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Protection by oestradiol against the development of cardiovascular changes associated with monocrotaline pulmonary hypertension in rats

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1 We studied the effects of oestradiol 17β on the development of pulmonary vascular changes and right ventricular (RV) hypertrophy in response to monocrotaline in male Sprague-Dawley rats.

2 Rats were treated with either placebo or oestradiol 17β (10 mg) in the form of slow release pellets implanted subcutaneously 48 h before monocrotaline administration. Rats were injected with either saline or a single dose of monocrotaline (60 mg kg^{-1}, i.m.). Pulmonary vascular changes and RV hypertrophy were studied at 4 weeks following monocrotaline administration.

3 Monocrotaline induced a significant increase in the ratio of right ventricle (RV) to left ventricle-plus-septum (LV + S) weights. Monocrotaline-treated rats also showed significant myointimal proliferation in small pulmonary arteries, decrease of arterial numbers and increase in the number of abnormal alveolar macrophages.

4 Oestradiol 17β attenuated myointimal hyperplasia in pulmonary vessels, decreased the RV/(LV + S) ratio in monocrotaline-treated rats. Oestradiol 17β had no significant effect on control animals.

5 Oestradiol treatment prevented the increase in lung wet to dry weight ratio, observed 7 days post monocrotaline administration.

6 These results suggest that oestradiol 17β protects against the pulmonary vascular remodelling and RV hypertrophy associated with monocrotaline-induced pulmonary hypertension in the rat. Oestradiol also protects against microvascular leak observed in the early days of lesion.

Keywords: Oestrogen; monocrotaline; myointimal hyperplasia; right ventricular hypertrophy; pulmonary hypertension; microvascular leak

Introduction

Monocrotaline-induced pulmonary hypertension is an important model for the study of the pathogenesis of chronic inflammatory pulmonary conditions that involve the interstitium and vessels of the lung. Endothelial cell damage produced early by the metabolites of monocrotaline is thought to lead to the development of structural and functional changes (Butler, 1970; Roth & Reindel, 1991; Schultze *et al.*, 1991). A single monocrotaline injection causes pulmonary oedema, inflammatory lung parenchyma changes, pulmonary hypertension and right ventricular hypertrophy (Meyrick *et al.*, 1980; Ghodsi & Will, 1981; Roth & Reindel, 1991). The pulmonary vascular lesions are characterized by medial hyperplasia and hypertrophy of muscular pulmonary arteries and capillary endothelial cell changes (Meyrick *et al.*, 1980; Ghodsi & Will, 1981; Meyrick & Reid, 1982).

The development of monocrotaline-induced pulmonary hypertension appears to be sexually differentiated (Kiyatake *et al.*, 1992). Female rats treated with monocrotaline develop a lower degree of pulmonary hypertension than their male counterparts, suggesting a possible protective role of oestrogen in this animal model. Oestradiol has been shown to inhibit myointimal proliferation in the aorta of renal hypertensive rats (Wolinsky, 1972), prevent the development of atherosclerosis in the rabbit aorta (Fischer & Swain, 1985), and decrease the size of atherosclerotic plaque in monkeys and rabbits via changing the cholesterol metabolism (Kushwaha & Hazzard, 1981; Williams *et al.*, 1990). Oestradiol treatment also blunts pulmonary vascular responses to pressor agonists and inhibits hypoxic pulmonary vasoconstriction (Gordon *et al.*, 1986). However, the effect of oestrogen on the pathogenesis and development of different forms of pulmonary vascular injury, including monocrotaline-induced pulmonary hypertension remains unclear.

The purpose of this study was to evaluate the effect of oestrogen treatment on the structural changes in the right heart and pulmonary vasculature observed in monocrotaline-induced pulmonary hypertension in the rat.

Methods

Animals and treatment protocol

Ten week old male Sprague-Dawley rats (weighing between 250 to 300 g; Charles River Laboratories, Willmington, MA, U.S.A.) were treated with a single intramuscular injection of saline or monocrotaline (60 mg kg^{-1}). An aqueous solution of monocrotaline was prepared as described by Hayashi *et al.* (1967) wherein, 200 mg was dissolved in 1.2 ml of 1 N HCl, diluted with distilled water to about 5 ml and neutralized with 0.5 N NaOH; the volume was then adjusted to 10 ml with distilled water.

All animals were treated with either placebo or oestradiol-17β (10 mg) in the form of slow release pellets, implanted subcutaneously 2 days before monocrotaline injection. Pellets were purchased from Innovative Research of America (Toledo, OH, U.S.A.) and were fused and compressed individually with filter material including cholesterol, microcrystalline cellulose, α-lactose, di- and tricalcium phosphate, calcium and magnesium stearate, and stearic acid (Osborne *et al.*, 1985). The pellet doses were chosen so as to sustain rat blood oestradiol levels of about 10 ng ml^{-1}. The development of pulmonary vascular changes and right ventricular (RV)

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hypertrophy both in the presence and absence of oestradiol treatment was studied at 4 weeks following monocrotaline administration. All of the animals were housed at constant temperature and kept in a 12 h light-dark cycle in the Research Resources Facility of Georgetown University Medical Center, where they had free access to food and water.

Preparation of tissues

At the end of the study period, rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). The trachea was cannulated and rats were ventilated with room air at a tidal volume of 2.5 ml and a frequency of 60 breaths min^{-1}, using a small animal respirator (Model 680, Harvard Apparatus Co., Dover, MA, U.S.A.). A blood sample was collected from the abdominal aorta for the determination of plasma oestradiol levels by radioimmunoassay (Jurjen *et al.*, 1975). Lungs were perfused *in situ* from the right ventricle with heparinized (heparin sodium, 5 units ml^{-1}) lactated Ringer solution for 10 min at a pressure of 20 mmHg, followed by Karnovsky's fixative (Karnovsky, 1956) for 20 min. The trachea was then perfused with the same fixative at a pressure of 25 cmH_2O for 10 min. The lungs and heart were removed en bloc. Tissues from both lungs were immersed in the fixative overnight, then transferred to the 0.1 M sodium-cacodylate buffer (pH 7.3) and stored at 4°C for 1 week before structural study.

The development of pulmonary hypertension was confirmed by right ventricular hypertrophy which was expressed as an increase in right ventricular free wall (RV)/left ventricle-plus-septum (LV + S) in the absence of a change in (LV + S) weight (Fulton *et al.*, 1952). Both atria, valves and fatty tissue of the heart were trimmed from the ventricles. The RV was separated from the LV + S, which were blotted and weighed separately.

Morphology

Histological sections were prepared from lung tissues and stained with haematoxylin-eosin as well as with a multiple stain (ethylene alcohol, methyl alcohol, basic fuchsin, and toluidine blue) to demonstrate internal and external elastic laminae for morphometric studies. The number of abnormal alveolar macrophages was also determined.

Measurement of medial thickness was made on muscular arteries with an external diameter of 30–100 and 100–200 μm respectively, with a computerized morphometer (The Morphometer, Woods Hole Educational Associates, Woods Hole, MA, U.S.A.). For each artery, medial thickness was expressed as:

(external diameter – luminal diameter)/external diameter × 100

The external diameter is the distance between and including the two external elastic laminae intersected by the diameter. Because vessels were often not perfectly circular in cross-section, each vessel was measured twice at right angles to the first measurements. In each section, ten vessels were measured, and the average was calculated. To determine the number of abnormal alveolar macrophages and small arteries (external diameter < 100 μm), macrophages, arteries and alveoli were counted in 10 consecutive fields at × 500 magnification in each lung section and expressed per 100 alveoli (Meyrick *et al.*, 1980; Ono & Voelkel, 1991).

Histological evaluation was carried out using a double blind study, in which the treatment status was unknown to the investigators.

Determination of lung perivascular oedema

In one group of animals, 7 days following monocrotaline or vehicle administration, lungs were excised as previously described, and weighed immediately to the nearest mg to determine wet weights. Lung samples were then allowed to stand at room temperature until a stable dry weight was obtained. The extent of lung oedema was quantified by the ratio of wet to dry weight (Sugita *et al.*, 1983). These values were compared to those obtained from oestradiol-treated animals.

Statistical analysis

All data were expressed as means ± standard deviations (s.d.). Data were analysed by unpaired Student's *t* test and Fisher's Multiple Comparison Test, to determine significant differences between various experimental groups.

Significance was achieved at $P < 0.05$.

Materials

Crotaline was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; and heparin sodium Organon Inc., West Orange, NJ, U.S.A.

Results

Animal behaviour and body weight changes

Monocrotaline-treated (M) rats showed loss of appetite and failed to gain weight 4 weeks after injection. Two monocrotaline-treated rats became lethargic after 3 weeks and were killed with a high dose of pentobarbitone sodium (100 mg kg^{-1}). Subsequent autopsy showed severe pulmonary oedema. Body weight loss was less in oestrogen-treated animals, and these animals did not show any significant change in behaviour. Oestradiol-treated control (E) animals however, exhibited a significant decrease in body weight as compared to placebo controls (C) (Figure 1). Plasma oestradiol levels in oestradiol-treated animals ranged between 8.1 and 14.6 ng ml^{-1}.

Structural studies

Right ventricular hypertrophy and heart weight index As shown in Table 1, monocrotaline induced a significant increase in the RV/(LV + S) ratio at 4 weeks (0.48 ± 0.07 vs 0.29 ± 0.04 in C, $P < 0.05$). Oestradiol treatment protected against monocrotaline-induced increase in ventricular ratio, but had no effect in placebo-treated animals. There was no significant difference in the (LV + S)/body weight ratio among all groups.

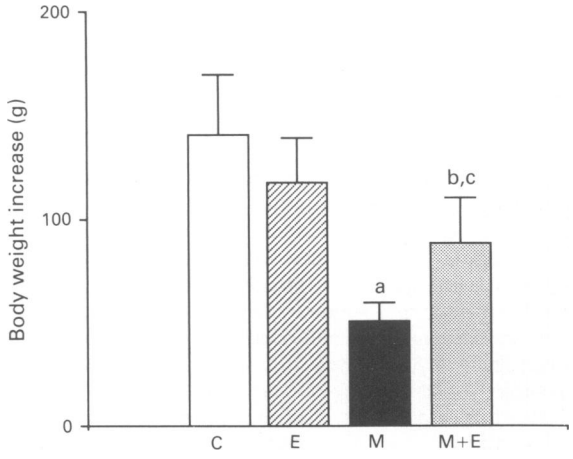

Figure 1 Effect of oestradiol 17β treatment on body weight gain in monocrotaline-treated male rats. Measurements were made 4 weeks following monocrotaline administration. C: control. M: monocrotaline. E: oestradiol. M + E: monocrotaline + oestradiol-treated rats. Each value represents the mean ± s.d. from 4 to 6 animals. $^{a}P < 0.001$ vs C; $^{b}P < 0.02$ vs M and $^{c}P < 0.02$ vs C.

Table 1 Effect of oestradiol 17β on right ventricular hypertrophy in monocrotaline-treated male rats

	RV/(LV + S)	*(LV + S)/BW* ($\times 10^{-3}$)
C ($n = 6$)	0.29 ± 0.01	2.15 ± 0.36
E ($n = 6$)	0.28 ± 0.05	2.13 ± 0.30
M ($n = 4$)	0.48 ± 0.07[ab]	2.08 ± 0.15
M + E ($n = 6$)	0.35 ± 0.05	2.19 ± 0.14

C: control. M: monocrotaline. E: oestradiol. M + E: monocrotaline + oestradiol-treated rats. BW: body weight. LV: left ventricle. RV: right ventricle. S: septum.
[a]$P < 0.005$ vs C; [b]$P < 0.01$ vs M + E.

Myointimal proliferation Monocrotaline induced a 2 fold medial thickening in small muscular arteries with external diameter of less than 200 μm. The percentage myointimal thickening was more significant in those arteries with an external diameter of 30 to 100 μm (13.9 ± 2.1 in M vs 7.1 ± 1.5 in C; $P < 0.05$). Oestradiol significantly prevented monocrotaline-induced medial hypertrophy (Figure 2) 4 weeks after treatment (9.2 ± 1.9 in M + E; $P < 0.01$ vs M), but had no significant effect on placebo-treated animals (Table 2).

Abnormal alveolar macrophages and arterial numbers Sections of lung tissue from monocrotaline-treated rats showed an increase in number of inflammatory cells in lung parenchyma with perivascular infiltration. Lung histology showed increased numbers of foamy alveolar macrophages (50.9 ± 14.1/100 alveoli) in intra-alveolar spaces (Table 2). Oestradiol significantly reduced the numbers of abnormal alveolar macrophages induced by monocrotaline (30.9 ± 10.7/100 alveoli; $P < 0.05$). Monocrotaline treatment also slightly reduced the alveolar numbers in each × 500 magnification field. However, Figure 3 shows that monocrotaline-treated rats had the least arterial (external diameter < 100 μm) density among the four groups studied. Oestradiol 17β significantly protected against the monocrotaline-induced decrease in arterial density (3.6 ± 0.07 in M vs 5.1 ± 1.1/100 alveoli in M + E; $P < 0.05$).

Microvascular leak Monocrotaline induced a significant increase in water content of the lung. The wet to dry weight changed from 5.31 ± 0.06 in C to 5.73 ± 0.07 in M ($P < 0.001$) after 7 days. Oestradiol 17β treatment had no effect on basal lung weight ratio but significantly ($P < 0.01$) inhibited the increase in water content induced by monocrotaline (Figure 4). There was no significant change in lung dry weight to body weight ratio in all groups of study.

Discussion

This study shows that oestradiol significantly improves survival and prevents right ventricular hypertrophy as well as medial hypertrophy in small pulmonary arteries of monocrotaline-treated rats. These data suggest that oestradiol may protect against the development of monocrotaline-induced pulmonary hypertension.

Monocrotaline induces a dose-dependent injury to pulmonary vascular endothelium, leading to a time-related progression of medial proliferation in pulmonary vessels, as well as right ventricular hypertrophy and pulmonary hypertension (Ghodsi & Will, 1981; Meyrick & Reid, 1982; Roth & Reindel, 1991). The delayed and progressive nature of the injury to endothelial cells seems enigmatic, since reactive metabolites of monocrotaline bind rapidly to tissue macromolecules or are rapidly inactivated in aqueous environments

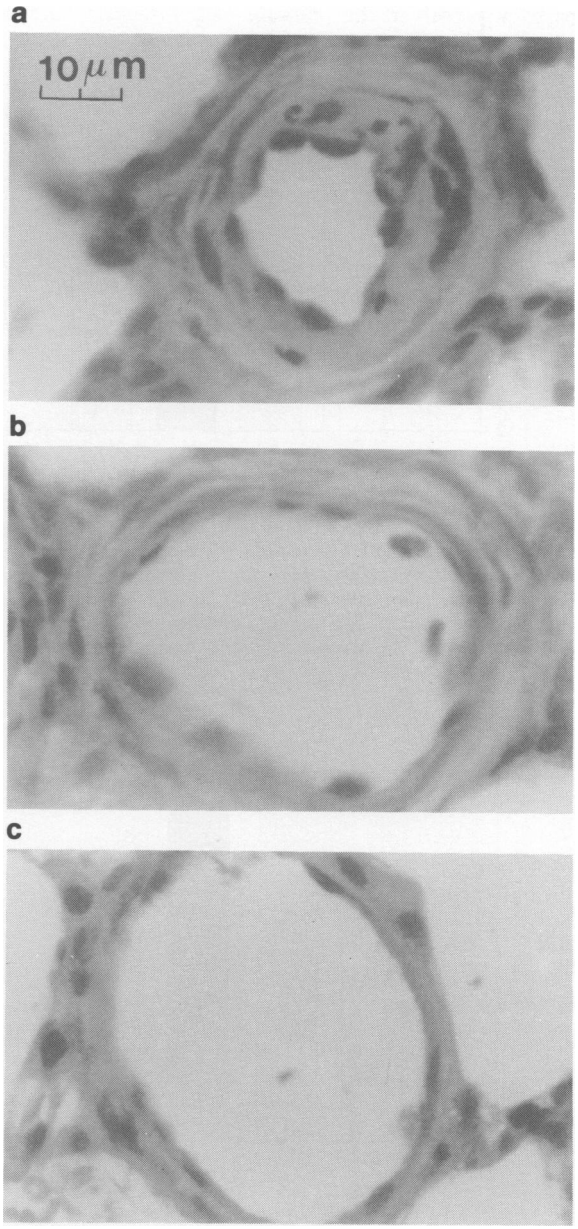

Figure 2 Cross-section (× 500) of small pulmonary artery from lungs of monocrotaline (a), monocrotaline + oestradiol-treated (b), and control (c) rats at 4-weeks following monocrotaline injection, showing the protective effect of oestrogen on pulmonary vascular hypertrophy induced by monocrotaline.

Table 2 Effect of oestradiol 17β on monocrotaline pneumotoxicity in male rats

	MT (%) 30 to 100 μm	*MT* (%) 100 to 200 μm	*Alveoli /field*	*Macro /100 alveoli*
C ($n = 6$)	7.1 ± 1.5	5.9 ± 1.2[a]	23.8 ± 4.8	0
E ($n = 6$)	6.6 ± 1.0	5.7 ± 1.4	22.7 ± 5.1	0
M ($n = 4$)	13.9 ± 2.1[bc]	11.0 ± 2.6	18.4 ± 4.0	50.9 ± 14.1
M + E ($n = 6$)	9.2 ± 1.9	6.6 ± 1.8[a]	22.0 ± 5.0	30.9 ± 10.7

The parameters measured are: % medial thickness (MT%) of small pulmonary arteries (external diameters of 30–100 μm and 100–200 μm), number of alveoli per magnification field (× 500) and number of abnormal alveolar macrophages per 100 alveoli. C: control. M: monocrotaline. E: oestradiol. M + E: monocrotaline + oestradiol-treated rats.
[a]$P < 0.02$ vs M; [b]$P < 0.005$ vs C; [c]$P < 0.01$ vs M + E.

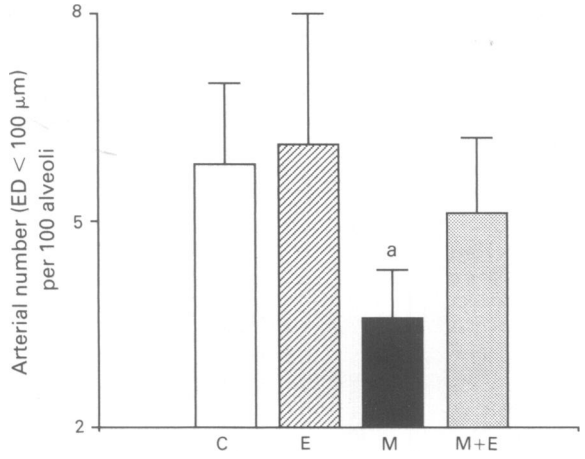

Figure 3 Effect of oestradiol 17β on arterial number in monocrotaline-treated male rats. The number of small pulmonary arteries with external diameter (ED) $<100\,\mu m$ is measured. C: control. M: monocrotaline. E: oestradiol. M + E: monocrotaline + oestradiol-treated rats. Oestradiol treatment (M + E) significantly protected against the decrease in number of arteries observed in monocrotaline-treated rats. Each value represents mean ± s.d. from 4 to 6 animals. $^aP<0.02$ vs C.

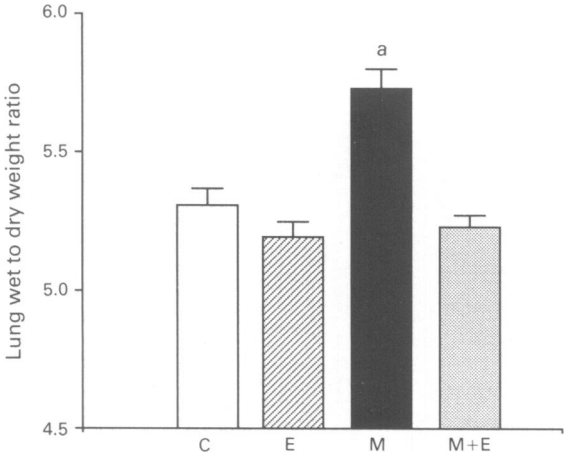

Figure 4 Effect of oestradiol 17β on pulmonary oedema in monocrotaline-treated male rats. Oedema is measured as the lung wet to dry weight ratio, 7 days post monocrotaline administration. C: control. M: monocrotaline. E: oestradiol. M + E: monocrotaline + oestradiol-treated rats. Oestradiol treatment protected against monocrotaline-induced increase in lung water content. Each value represents the mean ± s.d. from 4 to 6 animals. $^aP<0.01$ vs M + E.

(Roth & Reindel, 1991). In fact, it has been suggested that monocrotaline metabolites may not be directly toxic to endothelial cells but may trigger some indirect mechanisms, such as production and release of endogenous mediators of injury (e.g. leukotrienes, cytokines) (Rosenberg & Rabinovitch, 1988; Ito *et al.*, 1988; Ono & Voelkel, 1991; 1992), or inhibit production of endothelium-dependent relaxing factor and cytoprotective compounds like prostacyclin.

We find that smaller arteries displayed greater medial proliferation than the larger ones (Table 2), which is in accordance with previous reports (Meyrick & Reid, 1982; Ono & Voelkel, 1991). We also find that oestradiol significantly reduced right ventricular hypertrophy (Table 1) and decreased pulmonary vascular medial thickening in oestradiol-treated monocrotaline rats. Since there is, in general, a positive correlation between progressive pulmonary hypertension, thickening of the medial wall of the small pulmonary vessels, and right ventricular hypertrophy (Meyrick *et al.*, 1980; Ghodsi & Will, 1981) it is possible that a reduction of pulmonary hypertension and RV hypertrophy by oestradiol treatment might also be a consequence of the reduction in lung vessel thickening.

In addition, the histology of monocrotaline-induced pulmonary hypertension showed profuse inflammatory cell infiltration in the lung parenchyma and abnormal macrophages in alveolar spaces. Oestrogen treatment however, reduced the number of abnormal alveolar macrophages per field (Table 2). Natural and synthetic oestrogens are known to stimulate phagocytosis and clearance of intravenously administered particles by tissue elements of the mononuclear phagocyte system in mice (Loose & Diluzio, 1976; Boorman *et al.*, 1980). It is not clear whether oestrogen decreases inflammation in our model by a direct anti-inflammatory effect or indirectly via attenuating monocrotaline toxicity.

There are several possible mechanisms by which oestradiol may act to attenuate the monocrotaline-induced pulmonary vascular remodelling. Firstly, oestradiol may stimulate angiogenesis (Wolinsky, 1972; Khosla *et al.*, 1981) and hence increase the overall cross-sectional area of pulmonary vascular bed, changing the flow-resistive characteristics of the pulmonary vasculature in monocrotaline-treated animals.

Secondly, oestradiol could decrease the smooth muscle mass in pulmonary vessels, as demonstrated in systemic arteries of hypertensive rats (Wolinsky, 1972), thus leading to a blunted response of monocrotaline-induced myointimal proliferation. We have observed that oestradiol causes a significant attenuation of cardiac transplant myointimal proliferation (Foegh *et al.*, 1987), and inhibition of [^{3}H]-thymidine uptake in arterial segments from the left anterior descending coronary artery of the pig, *in vitro* (unpublished observation). On the other hand, we have also shown that in pulmonary vessels oestradiol potentiates [^{3}H]-thymidine uptake by vascular smooth muscle cells. This mitogenic effect of oestrogen however, is abolished in presence of an intact endothelium, suggesting that endothelial injury or dysfunction may be important (Farhat *et al.*, 1992).

Thirdly, oestradiol has been shown to cause arterial dilatation in several systemic vascular beds (Rosenfeld *et al.*, 1976), and to alter the response of isolated systemic vessels to various vasoconstrictor agents (Colucci *et al.*, 1982). *In vivo* studies also show a blunted pulmonary vascular reactivity to hypoxia, angiotensin II and prostaglandin $F_{2\alpha}$ during pregnancy (Moore & Reeves, 1980; Fuchs *et al.*, 1982; Sylvester *et al.*, 1985), and following oestradiol treatment (Wetzel *et al.*, 1984). Increased production of prostacyclin or other endothelial derived vasodilators may be implicated in this effect (Resnick, 1981). On the other hand, oestradiol 17β has been shown to increase contractions and increase vascular sensitivity to catecholamines in bovine isolated radial arteries (Chan & Kalsner, 1982), rabbit saphenous veins (Rorie & Muldoon, 1979) and rat small mesenteric arteries (Colucci *et al.*, 1982). We have also observed that oestradiol treatment enhances pulmonary vascular response of the rat isolated perfused lung to the thromboxane-mimetic, U46619 (Farhat & Ramwell, 1992). These data suggest that oestrogen plays a role in the regulation of the pulmonary vascular reactivity, and that the effect of oestrogen may depend on the identity of the vessel in the experimental model, as well as on the nature of the agonist used.

On the other hand, endothelial injury by monocrotaline results in increased permeability, perivascular oedema (Plestina & Stoner, 1972; Roth & Reindel, 1991) and increased lung weight which occurs within a few hours after monocrotaline administration (Reindel *et al.*, 1990). Evidence indicates that the structural and functional changes observed at a later stage in the pulmonary arteries and right ventricle may be secondary to those earlier cellular or subcellular events (Ghodsi & Will, 1981; Roth & Reindel, 1991). Our data show that oestradiol treatment prevents monocrotaline-induced microvascular leakage, suggesting that oestradiol may protect against endothelial injury by monocrotaline. Endothelial damage is not caused directly by monocrotaline

but by its metabolic products. Monocrotaline is converted to reactive pyrroles by the mixed function oxidase system of the liver (Hilliker *et al.*, 1983). Oestradiol may have a direct inhibitory effect on the conversion of monocrotaline to its toxic metabolite, monocrotaline pyrrole. Kiyatake *et al.* (1992) have shown that liver homogenates from female rats produced significantly lower concentrations of monocrotaline pyrrole than those from males. This was associated with a lower right ventricular pressure observed in female rats suggesting a gender difference in the severity of monocrotaline-induced pulmonary hypertension.

In conclusion, chronic treatment with oestradiol significantly attenuates pulmonary vascular remodelling and right ventricular hypertrophy in monocrotaline pulmonary hypertension in the rat. Our data suggest that oestradiol may exert its protective effect by preventing monocrotaline-induced endothelial injury. This is the first indication of a protective effect of oestrogen in this animal model and may open new avenues to understanding the role of oestrogen in other forms of pulmonary vascular injury.

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