Disruption of Endothelial-Cell Caveolin-1α/Raft Scaffolding During Development of Monocrotaline-Induced Pulmonary Hypertension

Rajamma Mathew, MD; Jing Huang, MD; Mehul Shah, MD; Kirit Patel, BS; Michael Gewitz, MD; Pravin B. Sehgal, MD, PhD

Background—In the monocrotaline (MCT)-treated rat, there is marked stimulation of DNA synthesis and megalocytosis of pulmonary arterial endothelial cells (PAECs) within 3 to 4 days, followed by pulmonary hypertension (PH) 10 to 14 days later. Growing evidence implicates caveolin-1 (cav-1) in plasma membrane rafts as a negative regulator of promitogenic signaling. We have investigated the integrity and function of endothelial cell–selective cav-1/raft signaling in MCT-induced PH.

Methods and Results—Although PH and right ventricular hypertrophy developed by 2 weeks after MCT, a reduction in cav-1 levels in the lung was apparent within 48 hours, declining to ~30% by 2 weeks, accompanied by an increase in activation of the promitogenic transcription factor STAT3 (PY-STAT3). Immunofluorescence studies showed a selective loss of cav-1 and platelet endothelial cell adhesion molecule-1 in the PAEC layer within 48 hours after MCT but an increase in PY-STAT3. PAECs with cav-1 loss displayed high PY-STAT3 and nuclear immunostaining for proliferating cell nuclear antigen (PCNA). Biochemical studies showed a loss of cav-1 from the detergent-resistant lipid raft fraction concomitant with hyperactivation of STAT3. Moreover, cultured PAECs treated with MCT-pyrrole for 48 hours developed megalocytosis associated with hypo-oligomerization and reduction of cav-1, hyperactivation of STAT3 and ERK1/2 signaling, and stimulation of DNA synthesis.

Conclusions—MCT-induced disruption of cav-1 chaperone and scaffolding function in PAECs likely accounts for diverse alterations in endothelial cell signaling in this model of PH. (Circulation. 2004;110:1499-1506.)

Key Words: endothelium ▪ hypertension, pulmonary ▪ interleukins ▪ signal transduction ▪ transcription factors

Pulmonary hypertension (PH) is a progressive disease with high morbidity and mortality.1 Because the clinical manifestations of PH occur long after the initial injury, experimental models provide an opportunity to investigate the initiating mechanisms of this disease process. The monocrotaline (MCT)-treated rat is a well-established experimental model of PH.2–4 This plant alkaloid is biotransformed in the liver to pyrrolic metabolites.5 The bioactive derivatives produced in the liver have a half-life of ~3 seconds and thus significantly affect mainly the pulmonary arterial endothelium.5 Within 24 to 48 hours, there is evidence of endothelial cell damage6 and a pulmonary vascular leak.2 This is followed within 3 to 4 days by a 3- to 5-fold stimulation of pulmonary arterial endothelial cell (PAEC) DNA synthesis and megalocytosis resulting from abortive cytokinesis7–12. These hypertrophic endothelial cells are thought to secrete growth- and motility-promoting factors such as platelet-derived growth factor-B, transforming growth factor-β, and interleukin-6 (IL-6), which contribute to the migration and megalocytosis of underlying smooth muscle cells.10–14 On its abluminal side, increased deposition of insoluble elastin and collagen10 and migration and megalocytosis of smooth muscle cells are observed.3,7–10 On the luminal side, there is an increase in adhesiveness of the endothelial cell surface to cellular elements in the bloodstream.15

The initial in vivo phenotypic changes in PAECs in lungs of MCT-treated rats have been extensively studied in cell culture. Briefly, within 48 hours, monocrotaline pyrrole (MCTP)-treated PAECs show a marked increase in cell size, enlarged nuclei, and increased and continuing DNA synthesis, leading to hypertetraploidy.5,16,17 Vascular smooth muscle cells in culture show minimal changes.8

The molecular mechanism that initiates PAEC DNA synthesis and megalocytosis in the MCT-treated rat is largely unclear. Recent data have emphasized the role of IL-6 in the development of PH in this model.8 An increase in IL-6 in the rat lung is observed within 48 hours of
MCT, IL-6 activates promitogenic signaling, including activation of the transcription factor STAT3 and the ras-raf-MAPK pathway. IL-6 signaling involves specialized cholesterol-rich detergent-resistant plasma membrane microdomains called rafts. Raft microdomains represent preassembled complexes of cytokine and growth factor receptors, their associated signaling molecules with scaffolding proteins such as the caveolins (cav). Cav-1, a major protein component in rafts/caveolae, is largely a negative regulator of mitogenic signaling.

Interestingly, a 25% reduction in the number of caveolae per unit endothelial cell volume has been reported in MCT-treated rat lungs (see Reference 11, Table IV). In mouse and rat lungs, the cav-1α isoform but not the N-terminal truncated cav-1β isoform is selectively found in endothelial cells, whereas the reverse is true for the alveolar epithelium. Compared with the endothelium, vascular smooth muscle cells express minimal cav-1α.

In the present study, we investigated whether the MCT-induced disruption of cav-1/raft integrity and consequent hyperactivation of promitogenic signaling results in early endothelial dysfunction followed by PH. While this study was in progress, a causal role for loss of cav-1 in the development of PH in the mouse was established by the observation that homozygous deletion of the murine cav-1 molecule-1 (PECAM-1), cav-1 and von Willebrand factor receptors, their associated signaling molecules with scaffolding proteins such as the caveolins (cav), Cav-1, a major protein component in rafts/caveolae, is largely a negative regulator of mitogenic signaling.

Methods

Monocrotaline Administration and Hemodynamic Function
Male Sprague-Dawley rats (age, 6 to 7 weeks) weighing 125 to 150 g (Charles River) were given 1 subcutaneous injection of MCT (60 mg/kg), and pulmonary artery pressure and right ventricular hypertrophy were assessed at 48 hours, 1 week, and 2 weeks after MCT and in controls as described previously.

Western Blot Analyses
Protein aliquots (50 μg) of respective lung extracts were Western blotted, and cav-1α (sc-894), STAT3, tyrosine-phosphorylated STAT3 (PY-STAT3), and glucose-regulated protein 58 (GRP58, also called ER-60 and ERp57) antigens were detected as described previously.

Double-Label Immunofluorescence Studies
Formaldehyde-fixed, paraaffin-embedded sections (6 microns thick) of lung tissue were probed in double-label immunofluorescence assays for endothelial-selective cav-1α and von Willebrand Factor (vWF), cav-1α and platelet endothelial cell adhesion molecule-1 (PECAM-1), cav-1α and PY-STAT3, cav-1α and proliferating cell nuclear antigen (PCNA), and PY-STAT3 and PCNA using respective rabbit and goat polyclonal antibodies or murine monoclonal antibodies and corresponding AlexaFluor 488-, AlexaFluor 594-, CY3-, or rhodamine-tagged secondary antibodies. Images were collected with a MRC 1024 ES (Bio-Rad) confocal microscopy system at a magnification of ×180 (Figures 2 and 3), ×270 (Figure 4A), or ×450 (Figure 4B and 4C). Immunostaining intensities were quantified (10 to 12 arteries per section and 3 to 4 animals in each group) by use of a pixel histogram procedure (Adobe Photoshop 7.0) and expressed in arbitrary luminosity units.

Membrane Fractionation and Isolation of Rafts
Triton X-100–resistant (0.05%) membrane rafts were isolated from sonicated and homogenized lung tissue by equilibrium sucrose-density flotation by use of the method of Lafont and Simons as modified.

Seven fractions were collected from each gradient corresponding to the visible band at the very top (fraction 1), just above the 13/43% sucrose interface (fraction 2), at the 13/43% interface (fraction 3), just below the 13/43% interface (fraction 4), at the 43/60% interface (fraction 5), an aliquot from the 60% loading region (fraction 6), and the pellet (fraction 7). Fractions 2, 3, 4, 5, and 7 were diluted in cold Triton-solubilizing buffer, resuspended at 15 000g for 20 minutes, and resuspended in 200 μL solubilizing buffer. Aliquots were assayed for the plasma membrane marker 5′ nucleotidase (5′ ND) (Sigma) and for various proteins by SDS-PAGE and Western blotting. Fraction 3 is the detergent-resistant lipid raft fraction.

PAEC Cell Culture, MCTP Treatment, and Cell Fractionation
Growth and fractionation of primary bovine PAECs (BPAECs) into the “PI5” membrane fraction, the cytosolic “S100” fraction, and the “NE” nuclear extract were carried out as described earlier. MCTP was prepared by use of the procedure of Mattocks et al. Protein-matched aliquots of the respective cellular fractions were Western blotted for cav-1α, STAT3, STAT1, PY-STAT3, ERK1/2, and P-ERK1/2. DNA-shift assays for STAT-specific binding in nuclear extracts were carried out with the SIE oligonucleotide probe, and the SIF-A band observed (as in Figure 6A) was verified to be due to PY-STAT3 homodimers through the use of an antibody supershift assay (data not shown). Phase contrast confirmed megalocytosis of MCTP-treated BPAECs; respective cultures in 6-well plates were double-immunostained for cav-1 and PCNA after methanol-acetone fixation.

Cav-1α Oligomerization Status
This status was assessed by treating respective BPAEC cellular fractions with n-octylglucoside (50 mmol/L) and subjecting the sample to velocity sedimentation as described earlier.

Statistical Analyses
Statistical analyses were carried out with Student’s t test (for paired samples, 2 tailed; each variable group was compared pairwise with controls). Additionally, we used 1-Way ANOVA and the Tukey-Kramer multiple comparison test.

Results

Progressive Loss of Cav-1α and Increased Tyr-Phosphorylation of STAT3 in Lungs
Figure 1A and 1B show the occurrence of significant PH and right ventricular hypertrophy by 2 weeks in rats administered MCT. Remarkably, Western blotting data showed a reduction in the endothelial cell–selective cav-1α isoform beginning as early as 48 hours after MCT and declining to 30% of control levels by 2 weeks (Figure 1C and 1E). During this time, there was reciprocal activation of STAT3 as indicated by increased levels of PY-STAT3 (Figure 1D and 1E). MCT had little effect on the levels of total STAT3 or of GRP58, a protein known to be derivatized by MCT pyrrole in cell culture (Figure 1E).
Consistent with previous reports, little cav-2 or cav-3 protein was detected (data not shown).

**Immunofluorescence Studies of Cav-1α, vWF, and PECAM-1, in PAECs**

As expected, the most intense cellular immunostaining for cav-1α colocalized with vWF, a marker for endothelial cells (Figure 2A). There was a progressive loss of cav-1α beginning as early as 48 hour post-MCT in the intimal layer (Figure 2). In contrast, there was little alteration in vWF (Figure 2). The lack of alteration in vWF suggests that the integrity of PAEC per se was maintained during development of PH.

Figure 3 confirms the early and sustained loss of cav-1α in PAEC, and also shows the concomitant loss of PECAM-1, another plasma membrane-spanning scaffolding protein which negatively regulates diverse Tyr-kinase signaling pathways, including STAT3. Compared with the marked loss of cav-1α in arterial segments, there was little alteration of cav-1α in the endothelium of pulmonary venous segments (Figure 4A).

**Disruption of Cav-1α/Raft Association and Modulation of cav-1α/Raft/STAT3 Signaling**

Figure 5A (control) confirms a major distribution of cav-1α in the low-density raft fraction at the 13/43% sucrose interface (fraction 3). Figure 5A (MCT groups) and Figure 5E taken together show a selective and marked loss (>90%) of cav-1α but not of the 5′ ND plasma membrane marker from the raft fraction (fraction 3) during development of PH.

Figure 5B and 5E shows that activated Tyr-phosphorylated STAT3 (PY-STAT3) was associated with the raft fraction as early as 48 hours after MCT, with sustained and increasing PY-STAT3/raft association throughout the 2-week period. Additionally, Figure 5C and 5E shows a rapid and sustained recruitment of the transcription factor STAT3 per se to the raft fraction beginning within 48 hours (see fraction 3 at 48 hours after MCT) and that of STAT1 (Figure 5E and data not shown). In contrast, the gp130 signal-transducing chain of the IL-6 receptor, which was found in both raft (fraction 3) and nonraft (fraction 5) membrane fractions, was progressively downregulated (Figure 5D and 5E). The association of GRP58 with the raft fraction was largely unchanged (Figure 5E and data not shown).

**Inverse Relationship Between Cav-1α Loss and PY-STAT3 and Nuclear PCNA at the Single Endothelial Cell Level In Vivo**

Figure 4B replicates the loss of cav-1α in the PAEC layer 48 hours after MCT, with a concomitant increase in
PY-STAT3 at the cellular level. Importantly, Figure 4C shows that endothelial cells with high PY-STAT3 showed nuclear immunostaining for PCNA, indicative of active DNA synthesis. In additional controls, we have confirmed the absence of PCNA staining in high-cav-1/H9251-containing PAECs from untreated rats and the presence of PCNA immunostaining in low-cav-1/H9251-containing PAECs from rats 48 hours after MCT (data not shown).

**Effects of MCTP on PAEC in Culture**

Treatment of BPAECs in culture with MCTP (100 mmol/L) for 48 hours produced megalocytosis (not shown). Figure 6A shows that MCTP-treated BPAECs displayed (1) a reduction in cav-1α in the P15 membrane fraction, (2) cytosolic activation of phospho-ERK1/2, and (3) increased nuclear translocation of STAT3 and STAT1. Additionally, IL-6 stimulation of MCTP-treated cells led to hyperactivation of STAT3 signaling (increased Western-blottable nuclear PY-STAT3 and “SIF-A” DNA binding activity in nuclear extracts) and further activation of phospho-ERK1/2. Figure 6B shows that cav-1 in the detergent-resistant fraction derived from MCTP-treated cells banding at the 13/43% sucrose interface was hypo-oligomeric (n<8-mer compared with n>20-mer in control). This low-density membrane fraction from MCTP-treated cells but not from untreated cells was enriched in the Golgi marker GM130 (data not shown), indicating that MCTP blocks cav-1 trafficking and assembly in the Golgi compartment.27,33 Figure 6C illustrates the inverse relationship between cav-1α and DNA synthesis (nuclear accumulation of PCNA indicative of cells in S phase) in MCTP-treated cells. Overall, >90% of the cells showing nuclear PCNA immunostaining were low-cav-1α-expressing cells.

**Discussion**

Our studies show that administration of MCT results in a rapid reduction of cav-1α expression in rat lungs with a reciprocal activation of PY-STAT3 and of DNA synthesis before the development of PH. The loss of cav-1α was most marked in the detergent-resistant raft fractions. The loss of cav-1 was also associated with a parallel loss of
PECAM-1, another scaffolding protein and an endothelial cell marker. Interestingly, PECAM-1 also negatively regulates STAT3.32 However, vWF, another marker for the endothelial cells which is contained in Weibel-Palade bodies,30 was not altered, indicating that MCT-induced injury is confined mainly to the raft/scaffolding component of the endothelial cells. The inverse relationship between cav-1α and DNA synthesis and activation of promitogenic endothelial cell signaling, including hyperactivation of PY-STAT3, P-ERK1/2, and PY-STAT3 DNA binding activity, was confirmed in MCTP-treated PAECs in culture. The low-density membrane fraction from these cells showed hypo-oligomerization of cav-1 (Figure 6B), and this fraction was enriched in the Golgi scaffolding protein GM130 (data not shown). Mechanistically, the hypo-oligomerized state of cav-1α in detergent-resistant membranes from MCTP-treated PAECs suggests a block in trafficking of cav-1α through the Golgi compartment.27,34 An MCTP-induced Golgi-block would shunt cav-1α away from the plasma membrane, resulting in enlarged Golgi and enlarged intracellular membranes as has been observed in vivo.10,11 Thus, an MCT-induced Golgi block may be the subcellular mechanism leading to cav-1α loss, hyperactivation of promitogenic signaling, and DNA synthesis, yet a block at the Golgi fragmentation control point at the G2+M border53 results in hyperploidy and the megalocytotic cell phenotype8,9,16,17 and endothelial cell dysfunction.

Cav-1, the major protein constituent of caveolae, has emerged as a “master regulator” of signaling in endothelial cells.35 Cav-1 multimers in raft/caveolae provide a scaffold for colocalization of cytokine and growth factor receptors, together with their signaling molecules.19–23,34 Cav-1 serves both a positive chaperone function in ferrying receptors to plasma membrane raft domains and a negative function attenuating signaling by sequestering activated signaling intermediates.19–23,34 Loss of cav-1α resulting in hyperactivation of STAT3 in the MCT model is significant because STAT3 has been shown to be involved in cell proliferation.36 Furthermore, the upregulation of cav-1α is reported to attenuate epidermal growth factor signaling.29 Cav-1 also negatively regulates endothelial nitric oxide synthase (eNOS) activity, but cav-1 chaperone function is
needed to deliver eNOS to the plasma membrane caveolae. Thus, a reduction in cav-1 secondary to a trafficking block at the Golgi could adversely affect the eNOS activity, resulting in low bioavailability of NO, an underlying feature of MCT-induced PH. Interestingly, the endothelial cell angiopoietin-1 receptor Tie2, which is associated with cav-1, is also reported to be decreased in MCT-induced PH. Thus, the cav-1 loss is associated not only with the activation of promitogenic pathways such as PY-STAT3, ERK1/2, and DNA synthesis but also with negative regulation of eNOS and Tie2 pathways. All these factors individually or in concert are capable of participating in the pathogenesis of PH.

To summarize, the MCT-induced loss of cav-1α chaperone and scaffolding function in PAECs may provide an underlying mechanism for diverse alterations in endothelial cell signaling in this model of PH.

**Note Added in Proof**

Historically, endothelial cell proliferation induced by pyrrolizidine plant alkaloids was reported by Davidson, the marked enlargement of target cell types was reported by Harris et al., and the term “megalocytosis” was introduced to describe this phenotype by Bull.

**Acknowledgments**

This work was supported in part by research grant HL–73301 from the National Heart Lung and Blood Institute, National Heart, Lung, and Blood Institute, National Heart, Lung, and Blood Institute.
Institutes of Health. We thank Dr Susan Olson for BPAEC cell stocks and Joanne Abrahams for expert help in preparing the figures.

References


Disruption of Endothelial-Cell Caveolin-1α/Raft Scaffolding During Development of Monocrotaline-Induced Pulmonary Hypertension
Rajamma Mathew, Jing Huang, Mehul Shah, Kirit Patel, Michael Gewitz and Pravin B. Sehgal

_Circulation_. 2004;110:1499-1506; originally published online September 7, 2004; doi: 10.1161/01.CIR.0000141576.39579.23
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/11/1499

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/