Pyrrolidine dithiocarbamate restores endothelial cell membrane integrity and attenuates monocrotaline-induced pulmonary artery hypertension

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Pyrrolidine dithiocarbamate restores endothelial cell membrane integrity and attenuates monocrotaline-induced pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol 294: L1250–L1259, 2008. First published April 4, 2008; doi:10.1152/ajplung.00069.2007.—Monocrotaline (MCT)-induced pulmonary artery hypertension (PAH) in rats is preceded by an inflammatory response, progressive endothelial cell membrane disruption, reduction in the expression of caveolin-1, and reciprocal activation of STAT3 (PY-STAT3). Superoxide and NF-κB have been implicated in PAH. To evaluate the role of caveolin-1, membrane disruption, reduction in the expression of caveolin-1, and NF-κB activation despite attenuation of PAH. Increased superoxide chemiluminescence at 2 wk and reciprocal activation of PY-STAT3 plays a key role in the MCT-induced PAH.

caveolin-1; inhibitory κBα; inflammation; PY-STAT3; Tie2

DESPITE MAJOR ADVANCES MADE in the understanding of the pathogenesis of pulmonary artery hypertension (PAH), the precise molecular mechanism remains elusive. Both inflammation and oxidant stress have been implicated in the pathogenesis of PAH. Perivascular infiltration with inflammatory cells is evident in plexiform lesions in the lungs of patients with primary PAH, and the levels of proinflammatory cytokines such as IL-1 and IL-6 are elevated in the serum (22, 47). In addition, the occurrence of PAH in patients suffering from systemic inflammatory diseases is not uncommon (14). Monocrotaline (MCT)-induced PAH in rats is preceded by an inflammatory response in the lungs and is associated with an early and progressive increase in the expression of IL-6 mRNA and IL-6 bioactivity in the lungs (6). IL-6 is known to activate STAT3, a transcription factor that positively regulates cell growth and proliferation. Tyrosine phosphorylation of STAT3 is required for STAT3 dimerization and subsequent translocation to nucleus where it binds with DNA and modulates gene expression. Its activation has been implicated in cell proliferation (8, 21). Indeed, our previous work (33) showed a marked activation of STAT3 (phosphorylation at Tyr705, PY-STAT3) coupled with an early and progressive loss of caveolin-1 expression in rat lungs with MCT-induced PAH. Furthermore, caveolin-1−/− mice show activation of PY-STAT3 and a propensity to develop PAH (49). Importantly, several forms of PAH including MCT-induced PAH are reported to be associated with significant reduction in the expression of caveolin-1 (1, 24, 33).

Caveolin-1 is a major protein constituent of flask-shaped caveolae found on the surface of a variety of cells including endothelial cells. Caveolin-1 interacts with and modulates numerous signaling molecules that reside in or are recruited to caveolae. Caveolin-1 inhibits the activation of proliferative pathways, regulates cell cycle, and modulates apoptosis (15, 19, 27). We (33, 34) have shown that, in addition to caveolin-1, other membrane proteins such as platelet-endothelial cell adhesion molecule-1 (PECAM-1) and Tie2 that localize in the caveolae show parallel reduction indicating a generalized disruption of the caveolar membrane integrity caused by MCT. Tie2 has been implicated in the pathogenesis of PAH (46). It is worth noting here that von Willebrand factor (vWF), which is localized in the Weibel-Palade bodies of endothelial cells, are not affected up to 2 wk post-MCT (33). These observations suggest that the endothelial cell membrane integrity plays a key role in maintaining vascular health.

Oxidant injury is known to perturb endothelial cell membrane integrity, and the evidence of oxidant injury has been reported in the presence of established PAH in humans and in the MCT-induced PAH in rats (7, 32). Furthermore, the activation of NF-κB has been reported in primary PAH and in the MCT model of PAH (23, 42). Both oxidant injury and inflammation are known to activate NF-κB, which regulates the expression of several genes involved in inflammation including IL-1 and IL-6 and also mediates proliferative responses. Inactive NF-κB exists in the cytoplasm in association with inhibitory (I)-κB family of proteins such as I-κBα. The release from the inhibitors is critical for the translocation of NF-κB to the nucleus to induce transcription of targeted genes (2, 4, 28, 48). The activation of NF-κB is considered essential for proliferation of cultured smooth muscle cells, and IL-6 is known to activate STAT3, a transcription factor that positively regulates cell growth and proliferation. Tyrosine phosphorylation of STAT3 is required for STAT3 dimerization and subsequent translocation to nucleus where it binds with DNA and modulates gene expression. Its activation has been implicated in cell proliferation (8, 21). Indeed, our previous work (33) showed a marked activation of STAT3 (phosphorylation at Tyr705, PY-STAT3) coupled with an early and progressive loss of caveolin-1 expression in rat lungs with MCT-induced PAH. Furthermore, caveolin-1−/− mice show activation of PY-STAT3 and a propensity to develop PAH (49). Importantly, several forms of PAH including MCT-induced PAH are reported to be associated with significant reduction in the expression of caveolin-1 (1, 24, 33).

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and its activation has been reported in the balloon-induced injury of arteries in rats (5, 9). In addition, overexpression of mutated IκBα or administration of antisense p65 (a subunit of NF-κB) inhibits vascular smooth muscle cell proliferation and intimal hyperplasia (3, 50).

Based on these observations, we hypothesized that MCT-induced disruption of endothelial cell membrane integrity results in a reduction in caveolin-1 expression and reciprocal activation PY-STAT3, leading to cell proliferation, pulmonary vascular remodeling, and PAH. The aim of this study was to define the relationship between the cell membrane damage and resulting reduction in the expression of caveolin-1 and activation of PY-STAT3 and also to examine the role of superoxide in the MCT model of PAH. MCT-injected rats were treated with pyrrolidine dithiocarbamate (PDTC), a low molecular weight thiol compound with an ability to traverse cell membrane. PDTC is known to inhibit inflammation and NF-κB activation in vitro and in vivo (10, 29). Our preliminary studies showed that the PDTC treatment started on day 1 significantly attenuated MCT-induced PAH coupled with restoration of caveolin-1 expression and inhibition of PY-STAT3. In the present study, in addition to PDTC treatment starting on day 1, we added two more groups. One group was started PDTC treatment on day 3 to ascertain whether the observed beneficial effects of PDTC were based on its action on the lungs and not on the liver affecting MCT metabolism. The second group received PDTC 2 wk post-MCT to examine whether PDTC could reverse PAH or halt its progression. The time-dependent effect of PDTC on the membrane integrity, expression of caveolin-1, Tie2, and IκBα, the activation of transcription factors, and superoxide generation on the MCT-induced PAH were examined.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 150–175 g were obtained from Charles River Laboratories (Wilmington, MA). Rats were allowed to acclimatize in the animal facility for 5 days before the start of experimental protocols and had free access to laboratory chow and water. Protocols were approved by the Institutional Animal Care and Use Committee and conform to the guiding principles for the use and care of laboratory animals of the American Physiological Society and the National Institutes of Health. Rats received a single subcutaneous injection of 60 mg/kg MCT (Trans World Chemicals, Rockville, MD) as described previously.

PDTC Treatment

There were four groups of rats that received PDTC treatment. In group 1, rats received MCT and daily subcutaneous injection of PDTC (50, 100, or 200 mg/kg for 2 wk) starting on day 1. A subset of rats receiving PDTC 100 mg·kg\(^{-1}\)·day\(^{-1}\) was examined at 1 wk post-MCT. In group 2, rats were started on PDTC on day 3 post-MCT. Our initial results with group 1 showed complete reversal of PAH with 100 mg/kg PDTC. Therefore, in group 2, rats were treated with 100 or 200 mg/kg PDTC for 2 wk. In group 3, rats were started on PDTC (200 mg·kg\(^{-1}\)·day\(^{-1}\)) on day 14 when PAH was already established. We opted to use a higher dose (200 mg·kg\(^{-1}\)·day\(^{-1}\)) in this group because we hypothesized that the anti-inflammatory property of PDTC was unlikely to reverse or halt the progression of PAH. The inflammatory response is an early phenomenon in the MCT model of PAH. The endothelial damage occurs within 48 h of MCT injection. To evaluate the effects of PDTC alone, a group of control rats (group 4, \(n = 9\)) were treated with 50, 100, or 200 mg/kg PDTC (Sigma, St. Louis, MO) per day (3 rats in each group). PDTC was freshly dissolved in normal saline (pH 7.3) each day. True controls did not receive PDTC.

Measurements of Pulmonary Artery Pressure

Pulmonary artery pressure (PAP) was measured using methods described previously (33). Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). Through an incision in the neck, the trachea was exposed and cannulated with PE-240 tubing, and the rat was ventilated in room air at a rate of ~70–80 breaths/min with a tidal volume of 0.83 ml per 100 g body wt. The chest was opened, PE-50 tubing was inserted into the right ventricle (RV), and the pressure was recorded on a Grass polygraph (model 7E). Right ventricular systolic pressure was used as PAP. At the end of the pressure measurements, the lungs were perfused with autoclaved normal saline to remove blood. Main and hilar pulmonary artery segment and aorta were removed and cleaned of adventitia and placed in separate vials containing Krebs solution buffered with 10 mM HEPES-NaOH (pH 7.4) for measurement of superoxide generation. Right lung was removed, quickly frozen in liquid N\(_2\), and stored at −80°C for protein extraction. Left lung and heart were placed in 10% buffered formaldehyde.

Assessment of Right Ventricular Hypertrophy

A week later, the heart was removed from formaldehyde, and atria were trimmed. The free wall of the RV was separated from the left ventricle and the septum (LV). The ratio of RV/LV was calculated to assess right ventricular hypertrophy (RVH).

Measurement of Superoxide

Superoxide generation from pulmonary arteries (main and hilar) and aorta from rats (48 h and 1, 2, and 4 wk post-MCT and from MCT+PDTC treated groups) was measured as previously described (16). Briefly, the arteries were placed in plastic scintillation minivials containing 5 μM lucigenin, and chemiluminescence was measured in a liquid scintillation counter (LS 6000 IC, Beckman Instruments) at ~37°C. The data are expressed as counts/min per milligram of arterial tissue after background subtraction.

Protein Extraction and Western Blot Analysis

The lung tissue was homogenized in a buffer containing 0.1 M PBS (pH 7.4), 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS, 10 μl/ml PMSF (10 mg dissolved in 1 ml of isopropanol), 25 μg/ml aprotinin, and 25 μg/ml leupeptin. PMSF (10 μl/ml) was added to homogenates placed on ice for 30 min and centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentrations in the supernatants were analyzed by using a kit from Bio-Rad and serum albumin as standard. Fifty micrograms of protein was loaded and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat milk powder in Tris-buffered saline with Tween buffer (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) overnight at 4°C. Membranes were then incubated with IκBα (sc-371, 1:500; Santa Cruz Biotechnology), endothelium-specific caveolin-1e (sc-897, 1:4,000, Santa Cruz Biotechnology) or Tie2 (sc-324, 1:500, Santa Cruz Biotechnology) or STAT3 (sc-337, 1:5,000, BD Biosciences) for 1 h and 20 min at room temperature or with PY-STAT3 (1:1,000; no. 9131, Cell Signaling Technology) overnight at 4°C. The membranes were washed for 10 min ×3 with TBST and incubated with appropriate horseradish peroxidase secondary antibody (anti-rabbit antibody: 1:5,000 IκBα, caveolin-1, Tie2, and PY-STAT3; anti-mouse antibody: 1:2,000 for β-actin and STAT3) for 1 h at room temperature and then washed for 10 min ×3 with TBST. The protein bands were visualized by chemiluminescence (Amersham ECL Western Blotting...
Detection System, Amersham Biosciences). Membranes were reprobed with β-actin (A5441, Sigma) to assess the protein loading. PY-STAT3 blots were reprobed with STAT3 antibody. The relative expression of the proteins was quantified using densitometric scanning and expressed as percent of controls.

**EMSA**

*Nuclear extraction.* The lung tissue was snap-frozen in liquid N2 and stored at −80°C until use. Nuclear extracts from lung tissue were prepared as previously described (41). In brief, lungs were pulverized under liquid N2 and then Dounce homogenized with six strokes using a type B pestle in a low-salt buffer (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1× protease inhibitor cocktail; P8340, Sigma). The homogenate was then centrifuged twice (30 s; 4°C; 500 g) with the supernatant carefully retained to remove debris and avoid the pellet. Following this, the samples were incubated on ice for 5 min to allow swelling of the nuclei. The nuclei were pelleted by centrifugation (5 min; 4°C; 3,500 g) and lysed on ice for 20 min in a high-salt solution (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1× protease inhibitor cocktail) for 20 min. The lysed nuclear mixture was centrifuged at 16,000 g for 30 s. The supernatant was aliquoted and snap-frozen in dry ice and ethanol and stored at −80°C until further use.

*Mobility shift assay.* NF-κB nucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ was annealed and 5′-end-labeled with [γ-32P]ATP (3,000 Ci/mmol) using T4 polynucleotide kinase according to standard procedure. Binding reactions were performed by preincubation of nuclear extract protein (30 μg) in HEPES (pH 7.9, 12 mM), KCl (60 mM), MgCl2 (4.0 mM), EDTA (1.0 mM), glycerol (12%), DTT (1.0 mM), and poly(dI-dC) (200 ng) at room temperature for 10 min followed by the addition of the double-stranded [32P]-labeled oligonucleotide (50,000 cpm) and a second incubation at room temperature for 30 min. Fifteen microliters of reaction mixture was loaded onto each lane of a nondenaturing polyacrylamide-bis-acrylamide gel (5%) prepared in 0.5× Tris-borate-EDTA (45 mM Tris, 45 mM boric acid, and 0.1 M EDTA), and electrophoresis was performed at room temperature for 1.5–2 h at 100 V. The gel was dried before being exposed in a phosphor screen cassette. Band intensities were imaged using a STORM 840 Phosphimager.

**Lung Histology**

Midsections from the left lungs preserved in 10% buffered formaldehyde were processed for microscopic examination by paraffin embedding. Five micrometer-thin sections were stained with elastic van Gieson method for the identification of pulmonary arteries with elastic laminae.

**Statistical Analysis**

The data are expressed as means ± SE. For statistical analysis, we used one-way ANOVA followed by Scheffe’s multiple comparison tests. Differences were considered statistically significant at P < 0.05.

**RESULTS**

The percent weight gain at the end of 2 wk in the MCT group was significantly lower compared with the controls (controls, 82% ± 9%, n = 8 vs. MCT, 50% ± 3%, n = 10; P < 0.05). The weight gain in MCT+PDTC group 1 (MCT+PDTC, 50 mg/kg, 33% ± 4%, n = 8; MCT+PDTC, 100 mg/kg, 43% ± 5%, n = 7; MCT+PDTC, 200 mg/kg, 42% ± 4%, n = 6; started on day 1) was comparable to that in the MCT group. In group 2, rats (PDTC, 100 mg/kg, started on day 3, n = 5) showed similar weight gain (31% ± 2.3%) as the MCT group. The weight gain in rats 4 wk post-MCT (n = 3) was only 31% ± 1%, with a further loss during the last 2 days before experiment. The comparable controls gained 99% ± 14% (n = 4). Rats started PDTC treatment 2 wk post-MCT also showed low weight gain (36% ± 4%, n = 5). PDTC treatment alone had no adverse effect on weight gain in the controls.

**PAP and RVH**

As shown in Fig. 1, A and B, rats developed significant PAH and RVH at 2 wk with a further progression at 4 wk post-MCT. At 48 h and 1 wk post-MCT, there was no evidence of PAH or RVH.

**Reduction in the Expression of Caveolin-1 and Tie2 Proteins**

Figure 2 (a typical blot and a bar graph) shows a significant progressive reduction in the expression of caveolin-1 and Tie2 seen as early as 1 wk post-MCT. The expression of β-actin was not altered.

**Reduction in the Expression of I-κBα Protein and NF-κB Activation**

Significant reduction in the expression of I-κBα was observed at 2 and 4 wk post-MCT (Fig. 3, left) and also at 3 wk post-MCT (Fig. 3, right).
post-MCT (data not included). Control rats showed a low level of DNA binding activity. In contrast, EMSA revealed a significant increase in NF-\(\kappa\)B DNA binding at 2 wk post-MCT when PAH is first observed followed by a significant reduction at 3 and 4 wk post-MCT despite persistent PAH (Fig. 3, right).

**Superoxide Generation**

There was a significant increase in lucigenin chemiluminescence in pulmonary arteries at 2 wk post-MCT compared with the controls, indicating increased superoxide generation (Fig. 4). At 48 h and 1 wk post-MCT, there was no increase in lucigenin chemiluminescence. Importantly, lucigenin chemiluminescence was not increased at 4 wk post-MCT despite increased PAP. MCT did not affect the superoxide chemiluminescence in the aortic tissue at the time periods studied.

**Activation of PY-STAT3**

A representative blot and a bar graph (Fig. 5) show progressive activation of PY-STAT3 in the lungs from 1 to 4 wk post-MCT.

**PDTC Treatment**

Effect of PDTC on MCT-induced PAH and RVH. As depicted in Fig. 6, A and B, treatment with PDTC (100 mg/day \(\times\) 14 days) started on days 1 and 3 normalized PAP and RVH. However, PDTC did not reverse the established PAH or halt its progression. Treatment with 50 mg of PDTC started on day 1 (\(n = 5\)) significantly lowered PAP (25 ± 2.6 mmHg; \(P < 0.05\) vs. controls and 2 wk post-MCT) and normalized RV/LV ratio (0.026 ± 0.012). A dose of 200 mg/kg PDTC started on day 1 normalized PAP (19 ± 1.3 mmHg, \(n = 9\)) and RV/LV ratio.

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**Fig. 2.** A representative Western blot and a bar graph showing the expression of Tie2 (a membrane protein), caveolin-1, and beta-actin in the lungs of controls (lane 1) and 48 h and 1, 2, and 4 wk post-MCT (lanes 2–5). There is progressive loss of caveolin-1 and Tie2 expression in MCT-treated rat lungs. \(*P < 0.05\) vs. respective controls (\(n = 3–12\)).

**Fig. 3.** A representative Western blot showing the expression of inhibitory (I)-NF-\(\kappa\)B and beta-actin (lane 1 = control, lanes 2–5 = 48 h and 1, 2, and 4 wk post-MCT). The bar graph shows a significant reduction in the I-NF-\(\kappa\)B expression is seen at 2 and 4 wk post-MCT. \(*P < 0.05\) vs. controls (\(n = 3–12\)). Right shows activation of NF-\(\kappa\)B binding by MCT treatment (lanes 1–6: control, 48 h, and 1, 2, 3, and 4 wk post-MCT, respectively). Note that since NF-\(\kappa\)B activation was significantly reduced at 4 wk post-MCT, we included a 3-wk post-MCT sample. This figure clearly shows that NF-\(\kappa\)B activation occurs transiently at 2 wk post-MCT.

**Fig. 4.** Superoxide chemiluminescence in pulmonary artery (Pulm. Art) and aortic tissue from control rats and 48 h and 1, 2, and 4 wk post-MCT. There is significant increase in the superoxide chemiluminescence in pulmonary arterial tissue at 2 wk post-MCT. At 4 wk post-MCT, despite PAH, chemiluminescence is not increased. MCT does not alter chemiluminescence in aortic tissue. \(*P < 0.05\) vs. controls (\(n = 3–12\)).

**Fig. 5.** A representative blot and a bar graph (Fig. 5) show progressive activation of PY-STAT3 in the lungs from 1 to 4 wk post-MCT.
showed dose-dependent restoration of caveolin-1 (77% \( \times \) H11006 (Fig. 7). Treatment with 50 and 200 mg/kg PDTC (endothelial cell membrane proteins caveolin-1 and Tie2 expression.

Neither MCT nor PDTC had any effects on LV (mg)/final body weight (g) ratio in any of the groups studied (data not included).

Effect of PDTC on MCT-induced reduction in caveolin-1 and Tie2 expression. PDTC treatment (100 mg·kg\(^{-1}\)·day\(^{-1}\)) started on days 1 and 3 significantly restored the expression of endothelial cell membrane proteins caveolin-1 and Tie2 (Fig. 7). Treatment with 50 and 200 mg/kg PDTC (day 1) also showed dose-dependent restoration of caveolin-1 (77% \( \pm \) 5%, 95% \( \pm \) 4%) and Tie2 (42% \( \pm \) 5.3%, 107% \( \pm \) 14%), respectively. As shown in Fig. 7, PDTC treatment started at 2 wk post-MCT failed to reverse or halt MCT-induced progressive loss of caveolin-1 and Tie2.

Effect of PDTC on MCT-induced I-kB\(\alpha\) degradation. PDTC treatment started on days 1 and 3 post-MCT completely reversed MCT-induced degradation of I-kB\(\alpha\). PDTC started on day 14 post-MCT did not attenuate I-kB\(\alpha\) degradation (Fig. 8, left). PDTC in controls did not affect the expression of I-kB\(\alpha\) (lane 2). Treatment with 50 and 200 mg/kg PDTC (day 1) showed dose-dependent prevention of MCT-induced degradation of I-kB\(\alpha\) (60% \( \pm \) 7% and 93% \( \pm \) 5%, respectively).

Effect of PDTC on NF-\kappaB activation. As shown in Fig. 8, right, treatment with PDTC had no effect on NF-\kappaB activation irrespective of PAP. This observation strongly suggests that NF-\kappaB may not have any significant role in the pathogenesis of MCT-induced PAH. Interestingly, there is a discrepancy between NF-\kappaB activation and I-kB\(\alpha\) expression at 4 wk post-MCT.

Effect of PDTC on superoxide chemiluminescence. Increased lucigenin chemiluminescence is observed in pulmonary arterial tissue only at 2 wk post-MCT, which is reversed by PDTC treatment started on days 1 and 3 post-MCT (Fig. 9). Treatment with 50 and 200 mg/kg PDTC (day 1) also completely reversed MCT-induced superoxide generation (543 \( \pm \) 184, \( n = 5 \) and 648 \( \pm \) 172 cpm/mg tissue, \( n = 3 \), respectively). Rats started on PDTC treatment for 2 wk at 2 wk post-MCT showed normal lucigenin chemiluminescence in pulmonary arterial tissue. This is not surprising because at 4 wk post-MCT, despite the presence of PAH superoxide, generation was within normal limits. Neither MCT nor PDTC had any effect on lucigenin chemiluminescence in aortic tissue.

Effect of PDTC on PY-STAT3 activation. As shown in Fig. 10, PDTC treatment started on days 1 and 3 post-MCT completely inhibited PY-STAT3 activation. Not surprisingly, PDTC started at 2 wk failed to prevent PY-STAT3 activation, which is consistent with the hemodynamic data. Treatment with 50 and 200 mg/kg PDTC (day 1) inhibited PY-STAT3 activation in a dose-dependent manner (220% \( \pm \) 34% and 115% \( \pm \) 5%, respectively, compared with the controls, 100%, and 2 wk post-MCT, 480% \( \pm \) 37%).

Effect of PDTC at 1 wk post-MCT. At 1 wk post-MCT, there is no evidence of PAH or RVH (Fig. 1). PDTC (\( n = 3 \)) had no effect on PAP (19 \( \pm \) 0.7 mmHg) or on RV/LV ratio (0.25 \( \pm \) 0.08). PDTC significantly restored the expression of caveolin-1 (control \( = 100\% \), MCT \( = 44\% \), \( P < 0.05 \) vs. controls; MCT + PDTC \( = 108\% \pm 16\% \)) and Tie2 (control \( = 100\% \), MCT \( = 46\% \pm 5\% \), \( P < 0.05 \) vs. controls; MCT + PDTC \( = 97\% \pm 3\%) and significantly inhibited PY-STAT3 activation (control \( = 100\% \), MCT \( = 326\% \pm 40\% \), \( P < 0.05 \) vs.

\( (0.24 \pm 0.007) \). PDTC alone (all doses) had no effect on PAP (18.8 \( \pm \) 1 mmHg, \( n = 9 \)) or on RV/LV ratio (0.23 \( \pm \) 0.05). Neither MCT nor PDTC had any effects on LV (mg)/final body weight (g) ratio in any of the groups studied (data not included).

Fig. 5. A representative Western blot and bar graph depicting PY-STAT3 activation and the expression of STAT3 in rat lungs. MCT induces progressive increase in the activation of PY-STAT3. Lanes 1–5 depict PY-STAT3/STAT3 in control and 48 h and 1, 2, and 4 wk post-MCT. \(*P < 0.05 \) vs. controls; **\( P < 0.05 \) vs. controls and 1 wk post-MCT; ***\( P < 0.05 \) vs. controls and 1 and 2 wk post-MCT (\( n = 3–12 \)).

Fig. 6. Bar graphs depicting PAP (A) and RV/LV (B) ratio in controls 2 and 4 wk post-MCT and in rats treated with pyrroline diethiocarbamate (PDTC; 100 mg·kg\(^{-1}\)·day\(^{-1}\) \( \times \) 14 days) starting on day 1 (MCT + PDTC day 1) and day 3 (MCT + PDTC day 3) and PDTC (200 mg·kg\(^{-1}\)·day\(^{-1}\) \( \times \) 14 days) starting on day 14 post-MCT (MCT + PDTC day 14). There is significant reduction in PAP and RV/LV ratio in rats receiving PDTC from day 1 and 3. PDTC treatment started on day 14 did not attenuate MCT-induced PAH or RVH. \(*P < 0.05 \) vs. controls (\( n = 3–12 \)).
controls; MCT + PDTC = 150% ± 15%). At 1 wk post-MCT, I-κBα levels were slightly lower than the controls, but it did not reach statistical significance. PDTC treatment did show improvement in the expression of I-κBα (control = 100%, MCT = 77% ± 13%, MCT + PDTC = 97% ± 14%; P = not significant).

**Effect of PDTC on pulmonary vascular remodeling.** As shown in Fig. 11, at 48 h and 1 wk post-MCT, the thickness of pulmonary arteries is comparable to that of the control. There is increased medial thickening at 2 and 4 wk post-MCT. PDTC treatment started on days 1 and 3 inhibited MCT-induced medial wall thickness of pulmonary arteries. PDTC treatment started at day 14 had no effect of MCT-induced vascular remodeling.

**DISCUSSION**

The principal findings of the present study are that the MCT-induced PAH is associated with 1) extensive pulmonary endothelial cell membrane damage as evidenced by a significant reduction in the expression of membrane-bound proteins such as caveolin-1 and Tie2 in the lungs; 2) early and progressive activation of PY-STAT3, a proliferative transcription factor; and 3) a significant reduction in the expression of I-κBα in the lungs at 2 and 4 wk post-MCT in the presence of PAH. 4) Interestingly, NF-κB activation occurred only at 2 wk post-MCT at the onset of PAH, but during sustained PAH (3 and 4 wk post-MCT) NF-κB activation was significantly attenuated despite progressive reduction in the level of expression of I-κBα. 5) Similarly, increased lucigenin chemiluminescence in pulmonary arteries occurred only at 2 wk post-MCT with normalization at 4 wk, and thus NF-κB activation and superoxide may not have a significant role in the pathogenesis of MCT-induced PAH. 6) PDTC treatment started on days 1 and 3 restored membrane integrity, rescued membrane proteins including caveolin-1 with concomitant inhibition of PY-STAT3 activation, and attenuated PAH, RVH, and pulmonary vascular remodeling at 2 wk post-MCT. 7) In addition, PDTC restored the expression of I-κBα and inhibited superoxide generation in pulmonary arteries at 2 wk post-MCT. 8) PDTC at 1 wk post-MCT rescued membrane proteins and inhibited PY-STAT3 activation; and 9) PDTC started at 2 wk post-MCT did not restore membrane integrity or inhibit PY-STAT3 acti-
lack of caveolin-1 display abnormalities in proliferation both in vivo and in vitro (18, 19, 39). Interestingly, recent studies have shown increased caveolin-1 expression in pulmonary arterial smooth muscle cells obtained from patients with idiopathic PAH contributing to increased capacitive Ca\(^{2+}\) entry and DNA synthesis (38). Thus, the activity of caveolin-1 is cell specific and may also depend on the disease state of smooth muscle cells. In human primary PAH, a reduction in the expression of caveolin-1 is observed exclusively in the plexiform lesions seen in the advanced stages of PAH (2), underscoring a role for caveolin-1 dysregulation in PAH.

We (33) have recently shown that MCT-induced endothelial caveolin-1 reduction in pulmonary arteries is accompanied by a reciprocal activation of PY-STAT3. In addition to PY-STAT3, immunostaining for proliferating cell nuclear antigen was observed in pulmonary endothelial cells lacking caveolin-1 (33). The activation of PY-STAT3 accompanying a reduction in caveolin-1 expression has also been reported in the myocardial infarction model of PAH in rats (24). Not surprisingly, PY-STAT3 is activated in caveolin-1\(^{-/-}\) mice (49). STAT3 resides in the cytoplasm of quiescent cells. On activation by cytokines or growth factors, phosphorylated (serine or tyrosine) STAT3 translocates to nucleus to induce gene transcription. STAT3 activation is thought to play an important role in cell proliferation, differentiation, inflammation, apoptosis, and wound repair (11, 12). Jasmin et al. (25) have recently shown that caveolin-1 acts as a suppressor of STAT3 by inhibiting the JAK-STAT signaling pathway. Thus, the activation of STAT3 accompanying endothelial caveolin-1 loss may be an upstream regulator of cell proliferation and vascular remodeling in PAH. Recent studies have shown that hypoxia-

![Graph showing superoxide chemiluminescence in pulmonary arterial and aortic tissue](image)

**Fig. 9.** This bar graph depicts superoxide chemiluminescence in pulmonary arterial and aortic tissue from control rats, 2 and 4 wk post-MCT, PDTC treatment started on day 1 (MCT+PDTC day1), day 3 (MCT+PDTC day3), and day 14 (MCT+PDTC day14). Significant increase in superoxide is observed in pulmonary artery tissue only at 2 wk post-MCT. PDTC treatment started on days 1 and 3 significantly reduced chemiluminescence. At 4 wk, post-MCT and PDTC treatment started on 14 d does not exhibit increased superoxide generation. Neither MCT nor PDTC had any effect on aortic tissue. \(^*P<0.05\) vs. controls (n = 3–12).

![Graph showing PY-STAT3 activation](image)

**Fig. 10.** A representative Western blot and bar graph depicting PY-STAT3 activation and STAT3 expression in rat lungs from control (lane 1), 2 wk post-MCT (lane 2), MCT+PDTC started on day 1 (lane 3, MCT+PDTC day1) and day 3 (lane 4, MCT+PDTC day3), 4 wk post-MCT (lane 5), and PDTC treatment started on day 14 post-MCT (lane 6, MCT+PDTC day14). Significant activation of PY-STAT3 is present at 2 and 4 wk post-MCT. There is significant reduction in PY-STAT3 activation in MCT+PDTC day 1 and MCT+PDTC day 3 groups. PDTC treatment started on day 14 did not inhibit MCT-induced PY-STAT3 activation. \(^*P<0.05\) vs. controls (n = 3–12).
induced PAH is associated with progressive activation of PY-STAT3 (36), and, furthermore, endothelial cells harvested from patients with idiopathic PAH show PY-STAT3 activation (30). These observations strongly support a role for PY-STAT3 activation in the pathogenesis of PAH.

Superoxide has been implicated in the pathogenesis of PAH. Our present studies reveal that the increased superoxide chemiluminescence in pulmonary arteries is a transient phenomenon. It is observed only at 2 wk post-MCT at the onset of PAH but not at 4 wk post-MCT in the presence of sustained PAH. Thus superoxide does not appear to participate in the initiation or progression of MCT-induced PAH. It is likely that superoxide generation increases transiently at the start of PAH, and PDTC treatment started at days 1 and 3 inhibits the MCT-induced increased superoxide chemiluminescence.

The activation of NF-κB has been reported in primary PAH in humans and MCT-induced PAH in rats (23, 42). NF-κB controls cell proliferation and differentiation and regulates the expression of cytokines. The activation of NF-κB is a common endpoint of various signal transduction pathways (17, 44). Recently, Sawada et al. (43) have shown that PDTC treatment from day 3 to 16 post-MCT results in significant attenuation of PAH and decrease in inflammatory markers such as VCAM-1 and ED1. In addition, PDTC significantly decreased the number of cells stained with phosphorylated p65 (a subunit of NF-κB) in the lung sections of rats injected with MCT. In our studies, NF-κB activation followed the same pattern as superoxide chemiluminescence. NF-κB DNA binding occurred only at 2 wk post-MCT, and NF-κB activation persisted despite PDTC-induced attenuation of PAH and inhibition of superoxide generation. It is worth noting here that NF-κB DNA binding is significantly reduced at 4 wk post-MCT despite persistent PAH and I-κBα loss. Thus NF-κB activation is independent of PAP, and it may not have any significant role in the pathogenesis of MCT-induced PAH. The reason for discrepancy between our results and the studies of Sawada et al. (43) is not clear. It may be dependent on differences in the methods employed. Significant reduction in the expression of I-κBα was present at 2 and 4 wk post-MCT. I-κBα is an inhibitory protein of NF-κB. It is interesting that at 4 wk post-MCT, despite significant degradation of I-κBα, NF-κB DNA binding is low. The loss of I-κBα may be the result of extensive and progressive endothelial cell damage observed in the MCT model of PAH. Our previous studies (33) as well as the present one show that PY-STAT3 is activated by 1 wk post-MCT, before the development of PAH, whereas a significant reduction in the expression of I-κBα occurs at 2 wk post-MCT. Interestingly, in some cell systems, STAT3 is a requirement for NF-κB activation and nuclear translocation of NF-κB (13), and superoxide also can activate NF-κB. Furthermore, NF-κB is thought to play a crucial role in smooth cell proliferation observed in balloon-induced arterial injury (5, 9). In contrast, our results show that NF-κB activation is independent of PAP, superoxide generation, I-κBα expression, and PY-STAT3 activation. Further studies are required to assess the role of NF-κB or the lack of it in the MCT model.

In vivo and in vitro studies have shown PDTC to be an inhibitor of NF-κB activation by various stimuli. However, the precise mechanism by which PDTC inhibits NF-κB activation remains controversial. Recent studies have shown that in some cell systems PDTC acts as an anti-inflammatory agent inhibiting IL-6-mediated STAT3 activation (20). In the present study, PDTC in a dose-dependent manner rescued endothelial cell membrane integrity as evidenced by reversal of MCT-induced destruction/degredation of plasmalemmal membrane proteins such as caveolin-1 and Tie2. These results indicate that it is the anti-inflammatory activity of PDTC that resulted in the restoration of membrane integrity. This concept is further supported by our present data showing restoration of membrane proteins and inhibition of PY-STAT3 by PDTC at 1 wk post-MCT. Furthermore, Sawada et al. (43) have shown that PDTC treatment significantly reduces inflammatory markers in the lungs of rats injected with MCT. The role of caveolin-1 in PAH is further supported by recent studies by Jasmin et al. (26) showing that administration of cell-permeable caveolin-1 peptide inhibits the activation of PY-STAT3 and attenuates MCT-induced PAH. Thus it is likely that the inflammatory response to MCT results in the disruption of

![Fig. 11. Representative lung sections depicting pulmonary arteries (>95 μm) in control (A), 48 h post-MCT (B), 1 wk post-MCT (C), 2 wk post-MCT (D), MCT+PDTC treatment started on day 1 (E) and on day 3 (F), 4 wk post-MCT (G), and MCT+PDTC started on day 14 (H). Pulmonary arteries appear normal at 48 h and 1 wk post-MCT (B and C). At 2 and 4 wk post-MCT (D and G), there is significant increase in medial thickness compared with the control. PDTC treatment started on days 1 and 3 (E and F) show significant reduction in the MCT-induced medial wall thickening, whereas PDTC treatment started at 14 days post-MCT had no effect on MCT-induced vascular remodeling. The histological picture is consistent with hemodynamic data.](http://ajplung.physiology.org/)
endothelial cell membrane integrity, and loss of caveolin-1 and reciprocal activation of PY-STAT3 play a crucial role in the MCT model of PAH.

In the present study, early sustained treatment with PDTC prevented degradation of I-κBα protein and inhibited superoxide chemiluminescence in addition to rescuing membrane integrity and attenuation of PAH. Interestingly, superoxide chemiluminescence and NF-κB activation occur transiently at 2 wk post-MCT, and, in the present study, PDTC treatment had no effect on NF-κB activation. Therefore, the rescue of caveolin-1 and its antiproliferative activity resulting in the attenuation of PAH may have had a greater role in modulating the I-κBα expression and superoxide chemiluminescence. The anti-inflammatory role of PDTC seems to have restored membrane integrity and attenuated PAH. PDTC, however, does not reverse or halt the progression of MCT-induced disruption of membrane integrity, loss of caveolin-1, or PY-STAT3 activation once PAH is already established. Nitric oxide (NO) plays an important physiological role in inhibiting the activation of STAT3 and downregulating proinflammatory cytokines. In this context, it is important to note that most forms of PAH including MCT-induced PAH show a significant reduction in NO bioavailability. Treatment with exogenous NO not only attenuates PAH and RVH in the MCT model, but also preserves the expression of caveolin-1 protein (31, 35). Since NO has an anti-inflammatory role, one of the mechanisms by which NO attenuates MCT-induced PAH may be via inhibition of inflammation and stabilization of endothelial plasmalemmal membrane resulting in the rescue of caveolin-1. These data suggest the antiproliferative properties of caveolin-1 may have a significant role in attenuating PAH.

In summary, the novel observation in this study is that the inflammatory response of MCT is associated with the disruption of endothelial cell membrane integrity, early and progressive loss of membrane proteins such as caveolin-1, Tie2, and I-κBα, and PY-STAT3 activation. Superoxide generation and NF-κB activation occur transiently at 2 wk post-MCT. Treatment with PDTC started early (on days 1 and 3) restored membrane integrity, rescued caveolin-1, Tie2, and I-κBα, and inhibited PY-STAT3 activation, leading to the attenuation of PAH. PDTC had no effect on established PAH. We conclude that protecting and/or restoring the endothelial cell membrane integrity may be a useful therapeutic approach in treatment of PAH, particularly that which is associated with inflammation, infection, and autoimmune diseases.

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