basal eNOS enzymatic activity.

Key words: caveola, caveolin protein, endothelial nitric oxide synthase (eNOS) enzyme, human umbilical-vein endothelial cells (HUVEC), transforming growth factor- β (TGF- β) receptor, vascular endothelium-dependent relaxation.

INTRODUCTION

Caveolae are non-clathrin-coated invaginations of the cell membrane, rich in cholesterol and sphingolipids and supported by a caveolin protein scaffold [1]. Cav-1 (caveolin-1)-supported caveolae are present in most mammalian cells [1], whereas Cav-2 is preferentially expressed in adipose tissue [2]. Muscle cells (smooth, skeletal and cardiac) express morphologically and functionally distinct caveolae supported by a Cav-3 scaffold [3]. The membrane of caveolae has been characterized as belonging to the lipid raft domains, based on its composition and tendency to float higher in density-gradient centrifugation than most of the cell membranes. Initially described for their role in transcytosis, caveolae also serve as an important nexus of intracellular signal transduction [1]; caveolae contain nearly all of the tyrosine kinase activity of the cell membrane, including growth factor receptor tyrosine kinases [such as VEGFR (vascular endothelial growth factor receptor)-1 and -2, PDGFR (platelet-derived growth factor receptor), EGFR (epidermal growth factor receptor) and insulin receptor], other receptors [e.g. muscarinic cholinergic receptors, inositol trisphosphate receptors, bradykinin receptors, β -adrenergic receptor, CCK (cholecystokinin) receptor and tissue factor receptor], as well as important secondary messengers and substrates [e.g. heterotrimeric G-proteins, eNOS (endothelial nitric oxide synthase), calmodulin, Src-family and other non-receptor tyrosine kinases, phosphoinositides, adenylate cyclase, Ca²⁺-dependent ATPase, Ras, Raf-1, Shc (Src-homology type 2 domains), DAG (diacylglycerol), PKC α (protein kinase C α) and mitogen-activated protein kinases] [1].

Recent evidence indicates that localization to caveolae serves as an important regulator of the activity of many signalling molecules. For example, eNOS activity is inhibited by recombinant Cav-1 scaffolding domain [4] and PDGFR autophosphorylation is inhibited by either Cav-1 or Cav-3 (but not by Cav-2) [5]. In addition, many growth factor receptors that depend on dimerization for their function must localize to caveolae in order to be active; the extremely low expression levels of most growth factor receptors on the cell surface would render impossible the hypothesis that a lone receptor could locate a partner for dimerization, unless these receptors were all concentrated within microdomains such as caveolae [6]. Razani et al. used a model of transfected NIH-3T3 cells to demonstrate that T β RI [TGF (transforming growth factor)- β receptor-I] localizes to caveolae and that the scaffolding domain of Cav-1 binds to and inhibits the activity of T β RI, analogous to the role of Cav-1 in the regulation of eNOS activity [7].

One of the genes up-regulated by treatment of endothelial cells with TGF- β 1 is eNOS, which is an important regulator of vascular tone. After treatment of HUVEC (human umbilical-vein endothelial cells) with 5 ng/ml TGF- β 1 for 4 h, eNOS mRNA levels increased [8]. Having considerable homology to cytochrome P-450 NADPH oxidase [9], eNOS functions as a dimer, catalysing the oxidation of two molecules of NADPH in order to reduce two molecules of arginine to citrulline and NO. Dimerization of eNOS is essential for this catalytic function, since there is no electron path from the NADPH oxidase domain of eNOS to the arginine-binding domain on the same mer; rather, each mer passes the electron across a haem bridge to the arginine of the

Abbreviations used: Ach, acetylcholine; Cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical-vein endothelial cells; MBS, morpholinoethanesulphonic acid-buffered saline; TBS, Tris-buffered saline; TEM, transmission electron microscopy; TGF, transforming growth factor; T β R, TGF- β receptor.

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opposite mer [10]. In the absence of appropriate cofactors [Hsp90 (heat-shock protein 90), Akt/PKB, flavins FAD and FMN, calmodulin, tetrahydrobiopterin and arginine], the NADPH oxidase domain of eNOS serves as a superoxide synthase [11,12]. Under normal, physiological conditions, NO produced by eNOS rapidly diffuses from the endothelial cells into the underlying smooth muscle cells [13], and may be carried some distance farther as peroxynitrite or as nitrosylated proteins [14].

The first aim of the present study was to demonstrate localization of native TGF receptors to caveolae in normal human endothelial cells. Initial studies investigating the localization of TGF receptors to caveolae revealed an interaction between TGF receptors and eNOS. In light of the important roles of both Cav-1 and TGF- β 1 signalling in regulating vascular tone through eNOS, these results suggested the existence of a potential, physiologically important regulatory mechanism not previously described in the literature. Therefore a second aim of the present study was to investigate the functional consequences of this interaction for eNOS function in endothelial cells and explore the implications of this novel, TGF- β 1-mediated signalling pathway.

EXPERIMENTAL

Materials

HUVEC, EGM-2 media, Hanks balanced salt solution and trypsin-EDTA were obtained from Clonetics (Gaithersburg, MD, U.S.A.); tissue culture plasticware was from Corning (Corning, NY, U.S.A.); non-fat dry milk was from Carnation (Vevey, Switzerland); nitrocellulose membranes were from Schleicher and Schuell (Keene, NH, U.S.A.); polyclonal antisera to clathrin, FLAG tag and T β RI and T β RII were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); monoclonal antibodies to eNOS (clone 3) and Cav-1 (clone C060), polyclonal antisera to caveolin, and positive control (RSV 3T3 and human endothelial) cell lysates (for caveolin and eNOS respectively) were from Transduction Laboratories (San Diego, CA, U.S.A.); monoclonal antibodies to calmodulin were from Upstate Biotechnology (Lake Placid, NY, U.S.A.); polyclonal antisera to phospho-eNOS (pSer¹¹⁷⁷) was from Cell Signaling Technology (Beverly, MA, U.S.A.); antibody affinity gel beads were from ICN/Cappel (Aurora, OH, U.S.A.); glutaraldehyde, benzoin methyl ether, osmium tetroxide and LR Gold acrylic resin were from Ted Pella (Reading, CA, U.S.A.); uranyl acetate was from Fluka (St. Gallen, Sweden); ³H-arginine was from Amersham Biosciences (Piscataway, NJ, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.) unless otherwise indicated.

Cell culture

HUVEC (passage 1–4) were subcultured using trypsin-EDTA solution, reseeded at a density of 1:4 and cultured to confluence in Medium 199 [supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 20 units/ml heparin and 50 mg/l endothelial cell mitogen], in polystyrene tissue culture flasks that had been precoated with 1.5 % (w/w) gelatin by adsorption.

Extraction of caveolae and related lipid rafts

Upon reaching confluence, HUVEC were lysed to extract caveolae, as described previously [15,16]. Briefly, HUVEC were maintained under standard cell culture conditions or treated with 5 ng/ml TGF- β 1 for 20 min. At the end of this time period, cells were scraped into 1 ml of PBS, and pelleted by centrifugation. The resulting cell pellet was mixed with 500 ml of sodium carbonate (500 mM, pH 11) and homogenized using both a hand-held Pellet Pestle grinder for three pulses of 20 s each, on ice, and a PRO 500

tissue homogenizer at 50 % speed, for three pulses of 10 s each, with 15 s on ice between pulses. The homogenized cell suspension was mixed with an equal volume of 90 % (w/w) sucrose in MBS (morpholinoethanesulphonic acid-buffered saline), and a discontinuous (45:35:4, by percentage) sucrose gradient was laid on top of the homogenized cell lysates. The resulting gradient was subjected to centrifugation (200 000 g) in a Beckman SW-41 rotor under gentle acceleration and deceleration at 16 °C for 16–20 h. After centrifugation, the gradient was fractionated into 12 × 1 ml aliquots from top to bottom; density fractions were either used immediately for immunoprecipitation and/or Western blotting or snap-frozen in dry ice and methanol at –70 °C and stored at –80 °C until use.

Immunoprecipitation

Because no single method for isolation of lipid rafts is definitive for complete recovery and purification of caveolae, an immunoprecipitation technique [17] was used to confirm the results of the density-gradient preparations. HUVEC were either maintained under standard cell culture conditions or treated with 5 ng/ml TGF- β 1 for 20 min, then prepared in 500 mM sodium carbonate (pH 11) by dual-speed homogenization (as described for extraction of caveolae), before being mixed with an equal volume of MBS to lower the pH. These homogenized lysates were mixed with 10 μ l of antisera directed to any one of the following: Cav-1, eNOS, T β RI, T β RII, FLAG tag and calmodulin (the latter two as non-immune controls) at 4 °C, overnight. The lysates were subsequently incubated in the presence of 50 μ l of antibody affinity gel at 4 °C for 1 h to precipitate antibody/protein complexes. The complexes on the gel were washed four times with 50 % MBS/50 % 500 mM sodium carbonate, then eluted from the antibody affinity gel by boiling in 20 μ l of 3 × Laemmli denaturing sample buffer [150 mM Tris/HCl (pH 6.8), 6 % (v/v) SDS, 30 % (v/v) glycerol, 0.01 % (v/v) bromophenol blue, 15 % (v/v) β -mercaptoethanol] for 5 min, and quenched on ice; the eluted protein in 3 × sample buffer was diluted to 1 × with 40 μ l of 50 % MBS/50 % 500 mM sodium carbonate, and boiled for a further 5 min. These samples were again quenched on ice, centrifuged at 10 000 g in an Eppendorf microcentrifuge to remove the affinity gel and insoluble precipitates, and the supernatant was loaded on to 12.5 % (w/v) polyacrylamide gels for electrophoresis and Western blotting (using antisera to Cav-1, eNOS, T β RI and T β RII).

Gel electrophoresis

Density fractions from HUVEC were analysed by Western blotting (by the method of Laemmli), using standard laboratory procedures. Briefly, 40 μ l of each density fraction was mixed with 20 μ l of 3 × Laemmli denaturing sample buffer and vortex-mixed for 10 s; this mixture was boiled for 5 min, then quenched on ice for 1 min. Equal volumes of each density fraction were loaded on to each lane of a 1.5 mm-thick polyacrylamide gel; 8 % polyacrylamide gels were used to analyse eNOS (molecular mass 144 kDa) and T β RII (molecular mass 77 kDa), and 12.5 % polyacrylamide gels were used to analyse T β RI (molecular mass 44 kDa) and Cav-1 (molecular mass 22 kDa). After electrophoresis, proteins were subsequently blotted on to nitrocellulose membranes, and transfer was confirmed by staining with 0.1 % Ponceau Red S in 2 % (v/v) acetic acid for 5 min; Ponceau Red S was washed off with deionized water before Western blotting.

Western blotting

Washed membranes were blocked in blocking buffer BLOTTO [TBS (Tris-buffered saline) containing 0.1 % Tween 20 and 5 %,

w/w, non-fat dry milk] for 2 h, then incubated with antibodies to either phospho-eNOS (pSer¹¹⁷⁷; 1:1000), Cav-1 (1:500), T β RI (1:1000), T β RII (1:500), calmodulin (1:200), FLAG tag (1:1000), clathrin (1:200) or eNOS (1:1000) in BLOTTO at 4°C, overnight. Membranes were washed four times with TBS containing 0.1 % Tween 20, and subsequently incubated in BLOTTO with horseradish peroxidase-conjugated secondary antibodies (1:3000) to either rabbit [phospho-eNOS (pSer¹¹⁷⁷), T β RI, T β RII, calmodulin, FLAG tag, clathrin and Cav-1] or mouse (Cav-1 and eNOS) IgG at room temperature (20°C) for 2 h, then washed four times with TBS containing 0.1 % Tween 20. Labelled bands were visualized using a SuperSignal West Pico chemiluminescent reagent (according to the manufacturer's instructions), and a Kodak XAR-5 film. The identities of the bands visualized in the Western blots were confirmed by comparison with molecular-mass standards and/or positive control lysates provided by the manufacturer of the antibody (results not shown).

Cell fixation for electron microscopy

Immunogold electron microscopy was carried out with slight modifications to the procedures published by Berryman and Rodewald [18] and Reiner et al. [19]. Briefly, HUVEC were either maintained under control conditions or treated with 5 ng/ml TGF- β 1 for 20 min. After these treatments, the cells were pre-fixed in an excess of ice-cold 4 % (w/v) paraformaldehyde/0.05 % (v/v) glutaraldehyde in PBS for 2 min on ice; the fixative was aspirated and the cells were then scraped from the culture vessels in 400 μ l of fresh fixative. Fixed cells were pelleted by centrifugation at 10 000 *g* in 0.4 ml microcentrifuge tubes in an Eppendorf microcentrifuge at 4°C. Cell pellets were cut from the microcentrifuge tubes and fixed in a further excess of 4 % paraformaldehyde/0.05 % glutaraldehyde in PBS, on ice, for a total fixation time of 2 h. At the end of this time period, the cell pellets were washed three times with 4 % paraformaldehyde in PBS and stored in this solution at 4°C until processed for embedding.

Electron microscope embedding

Cell pellets were either fixed and stained in 0.5 % tannic acid in 3.5 % sucrose/0.5 mM CaCl₂ in PBS (without washing out residual paraformaldehyde; [19]) on ice for 4 h or processed without tannic acid staining [18]. After or without tannic acid staining, cell pellets were washed with three changes of 3.5 % sucrose/0.5 mM CaCl₂ in PBS on ice for 2 h to remove residual paraformaldehyde; the remaining aldehydes were quenched with 50 mM NH₃Cl/3.5 % sucrose/0.5 mM CaCl₂ in PBS on ice for 1 h. Phosphate ions were removed by four rinses in 3.5 % sucrose/0.1 M sodium maleate buffer on ice for 10 min, and cells were stained with 4 % (w/v) uranyl acetate in 3.5 % sucrose/0.1 M sodium maleate buffer at 4°C for 3 days.

Uranyl acetate-stained pellets were dehydrated by graded aqueous acetone (50 % on ice for 45 min; 70 % at -20°C for 45 min; and 90 % at -20°C for 45 min), then infiltrated with graded LR Gold resin in five stages, followed by three stages of infiltration with LR Gold resin containing 0.5 % benzoin methyl ether; resin was cured at -20°C under UV light for 48 h.

Thin sections (100 nm) were cut from the resulting embedded cell pellets and fixed to flame-carbon-coated #200 pure nickel grids. The grids were dried overnight before immunogold staining.

Immunogold staining

Grids to be immunogold-stained were pre-wet by floatation on TBS for 5 min, then preblocked on 3 % (w/v) BSA in TBS for 1 h. The grids were immunostained on solutions of 1:150 poly-

clonal antisera/Cav-1, 1:250 (without tannic acid) or 1:10 (with tannic acid) polyclonal antisera/T β RI, 1:50 monoclonal antibodies/Cav-1 and 1:50 monoclonal antibodies/eNOS in 1 % BSA in TBS either at room temperature for 2 h (T β RI) or at 4°C overnight. Grids were washed five times with 1 % BSA in TBS at room temperature for 3 min each time, then exposed to 1:50 anti-rabbit or anti-mouse antisera conjugated with 10 nm colloidal gold particles in 1 % BSA in TBS at room temperature for 1 h. Grids were washed five times with TBS at room temperature for 3 min each time, and fixed in 2 % (v/v) aqueous glutaraldehyde for 5 min, then rinsed in deionized water and allowed to dry.

Grids were post-stained in osmium tetroxide vapour for 30 min, then rinsed in deionized water and allowed to dry. Further post-staining was accomplished by floating the grids first on drops of aq. 4 % uranyl acetate for 10 min, followed by rinsing and drying, and then on drops of Reynold's lead citrate (surrounded by a ring of sodium hydroxide) for 1 min. The grids were immediately given two final rinses in 0.2 μ M filtered, deionized, degassed water for 10 s each time, and allowed to dry. The grids were examined using a Jeol JEM-100CX electron microscope and photographed on Kodak films.

Ultrastructural localization of eNOS

To determine whether treatment of HUVEC with TGF- β 1 resulted in expulsion of eNOS from the caveolae, nine random TEM (transmission electron microscopy) fields were selected and photographed, in a double-blinded manner, from numerically coded grids containing cells that had been either maintained under control conditions or treated with 5 ng/ml TGF- β 1 for 20 min. For each micrograph, a large area including the cell surface was chosen, and gold particles (labelling eNOS) were counted as being associated with caveolae, or not. Subsequently, the number of particles associated with caveolae were normalized to caveolar area, and the number of particles not associated with caveolae were normalized to non-caveolar, cytoplasmic areas of the micrograph. Results were double-blind grouped by numerical code and analysed by Student's *t* test.

Enzymatic activity of eNOS

To measure eNOS activity, HUVEC were cultured to confluence, then either maintained under standard cell culture conditions or treated with 5 ng/ml TGF- β 1 and 100 ng/ml Ach (acetylcholine), and/or a combination of the two, for 20 min. At the end of this time period, cellular activity was stopped by rinsing the HUVEC monolayer in ice-cold PBS, followed by removal of the cells from their culture dishes using ice-cold, 500 mM EDTA in PBS. Detached cells were collected by scraping in EDTA in PBS and pelleted by centrifugation at 10 000 *g* in an Eppendorf microcentrifuge. Cell pellets were either flash-frozen in liquid nitrogen and stored at -80°C before lysis or immediately disrupted by rapid pipetting in a lysis buffer comprising 25 mM Tris/HCl, 1 mM EDTA and 1 mM EGTA solution, for analysis using a nitric oxide synthase assay kit (Calbiochem, San Diego, CA, U.S.A.) according to the manufacturer's instructions. Briefly, cell lysates were centrifuged to pellet the insoluble protein and membrane fraction, which was subsequently resuspended in lysis buffer, and an aliquot was added to a reaction mixture consisting of 40 mM Tris/HCl, 4.8 μ M tetrahydrobiopterin, 1.6 μ M FAD, 1.6 μ M FMN, 1.25 mM NADPH, 0.025 μ Ci/ μ l [³H]arginine and 0.75 mM CaCl₂ on ice. The reaction samples were incubated at room temperature for 30 min, at which time the reaction was stopped by the addition of 400 μ l of 50 mM Hepes and 5 mM EDTA. Unchanged [³H]arginine was removed using a column of

Figure 1 Cav-1-rich lipid raft membrane fractions from HUVEC contain eNOS, TβRI and TβRII

(A) Equal volume aliquots of 12 sucrose-gradient density fractions from HUVEC were analysed by Western blotting, using specific antibodies directed to clathrin, Cav-1, eNOS, TβRI and TβRII. The caveolar fraction (lane 5), identifiable by the presence of Cav-1 (Cav-1 band), contains eNOS (eNOS band), as well as the TGF receptors TβRI (TβRI band) and TβRII (TβRII band). In contrast, clathrin (clathrin band) was localized exclusively to fraction 9. Proteins were identified both by immunoreactivity with specific antibodies and by molecular mass. (B) Compared with controls (A), HUVEC treated with 5 ng/ml TGF-β1 for 20 min exhibited similar amounts of Cav-1, eNOS, TβRI or TβRII localizing to fraction 5 (B, lane 5) of these gradients, and did not induce such localizations to the irrelevant fraction 7 (B, lane 7), nor to the clathrin pathway in fraction 9 (B, lane 9).

equilibrated ionic resin, and the radioactivity of the [³H]citrulline in the eluate was measured using Lumi-Safe scintillation fluid and a scintillation counter. Arginine was eluted from the resin using 0.5 M NH₄Cl and was also measured to ensure that samples had sufficient [³H]arginine for the reaction. Protein levels in each of these lysates were measured in triplicate using a BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.) and following the manufacturer's instructions, and [³H]citrulline counts were normalized to these total protein measurements.

RESULTS

Density-gradient centrifugation

Density-gradient centrifugation of ground, homogenized HUVEC resulted in a flocculent layer near the interface of the 5 and 35 % sucrose layers of the gradient, which corresponded to the fifth fraction from the top. The characteristics and location of this layer were in agreement with the literature descriptions of caveolae-enriched lipid raft fractions isolated by detergent-free sucrose gradient centrifugation [15,16].

Western blotting of HUVEC

Density gradients from HUVEC were used in Western-blot analyses to investigate the caveolar localization of TGF receptor superfamily molecules in normal, untransfected, human endothelial cells. Western blotting of density-gradient fractions of these cells demonstrated that Cav-1 was found primarily in density fraction 5 of the gradients (Figure 1A, lane 5), and not substantially in other density fractions (Figure 1A, lanes 1–4 and 6–12); in contrast with methods that employ detergent lysis, the method we used does not result in the appearance of lipid raft fractions in the lower portions of the gradient [15]. Specifically, Cav-1 (Figure 1A, Cav-1 band) was found in fraction 5, and not in

Figure 2 Immunoprecipitation of caveolar components from HUVEC

HUVEC lysates were immunoprecipitated using monoclonal and polyclonal antibodies directed to Cav-1, TβRI, TβRII and eNOS. These proteins were then visualized by Western blotting using the same antibodies. (A) An immunoreactive band corresponding to the molecular mass of Cav-1 (Cav-1 band) was observed to co-precipitate with TβRI and with TβRII. Immunoreactive bands corresponding to the molecular mass of eNOS (eNOS band) were found to have co-precipitated with TβRI, TβRII and Cav-1. In contrast, after treatment of HUVEC with 5 ng/ml TGF-β1 for 20 min (marked by + below the lane), co-precipitation of eNOS with TβRI, TβRII and Cav-1 was diminished (eNOS band). (B) No change in the total amount of these proteins was observed after treatment of the cells with TGF-β1. These data provided evidence for TGF-β1-mediated interactions of TβRI, TβRII and Cav-1 with eNOS, within caveolae.

other, nearby fractions (a faint band for Cav-1 was, however, found in fraction 9; Figure 1A, Cav-1 band, lane 9). In addition, eNOS, which has previously been reported to localize specifically to caveolae [20], was predominantly found in fraction 5 (Figure 1A, eNOS band, lane 5); again, there was a very faint band for eNOS in fraction 9 (Figure 1A, eNOS band, lane 9). Similarly, both TβRI and TβRII were found exclusively in fractions 4 and 5, demonstrating that these proteins localize to lipid raft fractions (Figure 1A, TβRI and TβRII bands respectively). It should be noted that it was not possible to visualize native TβRs in untransfected, untransformed human cells with the protein from less than 1.5 million, low-passage ($P \leq 3$) cells, and still required very long exposure times on film; this result suggests that TGF receptors occur at very low copy numbers in normal, human endothelial cells. In contrast with Cav-1 and TβRs, clathrin was found exclusively in fraction 9 (Figure 1A, clathrin band, lane 9).

Compared with controls (Figure 1A), HUVEC treated with 5 ng/ml TGF-β1 for 20 min (Figure 1B, lanes 5 and 7) did not demonstrate any substantial change in the amount of Cav-1, eNOS, TβRI or TβRII associated with density fraction 5 (Figure 1B, lane 5). Similarly, no change was noted in the amount of these proteins in an irrelevant fraction with density close to that of the lipid rafts and caveolae (a control for poor fractionation; Figure 1B, lanes 7), nor in the clathrin pathway-associated fraction (lanes 9).

Immunoprecipitation

In order to confirm the results obtained with density-gradient centrifugation, intact Cav-1-rich lipid rafts were isolated from detergent-free HUVEC lysates by immunoprecipitation [17]. Immunoprecipitation of Cav-1 (using either monoclonal or polyclonal antibodies) from lysates of HUVEC that had been maintained under control conditions resulted in the co-precipitation of TβRI and eNOS (Figure 2A). In addition, immunoprecipitation using polyclonal antisera directed to either TβRI or TβRII also co-precipitated Cav-1 (Figure 2A, Cav-1 band) as well as TβRI, TβRII and eNOS as a group (Figure 2A); these results suggested that Cav-1, TβRI and TβRII and eNOS were all linked.

Figure 3 Transmission electron micrographs of caveolae in HUVEC

HUVEC were either maintained under standard cell culture conditions (not shown) or treated with 5 ng/ml TGF- β 1 for 20 min, and were subsequently processed by the methods of Berryman and Rodewald [18] and Reiner et al. [19]. Caveolae could be distinguished in these preparations either by their characteristic, flask-shaped morphology and 100 nm diameter (**A**, arrows) or by specific immunogold labelling for Cav-1 (**A**, arrowheads); addition of tannic acid to the fixed cell pellet allowed concurrent visualization of both caveolar morphology and Cav-1 immunolabelling in the same section (**A**). Immunogold labelling for eNOS (**B**, arrows and arrowheads) could be observed both concurrent with (**B**, arrows) and separately from (**B**, arrowheads) caveolae in tannic acid-fixed HUVEC. There was no difference between the localization of eNOS in HUVEC maintained under control conditions and in HUVEC treated with 5 ng/ml TGF- β 1 (results not shown). Due to divergent requirements for staining, T β Rs could not be visualized concurrently with definitive caveolae by TEM (results not shown). Magnifications, $\times 36\,000$.

Interestingly, when HUVEC were treated with 5 ng/ml TGF- β 1 for 20 min, co-precipitation of eNOS with this cluster was greatly diminished (Figure 2A, eNOS band). Neither Cav-1, T β R1, T β R2, eNOS nor calmodulin were co-immunoprecipitated with isotype-matched anti-FLAG antibodies used as a non-immune control (results not shown). Immunoprecipitation of calmodulin only resulted in visualization of calmodulin (results not shown). These controls suggested that the observed co-precipitations of Cav-1, eNOS, T β R1 and T β R2 were specific interactions between the blotted and precipitated proteins. Total levels of these proteins (as measured by immunoprecipitation, followed by Western blotting with the same antibodies) were not substantially altered by treatment of HUVEC with 5 ng/ml TGF- β 1 for 20 min (Figure 2B). Given that 20 min is too short a time period for *de novo* protein synthesis, the observed decrease in T β R- and Cav-1-associated eNOS after treatment of the cells with TGF- β 1 was probably due to changes in protein–protein interactions, rather than to protein turnover.

Electron microscopy

Transmission electron microscopy was used to determine the localization of suspected caveolar proteins to morphologically recognizable caveolae. Caveolae were readily visualized in thin sections of HUVEC, using TEM, either by their characteristic 100 nm size and flask-shaped morphology (Figure 3A, arrows) or by the presence of immunogold beads marking anti-Cav-1 antibodies (Figure 3A, arrowheads); addition of tannic acid to the cell pellets during the fixation and embedding procedure [19] resulted in improved visualization of caveolar morphology and the ability to see caveolae in areas with sufficient antigenic quality to permit immunogold labelling (Figure 3A). Cav-1 could be detected with either polyclonal (Figure 3A, arrowheads) or monoclonal (results not shown) antibodies against Cav-1. Matched non-immune control antisera did not produce significant immunogold labelling, demonstrating specificity of these antibodies (results not shown).

Similarly, substantial immunogold labelling of T β R1 could be clearly visualized in tannic acid-free thin sections of HUVEC, although sections with sufficient morphological preservation to identify caveolae did not have adequate antigenicity to immunogold label T β R1 (results not shown). Addition of tannic acid to

the samples, while permitting visualization of caveolar location and morphology, resulted in the loss of immunogold labelling for T β R1 (results not shown); it was not, therefore, possible to visualize both caveolae and T β R1 simultaneously, and to demonstrate specific localization of T β R1 to caveolae by electron microscopy, using the available methods.

In contrast, localization of eNOS to caveolae was readily demonstrated in thin sections of tannic acid-stained HUVEC; eNOS (Figure 3B, arrows) was not, however, localized specifically to caveolae (Figure 3B, arrowheads), but was present diffusely throughout the cytoplasm and inside organelles such as mitochondria. Labelling with isotype-matched, non-immune antisera did not produce this diffuse labelling, suggesting that the labelling was, in fact, specific for eNOS. Random fields (nine per condition) were selected from HUVEC either maintained under control conditions or treated with 5 ng/ml TGF- β 1 for 20 min; the localization of immunogold beads to caveolae was counted and normalized to the caveolar area as described in the Experimental section. Although caveolae showed substantial labelling of eNOS, the gold particle count per caveolar area versus gold particle count per non-caveolar area was essentially similar (Figure 3B). TGF- β 1 treatment of the cells had no effect on this pattern of labelling (results not shown).

Measurements of eNOS activity

A wide range of cofactors found in caveolae is required for eNOS enzymatic activity; for this reason, it could be hypothesized that expulsion of eNOS from association with caveolae (and, thus, from essential cofactors) would have an inhibitory effect on enzymatic activity. However, an excitatory effect could also be defended, since Cav-1 is a native inhibitor of NO synthesis by eNOS; this suggests that dissociation of eNOS from Cav-1 in the caveolae may increase eNOS activity. Because the likely effects of the observed dissociation of eNOS from caveolae were not clearly evident from the literature, we measured the functional effects of treatment of HUVEC with TGF- β 1 on eNOS activity.

Treatment of HUVEC with 5 ng/ml TGF- β 1 for 5 min resulted in a significant decrease in phosphorylation of eNOS on Ser¹¹⁷⁷ (Figure 4); phosphorylation of this residue has been reported in the literature to regulate eNOS enzymatic activity [21]. Direct measurement of eNOS activity levels showed that, compared with

Figure 4 Phosphorylation of eNOS on Ser¹¹⁷⁷ is reduced after treatment of HUVEC with 5 ng/ml TGF- β 1 for 5 min

Compared with HUVEC that were maintained under control conditions (lane C and black bar), cells treated with 5 ng/ml TGF- β 1 (lane T and white bar) for 5 min demonstrated reduced eNOS phosphorylation on a serine residue (Ser¹¹⁷⁷) that regulates enzymatic activity (eNOS pSer¹¹⁷⁷ band); band densities were normalized to total eNOS (total eNOS band) using Adobe Photoshop. Normalized band intensities are expressed as fold difference from the average for control samples (mean $\pm$ S.D.; $n = 4$). * $P < 0.05$, significantly different from controls, by Student's t test.

Table 1 Treatment of HUVEC with TGF- β 1 for 20 min resulted in reduced eNOS enzymatic activity

NOS activity of HUVEC lysates was analysed by [³H]arginine conversion assay using a commercially available kit and following the manufacturer's instructions. Data are expressed as c.p.m. of [³H]citrulline (a surrogate for NO production). Compared with controls, HUVEC treated with 5 ng/ml TGF- β 1 for 20 min exhibited reduced eNOS enzymatic activity. HUVEC treated with 100 ng/ml Ach for 20 min did not differ significantly from cells treated with both TGF- β 1 and Ach (TGF- β 1/Ach).

	Control (c.p.m.)	TGF- β 1 (c.p.m.)	Ach (c.p.m.)	TGF- β 1/Ach (c.p.m.)
Average	3172	1674*	11599	11849
S.D.	1183	897	1011	3106

* Significantly different from control at $P < 0.009$; $n = 6$.

controls, HUVEC that had been exposed to 5 ng/ml TGF- β 1 for 20 min demonstrated significantly ($P < 0.02$) reduced conversion of [³H]arginine into [³H]citrulline (Table 1). Furthermore, treatment of HUVEC with either 100 ng/ml Ach (Table 1) or 1 μ M A23187 calcium ionophore (results not shown) resulted in increased conversion of [³H]arginine into [³H]citrulline; these effects could be blocked by the addition of 100 μ M NOLA, a competitive antagonist of arginine binding to eNOS (results not shown). Compared with HUVEC that had been treated with 100 ng/ml Ach, however, HUVEC that had been treated with both 100 ng/ml Ach and 5 ng/ml TGF- β 1 together did not demonstrate decreased conversion of [³H]arginine into [³H]citrulline (Table 1).

DISCUSSION

Razani et al. [7] have demonstrated that T β R1 localized to caveolae in NIH-3T3 cells through several elegant two-hybrid experiments. Later, Di Guglielmo et al. [22] demonstrated localization of some fraction of a transfected T β R2-HA (where HA stands for haemagglutinin) construct to caveolae and lipid rafts

in Mv1Lu cells [22]. In addition, earlier work provided evidence that TGF- β 1-induced generation of inositol phosphoglycan was processed through caveolae [23,24]. Although other laboratories have demonstrated the presence of native T β Rs in lipid rafts in some transformed cell lines of animal origin, the present study is the first to show that the native receptors localize to Cav-1-rich lipid rafts in normal human cells, or in endothelial cells. Razani et al. [7] specifically reported that they were unable to detect T β R2 in their Western-blot analyses of lipid raft-enriched fractions of a sucrose density gradient [7]. However, for dimerization of T β R1 with T β R2 to occur, it would be necessary for both receptors to be in the same place (namely, within caveolae). In the present study, it was necessary both to pool 225 cm² ($3 \times$ T-75 flasks) of confluent HUVEC per density gradient, and to use only very low passage (passages 2–3) HUVEC, in order to detect any T β R2 in any density fraction. Taken together, these results may suggest that T β R2 is a rare species in the cell membrane in endothelium and may serve as a rate-limiting regulator of TGF signalling; alternatively, localization of T β R2 to caveolae (and, thus, to T β R1) may occur only in response to a specific activating signal present under basal conditions in endothelial and Mv1Lu cells, but not in some other cell types. In either case, the results of the present study suggest that specific localization of T β R2 to caveolae may not be ubiquitous, but has a specific purpose in some cells.

The present study provided biochemical evidence from both density-gradient (Figure 1) and immunoprecipitation (Figure 2) methods that TGF- β 1 stimulation was not required for localization of T β R1 to Cav-1-rich lipid raft domains in HUVEC, but may be constitutive in these cells. In contrast, the biochemical results of Razani et al. [7] suggested that localization of T β R1 to caveolae could be abolished by stimulation of the cells with TGF- β 1. Our attempts to address this discrepancy through definitive electron microscope techniques were not successful, due to the conflicting requirements for visualization of caveolae and of T β R1. There are, however, several possible explanations for this discrepancy. First, the shorter (5–20 min) time course used in the present study differed from the 45 min time course used by Razani et al. [7]; thus, while the present results captured comparatively early events (namely the dephosphorylation, de-association from Cav-1, and reduced enzymatic activity, of eNOS), they may not have caught late events, such as internalization, dissolution and recycling of caveolae. Furthermore, as noted above, the present results were obtained using normal, human endothelial cells, whereas the work of Razani et al. [7] was performed with immortalized, transfected 3T3 cells. It is interesting to note that small amounts of T β Rs, and even Cav-1, were found in fraction 9, which corresponds to clathrin-coated pits; while being far from conclusive evidence, this finding does suggest the possibility that caveolar components may cycle through clathrin-mediated endocytosis as well as caveolae-mediated endocytotic pathways in these cells [22]. Nevertheless, it remains possible that constitutive localization of T β R1 to caveolae is cell-type-specific, or perhaps is rapidly lost with increasing passage in *in vitro* culture. The biochemical results of the present study do, however, suggest that localization of T β R1 to caveolae is constitutive in healthy HUVEC, and is not affected by stimulation of the cells with TGF- β 1 within the time frame of its interactions with eNOS. If this association is indeed permanent in endothelial cells, then it is likely that any regulatory or signalling cascade involving internalization of T β Rs will differ between endothelial cells and some other cell types.

The results of the present study have demonstrated a novel interaction between T β R1 and eNOS, with functional consequences on NO synthesis by HUVEC. Specifically, treatment of HUVEC with 5 ng/ml TGF- β 1 for 20 min resulted in expulsion of eNOS

from association with caveolar proteins (namely Cav-1, T β RI and T β RII; Figure 2). Treatment of HUVEC with 5 ng/ml TGF- β 1 for time periods of 5 min to 24 h resulted in decreased eNOS phosphorylation (Figure 4) and eNOS enzymatic activity levels (Table 1). The comparatively short time course of these responses (as low as 5 min for decreased phosphorylation) would leave no time for mRNA or protein synthesis in response to TGF- β 1; taken together with the loss of co-precipitation of eNOS with T β RI after treatment of the cells with TGF- β 1 (Figure 2), and the rapid loss of eNOS phosphorylation (Figure 4), without changes in total eNOS protein (Figures 2–4), these results suggest that this interaction is regulatory on eNOS enzymatic function, rather than being mediated by regulation of mRNA or protein synthesis, or by regulation of protein turnover.

The current model of the regulation of eNOS function implies that association with Cav-1 maintains eNOS in an inactive state; eNOS activation concurrent with dissociation from Cav-1 [25]. The literature is unclear, however, whether dissociation of eNOS from Cav-1 may directly activate eNOS [26] or will permit eNOS to associate with stimulatory cofactors [27,28]. The results of the present study provide evidence that dissociation of eNOS from Cav-1 is not, by itself, an activating event; specifically, treatment of HUVEC with TGF- β 1 for 20 min results in dissociation of eNOS from Cav-1 (Figure 2), yet causes decreased eNOS activity in these cells (Figure 4 and Table 1). This result could suggest that T β RI-mediated expulsion of eNOS from association with caveolar proteins results in removal of eNOS from a number of caveolar-associated cofactors required for enzymatic activity (e.g. FAD, FMN, tetrahydrobiopterin, calmodulin, Akt etc.), possibly by altering the ultrastructural, subcaveolar localization of eNOS, although not by expulsion of eNOS from the caveolae (Figure 3). These results suggest that the TGF- β 1-mediated interaction of T β RI and eNOS results in the transfer of eNOS to an inactive state. This inactive state is not, however, refractory to activation by conventional stimuli; specifically, treatment of HUVEC with 100 ng/ml Ach for 20 min resulted in increased eNOS activity (as measured by [3 H]arginine to [3 H]citrulline conversion assay) both in the presence and absence of 5 ng/ml TGF- β 1 (Table 1). Taken together, these results would suggest that the role of T β RI is more likely in the fine tuning of basal vascular tone, rather than in modulating large, Ach-mediated changes in vascular diameter.

Surprisingly, the transmission electron micrograph results of the present study demonstrated that eNOS, although sometimes present in the caveolae, is not localized to the caveolae exclusively, and is also present in non-caveolar cytoplasm (Figure 3); these studies were exhaustively repeated to confirm this result. The fact that eNOS is not exclusively associated with caveolae in cross-sections of intact cells is in marked contrast with the density-gradient centrifugation results (Figure 1), which showed a near-exclusive co-fractionation of both eNOS and Cav-1 to lipid raft fractions. Although some of this extra-caveolar eNOS observed by TEM may represent mitochondrial NOS (which is cross-reactive with the monoclonal antibodies directed to eNOS used in the present study [29]), most of it shows no apparent association with any feature having the ultrastructural appearance of mitochondria. One possible explanation is that eNOS may, even separate from organized caveolae, exist in association with Cav-1 and a raft fraction with a sufficiently buoyant quantity of sphingolipids and cholesterol to co-purify with Cav-1-rich lipid raft fractions. In addition, the homogenization of the cells during fractionation may have also resulted in a scavenging effect, in which all eNOS that was physically separate from the caveolae of intact cells became attached to those caveolae once the cell structure was disrupted and homogenized. Such an artifact would confound any attempt

to study the caveolar composition or function using existing biochemical methods, and gives further support to the decision to utilize multiple, potentially redundant experimental methods to validate the results of the present study; furthermore, since caveolae are smaller than the limit of visible light microscopy, these results suggest that TEM studies of cross-sections of intact cells are essential for any investigation of the caveolar composition or function. Nevertheless, substantial inhibition of eNOS phosphorylation (Figure 4) and function (Table 1) occurred after stimulation of HUVEC with 5 ng/ml TGF- β 1 for 20 min. Whereas the enzymatic activity assay (Table 1) was performed on homogenized cell lysates and thus may have artificially brought T β Rs and Cav-1 into contact with eNOS, the inhibition of eNOS phosphorylation by TGF- β 1 occurred in intact, living cells, confirming that T β Rs possess mechanisms to down-regulate eNOS function *in vivo*. It should be noted that changes in enzymatic phosphorylation and activity were in the range of 50%. If the extent of translocation of eNOS out of caveolae was also in the range of 50%, it might have been below the quantitative sensitivity of immunogold electron microscopy; thus, we cannot firmly conclude that there was no ultrastructural translocation of eNOS, only that we did not find any evidence for such a mechanism.

In summary, the results of the present study demonstrated localization of TGF receptors T β RI and T β RII to Cav-1-rich lipid raft domains in normal, untransfected human endothelial cells and, furthermore, that T β RI interacts with eNOS within these domains; treatment of HUVEC with physiological levels of TGF- β 1 for as little as 5 min results in expulsion of eNOS from association with caveolar proteins and in the dephosphorylation and inactivation of eNOS. This inactivation can be overcome by treatment of HUVEC with Ach. The present study has, therefore, elucidated a novel signalling pathway by which TGF- β 1 can regulate NO production (and therefore, perhaps vascular tone and cell proliferation) in HUVEC under conditions approximating the basal state of normal vascular physiology.

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