Peptide-Stimulation Enhances Compartmentalization and the Catalytic Activity of Lung Endothelial NOS

Tarun E. Hutchinson², Sudeep Kuchibhotla², Edward R. Block^{1,2} and Jawaharlal M. Patel^{1,2}

¹Research Service, Malcom Randall Department of Veterans Affairs Medical Center and ²Department of Medicine, University of Florida College of Medicine, Gainesville

Key Words

Endothelial Cells • Compartmentalization • Plasma membrane • Golgi Complex • Caveolae • Vasorelaxation

Abstract

We reported that an 11 amino acid synthetic peptide (P1) activates lung endothelial cell nitric oxide synthase (eNOS) independent of its change in expression and/or phosphorylation. Since caveolae/eNOS dissociation is known to enhance the catalytic activity of eNOS, we examined whether P1-mediated increase of eNOS activity is associated with caveolae/cholesterol modulation, increased caveolin-1 phosphorylation, and intracellular compartmentalization of eNOS in pulmonary artery endothelial cells (PAEC). PAEC were incubated with or without (control) P1 or cholesterol modulators/caveolae disruptors, cholesterol oxidase (CHOX) and methyl- β -cyclodextrin (CD), for 1 h at 37°C. After incubation cells were used for: i) immunoprecipitation, ii) isolation of plasma membrane (PM)-, Golgi complex (GC)-, and non-Golgi complex (NGC)-enriched fractions, iii)

immunofluorescence confocal imaging, and iv) electron microscopy for localization and/or eNOS activity. P1, CHOX, and CD-stimulation caused dissociation of eNOS from PM with increased localization to GC and/or NGC. P1 and CHOX significantly increased eNOS activity in PM and GC and CD-stimulation increased eNOS activity localized only in GC. P1 increased phosphorylation of caveolin-1 in intact cells and GC fraction. Immunofluorescence and/or immunogold labeled imaging/electron microscopy analysis of P1-, CHOX-, and CD-stimulated intact cells confirmed eNOS/caveolae dissociation and translocation of eNOS to GC. These results suggest that: i) P1-stimulation translocates eNOS to GC and enhances the catalytic activity of eNOS in both the PM and GC fractions of PAEC, ii) CHOX- but not CD-mediated caveolae and/or cholesterol modulation mimics the effect of P1-stimulated compartmentalization and activation of eNOS in PAEC, and iii) P1-stimulated caveolae/cholesterol modulation, phosphorylation of caveolin-1, and activation of eNOS is physiologically relevant since P1 is known to enhance NO/cGMP-dependent vasorelaxation in the pulmonary circulation.

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
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1015-8987/09/0246-0471\$26.00/0

Accessible online at:
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J.M.Patel, Ph.D
Research Service (151), VA Medical Center
1601 S.W. Archer Road, Gainesville, FL, USA 32608-1197 (USA)
Tel. +1 352-376-1611 Ext. 6448, Fax +1 352-379-7421,
E-Mail Pateljm@medicine.ufl.edu

Introduction

Vascular endothelial cell NOS (eNOS) is a member of a novel family of enzymes containing heme oxygenase and cytochrome P450 reductase domains that catalyze the oxidative metabolism of L-arginine to generate NO [1]. The catalytic activity of eNOS is regulated by multiple post-transcriptional mechanisms involving a variety of factors including phosphorylation state [2, 3], active site cysteine modulation in oxygenase and reductase domains of eNOS [4-7], myristoylation/palmitoylation [8-10], and by protein:protein interaction including caveolin:eNOS association-dissociation [3, 5, 11, 12]. A family of three proteins, Caveolin-1,-2,-3, serve as marker proteins of caveolae, flask-shaped invaginations of plasma membranes with distinct lipid rafts enriched in cholesterol in various cell types [13-16]. In vascular endothelial cells, caveolin plays a critical role in the regulation of eNOS activity. For example, several studies have shown that in caveolae, caveolin:eNOS association or dissociation is linked with inhibition or stimulation, respectively, of the enzyme activity [4, 17-21]. Similarly, modulation of caveolin and/or caveolae lipid raft components has also been shown to alter the catalytic activity of eNOS [22, 23]. For example, lipid modulations such as acylation/deacylation of eNOS by myristic and palmitic acids have been shown to selectively target eNOS to cholesterol-rich caveolae microdomains or to the intracellular trans-Golgi complex [13, 14, 24]. Increased caveolin-1 phosphorylation promotes internalization of caveolae as well as dissociation of caveolae-associated enzymes including eNOS [25, 26]. Cholesterol modulation can compromise functional integrity of caveolae since caveolin is a cholesterol binding protein [27, 28]. The caveolae disruptors and/or cholesterol modulators such as methyl- β -cyclodextrin (CD), filipin, and cholesterol oxidase (CHOX) [8-31] have been shown to modulate activities of multiple enzymes in diverse cell types including vascular endothelial cells [13-15, 32-34].

We recently reported that an eleven amino acid (626-SSWRRKRKESS-636) synthetic peptide (P1) derived from the reductase domain of eNOS protein enhances the catalytic activity of eNOS, NO release, cGMP production, and NO/cGMP-dependent vasorelaxation in multiple models including isolated pulmonary artery segments and pulmonary artery endothelial cell (PAEC)/smooth muscle cell co-cultures and that P1 activation of eNOS is independent of expression and phosphorylation of eNOS protein [35]. The molecular events associated with P1-mediated activation of eNOS remain to be

determined. The primary focus of this study was to determine whether: i) P1-stimulated eNOS activation involves its dissociation from caveolae, phosphorylation (Tyr 14) of caveolin-1, and translocation to intracellular sites such as the Golgi complex (GC) or non-Golgi complexes (NGC), and ii) cholesterol modulating and/or disrupting agents CHOX and CD mimic the effect of P1 on eNOS dissociation, compartmentalization, and/or activation in lung PAEC.

Materials and Methods

Antibodies and reagents

Primary antibodies for immunoblot, immunofluorescence and immunogold labeled images were commercially obtained as mouse monoclonal anti-eNOS/NOS type III; rabbit polyclonal anti-caveolin (BD Transduction Laboratories, San Jose, CA); mouse anti p-caveolin-1 (Tyr 14) (BD Transduction Laboratories, San Jose, CA); rabbit polyclonal anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA); and rabbit polyclonal anti-mannosidase II (Abcam, Cambridge, MA). Secondary antibodies (peroxidase conjugated-, Rhodamine Red™-X-conjugated-, Fluorescein (FITC)-conjugated-donkey anti-mouse and donkey anti-rabbit IgG) were obtained from Jackson Immuno Research Laboratories (West Grove, PA), CD and *microbial* CHOX was obtained from Sigma Chemicals (St. Louis, MO). All other reagents were obtained from Bio-Rad Laboratories (Hercules, CA), Santa Cruz Biotechnology (Santa Cruz, CA), and Fisher Scientific (Orlando, FL).

Cell culture and treatment

PAEC were obtained by collagenase treatment from the main PA of 6 to 7 month-old pigs. Endothelial cells were propagated in monolayer culture as previously described [36]. Cells grown to confluence in 100-mm or 35-mm clear glass bottom dishes were used for cell fractionation, confocal microscopy, and electron microscopy. In each experiment, cells were studied 1 or 2 days after confluence at passages 3-5 and were matched for cell line, passage number, and days after confluence.

PAEC (8 X 100 mm dishes for each treatment) were incubated with or without (control) the presence of P1 (100 μ M), CHOX (0.1 U/mL), and CD (1 mM) in RPMI 1640 for 1 h at 37°C. After treatment cells were used for preparation of plasma membrane (PM), Golgi complex (GC), and non-Golgi complex (NGC) enriched fractions, immunofluorescence analysis, electron microscopy, and immunoprecipitation.

Preparation of PM, GC, and NGC enriched fractions

The cellular fractions (PM, GC and NGC) were prepared as previously described by Yang et al. [37] and Hu et al. [38] with slight modification. In brief, after incubation, cells were washed, scraped in 10 mM Tris-HCl, pH 7.4, centrifuged at 3000 rpm, and then resuspended in 2 ml of ice-cold homogenizing buffer (10 mM Tris-HCl, pH 7.4) containing protease inhibitor

cocktail set III (AEBSF, Hydrochloride 10 mM Aprotinin, Bovine Lung lyophilized 8 μ M, Bestatin 500 μ M, E-64 150 μ M, Leupeptin, Hemisulfate 200 μ M, Pepstatin A 100 μ M, Calbiochem). The cells were homogenized using a glass dounce homogenizer and lysed by gentle sonication with 15% output for 15 sec. The cell lysates were adjusted to 1.4 M sucrose solution by adding 2.55 M sucrose (prepared in 2 mM EDTA and 10 mM Tris-HCl, pH 7.4). A four step sucrose density gradient (1.6, 1.4, 1.2, and 0.8 M) was prepared and centrifuged for 2.5 h at 25,000 rpm in an SW-40 rotor (Beckman Coulter Optima, L-70K ultracentrifuge) at 4°C. Three fractions at the gradient interphase junctions of 0.8 M/ 1.2 M, 1.2 M/1.4 M and 1.4 M/1.6 M were collected as PM-, GC-, and NGC-enriched fractions, respectively (The purity of isolated fractions was assessed by immunoblot analysis using marker proteins and designated as PM-, GC-, and NGC-enriched fractions). The collected fractions were diluted with three volumes of homogenizing buffer and centrifuged at 100,000g for 1 h in a Ti-70 rotor (Beckman Coulter Optima, L-70K ultracentrifuge) at 4°C. The resulting pellets were resuspended in desired buffer as required for Western blots, enzyme assays, and protein concentration determination.

Immunoblotting analysis

Each PM-, GC- and NGC-enriched fractions (pellets) were solubilized in four equal volumes of PBS (pH 7.4), mixed with one volume of 5X loading buffer (Fermentas #R0891), vortexed, and boiled for 10 min. The equal volumes of protein fractions were loaded on gradient Gel 4-20%, and resolved in Criterion cell BIORAD at 200 volts for 50 min, followed by electroblotting in Criterion blotter BIORAD by transferring resolved protein onto nitrocellulose (NC) membranes at 100 volts for 60 min. The NC membranes were washed three times in Tris-Tween buffered saline; 0.1% Tween 20 (TTBS) and blocked by 5% non-fat dry milk (NFDm) in TBS for 1 h. The NC membranes were washed thrice with TTBS for 30 min, and the washed membranes were incubated overnight with primary antibody (anti-eNOS/NOS type III or rabbit polyclonal anti-caveolin or mouse anti p-caveolin-1(Tyr 14) or rabbit polyclonal anti-vimentin or rabbit polyclonal anti-mannosidase II) diluted in 1% NFDm-TBS as prescribed by suppliers. Prior to incubation with secondary antibody the NC membranes were washed thrice for 30 min in TTBS and incubated with desired horseradish peroxidase-conjugated secondary antibody diluted in 1% NFDm-TBS as prescribed by suppliers for 2 h. Proteins reactive with primary antibody were visualized with chemiluminescence technique using luminol reagents.

Immunofluorescence studies

To identify translocation of eNOS from PM to intracellular compartments (GC and NGC), fluorescence micrographs of PAEC were obtained by confocal microscopy. After incubation, cells were washed with 1 ml phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 2 h at 4°C or with 100% methanol (kept at -20°C) for 30 min at 4°C in the case of mannosidase II localization, washed with 1 ml 3X PBS, permeabilized with 0.1% Triton X-100 for 30 min at 4°C, and washed again with 3X PBS. Non

specific sites were blocked by incubation with 5% BSA in PBS for 1 h at 4°C. The cells were washed thrice with PBS, incubated overnight at 4°C with mouse anti-eNOS antibody (dilution 1:100) in PBS containing 1.5% BSA, pH 7.4, for single labeling and/or in combination with rabbit anti-vimentin antibody (dilution 1:200) or rabbit anti-mannosidase II antibody (dilution 1:200) for double labeling. After 2 h incubation at 4°C in the dark, cells were incubated with secondary antibody donkey anti-mouse IgG (labeled with FITC; dilution 1:100 for each) for single labeling and/or in combination with donkey anti-rabbit IgG (labeled with Rhodamine red; dilution 1:100 for each) for double labeling.

The cells were observed and analyzed at wavelength 488 nm for FITC and 543 nm for Rhodamine red labeling using a Zeiss LSM 510 inverted confocal microscope and images were captured through 100X oil immersion objective, keeping all the conditions of microscope and settings of software identical for stimulated (P1, CHOx, and CD) as well as for non-stimulated cells. The images were enhanced by increasing/decreasing same amount (%) of brightness and contrast from all exposures in each set.

Cell processing for electron microscopy

To identify and confirm the translocation of eNOS from the PM to intracellular compartments, after incubation, cells (2 x 100 mm dishes for each treatment) were scraped in PBS (pH 7.4), centrifuged at 3000 rpm, and the resulting pellet was preserved by immersion in 4% paraformaldehyde, 0.25 % glutaraldehyde in PBS overnight at 4°C as previously described [39]. The cell pellets were washed in PBS, treated with 0.1M NH₄Cl, then dehydrated in a graded series of ethanols and embedded in Lowicryl K4M (Electron Microscopy Sciences, Ft. Washington, PA). Lowicryl polymerization was carried out under ultraviolet light for 24 h at -20°C and then for 48 h at room temperature. Samples containing well-preserved cells were selected after light microscopic examination of 0.5 μ m-thick sections stained with toluidine blue. Ultrathin sections of the samples were mounted on Formvar/carbon-coated nickel grids for immunogold cytochemistry.

Immunogold labeling

The immunogold labeling of ultrathin sections on nickel grids was performed by floating the grids on droplets of solution at room temperature. In brief, the grids were exposed to 0.1 M NH₄Cl for 1 h, rinsed with PBS, treated with the blocking solution for 30 min, washed with the incubation buffer, and then incubated in a humidified chamber overnight at 4°C with either the affinity-purified primary mouse anti-eNOS antibody (dilution 1:100) or rabbit anti-caveolin antibody (dilution 1:200) in the incubation solution. The sections were washed and exposed for 1.5 h to goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies, respectively, conjugated to ultra small colloidal gold particles (Aurion Ultra Small, EM Grade, Electron Microscopy Sciences, Ft. Washington, PA) diluted in the incubation solution. The sections were washed with the incubation buffer, then with PBS, and postfixed with 1.25% glutaraldehyde in PBS. Postfixed sections were washed with PBS, distilled water, and silver enhanced by incubating sections

with agitation in Aurion SE-EM silver enhancement solution (Electron Microscopy Sciences) at room temperature for 45 min. After washing with distilled water, sections were counterstained with saturated uranyl acetate. The following solutions were used: an incubation solution, 0.2% acetylated BSA (Aurion BSA-c, Electron Microscopy Sciences) and 10 mM NaN₃, in PBS, pH 7.4, and a blocking solution, 5% BSA, 0.1% cold-water fish-skin gelatin, and 5% normal goat serum, in PBS.

Ultrathin sections were examined using a Zeiss EM10A or Hitachi 7600 transmission electron microscope, the images were magnified from 10,000X to 25,000X, and cells with well defined structural integrity were chosen to count the gold particles in each grid. The gold particles found on the peripheral areas of cells were considered associated with the PM caveolar proteins, and the gold particles present away from the peripheral margins and localized towards the center of the cells in the perinuclear area were considered present in the cytoplasmic matrix. Gold particles were counted manually using two sets of grids for each treatment.

Immunoprecipitation Assay

To confirm eNOS/caveolae dissociation, cells (10 X 100 mm dishes for each treatment) were scraped, centrifuged at 3000 rpm for 10 min at 4°C, and pellets were resuspended in 1 mL of cold lysis buffer (50mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% NP-40) in 1.5 mL centrifuged tubes. After incubation on ice for 1h with occasional mixing, cells were centrifuged at 10000 x g for 10 min at 4°C, supernatants were collected and divided into two sets (450 µl each), and 50 µl of washed Protein A slurry (washed thrice in 450 µl lysis buffer at 10000 x g for 10 min at 4°C) was added and incubated on ice for 30 min with occasional mixing. After incubation, lysates were centrifuged at 10000 x g for 10 min at 4°C, and supernatants were collected carefully without disturbing the pellets. One set of precleared cell lysates was immunoprecipitated overnight at 4°C with 10µg of rabbit-anti-caveolin antibody and another set with 10 µg of rabbit-anti eNOS. After incubation, Protein A slurry was added to immunoprecipitated cell lysates, incubated on ice for 1h with occasional mixing, and then centrifuged at 10000 x g for 10 min at 4°C. Supernatant were then discarded, and 100µL of SDS-PAGE sample buffer was added to the saved pellets, heated to 100°C, and centrifuged at 10000xg for 5 min. Supernatants containing equal amounts (5 µg) of eNOS and caveolin immunoprecipitated proteins representing total eNOS and caveolin-associated eNOS, respectively, were fractionated by SDS-PAGE and immunodetected using mouse anti-eNOS antibody. Immunoblots for both, total eNOS protein as well as for caveolin-associated eNOS protein were developed using the same gel, transferred to NC membranes, and visualized by chemiluminescence. The optical densities (ODu mm⁻²) of the blots were measured, and the ratio of caveolin- associated eNOS to total eNOS was determined.

To evaluate P1-mediated phosphorylation of caveolin-1 (Tyr 14) in PAEC, the cells (10 X100 mm dishes) were treated with and without P1 for 1h at 37°C, and cell lysate proteins were immunoprecipitated with rabbit anti-caveolin antibody and immunoblotted for mouse anti-p-caveolin-1

(Tyr 14) antibody as discussed above. The relative optical densities (ODu mm⁻²) of the blots were measured.

Measurement of eNOS activity

eNOS activity was measured by monitoring the formation of L-[3H]citrulline from L-[3H] arginine in PM-, GC-, and NGC-enriched fractions isolated from PAEC treated with or without the presence of P1, CHOX, or CD. The membrane fraction proteins (100–200 µg each) were incubated in 0.4 ml of Tris HCl buffer containing 1 mM NADPH, 100 nM calmodulin, 10 µM tetrahydrobiopterin, and 5 µM combined L-arginine and purified L-[3H]arginine for 30 min at 37°C [40]. Purification of L-[3H]arginine and measurement of L-[3H]citrulline formation were carried out as previously described [40].

Statistical analysis

The data were subjected to statistical analysis and expressed as mean ± S.E.M. (Standard Error of Mean). Significance for the effect of P1 on eNOS activity, and the difference between optical densities of immunoblots of PAEC fractions were determined by ANOVA and a Student's paired *t*-test. A value of *p* <0.05 was considered statistically significant [41].

Results

Identification of PM-, GC-, and NGC-enriched fractions in PAEC

Figure 1 shows sucrose density gradient analysis of PM-, GC-, and NGC-enriched fractions isolated from control PAEC. To determine relative purity of PM and GC fractions, Western blot analysis was performed using specific protein marker antibodies for caveolin-1 (PM) and mannosidase II (GC), respectively. Anti-vimentin antibody as an intracellular protein marker was used to characterize the NGC fraction. Immunoblot and densitometric analyses in Fig. 1, panels A and B, show high levels of PM marker protein caveolin-1 and GC marker protein mannosidase II, respectively, whereas panel C shows relatively high levels of vimentin as an intracellular protein marker in NGC followed by GC fractions but not in PM fraction.

P1, CHOX, and CD-stimulation translocate eNOS to intracellular compartments in PAEC

Figure 2A shows representative Western blots of eNOS protein levels in PM, GC, and NGC fractions isolated from control, P1-, CHOX-, and CD-stimulated PAEC. Because Western blots for each treatment represent independent experiments, densitometric analysis of blots was performed by measuring the relative density ratios of GC/PM and NGC/PM spots where PM

Fig. 1. Identification of plasma membrane (PM), Golgi complex (GC), and non-GC (NGC) enriched fractions. PM, GC, and NGC fractions were isolated from control cells by sucrose density gradient as described in Methods. Relative purity of PM, GC, and NGC fractions was determined by Western blot analysis using selective marker proteins, anti caveolin (panel A) for PM, anti-mannosidase II (panel B) for GC, and intracellular marker anti-vimentin (panel C) for GC and NGC proteins. Densitometric analysis of two separate Western blots is shown in the respective panels A, B, and C as % specific Optical Density unit (ODu) mm⁻².

Fig. 2. Effect of P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulation on compartmentalization of eNOS in PAEC. Cells were incubated with or without (control, CON) P1 (100 μ M), CHOX (0.1 U/mL), and CD (1 mM) for 1 h. After treatment, plasma membrane (PM), Golgi complex (GC), and non-GC (NGC) fractions were isolated as described in Methods. Panel A shows representative Western blots of eNOS protein levels in PM, GC, and NGC fractions from CON, P1, CHOX, and CD-stimulated cells. Densitometric analysis of three separate blots in each treatment group was performed using relative ratios of PM to GC and NGC with PM = 1 (panel B) and percent density of each spot in specific treatment group (panel C) as described in Methods. * $P < 0.05$, and # $P < 0.05$ vs respective fraction in CON (panel B). * $P < 0.05$ vs PM in CON; ** $P < 0.05$ vs GC, and # $P < 0.05$ vs NGC in CON (panel C). Data are the means $\pm$ SEM of 3 separate experiments

density = 1 (panel B). Relative levels of eNOS determined by density ratio indicate increased translocation of eNOS to GC and NGC fractions of P1-stimulated cells and to the GC fraction of CHOX and CD-treated cells. Panel C shows densitometric analysis as a percent density of

individual spots in each treatment group. P1, CHOX, and CD treatments resulted in reduction of eNOS protein content in PM fractions with associated increase of eNOS protein levels in NGC of P1-stimulated cells and in GC of CHOX and CD-stimulated cell fractions.

Fig. 3. Effect of P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulation on the catalytic activity of compartmentalized eNOS. Cells were incubated with or without (control, CON) P1 (100 μ M), CHOX (0.1 U/mL), and CD (1 mM) for 1 h. After treatment, plasma membrane (PM), Golgi complex (GC), and non-GC (NGC) fractions were isolated and eNOS activity was determined as described in Methods. * $P < 0.05$, and ** $P < 0.05$ vs respective fractions in CON; # $P < 0.05$ vs GC fraction in P1, and CHOX. Data represent mean $\pm$ SEM ($n = 4$) for each fraction and treatment group.

Fig. 4. Immunofluorescence localization of eNOS in P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulated PAEC. Cells were incubated with or without (control, CON) P1 (100 μ M), CHOX (0.1 U/mL), and CD (1 mM) for 1 h. After incubation, cells were immuno-labeled using mouse anti-eNOS antibody (1:100 dilution) followed by secondary FITC labeled donkey anti-mouse IgG antibody as described in Methods. The images were captured through 100x oil immersion objective using Zeiss LSM 510 inverted confocal microscope. Immunofluorescence image shows localization of eNOS (arrows) in the PM region of control cells (CON) whereas P1, CHOX, and CD-stimulation enhanced compartmentalization of eNOS to perinuclear region (arrow heads) in PAEC.

Fig. 5. Immunogold localization of eNOS and caveolin in P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulated PAEC. Immunogold labeling of ultrathin sections on nickel grids was performed and sections were incubated with affinity purified primary mouse anti-eNOS (1:100 dilution, panel A) and rabbit anti-caveolin (1:200 dilution, panel B) antibodies, followed by secondary goat anti-mouse IgG and goat anti-rabbit IgG antibodies, respectively, conjugated with ultra small colloidal gold particles as described in Methods. Immunogold labeling shows localization of caveolin and eNOS in PM/caveolae region (arrows) in control (CON) cells. P1, CHOX, and CD-stimulation resulted in increased migration of caveolin and eNOS to perinuclear compartments (arrow heads) in PAEC.

P1-stimulation enhances the catalytic activity of eNOS in PM and GC fractions

To determine whether P1-mediated increased catalytic activity of eNOS is associated with its

A	Number of cells counted	Number of eNOS-labeled gold particles	
Treatment	(Total gold particles)	Peripheral region	Perinuclear region
Non-Stimulated	36 (1651)	708 (42.88%)	943 (57.12%)
P1-Stimulated	27 (1063)	386 (36.31%)	677 (63.69%)
CHOX-Stimulated	26 (956)	309 (32.32%)	647 (67.68%)
CD-Stimulated	32 (1201)	288 (23.98%)	913 (76.02%)
B	Number of cells counted	Number of caveolin-labeled gold particles	
Treatment	(Total gold particles)	Peripheral region	Perinuclear region
Non-Stimulated	25 (2040)	937 (45.93%)	1103 (54.07%)
P1-Stimulated	23 (1402)	591 (42.15%)	811 (57.85%)
CHOX-Stimulated	18 (1251)	469 (37.49%)	782 (62.51%)
CD-Stimulated	21 (1438)	416 (28.93%)	1022 (71.07%)

Table 1. Quantitative Analysis of EM Micrographs for P1, CHOX, and CD-Stimulated Translocation of eNOS and Caveolin from Peripheral to Perinuclear Compartment in Lung EC. Cell monolayers were treated with P1 (100 μ M), CHOX (0.1 U/ml, and CD (1 mM) in RPMI 1640 for 1 h. Immunogold labeling of ultrathin sections on nickel grids was performed and sections were incubated with affinity purified primary mouse anti-eNOS (1:100 dilution) and rabbit anti-caveolin (1:200 dilution) antibodies, followed by the secondary goat anti-mouse IgG and goat anti-rabbit IgG antibodies, respectively, conjugated with ultra small colloidal gold particles as described in Methods. eNOS (A) and caveolin (B) immuno-gold-labeled particles were counted in morphologically intact cells with defined peripheral and perinuclear regions.

translocation to intracellular compartments and whether the caveolae/cholesterol modulators CHOX and CD mimic the effects of P1, we examined the catalytic activity of eNOS in PM, GC, and NGC fractions isolated from P1, CHOX, and CD treated cells. As shown in Fig. 3, the catalytic activity of eNOS was significantly elevated in PM fractions from P1- and CHOX-stimulated cells and in GC fractions of P1, CHOX, and CD-stimulated cells. Despite increased translocation of eNOS protein from PM to GC/NGC fractions of P1 and GC fraction of CHOX- and CD-stimulated cells (Fig. 2), the increased level of eNOS activity was much greater in PM than in GC and/or NGC fractions of P1 and CHOX-stimulated cells.

Immunofluorescence analysis indicates P1, CHOX, and CD-stimulation translocated eNOS to intracellular compartments

Immunofluorescence micrographs using anti-eNOS antibody (Fig. 4) show localization of eNOS in PM (arrows) and intracellular compartments (arrow heads). Relatively dominant localization of eNOS was observed in the PM of control cells. Intense immunostaining of eNOS in the perinuclear region associated with reduction of eNOS in PM of P1, CHOX, or CD-stimulated cells indicates translocation of eNOS from PM to intracellular compartment.

Electron microscopy indicates P1, CHOX, and CD-disruption of caveolae are associated with compartmentalization of eNOS in PAEC

To demonstrate whether P1-mediated caveolae/cholesterol disruption is involved in translocation of eNOS to the intracellular compartment, the effects of known caveolae/cholesterol disrupting agents CD/CHOX, respectively, were examined using transmission electron microscopy and immunogold cytochemistry of caveolin and eNOS in P1, CHOX, and CD-stimulated cells. Fig. 5 shows electron micrographs of immunogold-labeled eNOS (panel A) and caveolin (panel B), a caveolae marker protein in PAEC. eNOS and caveolin proteins are localized to the peripheral region representing the presence of caveolae in PAEC. In contrast, stimulation of cells with P1, CHOX, or CD resulted in translocation of eNOS and caveolin from peripheral to perinuclear compartments. Quantitative analysis of eNOS and caveolin gold-labeled particles confirmed increased translocation of eNOS and caveolin from peripheral to perinuclear compartments of P1, CHOX, and CD-stimulated cells (Table 1).

P1-, CHOX, and CD-stimulation result in eNOS compartmentalization in PAEC

To confirm whether P1, CHOX, and CD-mediated caveolae modulation is associated with eNOS translocation

Fig. 6. Confocal images of P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulation and intracellular localization of eNOS to GC compartment in PAEC. Cells were incubated with or without (control, CON) P1 (100 μ M), CHOX (0.1 U/ml), and CD (1 mM) for 1 h. After incubation, cells were immuno-labeled singly using mouse anti-eNOS antibody (1:100 dilution) or in combination with rabbit anti-vimentin (1:200 dilution) (panel A) or rabbit anti-mannosidase II (1:200 dilution) (panel B) antibodies followed by secondary FITC labeled donkey anti-mouse IgG antibody (1:100 dilution) for single labeling and with Rhodamine red-labeled donkey anti-rabbit IgG (1:100 dilution) as described in Methods. The images were captured through 100x oil immersion objective using Zeiss LSM 510 inverted confocal microscope. Immuno-fluorescence image shows localization of eNOS/vimentin, and eNOS/mannosidase II. In each panel arrows indicate localization to plasma membrane and arrow heads indicate perinuclear region in the PAEC.

in intracellular compartments, confocal immunofluorescence microscopy was performed using intracellular (NGC) and GC selective marker proteins vimentin- and mannosidase-II-specific antibodies, respectively. As shown in Fig. 6 panel A and B, eNOS (green) is localized to peripheral or PM region (arrows) and vimentin and/or mannosidase-II (red) is more localized to intracellular compartments (arrow heads). Merge images of eNOS and vimentin and/or mannosidase-II indicate more selective co-localization of eNOS in the intracellular compartment of control cells. In contrast, P1, CHOX, and CD-stimulation resulted in reduction of peripheral and increased intracellular eNOS immuno labeling in the perinuclear compartment. Vimentin was used as an additional intracellular marker protein to confirm the P1, CHOX, and CD-mediated effect on

eNOS translocation and same has been shown by enhanced colocalization of eNOS with vimentin in Fig. 6 panel A, where P1, CHOX, and CD-stimulation enhanced translocation of eNOS to intracellular compartments. To demonstrate more selective localization of eNOS in the sub-intracellular compartment, anti-mannosidase-II-specific antibody was used as a GC marker. Fig. 6 panel B shows P1, CHOX, and CD-mediated increased translocation of eNOS to the intracellular compartment which, for the most part, is co-localized with GC.

P1, CHOX, and CD-stimulation dissociate eNOS from caveolin in PAEC

To confirm whether translocation of eNOS to intracellular compartments (Fig. 4) is due to eNOS/caveolin dissociation after P1, CHOX, and CD-

Fig. 7. Immunoblots of immunoprecipitated proteins by caveolin and eNOS antibodies shows dissociation of eNOS from caveolin in P1, cholesterol oxidase (CHOX), and methyl- β -cyclodextrin (CD)-stimulated PAEC. The cells were stimulated with and without P1, CD and CHOX and cell lysates were immunoprecipitated with rabbit anti-eNOS and rabbit anti-caveolin antibodies as discussed in Material and Methods. Proteins immunoprecipitated by eNOS and caveolin antibodies were fractionated by SDS-PAGE and probed with eNOS antibody. Panels A and B show immunoblots of eNOS- and caveolin-immunoprecipitates probed with mouse-anti-eNOS antibody representing total eNOS and caveolin-associated eNOS, respectively. Panel C shows the ratio of optical densities of caveolin associated eNOS to the total eNOS for control (Con), P1, CHOX and CD-stimulated PAEC.

stimulation, immunoprecipitations were performed using anti eNOS and anti-caveolin antibodies as described in Methods section. As shown in Fig. 7 panel A, total eNOS content is comparable in control and P1, CHOX, and CD-stimulated cells. In contrast, caveolin associated eNOS content (panel B) was reduced in P1, CHOX, and CD-stimulated cells compared to control. Densitometric analysis of Western blots determined by ratio of caveolin-associated eNOS to total eNOS indicates significant reduction in caveolin-associated eNOS in P1, CHOX, and CD-stimulated cells (panel C).

P1 stimulation enhanced phosphorylation of caveolin-1 (Tyr 14) in PAEC

Since increased phosphorylation of caveolin-1 can enhance caveolae/eNOS dissociation and activity of eNOS [25, 26], we examined whether P1-stimulation promotes phosphorylation (Tyr 14) of caveolin-1 in intact cells and compared this to the P1-mediated phosphorylation of caveolin-1 in isolated PM, GC, and NGC fractions. As shown in Fig. 8 (panel A), total caveolin content in caveolin-immunoprecipitated control and P1-stimulated intact cells is comparable. In contrast, p-caveolin-1 content was increased in P1-stimulated cells compared to control cells. Densitometric analysis of Western blots indicates increased p-caveolin-1 content in P1-stimulated cells. Fig. 8 (panel B) shows p-caveolin-1 content in PM, GC, and NGC fractions isolated from control and P1-stimulated cells. Western blots show localization of p-caveolin-1 contents in GC and NGC but not in PM fractions of both the control and P1-stimulated cells. However, P1-stimulation increased p-caveolin-1 content in the GC fraction but not in the NGC fraction compared to respective fractions from controls. Densitometric analysis of Western blots indicate

Fig. 8. Immunoblots of caveolin-immunoprecipitated PAEC (panel A) and isolated plasma membrane (PM), Golgi complex (GC), and non-GC (NGC) enriched fractions (panel B) show phosphorylation of Cav-1 (Tyr 14). Cells stimulated with or without P1 were used for immunoprecipitation with anti-caveolin antibody or for isolation of PM, GC, and NGC enriched fractions as described in Materials and Methods. Proteins immunoprecipitated by caveolin antibody and PM, GC, and NGC fraction proteins were fractionated by SDS-PAGE and probed with anti-p-Cav-1 (Tyr 14) antibody. Panels A shows representative Western blots of caveolin-immunoprecipitates probed with anti-p-Cav-1 antibody representing p-Cav-1 (Tyr 14) and total caveolin from two separate experiments. Panel B shows representative Western blots of two separate experiments PM, GC, and NGC fractions probed with anti-p-Cav-1 (Tyr 14) antibody. Densitometric analyses of two separate blots shown in panels A and B were performed using the relative optical densities of p-Cav-1 (Tyr 14) for control (CON) or P1-stimulated PAEC or PM, GC, and NGC fractions, respectively.

p-caveolin-1 content in GC but not NGC fractions from P1-stimulated cells.

Discussion

The primary findings of the present study demonstrate that P1-mediated increased catalytic activity of eNOS is associated with change in caveolae/cholesterol dynamics, increased phosphorylation of caveolin-1 (Tyr 14), and the translocation of eNOS to intracellular compartments in PAEC. P1-stimulation resulted in differential translocation of eNOS protein between the PM and intracellular compartments of PAEC. Western blot analysis indicated that P1-mediated decrease in eNOS protein content in the PM fraction was associated with increased localization of eNOS to GC and NGC fractions and increased phosphorylation of caveolin-1 (Tyr 14). P1-stimulation enhanced the catalytic activity of eNOS in both the PM and GC fractions. However, despite reduction of eNOS in the PM fraction and increased translocation to the GC compartment, the catalytic activity of eNOS was greater in the PM than in the GC fraction of P1-stimulated PAEC. Our results demonstrate that P1-stimulated translocation and activation of eNOS was mimicked by the caveolae-cholesterol modulator CHOX more than the caveolae disrupting agent CD.

Previous *in vivo* and *in vitro* studies have suggested the existence of two distinct pools of eNOS localized to the PM or caveolar region and the perinuclear or GC region in vascular endothelial cells [42]. A wide range of external stimuli including agonists, shear stress, and hormones are known to influence translocation of eNOS from the PM/caveolae region to the perinuclear/GC compartments via multiple mechanisms including modulation of calcium homeostasis, myristoylation/palmitoylation, and increased phosphorylation of eNOS and caveolin-1 (Tyr 14) leading to dissociation of eNOS from its negative regulator caveolin in endothelial cells [42-45]. The results of the present study using multiple techniques including cell fractionation, intact cell immunofluorescence, and electron microscopy demonstrate that the change in caveolae-cholesterol dynamics plays a critical role in P1-mediated translocation and activation of eNOS in PAEC. Increased eNOS/caveolin dissociation and translocation of eNOS from its peripheral location to the intracellular GC compartment in isolated fractions from P1, CHOX and CD-stimulated cells is supported by confocal and electron microscopy

images and immunoprecipitation in intact cells. Despite increased translocation of eNOS to the GC compartment, the catalytic activity of eNOS in the GC fraction was limited due to increased translocation of its negative regulator caveolin and/or phosphorylated-caveolin-1 (Tyr 14) to the GC compartment. This accounts for why the catalytic activity of eNOS remaining in the PM was greater than in the GC despite translocation of eNOS from the PM to the GC. P1-, CHOX-, and CD-mediated increased translocation and activity of eNOS appear to be linked with changes in caveolae/cholesterol dynamics. Since caveolae contain >4-fold more cholesterol content than the surrounding PM in human cells [46] and since caveolin is a cholesterol binding protein [27, 28], the integrity of caveolae and its associated proteins is dependent on cholesterol content. For example, oxidation and/or modulation of cholesterol or disruption of caveolae are known to compromise caveolar integrity and associated protein function [31].

The catalytic activity of eNOS is regulated by multiple factors including the expression and phosphorylation of eNOS protein [2, 3, 4]. P1-mediated translocation and activation of eNOS appears to be unique from other known activating mechanisms. For example, P1-mediated activation of eNOS is independent of eNOS expression and/or phosphorylation [6]. CHOX-mediated modulation of cholesterol mimics the effect of P1-mediated increased translocation of eNOS to the GC fraction and activation of eNOS in the PM and GC fractions. Although the caveolae disrupting agent CD also increased translocation of eNOS to the GC fraction, increased catalytic activity was observed only in the GC fraction. The effects of CHOX and CD are distinctly different on caveolae/cholesterol dynamics. CHOX through oxidation of cholesterol most likely modulates the caveolin/eNOS interaction since caveolin is a cholesterol binding protein without affecting caveolar integrity [31]. In contrast, CD is known to deplete cholesterol by disrupting caveolae structure [32].

The precise molecular events associated with P1- and CHOX-mediated enhanced catalytic activity of PM associated eNOS remain to be determined. However, the increased catalytic activity of eNOS in PM fraction despite reduction in PM associated eNOS protein level in P1- and CHOX-stimulated cells suggests changes in caveolin/cholesterol association and/or modulation of caveolin:eNOS interaction and/or increased translocation of phosphorylated Cav-1 (Tyr 14) from caveolae may contribute to increased catalytic activity. The precise mechanism of P1-mediated modulation of caveolae/

cholesterol modulation, phosphorylation of Cav-1 (Tyr 14), and eNOS activation remains to be determined. However, P1-mediated translocation and activation of eNOS to distinct cellular compartments may be physiologically significant. For example, a recent report suggests that translocation of eNOS and its functional responses were distinctly different in dermal microvascular endothelial cells stimulated with platelet-activating factor (PAF) and acetylcholine (Ach) primarily due to differential activation of PAF- and Ach-stimulated signaling events [45]. The results of our study indicate an additional mechanism by which external stimuli modulate eNOS compartmentalization and selective activation of eNOS via modulation of caveolae/cholesterol dynamics.

Acknowledgements

We thank Mr. Humberto Herrera and Lin Ai for their excellent tissue culture and technical assistance. We also thank the staff of the University of Florida College of Medicine Electron Microscopy Core Facility, particularly Dr. Jill Verlander Reed and Ms. Wencui Zheng, for advice and expert technical assistance in performing the immunogold studies. This work is supported in part by a Department of Veterans Affairs Merit Review proposal and by National Heart, Lung, and Blood Institute grants HL-85113 and HL-68666 (to J.M.Patel).

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