Dependence of Golgi apparatus integrity on nitric oxide in vascular cells: implications in pulmonary arterial hypertension

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Lee JE, Patel K, Almodóvar S, Tuder RM, Flores SC, Sehgal PB. Dependence of Golgi apparatus integrity on nitric oxide in vascular cells: implications in pulmonary arterial hypertension. Am J Physiol Heart Circ Physiol 300: H1141–H1158, 2011. First published January 7, 2011; doi:10.1152/ajpheart.00767.2010.—Although reduced bioavailability of nitric oxide (NO) has been implicated in the pathogenesis of pulmonary arterial hypertension (PAH), its consequences on organellar structure and function within vascular cells is largely unexplored. We investigated the effect of reduced NO on the structure of the Golgi apparatus as assessed by giantin or GM130 immunofluorescence in human pulmonary arterial endothelial (HPAECs) and smooth muscle (HPASMCs) cells, bovine PAECs, and human EA.hy926 endothelial cells. Structure was also investigated in cells in tissue sections of pulmonary vascular lesions in idiopathic PAH (IPAH) and in macaques infected with a chimeric simian immunodeficiency virus containing the human immunodeficiency virus (HIV)-nef gene (SHIV-nef) with subcellular three-dimensional (3D) immunoinaging. Compounds with NO scavenging activity including 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), methylene blue, N-acetylcysteine, and hemoglobin markedly fragmented the Golgi in all cell types evaluated as did monocrotaline pyrrole, while L-arginine substrate, or enzymatic “uncoupling” of NADPH dynamin 2, and was accompanied by depletion of α-soluble N-ethylmaleimide-sensitive factor (NSF) acceptor protein (α-SNAP) from Golgi membranes and codispersal of the SNAP receptor (SNARE) Vti1a with giantin. Golgi fragmentation was confirmed in endothelial and smooth muscle cells in pulmonary arterial lesions in IPAH and the SHIV-nef-infected macaque with subcellular 3D immunoinaging. In SHIV-nef-infected macaques Golgi fragmentation was observed in cells containing HIV-nef-bearing endosomes. The observed Golgi fragmentation suggests that NO plays a significant role in modulating global protein trafficking patterns that contribute to changes in the cell surface landscape and functional signaling in vascular cells.

nitric oxide scavenging; Golgi fragmentation; subcellular three-dimensional immunoimaging; giantin; GM130; p115; α-soluble N-ethylmaleimide-sensitive factor acceptor protein; Vti1a; endothelial nitric oxide synthase mislocalization

IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION (IPAH) is a progressive disease with high morbidity and mortality (57, 69, 71). PAH is characterized by vascular remodeling that results in the formation of proliferative, obliterative, and plexiform vascular lesions packed with enlarged and vacuolated endothelial and smooth muscle cells resistant to apoptosis (57, 64, 69, 71). IPAH as well as PAH with several etiologic origins are now recognized: BMPRII mutations in familial PAH, hypoxia, human immunodeficiency virus (HIV) infection, scleroderma, and other autoimmune diseases, sickle-cell anemia, ingestion of plant alkaloids [now incorporated into the widely used monocrotaline (MCT)/rat model of PAH], and ingestion of anorexic agents (18–20, 30, 57, 69, 71). The disease involves changes in the cell surface landscape of pulmonary vascular cells as reflected in alterations of multiple cell surface receptors, cell surface signaling molecules and pathways, cytokine and growth factor secretion, enhanced prothrombogenicity of the luminal surfaces, smooth muscle cell migration, and the chemotactic infiltration of immune-competent cells (28, 33, 57, 64, 71, 75). Over the years different investigators have emphasized one or another mechanistic aspect in the pathogenesis of PAH (29, 30, 33, 57, 69, 71, 75). Largely missing from prior studies is an attempt to understand how changes in subcellular organellar and membrane trafficking pathways may provide a unified view about the pathogenesis of this disease (28, 64).

Numerous studies have implicated decreased bioavailability of nitric oxide (NO) and/or a decreased responsiveness to NO in the pathogenesis of PAH (18–20, 78). NO has been investigated in terms of its effects on pulmonary arterial vasodilation, inhibition of smooth muscle cell proliferation and migration, and antiplatelet effects (reviewed in Ref. 78). As summarized by Zuckerbraun et al. (78), decreased bioavailability of NO in PAH may occur secondary to impaired formation [due to reduced endothelial nitric oxide synthase (eNOS) levels, reduced l-arginine substrate, or enzymatic “uncoupling” of eNOS] or increased consumption [by reactive oxygen species (ROS) and NO scavengers such as hemoglobin (Hb)]. Inhalation of NO gas or nebulized sodium nitrite (which generates intravascular NO) has been used as a modality of PAH therapy in the clinic and in experimental models (4, 20, 78). However, the subcellular structural changes elicited in human pulmonary vascular cells due to reduced NO bioavailability and the amelioration of such defects in response to NO donors remain largely unexplored.

In mammalian cells the juxtanuclear Golgi apparatus (6, 13, 21, 42, 51) commonly consists of multiple stacks of three to eight flattened cisternae interconnected by reticulotubular membranes (the “Golgi ribbon”). Cajal reported in 1914 that there was one compact Golgi apparatus per cell in endothelial cells (see Figs. 6 and 7 in Ref. 6). Cajal also recognized that hypoxia leads to Golgi fragmentation in neuronal cells (see Fig. 50 in Ref. 6). In 2007, we confirmed this effect of hypoxia on the Golgi apparatus in bovine pulmonary arterial endothelial
cells (BPAECs) in culture (46). Although recent investigators have studied the association of the cytosolic protein eNOS with the cytosolic face of Golgi membranes upon myristoylation and modulation of the NO-generating function of eNOS upon phosphorylation, trafficking to the plasma membrane, and interactions with caveolin-1 and heat shock protein 90 (14, 24, 55), the contribution of NO per se to the integrity of the structure and function of the Golgi ribbon is largely unexplored.

In previous studies we reported that BPAECs in culture exposed to MCT pyrrole (MCTP), hypoxia, or NO scavenging developed an enlarged circumnuclear Golgi apparatus accompanied by an increase in cell size or “megalocytosis” (27, 43, 46, 65, 66). This phenotype was also characterized by sequestration of vesicle tethers, various soluble N-ethylmaleimidesensitive factor (NSF) acceptor protein receptor (SNARE) proteins, and α-soluble NSF acceptor protein (α-SNAP) in the enlarged Golgi membranes, the cytoplasmic mislocalization of eNOS, and a hypo-NO state (27, 43, 46, 65, 66). Furthermore, we confirmed Golgi dysfunction in pulmonary arterial endothelial and smooth muscle elements in the MCT/rat model (65) and in the proliferative, obliterative, and plexiform lesions in IPAH (62). In the MCT/rat model loss of plasma membrane raft caveolin-1 and Golgi fragmentation were evident in pulmonary arterial endothelial cells (PAECs) within 4 days, i.e., at a time prior to development of PAH, which usually takes 10–14 days (38, 66). Moreover, increased amounts of the Golgi tethers/scaffolding proteins giantin and p115 were observed in cellular elements in IPAH and in PAH-like pulmonary arterial lesions in macaques infected with the recombinant chimeric simian immunodeficiency virus (SIV) containing the HIV-nef gene (SHIV-nef) (64). The overall hypothesis is that dysfunctions in Golgi-dependent trafficking underlie the development of pulmonary vascular lesions and that the global alterations in the cell surface landscape are caused by defects in proteins that mediate intracellular vesicular trafficking (the tethers, SNAREs, SNAPs, and NSF) (61–63). Such disruptions would impair delivery of vasorelevant receptors to the vascular cell surface and lead to defects in endosome/caveosome-associated inward signaling by multiple different vasoactive ligands as well as affecting cellular entry into mitosis, cell proliferation, cell and nuclear size, and cell migration (28, 61a–64).

In the present study we used immunofluorescent tagging of Golgi tethers/scaffolding proteins (giantin, GM130, or p115 in different experiments) together with quantitative objective image analysis techniques to investigate the interplay between NO and the integrity of the Golgi apparatus in human pulmonary vascular cells (endothelial and smooth muscle), in an immortalized human umbilical vein endothelial cell line (EA.hy926) in culture, as well as in BPAECs. Additionally, we investigated the occurrence of the Golgi fragmentation signature in single cells in tissue sections of pulmonary vascular lesions in IPAH and in the SHIV-infected macaque model, using subcellular three-dimensional (3D) immunomapping methods.

**MATERIALS AND METHODS**

**Chemical reagents.** 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide (c-PTIO) was purchased from BioMol International (Plymouth Meeting, PA), methylene blue (MB) from Fisher Scientific (Fair Lawn, NJ), and sildenafil citrate from Santa Cruz Biotechnology (Santa Cruz, CA), while MCT, diethylamine NONOate sodium salt hydrate (NONOate), cytotoxic αglobin (Hb), ferrous Hb, N-acetylcysteine (NAC), Tempol, imidrubic E804, dynasore, paclitaxel, fasudil, and Y-27632 were all from Sigma-Aldrich (St. Louis, MO) and H2O2 from J. T. Baker (Phillipsburg, NJ). MB and Hb stock solutions were held under a stream of nitrogen for 15 min just before their use. MCTP was prepared from MCT by the method of Mattocks et al. (40) as described by us previously (43–46, 65, 66).

**Pulmonary vascular and EA.hy926 cells in culture.** Primary human pulmonary arterial endothelial (HPAECs) and smooth muscle (HPASMCs) cells were purchased from Clonetics (San Diego, CA). Both were seeded into T-25, T-75, or six-well plates coated with fibronectin, collagen, and bovine serum albumin (1 µg/ml, 30 µg/ml, and 10 µg/ml, respectively in coating medium) (2). HPAECs were grown in medium 200 supplemented with low-serum growth supplement (LSGS; Cascade Biologics, Carlsbad, CA) and were used between passages 4 and 10. HPASMCs were grown in medium 231 containing smooth muscle growth supplement (SMGS; Cascade Biologics). For differentiation, HPASMC cultures were switched to medium 231 supplemented with smooth muscle differentiation supplement (SMDS; Cascade Biologics). HPAECs were verified by immunofluorescence assays to be positive for expression of von Willebrand factor (vWF) and negative for smooth muscle α-actin (SMA); conversely, HPASMCs were verified to be vWF negative and SMA positive (data not shown). Primary BPAECs were used as previously described (28, 43–46, 65, 66). EA.hy926 cells, derived from primary human umbilical vein endothelial cells immortalized by fusion with A549 cells (10, 11) were obtained from Dr. Michel Schwartzman (Dept. of Pharmacology, New York Medical College) and were grown in DMEM supplemented with 10% (vol/vol) fetal bovine serum and 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (HAT). EA.hy926 cells remain vWF positive and display functional characteristics of endothelial cells over long-term passage (10, 11). HPAECs and BPAECs were plated respectively in coated or uncoated six-well plates (3–4 × 104 cells/well) and allowed to recover for 2 days before experimental exposures. HPASMCs were plated in coated six-well plates (3–4 × 104 cells/well) in growth medium for 1 day and switched to differentiation medium for 2 days before experimental exposures. EA.hy926 cells were handled similar to BPAECs. Reagents were added at the indicated concentrations and combinations described in figures with daily replenishment. MCTP in dimethylformamide was added once directly into the culture medium with a rapid swirling motion, and cultures were refreshed with MCTP-free medium daily thereafter. Phase contrast microscopy was carried out daily and at the conclusion of each experiment with a Nikon Diaphot Microscope and a Nikon Coolpix digital camera.

**Small interfering RNA transfections.** Small interfering RNA (siRNA) to human NOS3 (sc-36093) and control scrambled siRNA-A (sc-36093) were purchased from Santa Cruz Biotechnology and used to transfect HPAECs in six-well plates according to the manufacturer’s protocol. Cells were first transfected 1 day after plating, the transfection was repeated 3 days after plating, and the cultures were evaluated 5 days after plating (i.e., ~96 h after initial siRNA transfection). Side-by-side cultures from the same six-well plate were used for immunofluorescence imaging or for preparation of whole cell extracts with 50 µl of a 0.1% SDS-0.5% Triton X-100 buffer (63, 64) per well for Western blot analyses.

**Plasmid transfections.** Constitutive expression plasmids for green fluorescent protein (GFP)-tagged wild-type (WT) dynamin-2 (WT Dyn2-GFP) or the dominant-negative K44A mutant (K44A Dyn2-GFP) were a gift from Dr. Mark A. McNiven (Mayo Clinic, Rochester, MN; Ref. 47). Expression vectors (1 µg DNA/well in 6-well cultures) were transfected with PolyFect transfection reagent (Qiagen, Valencia, CA) 1 day before experimental use.
Immunofluorescence microscopy of cells in culture. Immunofluorescence microscopy was carried out as described previously (28, 43–46, 64–66). Images were collected with either 1) a Nikon Eclipse 50i epifluorescence microscope equipped with an RGB charge-coupled device (CCD) camera and RS Image 1.9.2 software (Roper Scientific, Tucson, AZ) or 2) a Zeiss AxiosImager M2 epifluorescence motorized microscopy system with Zeiss W N-Achromplan ×40/NA0.75 and Zeiss EC Plan-Neofluor ×100/NA1.3 oil objectives equipped with a high-resolution RGB HRc AxioCam camera and AxioVision 4.8.1 software. Controls included secondary antibodies alone, peptide competition assays, and multiple different antibodies toward the same antigen (43–46, 61–63). All data within each experiment were collected at identical imaging settings.

Quantitative image analyses of Golgi structural parameters. Structures within the Golgi apparatus were quantitated with ×40 immunofluorescence images of the Golgi scaffolding protein giantin and the McMaster Biophotonics Facility version of NIH ImageJ software and respective utility plugins (available as free downloads from www.machiophotonics.ca/imageJ/). The giantin images were converted to 16-bit grayscale images and subjected to software-driven segmentation analysis with automatic machine-set Otsu thresholding (50) (thus eliminating subjective investigator bias) followed by particle parameter enumeration analysis (size exclusion set at minimum of 20 pixel²). Superimposition of DAPI nuclear images allowed identification of cell numbers and enumeration of Golgi fragments per cell (compact: 1 fragment by objective Otsu thresholding; dispersed: multiple different fragments toward the same antigen). The giantin images were converted to 16-bit grayscale images and subjected to software-driven segmentation analysis with automatic machine-set Otsu thresholding (50) (thus eliminating subjective investigator bias) followed by particle parameter enumeration analysis (size exclusion set at minimum of 20 pixel²). Superimposition of DAPI nuclear images allowed identification of cell numbers and enumeration of Golgi fragments per cell (compact: 1 fragment by objective Otsu thresholding; dispersed: >1 fragment). Four Golgi structure parameters could then be derived: percentage of cells with fragmented Golgi, Golgi fragments/cell, Golgi area/cell (1 sq. pixel unit = 0.0256 m²), and Golgi area/fragment (1 sq. pixel unit = 0.0256 m²). Typically 200–400 cells (range 37–1,075) derived from two to five independent experiments were imaged and quantitated for each variable (n in Figs. 1–10 = no. of cells evaluated for the designated variable); all data are expressed as means ± SE.

Quantitation of nuclear size and cell size. The nuclei displayed with DAPI in each ×40 image frame were thresholded and then assessed with the NIH ImageJ particle counter with the minimum size set at 500 pixels to give number of nuclei counted (a) and total area comprising the DAPI stain (b). The mean area per nucleus (b/a) was derived from these data for each image frame. Mean cell size was estimated from the image area of a confluent sheet of cells in an image frame divided by the number of DAPI-stained nuclei observed in that frame, with the occasional binucleate or trinucleate cell determined visually by respective protein immunofluorescence (for giantin or tubulin, etc) and counted as one cell.

Subcellular 3D immunoinaging of single cells in sections of archived lung tissue from patients with PAH and from macaques infected with SHIV-nef or SIV. Serial paraffin block sections (5 μm) of formalin-fixed archived lung tissue, from J patients with IPAH and respective control subjects without PAH (n = 6 each) archived at the Johns Hopkins University School of Medicine (Baltimore, MD) according to protocols reviewed and approved by the Institutional Review Board and 2) macaques infected with SHIV-nef (n = 4), infected with native SIV (n = 3), or left uninfected (n = 1) housed at the California National Primate Research Center according to protocols reviewed and approved by the Institutional Animal Care and Use Committee, and processed previously for multicolor immunofluorescence imaging of the Golgi tethers giantin and p115, HIV-nef, and DAPI to mark nuclei (Ref. 64 and Supplemental Tables 1 and 2 therein) were reimaged with the Zeiss AxiosImager M2 microscopy system, the ×100 oil objective, and the HRc AxioCam camera in a 1,388 × 1,040-pixel high-speed color capture mode using z-stack data acquisition software (typically stacks of 15 or 21 slices 0.3 μm apart). The imaged stacks were subjected to iterative deconvolution (up to 30 cycles) and 3D reconstruction with the respective Deconvolve and Inside4D modules in the Zeiss Axiovision 4.9.1 software. The Inside4D module allows multicolor visualization of structures within 3D volume space from any perspective, the rotation of this volume around any axis, and the conversion of such rotation sequences into video representations.

Antibody reagents. Rabbit polyclonal antibodies (PABs) to giantin were purchased from Abcam (Cambridge, MA) and those to p115, eNOS, and STAT5b and murine MABs to Vti1a (vesicle transport interaction with t-SNAREs homolog 1a), α-SNAP, GS28 (Golgi SNARE 28 kDa), and β-tubulin were from Santa Cruz. Murine MAB to GM130 (Golgi Matrix 130-kDa protein) was purchased from BD Biosciences (Eugene, OR). Rabbit PAB to human vWF was purchased from Dako Cytomation (Carpinteria, CA), while murine MAB to HIV-nef was from Advanced Biotechnologies (Columbia, MD) and that to SMA was from Sigma-Aldrich. Respective AlexaFluor 488- and AlexaFluor 594-tagged secondary donkey antibodies to rabbit or mouse IgG were from Invitrogen Molecular Probes (Eugene, OR).

Statistical analyses. Statistical analyses were carried out with the two-tailed Student’s t-test in Microsoft Excel software; for significance evaluation each individual culture (irrespective of the number of cells evaluated therein) was taken as one independent observation. Additionally, linear regression and ANOVA were used for comparisons between respective experimental groups and their matched controls using analysis of variance (ANOVA) tests (1 way and repeated measures). Post hoc comparisons were carried out with the Tukey-Kramer multiple comparison test, with α set at 0.05.

RESULTS

Nitric oxide is required for structural integrity of Golgi ribbon in HPAECs and HPASMCs in culture. To examine whether the structural integrity of the Golgi ribbon depends on NO in human pulmonary vascular cells, HPAECs and HPASMCs were exposed to the NO scavengers c-PTIO and MB. For comparison, cultures exposed to the bioactive MCTP were included in each experiment. We previously confirmed, using live single-cell 4,5-diaminofluorescein diacetate (DAF-2DA) imaging, that c-PTIO and MCTP reduce caveolar NO in endothelial cell cultures (43, 46). In preliminary experiments Golgi fragmentation was observed within 1 day of MCTP or c-PTIO exposure and increased progressively over 4 days (Supplemental Fig. S1A). This fragmentation was apparent when either giantin or a different Golgi scaffolding protein/tether (GM130) was imaged (Supplemental Fig. S1B). Thus for most cell culture experiments we selected a 2-day reagent exposure time and used immunotagging for giantin using a PAb. The advantage of immunotagging for giantin was that the same PAb reagent for giantin could also be used for immunoinaging Golgi elements in single cells in sections of archived formalin-fixed lung tissue (as in Figs. 8 and 9).

Figure 1, A and B, show the images and quantitation of Golgi fragmentation in HPAECs exposed to the NO scavengers c-PTIO and MB compared with MCTP, MCTP, c-PTIO and MB markedly fragmented the Golgi apparatus. Compared with MCTP, c-PTIO and MB fragmented the Golgi apparatus into a larger number of smaller fragments. Figure 1, C and D, show a similar increase in Golgi fragmentation upon exposure of HPASMC cultures to c-PTIO, MB, and MCTP. This fragmentation was reversible upon washout of the NO scavenger but.....
Fig. 1. Structural changes produced in the Golgi apparatus in human pulmonary arterial endothelial (HPAECs) and smooth muscle (HPASMCs) cells in culture after exposure to the nitric oxide (NO) scavengers 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) and methylene blue (MB) compared with that produced by monocrotaline pyrrole (MCTP). Respective cultures in 6-well plates were exposed to MCTP (M; single exposure to ~50 μM equivalent), c-PTIO (P; 50 μM), or MB (MB; 10 μM) or left untreated (U). The medium was refreshed daily and included the addition of fresh c-PTIO and MB. In the experiment illustrated, the cultures were fixed 2 days after the start of treatment, immunostained for gian tin, and stained with DAPI to display nuclei. Structural changes in the Golgi apparatus were quantitatively evaluated with the objective Otsu thresholding and segmentation approach as described in MATERIALS AND METHODS to yield % cells with Golgi fragmentation (1; Otsu fragment number >1), number of Golgi fragments per cell (2), Golgi area per cell (3; in pixel²) and Golgi area per fragment (4; in pixel²). Data in A and B pool 3 independent experiments using HPAECs (means ± SE); those in C and D pool 2 independent experiments using HPASMCs (n = no. of cell images analyzed in this and subsequent figures corresponding to each experimental condition; this number is taken as 100% in B1 and D1). Scale bars, 10 μm. *P < 0.05 compared with untreated cultures.
not upon washout of MCTP (data not shown). Supplemental Figure S2A, top, shows that the NO donor NONOate blocked the Golgi fragmentation produced by c-PTIO and MB in HPAEC cultures. Moreover, Supplemental Fig. S2A shows that c-PTIO dispersed β-tubulin concomitant with Golgi fragmentation and NONOate reversed both. However, the combination of NONOate with MB did not reorganize β-tubulin in the centrosome region even though the Golgi was now rendered compact (Supplemental Fig. S2A). Supplemental Figure S2B shows that a combination of MCTP and c-PTIO preserved β-tubulin organization even though the extent of Golgi fragmentation was marked, while Supplemental Fig. S2C shows that Golgi fragmentation produced by c-PTIO plus MCTP persisted even in the presence of a tubulin stabilizer such as paclitaxel. Taken together these data show that NO deficiency induced Golgi fragmentation (this was blocked by the NO donor NONOate) and that, mechanistically, the Golgi fragmentation was independent of changes in β-tubulin organization. Moreover, as previously reported for MCTP (65, 66), both c-PTIO and MB elicited an increase in cell and nuclear size in endothelial cells (Supplemental Fig. S3A); however, this effect was less apparent in smooth muscle cells (Supplemental Fig. S3B).

Figure 2A shows that additional compounds with NO-scavenging activity such as NAC and Hb (reviewed in Ref. 51 and citations therein), but not the inactive ferrous Hb, also induced Golgi fragmentation. In contrast, LY-83583, an inhibitor of the NO-dependent soluble guanylate cyclase, and Tiron and Tempol, scavengers of ROS, were unable to induce Golgi fragmentation (Fig. 2A). Sildenafil, a cGMP phosphodiesterase inhibition was independent of changes in cell and nuclear size. Neither dynasore nor indirubin E804 affected the ability of c-PTIO to fragment the Golgi apparatus (Supplemental Fig. S4A). Dynamin Y-27632, Rho A kinase inhibitors, behaved similarly (data not shown). Therefore, NAC, which is typically reported to be an antioxidant and a ROS and NO scavenger (see Ref. 51 and citations therein), has also been attributed a “prooxidant” property at high concentrations, we investigated the concentration dependence of its ability to fragment the Golgi apparatus: 10 mM produced maximal fragmentation (data not shown). Moreover, the NO donor NONOate reduced the ability of NAC to fragment the Golgi, suggesting that the observed Golgi fragmentation produced by NAC was attributable to its NO-scavenging abilities (data not shown). Furthermore, we investigated the effect of an oxidant such as H$_2$O$_2$ on Golgi structure. H$_2$O$_2$ did not lead to Golgi fragmentation (Supplemental Fig. S4B).

The ability of Hb to elicit Golgi fragmentation in endothelial cells was also inhibited by NONOate (Fig. 2, B and C) and was accompanied by evidence of the endocytic uptake of Hb by the endothelial cells (Supplemental Fig. S5). Scavenging of NO by Hb also led to increased cell size in HPAECs but with little change in nuclear size (Supplemental Fig. S6). NONOate inhibited the increase in cell size produced upon exposure of HPAECs to Hb (Supplemental Fig. S6).

The Golgi fragmentation effect of the NO scavenger c-PTIO was not restricted to pulmonary vascular cells but was more general. The data in Fig. 3A show that Golgi fragmentation was also produced in human umbilical vein endothelial cell-derived immortalized EA.hy926 cells exposed to c-PTIO. Remarkably, eNOS was mislocalized away from the plasma membrane with trapping of eNOS in the Golgi fragments in EA.hy926 cells exposed to c-PTIO (Fig. 3B).

**Knockdown of eNOS in HPAECs leads to Golgi fragmentation.** eNOS expressed from the NOS3 gene is the major source of constitutive NO in endothelial cells (14, 24, 55). Thus eNOS expression was selectively knocked down in HPAECs with an siRNA approach, and the integrity of the Golgi apparatus and changes in cell and nuclear size were investigated. Figure 4A shows that a >90% selective knockdown of eNOS was achieved in HPAECs transfected with NOS3 siRNA oligonucleotides compared with cultures that received the control scrambled siRNA. Moreover, 96 h after NOS3 siRNA transfection there was a dramatic reduction in eNOS immunofluorescence in HPAECs (Fig. 4B) accompanied by a marked fragmentation of the Golgi apparatus (Fig. 4, A and C). Additionally, both cell and nuclear size were increased after NOS3 siRNA transfection into HPAECs (Supplemental Fig. S3C).

**Functional implications of Golgi fragmentation due to reduced NO.** Vitril, a SNARE protein involved in vesicular trafficking across the Golgi apparatus (32), was found scattered with the Golgi fragments after NO was scavenged in both HPAECs and HPASMCs (Fig. 5, A and B). Moreover, α-SNAP, a protein that associates with all trans-SNARE complexes to recruit the ATPase NSF for subsequent “resolution” of these complexes and is thus required in all vesicular trafficking (5, 63), was depleted from the Golgi membrane fragments and instead found in vesicular structures dispersed in the cytoplasm after NO was scavenged (Fig. 5, C and D). These alterations in trafficking proteins were selective in that the SNARE GS28 was not affected (data not shown).

**The GTPase dynamin 2 participates in Golgi fragmentation.** The process of Golgi fragmentation involves the scission or pinching of membrane elements from Golgi ministacks/cisternae (5, 54). The GTPase Dyn2 has been shown to be involved in the scission or pinching of endocytic and caveolar vesicles from the plasma membrane and, more recently, in a similar effect on Golgi vesicles (5, 67, 73). Dyn2 activity in caveolar endocytosis is enhanced by src-mediated Tyr phosphorylation (67). We investigated whether Golgi fragmentation due to reduced NO bioavailability required Dyn2 and whether this process might involve src-mediated Tyr phosphorylation.

Figure 6, A and B, show that transfection of endothelial cells with expression vectors for GFP-tagged WT Dyn2 had little effect on NO-induced Golgi fragmentation. In contrast, transfection of endothelial cells with the dominant-negative K44A Dyn2-GFP reduced Golgi fragmentation (Fig. 6, C and D). The data in Supplemental Figs. S7 and S8 show that dynasore, an inhibitor of Dyn2 GTPase activity (23), reduced Golgi fragmentation but not the increase in cell or nuclear size. The data in Supplemental Figs. S9 and S10 show that indirubin E804, an inhibitor of src-mediated Tyr phosphorylation (48), also reduced Golgi fragmentation but not the increase in cell or nuclear size. Neither dynasore nor indirubin E804 affected already established Golgi fragmentation in endothelial cells exposed to c-PTIO, MCTP, or both 2 days earlier (data not shown). Thus Dyn2 was involved in the onset of Golgi fragmentation but not in its maintenance. Moreover, the mechanisms regulating Golgi fragmentation were independent of those regulating cell or nuclear size.
Fig. 2. Structural changes produced in the Golgi apparatus in bovine (B)PAECs or HPAECs in culture after exposure to various reagents including hemoglobin (Hb) and the NO donor NONOate. A: BPAEC cultures in 6-well plates were left untreated or exposed to MCTP (single exposure to ~50 μM equivalent), c-PTIO (100 μM), MB (10 μM), N-acetylcysteine (NAC; 80 mM), Hb (20 μM), ferrous Hb (20 μM), LY-83583 (5 μM), Tiron (5 mM), or Tempol (2 mM). The medium was refreshed 1 day later and included the addition of fresh reagents (except for MCTP). The cultures were fixed 2 days after the start of treatment and analyzed for % cells with Golgi fragmentation (Otsu fragment number). *P < 0.05 compared with untreated cultures. B: BPAEC cultures were exposed to Hb alone (10 or 20 μM) or to a combination of Hb and NONOate (NONO; 400 μM) for 2 days and analyzed for Golgi fragmentation. Scale bar, 10 μm. *P < 0.05 compared with untreated controls; **P < 0.05 compared with cells exposed to Hb at 10 μM; #P < 0.05 compared with cells exposed to Hb at 20 μM. C: HPAEC cultures were exposed to Hb alone (20 μM) or to a combination of Hb and NONOate (400 μM) for 2 days and analyzed for Golgi fragmentation. Scale bar, 10 μm. *P < 0.05 compared with untreated controls; #P < 0.05 compared with cells exposed to Hb at 20 μM.
Subcellular 3D immunoimaging of Golgi apparatus and trafficking mediator proteins in single cells in pulmonary vascular lesions. The occurrence of the Golgi fragmentation signature was investigated in single cells in proliferative, obliterator, and plexiform lesions in IPAH and in the SHIV-nef-infected macaque model with multicolor high-resolution subcellular 3D immunoimaging. The histological lesions selected for this 3D immunoimaging have been previously described with low-resolution imaging (64).

The subcellular 3D reconstruction technique was first evaluated on cells in HPAEC cultures exposed to c-PTIO or MCTP (Fig. 7). The image in Fig. 7A shows an intact Golgi ribbon in a compact Golgi apparatus in an untreated human endothelial cell, recalling the observation of Cajal in 1914 (Figs. 7 and 8 in Ref. 6). In contrast, the Golgi apparatus was enlarged and the Golgi ribbon fragmented after MCTP (Fig. 7B). The Golgi ribbon was markedly fragmented after c-PTIO, but with little apparent increase in Golgi area per cell (Fig. 7C). The 3D imaging data in Fig. 7 are representative of the differences between the effects of MCTP and c-PTIO in terms of the estimates of Golgi area per cell shown in Figs. 1B and 1D.

We then imaged the Golgi apparatus in single cells in a pulmonary arterial section from lungs of a patient without PAH (see Ctrl-A in Figs. 1 and 2 in Ref. 61 for histology and prior data) and in a proliferative lesion in IPAH (see PAH-B in Figs. 1 and 2 in Ref. 61 for histology and prior data). Figure 8 illustrates typical high-resolution 3D images, and
Supplemental Videos S1–S6 show rotation movies of the respective single-cells in Fig. 8. Whereas the giantin-tagged Golgi was compact in the control section in both the SMA-negative endothelial and SMA-positive smooth muscle cells (Fig. 8A), there was cellular enlargement and Golgi fragmentation in IPAH in both cell types within the lesion (Fig. 8B).

Figure 9 demonstrates similar observations in single cells in pulmonary vascular lesions in the SHIV-nef-infected macaque (35, 64). Previously we reported (64) increased giantin and p115 on a per-cell basis in HIV-nef-positive cells in the pulmonary vascular lesions. However, in the earlier study we were unable to delineate the respective subcellular structures involved. Figure 9, A, top, and B, top, confirm that giantin and p115, respectively, were observed in a single compact Golgi apparatus in pulmonary arterial cells in the luminal endothelium in SIV-infected macaques (the same sections as in Ref. 64). In contrast, Fig. 9, A, bottom, and B, bottom, show that giantin and p115 were markedly dispersed in cytoplasmic vesicular structures in cells in vascular lesions in SHIV-nef-infected macaques. Figure 9C demonstrates in high-resolution 3D that the giantin-tagged Golgi was fragmented specifically in cells that contained a swarm of HIV-nef-bearing endosomes in the cytoplasm. Supplemental
Videos S7–S9 are the 3D rotation renderings of the three still frames in Fig. 9C. The giantin-bearing Golgi fragments and the HIV-nef-bearing endosomes are clearly distinct cytoplasmic structures.

Supplemental Figure S11 confirms the increase in α-SNAP in vWF-positive and -negative cells in pulmonary vascular lesions in SHIV-nef-infected macaque and in IPAH. Figure 10A shows immunofluorescence for α-SNAP, Vti1a, and GS28 in one of the obliterative lesions in the SHIV-nef-macaque model, and Fig. 10B illustrates the respective representative single-cell 3D renderings. α-SNAP and Vti1a were markedly sequestered in cytoplasmic vesicular structures but not GS28. Figure 10, C and D, show that a similar pattern of cytoplasmic vesicular sequestration of α-SNAP and Vti1a but not GS28 was also observed in cells in a proliferative lesion in IPAH.

**DISCUSSION**

In the present study we show that the integrity of the Golgi apparatus in human vascular cells depends on NO. Exposure of cells to different reagents that scavenge NO (c-PTIO, MB, NAC, Hb) all led to fragmentation of the Golgi apparatus and to signature changes in the distribution of trafficking-mediator proteins (α-SNAP and Vti1a). None of the inhibitors tested (LY-83583 for soluble guanylate cyclase, sildenafil for phosphodiesterase 5A, fasudil and Y-27632 for Rho A kinase), ROS scavengers (Tiron and Tempol), or the oxidant H2O2 caused such fragmentation. That NONOate inhibited the onset of Golgi fragmentation due to c-PTIO, MB, NAC, and Hb highlights the critical role that NO plays in maintenance of Golgi structure. This was further substantiated by the demonstration that selective knockdown of eNOS in HPAECs using an siRNA approach resulted in marked Golgi fragmentation. We previously demonstrated (43, 46), using single-cell DAF-2DA assays, that exposure of PAEC cultures to c-PTIO and MCTP leads to a reduction in caveolar NO. In the present study we show that the process of Golgi fragmentation subsequent to reduced NO bioavailability required the GTPase Dyn2 but was dissociated from microtubule organization. Dyn2 is best known for its role in the scission or pinching of clathrin-coated endosomes from the cytosolic face of the plasma membrane (5, 49). In recent years Dyn2 has been implicated in trans-Golgi trafficking (73). Src-induced fragmentation of the Golgi apparatus in cancer cells has now been shown to be due to src-mediated Tyr phosphorylation (73). In the present stud-
ies the dominant-negative K44A mutant of Dyn2 inhibited the onset of Golgi fragmentation, as did the Dyn2-specific inhibitor dynasore. Moreover, indirubin E804, an inhibitor of src-dependent Tyr phosphorylation (28, 48), also inhibited Golgi fragmentation. In a manner consistent with the transient requirement for Dyn2 in the membrane pinching step (5), Dyn2 was not involved in the maintenance of the already fragmented Golgi.

The mammalian Golgi apparatus consists of sets of stacked cisternae, with each stack, or more accurately “ministack,” consisting of three to eight biochemically distinct cisternae linked laterally through reticulotubular structures to form the membrane network called the Golgi ribbon (13, 42, 54). The lateral cisternal membrane continuity forming the ribbon is thought to facilitate optimal processing conditions in biosynthetic pathways (54). The Golgi tethers/scaffolding proteins giantin, GM130, and p115 play a critical role in the association of coatomer protein complex I (COP-I) vesicles with the Golgi apparatus and endoplasmic reticulum (ER)-Golgi and intra-Golgi transport (42, 61). Moreover, tethers such as GM130 are required to maintain the integrity of the lateral connectivity between ministacks (54) and are also involved in regulating

Fig. 6. Golgi fragmentation requires the GTPase dynamin 2 (Dyn2). BPAEC cultures were transfected with constitutive expression vectors for wild-type green fluorescent protein (GFP)-tagged Dyn2 (WT Dyn2-GFP) (A and B) or K44A Dyn2-GFP (C and D) and then 1 day later exposed to MCTP, c-PTIO (100 μM), or their combination for another 2 days. Fixed cells were imaged for GFP, giantin, and DAPI and % cells with Golgi fragmentation. A and C show representative images; scale bars, 10 μm. *P < 0.05 in comparisons of GFP-positive transfected cells with respective GFP-negative untransfected cells (B and D).
other Golgi-dependent cellular events such as entry into mitosis, cell polarization, and directed cell migration (43). Vti1a is a Golgi-localized t-SNARE (32), GS28 is a resident Golgi SNARE that mediates ER-to-Golgi and intra-Golgi trafficking (74), while α-SNAP is involved in all vesicular trafficking (5, 63). NO scavenging resulted in marked fragmentation of the Golgi ribbon (no. of fragments per cell = 8–10) indicative of a loss of lateral connectivity between ministacks and a decrease in the Golgi area per cell as well as per fragment (Fig. 1). In contrast, the effect of MCTP was distinctive in that it involved a more modest fragmentation (mean no. of fragments per cell = 2–4) with a net increase in Golgi area per cell. We previously showed (28) that these morphological differences accompany functional differences in that the Golgi membranes can still accumulate C5-ceramide after MCTP exposure but can no longer accumulate C5-ceramide after c-PTIO. However, in both instances there was an inhibition of functional retrograde trafficking and endocytosis with respect to uptake of transferrin, acetyl-LDL, and cholera toxin. The underlying biochemical events culminating in these distinctive effects remain to be investigated.

At first glance there appears to be a relationship between Golgi fragmentation and increased cell and nuclear size upon NO scavenging (Supplemental Figs. S3 and S6). However, the relationships between the size of the Golgi apparatus, its fragmentation, cell size and nuclear size, and cell entry into mitosis are complex and different in different cell types (44, 45, 66). Nevertheless, changes in Golgi function were detectable in terms of increased caveolin-1 trapping by 6–8 h in BPAECs exposed to MCTP, which was prior to any alterations in cell or nuclear size becoming evident (65). Thus we inferred that the Golgi apparatus was a primary target for MCTP (65, 66), resulting in the subsequent disruption in the G2-to-M cell cycle checkpoint and the megalocytosis phenotype (65, 66).

In the case of NO scavenging by c-PTIO, the treated BPAECs and EA.hy926 cells, but not HPAECs or HPASMCs, showed increased transit into mitosis and increased cell proliferation accompanying the Golgi fragmentation (Ref. 28 and data not shown). MCTP blocked this cell cycle transit into mitosis (28). Thus, in BPAECs and EA.hy926 cells under conditions of NO scavenging, there was an increase in cell size and nuclear size as estimated on a culturewide basis, but cells did traverse through mitosis; the immediately postmitotic small cell doublets had fragmented Golgi but small nuclei (data not shown), suggesting an independence between Golgi fragmentation and cell and nuclear size per se. The latter inference is supported by the observations in Supplemental Figs. S7–S10 that dynasore and indirubin E804 reduced Golgi fragmentation due to a combination of MCTP + c-PTIO but not the increase in cell or nuclear size. Moreover, that the GTPase Dyn2 was involved in the process of Golgi fragmentation but not its maintenance (Fig. 6 and data not shown) also points to the independence of pathways leading to Golgi fragmentation upon NO scavenging from those affecting cell and nuclear size. However, it is clear that increased pulmonary vascular cell size (“enlarged,” “plump,” “vacuolated” cells) that collectively decreases the arterial lumen in lesions characteristic of IPAH is part of the pathobiology of this disease (reviewed in Refs. 63, 64, 71).

The technique of high-resolution single-cell 3D imaging of immunotagged subcellular proteins in lung tissue sections allowed us to dissect Golgi fragmentation in single cells in pulmonary vascular lesions in human IPAH and in the SHIV-nef-infected macaque model. Whereas the giantin-tagged Golgi apparatus was compact in endothelial and smooth muscle cells in disease-free pulmonary arteries, the Golgi was markedly fragmented in both the endothelial and smooth muscle cells found in PAH lesions. Similarly, whereas the giantin- or p115-tagged Golgi elements were compact in pulmonary vascular cells in an SIV-infected macaque, these were fragmented in cells in vascular lesions in the SHIV-nef-infected macaque. As with the observations in vascular cell cultures subjected to NO scavenging, there was increased cytoplasmic sequestration of α-SNAP and Vti1a but not GS28 in cells in lesions in the
SHIV-nef-infected macaque, suggesting an underlying mechanistic commonality.

High-resolution 3D immunoimaging of cells in pulmonary lesions in the SHIV-nef-infected macaque confirmed that swarms of HIV-nef-bearing endosomes were present in the very same cells with fragmented Golgi. These HIV-nef-bearing vesicular structures were distinct from the giantin-tagged Golgi elements, consistent with the notion that it is the circulating HIV-nef produced in SHIV-nef-infected macaques that is taken up by pulmonary vascular cells by endocytosis and this then triggers the Golgi changes (see Ref. 35 for a discussion of this mechanism). We were unable to detect plasma membrane-associated nef in the present immunostaining studies on archived macaque lung sections. It was demonstrated recently that exposure of porcine pulmonary artery rings and HPAECs in culture to HIV-nef protein for 24 h led to decreased vasorelaxation, decreased eNOS expression, decreased eNOS antigen by immunohistochemistry, and reduced NO bioavailability (9). That reduced NO bioavailability subsequent to endosomal uptake of HIV-nef might mechanistically underlie the Golgi fragmentation reported in Fig. 9C is an exciting possibility—one that can be tested in future studies using the subcellular 3D immunoimaging approach to investigate potential eNOS mislocalization in nef-positive cells in pulmonary vascular lesions.

Caliguiri and Tamm pioneered a line of research in the late 1960s and early 1970s showing that poliovirus associated with cellular “endomembranes” and used these cellular membrane locales for sites of virus replication and assembly (Refs. 7, 8; see extension of this work in Ref. 3). Subsequently, there has grown an extensive literature showing that cells infected with various viruses or transfected with expression plasmids for various viral proteins induce structural and functional changes in the Golgi apparatus (Refs. 3, 16, 36, 60 and citations therein). Other viral proteins traffic through the Golgi without affecting its structure or function—in fact, trafficking of a temperature-sensitive mutant of the G glycoprotein of vesicular stomatitis virus is used today as a major method of investigating Golgi apparatus function (5, 42, 54). However, in considering the SHIV-nef-infected macaque model, it is noteworthy that HIV and SIV at best minimally replicate, if at all, in vascular cells (1, 53). Marecki et al. (35) have previously pointed out that the nef present in pulmonary vascular cells was likely synthesized elsewhere, secreted, and then taken up by pulmonary endothelial cells. Thus the Golgi changes observed
Fig. 9. High-resolution subcellular 3D immunoimaging of Golgi apparatus fragmentation in pulmonary vascular lesions in macaques infected with chimeric simian immunodeficiency virus (SIV) containing human immunodeficiency virus (HIV)-nef gene (SHIV-nef). Single cells within pulmonary vascular lesions in SHIV-nef-infected macaques shown in Figs. 1 and 3 in Ref. 61 (SHIV-A and SHIV-D) were evaluated with subcellular 3D imaging of the Golgi tethers giantin (A) or p115 (B) and compared with that in SIV-infected macaques (SIV-F, -G, -H) (details and histology in Ref. 61). A and B: still frames of representative cells; scale bars, 4 μm. C: still frames of representative cells in triple-label (giantin, HIV-nef, and DAPI) subcellular 3D imaging of single cell in the pulmonary vascular (proliferative arterial lining and obliteratorive) lesions shown in Fig. 8B in Ref. 61 for macaque SHIV-A. Rotation videos of these 3 frames are in Supplemental Videos S7–S9. Scale bars, 4 μm.
in the SHIV-\textit{nef} macaque are not likely to be due to virus replication within the vascular cells. Furthermore, Tinkle and colleagues reported (70) that an HIV-1-transgenic mouse expressing an integrated provirus in which the \textit{gag}, \textit{pol}, and \textit{env} genes required for productive viral replication had been deleted spontaneously developed arterial proliferative and oblitative vasculopathy. The histological data in their Fig. 2d showing an oblitative lesion in the mesenteric artery and their Fig. 2g showing intimal arterial thickening are similar to the histological data in the SHIV-\textit{nef} macaque model (compare Fig. 2 panels in Ref. 70 with Fig. 1 in Ref. 64 and with present Supplemental Fig. S11). It is noteworthy that the HIV-\textit{nef} was intact in the transgenic mouse developed by Tinkle and colleagues, while genes required for virus replication had been deleted (70).

The literature concerning the involvement of eNOS, and thus NO, in pulmonary vascular remodeling in PAH appears controversial since some investigators have reported decreased eNOS antigen associated with PAECs in vascular lesions in PAH (18, 78) while others have reported unchanged or increased eNOS antigen levels (58, 72). We suggest an alternative explanation that may resolve the controversy—consideration of the subcellular mislocalization of eNOS in cells in pulmonary vascular lesions in PAH. In the MCT model we reported increased levels of eNOS in MCTP-treated PAECs, but this eNOS was sequestered in the Golgi and in cytoplasmic vesicular elements and therefore resulted in decreased caveolar NO production (by live-cell DAF-2DA assay) and reduced protein \textit{S}-nitrosylation (46). Figure 3B demonstrates the mislocalization of eNOS away from the plasma membrane accompanied by trapping in the Golgi fragments in EA.hy926 endothelial cells exposed to \textit{c}-PTIO. Thus exactly where within the endothelial cell the observed eNOS is located is critical to an understanding of NO biology and not just the amounts of eNOS protein per cell. It has been shown that the pulmonary circulations of homozygous and heterozygous eNOS-null mice are equally hyperresponsive to mild hypoxia even though the latter had 50% of normal eNOS protein (12). Moreover, eNOS-null mice do not develop PAH when exposed to NAC but do so when exposed to its \textit{S}-nitrosylated product (\textit{S}-nitroso-acetyl cysteine; Ref. 51). Parenthetically, a major source of NO in vascular smooth muscle cells is neuronal nitric oxide synthase (nNOS) (37); the subcellular localization of nNOS in PAH remains unexplored.

The timeline of changes in NO bioavailability observed in endothelial cells in culture uniformly exposed to MCTP and assayed with single-cell DAF-2DA imaging is rapid (within 2 days) (43, 46). However, the earliest increase in pulmonary arterial pressure is observed in the typical MCT/rat model by 10–14 days (see Ref. 38 for 1 example). It needs to be emphasized that in cell culture it is possible to expose 100% of the cells to a reagent. In contrast, the rat/MCT model has been optimized for rise in pulmonary arterial blood pressure 2–3 wk later and not specifically for synchronously affecting all 100% of the PAECs in the rat all at once (see Refs. 27, 41 and citations therein). Thus in our hands 37% of cells in a BPAEC culture exposed once to MCTP enter unscheduled DNA synthesis by 48 h (66), and in an earlier study Lappin et al. (27) showed that the vast majority of cells were affected with respect to DNA synthesis stimulation and cell cycle progression by MCTP within 12 h (see Figs. 2 and 3 in Ref. 27). However, in the same study, Lappin et al. (27) reported that after an MCT injection in the rat only 5–10% of PAECs show DNA synthesis by 4 days and thereafter (also see Ref. 41 for similar data). We posit that the intracellular structural Golgi changes triggered by hypoavailability of NO at the single-cell level within 48 h in a subset of the PAECs in the MCT/rat model in vivo are part of the initiation of the disease, with subsequent steps including alterations in the cell surface receptor landscape, increased cell proliferation and increased cell size, as well as increased secretion of cytokines and growth factors and vascular remodeling (see Refs. 28 and 61a) comprising the progression to the overt disease phenotype. This point is further emphasized by the observations of Palmer et al. (51), who showed the manifestations of initiating effects of NAC in BPAEC cell culture within 4 h and then reported that the earliest time that they observed PAH in the NAC-administered mouse (i.e., progression to disease) was at 1 wk after commencement of treatment, with maximal disease observed at 3 wk.

Several BMPRII mutants implicated in “initiating” the pathogenesis of familial PAH that develops decades later (71) have been reported to exhibit defective trafficking through the Golgi (59), and there is a selective decrease in eNOS antigen levels in arterial endothelium in lungs of \textit{BMPRII} mutant transgenic mice subjected to hypoxia (15). In cell culture, we have observed subcellular mislocalization of eNOS when endothelial cells were transfected with familial PAH disease-derived BMPRII mutants (Lee JE, Sehgal PB, unpublished observations). Investigations of aberrant trafficking interactions between BMPRII mutants and eNOS in single cells in disease tissues may prove amenable to study using subcellular 3D immunomaging techniques.

The complexity of the interplay between eNOS biology and PAH is emphasized by the observations in the \textit{cav-1} \textsuperscript{−/−} mouse, which spontaneously develops PAH concomitant with increased pulmonary tissue NO levels and increased Tyr nitration of protein kinase G (which impairs enzymatic activity) (77). In this model, a superoxide scavenger and also nitro-L-arginine methyl ester (L-NAME) reduced pulmonary vascular pathology (77). [Although Zhan et al. (76) reported increased NO production by lung tissue from patients with IPAH and increased protein nitration in whole lung tissue extracts, these investigators did not characterize NO bioavailability in PAECs per se; thus the data of Zhan et al. are not probative because in the lung it is the airway epithelium that contains the bulk of the
eNOS and the other NOS isoforms (Refs. 17, 26, 39, 68, 76; also confirmed by P. B. Sehgal in human and macaque lung sections in the present study, data not shown.) To add to the mechanistic complexity are observations showing that increased S-nitrosylation inhibited the activity of the ATPase NSF, which is involved in all vesicular trafficking (39), while S-nitrosylation of Dyn2, a GTPase involved in the pinching of vesicles during trafficking, enhanced its enzymatic activity (24). Moreover, eNOS itself is S-nitrosylated (46), although the influence of this modification on trafficking and function is not known. Additionally, eNOS/NOS3 is clearly established to have a second or modifier gene effect on the severity of polycystic disease of the kidney (polycystin-1 uses the conventional Golgi to plasma membrane trafficking pathway) (52) and cystic fibrosis (CFTR uses the unconventional ER-to-plasma membrane pathway that bypasses the Golgi) (22). Clearly, the influence of NO on membrane trafficking and subcellular structures can be expected to be intracellular location specific and process specific.

To summarize, the present data demonstrate that reduced NO bioavailability results in structural and functional changes in the Golgi apparatus within human pulmonary arterial endothelial and smooth muscle cells. The ability of NO to modulate Golgi apparatus integrity in vascular cells was more general in that it was also observed in the immortalized human endothelial cell line EA.hy926. The technique of subcellular 3D immunoimaging confirmed the signature occurrence of such Golgi fragmentation and cytoplasmic sequestration of trafficking mediator proteins α-SNAP and Vti1a within single cells in pulmonary vascular lesions in IPAH and in the SHIV-nef-infected macaque. The observed Golgi fragmentation portends global protein trafficking dysfunctions and widespread changes in the cell surface landscape, cell size, and functional signaling in vascular cells.

Note added in proof: The presence of NO in association specifically with the Golgi ribbon in HPAECs and EA.hy926 cells under basal conditions was directly confirmed using DAF-2DA imaging of live cells and high-resolution microscopy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


37. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O'Rourke B, Lowenstein JM, Pesner J, Wagnor DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell 115: 139–150, 2003.


66. Shaw PW, Afshar S, Gibson LL, Sherman TS, Kerecman JD, Grubb PH, Yoder BA, McCurnin DC. Developmental changes in nitric oxide...


