Propylthiouracil Attenuates Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats

Cheuk-Kwan Sun, MD, PhD; Chun-Man Yuen, MD*; Ying-Hsien Kao, PhD**; Li-Teh Chang, PhD†; Sarah Chua, MD††; Jiunn-Jye Sheu, MD‡; Chia-Hung Yen, PhD†‡; Sheung-Fat Ko, MD§; Hon-Kan Yip, MD†††

Background: Propylthiouracil (PTU) enhances nitric oxide production and inhibits smooth muscle cell proliferation, suggesting a possible role in the prevention of pulmonary arterial hypertension (PAH).

Methods and Results: The 30 male Sprague-Dawley rats were randomized to receive saline injection only (group 1), monocrotaline (MCT) (70 mg/kg) only (group 2) or MCT+0.1% PTU in drinking water (group 3) given on day 5 after MCT administration. By day 35, western blot showed lower connexin43 (Cx43) and membranous protein kinase C-ε expressions in the right ventricle (RV) of group 2 animals than in the other groups (all P<0.05). Conversely, Cx43 expression in the lung was higher in group 2 than in other groups (all P<0.02). Additionally, mRNA expressions of matrix metalloproteinase-9, tissue necrotic factor-α, and caspase-3 were higher, whereas Bcl-2 and endothelial nitric oxide synthase were lower, in the lungs and RV of group 2 rats than in the other groups (all P<0.05). Moreover, the numbers of alveolar sacs and lung arterioles were also reduced in group 2 than in other groups (all P<0.05), and RV systolic pressure and RV weight were increased in group 2 compared with other groups (all P<0.001).

Conclusions: PTU effectively attenuates complications associated with MCT-induced PAH. (Circ J 2009; 73: 1722–1730)

Key Words: Monocrotaline; Propylthiouracil; Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a devastating disease that can drastically limit physical capacity and seriously reduce life expectancy.1–4 Pathologic changes of PAH, characterized by vascular smooth muscle cell proliferation and obliteration of small pulmonary arteries,5 may ultimately lead to heart failure and death.3,4 Despite widespread use of state-of-the-art medical management against PAH during the past decade,6 the prognosis remains poor.4,8,9 Although the precise mechanistic basis of PAH is currently unclear, a process involving endothelial dysfunction and VSMC proliferation and migration is essential in the development of all stages of PAH, from initiation, progression, and finally to the evolution of complications.8–10

Monocrotaline (MCT), which is known to induce selective pulmonary endothelial injury in rats,8–12 causes pulmonary hypertension, interstitial pulmonary fibrosis, and proliferation of muscular intimal cells in pulmonary arterioles and fibroblasts in the alveolar walls at the capillary level.9,11–13 Although the pathologic changes in MCT-induced PAH and clinical PAH are not similar, because of differences in pathogenesis, the MCT-induced PAH model has been validated and widely accepted for PAH-related studies because of the consistent induction of selective pulmonary arteriolar endothelial damage.

In addition to its routine use in patients with hyperthyroidism, because of its thyroid-suppressing function, propylthiouracil (PTU) has also been shown to possess an antioxidant property.14–16 enhance nitric oxide (NO) production, inhibit VSMC proliferation and migration, as well as collagen production.15,16 Accordingly, PTU has recently been demonstrated to exhibit an anti-atherosclerotic effect.17,18 The aims of this study were, therefore, to determine whether PTU can also effectively attenuate MCT-induced PAH in the rat, in terms of alterations in pulmonary microvasculature and structure, as well as to investigate the underlying mechanisms of biological signaling.

Methods

Ethics

All animal experimental procedures were approved by the institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, DC, USA, revised 1996).
Animal Models of PAH
On day 0, 20 pathogen-free, adult male Sprague-Dawley (SD) rats, weighing 350–370 g (Charles River Technology, BioLASCO Co, Ltd, Taipei, Taiwan) were given a subcutaneous injection of MCT (70 mg/kg; Sigma, St Louis, MO, USA). On day 5, the MCT-treated animals were assigned to 2 experimental groups: group 2 (MCT alone, n=10) and group 3 [MCT fed with 0.1% PTU (Sigma) in drinking water (n=10)]. Another group of 10 SD rats (group 1) receiving neither MCT nor PTU treatment served as normal controls. PTU therapy was implemented immediately after the group assignment.

Rationale of the PTU Dosage
The dosage of the drug was according to previous descriptions.0,1,2 PTU in water 100 ml is equivalent to 0.27 mg/100 ml of drinking water, which was the average daily amount of water consumption for each rat in our study.

Hemodynamic Measurements
On day 35 after MCT administration, each rat was anesthetized with an intraperitoneal injection of chloral hydrate (35 mg/kg). After being shaved on the chest, each animal was endotracheally intubated with positive-pressure ventilation (180 ml/min) with room air using a small animal ventilator (SAR-830/A, CWE Inc, USA). The heart was exposed by left thoracotomy. A sterile 20-gauge, soft plastic-coated needle was inserted into the right ventricle (RV) and femoral artery of each rat to measure the systolic pressure (RVSP) and arterial pressure, respectively. The pressure signals were first transmitted to pressure transducers (UFI, model 1050, Morro Bay, CA, USA) and were then exported to a bridge amplifier (ML866 PowerLab/4/30 Data Acquisition Systems, ADInstruments Pty Ltd, Castle Hill, NSW, Australia) where the signals were amplified and digitized. The data were recorded and later analyzed with the Labchart software (ADInstruments). After hemodynamic measurements, the rats were euthanized and the hearts and lungs were harvested. For each animal, the RV weight, whole heart weight, and body weight (BW) were recorded and the ratios of RV to whole heart weight and of RV to BW were calculated. The left lung was fixed in 4% formaldehyde and then embedded in paraffin blocks. The right lung was cut into pieces, frozen in liquid nitrogen and stored at –80°C until future use. These methodologies were based on our recent studies.

Western Blot Analysis of Lung Tissue and RV Myocardium
To evaluate the effect of PTU therapy on the inhibition of overexpression of connexin43 (Cx43), protein aliquots (30 μg) of RV and lung were western-blotted for Cx43 according to manufacturer’s instructions. To determine the effect of PTU on protein kinase C (PKC)ε expression, protein aliquots (30 μg) of RV were western-blotted for PKCε in the membranous compartment of the RV.

Immunolabeling of Cx43 and Quantitative Image Data Analysis
Six serial sections of lung and RV tissues (3 longitudinal, 3 transverse) were prepared at 4-μm thickness by Cryostat (Leica CM3050S) for Cx43 immunolabeling according to our recent reports.2,3 To co-localize troponin I and Cx43 in the same sample, tissue sections were first incubated with a mixture of polyclonal anti-Cx43 (1:200) plus anti-Troponin I (1:200) for 24 h at 4°C, then incubated with anti-mouse FITC (1:200) and anti-rabbit rhodamine (1:200) for 30 min at room temperature.

Calculation of the integrated area (μm²) of Cx43 spots in the tissue sections was achieved using Image Tool 3 (IT3) image analysis software (UTHSCSA; Image Tool for Windows, Version 3.0, University of Texas, Health Science Center, San Antonio, TX, USA). Three selected sections for each animal were quantified. The number of pixels in each Cx43 spot per high-power field (HPF) was first determined, followed by summation of the pixel number obtained from 3 HPFs in each section. The mean pixels number per HPF for each animal was then determined by summation of all pixel numbers divided by 9. The mean area of Cx43 per HPF was obtained by adopting a conversion factor of 19.24 (1 μm² represented 19.24 pixels) as reported in our recent studies.2,3

Real-Time Quantitative Polymerase Chain Reaction (PCR) Analysis
Real-time PCR was conducted using a LightCycler TaqMan Master (Roche) in a single capillary tube according to the manufacturer’s guidelines for individual component concentrations. Forward and reverse primers were each designed in a different exon of the target gene sequence, eliminating the possibility of amplifying genomic DNA. A positive result was determined by identifying the threshold cycle value at which reporter dye emission appeared above background. If a fluorescence signal was not detected within 55 cycles, the sample was considered negative.

Distribution of Alveolar Sacs and Vessels in Lung Parenchyma
Immunohistochemical staining of α-smooth muscle actin (Sigma) was performed to determine the numbers of alveolar sacs and arterioles according to manufacturer’s instructions and our recent descriptions.2,3 Three lung sections from each rat were chosen and 3 randomly selected HPFs (×100) were analyzed in each section. The mean number per HPF for each animal was then determined by summation of all numbers divided by 9.

Statistical Analysis
Data are expressed as mean values (mean±SD). The significance in differences in the data simply between 2 groups was determined by t-test. The means among groups in Table were compared by 1-way ANOVA followed by Tukey multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC, USA). A P-value <0.05 was considered statistically significant.

Results

Hemodynamics, BW, Ratio of RV to Whole Heart Weight
As shown in Table, the initial and final BW on day 35 after MCT administration did not differ among the 3 groups. There was also no significant difference in whole heart weight between groups 1 and 3 or between groups 2 and 3. However, the whole heart weight was significantly higher in group 2 than in group 1. The RV weight was notably higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. The RVSP on day 35 was substantially higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. However,
the femoral arterial systolic blood pressure and 30-day mortality rate did not differ among the 3 groups.

mRNA Expressions of MMP-9, Tissue Necrotic Factor (TNF)-α, and eNOS in the Lung and RV
On day 35 following MCT treatment, the mRNA expressions of matrix metalloproteinase (MMP-9) (Figures 1A, B) and TNF-α (Figures 1C, D), 2 indexes of inflammatory status, in both lung tissue and RV were significantly higher in group 2 than in groups 1 and 3. However, the 2 parameters did not differ between groups 1 and 3. These findings suggest that PTU had an anti-inflammatory property. Conversely,
the mRNA expression of endothelial NO synthase (eNOS) in both lung tissue and RV was notably lower in group 2 than in groups 1 and 3, whereas it was similar between group 1 and group 3 (Figures 1E, F). These findings indicate significant anti-inflammatory and endothelium-protective effects of PTU therapy.

**mRNA Expressions of Caspase 3 and Bcl-2 in the Lung and RV**

On day 35 following MCT treatment, mRNA expression of caspase 3 (Figures 2A, B), an index of apoptosis, in both the lung and RV was significantly higher in group 2 than in groups 1 and 3. However, this mRNA expression was similar between group 1 and group 3. Conversely, Bcl-2 mRNA expression, an index of anti-apoptotic activity, was lower in group 2 than in groups 1 and 3, whereas it was similar between group 1 and group 3 (Figures 2C, D). These findings imply an anti-apoptotic action of PTU in the treatment of MCT-induced PAH in rats.

**PKC-ε Levels in Plasma Membrane and Cytosol of the RV**

Although western blot analysis demonstrated no significant difference in PKC-ε expression in the membrane compartment (Figure 3A) of the RV myocardium between groups 1 and 3, it was significantly lower in group 2 than in groups 1 and 3. Additionally, this PKC-ε expression in the cytosolic compartment (Figure 3B) was notably higher in group 2 than in groups 1 and 3 on day 35 after MCT administration. These findings imply that PTU treatment may trigger a translocation of PKC-ε from the cytosolic to the membranous domain for RV protection following MCT-induced PAH.
Cx43 Expression

Figures 4A–D shows the results of the immunofluorescence imaging study for Cx43 and quantification of integrated area (μm²) of clustered Cx43 spots in each group of RV tissue samples on day 35 following MCT treatment. The summation area of Cx43 was significantly lower in group 2 than in groups 1 and 3, whereas no notable difference was noted between groups 1 and 3 (Figure 4D). These findings indicate that PTU therapy was able to preserve Cx43 expression (ie, an index of gap junction integrity) in the RV.
following MCT-induced lung injury.

Cx43 protein expression in lung tissue (Figure 4E) was significantly higher in group 2 than in groups 1 and 3, whereas there was no notable difference between groups 1 and 3. These findings indicate that MCT administration elicited an early Cx43 protein overexpression, an index of smooth muscle proliferation in medial layer of vessels and trachea of lung that was attenuated by PTU treatment.

On the other hand, Cx43 protein expression in the RV (Figure 4F) was notably lower in group 2 than in groups 1 and 3, whereas no significant difference was noted between groups 1 and 3. These findings suggest that pressure overload in the RV, an indicator of MCT-induced PAH, markedly suppressed Cx43 expression in the RV. Besides, PTU therapy preserved Cx43 protein expression in the RV on day 35 after MCT treatment.

Quantitative Analysis of Small Arteriolar Density and Number of Alveolar Sacs in Lung Parenchyma

The number of small vessels (<100 μm) in lung parenchyma was substantially reduced in group 2 than in groups 1 and 3 on day 35 following MCT administration (Figure 5). However, the number of small vessels did not differ between groups 1 and 3. Moreover, histopathologic findings revealed...
that arteriolar wall thickness was notably increased in group 2 than in groups 1 and 3, and increased in group 3 compared with group 1.

Furthermore, microscopically the lung parenchyma was more crowded and the septum more thickened in group 2 than in groups 1 and 3, and in group 3 than in group 1, on day 35 following MCT treatment (Figure 6). In addition, the number of alveolar sacs was substantially lower in group 2 than in groups 1 and 3, and significantly lower in group 3 than in group 1 (Figure 6).

**Discussion**

The present study, which investigated the impact of PTU therapy on MCT-induced PAH in the rat, has several striking implications. First, MCT treatment elicited inflammatory responses and apoptosis in the lung and RV, which were effectively attenuated by PTU therapy. Second, PTU upregulated PKC-ε expression in the membrane compartment of RV myocardium and suppressed Cx43 expression in lung parenchyma. Finally, PTU effectively protected the lung against MCT-induced obliteration of microvasculature and destruction of parenchymal architecture. These microcirculatory and ventilatory improvements, in turn, reduce RV pressure overload and hypotrophy, which are key indexes of the severity of PAH. Therefore, PTU may be an attractive therapeutic alternative against PAH-associated pulmonary and cardiac complications.

An important finding in the current study was that not only were the mRNA expressions of TNF-α and MMP-9 markedly elevated in both lung tissue and RV, but the integrity of lung parenchymal architecture was also severely damaged following MCT treatment (Figures 5, 6). The findings suggest that MCT-induced PAH inflicts pulmonary and cardiac damage through eliciting inflammatory responses. In addition, a body of evidence has demonstrated an essential role of the involvement of inflammatory mediators in both endothelium and smooth muscle at all stages of atherosclerosis, from initiation, propagation, and the development of complications. Consistently, other studies have further identified that TNF-α, an inflammatory cytokine, directly participates in the activation of smooth muscle cell proliferation. Therefore, our proposal of inflammation-based PAH-induced organ damage is supported by those previous studies and may at least partially explain the PAH and RV hypotrophy observed in rats after MCT treatment in this study. Of importance was that PTU therapy significantly suppressed the mRNA expressions of TNF-α and MMP-9 and attenuated the elevation in RV pressure and subsequent myocardial hypertrophy.

Another interesting finding in this study was that MCT treatment significantly enhanced caspase-3 (ie, an index of cellular apoptosis) and suppressed Bcl-2 (ie, an index of anti-cellular apoptosis) mRNA expressions, which could be significantly reversed via PTU treatment. The effect of MCT-mediated PAH on cellular apoptosis in the rat model was identified in our recent studies, the results of which also strengthen the findings of the present study. Although inducing apoptosis of abnormal vascular cells is 1 of the therapeutic aims in treating PAH, apoptosis of pulmonary artery endothelial cells with loss of small vessels is also implicated as a feature in the pathogenesis of clinical PAH and in different animal models. A previous study has shown an increased level of vascular cell apoptosis in a pulmonary hypertension rat model 4 weeks after MCT administration. Moreover, it has been proposed that early endothelial cell apoptosis of muscular pulmonary arteries is required for the initiation of advanced vascular remodeling. Therefore, apoptosis in the abnormal VSMCs may occur at a later stage after therapeutic intervention against well-established PAH, whereas apoptosis of normal vascular endothelium and microvascular pericytes may happen at an early stage of PAH pathogenesis. Because a short-term, MCT-induced PAH animal model was used in this study to mimic early PAH development for an investigation of the therapeutic potential of prompt PTU treatment (ie, 5 days after MCT administration), it is speculated that the observed anti-apoptotic effect of PTU therapy was beneficial in suppressing the initial damage to normal vascular endothelium and hence the resulting vessel loss. This is compatible with the finding of another study that showed an elevated level of vascular cell apoptosis, together with increased smooth muscle cell proliferation, in a MCT-induced PAH rat model.

Moreover, the current study also revealed a remarkable MCT-induced downregulation in PKC-ε protein expression in RV myocardium, which was notably preserved by PTU therapy. Interestingly, previous studies have shown activation of PKC-ε during ischemic preconditioning, followed by its translocation to specific subcellular compartments, considered an important mechanism through which PKC-ε induces downstream signaling cascades and orchestrates protection. Our observation, therefore, is supported by previous studies and may explain the suppression of caspase 3 and preservation of Bcl-2 mRNA expressions in rat RV myocardium following PTU treatment in this study.

One of the most important findings in the present study was the remarkable Cx43 overexpression in the lung and Cx43 downregulation in the RV following MCT administration. The early expression of Cx43 has different meanings in the heart and the vasculature. Although the link between RV hypertrophy and downregulation of Cx43 expression has been identified, a strong association between Cx43 overexpression and the smooth muscle cell proliferative response to vascular injury during early development of atherosclerosis has also been reported. In the present study, the significant suppression and preservation of Cx43 expression in the lung and RV, respectively, following PTU treatment in the MCT-treated animals, therefore, signifies a protective role of PTU against PAH in the rat model.

On the other hand, eNOS mRNA expression, an index of NO production, in both lung tissue and the RV was found to be suppressed by PAH induction in this study. NO is a key vasodilatory molecule regulating vascular tone and which also has antiplatelet, anti-inflammatory, and antioxidant properties. The lack of eNOS has been demonstrated to be associated with accelerated vascular remodeling with high-flow stress. The suppressed eNOS expression, therefore, may at least partly explain the remarkable diminution in the number of small vessels and the aggravated arteriolar wall remodeling (Figure 5: thickened arterial wall) in the lung following MCT treatment in the current study. Importantly, the downregulation of eNOS mRNA expression in both the lung and RV was significantly reversed by PTU treatment. Interestingly, PTU has been recently established as possessing properties of increasing NO production, limiting VSMC proliferation, and being anti-atherosclerotic. The findings of the current inves-
tigation, in addition to reinforcing those of other recent studies, further elucidate the therapeutic potential of PTU in attenuating PAH and RV hypertrophy in the MCT-treated rat model. The proposed mechanisms underlying the potential effect of PTU therapy against MCT-induced PAH in rats have been summarized in Figure 7.

Study Limitations

First, unlike the MCT-induced inflammation that triggers the proliferation of pulmonary endothelial and VSMCs in the animal PAH model, it is believed that inflammation is not a main component in the pathogenesis of the clinical situation. However, emerging data begin to arouse the speculation that nonspecific inflammatory processes may be involved during the development of clinical PAH. Second, because only RV pressure and systemic arterial blood pressure were measured in this study without direct measurement of the pulmonary arterial pressure and tricuspid regurgitation pressure gradient, the hemodynamic efficacy of PTU in normalizing PAH cannot be established. Third, thyroid hormone supplementation was not given to the animals in this study to exclude the possible antithyroid effect of PTU on the results of the experiment, as previous studies have already demonstrated that the effects of PTU on the inhibition of VSMC proliferation and migration, NO-mediated endothelium-dependent vasodilatation, and reduction in VSMC collagen expression were independent of its antithyroid action. These mechanisms are essentially the same as those investigated in the current study. Furthermore, because the thyroid function test was not performed on the rats receiving PTU, it is unknown to what extent PTU influenced the release of thyroid hormone.

In conclusion, not only did this experimental study demonstrate a therapeutic application of PTU against PAH-associated pulmonary and cardiac injuries in a MCT-induced PAH rat model through improving the histologic integrity of the pulmonary architecture and microvasculature, it also elucidated the cellular and molecular pathologic mechanisms underlying MCT-induced PAH and the beneficial roles of PTU in these aspects, thereby stimulating further basic and clinical research in this field.

References


