Golgi, trafficking, and mitosis dysfunctions in pulmonary arterial endothelial cells exposed to monocrotaline pyrrole and NO scavenging

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Lee J, Reich R, Xu F, Sehgal PB. Golgi, trafficking, and mitosis dysfunctions in pulmonary arterial endothelial cells exposed to monocrotaline pyrrole and NO scavenging. Am J Physiol Lung Cell Mol Physiol 297: L715–L728, 2009. —Although the administration of monocrotaline (MCT) into experimental animals is in widespread use today in investigations of pulmonary arterial hypertension (PAH), the underlying cellular and subcellular mechanisms that culminate in vascular remodeling are incompletely understood. Bovine pulmonary arterial endothelial cells (PAECs) in culture exposed to monocrotaline pyrrole (MCTP) develop “megalocytosis” 18–24 h later characterized by enlarged hyperplastidic cells with enlarged Golgi, mislocalization of endothelial nitric oxide synthase away from the plasma membrane, decreased cell-surface/caveolar nitric oxide (NO), and hypo-S-nitrosylation of caveolin-1, clathrin heavy chain, and N-ethylmaleimide-sensitive factor. We investigated whether MCTP did in fact affect functional intracellular trafficking. The NO scavenger (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) and the NO donor diethylenetriamine NONOate were used for comparison. Both MCTP and c-PTIO produced distinctive four- to fivefold enlarged PAECs within 24–48 h with markedly enlarged/dispersed Golgi, as visualized by immunostaining for the Golgi tethers/matrix proteins giantin, GM130, and p115. Live-cell uptake of the Golgi marker C5 ceramide revealed a compact juxtanuclear Golgi in untreated PAECs, brightly labeled enlarged circumnuclear Golgi after MCTP, but minimally labeled Golgi elements after c-PTIO. These Golgi changes were reduced by NONOate. After an initial inhibition during the first day, both MCTP and c-PTIO markedly enhanced anterograde secretion of soluble cargo (exogenous vector-expressed recombinant horse-radish peroxidase) over the next 4 days. Live-cell internalization assays using fluorescently tagged ligands showed that both MCTP and c-PTIO inhibited the retrograde uptake of acetylated low-density lipoprotein, transferrin, and cholera toxin B. Moreover, MCTP, and to a variable extent c-PTIO, reduced the cell-surface density of all receptors assayed (LDLR, TfnR, BMPR, Tie-2, and PECAM-1/CD31). In an important distinction, c-PTIO enhanced mitosis in PAECs but MCTP inhibited mitosis, even that due to c-PTIO, despite markedly exaggerated Golgi dispersal. Taken together, these data define a broad-spectrum Golgi and subcellular trafficking dysfunction syndrome in endothelial cells exposed to MCTP or NO scavenging.

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endothelial nitric oxide synthase (eNOS) from the plasma membrane of megalocytotic PAECs with the aberrant sequestration of eNOS in the Golgi and in cytoplasmic vesicles. There was marked enlargement of the Golgi with marked accumulation of diverse vesicle trafficking tethers and soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) and SNAP receptors (SNAREs) in the Golgi in both bovine PAECs in culture and in PAECs in the MCT/rat model (30, 42, 53–55, 57). Various cargo molecules such as Cav-1, eNOS, and bone morphogenetic receptor type-2 (BMPR-2) were trapped, at least in part, in the Golgi (55). The mislocalization of eNOS was accompanied by reduced cell surface/caveolar production of NO as assayed by live-cell 4–5-diaminofluorescein diacetate (DAF-2DA) imaging in PAEC culture (42). Biochemically, this resulted in the hypo-S-nitrosylation of the trafficking mediator proteins Cav-1, clathrin heavy chain, and N-ethylmaleimide-sensitive factor and of eNOS itself (38). This raised the question of whether, consequently, functional endocytic and caveolar trafficking would be inhibited in MCTP-exposed PAECs.

In the present study, we investigated whether the MCTP produced hypo-S-nitrosylation of trafficking mediator proteins in PAECs (38) translated into defects in functional intracellular trafficking and Golgi function. For comparison, we included the NO scavenger (4-carboxyphenyl)-4,4,5,5-tetramethylimodazoline-1-oxyl-3-oxide (c-PTIO) and the NO donor diethylamine NONOate (NONOate; BioMol International, Plymouth Meeting, PA) was used at 100 μM and diethylamine NONOate sodium salt hydrate (NONOate; Sigma-Aldrich, St. Louis, MO) at 400 μM with daily refreshing of the culture medium containing c-PTIO and NONOate. Phase-contrast microscopy was carried out using a Nikon Diaphot microscope equipped with a Nikon Coolpix digital camera.

**Immunofluorescence microscopy.** Immunofluorescence assays were performed essentially as described previously (38–42, 55–57). Typically, PAECs in 6-well plates appropriately exposed to MCTP and/or c-PTIO and/or NONOate (see respective figure legends in RESULTS) were fixed using 4% cold paraformaldehyde for 1 h and permeabilized using 0.1% Triton X-100. Fixed cultures were then stained using various combinations of goat and rabbit pAbs and murine mAbs. Respective donkey AlexaFluor 488- or AlexaFluor 594-tagged secondary antibodies were used in this study (Molecular Probes, Eugene, OR). Images were collected using a Nikon Eclipse 50i epifluorescence microscopy system (objectives: low-magnification Nikon Plan 10×/NA 0.25 and high-magnification Nikon Plan 40×/NA 0.65) equipped with a red-green-blue charged-couple device (CCD) camera and RS Image 1.9.2 software (Roper Scientific, Tucson, AZ). In epifluorescence microscopy, nuclei were demarcated using DAPI (Sigma-Aldrich). Alternatively, images were collected using an MRC 1024 ES (Bio-Rad) confocal laser scanning microscopy system (objective: Olympus Plan 10×/NA 0.25) with a black-and-white CCD camera using the manufacturer’s software and then rendered in pseudocolor. All data within each experiment were collected at identical imaging settings. Controls included omission of the primary antibody and peptide competition assays and the use of multiple different antibodies toward the same antigen (see our previous Refs. 38–42, 55–57). Because of the limitations of sensitivity of the existing confocal microscope available for this project, confocal imaging was limited to data collection in the green channel (such as using AlexaFluor 488), while the epifluorescence microscope was used for data collection in the green, red (such as with AlexaFluor 594), and blue (DAPI) channels.

Endogenous receptors on the surface of PAECs were displayed by carrying out “surface-accessible immunostaining” after paraformaldehyde fixation but before permeabilization with detergent using antibodies to extracellular domains of respective receptors. After quantitative imaging, the immunostaining was repeated with Triton X-100 (0.1%) permeabilization to display distribution of all of the respective antigens within the cell.

**Live-cell quantitative horseradish peroxidase secretion assay.** Gene transfer assays for horseradish peroxidase (HRP) secretion were carried out essentially as described by Connolly et al. (9) and by us earlier (55). Briefly, 1-day-old PAEC cultures in 6-well plates were transfected with the constitutive expression vectors for secreted HRP (pSRα.ssHRP of Ref. 9; 2.5 μg/well in triplicate wells for each experimental variable) using the lipofectamine reagent (Polyfect, Qiagen, Valencia, CA) and the manufacturer’s protocol. As a baseline control, PAECs were transfected with pcDNA3.1 vector alone. After 1 day, the culture medium was harvested (“0” day samples; 1 ml per well) and MCTP or c-PTIO was added. Thereafter, the medium was harvested and replenished (1 ml each time) every day for 5 days with the daily addition of fresh c-PTIO. The harvested medium samples were assayed quantitatively for HRP in triplicate (9, 55), the baseline values derived from pcDNA3.1-transfected cells were subtracted, and the HRP secretion data for days 1 to 5 were normalized to the HRP in the medium of the same well in the “0” day sample.

**Live-cell imaging of caveolar NO using DAF-2DA fluorescence.** This was carried out using the membrane-permeant probe DAF-2DA as described previously (38, 42). Briefly, PAEC cultures in 6-well plates were washed with PBS and replenished with HBSS medium containing 0.1 mM l-arginine (Arg-HBSS). The cells were then loaded with DAF-2DA (10 μM) for 20–30 min at 37°C, and the fluorescence was imaged using the Nikon epifluorescence microscope and RS Image software as indicated in Immunofluorescence microscopy.

**Live-cell endocytic/caveolar trafficking assays.** Ligand uptake and internalization assays were carried out using AlexaFluor 594-tagged transferrin, AlexaFluor 594-tagged cholera toxin subunit B (CTB; recombinant), and AlexaFluor 488-tagged acetyl low density lipoprotein (AcLDL) purchased from Invitrogen Molecular Probes and the manufacturer’s respective protocols. Typically, PAEC cultures in 6-well or 35-mm plates were washed with warm PBS and replenished with 0.5 ml each of growth medium containing the respective tagged ligands (10 μM final concentration). The cultures were incubated at
Fig. 1. Golgi dysfunction in megalocytotic pulmonary arterial endothelial cells (PAECs) exposed to monocrotaline pyrrole (MCTP) or nitric oxide (NO) scavenging. One-day-old confluent PAEC cultures in 6-well plates were exposed to MCTP once (equivalent to $\sim 50 \mu M$ of active pyrrole) followed by daily replenishment with normal growth medium or to (4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (c-PTIO; 100 $\mu M$) with daily replenishment with c-PTIO-containing medium for 2 (A), 5 (B and D), or 4 days (C). A: cultures were imaged under phase contrast (i) and switched to Arg-HBSS medium at 4°C (1 ml/well) containing BODIPY TR C5 ceramide (5 $\mu M$) for 30 min. Cultures were then washed and replenished with Arg-HBSS at 37°C for 30 min. Fifteen minutes into the latter incubation, 4–5 diaminofluorescein diacetate (DAF-2DA; 10 $\mu M$) was added and cells were imaged 15 min later using epifluorescence microscopy (DAF-2DA in the green channel and C5 ceramide in the red channel). A, ii: same fields in the two colors; scale bars = 50 $\mu m$. Insets: $\times 4$ zoomed-in views from within the larger frame. Puncta of green DAF-2DA fluorescence seen in controls indicating surface/caveolar NO (white arrows; Ref. 43) are lost after MCTP and c-PTIO. B, C, and D: cultures were imaged under phase contrast, then fixed, permeabilized, and immunostained for the Golgi tether/scaffolding proteins giantin and p115 (using rabbit pAbs) or GM130 (using a murine mAb), as well as for $\beta$-actin (goat pAb) and respective different secondary Abs. DAPI was used for nuclear DNA staining. B, ii: giantin and GM130 images are of same cells. In B, percentage of cells with Golgi enlargement/dispersal was as follows: controls 14.4 ± 3.6 (means ± SE over all images collected; $n = 315$ cells), MCTP 93.5 ± 2.4 ($n = 41$ cells; $P < 0.001$), and c-PTIO 97.6 ± 1.6 ($n = 63$ cells; $P < 0.001$). Rounded cells apparent as doublets in B, i (phase contrast) in the c-PTIO-treated culture are cells traversing through mitosis (see Figs. 8 and 9). Scale bars = 50 $\mu m$. 

A

PAEC: Two days’ treatment

Control | MCTP | c-PTIO

(i)

Phase

(ii)

DAF2-DA

C5-ceramide

B

PAEC: Five days’ treatment

Control | MCTP | c-PTIO

(i)

Phase

(ii)

Giantin

GM130

p115

D

$\beta$-actin
This was carried out in endoplasmic reticulum, and mitochondria). min at 37°C before imaging or exposing cells to ligand for 30 min in the mental Fig. 3; supplemental data for this article are available online at surface plus the internalized ligand (see Figs. 5 and 6 and Supplemental Fig. 1A, ii, top row). As described previously (42), DAF-2DA fluorescence confirmed the loss of cell surface/caveolar NO after MCTP and after c-PTIO (Fig. 1A, i, ii, bottom row and Supplemental Fig. 1B, top row). Critically, 1) the changes in endothelial morphology and C5 ceramide uptake could be reduced by the NO donor diethylamine NONOate (Supplemental Fig. 1A, 1), and 2) the changes were selective in that the uptake and compartmental localization of LysoTracker, MitoTracker, or ER-Tracker was not affected by either MCTP or c-PTIO (data not shown). These data 1) confirm the selective enlargement of a functional Golgi in MCTP-treated PAECs and that the Golgi in such cells retains the ability to concentrate C5 ceramide, 2) show that NO scavenging inhibited uptake and transport of

RESULTS

PAEC megalocytosis and Golgi enlargement/dispersal after MCTP and NO scavenging. Given our previous data showing Golgi enlargement in MCTP-treated PAECs assayed after fixation and detergent permeabilization (55), we investigated whether the live-cell Golgi marker BODIPY TR C5 ceramide would be taken up by such cells and whether it would then be transported to and concentrated in the enlarged Golgi (C5 ceramide is taken up through endocytosis and is then localized to Golgi membranes via retrograde trafficking; Ref. 45). For comparison, PAEC cultures were exposed to the NO scavenger c-PTIO (100 μM). Figure 1A and Supplemental Figure 1A i summarize the live-cell C5 ceramide uptake data as well as several controls. Phase-contrast microscopy showed that MCTP-treated PAECs were enlarged and angular, while c-PTIO-treated cells were enlarged and cuboidal (Fig. 1, A, i, B, ii, and D, and Supplemental Fig. 1A, top row). Strikingly, while BODIPY TR C5 ceramide localization revealed a compact juxtanuclear Golgi in untreated PAECs, C5 ceramide brightly labeled the enlarged circumnuclear Golgi after MCTP but minimally labeled Golgi elements after c-PTIO (Fig. 1A, ii, bottom row and Supplemental Fig. 1B, top row). Given our previous data showing tethers/scaffolding proteins giantin, p115, and GM130 in PAECs exposed to MCTP or c-PTIO, immunofluorescence images derived from the experiments in Fig. 1, B and C, were quantitated as described in MATERIALS AND METHODS to obtain the respective integrated intensities per cell. Data are expressed in terms of fold change compared with respective means derived from control cells. *P < 0.05, compared with respective untreated controls.

Antibody and inhibitor reagents. Rabbit pAb to giantin was from Abcam (Cambridge, MA) and that to p115 was a gift from the late Dr. Dennis Shields (Albert Einstein College of Medicine; Ref. 38). Murine mAbs to GM130 (Golgi Matrix 130) were purchased from BD Biosciences (Eugene, OR). Rabbit pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAb to giantin was from Abcam (Cambridge, MA) and that to p115 was a gift from the late Dr. Dennis Shields (Albert Einstein College of Medicine; Ref. 38). Murine mAbs to GM130 (Golgi Matrix 130) were purchased from BD Biosciences (Eugene, OR). Rabbit pAbs to eNOS, cav-1, LDL receptor (LDLR), and transferrin receptor (TfnR) and goat pAbs to BMPR-2, Tie-2, platelet endothelial cell adhesion molecule (PECAM-1), and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Respective AlexaFluor 488-, AlexaFluor 594-, or AlexaFluor 647-tagged secondary donkey antibodies to rabbit, goat or mouse IgG were from Invitrogen Molecular Probes (Eugene, OR). The STAT3 inhibitor indirubin E804 (43) was purchased from Sigma-Aldrich.

Statistical analyses. NCSS 2007 Statistical Analysis Software (Kaysville, UT) was used for comparisons between respective experimental groups and their matched controls using ANOVA tests (one-way and repeated measures). Post hoc comparisons were carried out using the Newman-Keuls Multiple comparison test.
C₅ ceramide to the Golgi, and 3) show that the effects of both MCTP and c-PTIO were, at least in part, due to a hypo-NO state in that NONOate reduced the changes.

Despite the inhibition of C₅ ceramide uptake after c-PTIO treatment, the Golgi elements in such cells were markedly increased in amount but extensively dispersed in the cytoplasm (Fig. 1, B and C, Fig. 2, and Supplemental Fig. 2). Figure 1B, i and Figure 2 show that 5 days after exposing PAECs to either MCTP or c-PTIO there was a marked increase in cell size. This was accompanied by marked enlargement and cytoplasmic dispersal of the Golgi as assayed by immunostaining for either of the Golgi tethers/scaffolding proteins giantin, GM130, or p115 (Fig. 1, B, ii and C, and Fig. 2). Consistent with prior observations that Golgi dispersal typically precedes entry of cells into mitosis (60), c-PTIO-treated cultures showed rounded doublets characteristic of mitotic cells (Fig. 1B, i; also see Figs. 8 and 9). The data in Supplemental Fig. 2, A and B, confirm that NONOate reduced the Golgi enlargement and dispersal due to either of MCTP or c-PTIO, again implicating NO in the structural integrity of the Golgi apparatus in endothelial cells. Nevertheless, the effects of MCTP and c-PTIO on PAECs were distinctive in that β-actin was present in prominent stress fibers within the cell in MCTP-induced megalocytosis but accumulated at the cell periphery in c-PTIO-induced megalocytosis (Fig. 1D).

Fig. 3. Subcellular mislocalization of cav-1 and endothelial nitric oxide synthase (eNOS) in PAECs exposed to MCTP or c-PTIO. PAEC cultures in 6-well plates were exposed to MCTP or c-PTIO for 4 days as indicated in Fig. 1 legend. The fixed permeabilized cultures were then immunostained for GM130 (mAb), cav-1 (pAb), and eNOS (pAb) together with DAPI. A and B: each include high-magnification inserts zooming (×4) into indicated areas. Cav-1 and eNOS were localized to the plasma membrane in controls (arrowheads). Cav-1 was lost from the plasma membrane after MCTP or c-PTIO and sequestered in a cell-centric compartment (A). eNOS was lost from the plasma membrane after MCTP but was increased at intercellular membrane contacts after c-PTIO (B, arrowheads). Scale bars = 50 and 25 μm, respectively, in low- and high-magnification frames in A and 50 and 12.5 μm, respectively, in low- and high-magnification frames in B.
Functional changes in anterograde trafficking after MCTP or NO scavenging. The Golgi apparatus is the key nodal point in regulating the anterograde trafficking from the cell interior to the plasma membrane (3, 9, 37, 59, 67). Thus soluble cargo destined for secretion traverses through the Golgi as does membrane-associated cargo such as cav-1, eNOS, and cell surface receptors. Figure 3A shows that both MCTP and c-PTIO reduced the localization of cav-1 to the plasma membrane with increased sequestration in a GM130-positive Golgi compartment. However, although eNOS was sequestered in a cell-centric compartment after both MCTP and c-PTIO, it was markedly lost from the plasma membrane after MCTP but continued to be retained in the intercellular plasma membrane contacts after c-PTIO (Fig. 3B). In comparison, both MCTP and c-PTIO reduced the localization of PECAM-1/CD31 from intercellular plasma membrane contacts with sequestration in an intracellular compartment (see data in Fig. 7B, ii). Thus MCTP and c-PTIO each produce specialized phenotypic changes in PAECs that can be both similar (loss of cav-1 and PECAM-1 from plasma membrane after both) and dissimilar (retained eNOS in intercellular plasma membrane contacts after c-PTIO).

An extraordinary aspect of this specialized phenotype is revealed in the live-cell anterograde secretion assays in Fig. 4. Previously, we reported that PAECs expressing an engineered secretory form of recombinant HRP synthesized off a transiently transfected expression plasmid showed diminished secretion when exposed to MCTP for 1 day (Fig. 1 in Ref. 55). Yet, it is well known that such MCTP-treated cells survive and increase in size over the next several days and weeks (27, 28, 48, 49, 55, 57, 61, 66). Figure 4A illustrates the experimental protocol used to investigate the long-term secretion of HRP by PAECs derived from a single transient transfection of the cells with the constitutive expression plasmid. The data in Fig. 4B show that with both MCTP and c-PTIO, while there was an inhibition of HRP secretion during the first day compared with untreated controls (confirming our previous observation in Ref. 55), there was continued and sustained secretion of HRP by treated cells over the next 4 days even though this rapidly declined in untreated controls. This

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![Fig. 4](image_url)

**Fig. 4.** Prolonged and enhanced long-term anterograde secretion of soluble cargo [recombinant horseradish peroxidase (HRP)] by PAECs exposed to MCTP or c-PTIO. PAEC cultures (in triplicate per variable) were transfected with the ssHRP expression vector and exposed to MCTP or c-PTIO 1 day later, the culture medium (1 ml per well) was harvested for the next 5 days, and the cultures were then fixed for immunostaining (A). Each sample of culture medium was assayed in triplicate for peroxidase activity [expressed in arbitrary absorbance units (AU); Ref. 60]; values for background subtraction were generated from medium samples simultaneously harvested from PAEC cultures transfected with only the pCDNA3 vector. HRP activity data derived from each well during the 5-day experiment were normalized in terms of HRP activity obtained from that same well in the first 24-h period (time interval indicated as "0" in A); thus each well acted as its own transfection control. A: experimental protocol. B: summary of data obtained (means ± SE). C: confirmation of Golgi dispersal in cultures at the conclusion of this experiment (scale bar = 50 μm). *P < 0.01 in intraday comparison with respective untreated control at each time point; n = 9 at each time point for each variable (triplicate cultures per variable with each medium sample assayed in triplicate).
occurred even though the Golgi elements were markedly dispersed in the cytoplasm (Fig. 4C).

Functional changes in retrograde trafficking after MCTP or NO scavenging. The observed hypo-S-nitrosylation of clathrin heavy chain and cav-1 after MCTP or c-PTIO (38) suggested that there might be global dysfunction of endocytic pathways. We used ligand uptake and internalization assays to monitor the functional integrity of clathrin (using AcLDL and Tfn)- or cav-1 (using CTB)-endocytic pathways in live PAECs exposed to MCTP or c-PTIO using epifluorescence imaging methods. Such ligand uptake assays are typically carried out by binding the respective fluorescently tagged ligand to cells in culture at 4°C for 30 min, washing excess ligand away, and then assaying for internalization by shifting the temperature up to 37°C. However, in our hands, PAECs in culture tended to retract when exposed to 4°C for 30 min. Therefore, in addition to the customary binding in the cold protocol (as in Fig. 5D and Supplemental Fig. 3D), in most experiments we incubated

37°C, 30 min, wash, image in 5-10 min

A
AcLDL

B
Tfn

C
CTB

D
Tfn (4°C, 30 min, wash, then 37°C, 30 min, image)

E
Tfn (37°C, 90 min, wash, image)

Fig. 5. Epifluorescence imaging of functional retrograde uptake and internalization of acetylated low-density lipoprotein (AcLDL), transferrin (Tfn), and cholera toxin B (CTB) in PAECs exposed to MCTP and c-PTIO for 4 days. Respective fluorescent ligand uptake and internalization assays were carried out in PAEC cultures 4 days after commencement of MCTP or c-PTIO treatment using procedures summarized in MATERIALS AND METHODS with variations as indicated in the figure. Multiple epifluorescence images (×40 objective, n = 4–6 per variable) were collected in each experiment. A–E: representative illustrations of ×4 zoomed-in sections of representative image frames from separate experiments; full ×40 objective image frames corresponding to these experiments are illustrated in Supplemental Fig. 3. Scale bars = 12.5 μm.
PAEC cultures to tagged ligands at either 37°C for 30 min (as in Fig. 5, A–C, and Supplemental Fig. 3, A–C) to assay for localization of ligand soon after internalization or for 37°C for 90 min (as in Fig. 5E and Supplemental Fig. 3E) to assay for the eventual destination of the tagged ligand. The data in Supplemental Fig. 3, A–C (compilation of low-magnification images), and in Fig. 5, A–C (compilation of high-magnification images), and the quantitation summarized in Fig. 6 show that PAECs exposed to either MCTP and c-PTIO had reduced ligand uptake and internalization on a per unit cell surface area basis with each of the ligands assayed using 37°C for the 30-min incubation protocol. The reduction in Tfna uptake and internalization in MCTP-treated cells was also observed when assayed by binding ligand at 4°C followed by a 30-min chase at 37°C (Fig. 5D and Supplemental Fig. 3D). Moreover, in MCTP-treated cells the endocytic transit of ligands to the Golgi was incomplete in that Tfna did not reach the Golgi even after a 90-min labeling period (Fig. 5E and Supplemental Fig. 3E). Additionally, imaging of fluorescently tagged AcLDL uptake using the alternative approach of confocal imaging (Fig. 7, A and D, left) also confirmed a reduction in uptake and internalization in MCTP- and c-PTIO-treated PAECs.

Mechanistically, impaired ligand uptake and internalization could result from either a reduction in the cell surface density of the respective receptor or impaired endocytic transit from the plasma membrane to the cell interior or both. This question was investigated by quantitative imaging of the cell-surface accessible fluorescence fraction of the respective receptors after fixation with paraformaldehyde but without detergent permeabilization. The data in Fig. 7, B, i and C, i, confirm the effectiveness of this approach and show that MCTP and c-PTIO both reduced cell-surface accessible PECAM-1/CD31 immunostaining. Re-immunostaining after detergent permeabilization showed that while PECAM-1 was lost from intercellular membrane contact areas after MCTP and c-PTIO, this antigen was increasingly sequestered in cell-centric cytoplasmic vesicles but with a net loss (Fig. 7, B, ii and C, ii).

This surface-accessible immunostaining procedure was then used to assess the surface density of LDLR, TfnR, Tie-2, and BMPR-2. The data summarized in Fig. 7D show that MCTP reduced the surface density of LDLR, TfnR, Tie-2, and to a lesser extent that of BMPR-2. In comparison, c-PTIO had smaller effects, or not at all, on the surface densities of all of these receptors. Nevertheless, both MCTP and c-PTIO had equivalent inhibitory effects on functional LDLR, Tfn, and CTB uptakes (Fig. 6). Thus while the reduced uptake in MCTP-treated cells might be accounted for by reduced surface receptors, that due to c-PTIO cannot. Thus NO scavenging likely has additional inhibitory effects on endocytic transit from the plasma membrane to the cell interior.

**NO scavenging enhances mitosis in PAEC cultures.** Endothelial cell proliferation, i.e., an increase in endothelial cell number, is characteristic of vascular lesions and neointima development in PAH (46, 47, 62). The data in Fig. 8A show that NO scavenging led to increased entry of endothelial cells into mitosis and cell proliferation. The rounded cells in Fig. 8A, top row, after c-PTIO were not cells in apoptosis in that these cells were l annexin-V-FITC negative (38 and 2) and could be mechanically shaken off one culture and replated into a fresh one (Fig. 8A, bottom row). Additional data using DAPI to stain chromosomes confirmed an increase in mitotic figures after c-PTIO (not shown). This enhanced mitosis due to c-PTIO occurred concomitant with marked Golgi enlargement and dispersal (Fig. 8, B and C). The maturated, Golgi dispersal, and increased mitosis due to NO scavenging were all reversed by the STA3 inhibitor indirubin 804 (Fig. 8, B and C; Ref. 43). In contrast, while MCTP-treated cells were enlarged and megalocytotic with enlarged nuclei, these cells did not enter mitosis (Fig. 8A).

Figure 9 shows the results of an experiment in which PAECs were exposed to both MCTP and NO scavenging. Each of the MCTP and c-PTIO alone produced enlarged cells as did the combination (Fig. 9A). However, while c-PTIO alone clearly

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**Figure 7.** Confocal imaging assays of retrograde AcLDL uptake, of surface-accessible and total platelet endothelial cell adhesion molecule (PECAM-1)/CD31, and cell-surface accessible densities of LDLR, TfnR, BMPR-2 and Tie-2 in PAECs exposed to MCTP or c-PTIO for 4 days. A*: PAEC cultures in 6-well plates were exposed to MCTP or c-PTIO as indicated in Fig. 1 legend and AcLDL uptake was assayed (30 min at 37°C) and imaged using confocal microscopy and Bio-Rad imaging software. Scale bar = 60 µm. Quantification of this experiment is shown in D, left-most set. B: parallel cultures to those in A were fixed with 4% paraformaldehyde, immunostained for PECAM-1 without permeabilization, and imaged using confocal microscopy (i). Subsequently, these were treated with 0.1% Triton X-100 and PECAM-1 immunostaining and confocal microscopy was repeated (ii). Scale bar = 60 µm; the laser intensity used for imaging in ii was 30% of that in i. C: quantitation of surface-accessible and total PECAM-1 immunostaining per unit cell surface area in experiment in B. *P < 0.01 in comparisons with respective untreated control cells. D: PAECs in 6-well plates (at least in duplicate per variable) were exposed to MCTP or c-PTIO for 4 days and processed for LDL uptake (as in A) and surface-accessible immunostaining for LDLR, TfnR, bone morphogenetic receptor type-2 (BMPR-2), and Tie-2 after fixation but without permeabilization (as in B, i). Imaging was carried out using confocal microscopy (as = at least 4 images per variable). Quantitation (fluorescence intensity per unit cell surface area; mean ± SE) was carried out as indicated in C, i and normalized by taking respective mean values in control cultures as 100. *P < 0.05 in comparisons with respective untreated control cells; **P < 0.05 in comparisons between the MCTP and c-PTIO groups.
enhanced entry of cells into mitosis, the inclusion of MCTP together with c-PTIO inhibited this traverse into mitosis (Fig. 9A). Nevertheless, although each of the MCTP and c-PTIO alone enlarged the Golgi, albeit with different fine structural changes (enlargement with intact Golgi ribbon after MCTP and extensive cytoplasmic dispersal after c-PTIO), the combination of the two caused a dramatic and exaggerated cytoplasmic dispersal and fragmentation of the Golgi elements (Fig. 9B).

Thus 1) with respect to the Golgi, MCTP and c-PTIO must have distinct subcellular/biochemical targets, 2) MCTP has...
a distinct and unique effect on cell cycle traverse consisting of a premitosis block, and 3) NO scavenging stimulates mitosis.

**DISCUSSION**

The present data define a broad-spectrum subcellular dysfunction syndrome in PAECs exposed to either MCTP or NO scavenging characterized by multiple simultaneous functional changes in cell size, the Golgi apparatus, intracellular trafficking, surface receptors, and mitosis (Table 1). MCTP, an agent widely used to generate experimental models of PAH, was used as the index probe to uncover this subcellular dysfunction syndrome. Many of the effects of MCTP on trafficking were similar to those of NO scavenging. However, there were distinct differences in terms of the enlarged cell morphology including the disposition of β-actin fibers, the fine structure of Golgi enlargement/dispersal, and the entry into mitosis. Remarkably, the present data showing a 40–50% inhibition of the functional uptake of multiple different ligands (LDL, Tfn, and CTB) by bovine PAECs exposed to MCTP in cell culture recapitulate the observations of Gillis et al. (12) who reported in 1978 that pulmonary vessels in MCT-treated rats showed a
The morphological megalocytosis produced by c-PTIO in endothelial cells (an enlarged cuboidal cell phenotype) is virtually identical to that produced by small-interfering RNA-mediated downregulation of dynamin-2 (see Fig. 1B, top, in Ref. 22). Dynamin-2 is a GTPase involved in several critical aspects of intracellular membrane trafficking and one whose activity appears to require S-nitrosylation (22). Moreover, there are prior reports (21) showing that NO scavenging by c-PTIO, but not the inactive c-PTI, increases cell cycle traverse in endothelial cell cultures with increased accumulation of cells in G2/M. Additionally, c-PTIO (20 μM) increased cell proliferation (increased cell number) in cultures of human salivary gland neoplastic cells without any observable apoptosis (64). Conversely, various NO donors have been reported to inhibit cell cycle traverse, including that of vascular smooth muscle cells (56). The present data confirm the role of NO in regulating endothelial cell proliferation and entry into mitosis.

While NO scavenging increased the entry of PAECs into mitosis, MCTP had the opposite effect. MCTP, and other pyrrolizidine alkaloids, produce a unique block at a checkpoint that lies subsequent to the Golgi enlargement/dispersal stage but before nuclear dissolution and entry into mitosis (39, 57, 61, 66). MCTP-treated PAECs showed a loss of cdc2, the kinase required for entry into mitosis (39). Thus while many of the effects of MCTP on trafficking can be replicated by NO scavenging, MCTP generates a unique premitosis block despite stimulating continued DNA synthesis.

The relationships among eNOS expression, NO, and the pathogenesis and progression of PAH are confusing (reviewed in Ref. 23). On the one hand, idiopathic PAH in humans has been represented as an NO-deficiency disease state with the growing use of sildenafil, NO inhalation, and nebulized nitrates as therapeutic approaches in this disease (4, 10, 11, 13, 23, 29, 62, 68). On the other hand, varying data have been reported on the relationships between eNOS expression levels and NO activity (23 and citations therein). Nevertheless, we note that previous studies of eNOS expression in PAH (discussed in Ref. 23, and citations therein) dealt with eNOS expression in assays at the whole lung tissue or whole cell levels and not with respect to the exact subcellular localization of the eNOS protein. There is a similar controversy with respect to the role
of NO in the MCT/rat model. Roberts et al. (50) reported that NO inhalation ameliorated MCT-induced PAH and vascular changes in 8-day-old rat pups. Hill and Pearl (17) also reported attenuation of PAH by NO inhalation in the MCT/adult rat model. Moreover Stewart and colleagues (7, 69) reported amelioration of MCT-induced PAH by genetically engineered cell-based overexpression of eNOS in the pulmonary vascular bed. In contrast, other investigators (19, 31) have reported little or no effect of NO inhalation on PAH in the juvenile or adult rat administered MCT. Nevertheless, Maruyama et al. (31) did report that short-term inhalation of NO at least partially ameliorated PAH and that chronic 19-day inhalation of NO (at 40 ppm) did cause a statistically significant reduction in the percentage of muscularized arteries at the alveolar wall (Table 4 in Ref. 31). In our experiments, the NO donor NONOate delayed and reduced MCTP-induced megalocytosis but did not completely reverse it (Supplemental Figs. 1 and 2).

The sustained secretory phenotype of PAECs exposed to MCTP or c-PTIO (Fig. 4) is relevant to discussions of the pathogenesis of PAH in that many investigators have posited a similar contribution of stromal/perivascular cell types including infiltrating macrophages in the ultimate pathogenesis of PAH (30, 41).

Table 1. Similar and dissimilar subcellular changes in PAECs exposed to MCTP or NO scavenging with c-PTIO

<table>
<thead>
<tr>
<th>Feature</th>
<th>MCTP</th>
<th>c-PTIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>enlarged, angular</td>
<td>enlarged, cuboidal</td>
</tr>
<tr>
<td>Entry into mitosis</td>
<td>inhibited (inhibits that of c-PTIO)</td>
<td>enhanced</td>
</tr>
<tr>
<td>Nuclear size</td>
<td>increased</td>
<td>increased, reduced postmitosis</td>
</tr>
<tr>
<td>Cell surface NO (DAF2-DA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-nitrosylation*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>Clathrin heavy chain</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>NSF</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>eNOS</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>Golgi (giantin, GM130, p115)</td>
<td>increased, enlarged</td>
<td>increased, dispersed</td>
</tr>
<tr>
<td>Protein localizations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caveolin-1</td>
<td>reduced plasma membrane, some in Golgi (GM130)</td>
<td>reduced plasma membrane, some in Golgi (GM130)</td>
</tr>
<tr>
<td>eNOS</td>
<td>reduced plasma membrane, increased cell-centric</td>
<td>increased at intercellular contacts, cell-centric</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
<td>loss from intercellular contacts</td>
<td>loss from intercellular contacts</td>
</tr>
<tr>
<td>β-actin</td>
<td>increased stress fibers</td>
<td>cell-periphery</td>
</tr>
<tr>
<td>Live-cell secretion (ssHRP)†</td>
<td>initial inhibition, then enhanced, long-lived</td>
<td>initial inhibition, then enhanced, long-lived</td>
</tr>
<tr>
<td>Live-cell compartment labeling†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5 ceramide (Golgi)</td>
<td>increased</td>
<td>inhibited</td>
</tr>
<tr>
<td>LysoTracker</td>
<td>unchanged</td>
<td>unchanged</td>
</tr>
<tr>
<td>ER-Tracker</td>
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<td>unchanged</td>
</tr>
<tr>
<td>MitoTracker</td>
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<td>unchanged</td>
</tr>
<tr>
<td>Live-cell ligand uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcLDL</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>Transferrin</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>CTB</td>
<td>inhibited</td>
<td>inhibited</td>
</tr>
<tr>
<td>Surface-accessible receptor†</td>
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<td></td>
</tr>
<tr>
<td>LDLR</td>
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</tr>
<tr>
<td>Transferrin-R</td>
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<td>small reduction</td>
</tr>
<tr>
<td>PECAM-1 (CD31)</td>
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<td>reduced</td>
</tr>
<tr>
<td>BMPR-2</td>
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<td>small reduction</td>
</tr>
<tr>
<td>Tie-2</td>
<td>reduced</td>
<td>small reduction</td>
</tr>
</tbody>
</table>

*From Ref. 38. †Compared with untreated PAECs on a per unit cell surface basis.

To summarize, MCTP and NO scavenging produced a broad spectrum of subcellular functional and structural alterations in PAECs in culture (Table 1). These included not only the enlargement and dispersal of the Golgi but functional changes in anterograde and retrograde trafficking and in diverse vasorelevant cell surface receptors. In a discrete distinction, NO scavenging but not MCTP enhanced entry of PAECs into mitosis. The questions emanating from the present work are 1) is the Golgi dysfunction syndrome reported here of any relevance in human idiopathic PAH, and 2) is there a causal relationship between Golgi dysfunction and vasculopathies of PAH? The related submission (54a) addresses these questions.
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