Aberrant cytoplasmic sequestration of eNOS in endothelial cells after monocrotaline, hypoxia, and senescence: live-cell caveolar and cytoplasmic NO imaging

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Mukhopadhyay S, Xu F, Sehgal PB. Aberrant cytoplasmic sequestration of eNOS in endothelial cells after monocrotaline, hypoxia, and senescence: live-cell caveolar and cytoplasmic NO imaging. Am J Physiol Heart Circ Physiol 292: H1373–H1389, 2007. First published October 27, 2006; doi:10.1152/ajpheart.00990.2006.—We previously reported the disruption of caveolae/rafts, dysfunction of Golgi tethers, N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor proteins (SNAREs), and SNAPs, and inhibition of anterograde trafficking in endothelial cells in culture and rat lung exposed to monocrotaline pyrrole (MCTP) as a prelude to the development of pulmonary hypertension. We have now investigated whether Golgi blockade and eNOS sequestration are observed after hypoxia and senescence. Immunofluorescence data revealed that MCTP-induced “megalocytosis” of pulmonary arterial endothelial cells (PAEC) was accompanied by a loss of eNOS from the plasma membrane, with increased accumulation in the cytoplasm. This cytoplasmic eNOS was sequestered in heterogeneous compartments and partially colocalized with Golgi and endoplasmic reticulum (ER) markers, caveolin-1, NOSTRIN, and ER Tracker, but not LysoTracker. Hypoxia and senescence also produced enlarged PAEC, with dysfunctional Golgi and loss of eNOS from the plasma membrane, with sequestration in the cytoplasm. Live-cell imaging of caveolar and cytoplasmic NO with 4,5-diaminofluorescein diacetate (DAF-2DA) as probe showed a marked loss of caveolar NO after MCTP, hypoxia, and senescence. Although ionomycin stimulated DAF-2DA fluorescence in control PAEC, this ionophore decreased DAF-2DA fluorescence in MCTP-treated and senescent PAEC, suggesting localization of eNOS in an aberrant cytoplasmic compartment that was readily discharged by Ca²⁺-induced exocytosis. Thus monocrotaline, hypoxia, and senescence produce a Golgi blockade in PAEC, leading to sequestration of eNOS away from its functional caveolar location and providing a mechanism for the often-reported reduction in pulmonary arterial NO levels in experimental pulmonary hypertension, despite sustained eNOS protein levels.

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late vesicular trafficking (GM130, p115, giantin, golgin 84, clathrin heavy chain, syntaxin-4 and -6, Vti1a, Vti1b, GS15, GS27, GS28, SNAP23, and α-SNAP) in the enlarged Golgi organelle in MCTP-induced megalocytotic endothelial cells (unpublished observations). Immunofluorescence studies of lung tissue from MCT-treated rats confirmed enlargement of perinuclear Golgi elements in target lung cells as early as 4 days after MCT. These data provided the novel insight that MCT-induced PH represented a disease state involving dysfunction of Golgi tethers, SNAREs, and SNAPs and, thus, reduced trafficking of diverse vasoactive proteins to the vascular cell surface, with a marked backlog of such proteins in the ER/Golgi. An aberrant trapping of eNOS in the cytoplasm in PAEC would account for the often-reported observation of reduced nitric oxide (NO) in pulmonary arteries at early time points in the MCT model of PH (e.g., 2 wk after MCT), despite undiminished or even increased endothelial NO synthase (eNOS) levels (35, 25, 50).

Moreover, reports of enlargement and vacuolation of endothelial and smooth muscle cells in hypoxia-induced PH and senescent endothelial cells in culture with increased Golgi, ER, and Weibel-Palade bodies (exocytic vesicles) (16, 18, 22, 27, 28, 38, 39, 57, 62) suggested that disruption of membrane trafficking and resulting cellular enlargement/megaloctytosis may be a general pathogenetic mechanism in endothelial cell disease. We have now investigated 1) whether an MCTP-induced trafficking block affects eNOS subcellular localization and function and 2) whether Golgi blockade, disruption of vesicle tethers and SNAREs, and eNOS sequestration are observed in endothelial cells after hypoxia and senescence. Moreover, the functional consequences were investigated in live-cell imaging studies of caveolar and cytoplasmic NO. The data help extend the Golgi blockade hypothesis to endothelial cell disease states caused by MCT, hypoxia, and senescence.

MATERIALS AND METHODS

Cell Culture, Growth, and Fractionation

Growth of primary bovine PAEC in T-75 flasks, 10-cm petri dishes, or six-well plates has been described previously (34, 42, 43, 55). For experiments with MCT and under hypoxic conditions, cultures were used between passages 4 and 20. Senescent cultures, characterized by enlarged vacuolar cells, had markedly reduced growth rate and were generally used beyond passage 20. For hypoxia treatment, rapidly growing PAEC were incubated in 1.5% (vol/vol) oxygen for 4 days.

MCTP was prepared, stored, and added as described by Mattocks et al. (36) as reported by us previously (34, 42, 43, 55). In all experiments with MCTP, almost-confluent cultures of PAEC were treated with 0.4% (vol/vol) dimethylformamide (DMF; “control cultures”) or MCTP in DMF (equivalent to 200 μM MCT with 25–30% conversion to the active pyrrole). Megalocytosis began to develop within 12–18 h and was fully evident by 24–48 h. Cultures were usually used 4 days after addition of MCTP.

PAEC cultures were subjected to detergent-based fractionation in 100-mm plates sequentially into a Brij-58 cytoplasmic extract and a deoxycholate (DOC) cytoplasmic extract as described previously (42). Golgi elements and cytoplasmic vesicular fractions were purified as described by Xu and Shields (64) and as modified by us by addition of a second shallower gradient to the procedure to separate the various subclasses of eNOS-containing vesicles (11; unpublished observations). PAEC were harvested directly in 0.25 M sucrose-containing isotonic buffer with 10 mM TrisCl (pH 7.4) and 1 mM MgCl2 (64) and disintegrated with a Tekmar Tissumizer (15 s). Total protein was estimated in the postnuclear supernatant (PNS) using the Bradford reagent (Bio-Rad). Protein-matched amounts of the PNS derived from control or MCTP-treated PAEC were used to prepare Golgi membranes and cytoplasmic vesicles by sucrose gradient flotation as described by Xu and Shields. To obtain an approximately equal amount of total protein in the PNS for this purification, we typically used four cultures of 100-mm plates for controls and eight cultures for MCTP-treated cells. For analyses of the first gradient (a step gradient with ~6 ml of cell lysate adjusted to 1.4 M sucrose at the bottom, overlaid with ~4 ml of 1.2 M sucrose and ~1.5 ml of 0.8 M sucrose and centrifuged in a Beckman SW41Ti rotor at 35,000 rpm for 4.5 h), we collected 500-μl aliquots from the top. The band between 1.2 and 0.8 M sucrose represents the Golgi membranes, and the ER is at the bottom (64; unpublished observations). The Golgi band was collected, adjusted to 1.28 M sucrose, and floated up a second shallower gradient (a step gradient with the sample adjusted to 1.28 M sucrose at the bottom overlaid with ~0.66 ml each of 1.13, 1.05, 0.96, 0.85, and 0.5 M sucrose, respectively, and centrifuged in a Beckman SW50.1Ti rotor at 35,000 rpm for 18 h) for further separation of eNOS- and Cav-1-containing vesicles (11; unpublished observations). For analyses, fractions (600 μl) were collected from the top of the second gradient.

Immunofluorescence Analysis

Immunofluorescence was analyzed as described previously (34, 42, 43, 54, 55). Cells were fixed using 4% cold paraformaldehyde and permeabilized with 0.1% Triton X-100. Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes, Eugene, OR). Nuclei were visualized using 4′,6-diamino-2-phenylindole. Images were collected using a Leitz epifluorescence microscope system equipped with a black-and-white charge-coupled device camera and then rendered in pseudocolor or an MRC 1024 ES (Bio-Rad) confocal microscopy system with a black-and-white charge-coupled device camera and then rendered in pseudocolor. All data in each experiment were collected at identical image settings. Fluorescence intensity was measured using NIH Image J software.

Immunopanning Using Protein A Magnetic Beads

Protein A magnetic beads (New England Biolabs, Beverly, MA) were used for immunopanning essentially as described previously (55). Beads were blocked with 5% nonfat dry milk overnight and washed three times with PBS before incubation with samples. Protein-matched aliquots from respective cytoplasmic extracts were adjusted to 0.1% SDS in buffer containing 20 mM TrisCl, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and 1 mM PMSF (15, 42, 55) and incubated with respective rabbit polyclonal antibodies overnight at 4°C. Thereafter, preblocked and washed beads were added to the protein samples for 1 h at 4°C, washed six times with 0.1% SDS-containing buffer, and finally dissolved in Laemmli sample buffer for Western blot analysis. Immunopanning was also performed without SDS in the buffers.

Western Blot Analysis

Western blot analyses were carried out as described previously (34, 42, 43, 54, 55). NIH Image J software was used for quantitation.

Live-Cell Imaging Experiments

Imaging of caveolar and cytoplasmic NO using 4,5-diaminofluorescein diacetate fluorescence. Caveolar and cytoplasmic NO was imaged using the membrane-permeant probe 4,5-diaminofluorescein diacetate (DAF-2DA) as described previously (29, 41, 45). For fluorescence analysis, PAEC were plated in six-well plates and treated with MCTP in DMF or with DMF alone (as controls) 1 day after they were plated. Megalocytosis was allowed to develop over the next 4 days. The cultures were washed with PBS and replenished with...
Hanks’ balanced salt solution (HBSS) containing 0.1 mM L-arginine. The cells were loaded with 10 μM DAF-2DA for 30 min at 37°C. Basal fluorescence was measured using the MRC 1024 ES (Bio-Rad) confocal microscopy system (in the green channel). The cultures were then exposed to 3 μM ionomycin (53). To verify that DAF-2DA signals were derived from NO, cultures were treated with the inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM) for 72 h before imaging (48). For evaluating the effect of the exocytosis inhibitor phenylarsine oxide (PAO), cultures were treated first with 10 μM DAF-2DA and 15 min later with 10 μM PAO (33, 60), and data were collected after a total of 30 min of DAF-2DA loading. DAF-2DA and ionomycin were purchased from Calbiochem (San Diego, CA) and PAO and L-NAME from Sigma-Aldrich (St. Louis, MO).

Imaging of ER and lysosomal compartments. ER Tracker Red (glibenclamide-BODIPY FL) and Lyso Tracker Red (DND-99) were purchased from Molecular Probes (Eugene, OR) and used as directed by the manufacturer. ER Tracker was used at a final concentration of 1 μM and Lyso Tracker at 100 nM. Although Lyso Tracker was used in serum-containing medium or after transfer of cultures to HBSS (for coinaging with DAF-2DA), ER Tracker was always used in HBSS. There was no spillover between the red (ER Tracker or Lyso Tracker) and green (DAF-2DA) channels in confocal microscopy. Ionomycin was added as described above for DAF-2DA. For subsequent immunofluorescence analyses, cells were fixed after they were loaded as mentioned above and stained with or without permeabilization with 0.1% Triton X-100.

Antibodies and Other Reagents

Rabbit polyclonal antibodies to eNOS and Cav-1 and murine monoclonal antibody to Hsc70 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and murine monoclonal antibodies to GM130, clathrin heavy chain, 90-kDa heat shock protein (HSP90), BiP, KDEL, syntaxin-6, GS28, and Vti1a from BD Biosciences (Transduction Laboratory, San Diego, CA). Rabbit polyclonal antibody (PAb) to NOSTRIN was a gift from Prof. Werner Muller-Esterl (University of Frankfurt Medical School, Frankfurt, Germany), and PAb to p115 was a gift from Dr. Dennis Shields (Albert Einstein College of Medicine, New York, NY).

Statistical Analysis

Statistical analysis was carried out using two-tailed Student’s t-test and Microsoft Excel software.

RESULTS

Loss of Caveolar eNOS and Its Cytoplasmic Sequestration in MCTP-Treated PAEC

We previously showed a block in trafficking of proteins such as Cav-1 from the Golgi to plasma membrane caveolae/lipid rafts after exposure of endothelial cells to MCTP (34, 55). Inasmuch as the localization of eNOS to the cell surface/caveolae or to internal membranes or the cytosol plays a critical role in the regulation of eNOS enzymatic activity and NO production (65), we investigated the subcellular compartment(s) in which eNOS was localized in megalocytic endothelial cells. We used immunofluorescence, ER Tracker and Lyso Tracker labeling, and cell fractionation analyses to address this question.

A double-label immunofluorescence study of eNOS localization compared with cis-Golgi scaffolding protein and tether GM130 localization in endothelial cells is shown in Fig. 1. Parenthetically, GM130 is known to rapidly shuttle between the ER and the Golgi. In control cells, eNOS was present at the plasma membrane, as well as in the Golgi, together with GM130; however, in MCTP-treated megalocytic cells, a loss of eNOS from the cell surface and a large perinuclear accumulation of eNOS, only partially colocalizing with GM130, were observed. Additional double- and triple-label immunofluorescence studies were carried out using a variety of subcellular markers to define the non-GM130-containing cytoplasmic compartment into which eNOS was sequestered. Figures 2 and 3 summarize some of the data obtained. Although in all experiments, plasma membrane and discrete juxtanuclear eNOS (indicative of localization to caveolae and the Golgi) were present in untreated endothelial cells, there was a significant loss of eNOS from the cell surface in MCTP-treated megalocytic cells. After MCTP, the internally sequestered eNOS colocalized strongly with the ER marker KDEL (Fig. 2A) and clathrin heavy chain, a protein known to play a critical role in vesicular trafficking from the trans-Golgi (Fig. 2A). Figure 2B shows that, in addition to GM130, eNOS showed a

![Figure 1](http://ajpheart.physiology.org/)

Fig. 1. Cytoplasmic sequestration of endothelial nitric oxide synthase (eNOS) in monocrotaline (MCT) pyrrole (MCTP)-induced megalocytosis in pulmonary artery endothelial cells (PAEC). PAEC in 6-well plates were treated with MCTP or dimethylformamide (DMF), and megalocytosis was allowed to develop. On day 4, cells were fixed using the cold paraformaldehyde-Triton X-100 fixation protocol, and immunofluorescence analyses were performed using anti-eNOS polyclonal antibody (PAb) and a monoclonal antibody (MAb) against the cis-Golgi marker GM130. Scale bar, 50 μm.
high degree of colocalization with other Golgi markers such as syntaxin 6 (a trans-Golgi marker) and the Golgi SNAREs GS28 and Vti1a. However, as with GM130, the overlap between eNOS and these additional Golgi markers was only partial, indicating that although considerable eNOS was trapped in the Golgi, this is not the only cytoplasmic compartment into which eNOS was sequestered: the KDEL colocalization indicated that sites of eNOS sequestration included the ER.

Changes in the subcellular localization of eNOS suggested possible changes in the interaction of eNOS with protein partners such as Cav-1, HSP90 (10, 44), and the eNOS adaptor protein NOSTRIN (21). In megalocytotic endothelial cells, there were large accumulations of perinuclear Cav-1, as we reported earlier (34, 55), and HSP90 and eNOS largely, but not completely, colocalized with Cav-1 (Fig. 2A). In regions in the cytoplasm, eNOS was free of Cav-1 and vice versa (Fig. 2A). Additionally, partial colocalization of eNOS with HSP90 was observed in the perinuclear region. Immunofluorescence analysis of changes in the localization of NOSTRIN (Fig. 3) revealed that although localization of NOSTRIN changed after treatment with MCTP in endothelial cells (from diffuse...
cytoplasmic in controls to cell centric aggregates in megalocytic cells, suggestive of changes in membrane trafficking), NOSTRIN only partially colocalized with eNOS and GM130 (data not shown). Consistent with recent reports of the role of NOSTRIN in internalizing eNOS (21), we observed cytoplasmic vesicles containing NOSTRIN and eNOS (Fig. 3). However, cytoplasmic vesicles that contained NOSTRIN but were largely free of eNOS were also observed (Fig. 3). Thus, although there was clear evidence for redistribution of the eNOS adaptor NOSTRIN in megalocytosis to a cell centric location, eNOS and NOSTRIN were only partially colocalized.

**ER Tracker and Lyso Tracker Studies**

The colocalization of eNOS in megalocytic endothelial cells with the ER marker KDEL suggested that at least part of this sequestered eNOS may be trapped in the ER. Additionally, another relevant question was whether any of the eNOS was in lysosomes, because an eventual reduction in total eNOS levels has been reported in MCT-induced PH (19, 26, 61), and it might well be that the mislocalization of eNOS was a prelude to its lysosomal degradation. To answer these questions, we used a combination of live-cell imaging and immunofluorescence studies (Figs. 4 and 5).

In live cells, we observed a markedly increased lysosomal compartment in megalocytic endothelial cells (Fig. 4A). However, immunostaining for eNOS revealed a clear separation between the subcellular localization of eNOS and lysosomes (Fig. 4B, distinct separation between red lysosomal vesicles and green eNOS). Live-cell imaging of ER Tracker (Fig. 5) revealed increased ER in megalocytic cells, consistent with prior electron-microscopic data (1, 37). Immunostaining for eNOS revealed substantial, but not complete, colocalization between eNOS and ER Tracker.

Thus, in MCTP-induced megalocytosis, there was a loss of eNOS from the plasma membrane, with sequestration in heterogeneous cytoplasmic compartments, which partially overlapped the Golgi and the ER. These internal eNOS compartments were largely separate from lysosomes but included a Cav-1-positive subset.

**Cell Fractionation Studies**

Cell fractionation studies were carried out to isolate and characterize cytoplasmic eNOS-containing organelles. Figure 6A shows a two- to threefold increase in the total eNOS protein levels in cytoplasmic extracts of MCTP-treated PAEC, accompanied by increases in Cav-1 and NOSTRIN, but little change in HSP90 or the chaperones Hsc70 and GRP78/BiP.

The presence of this eNOS in cytoplasmic organelles in the postnuclear supernatant fraction of endothelial cells was investigated using two sequential flotation gradients to separate various components (11, 64; unpublished observations). These analyses are summarized in Fig. 6, B–D. First, there was an increase in eNOS and Cav-1 in cytoplasmic organelles after treatment with MCTP (the gradients in Fig. 6B were run using protein-matched aliquots of the postnuclear supernatants). eNOS was present near the bottom of each gradient, together with the ER marker BiP, in heterogeneous fractions up the gradient and peaked in fractions 5 and 6, together with Cav-1 in fraction 6. HSP90 cofractionated with eNOS at the bottom of the first gradient (together with the ER marker BiP). From previous characterizations (64; unpublished observations), fractions 5 and 6 at the 0.8 M-1.2 M sucrose interface are known to be enriched in Golgi membranes. Remarkably, although the lightest of these fractions (fraction 5) contained significant eNOS, its Cav-1 content was relatively depleted, and fractions 5 and 6 were free of HSP90. This separation between eNOS and Cav-1 was enhanced when fractions 5 and 6 from Fig. 6B were refloated up a shallower gradient (Fig. 6C). Thus there appeared to be at least several pools of eNOS in cytoplasmic vesicular elements: the heaviest fraction contained the ER marker BiP, intermediate density heterogeneous fractions contained Cav-1, a light fraction (fraction 6) was coincident with Cav-1 and the Golgi, and the lightest-density eNOS (fraction 5 in Fig. 6B, also see Fig. 6C) was depleted/devoid of Cav-1. These cell fractionation data confirm the heterogeneous cytoplasmic localization of eNOS deduced from the immunofluorescence data in Figs. 1–5.

The partial cofractionation of eNOS with Cav-1 led us to investigate whether there was significant protein-protein interaction between eNOS and Cav-1. We were unable to detect significant interaction between eNOS and Cav-1 in Brij-58 cytoplasmic extracts or DOC extracts in the presence of SDS in immunopanning assays (Fig. 6D,i). However, the DOC extracts, which contain proteins tightly associated with the outer nuclear membrane (and, thus, the circumnuclear ER), provided evidence for a subset of eNOS that was complexed with Cav-1 (Fig. 6D,ii), provided that SDS was omitted from the immunopanning assay. These data are consistent with an interaction between eNOS and Cav-1, at least in the tight circumnuclear ring of eNOS (extracted by DOC) observed by immunofluorescence of MCTP-treated cells (Figs. 1 and 2). Consistent with our cell fractionation data showing the presence of HSP90 with eNOS and Cav-1 only at the bottom of the first fractionation gradient, using our assay conditions, we did not observe substantial association of HSP90 with eNOS or Cav-1 in immunopanning (data not shown).

**Changes in the Golgi and in eNOS and Cav-1 Localization in Hypoxia and Senescence**

An ever-persistent question in the PH literature is the relevance of the MCT model to other experimental models such as hypoxia-induced PH and to human disease. We investigated changes in the Golgi and in eNOS under hypoxic and senescent conditions. Figure 7A shows the enlargement (megalocytosis) and vacuolation of PAEC 4 days after exposure to 1.5% (vol/vol) oxygen. Figure 7B shows the dramatic enlargement of the Golgi after hypoxia, with the altered localization of GM130, GS28, syntaxin 6, and p115 from a discrete juxtanuclear organelle to a circumnuclear distribution. As with MCTP, these changes were indicative of a major dysfunction of the Golgi organelle under hypoxic conditions in PAEC. Figure 7C shows the loss of eNOS from the plasma membrane and its perinuclear sequestration, together with circumnuclear GM130, GS28, syntaxin 6, and p115, under hypoxic conditions. Moreover, Cav-1 also accumulated in the circumnuclear GM130-positive Golgi compartment under hypoxic conditions. These changes in the Golgi and in eNOS localization after hypoxia are similar to those induced by MCTP.

Data in Fig. 8 show changes in the Golgi and in eNOS localization in senescent PAEC. Figure 8A shows megalocyto-
Fig. 4. Cytoplasmic eNOS in MCTP-induced megalocytosis is separate from lysosomes. A: control and MCTP-treated PAEC in 6-well plates were loaded with Lyso Tracker, and live cells were imaged. B: Lyso Tracker-loaded cells were fixed with cold paraformaldehyde and stained with anti-eNOS PAb (without permeabilization). Similar results were obtained with permeabilization with 0.1% Triton X-100 (not shown). Scale bars, 50 μm.
Fig. 5. Partial colocalization of cytoplasmic sequestered eNOS with ER in MCTP-induced megalocytosis. A: control and MCTP-treated PAEC in 6-well plates were loaded with ER Tracker in Hanks’ balanced salt solution (HBSS), and live cells were imaged. B: ER Tracker-loaded cells were fixed without permeabilization and stained with anti-eNOS PAb. Scale bars, 50 μm.
tosis in senescent PAEC, with a large number of enlarged vacuoles. The Golgi tethers and SNAREs GM130, GS28, Vti1a, syntaxin 6, p115, and giantin change from discrete juxtanuclear to circumnuclear structures. Figure 8C shows the sequestration of eNOS with GM130, GS28, syntaxin 6, p115, and Cav-1 in senescent cultures. eNOS also colocalizes with Cav-1 in the cytoplasm. Thus Golgi dysfunction and the cytoplasmic sequestration of eNOS in PAEC are observed in endothelial cells not only after MCTP but also after hypoxia and senescence.

Functional Consequences of Altered eNOS Localization: Live-Cell Imaging Studies of Caveolar and Cytoplasmic NO

It is known that the enzymatic activity of eNOS is exquisitely regulated by subcellular location (65). What are the functional consequences of the loss of plasma membrane eNOS and its internal sequestration after MCTP, hypoxia, and senescence? We used live-cell imaging with the cell-permeable NO reporter DAF-2DA to investigate the subcellular location of NO production in PAEC after MCTP, hypoxia, and senescence. Our observations are summarized in Figs. 9–11.

Figure 9A shows that control PAEC exhibited discrete regions of caveolar NO production (DAF-2DA signals) at the plasma membrane (arrowheads). Additionally, there was a loss of DAF-2DA signals from MCTP-treated cultures after stimulation with ionomycin, which is investigated further in Figs. 10 and 11. Therefore, a functional consequence of MCTP-induced megalocytosis of PAEC is decreased NO production in cell surface caveolae.

As expected, the NO fluorescence in control PAEC increased in intensity after stimulation of eNOS with ionomycin (Fig. 9B), and the basal and ionomycin-stimulated increases were inhibited by L-NAME (Fig. 9C and D). In contrast, Fig. 9B shows a loss of DAF-2DA signals from MCTP-treated cultures after stimulation with ionomycin, which is investigated further in Figs. 10 and 11. Therefore, a functional consequence of MCTP-induced megalocytosis of PAEC is decreased NO production in cell surface caveolae.

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The absence of caveolar DAF-2DA signals in MCTP-treated cultures compared with controls is again evident in Fig. 9E.

The similarities of the effects of MCTP, hypoxia, and senescence extended to NO production and its regulation. Cell surface caveolar DAF-2DA fluorescence was absent after senescence and hypoxia (Fig. 10, A and B). As with MCTP, although there was some overlap of the internal DAF-2DA signal in senescent cultures with the ER, the bulk of it was free of ER and lysosomes (Fig. 10C).

**Fig. 7.** Endothelial cell megalocytosis and cytoplasmic trapping of eNOS with Golgi tethers and N-ethylmaleimide-sensitive factor-attachment protein receptor proteins (SNAREs) after hypoxia. PAEC in 6-well plates were incubated in 1.5% (vol/vol) oxygen (hypoxia) or room air, and phase contrast images were obtained on day 4 (A). B and C. cells were then fixed using cold paraformaldehyde-Triton X-100 and stained for eNOS, Cav-1, Golgi tethers (GM130, p115), and SNAREs (syntaxin 6, GS28), singly (B) or in pairs (C). Scale bars, 50 μm (A and C) and 25 μm (B).
Exposure to ionomycin led to a dynamic and rapid loss of DAF-2DA fluorescence from MCTP-treated and senescent cultures (Figs. 9B and 10D). To investigate the nature of this loss, we performed live-cell time-course experiments (Fig. 10, D–F). Although the addition of ionomycin caused a large increase in the mean DAF-2DA fluorescence per cell in control PAEC cultures (DAF-2DA fluorescence was 63.7% higher at 15 min after ionomycin than at time 0 in control cultures, \( P < \))

Fig. 8. Endothelial cell megalocytosis and cytoplasmic trapping of eNOS with Golgi tethers and SNAREs and Cav-1 after senescence. Senescent PAEC that had undergone replicative growth arrest were plated in 6-well plates; control cultures were “young” PAEC (passages 4–20) that had been plated contemporaneously. A: phase contrast micrographs of respective culture. B and C: cultures were fixed with cold paraformaldehyde-Triton X-100 and stained for eNOS, Cav-1, Golgi tethers (GM130, p115, gian tin), and SNAREs (syntaxin 6, GS28, and Vti1a) singly (B) or in pairs (C). Scale bars, 50 \( \mu \)m.
in MCTP-treated and senescent cultures, mean fluorescence intensities per cell decreased \[\text{DAF-2DA fluorescence in MCTP-treated cultures was 71.4\% lower at 15 min after ionomycin than at time 0 (}\text{P} < 0.001\text{)}\] and DAF-2DA fluorescence in senescent cultures was 24.3\% lower than at time 0 \[\text{(P < 0.05)}\]. Additionally, at time 0, mean fluorescence intensity per cell was 49.7\% greater in MCTP-treated cultures than in control cultures \[\text{(P < 0.001)}\]. However, although the mean fluorescence intensity per cell in MCTP-treated and senescent cultures decreased after ionomycin, we observed a circumnuclear DAF-2DA fluorescent ring in these cultures that persisted and did not decrease over time. This signal appeared to be in the same subcellular location as the cytoplasmically sequestered eNOS in MCTP-induced megalocytosis and in senescence (Figs. 1–5 and 8).

What was the cause of this decrease in the DAF-2DA fluorescence intensity in megalocytosis? Ionomycin is a Ca\(^{2+}\) ionophore, and treatment with such ionophores induces massive and very rapid exocytosis (3, 7, 9, 24). Under phase-contrast microscopy, after treatment with ionomycin, several very large vesicles were observed in MCTP-treated cultures; these vesicles appeared to fuse with the plasma membrane (data not shown). The compound PAO has been used to block Ca\(^{2+}\)-induced exocytosis (33, 60). Pretreatment of MCTP-treated cultures with PAO prevented the ionomycin-induced exocytosis and loss of DAF-2DA signals (Fig. 10, F and G).

Although the addition of ionomycin caused a large increase in the mean DAF-2DA fluorescence per cell in control PAEC (DAF-2DA fluorescence was 36.94\% higher 30 min after addition of ionomycin than at time 0 in control cultures, \text{P} < 0.05), DAF-2DA fluorescence of MCTP-treated cultures decreased by 70.3\% in the same time interval \[\text{(P < 0.001)}\], whereas addition of PAO to the MCTP-treated cultures before ionomycin treatment prevented this decrease in the mean DAF-2DA fluorescence per cell in control PAEC (DAF-2DA fluorescence was 36.94\% higher 30 min after addition of ionomycin than at time 0 in control cultures, \text{P} < 0.05). Pretreatment of MCTP-treated cultures with PAO prevented the ionomycin-induced exocytosis and loss of DAF-2DA signals (Fig. 10, F and G).

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Although the addition of ionomycin caused a large increase in the mean DAF-2DA fluorescence per cell in control PAEC (DAF-2DA fluorescence was 36.94\% higher 30 min after addition of ionomycin than at time 0 in control cultures, \text{P} < 0.05), DAF-2DA fluorescence of MCTP-treated cultures decreased by 70.3\% in the same time interval \[\text{(P < 0.001)}\], whereas addition of PAO to the MCTP-treated cultures before ionomycin treatment prevented this decrease in the mean DAF-2DA fluorescence per cell in control PAEC (DAF-2DA fluorescence was 36.94\% higher 30 min after addition of ionomycin than at time 0 in control cultures, \text{P} < 0.05). Pretreatment of MCTP-treated cultures with PAO prevented the ionomycin-induced exocytosis and loss of DAF-2DA signals (Fig. 10, F and G).
DAF-2DA in MCTP-induced megalocytosis, whereas it did not cause such an effect in control cultures. This result further shows that the specific cytoplasmic sequestration of eNOS in megalocytotic PAEC is aberrant and is in a compartment(s) that can be rapidly exocytosed on exposure to ionomycin.

Previous studies had identified lysosomes as comprising one compartment that was rapidly exocytosed in response to increased intracellular Ca$^{2+}$ (24). Yet our data showed that eNOS and NO fluorescence were separable from the lysosomal compartment (Figs. 4 and 10C). To investigate this apparent discrepancy, we used two-color live-cell imaging of PAEC loaded with DAF-2DA as well as with Lyso Tracker or ER Tracker (Fig. 11). The data (Fig. 11A) show the dramatic and rapid loss of the lysosomal compartment in control, MCTP-treated, and senescent PAEC on exposure to ionomycin. In comparison, the loss of DAF-2DA fluorescence was distinctly slower (control PAEC showed an increase of this fluorescence), and ionomycin did not affect fluorescence of ER Tracker (Fig. 11B). Taken together, these data show that although the cytoplasmic NO-producing compartment in megalocytotic PAEC is aberrant, it is not lysosomal (consistent with data in Fig. 4).

**DISCUSSION**

MCT, hypoxia, and senescence produce a Golgi blockade in PAEC, characterized by the loss of cell surface/caveolar eNOS and its sequestration in aberrant heterogeneous subcellular elements including, but not limited to, the Golgi and the ER (Fig. 12). This cytoplasmically sequestered eNOS ...
includes Cav-1-positive elements but is largely separate from lysosomes. Live-cell imaging of NO showed the loss of caveolar NO in PAEC after MCT treatment, hypoxia, and senescence. Thus the location of eNOS within an endothelial cell and its function are dramatically altered in megalocytosis.

Although it is known that MCT-induced PH is associated with a decrease in NO production from the pulmonary vasculature (46), there have been conflicting reports about changes in levels of eNOS protein after MCT administration. Although MCT treatment is known to increase eNOS mRNA (46, 61), levels of eNOS protein have been reported to increase (50), not

Fig. 11. Live-cell subcellular localization of cytoplasmic NO production in megalocytotic endothelial cells is separable from lysosomes but partially colocalizes with the ER. PAEC in 6-well plates were cultured as described in the legend of Fig. 10, C and D. Respective cultures were loaded with DAF-2DA and either Lyso Tracker or ER Tracker. After completion of baseline fluorescence measurements, cultures were exposed to 3 μM ionomycin, and 2-color images were obtained using the time-series software at 1-min intervals for 15–20 min. Scale bars, 25 μm.
change (35, 25) or primarily decrease (19, 26, 61). The present data demonstrating loss of cell surface/caveolar eNOS and its cytoplasmic sequestration in megalocytosis, despite sustained and even elevated total eNOS protein levels per cell, provide a mechanistic basis for understanding the consistent observation of a loss of NO in the pulmonary vasculature. In endothelial cells, eNOS localized to the plasma membrane is most effective in the production of NO (65) and is more responsive to agonists such as Ca\textsuperscript{2+} and Akt (65). Although eNOS localized to the cis-Golgi can be upregulated by phosphorylation (12, 23), that in the trans-Golgi has been reported to be an ineffective producer of net NO (12). Also, mutants of eNOS leading to a cytosolic localization are also known to be less efficacious in the production of NO than membrane-localized eNOS (6). Moreover, NO produced intracellularly reacts very quickly and has a very limited region of diffusion (14). Thus, although ER/Golgi-localized eNOS can be stimulated to produce NO, its contribution to vascular NO level is limited, with cell surface/caveolar NO being the major contributor. Thus our data showing not only the loss of eNOS protein from the cell surface but also the loss of caveolar NO in live-cell imaging studies in MCTP-treated PAEC are consistent with the reported in vivo reduction in NO levels in pulmonary arteries in MCT-induced PH.

Prior studies from this laboratory have drawn attention to the disruption of subcellular trafficking and a Golgi blockade (Fig. 12) in MCTP-treated lung endothelial and epithelial cells in cell culture and in vivo (34, 42, 45, 55). However, an often-heard critique of the MCT-PH model concerns its relevance to other models of PH, such as that resulting from hypoxia, and to clinical disease. We previously suggested that disruption of intracellular membrane trafficking may indeed be a common underlying mechanism in various forms of PH (43; unpublished observations). The literature clearly provides a basis for this suggestion. Although the term megalocytosis as such has not been used, except in the context of the pyrrolizidine alkaloids (4; for review see Ref. 55), histological descriptions of “plump” and “enlarged” endothelial and smooth muscle cells with increased vacuolation, Golgi stacks, and ER in hypoxic and primary PH can be found (16, 18, 22, 27, 38, 39, 57). Meyrick and Reid (39) report “hypertrophied smooth muscle cells [with] a significant increase in relative areal proportions of Golgi apparatus and rough sarcoplasmic reticulum” in the tunica media of the hilar pulmonary artery of rats with hypoxia-induced PH. Their description of subcellular changes in the intima of hilar arteries of rats with hypoxic PH is as follows: “[the] thickened endothelial cells [have] a more extensive Golgi apparatus than normal and many have swollen cisternae of rough endoplasmic reticulum” (38). Increases in Golgi stacks in pericytes in hypoxic PH in rats have also been described (38). Increased vacuolation and increased Golgi stacks and rough ER profiles in the intima (endothelium) and media (smooth muscle) were reported by Jaenke and Alexander (22) in hypoxia-induced PH in bovine species. Particularly noteworthy are reports of increased accumulations of Weibel-Palade bodies in endothelial cells in rats exposed to neonatal hypoxia (27), which clearly points to inhibited anterograde vesicular trafficking (these bodies are exocytotic vesicles). Similar increases in Weibel-Palade bodies have been described in human primary PH (18). An abundance of rough ER in the endothelium in plexiform lesions of primary PH has also been recorded (57). Moreover, in hypoxic PH, Murata et al. (44) showed decreased DAF-2DA fluorescence and vascular relaxation in pulmonary arteries from hypoxic animals, with perinuclear accumulation of eNOS, together with Cav-1, in the Golgi. Additionally, increased Weibel-Palade bodies and enlargement of cells with increased ER and lysosomes have been a part of the literature of senescent endothelial cells (28, 62), again suggestive of an anterograde trafficking block. There are clear known relations between age and the development of familial PH in humans (52) and between age and the loss of plasma membrane eNOS in aged rats (56). The clinical syndrome of familial PH most commonly presents in the third decade in women and in the fourth decade in men (52). Moreover, familial PH shows genetic anticipation, i.e., the disease appears at an earlier age in offspring of patients (52). In the rat model of aging, Smith et al. (56) report a loss of plasma membrane eNOS in the aortic endothelium of aged rats, with decreased NO-dependent aortic vasodilation.

Our experiments with hypoxic and senescent endothelial cells provide experimental evidence for changes in vesicular trafficking, i.e., the Golgi tethers and SNAREs with consequent mislocalization of critical vasorelevant proteins such as eNOS and Cav-1. Functionally, this mislocalization of eNOS in hypoxia and senescence was associated with a loss of NO production at cell surface caveolae, similar to our observations with MCTP. Thus our data show that cellular enlargement or megalocytosis resulting from MCT, hypoxia, or senescence derive from the same underlying mechanism: a Golgi blockade accompanied by defective vesicular trafficking (Fig. 12). The observation that ionomycin was able to induce rapid exocytosis of eNOS and DAF-2DA fluorescence in MCTP-treated and senescent cultures, but not in control PAEC, further emphasizes the aberrant cytoplasmic location of eNOS in megalocytosis.

In conclusion, MCT, hypoxia, and senescence lead to a disruption of vesicular trafficking and Golgi function in endothelial cells, characterized by the aberrant sequestration of eNOS in the cytoplasm and a loss of caveolar NO production. A disruption of the function of vesicle tethers, SNAREs, and SNAPs and Golgi blockade underlie all three situations (Fig. 12).
NOTE ADDED IN PROOF:
Sessa and colleagues reported very recently that Golgi-targeted mutants of eNOS produced NO locally within this subcellular compartment and thus modified the Cys-rich and redox-sensitive intracellular NSF by S-nitrosylation (Iwakiri Y, Satoh A, Chatterjee S, Toonre DK, Chalouni CM, Fulton, D, Groszmann RJ, Shah VH, Sessa WC. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein S-nitrosylation and protein trafficking. Proc Natl Acad Sci USA 103: 19777–19782, 2006). They showed that a consequence was a block in trafficking through the Golgi of cargo proteins such as the vesicular stomatitis virus G protein. Thus, from the point of view of the present article, we suggest that the aberrantly sequestered eNOS after MCTP, hypoxia or senescence would generate intracellular NO, which would further inhibit NSF and membrane trafficking through the Golgi in a self-reinforcing inhibitory loop.

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