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Pyrrolidine Dithiocarbamate Restores Endothelial Cell Membrane Integrity and Attenuates Monocrotaline-Induced Pulmonary Artery Hypertension

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Abstract

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- κ B have been implicated in PAH. To evaluate the role of caveolin-1, PY-STAT3 activation and superoxide in PAH, MCT-injected rats were treated daily with pyrrolidine dithiocarbamate (PDTC, starting on day 1, 3 and 14 $\times$ 2 wks) an inhibitor of inflammation and NF- κ B activation. Hemodynamic data, the expression of inhibitory (I)- κ B α , caveolin-1 and Tie2 (a membrane protein), activation of PY-STAT3 and NF- κ B, and superoxide chemiluminescence were examined. Rats developed progressive PAH at 2 wks post-MCT. There were progressive reduction in the expression of caveolin-1, Tie2 and activation of PY-STAT3 in the lungs. Reduction in I- κ B α expression was present at 2 and 4 wks post-MCT. Superoxide chemiluminescence and NF- κ B activation were observed only at 2 wks post-MCT and both decreased by 4 wks post-MCT despite progressive PAH. PDTC (starting on day 1 and 3) rescued caveolin-1 and Tie2, reversed MCT-induced PY-STAT3 activation, and attenuated PAH. In addition, PDTC restored I- κ B α expression and reduced superoxide chemiluminescence at 2 wks, but did not inhibit NF- κ B activation despite attenuation of PAH. PDTC had no effect on established PAH. Increased superoxide chemiluminescence and NF- κ B activation appear to be a transient phenomenon in the MCT model. Thus, the disruption of endothelial cell membrane integrity resulting in cav-1 loss and reciprocal activation of PY-STAT3 plays a key role in the MCT-induced PAH.

Keywords

Caveolin-1; I- κ B α ; Inflammation; PY-STAT3; Tie2

INTRODUCTION

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 interleukin (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 signal transducer and activator of transcription (STAT) 3, a transcription factor which 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 showed a marked activation of STAT3 (phosphorylation at Tyr 705, PY-STAT3) coupled with an early and progressive loss of caveolin-1 expression in rat lungs with MCT-induced PAH [33]. 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 [2,24,33].

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 have shown that in addition to caveolin-1, other membrane proteins such as PECAM-1 and Tie2 that localize in the caveolae show parallel reduction indicating a generalized disruption of the caveolar membrane integrity caused by MCT [33,34]. Tie2 has been implicated in the pathogenesis of PAH [46]. It is worth noting here that vWF which is localized in the Weibel Palade bodies of endothelial cells are not affected up to 2 wks 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- κ B has been reported in primary PAH and in the MCT model of PAH [23,42]. Both oxidant injury and inflammation are known to activate nuclear factor (NF)- κ B which regulates the expression of several genes involved in inflammation including IL-1 and IL-6, and also mediates proliferative responses. Inactive NF- κ B exists in the cytoplasm in association with inhibitory (I)- κ B family of proteins such as I- κ B α . The release from the inhibitors is critical for the translocation of NF- κ B to the nucleus to induce transcription of targeted genes [1,4,28,48]. The activation of NF- κ B is considered essential for proliferation of cultured smooth muscle cells, and its activation has been reported in the balloon-induced injury of arteries in rats [5,9]. In addition, over expression of mutated I- κ B α or administration of antisense p65 (a subunit of NF- κ B) inhibits vascular smooth muscle cell proliferation and intimal hyperplasia [3,50].

Based on these observations we hypothesize 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- κ B 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 2 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 wks 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 I- κ B α , 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 monocrotaline (MCT, Trans World Chemicals, Rockville, MD) as described previously.

PDTC Treatment

There were 4 groups of rats that received PDTC treatment. *Group 1*: In this group, rats received MCT and daily subcutaneous injection of PDTC (50, 100 or 200 mg/kg for 2 wks,) starting on day 1. A subset of rats receiving PDTC 100 mg/kg/day was examined at 1 wk post-MCT. *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 wks. *Group 3*: Rats were started on PDTC (200 mg/kg/day) on day 14, when PAH was already established. We opted to use a higher dose (200 mg/kg/day) 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 hrs of MCT injection. *Group 4*: To evaluate the effects of PDTC alone, a group of control rats (n=9) were treated with 50, 100, 200 mg/kg PDTC/day (3 rats in each group). PDTC (Sigma Co, St Louis, MO) was freshly dissolved in normal saline (pH 7.3) each day. True controls did not receive PDTC.

Measurements of Pulmonary Artery Pressure (PAP)

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 PE240 tubing, and the rat ventilated in room air at a rate of approximately 70–80 breaths per minute with a tidal volume of 0.83 ml/100 g body weight. The chest was opened and PE50 tubing inserted into the right ventricle 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 10mM HEPES.NaOH (pH7.4) for measurement of superoxide generation. Right lung was removed, quickly frozen in liquid N₂ and stored at –80°C for protein extraction. Left lung and heart were placed in 10% buffered formaldehyde.

Assessment of Right Ventricular Hypertrophy (RVH)

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

Measurement of Superoxide

Superoxide generation from pulmonary arteries (main and hilar) and aorta from rats (48h, 1, 2 and 4 wks 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 μ M lucigenin and chemiluminescence measured in a liquid scintillation counter (LS6000IC, Beckman Instruments) at $\approx 37^\circ$ C. The data are expressed as counts per minute per milligram of arterial tissue after background subtraction.

Protein Extraction and Western Blot Analysis

The lung tissue was homogenized in a buffer containing 0.1M PBS (pH 7.4), 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS, 10 μ l/ml phenylmethylsulfonyl fluoride (PMSF, 10 mg dissolved in 1 ml of isopropanol), 25 μ g/ml aprotinin, and 25 μ g/ml leupeptin. PMSF (10 μ l/ml) was added to homogenates placed on ice for 30 minutes, and centrifuged at 14,000 rpm for 20 minutes at 4° C. Protein concentrations in the supernatants were analyzed by using a kit from Bio-Rad and serum albumen as standard. Fifty μ g of protein was loaded and separated on a 10% sodium SDS-polyacrilamide gel (Mini Protean-II, Bio-Rad) and transferred to nitrocellulose membrane (Hybond ECL, Amersham Biobiosciences, MA) using Semi Dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% non fat milk powder in Tris-Buffered-Saline with Tween buffer (TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) overnight at 4° C. Membranes were then incubated with I- κ B α (sc 371, 1:500, Santa Cruz Lab, CA), endothelium-specific caveolin-1 α (sc 897, 1:4000, Santa Cruz) or Tie2 (sc 324, 1:500, Santa Cruz) or STAT3 (1:5000, BD Bioscience) for 1 h and 20 min at room temperature or with PY-STAT3 (1:1000, #9131, Cell Signaling Technology) overnight at 4° C. The membranes were washed for 10 min X3 with TBST and incubated with appropriate horseradish peroxidase secondary antibody (anti-rabbit antibody, 1:5000 for I- κ B α , cav-1, Tie2 and PY-STAT3, and anti-mouse antibody 1:2000 for β -actin and STAT3) for 1 hour at room temperature, then washed for 10 min X3 with TBST buffer. The protein bands were visualized by chemiluminescence (Amersham ECL, Western Blotting Detection System, Amersham Biosciences). Membranes were re-probed with β -actin (A5441, Sigma, St Louis, MO) to assess the protein loading. PY-STAT3 blots were re-probed with STAT3 antibody. The relative expression of the proteins was quantified using densitometric scanning and expressed as % of controls.

Electrophoretic Mobility Shift Assay (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 “B” pestle in a low salt buffer (0.6% Nonidet-40, 150mM NaCl, 10mM HEPES pH 7.9, 1mM EDTA, 1 mM dithiothreitol, 0.5mM PMSF, 1 $\times$ protease inhibitor cocktail (Sigma P8340). The homogenate was then centrifuged twice (30s; 4° C; $500 \times g$) with the supernatant carefully retained to remove debris and avoid the pellet. Following this, the samples were incubated on ice for 5 minutes to allow swelling of the nuclei. The nuclei were pelleted by centrifugation (5 min; 4° C; $3500 \times g$) and lysed on ice for 20 minutes, 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 dithiothreitol, 1 $\times$ protease inhibitor cocktail for 20 min. The lysed nuclear mixture was centrifuged at 16,000g for 30 sec. The supernatant was aliquoted and snap frozen in dry ice and ethanol, and stored at -80° C until further use.

Mobility Shift Assay—NF- κ B nucleotide 5'-AGT TGA GGG GAC TTT CCC AGG C 3' was annealed and 5'-end-labeled with [³²P] ATP (3000 Ci/mmol) using T4 polynucleotide kinase according to standard procedure. Binding reactions was performed by preincubation of

nuclear extract protein (30 μ g) in HEPES (pH 7.9, 12 mM), KCl (60 mM) $MgCl_2$ (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 ^{32}P -labeled oligonucleotide (50000 cpm) and a second incubation at room temperature for 30 min. Fifteen μ l of reaction mixture was loaded onto each lane of a nondenaturing polyacrylamide-bisacrylamide gel (5%) prepared in 0.5X Tris-Borate/EDTA (Tris 45 mM, boric acid 45 mM and EDTA 0.1M) and electrophoresis was performed at room temperature for 1.5–2 h at 100 volts. The gel was dried before being exposed in a phosphor screen cassette. Band intensities were imaged using a Storm 840 Phosphorimager.

Lung Histology—Mid sections from the left lungs preserved in 10% buffered formaldehyde were processed for microscopic examination by paraffin embedding. Five μ m thin sections were stained with elastic van Giesen method for the identification of pulmonary arteries with elastic laminae.

Statistical Analysis—The data are expressed as means $\pm$ SEM. For statistical analysis we used one way analysis of variance 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 weeks in the MCT group was significantly lower compared with the controls (controls, $82 \pm 9\%$, $n=8$ vs MCT, $50 \pm 3\%$, $n=10$, $P < 0.05$). The weight gain in MCT+PDTC group 1 (MCT+PDTC 50 mg/kg, $33 \pm 4\%$, $n=8$, MCT+PDTC 100 mg/kg, $43 \pm 5\%$, $n=7$, MCT+PDTC 200 mg/kg, $42 \pm 4\%$, $n=6$, 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 ($31 \pm 2.3\%$) as the MCT group. The weight gain in rats 4 wks post MCT ($n=3$) was only $31 \pm 1\%$, with a further loss during the last 2 days before experiment. The comparable controls gained $99 \pm 14\%$ ($n=4$). Rats started PDTC treatment 2 wks post-MCT also showed low weight gain ($36 \pm 4\%$, $n=5$). PDTC treatment alone had no adverse effect on weight gain in the controls.

PAP and RVH

As shown in Figure 1A and 1B rats developed significant PAH and RVH at 2 wks with a further progression at 4 wks post-MCT. At 48h 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- κ B α Protein and NF- κ B Activation

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

Superoxide Generation

There was a significant increase in lucigenin chemiluminescence in pulmonary arteries at 2 wks post-MCT compared with the controls, indicating increased superoxide generation (Fig 4). At 48h and 1 wk post MCT, there was no increase in lucigenin chemiluminescence. Importantly, lucigenin chemiluminescence was not increased at 4 wks post-MCT despite increased pulmonary artery pressure. 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 (Figure 5) show progressive activation of PY-STAT3 in the lungs from 1 to 4 wks post-MCT.

PDTC Treatment

Effect of PDTC on MCT-Induced PAH and RVH—As depicted in Figure 6 (A and B), treatment with PDTC (100 mg/day $\times$ 14 days) started on day 1 and day 3 normalized PAP and RVH. However, PDTC did not reverse the established PAH or halt its progression. Treatment with 50 mg PDTC started on day 1 (n=5) significantly lowered pulmonary artery pressure (25 ± 2.6 , mmHg, $P < 0.05$ vs controls and 2 wks 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 (0.24 ± 0.007). PDTC alone (all doses) had no effect on pulmonary artery pressure (18.8 ± 1 mmHg, n=9) or on RV/LV ratio (0.23 ± 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).

Effect of PDTC on MCT-Induced Reduction in Caveolin-1 and Tie2 Expression—PDTC treatment (100 mg/kg/day) started on day 1 and day 3 significantly restored the expression of endothelial cell membrane proteins caveolin-1 and Tie2 (Figure 7). Treatment with 50 and 200 mg/kg PDTC (day1) 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 Figure 7, PDTC treatment started at 2 wks post MCT failed to reverse or halt MCT-induced progressive loss of caveolin-1 and Tie2.

Effect of PDTC on MCT-Induced I- κ B α Degradation—PDTC treatment started on day 1 and 3 post-MCT completely reversed MCT-induced degradation of I- κ B α . PDTC started on day 14 post-MCT did not attenuate I- κ B α degradation (Fig. 8, left panel). PDTC in controls did not affect the expression of I- κ B α (lane 2). Treatment with 50 and 200 mg/kg PDTC (day1) showed dose-dependent prevention of MCT-induced degradation of I- κ B α ($60 \pm 7\%$ and $93 \pm 5\%$ respectively).

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

Effect of PDTC on Superoxide Chemiluminescence—Increased lucigenin chemiluminescence is observed in pulmonary arterial tissue only at 2 wks post-MCT which is reversed by PDTC treatment started on day 1 and day 3 post-MCT (Figure 9). Treatment with 50 and 200 mg/kg PDTC (day1) also completely reversed MCT-induced superoxide generation (543 ± 184 , n=5, and 648 ± 172 , cpm/mg tissue, n=3 respectively). Rats started on PDTC treatment for 2 wks at 2 wks post-MCT showed normal lucigenin chemiluminescence in

pulmonary arterial tissue. This is not surprising because at 4 wks 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 figure 10, PDTC treatment started on day1 and 3 post-MCT completely inhibited PY-STAT3 activation. Not surprisingly, PDTC started at 2 wks failed to prevent PY-STAT3 activation which is consistent with the hemodynamic data. Treatment with 50 and 200 mg/kg PDTC (day1) inhibited PY-STAT3 activation in a dose-dependent manner ($220 \pm 34\%$ and $115 \pm 5\%$ respectively, compared to the controls 100% and 2 wks post-MCT $480 \pm 37\%$).

Effect of PDTC at 1 wk post-MCT—At one wk post-MCT there is no evidence of PAH or RVH (Figure 1). PDTC (n=3) had no effect on PAP (19 ± 0.7 mmHg) or on RV/LV ratio (0.25 ± 0.08). PDTC significantly restored the expression of caveolin-1 (control=100%, MCT= $44 \pm 8\%$ *, MCT+PDTC = $108 \pm 16\%$, * = $P < 0.05$ vs controls), and Tie 2 (control=100%, MCT= $46 \pm 5\%$ *, MCT+PDTC = $97 \pm 3\%$, * = $P < 0.05$ vs controls); and significantly inhibited PY-STAT3 activation (control=100%, MCT= $326 \pm 40\%$ *, MCT+PDTC = $150 \pm 15\%$, * = $P < 0.05$ vs controls). At one wk post-MCT, I- κ B α levels were slightly lower than the controls, but it did not reach statistical significance. PDTC treatment did show improvement in the expression of I- κ B α (control=100%, MCT= $77 \pm 13\%$, MCT+PDTC = $97 \pm 14\%$, P=ns).

Effect of PDTC on Pulmonary Vascular Remodeling—As shown in Fig. 11, at 48h 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 wks post-MCT. PDTC treatment started on day1 and on day3 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, 3) a significant reduction in the expression of I- κ B α in the lungs at 2 and 4 wks post-MCT in the presence of PAH, 4) Interestingly, NF- κ B activation occurred only at 2 wks post-MCT at the onset of PAH, but during sustained PAH (3 and 4 wks post-MCT) NF- κ B activation was significantly attenuated despite progressive reduction in the level of expression of I- κ B α . 5) Similarly, increased lucigenin chemiluminescence in pulmonary arteries occurred only at 2 wks post-MCT with normalization at 4 wks. Thus, NF- κ B activation and superoxide may not have a significant role in the pathogenesis of MCT-induced PAH, 6) PDTC treatment started on day 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 wks post-MCT, 7) In addition, PDTC restored the expression of I- κ B α and inhibited superoxide generation in pulmonary arteries at 2 wks post-MCT, 8) PDTC at one wk post-MCT rescued membrane proteins and inhibited PY-STAT3 activation, 9) PDTC started at 2 wks post-MCT did not restore membrane integrity or inhibit PY-STAT3 activation; and it failed to reverse or halt the progression of established PAH.

Inflammation is thought to play a major role in vascular diseases including MCT-induced PAH. We have previously shown that the pulmonary endothelial cell damage occurs within 48h of MCT injection, and is accompanied by an early and progressive upregulation of IL-6 mRNA, an increased biological activity of IL-6, and the activation of PY-STAT3 in pulmonary arteries [6,33]. The transcription factor STAT3 mediates cell growth and differentiation, and is known

to be activated by IL-6 [21]. Importantly, the activation of PY-STAT3 is associated with concomitant reduction in the expression of caveolin-1. In addition, MCT-induced PAH is associated with a progressive loss of other endothelial plasmalemmal membrane signaling protein molecules such as PECAM-1 and Tie2 in tandem with caveolin-1. These changes occur before the development of PAH [33,34]. Thus, the disruption of endothelial caveolar membrane integrity with a loss of multiple signaling molecules residing in the caveolae is an early and a progressive phenomenon in the MCT model of PAH. Interestingly, recent studies have implicated Tie2 in the pathogenesis of PAH [46]. In the MCT model the loss of Tie2 appears to be a part of a generalized endothelial cell membrane disruption.

Caveolin-1, a 21–24 kD protein is a major resident scaffolding protein constituent of caveolae, and is known to interact with and modulate numerous signal transducing pathways [37,40, 45]. Caveolin-1, also known as a tumor suppressor factor, inhibits cell proliferation by inhibiting various cell proliferative pathways including STAT3; and it plays a pivotal role in the modulation of cell cycle progression. Therefore, the reduction in caveolin-1 is likely to deregulate cell proliferation leading to vascular remodeling and PAH. A reduction in the expression of caveolin-1 has been reported in several forms of PAH [2,24,33,49]. In addition, arterial smooth muscle cells lacking 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 capacitative 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 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. Upon 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 janus kinase (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-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 wks post-MCT at the onset of PAH but not at 4 wks 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 day 1 and 3 inhibits the MCT-induced increased superoxide chemiluminescence.

The activation of NF- κ B has been reported in primary PAH in humans and MCT-induced PAH in rats [23,42]. NF- κ B controls cell proliferation and differentiation, and regulates the expression of cytokines. The activation of NF- κ B 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- κ B) in the lung sections of rats injected with MCT. In our studies, NF- κ B activation followed the same pattern as superoxide chemiluminescence. NF- κ B DNA binding occurred only at 2 wks post-MCT; and NF- κ B activation persisted despite PDTC-induced attenuation of PAH and inhibition of superoxide generation. It is worth noting here that NF- κ B DNA binding is significantly reduced at 4 wks post-MCT despite persistent PAH and I- κ B α loss. Thus, NF- κ B activation is independent of pulmonary artery pressure, 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- κ B α was present at 2 and 4 wks post-MCT. I- κ B α is an inhibitory protein of NF- κ B. It is interesting that at 4 wks post-MCT, despite significant degradation of I- κ B α , NF- κ B DNA binding is low. The loss of I- κ B α may be the result of extensive and progressive endothelial cell damage observed in the MCT model of PAH. Our previous studies as well as the present one show that PY-STAT3 is activated by 1 wk post-MCT, before the development of PAH [33], whereas a significant reduction in the expression of I- κ B α occurs at 2 wks post-MCT. Interestingly, in some cell systems, STAT3 is a requirement for NF- κ B activation and nuclear translocation of NF- κ B [13]; and superoxide also can activate NF- κ B. Furthermore, NF- κ B 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- κ B activation is independent of PAP, superoxide generation, I- κ B α expression and PY-STAT3 activation. Further studies are required to assess the role of NF- κ B or the lack of it in the MCT model.

In-vivo and in-vitro studies have shown PDTC to be an inhibitor of NF- κ B activation by various stimuli. However, the precise mechanism by which PDTC inhibits NF- κ B activation remains controversial. Recent studies have shown that in some cell system 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/degradation 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 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 endothelial cell membrane integrity, 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- κ B α protein and inhibited superoxide chemiluminescence, in addition to rescuing membrane integrity and attenuation of PAH. Interestingly superoxide chemiluminescence and NF- κ B activation occur transiently at 2 wks post-MCT and in the present study PDTC treatment had no effect on NF- κ B 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- κ B α 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 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 physiologic role in inhibiting the activation of STAT3 and down-regulating 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- κ B α , and PY-STAT3 activation. Superoxide generation and NF- κ B activation occur transiently at 2 wks post-MCT. Treatment with PDTC started early (on day 1 and day 3) restored membrane integrity, rescued caveolin-1, Tie2 and I- κ B α 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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Figure 1.

Bar graphs depicting significant PAP (A) and increased right ventricle (RV)/left ventricle and the septum (LV) (Fig. B) at 2 and 4 wks post-MCT. There is no PAH or RVH at 48h and 1 wk post-MCT (n=3–12).. * = $p < 0.05$ vs controls.

Figure 2.

A representative western blot and a bar graph showing the expression of Tie2, caveolin-1 and β -actin in the lungs of controls (lane 1) and 48h, 1, 2 and 4 wks 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).

Figure 3.

A representative western blot showing the expression of I-κBα and β-actin (Lane 1= control, lanes 2–5 = 48 h, 1, 2 and 4 wks post-MCT). The bar graph shows a significant reduction in the I-κBα expression is seen at 2 and 4 wks post-MCT. * = $p < 0.05$ vs controls ($n = 3-12$). The right panel shows activation of NF-κB binding by monocrotaline treatment (lanes 1–6: control, 48h, 1, 2, 3 and 4 wks post-MCT respectively). Note: since NF-κB activation was significantly reduced at 4 wks post-MCT, we included a 3 wks post-MCT sample. This figure clearly shows that NF- κB activation occurs transiently at 2 wks post-MCT.

Figure 4.

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

Figure 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, 48h, 1, 3, and 4 wks post-MCT. * = $p < 0.05$ vs controls, ** = $P < 0.05$ vs controls and 1 wks post-MCT, *** = $P < 0.05$ vs controls, 1 and 2 wks post-MCT ($n = 3-12$).

Figure 6.

Bar graphs depicting PAP (A) and RV/LV (B) ratio in controls, 2 and 4 wks post-MCT and in rats treated with PDTC (100 mg/kg/day $\times$ 14 days) starting on day 1 (MCT+PDTC day1) and 3 (MCT+PDTC day3), and PDTC (200 mg/kg/day $\times$ 14 days) starting on day 14 post-MCT (MCT+PDTC day14). There is significant reduction in PAP and RV/LV ratio in rats receiving PDTC from day 1 and day 3. PDTC treatment started on day 14 did not attenuate MCT-induced PAH or RVH. * = $p < 0.05$ vs controls (n = 3-12).

Figure 7.

A representative western blot showing the expression of caveolin-1, Tie2 and β -actin in lane 1 (control), lane 2 (PDTC control) lane 3 (2 wks post-MCT), lane 4 (MCT+ PDTC started on day 1), lane 5 (MCT+ PDTC started on day 3), lane 6 (4 wks post-MCT) and lane 7 (MCT+ PDTC started on day 14). PDTC in controls did not alter caveolin-1 or Tie2 expression (lane 2). Bar graph shows significant reduction in the expression of caveolin-1 and Tie2 in rat lungs 2 and 4 wks post-MCT. PDTC treatment started on day 1 (MCT+PDTC day1) and 3 (MCT +PDTC day3) rescued caveolin-1 and Tie2 expression. In contrast, PDTC treatment started at 2 wks post-MCT for 2 wks (MCT+PDTC day14) had no effect on MCT-induced loss of caveolin-1 and Tie2. * = $P < 0.05$ vs Controls, # = $P < 0.05$ vs controls and MCT ($n = 3-12$).

Figure 8.

A representative western blot showing the expression of I-κBα in lane 1 (control), lane 2 (PDTC control), lane 3 (2 wks post-MCT), lane 4 (MCT+ PDTC started on day 1), lane 5 (MCT+ PDTC started on day 3), lane 6 (4 wks post-MCT) and lane 7 (MCT+ PDTC started on day 14). PDTC in controls did not alter I-κBα expression (lane 2). Bar graph depicts significant reduction in the expression of I-κBα at 2 and 4 wks post-MCT. PDTC treatment started on day 1 (MCT+PDTC day1) and 3 (MCT+PDTC day3) normalized I-κBα expression. PDTC started on day 14 (MCT+PDTC day14) had no effect on MCT-induced reduction in the expression of I-κBα. * = $P < 0.05$ vs controls. (n = 3–12).

Activation of NF-κB binding by monocrotaline treatment is depicted in lane 1 (PDTC control), lane 2 (2 wks post-MCT), lane 3 and 4 (MCT+ PDTC treatment started on day1 and 3 respectively) and lane 5 (MCT+PDTC treatment started on day 14). NF-κB activation is present at 2 wks post-MCT despite PDTC (day 1 and 3)-induced restoration of I-κBα, and attenuation of PAH and RVH. NF-κB activation was not present in lungs from rats started on PDTC at day 14 post-MCT. This is not surprising because NF-κB activation is attenuated at 4 wks post-MCT.

Figure 9.

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

Figure 10.

A representative western blot and bar graph depicting PY-STAT3 activation and STAT3 expression in rat lungs from control (lane 1), 2 wks post-MCT (lane 2), MCT + PDTC started on day 1 (lane 3, MCT+PDTC day1) and day 3 (lane 4, MCT+PDTC day3), 4 wks post-MCT (lane 5), and PDTC treatment started on day 14 post-MCT (lane 6, MCT+PDTC day 14). Significant activation of PY-STAT3 is present at 2 and 4 wks post-MCT. There is significant reduction in PY-STAT3 activation in MCT+PDTC day1 and MCT+PDTC day3 groups. PDTC treatment started on day 14 did not inhibit MCT-induced PY-STAT3 activation. * = $P < 0.05$ vs controls ($n = 3-12$).

Figure 11.

Representative lung sections depicting pulmonary arteries ($>95\ \mu\text{m}$) in control (A), 48h post-MCT (B), 1wk post-MCT (C), 2 wks post-MCT (D), MCT+PDTC treatment started on day 1 (E) and on day 3 (F), 4 wks post-MCT (G) and MCT+PDTC started on day 14 (H). Pulmonary arteries appear normal at 48h and 1 wk post-MCT (B and C). At 2 and 4 wks post-MCT (D and G) there is significant increase in medial thickness compared with the control. PDTC treatment started on day 1 and on day 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.