Oestradiol ameliorates monocrotaline pulmonary hypertension via NO, PGI₂ and ET-1 pathways

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Authorship
All authors actively participated in the study and in the review and approval of the manuscript. P.Y., W-H.W. and Z-C.J. contributed to drafting of the manuscript and to study concept and design; P.Y., W-H.W, L.G. and H-Y.M. contributed to data acquisition; P.Y., W-H.W., D.L. and Z-C.J. contributed to data analysis and interpretation; Z-L.Z. and Z-C.J. contributed to critical revision of the manuscript for important intellectual content; P.Y., W-H.W., D.L. and Z-C.J. contributed to statistical analysis; D.L. and Z-C.J. contributed to study supervision; and Z-C.J. contributed to acquisition of funding.
ABSTRACT

Pulmonary hypertension (PH) continues to be a serious clinical problem with high mortality. As oestrogen is a potential vasodilator of the pulmonary circulation, this study examined the mechanisms by which 17β-oestradiol improves monocrotaline (MCT)-induced PH.

Female Sprague-Dawley rats underwent bilateral ovariectomy or sham operations. The rats received MCT (50 mg/kg) and were treated with 17β-oestradiol (1 mg/kg/day) for 5 weeks or only from Week 4 to Week 5. Plasma 17β-oestradiol concentrations were decreased in sham-operated, MCT-treated rats compared with sham-operated rats (17.7 ± 4.7 vs 50.3 ± 15.4 pg/mL; p = 0.029). The 17β-oestradiol anabolic enzyme cytochrome P450 (CYP) 19 was decreased by MCT treatment, while the catabolic enzymes CYP 1A1 and 1B1 were increased. Ovariectomized and MCT-treated rats had more severe PH. 17β-oestradiol suppressed pulmonary arterial smooth muscle cell proliferation and macrophage infiltration, and enhanced apoptosis by increasing nitric oxide and prostacyclin levels and reducing endothelin-1 levels. PI3K and Akt phosphorylations were markedly increased but were inhibited by 17β-oestradiol treatment in PH rats.

Oestrogen deficiency may aggravate development of PH. 17β-oestradiol improved PH via activation of the PI3K/Akt pathway to regulate nitric oxide, prostacyclin and endothelin-1 expression.

KEYWORDS: Pulmonary hypertension, 17β-oestradiol, metabolic enzymes, oestrogen receptors
INTRODUCTION

Pulmonary hypertension (PH) is a progressive disorder with a poor prognosis. It is characterized by elevated pulmonary arterial pressure and right ventricular hypertrophy (RVH) [1]. The pathological changes of PH include endothelial injury, pulmonary arterial smooth muscle cell (PASMC) proliferation, and migration of inflammatory cells. Currently, there is no ideal pharmacological agent to reverse advanced PH.

Sex hormones, especially oestradiol (17β-oestradiol; E2), appear to improve experimental PH [2,3]. E2 is converted from testosterone by the cytochrome P450 (CYP)19 enzyme, aromatase. E2 is subsequently converted to multiple metabolites via several pathways, among which CYP1A1 and CYP1B1 are key catabolic enzymes [3]. Experimental studies using a chronic hypoxia- or monocrotaline (MCT)-induced PH model have consistently demonstrated that administration of E2 exerts protective effects on the progression of PH [2,3]. Nitric oxide (NO), prostacyclin (prostaglandin I2; PGI2) and endothelin-1 (ET-1) are the key vasoactive mediators and are therapeutic targets in patients with PH [4]. In pulmonary artery endothelial cells, oestrogen acutely stimulates NO and NO synthase (NOS) release [5,6]. Oestrogen upregulates PGI2 in ovine fetal pulmonary artery endothelium and human umbilical vein endothelial cells, and oestrogen receptor-β (ERβ) has been shown to mediate cyclooxygenase-2 (COX-2) expression in human placental villous endothelial cells [7-9]. A recent study has shown that oestrogen attenuates hypoxia-induced pulmonary ET-1 gene expression in lung tissue of adult female rats [2]. However, plasma E2 concentrations, changes of E2 metabolizing enzymes, and the mechanism by which E2 improves PH have not been clarified.

In the present study, we examined plasma E2 concentrations, changes in E2 metabolizing enzymes and oestrogen receptors, and the effects of E2 on NO, endothelial NOS (eNOS), PGI2, COX-2, ET-1, and endothelin-converting enzyme (ECE) expression, and on pulmonary proliferation, apoptosis and inflammation. We also investigated whether E2 deficiency is an influential factor in the progression of PH.

METHODS

Animal models
Female Sprague-Dawley rats (180-200 g) were provided by the Shanghai SLAC laboratory animal center. All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tongji University and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 8523, revised 1996). Rats were randomly divided into the following 8 groups (n = 12 for each group; fig. 1): (1) sham-operated, saline-treated, vehicle (vegetable oil) group (S); (2) sham-operated, MCT-treated, vehicle group (S+M); (3) sham-operated, MCT-treated, chronic E2 pretreatment (preventive therapy) group [S+M+E (P)]; (4) sham-operated, MCT-treated, acute E2 treatment group [S+M+E (T)]; (5) ovariectomized, saline-treated, vehicle group (O); (6) ovariectomized, MCT-treated, vehicle group (O+M); (7) ovariectomized, MCT-treated, chronic E2 pretreatment (preventive therapy) group [O+M+E (P)]; and (8) ovariectomized, MCT-treated, acute E2 treatment group [O+M+E (T)].

Rats were anesthetized with 60 mg/kg sodium pentobarbital given intraperitoneally, and a bilateral ovariectomy or sham operation was performed. After a 1-week recovery period, rats received a single subcutaneous injection of MCT (50 mg/kg, Sigma) or 0.9% saline at the same volume as the MCT injection. In the S+M+E (P) and O+M+E (P) groups, animals were treated for 5 weeks with E2 (1 mg/kg/day, Sigma), starting on the day of the MCT injection. In the S+M+E (T) and O+M+E (T) groups, rats received vehicle until Week 4 after MCT injection and were then treated with E2 (1 mg/kg/day) in the fifth week. Other groups received vehicle for 5 weeks after MCT or saline injection. At the end of the fifth week, rats were sacrificed and the plasma and lung tissues were harvested and stored at −80°C until use.

**Haemodynamic analyses**

After rats were anaesthetized, and a tracheotomy was performed, a polyethylene catheter connected to a pressure transducer was inserted into the right external jugular vein and threaded into the right ventricle and pulmonary artery to measure right ventricular systolic pressure (RVSP) and mean pulmonary artery pressure (mPAP) by a polygraph system (Power Lab 8/30, Australia). Another polygraph catheter was inserted into the left carotid artery to measure cardiac output (CO) by thermodilution. Pulmonary vascular resistance (PVR) was calculated using the following formula: PVR = mPAP/CO.
Right ventricular hypertrophy evaluation

The right ventricular (RV) free wall was dissected from the left ventricular septum (LV+S) and weighed separately. The degree of right ventricular hypertrophy (RVH) was determined by the weight ratios RV/(LV+S) and RV (mg)/BW (g).

Measurement of plasma E2 concentrations

Plasma samples were collected after haemodynamic analyses. Plasma E2 concentrations were determined in duplicate using the Estradiol EIA kit (Cayman Chemical Company, USA) [10].

Histologic analyses and morphometry

After the haemodynamic measurements, lung tissue was prepared for morphometric analyses using the barium injection method [11]. Morphometric analyses were performed in pulmonary arteries with an external diameter of 50 to 100 μm. The medial wall thickness was calculated by the following formula: Medial thickness (%) = medial wall thickness/external diameter × 100. For quantitative analyses, 30 vessels from each rat were counted and the average was calculated. At × 400 magnification, 80 small pulmonary vessels of each animal ranging from 10 to 50 μm in external diameter were evaluated for muscularization [12].

Proliferating cells were evaluated by proliferating cell nuclear antigen (PCNA) staining (Dako) and apoptotic cells by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method (Apoptosis Detection Kit, Wako). Inflammatory cells were evaluated by ED-1 (analogue of CD68) staining (Santa Cruz Biotechnology). The number of PCNA and TUNEL-positive cells in 10 fields for each section was quantitatively evaluated as a percentage of the total cells at a magnification of × 400 in a blind manner [13]. The number of ED-1–positive cells was counted in 30 fields [14].

Assay of NO production in the lung

NO production in cells was measured by the Griess method, as indicated on the NO assay kit (Beyotime Biotech Inc., Jiangsu, China). Nitrite levels were corrected by protein measurements, and data were shown as a percentage of control.
Measurement of lung PGI₂ concentrations
The amount of lung PGI₂ produced, calculated as the concentration of the stable hydrolysis product 6-keto-prostaglandin F₁α (6k-PGF₁α), was determined in duplicate using a Prostacyclin EIA kit (Enzo Life Science, USA) [8].

Measurement of lung ET-1 concentrations
Lung ET-1 concentrations were determined in duplicate by the Endothelin-1 EIA kit (Enzo Life Science, USA) [15].

Western blot analyses
Lungs were lysed in 1 × sodium dodecyl sulphate (SDS) supplemented with proteinase inhibitor at a dilution of 1:25. Thirty micrograms of protein lysate was electrophoresed on a 12% polyacrylamide SDS gel and transblotted onto a PVDF membrane at 270 mA for 90 min. The membranes were blocked with 5% skim milk in Tris-buffered 170 saline (TBS) and 0.1% Tween (TBS/Tween) for 1 hour at room temperature with gentle rocking, and then incubated with primary antibodies at 4°C overnight. After three washes with TBS/Tween, the membranes were incubated with secondary anti-mouse/goat antibody (1:2000) for 1 hour at room temperature. The relative protein level was normalized by intensity of β-actin.

Statistical analyses
Data from multiple experiments were expressed as means ± SEM. Data were analyzed by student's t-tests or one-way analyses of variance (ANOVA). When significant differences were detected, individual mean values were compared by post-hoc tests (Bonferroni correction) that allowed for multiple comparisons. Where appropriate, Kruskal-Wallis ANOVA by ranks was performed on non-parametric data. A p-value of < 0.05 was considered statistically significant.

RESULTS
Improvement of haemodynamic parameters and RVH in MCT-induced PH by E₂
Pulmonary haemodynamic evaluations showed that the MCT-treated groups developed severe
PH, with increased RVSP, mPAP, PVR, RV/(LV+S) weight ratio and RV (mg)/BW (g) ratio, and decreased CO compared with the sham-operated (S) or ovariectomized (O) groups (figs 2a-f). Interestingly, ovariectomized, MCT-treated (O+M) rats exhibited higher RVSP and PVR and lower CO compared with sham-operated, MCT-treated (S+M) rats (figs 2a, 2c and 2d).

In the chronic pretreatment groups, E2 significantly attenuated the elevation of RVSP, mPAP, PVR, RV/(LV+S) weight ratio and RV (mg)/BW(g) ratio in sham-operated, MCT-treated and E2 (pretreatment) [S+M+E (P)] rats, and also reduced PVR in ovariectomized, MCT-treated and E2 (pretreatment) [O+M+E (P)] rats compared with MCT-treated rats not given E2 (figs 2a, 2b and 2d-f). In these groups, E2 also significantly enhanced CO in both S+M+E (P) rats and O+M+E (P) rats (fig. 2c).

In the acute treatment groups, E2 reduced PVR and increased CO in sham-operated, MCT-treated and E2 (treatment) rats [S+M+E (T)] and ovariectomized, MCT-treated and E2 (treatment) [O+M+E (T)] rats compared with MCT-treated rats not given E2 (figs 2c and 2d).

**Reversal of pulmonary vascular remodeling, pulmonary proliferation, apoptosis and inflammation by E2**

Fully muscularized vessels and medial wall thickness were significantly enhanced in response to MCT compared with the control groups, and obviously reduced in the pretreatment groups. In addition, there was a marked increase of normally non-muscularized vessels in MCT-treated groups with E2 pretreatment and acute treatment (figs 2g and 2h).

PCNA expression in vascular smooth muscle cells (VSMCs) was increased in the MCT-treated groups, and was prevented by E2 (figs 3a and 3b). Macrophage recruitments were increased in the MCT-treated groups, and were markedly suppressed by E2 treatment (figs 3c and 3d). E2 also significantly enhanced VSMC apoptosis (figs 3e and 3f). The percentages of TUNEL-positive cells were significantly increased in the E2 groups compared with the saline-treated, normal groups and the MCT-treated groups (figs 3e and 3f).

**Plasma E2 concentrations, oestrogen receptors and expressions of CYP19, CYP1A1, CYP1B1 in the lung**

Plasma E₂ concentrations were significantly reduced in sham-operated, MCT-treated (S+M) rats compared with sham operated (S) rats (fig. 4a). There was almost no E₂ in ovariectomized (O) rats or in ovariectomized, MCT-treated (O+M) rats. After the long-term E₂ exposure, plasma E₂ concentrations were dramatically increased in the sham-operated, MCT-treated and E₂ (pretreatment) [S+M+E (P)] rats and in ovariectomized, MCT-treated and E₂ (pretreatment ) [O+M+E (P)] group, and both were similar to levels in the sham-operated (S) rats. After the short-term E₂ exposure, plasma E₂ concentrations were significantly increased in the sham-operated, MCT-treated and E₂ (treatment) [S+M+E (T)] rats, and were similar to levels in the S rats; E₂ concentrations were also increased in the ovariectomized, MCT-treated and E₂ (treatment) [O+M+E (T)] groups compared with in the O rats, but were considerably less than levels in the S rats.

Pulmonary ERα protein levels were decreased in MCT-treated rats (figs 4b and 4c) but ERβ protein levels were not changed (figs 4b and 4d). The expression of CYP19 protein in the lung was significantly decreased by MCT. Conversely, the expression of pulmonary CYP1A1 and CYP1B1 proteins was significantly increased in MCT-treated rats (figs 4b and 4e-g).

**Reversal of pulmonary NO, PGI₂, and ET-1 concentrations by E₂**

Changes in NO and PGI₂ concentrations in lung are shown in Figures 4a and 4b. There were marked reductions of NO and PGI₂ concentrations in MCT-treated PH rats compared with controls. Notably, ovariectomized, MCT-treated (O+M) rats showed much lower concentrations of NO and PGI₂ than sham-operated, MCT-treated (S+M) animals. NO and PGI₂ levels were significantly increased by E₂ pretreatment and acute treatment (figs 5a and 5b).

In the MCT-treated groups, lung ET-1 concentrations were increased in comparison with controls (fig. 5c). In addition, O+M rats exhibited higher levels of ET-1 than S+M rats (fig. 5c). Pretreatment with E₂ inhibited the elevation of lung ET-1 concentrations in the sham-operated and ovariectomized, MCT-treated rats [S+M+E (P) and O+M+E (P) groups] (fig. 5c). Acute treatment with E₂ also significantly suppressed the expression of lung ET-1 in the sham-operated and ovariectomized, MCT-treated rats [S+M+E (T) and O+M+E (T) groups] compared with the MCT-treated animals (fig. 5c).
**Reversal of eNOS, COX-2, and ECE concentrations by E₂**

Changes in eNOS and COX-2 concentrations in the lung are shown in Figures 5d to f. ENOS and COX-2 proteins were significantly reduced in MCT-treated rats, and both eNOS and COX-2 protein levels were markedly increased by E₂ pretreatment (figs 5d to f).

Lung ECE levels were increased by MCT treatment (figs 5d and 5g). Pretreatment with E₂ inhibited the elevation of lung ECE proteins (figs 5d and 5g).

**Expression of PI3K and Akt phosphorylations in the lung**

The expression of PI3K and Akt phosphorylations were significantly increased in the MCT-treated groups, and both were markedly inhibited by E₂ treatment (figs 6a-c).

**DISCUSSION**

The novel findings of the present study are that E₂ treatment improved PH via multiple different pathways. Suppressed metabolizing enzyme-mediated E₂ in the lung may play an important role in the pathogenesis of PH. The study showed that: (1) plasma E₂ concentrations were significantly decreased in MCT-treated rats; (2) oestrogen receptors and some metabolizing enzymes were altered by MCT treatment; (3) E₂ prevented the development of PH and RVH, suppressed PASMC proliferation and macrophage infiltration, enhanced PASMC apoptosis, reduced pulmonary ET-1 expression, and enhanced pulmonary NO and PGI₂ expression; and (4) E₂ may reduce PI3K/Akt activation to play a protective role. These finding suggest that endogenous oestrogen deficiency may play an important role in the pathogenesis of MCT-induced PH, and that E₂ may ameliorate the progression of PH by combining with ERβ to inhibit PI3K/Akt activation to regulate the production of ET-1, PGI₂, NO and their enzymes.

Although PAH is a disease that occurs predominantly in females [16-17], Shapiro and colleagues [16] reported that men with PAH had a higher mPAP and mean right atrial pressure at diagnosis, and that men aged ≥60 years had lower survival rates compared with women aged ≥60 years. Similar female predominance and survival data were reported in the French registry [17,18]. These data indicate that oestrogen may be the protective factor.
However, oestrogen exposure has been associated with PAH [19], and oestrogen may exert detrimental pro-proliferative and pro-inflammatory effects in pulmonary vascular tissue [20]. Nevertheless, there is significant evidence that gender is a profound modifier of pathogenesis and survival in PAH, and the potential role of oestrogen needs to be explored further. We investigated whether E2 deficiency is an influential factor in the progression of PH, and also whether its replacement would reverse some of these effects. We evaluated the impact of E2, E2 deficiency, and E2 replacement upon the development of PH in MCT-treated female rats to determine whether this may provide new ideas for exploring the treatment of patients with PH.

The relationship between oestrogen and PH has received adequate attention in the literature recently. Experimental studies have demonstrated that E2 improves PH, but there have been no reports of changes in plasma E2 concentrations in MCT-treated rats. In this study, we found that plasma E2 concentrations were decreased by MCT treatment, which may give rise to a less protective role of E2 in pulmonary arteries. Possible explanations include changes in E2 metabolizing enzymes. Interestingly, our findings suggest that CYP19 is decreased and CYP1A1 and CYP1B1 are increased in MCT-induced PH. Whether these changes in metabolic enzymes lead to abnormal E2 concentrations requires further confirmation. Recently, Austin and colleagues demonstrated that altered oestrogen metabolism through polymorphisms in CYP1B1 may modify the risk of developing familial PH in female BMPR2 mutation carriers [21].

E2 exerts its effects mainly via ERα and ERβ. Both receptor subtypes are present in the lungs. An ERβ-mediated action of oestrogen has recently been reported in male rats with MCT-induced PH [22]. This study showed that a selective ERβ agonist is able to rescue PH as efficiently as E2, and that E2 fails to ameliorate PH in the presence of an ERβ-selective antagonist. The data from this study strongly support the notion that the rescue action of E2 is mainly mediated through ERβ in MCT-induced PH [22]. Lahm and colleagues [23] reported that a non-selective ER inhibitor and ERα- and ERβ-specific antagonists opposed the effects of E2; however, they considered that the important effects of E2 on functional endpoints in hypoxic PH were predominantly mediated by ERα. Therefore, evaluation of ER expression in PH was considered important. The data of Lahm et al. [23] indicate that hypoxia increased
ERβ but not ERα lung vascular expression. In contrast, our results showed that MCT inhibited ERα but had no effect on ERβ proteins in the lung. Interestingly, MCT-regulated ER protein expressions were not in line with hypoxia. This difference may reflect distinct mechanisms of E₂ signaling in MCT-induced PH compared with hypoxia-induced PH. Future studies will need to explore the mechanisms of altered ER levels in PH.

Previous studies have demonstrated that E₂ attenuates the development of experimental PH by investigating survival rates and haemodynamic parameters such as mPAP, RVSP and RV/(LV+S) weight ratio [22,24]. However, these parameters do not comprehensively evaluate the clinical severity of PH. RVSP and mPAP do not consistently increase with progression of the disease and may decrease in severe PH. CO and PVR are important parameters and more accurately reflect disease severity and prognosis. Thus, we also assessed CO and PVR in this study. Ovariectomized, MCT-treated rats had more severe PH than sham-operated, MCT-treated rats, and E₂ treatment improved PH. Thus, E₂ deficiency may be a risk factor for PH.

PH is characterized by enhanced PASMC proliferation, constriction, inflammation and resistance to apoptosis, all of which contribute to increased pulmonary artery wall thickness, resistance, and, therefore, pressure. In the present study, E₂ pretreatment suppressed PASMC proliferation and macrophage infiltration in MCT-treated rats, consistent with the findings of a previous study [22]. In this study, E₂ administration enhanced PASMC apoptosis in PH. Indeed, pretreatment and acute treatment with E₂ induced a marked improvement of pulmonary artery muscularization and medial wall thickness in MCT-induced PH rats.

Endothelial injury and dysfunction is a major feature of MCT-induced PH. The perturbation between vasodilators and vasoconstrictors in PH is probably a consequence of pulmonary endothelial injury. NO, PGI₂ and ET-1 are key vasoactive mediators and are therapeutic targets in patients with PH. In previous studies and in the present study, decreased NO and eNOS, PGI₂ and COX-2 levels and increased ET-1 and ECE levels have been noted in PH [15,25,26]. Gonzales et al. [5] observed that endothelium-dependent vasodilation in the lung was enhanced by chronic E₂ treatment. In fetal pulmonary artery endothelial cells, E₂-stimulated eNOS activity was fully inhibited by the oestrogen receptor antagonist tamoxifen and ICI-182,780 [6]. Although this study found that eNOS protein and activity was
upregulated in the pulmonary circulation by E2, the authors did not investigate the role of E2 during hypoxia or MCT administration. However, Resta et al. [24] have suggested that E2 exerts a protective influence in hypoxic PH but that this protection was not likely to be a function of increased eNOS expression. Therefore, E2 regulation of eNOS expression remains controversial. We further demonstrated upregulation of eNOS and NO by E2 in the lung in MCT-induced PH rats. These findings are in agreement with previous reports [5,6]. Although the mechanisms by which E2 increases eNOS expression have not been clearly elucidated, epigenetic abnormalities may be an explanation.

Studies in both animals and humans indicate that during pregnancy, there is a marked elevation of PGI2 metabolite levels in both blood and urine [27]. Postmenopausal women receiving oestrogen replacement therapy show increased urinary excretion of PGI2 [28]. Sobrino and colleagues [8] have reported that E2 dose-dependently increased PGI2 production and COX-1 and PGI2 synthase expression without affecting COX-2 expression in human umbilical vein endothelial cells. However, we observed that pulmonary PGI2 production and COX-2 expression were increased by E2 in MCT-treated rats. One possible reason is that MCT potentially has a direct influence on the regulation of COX-2 by E2; another possible reason is that the adjustment mechanisms of E2 may be cell-specific. It is known that oestrogen modulates both ET-1 gene expression and production [29]. Our data indicate that E2 not only regulates eNOS and COX-2 expressions, but also reduces ECE levels in MCT-treated rats.

An effect of E2 on the PI3K/Akt pathway has been demonstrated in other organ systems or cells [30,31]. However, the precise mechanisms of its effects are not entirely clear and it has not yet been determined whether these effects play a role in the pulmonary vasculature as well. In the present study, we observed that phosphorylated PI3K and Akt were increased by MCT treatment and that E2 administration reversed the increases. In previous studies in rat aortic rings, LY 294002, a specific inhibitor of the PI3K/Akt/PKB pathway, and Nω-nitro-L-arginine-methyl ester, a nitric oxide synthase inhibitor, reduced E2-induced vasorelaxation. The data showed that E2 exerted a vasorelaxant effect mediated by eNOS activation through Akt/PKB-dependent mechanisms in rat aortic rings [32]. In other studies, 2-methoxyestradiol, an E2 metabolite, inhibited neointima formation and human aortic SMC
growth by regulating phosphorylated Akt or other pathway proteins to enhance COX-2 expression [33]. Dehydroepiandrosterone, a progenitor for E₂, has been found to have beneficial or harmful effects relevant to the vascular endothelium by altering the balance between PI3K and MAPK-dependent signaling in bovine aortic endothelial cells [34]. A similar effect of E₂ was reported on ERK1/2 pathway in hypoxic animals [23]. In this study, hypoxia increased ERK1/2 expression, and E₂-treated hypoxic animals exhibited reduced ERK1/2 activation. Thus, E₂ may regulate the expression of NO, PGI₂ and ET-1 through the PI3K/Akt pathway in MCT-treated rats.

**Limitations**

Taken together, these findings provide good evidence of the importance of E₂ in regulating the development of PH. We cannot, however, exclude other potential mechanisms of enhanced hypertrophy being modulated by E₂. As we did not administer an oestrogen receptor antagonist in conjunction with E₂, potential downstream signaling mechanisms by which E₂ may regulate the three vasoactive mediators will be the subject of future investigations in pulmonary artery endothelial cells and PASMC. We cannot definitively state whether or not E₂ metabolizing enzymes affect the progression of PH. In addition, we could not evaluate androgens or estrogen metabolite and E₂ levels over the course of the rats’ lives prior to the end of the cross-sectional measurements.

**Conclusions**

These results indicate that E₂ effectively improved PH by various mechanisms, including suppressing PASMC proliferation and macrophage infiltration, enhancing PASMC apoptosis, and probably mediating the ER/PI3K/Akt pathway, regulating pulmonary eNOS, COX-2, and ECE levels to rebalance ET-1, NO and PGI₂ expression. An E₂ deficiency mediated by changes in metabolizing enzymes may increase the risk of PH. These findings may have significant clinical implications in patients with PH.

**SUPPORT STATEMENT**

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**Figure legends**

**FIGURE 1.** Experimental protocol (n = 12 per group). A bilateral ovariectomy (O) or sham (S) operation was performed at week −1. Rats were injected with MCT or 0.9% saline at Week 0. The thick horizontal lines represent the duration of each experimental group. Both the S+M+E (P) and O+M+E (P) groups received E2 from Week 0 to Week 5. Both the S+M+E (T) and O+M+E (T) groups were treated with vehicle (vegetable oil) from Week 0 to Week 4 and E2 only from Week 4 to Week 5. Other groups received vehicle for 5 weeks.

**FIGURE 2.** Reversal of pulmonary haemodynamic parameters, RVH, and pulmonary vascular remodeling by E2 administration in MCT-treated rats. Changes of RVSP, mPAP, CO, PVR, RV/(LV+S) ratio, RV(mg)/BW(g) ratio, the degree of medial wall thickness, and muscularization of small pulmonary arteries in all groups are shown (n = 6 each). a-g: Preventive E2 therapy reversed the changes in RVSP, mPAP, CO, PVR, RV/(LV+S) ratio, RV(mg)/BW(g) ratio and the degree of medial wall thickness in MCT-treated groups. Acute E2 treatment also reversed the changes of CO and PVR in PH rats. Medial thickness (%) = medial wall thickness/external diameter × 100. Error bars represent SE. **p < 0.01 vs S rats; #p < 0.05 and ##p < 0.01 vs S+M rats; &&p < 0.01 vs O rats; $p < 0.05 and $$p < 0.01 vs O+M rats. E2 treatment improved pulmonary vascular remodeling by reversing the degree of medial wall thickness (g) as well as muscularization (h) of small pulmonary arteries.
Percentages of non-muscularized (N), partially muscularized (P), or fully muscularized (F) pulmonary arteries are shown in panel h. Error bars represent SE. **p < 0.01 vs S animals N pulmonary arteries; "p < 0.05 and ""p < 0.01 vs S+M animals N pulmonary arteries; &&p < 0.01 vs O animals N pulmonary arteries; $$p < 0.01 vs O+M animals N pulmonary arteries; **p < 0.01 vs S animals F pulmonary arteries; +p < 0.05 and ++P < 0.01 vs S+M animals F pulmonary arteries; &&p < 0.01 vs O animals F pulmonary arteries; §§p < 0.01 vs O+M animals F pulmonary arteries.

FIGURE 3 E2 treatment improved pulmonary vascular proliferation, inflammation and apoptosis in PH. Histology illustrations of pulmonary arteries in the groups (n = 6 each) are shown. MCT-induced increases in PCNA-positive cells (arrows, a and b) and ED-1-positive macrophages (arrows, c and d) were prevented in the E2 treatment groups. TUNEL-positive cells were increased in the E2 treatment groups (arrows, e and f). Error bars represent SE. **p < 0.01 vs S animals; "p < 0.05 and ""p < 0.01 vs S+M animals; &&p < 0.01 vs O animals; $$p < 0.01 vs O+M animals.
**FIGURE 4** Plasma E2 concentrations, oestrogen receptors and metabolizing enzyme levels in MCT-induced PH. 

**a:** Plasma E2 concentrations were reduced in PH (n = 8 each). 

**b:** Representative immunoblots of lung lysates with anti-ERα, anti-ERβ, anti-CYP19, anti-CYP1A1, anti-CYP1B1 and anti-β-actin (n = 6 each). 

**c** and **d:** The expressions of ERα were significantly reduced in the MCT-treated groups, but ERβ proteins were not markedly changed in MCT-treated rats. 

**e:** CYP19 was significantly suppressed in MCT-treated rats. 

**f** and **g:** CYP1A1 and CYP1B1 were markedly increased in MCT-treated rats. Error bars represent SE. *p < 0.05 and **p < 0.05 vs S animals.
FIGURE 5  E₂ treatment regulated pulmonary NO, PGI₂, and ET-1 concentrations and their enzymes in MCT-treated rats. a, b and e show the changes in lung NO, PGI₂, and ET-1 concentrations in all groups (n = 6 each). d: Representative immunoblots of lung lysates with anti-eNOS, anti-COX-2, anti-ECE and anti-β-actin in S, S+M, and S+M+E (P) groups (n = 6 each). e and f show that E₂ enhanced the expression of pulmonary eNOS and COX-2 in PH. g: E₂ suppressed the expression of pulmonary ECE in MCT-induced PH. Error bars represent SE. *p < 0.05 and **p < 0.01 vs S animals; #p < 0.05 and ##p < 0.01 vs S+M animals; &p < 0.05 and &&p < 0.01 vs O animals; $p < 0.05 and $$p < 0.01 vs O+M animals.
FIGURE 6  E2 treatment regulated pulmonary PI3K and Akt phosphorylations in MCT-treated rats. a: Representative immunoblots of lung lysates with anti-p-PI3K, anti-PI3K, anti-p-Akt, anti-Akt and anti-β-actin in S, S+M, and S+M+E (P) groups (n = 6 each). b and c: E2 significantly increased pulmonary PI3K phosphorylations and Akt phosphorylations in MCT-induced PH. Error bars represent SE. *p < 0.05 vs S animals, #p < 0.05 vs S+M animals.