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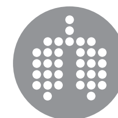

Oestradiol ameliorates monocrotaline pulmonary hypertension *via* NO, prostacyclin and endothelin-1 pathways

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ABSTRACT: Pulmonary hypertension 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 pulmonary hypertension.

Female Sprague–Dawley rats underwent bilateral ovariectomy or sham operations. The rats received MCT (50 mg·kg^{-1}) and were treated with 17β-oestradiol (1 mg·kg^{-1} per day) for either 5 weeks or only from week 4 to week 5. Plasma 17β-oestradiol concentrations were decreased in sham-operated, MCT-treated rats when compared with sham-operated rats (17.7±4.7 *versus* 50.3±15.4 pg·mL^{-1}; 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. Ovariectomised and MCT-treated rats had more severe pulmonary hypertension. 17β-oestradiol suppressed pulmonary arterial smooth muscle cell proliferation and macrophage infiltration, and enhanced apoptosis by increasing nitric oxide (NO) and prostacyclin (prostaglandin (PG)I$_2$) levels and reducing endothelin (ET)-1 levels. Phosphoinositide-3-kinase (PI3K) and Akt phosphorylations were markedly increased, but were inhibited by 17β-oestradiol treatment in rats with pulmonary hypertension.

Oestrogen deficiency may aggravate development of pulmonary hypertension. 17β-oestradiol improved pulmonary hypertension *via* activation of the PI3K/Akt pathway to regulate NO, PGI$_2$ and ET-1 expression.

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

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Pulmonary hypertension (PH) is a progressive disorder with a poor prognosis. It is characterised by elevated pulmonary arterial pressure (PAP) 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 (E$_2$)), appear to improve experimental PH [2, 3]. E$_2$ is converted from testosterone by the cytochrome P450 (CYP)-19 enzyme, aromatase. E$_2$ is subsequently converted to multiple metabolites *via* several pathways, in which CYP-1A1 and CYP-1B1 are key catabolic enzymes [3]. Experimental studies using a chronic hypoxia- or monocrotaline (MCT)-induced PH model have consistently demonstrated that the administration of E$_2$ exerts protective effects against the progression of PH [2, 3]. Nitric oxide (NO), prostacyclin (prostaglandin (PG)I$_2$) 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 PGI$_2$ in ovine fetal pulmonary artery endothelium and human umbilical vein endothelial cells, and oestrogen receptor (ER)-β has been shown to mediate cyclooxygenase (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 the lung tissue of adult female rats [2]. However, plasma E$_2$ concentrations, changes of E$_2$ metabolising enzymes and the mechanism by which E$_2$ improves PH have not been clarified.

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In the present study, we examined plasma E_2 concentrations, changes in E_2 metabolising enzymes and oestrogen receptors, and the effects of E_2 on NO, endothelial NOS (eNOS), PGI_2, COX-2, ET-1, endothelin-converting enzyme (ECE) expression, and on pulmonary proliferation, apoptosis and inflammation. We also investigated whether E_2 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 Laboratory Animal Commission laboratory animal centre (Shanghai, China). All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tongji University (Shanghai, China) 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 eight 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 E_2 pretreatment (preventive therapy) group (S+M+E (P)); 4) sham-operated, MCT-treated, acute E_2 treatment group (S+M+E (T)); 5) ovariectomised, saline-treated vehicle group (O); 6) ovariectomised, MCT-treated vehicle group (O+M); 7) ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group (O+M+E (P)); and 8) ovariectomised, MCT-treated, acute E_2 treatment group (O+M+E (T)).

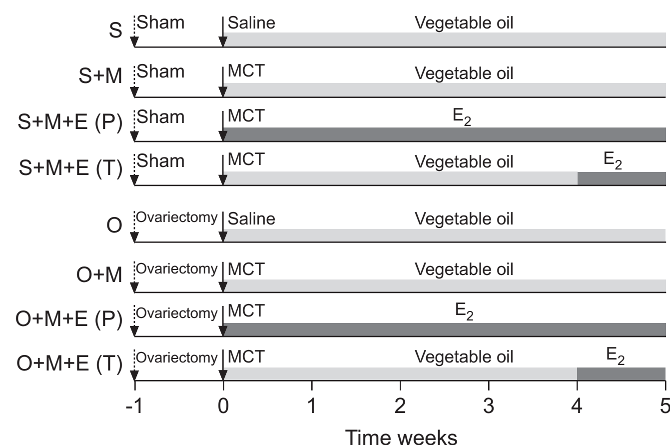

FIGURE 1. Experimental protocol (n=12 per group). A bilateral ovariectomy or sham operation was performed at week -1. Rats were injected with 50 mg·kg^{-1} monocrotaline (MCT) or 0.9% saline at week 0. S: sham-operated, saline-treated vehicle (vegetable oil) group; S+M: sham-operated, MCT-treated vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic 17β-oestradiol (E_2) pretreatment (preventive therapy) group; S+M+E (T): sham-operated, MCT-treated, acute E_2 treatment group; O: ovariectomised, saline-treated vehicle group; O+M: ovariectomised, MCT-treated vehicle group; O+M+E (P): ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; O+M+E (T): ovariectomised, MCT-treated, acute E_2 treatment group. Both the S+M+E (P) and O+M+E (P) groups received 1 mg·kg^{-1} E_2 daily from week 0 to week 5. Both the S+M+E (T) and O+M+E (T) groups were treated with vehicle (vegetable oil) daily from week 0 to week 4 and E_2 only daily from week 4 to week 5. Other groups received vehicle daily for 5 weeks. The shaded bars represent the duration of each experimental setting.

Rats were anaesthetised with 60 mg·kg^{-1} sodium pentobarbital (Sigma, Saint Louis, MO, USA) given intraperitoneally, and a bilateral ovariectomy or a sham operation was performed. After a 1-week recovery period, rats received a single subcutaneous injection of MCT (50 mg·kg^{-1}; Sigma, Chengdu, China) 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 E_2 (1 mg·kg^{-1} per 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 E_2 (1 mg·kg^{-1} per day) throughout week 5. Other groups received vehicle for 5 weeks after MCT or saline injection. At the end of week 5, rats were sacrificed and the plasma and lung tissues were harvested and stored at -80°C until use.

Haemodynamic analyses

After rats were anaesthetised and a tracheotomy 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 PAP (mPAP) by a polygraph system (Power Lab 8/30; AD Instruments, Sydney, 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 RVH was determined by the weight ratios RV/(LV+S) and RV(mg)/body weight (BW)(g).

Measurement of plasma E_2 concentrations

Plasma samples were collected after haemodynamic analyses. Plasma E_2 concentrations were determined in duplicate using the Estradiol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) [10].

Histological 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 50–100 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 muscularisation [12].

Proliferating cells were evaluated by proliferating cell nuclear antigen (PCNA) staining (Dako) and apoptotic cells by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) method (Apoptosis Detection Kit; Wako). Inflammatory cells were evaluated by ED-1 (analogue of CD68) staining (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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

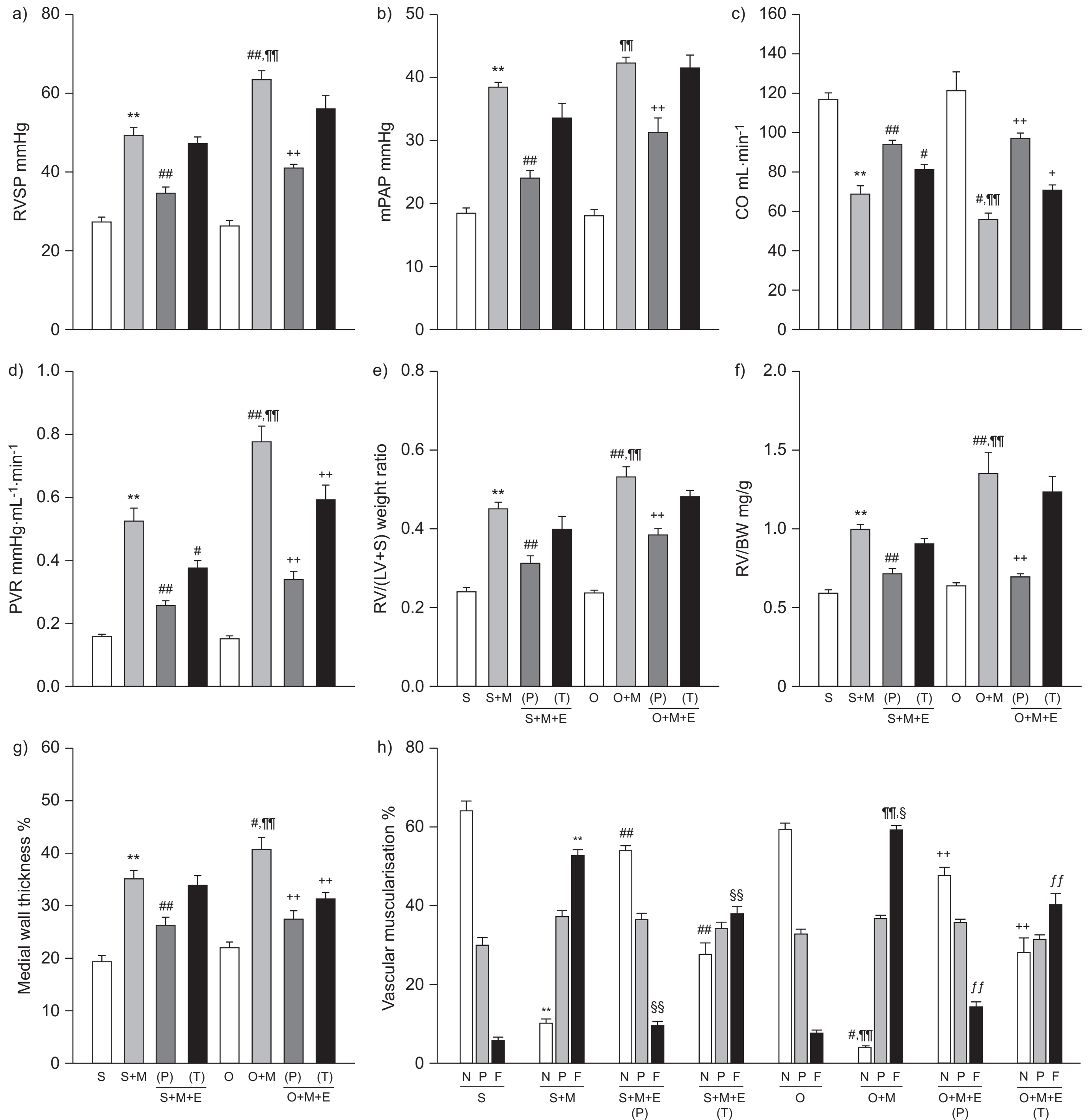

FIGURE 2. Reversal of pulmonary haemodynamic parameters, right ventricular hypertrophy and pulmonary vascular remodelling by the administration of 17β-oestradiol (E_2) in monocrotaline (MCT)-treated rats. Changes of a) right ventricular systolic pressure (RVSP), b) mean pulmonary artery pressure (mPAP), c) cardiac output (CO), d) pulmonary vascular resistance (PVR), e) right ventricular (RV) wall/left ventricular septum (LV+S) ratio, f) RV(mg)/body weight (BW)(g) ratio, g) the degree of medial wall thickness and h) muscularisation of small pulmonary arteries in all groups are shown ($n=6$). S: sham-operated, saline-treated, vehicle (vegetable oil) group; S+M: sham-operated, monocrotaline (MCT)-treated, vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; S+M+E (T): sham-operated, MCT-treated, acute E_2 treatment group; O: ovariectomised, saline-treated, vehicle group; O+M: ovariectomised, MCT-treated, vehicle group; O+M+E (P): ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; O+M+E (T): ovariectomised, MCT-treated, acute E_2 treatment group. a–g) preventive E_2 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 E_2 treatment also reversed the changes in CO and PVR in rats with pulmonary hypertension. Medial thickness: medial wall thickness/external diameter × 100. Error bars represent SE. **: $p<0.01$ *versus* S rats; #: $p<0.05$ and ##: $p<0.01$ *versus* S+M rats; ¶¶: $p<0.01$ *versus* O rats; +: $p<0.05$ and ++: $p<0.01$ *versus* O+M rats. E_2 treatment improved pulmonary vascular remodelling by reversing the degree of medial wall thickness as well as muscularisation of small pulmonary arteries. (continued next page)

FIGURE 2 continued. h) Percentage of non-muscularised (N), partially muscularised (P) and fully muscularised (F) pulmonary arteries are shown. Error bars represent SE. **: $p<0.01$ *versus* S animals N pulmonary arteries; #: $p<0.05$ and ##: $p<0.01$ *versus* S+M animals N pulmonary arteries; ¶¶: $p<0.01$ *versus* O animals N pulmonary arteries; ++: $p<0.01$ *versus* O+M animals N pulmonary arteries; **: $p<0.01$ *versus* S animals F pulmonary arteries; §: $p<0.05$ and §§: $p<0.01$ *versus* S+M animals F pulmonary arteries; ¶¶: $p<0.01$ *versus* O animals F pulmonary arteries; ƒƒ: $p<0.01$ *versus* O+M animals F pulmonary arteries.

of $\times 400$, 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 levels.

Measurement of lung PGI_2 concentrations
The amount of lung PGI_2 produced, calculated as the concentration of the stable hydrolysis product 6-keto-prostaglandin (6k-PG)F1α, was determined in duplicate using a Prostacyclin EIA kit (Enzo Life Science, Farmingdale, NY, 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) [15].

Western blot analyses
Lungs were lysed in $\times 1$ sodium dodecyl sulphate (SDS) supplemented with proteinase inhibitor at a dilution of 1:25. 30 μg of protein lysate was electrophoresed on a 12% polyacrylamide SDS gel and transblotted onto a polyvinylidene fluoride membrane at 270 mA for 90 min. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) and 0.1% Tween (TBS/Tween) for 1 h 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 h at room temperature. The relative protein level was normalised by intensity of β-actin.

Statistical analyses
Data from multiple experiments were expressed as means ± SEM. Data were analysed by t-tests or 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 E_2
Pulmonary haemodynamic evaluations showed that the MCT-treated groups developed severe PH, with increased RVSP, mPAP, PVR, RV/(LV+S) weight ratio and RV/BW ratio, and decreased CO compared with the sham-operated or ovariectomised groups (fig. 2a–f). Interestingly, ovariectomised, MCT-treated (O+M) rats exhibited higher RVSP and PVR and lower CO compared with sham-operated, MCT-treated (S+M) rats (fig. 2a, c and d).

In the chronic pretreatment groups, E_2 significantly attenuated the elevation of RVSP, mPAP, PVR, RV/(LV+S) weight ratio and RV/BW ratio in sham-operated, MCT-treated and E_2 (pretreated) (S+M+E (P)) rats, and also reduced PVR in ovariectomised, MCT-treated and E_2 (pretreated) (O+M+E (P)) rats compared with MCT-treated rats not given E_2 (fig. 2a, b, d–f). In these groups, E_2 also significantly enhanced CO in both S+M+E (P) rats and O+M+E (P) rats (fig. 2c).

In the acute treatment groups, E_2 reduced PVR and increased CO in sham-operated, MCT-treated and E_2 (treated) rats (S+M+E (T)) and ovariectomised, MCT-treated and E_2 (treated) (O+M+E (T)) rats compared with MCT-treated rats not given E_2 (fig. 2c and d).

Reversal of pulmonary vascular remodelling, pulmonary proliferation, apoptosis and inflammation by E_2
Fully muscularised 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-muscularised vessels in MCT-treated groups with E_2 pretreatment and acute treatment (fig. 2g and h).

PCNA expression in vascular smooth muscle cells was increased in the MCT-treated groups, and was prevented by E_2 (fig. 3a and b). Macrophage recruitments were increased in the MCT-treated groups, and were markedly suppressed by E_2 treatment (fig. 3c and d). E_2 also significantly enhanced vascular smooth muscle cells apoptosis (fig. 3e and f). The percentages of TUNEL-positive cells were significantly increased in the E_2 groups compared with the saline-treated normal groups and the MCT-treated groups (fig. 3e and f).

Plasma E_2 concentrations, oestrogen receptors and expressions of CYP19, CYP1A1 and CYP1B1 in the lung
Plasma E_2 concentrations were significantly reduced in S+M rats compared with S rats (fig. 4a). There was almost no E_2 in O rats or in O+M rats. After the long-term E_2 exposure, plasma E_2 concentrations were dramatically increased in the S+M+E (P) rats and in O+M+E (P) group, and both were similar to levels in the S rats. After the short-term E_2 exposure, plasma E_2 concentrations were significantly increased in the S+M+E (T) rats, and were similar to levels in the S rats; E_2 concentrations were also increased in the O+M+E (T) group compared with in the O rats, but were considerably less than levels in the S rats.

Pulmonary ER-α protein levels were decreased in MCT-treated rats (fig. 4b and c) but ER-β protein levels were not changed (fig. 4b and d). The expression of CYP-19 protein in the lung was significantly decreased by MCT. Conversely, the expression of pulmonary CYP-1A1 and -1B1 proteins was significantly increased in MCT-treated rats (fig. 4b and e–g).

Reversal of pulmonary NO, PGI_2 and ET-1 concentrations by E_2

Changes in NO and PGI_2 concentrations in lung are shown in figure 5a and b. There were marked reductions of NO and PGI_2 concentrations in MCT-treated PH rats compared with controls. Notably, ovariectomised, MCT-treated (O+M) rats showed much lower concentrations of NO and PGI_2 than sham-operated, MCT-treated (S+M) animals. NO and PGI_2 levels were significantly increased by E_2 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 E_2 inhibited the elevation of lung ET-1 concentrations in the sham-operated and ovariectomised, MCT-treated rats (S+M+E (P) and O+M+E (P) groups) (fig. 5c). Acute treatment with E_2 also significantly suppressed the expression of lung ET-1 in the sham-operated and ovariectomised, 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 E_2

Changes in eNOS and COX-2 concentrations in the lung are shown in figures 5d–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 E_2 pretreatment (fig. 5d–f).

Lung ECE levels were increased by MCT treatment (fig. 5d and g). Pretreatment with E_2 inhibited the elevation of lung ECE proteins (fig. 5d and g).

Expression of phosphoinositide-3-kinase and Akt phosphorylations in the lung

The expression of phosphoinositide-3-kinase (PI3K) and Akt phosphorylations were significantly increased in the MCT-treated groups and both were markedly inhibited by E_2 treatment (fig. 6a–c).

DISCUSSION

The novel findings of the present study are that E_2 treatment improved PH *via* multiple differing pathways. Suppressed metabolising enzyme-mediated E_2 in the lung may play an important role in the pathogenesis of PH. The study showed that: 1) plasma E_2 concentrations were significantly decreased in MCT-treated rats; 2) oestrogen receptors and some metabolising enzymes were altered by MCT treatment; 3) E_2 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 PGI_2 expression; and 4) E_2 may reduce PI3K/Akt activation in order to play a protective role. These findings suggest that endogenous oestrogen deficiency may play an important role in the pathogenesis of MCT-induced PH, and that E_2 may ameliorate the progression of PH by combining with ER-β to inhibit PI3K/Akt activation to regulate the production of ET-1, PGI_2 and NO and their enzymes.

Although pulmonary arterial hypertension (PAH) is a disease that occurs predominantly in females [16–17], SHAPIRO *et al.* [16] reported that males with PAH had higher mPAP and mean right atrial pressure at diagnosis, and that males aged $\geqslant 60$ years had lower survival rates compared with females aged $\geqslant 60$ 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 sex is a profound modifier of pathogenesis and survival in PAH, and the potential role of oestrogen needs to be explored further. We investigated whether E_2 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 E_2, E_2 deficiency and E_2 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 E_2 improves PH, but there have been no reports of changes in plasma E_2 concentrations in MCT-treated rats. In this study, we found that plasma E_2 concentrations were decreased by MCT treatment, which may give rise to a less protective role of E_2 in pulmonary arteries. Possible explanations include changes in E_2 metabolising enzymes. Interestingly, our findings suggest that CYP-19 is decreased and CYP-1A1 and CYP-1B1 are increased in MCT-induced PH. Whether these changes in metabolic enzymes lead to abnormal E_2 concentrations requires further confirmation. Recently, AUSTIN *et al.* [21] demonstrated that altered oestrogen metabolism through polymorphisms in CYP-1B1 may modify the risk of developing familial PH in female *BMPR2* mutation carriers.

E_2 exerts its effects mainly *via* ER-α and ER-β. Both receptor subtypes are present in the lungs. An ER-β-mediated action of oestrogen has recently been reported in male rats with MCT-induced PH [22]. This study showed that a selective ER-β agonist is able to rescue PH as efficiently as E_2, and that E_2 fails to ameliorate PH in the presence of an ER-β-selective antagonist. The data from this study strongly support the notion that the rescue action of E_2 is mainly mediated through ER-β in MCT-induced PH [22]. LAHM *et al.* [23] reported that a non-selective ER inhibitor and ER-α- and ER-β-specific antagonists opposed the effects of E_2; however, they considered that the important effects of E_2 on functional end-points in hypoxic PH were predominantly mediated by ER-α. Therefore, evaluation of ER expression in PH was considered important. The data of LAHM *et al*. [23] indicate that hypoxia increased ER-β, but not ER-α, lung vascular expression. In contrast, our results showed that MCT inhibited ER-α, but had no effect on ER-β proteins in the lung. Interestingly, MCT-regulated ER protein expressions were not in line with those seen in hypoxia. This difference may reflect distinct mechanisms of E_2 signalling 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 E_2 attenuates the development of experimental PH by investigating survival rates and haemodynamic parameters such as mPAP, RVSP and

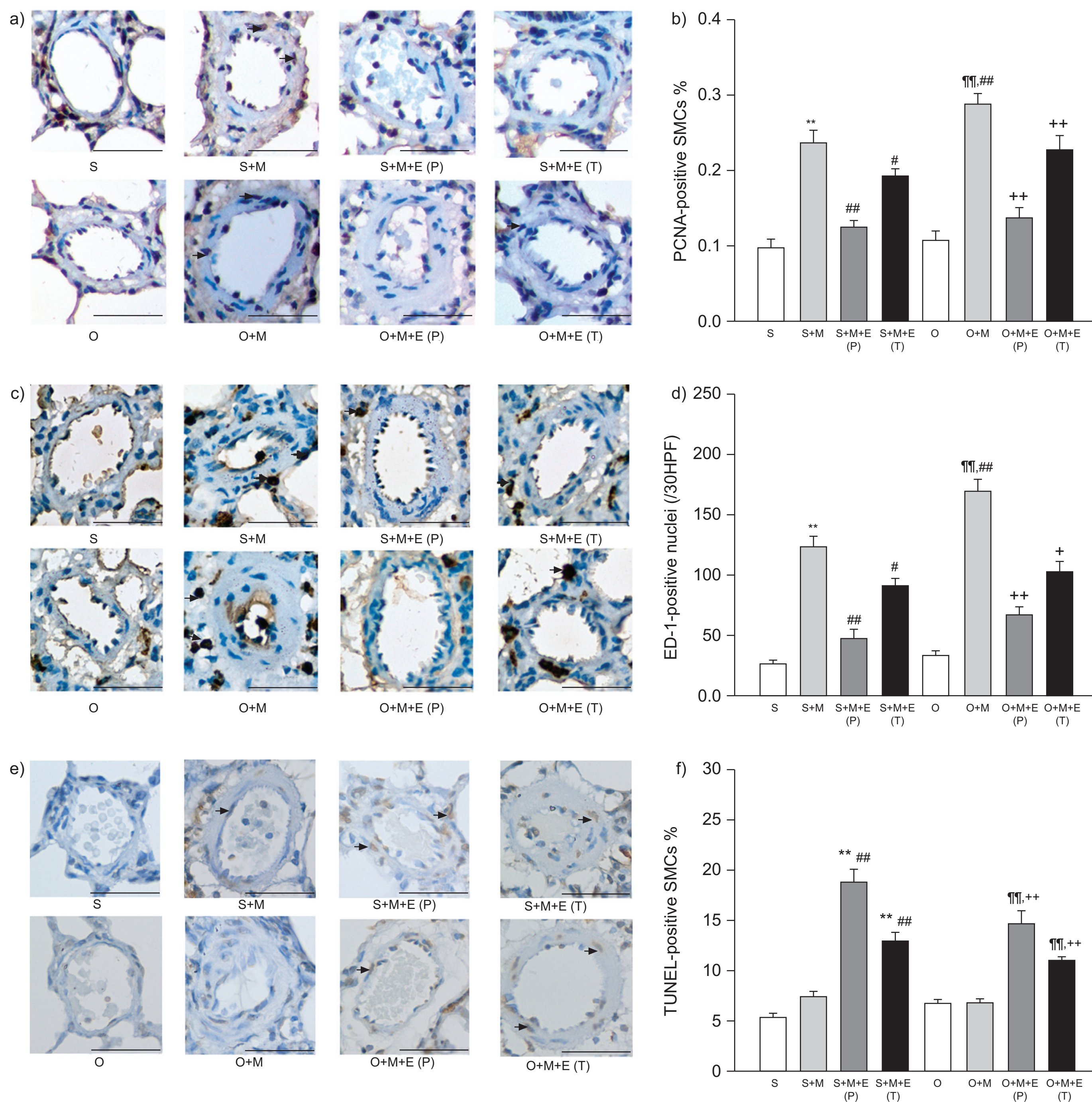

FIGURE 3. Treatment with 17β-oestradiol (E_2) improved pulmonary vascular proliferation, inflammation and apoptosis in pulmonary hypertension. Histology illustrations of pulmonary arteries in the groups (n=6 each) are shown. Monocrotaline (MCT)-induction increases in proliferating cell nuclear antigen (PCNA)-positive smooth muscle cells (SMC) (arrows, a). ED-1-positive macrophages (arrows, c) were prevented in the E_2 treatment groups. e, f) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL)-positive cells were increased in the E_2 treatment groups (arrows, e). S: sham-operated, saline-treated, vehicle (vegetable oil) group; S+M: sham-operated, MCT-treated, vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; S+M+E (T): sham-operated, MCT-treated, acute E_2 treatment group; O: ovariectomised, saline-treated, vehicle group; O+M: ovariectomised, MCT-treated, vehicle group; O+M+E (P): ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; O+M+E (T): ovariectomised, MCT-treated, acute E_2 treatment group; HPF: high-power field. Error bars represent SE. **: $p<0.01$ *versus* S animals; #: $p<0.05$ and ##: $p<0.01$ *versus* S+M animals; ¶¶: $p<0.01$ *versus* O animals; +: $p<0.05$ and ++: $p<0.01$ *versus* O+M animals. Scale bars=50 μm.

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 the 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. Ovariectomised, MCT-treated rats had more severe PH than sham-operated, MCT-treated rats,

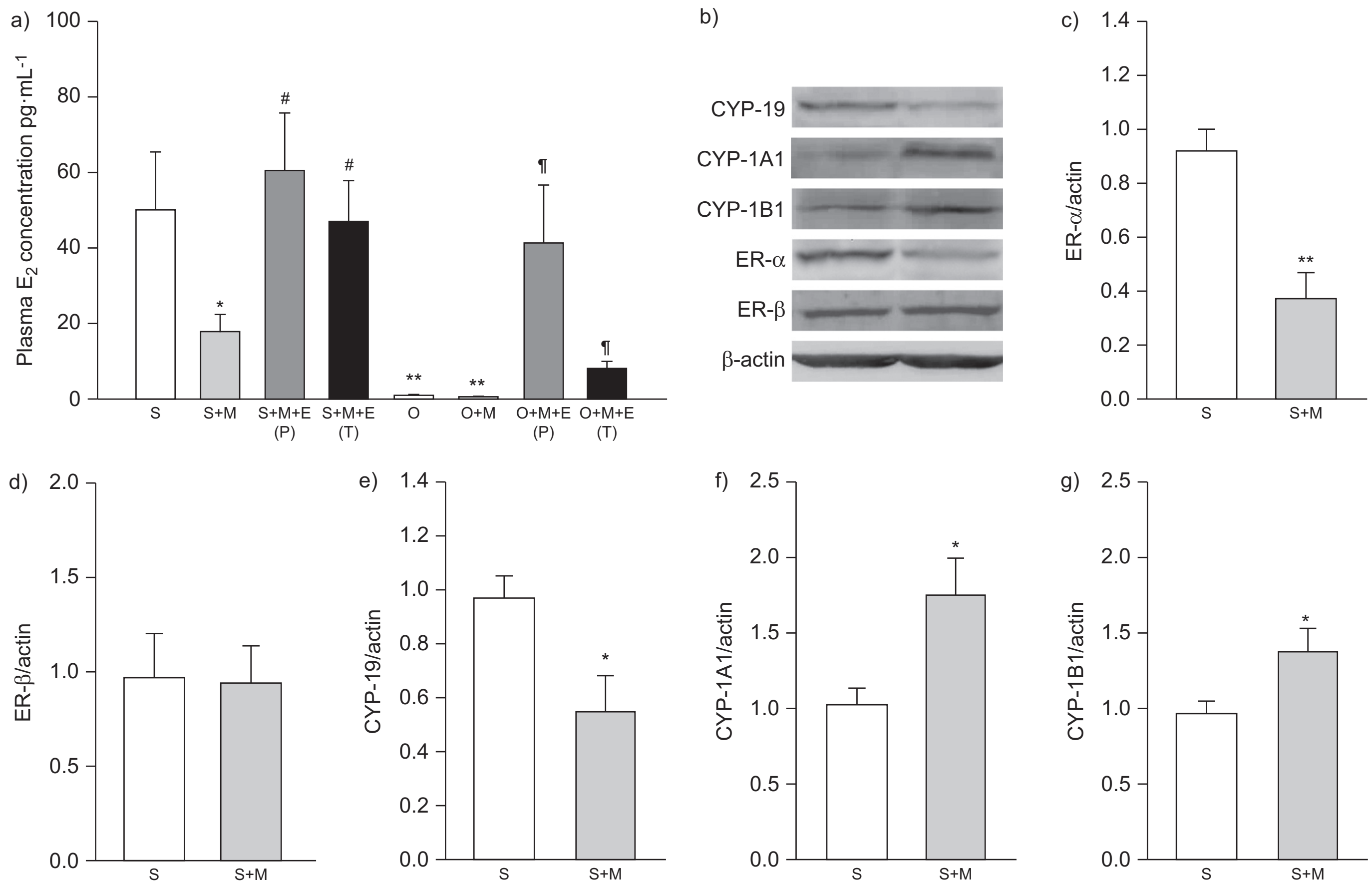

FIGURE 4. Plasma 17β-oestradiol (E_2) concentrations, oestrogen receptors and metabolising enzyme levels in monocrotaline (MCT)-induced pulmonary hypertension (PH). a) Plasma E_2 concentrations were reduced in PH (n=8 each). b) Representative immunoblots of lung lysates with anti-oestrogen receptor (ER)-α, anti-ER-β, anti-cytochrome P450 (CYP)-19, anti-CYP-1A1, anti-CYP-1B1 and anti-β-actin (n=6 each). c, d) The expression of ER-α was significantly reduced in the MCT-treated groups, but ER-β proteins were not markedly changed in MCT-treated rats. e) CYP-19 was significantly suppressed in MCT-treated rats. f, g) CYP-1A1 and -1B1 were markedly increased in MCT-treated rats. S: sham-operated, saline-treated, vehicle group; S+M: sham-operated, MCT-treated, vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic E_2 pretreatment group; S+M+E (T): sham-operated, MCT-treated, acute E_2 treatment group; O: ovariectomised, saline-treated, vehicle group; O+M: ovariectomised, MCT-treated, vehicle group; O+M+E (P): ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; O+M+E (T): ovariectomised, MCT-treated, acute E_2 treatment group. Error bars represent SE. *: $p<0.05$ and **: $p<0.01$ *versus* S animals; #: $p<0.05$ *versus* S+M animals; ¶: $p<0.01$ *versus* O+M animals.

and E_2 treatment improved PH. Thus, E_2 deficiency may be a risk factor for PH.

PH is characterised 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, E_2 pretreatment suppressed PASMC proliferation and macrophage infiltration in MCT-treated rats, consistent with the findings of a previous study [22]. In this study, by UMAR *et al*. [22], E_2 administration enhanced PASMC apoptosis in PH. Indeed, pretreatment and acute treatment with E_2 induced a marked improvement of pulmonary artery muscularisation 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, PGI_2 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, PGI_2 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 E_2 treatment. In fetal pulmonary artery endothelial cells, E_2- 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 E_2, the authors did not investigate the role of E_2 during hypoxia or MCT administration. However, RESTA *et al*. [24] have suggested that E_2 exerts a protective influence in hypoxic PH, but that this protection was not likely to be a function of increased eNOS expression. Therefore, E_2 regulation of eNOS expression remains controversial. We further demonstrated upregulation of eNOS and NO by E_2 in the lung in MCT-induced PH rats. These findings are in agreement with previous reports [5, 6]. Although the mechanisms by which E_2 increases eNOS expression have not been clearly elucidated, epigenetic abnormalities may be an explanation.

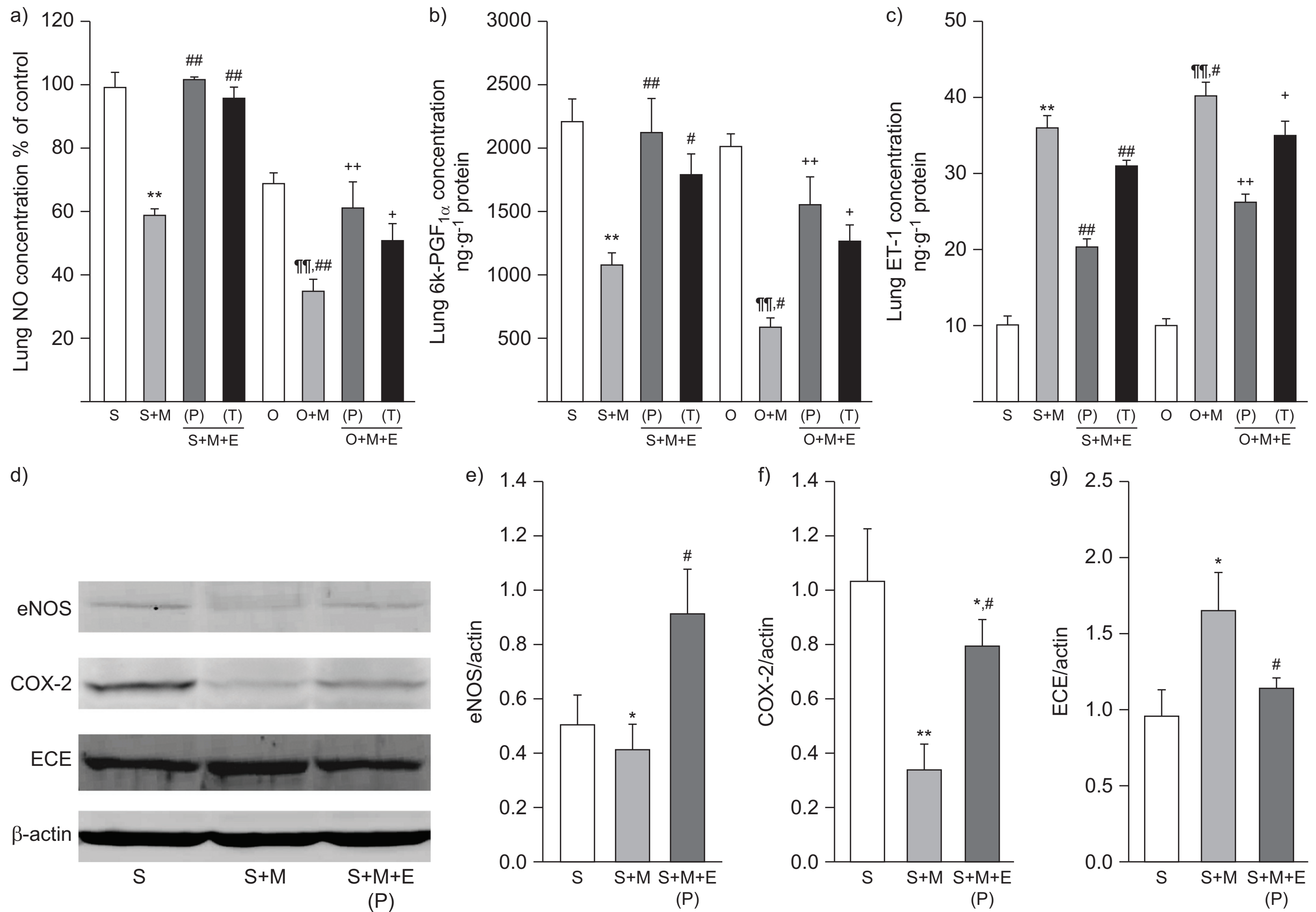

FIGURE 5. 17β-oestradiol (E_2) treatment regulated pulmonary nitric oxide (NO), prostaglandin (PG)I_2, and endothelin (ET)-1 concentrations and their enzymes in monocrotaline (MCT)-treated rats. Changes in lung a) NO, b) PGI_2 and c) ET-1 concentrations in all groups (n=6 each). d) Representative immunoblots of lung lysates with anti-endothelial NO synthase (eNOS), anti-cyclooxygenase (COX)-2, anti-endothelin-converting enzyme (ECE) and anti-β-actin (n=6 each). e, f) Show that E_2 enhanced the expression of pulmonary eNOS and COX-2 in pulmonary hypertension (PH). g) E_2 suppressed the expression of pulmonary ECE in MCT-induced PH. S: sham-operated, saline-treated, vehicle (vegetable oil) group; S+M: sham-operated, MCT-treated, vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; S+M+E (T): sham-operated, MCT-treated, acute E_2 treatment group; O: ovariectomised, saline-treated, vehicle group; O+M: ovariectomised, MCT-treated, vehicle group; O+M+E (P): ovariectomised, MCT-treated, chronic E_2 pretreatment (preventive therapy) group; O+M+E (T): ovariectomised, MCT-treated, acute E_2 treatment group. Error bars represent SE. *: $p<0.05$ and **: $p<0.01$ *versus* S animals; $^{\#}$: $p<0.05$ and $^{\#\#}$: $p<0.01$ *versus* S+M animals; ¶¶: $p<0.01$ *versus* O animals; $^{+}$: $p<0.05$ and $^{++}$: $p<0.01$ *versus* O+M animals.

Studies in both animals and humans indicate that during pregnancy there is a marked elevation of PGI_2 metabolite levels in both blood and urine [27]. Post-menopausal females receiving oestrogen replacement therapy show increased urinary excretion of PGI_2 [28]. SOBRINO *et al.* [8] have reported that E_2 dose-dependent increased PGI_2 production and COX-1 and PGI_2 synthase expression without affecting COX-2 expression in human umbilical vein endothelial cells. However, we observed that pulmonary PGI_2 production and COX-2 expression were increased by E_2 in MCT-treated rats. One possible reason is that MCT potentially has a direct influence on the regulation of COX-2 by E_2; another possible reason is that the adjustment mechanisms of E_2 may be cell-specific. It is known that oestrogen modulates both ET-1 gene expression and production [29]. Our data indicate that E_2 not only regulates eNOS and COX-2 expressions, but also reduces ECE levels in MCT-treated rats.

An effect of E_2 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 E_2 administration reversed the increases. In previous studies in rat aortic rings, LY 294002, a specific inhibitor of the PI3K/Akt pathway, and Nω-nitro-L-arginine-methyl ester, a NOS inhibitor, reduced E_2-induced vasorelaxation. The data showed that E_2 exerted a vasorelaxant effect mediated by eNOS activation through Akt/PKB-dependent mechanisms in rat aortic rings [32]. In other studies, 2-methoxyestradiol, an E_2 metabolite, inhibited neointima formation and human aortic smooth muscle cell growth by regulating phosphorylated Akt or other pathway proteins to enhance COX-2 expression [33]. Dehydroepiandrosterone, a

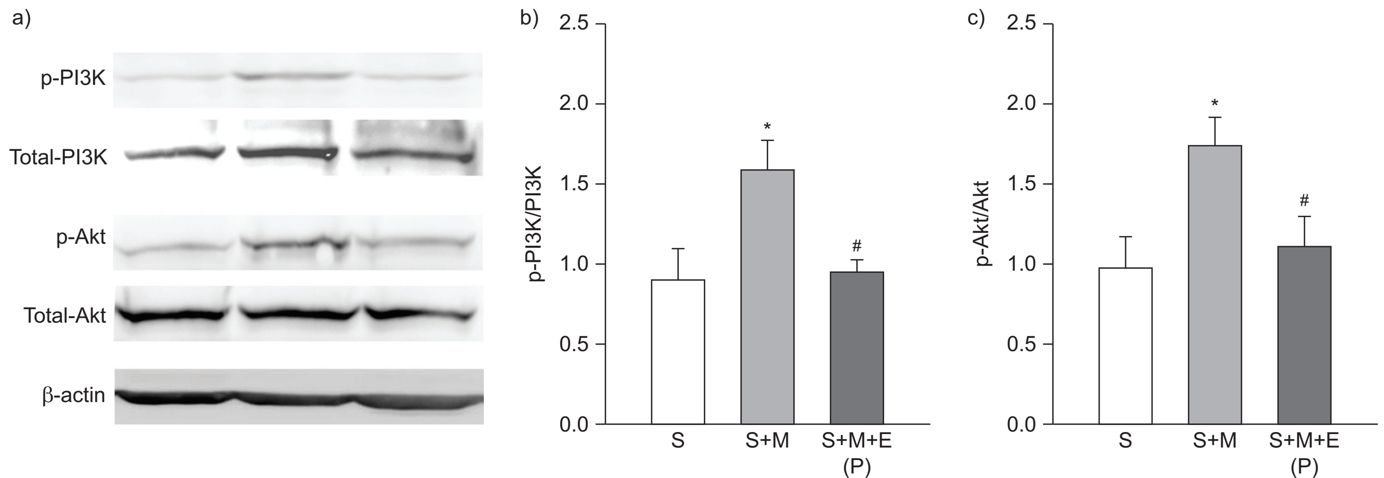

FIGURE 6. 17β-oestradiol (E_2) treatment regulated pulmonary phosphoinositide-3-kinase (PI3K) and Akt phosphorylations in monocrotaline (MCT)-treated rats. a) Representative immunoblots of lung lysates with anti-p-PI3K, anti-PI3K, anti-p-Akt, anti-Akt and anti-β-actin. b, c) E_2 significantly increased pulmonary PI3K phosphorylations and Akt phosphorylations in MCT-induced pulmonary hypertension. S: sham-operated, saline-treated vehicle group; S+M: sham-operated, MCT-treated vehicle group; S+M+E (P): sham-operated, MCT-treated, chronic E_2 pretreatment group; n=6 for each group. Error bars represent SE. *: $p<0.05$ *versus* S animals; #: $p<0.05$ *versus* S+M animals.

progenitor of E_2, has been found to have beneficial or harmful effects relevant to the vascular endothelium by altering the balance between PI3K and mitogen-activated protein kinase-dependent signalling in bovine aortic endothelial cells [34]. A similar effect of E_2 was reported on extracellular signal-related kinase (ERK)-1/2 pathway in hypoxic animals [23]. In this study, by LAHM *et al.* [23], hypoxia increased ERK-1/2 expression, and E_2-treated hypoxic animals exhibited reduced ERK-1/2 activation. Thus, E_2 may regulate the expression of NO, PGI_2 and ET-1 through the PI3K/Akt pathway in MCT-treated rats.

Limitations

Taken together, these findings provide good evidence of the importance of E_2 in regulating the development of PH. We cannot, however, exclude other potential mechanisms of enhanced hypertrophy being modulated by E_2. As we did not administer an oestrogen receptor antagonist in conjunction with E_2, potential downstream signalling mechanisms by which E_2 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 E_2 metabolising enzymes affect the progression of PH. In addition, we could not evaluate androgens or oestrogen metabolite and E_2 levels over the course of the rats' lives prior to the end of the cross-sectional measurements.

Conclusions

These results indicate that E_2 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 PGI_2 expression. An E_2 deficiency mediated by changes in metabolising enzymes may increase the risk of PH. These findings may have significant clinical implications in patients with PH.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

Conflict of interest information can be found alongside the online version of this article at www.ersjournals.com

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