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Altered function of pulmonary endothelium following monocrotaline-induced lung vascular injury in rats

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1 Based on the findings in the preceding paper we investigated the ability of pulmonary endothelial cells to metabolize prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and angiotensin I (AI), and to produce endothelium-derived relaxing factor (EDRF) following lung vascular injury induced by monocrotaline in rats. The isometric tension of pulmonary artery rings isolated from rats 3–5 weeks after an injection of monocrotaline or saline was measured. For comparison the responses to drugs of the artery denuded of endothelium by rubbing were tested.

2 Acetylcholine-induced relaxation of the rings precontracted by noradrenaline was diminished in the artery from moncrotaline-treated rats, depending on the duration after treatment. The diminution was comparable to that in the control artery denuded of endothelium.

3 The contractile response to $PGF_{2\alpha}$ was significantly augmented in the artery injured by monocrotaline. The similar augmentation was observed after the mechanical removal of endothelium in the control artery. Decrease of EDRF was not involved in the enhancement of contractile response to $PGF_{2\alpha}$ in the monocrotaline-injured artery.

4 AI caused a contraction, which was sensitive to captopril, in control rings, and also in moncrotaline-injured rings to a similar degree. Removal of endothelium from the control artery did not modify the response to AI or to AII.

5 These results suggest that the monocrotaline treatment of rats suppresses the ability of pulmonary endothelium to produce EDRF and to degrade prostaglandins. The relative resistance of the AI response to endothelial injury suggests that the existence of converting enzyme is not restricted to the endothelium.

Introduction

In the preceding paper (Ito *et al.*, 1988) we evaluated the changes of the metabolism of vasoactive autacoids in the pulmonary circulation of rats when the pulmonary vascular bed was injured by monocrotaline treatment. From the blood pressure response to exogenous autacoids it was suggested that the degradation of prostaglandin E_2 (PGE_2) was suppressed while the conversion of angiotensin I (AI) to angiotensin II (AII) and the degradation of bradykinin were little affected in rats receiving a single injection of monocrotaline. Although in these rats much of the endothelium survived after monocrotaline treatment, the data suggest that the endothelium of the lung vascular bed was functionally impaired by monocrotaline.

The lung vascular endothelium has multiple functions in addition to a role as a simple physical barrier between blood and vascular smooth cells. For example, it metabolizes certain vasoactive substances borne in blood (Bakhle & Vane, 1974; Said, 1982; Ryan, 1986), releases active substances into circulating blood (Gryglewski *et al.*, 1978; Moncada *et al.*, 1978; Schrör, 1985) and modulates smooth

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muscle contraction through endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980; Furchgott, 1984; Vanhoutte et al., 1986). Therefore, endothelial damage due to vascular diseases would be expected to have complicated effects on vascular smooth muscle contraction, and on local and systemic haemodynamics. In order to clarify how the function of the pulmonary endothelium is altered following a lung vascular injury, we examined the mechanical responses to vasoactive substances of pulmonary artery preparations isolated from rats receiving a single injection of monocrotaline.

Methods

Preparations

As in the preceding paper (Ito et al., 1988) male Wistar rats at 9 weeks age were given a single subcutaneous injection of monocrotaline ($90\,mg\,kg^{-1}$) or the same volume of saline. Three, four and five weeks after the injection, rats were killed by a blow on the neck. The intra- and extra-pulmonary arteries and the thoracic aorta were excised and ring preparations, 1 to 2 mm in width, were made with care to preserve the endothelial layer intact. Experiments were mainly done on the extra-pulmonary artery. Unless specified otherwise, 'pulmonary artery' in this text means the extra-pulmonary artery.

To test the effect of acute removal of the endothelium some rings from control rats were everted and the endothelial layer was rubbed with a cotton bud to remove the endothelial cells. Furthermore, for comparison, the thoracic aorta of rats and guinea-pigs, and intra-pulmonary arteries of calves and dogs were used.

Tension measurements

The preparations were suspended in an organ bath containing 5 ml of Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 at 37°C and incubated for 60 min with a resting tension of 1 g for equilibration. Then the muscles were contracted by hypertonically added 60 mM KCl and 1×10^{-6} M noradrenaline until reproducible responses were obtained. We ascertained that hypertonic KCl did not impair the ability of the endothelium to produce EDRF in rat pulmonary artery as reported for rabbit coronary artery by Griffith et al. (1984b). The tension was measured isometrically by a force-displacement transducer and recorded on a pen-writing oscillograph.

Histological examination

Excised pulmonary artery rings of various size were fixed in formalin and prepared for light microscopical examination as described in the preceding paper (Ito et al., 1988). In addition, to confirm that the rubbing procedure removed the endothelium completely, rings denuded of endothelium by rubbing were fixed in Karnovsky's solution, postfixed in osmium tetroxide and dried by the critical-point method. The specimens were coated with gold and observed with a scanning electron microscope (JSM-35C).

Solution and drugs

Krebs-Henseleit solution used for tension experiments had the following composition (mM); NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 10 (pH 7.4). Drugs used were monocrotaline (Wako Pure Chemicals), (−)-noradrenaline (NA; Tokyo Kasei), acetylcholine chloride (ACh; Nakarai Chemicals), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Upjohn), angiotensin I (AI), angiotensin II (AII; Peptide Research Foundation), indomethacin and quinacrine (Sigma). All drugs but indomethacin were dissolved in distilled water. Indomethacin was dissolved in 0.01 M Na_2CO_3 at a concentration of 10 mM and diluted in physiological solutions.

Statistics

Data are expressed as means with the standard error. Differences were evaluated by Student's t test at the level of $P < 0.05$.

Results

Responsiveness to agonists of pulmonary artery from rats after treatment with monocrotaline

As described in the accompanying paper (Ito et al., 1988), histological examination revealed degenerative or necrotic changes in the endothelial layer of pulmonary artery from rats at 3–5 weeks after an injection of monocrotaline. These injuries were observed in extrapulmonary arteries, and large and small intra-pulmonary arteries, and appeared to be equally severe in vessels from rats 3 to 5 weeks after treat-

Table 1 Responses to hypertonic KCl (60 mM) and noradrenaline (10^{-6} M) of extra-pulmonary arteries from rats at 4 weeks after an injection of saline or monocrotaline

	Developed tension by		
	KCl ($g\,mm^{-2}$)	*Noradrenaline* ($g\,mm^{-2}$)	n
Control			
Endothelium-intact	1.17 ± 0.20	1.00 ± 0.17	12
Endothelium removed	1.03 ± 0.20	1.10 ± 0.20	9
After monocrotaline treatment			
3 weeks	1.17 ± 0.23	1.27 ± 0.27	6
4 weeks	1.57 ± 0.23	1.50 ± 0.20	6
5 weeks	1.37 ± 0.37	1.20 ± 0.43	6

The endothelium of one group of control arteries was removed by rubbing. Contractions were elicited by agonists under 1 g of resting tension. Data are expressed as developed tension per cross sectional area.

ment. In addition, the medial layer also suffered from similar degenerative or necrotic injury.

As injury was also observed in the medial smooth muscle cell layer after monocrotaline treatment, we checked the responsiveness of arteries from monocrotaline-treated rats to KCl and NA. As summarized in Table 1, the contractile responses to KCl (60 mM) and NA (1×10^{-6} M) were not altered at any time after treatment with monocrotaline.

When the endothelium of control arteries was removed by rubbing, the removal was observed to be complete by scanning electron microscopy. Mechanical removal of the endothelium from control arteries did not modify the responsiveness to high potassium or NA (Table 1).

Ability of acetylcholine to induce endothelium-dependent relaxation

At first we examined the response to ACh of the extra-pulmonary artery rings precontracted by NA (1×10^{-6} M) to assess the endothelial ability to produce EDRF. ACh at 1×10^{-8} to 1×10^{-6} M induced a relaxation of a ring from control rats in a dose-dependent manner (Figures 1 and 2). After the endothelium was mechanically removed, however, ACh did not induce relaxation (Figure 2b). When tested on rings from rats given monocrotaline, ACh-induced relaxation was significantly depressed as shown in Figures 1 and 2a. The depression of the ACh-response developed slowly after the injection of monocrotaline. Percentage inhibition of ACh-responses over the entire dose range of 10^{-8} to 10^{-6} M was $56 \pm 4\%$, $77 \pm 5\%$ and $90 \pm 2\%$ at 3, 4 and 5 weeks after treatment, respectively. The inhibition at 5 weeks corresponded to that observed in rings, from which the endothelium had been removed mechanically ($92 \pm 1\%$). We also tested the response to ACh of intra-pulmonary artery and aortic rings isolated from rats at 4 weeks post-treatment. As observed in the extra-pulmonary artery, ACh induced only a small relaxation in the intra-pulmonary artery and the aorta of the treated rats whereas it caused a much greater relaxation in rings from control rats.

Contractile response to prostaglandin $F_{2\alpha}$

In the accompanying paper (Ito *et al.*, 1988), we used PGE_2 to examine the metabolism of prostaglandins in pulmonary circulation. In the experiment shown in Figure 3, we used $PGF_{2\alpha}$ instead of PGE_2 to test the metabolism of prostaglandins in vascular tissue injured by monocrotaline, since PGE_2 caused neither a sizable contraction nor relaxation in extra-pulmonary artery rings. $PGF_{2\alpha}$ caused a dose-dependent contraction in extra- and intra-pulmonary arteries and aortae. With $PGF_{2\alpha}$ at concentrations higher than 3×10^{-6} M for control pulmonary arteries or with concentrations higher than 1×10^{-6} M for endothelium-injured arteries the contraction was often transient and showed tachyphylaxis when $PGF_{2\alpha}$ was applied repeatedly. We applied $PGF_{2\alpha}$ cumulatively with a 5–10 min interval, as shown in Figure 3. In this case a significant desensitization did not develop because the response to cumulatively applied 1×10^{-5} M $PGF_{2\alpha}$ was almost the same as the response to a single application of 1×10^{-5} M. The threshold concentration of $PGF_{2\alpha}$ required to induce a contraction was smaller and the magnitude of contraction due to each concentration was larger in pulmonary artery rings from monocrotaline-treated rats than those from age-matched control rats (Figure 4). The enhancement of $PGF_{2\alpha}$-induced contraction was greater at 4 or 5 weeks post-treatment than at 3

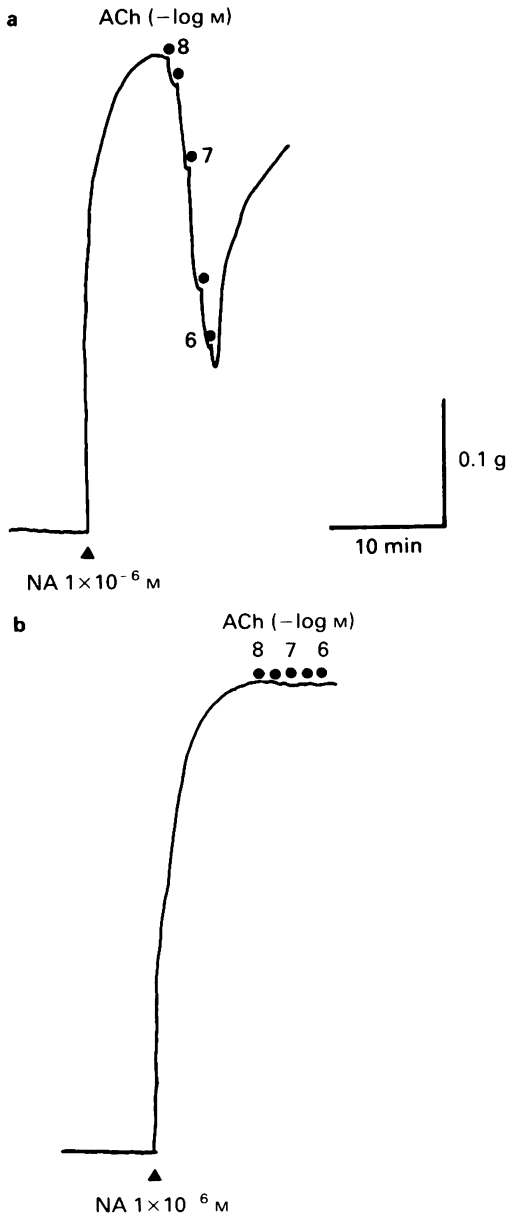

Figure 1 Examples of responses to cumulatively added acetylcholine (ACh) of the extra-pulmonary arteries precontracted by noradrenaline (NA) 10^{-6} M. (a) Response of the artery from a control rat; (b) response from a rat 4 weeks after an injection of monocrotaline.

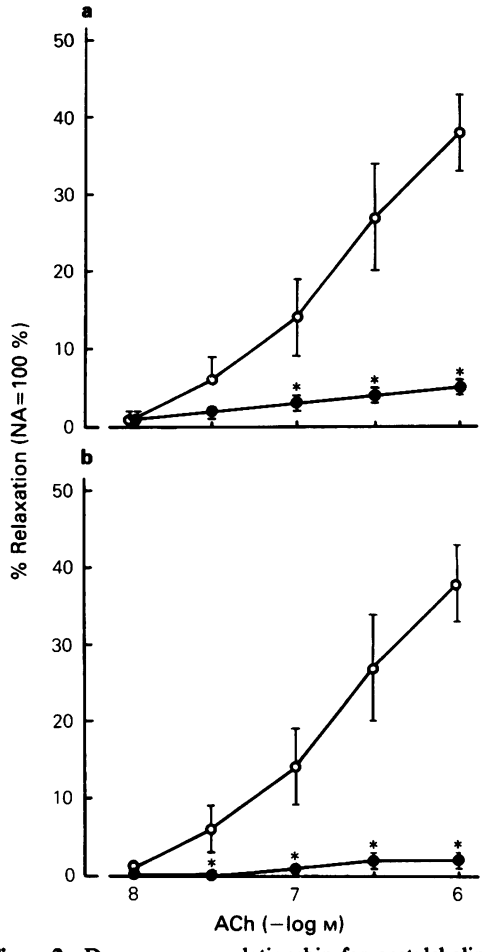

Figure 2 Dose-response relationship for acetylcholine (ACh)-induced relaxation of the pulmonary artery rings precontracted by noradrenaline (NA) 10^{-6} M; 100% on the ordinate scale represents the maximum contraction induced by NA, 1×10^{-6} M. $n = 6$. (a) Responses of arteries from rats at 4 weeks after an injection of saline (control, ○) or monocrotaline (●). The endothelium of control artery was preserved intact. $^*P < 0.05$ (vs. control) (b) Responses of arteries from control rats, of which endothelium was intact (○) or removed by rubbing (●). $^*P < 0.05$ (vs. endothelium intact)

weeks. When the endothelium of the pulmonary artery of control rats was removed by rubbing, the $PGF_{2\alpha}$-induced contraction was also enhanced (Figure 4b). However, the removal of the endothelium from the aorta did not enhance the contractile response to $PGF_{2\alpha}$.

In addition to its contractile action, $PGF_{2\alpha}$ might stimulate the release of EDRF or prostacyclin from the endothelium so that the endothelial injury caused by rubbing or monocrotaline might enhance the contractile response to $PGF_{2\alpha}$. This possibility was examined by two experiments. Firstly, $PGF_{2\alpha}$

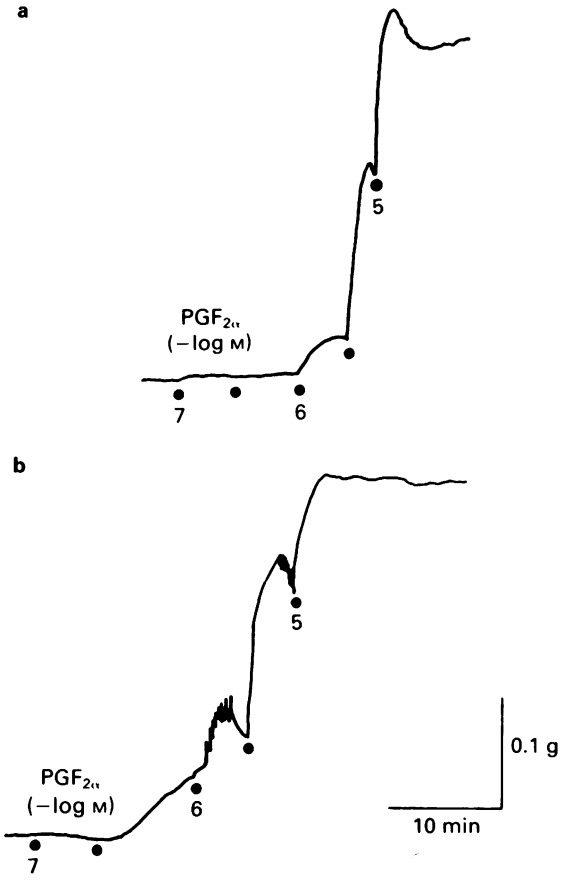

Figure 3 Examples of contractions induced by cumulatively added prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) of the pulmonary arteries from rats given saline (a) or monocrotaline (b) 4 weeks earlier.

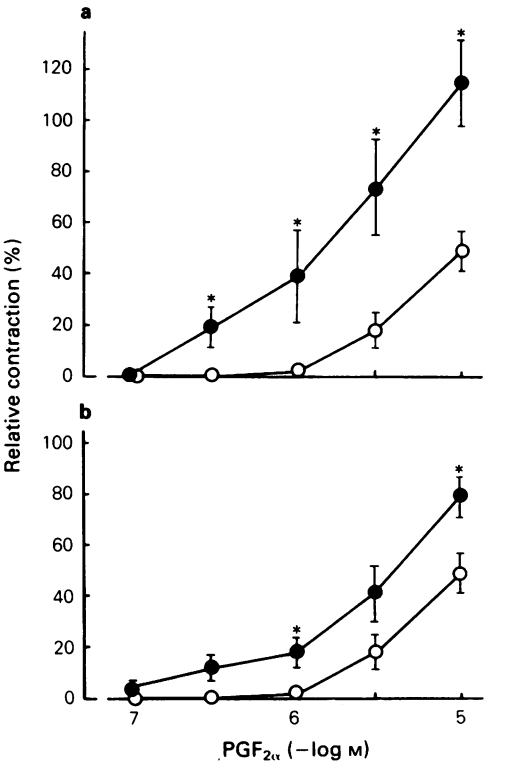

Figure 4 (a) Dose-response relationship for prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contraction of the pulmonary artery rings from rats given saline (control, ○) or monocrotaline (●) 4 weeks before. $n = 6$. * $P < 0.05$ (vs. control) (b) Dose-response relationship for $PGF_{2\alpha}$-induced contraction of the control artery rings of which the endothelium was intact (○) or removed by rubbing (●). $n = 6$. * $P < 0.05$ (vs. endothelium intact) 100% on the ordinate scale represents the maximal contraction induced by 60 mM KCl.

was cumulatively applied to pulmonary artery rings having intact endothelium and precontracted by NA. If $PGF_{2\alpha}$ stimulates the release of EDRF or prostacyclin, it should have caused a relaxation. However, $PGF_{2\alpha}$ consistently potentiated the NA-induced contraction without any sign of relaxation (data not shown). Secondly, the rings with endothelium intact and removed were pretreated with quinacrine (10^{-5} M) to suppress the production of EDRF (Furchgott & Zawadzski, 1980; Furchgott, 1984; Vanhoutte *et al.*, 1986) and then the response to $PGF_{2\alpha}$ was tested. After treatment with quinacrine, which completely prevented 1×10^{-7} or 1×10^{-6} M ACh-induced relaxation of NA-precontracted arteries, the response of rings without endothelium to $PGF_{2\alpha}$ was still larger than that of rings having intact endothelium. Indomethacin (3×10^{-5} M), which inhibits the generation of prostacyclin (Toda & Miyazaki, 1981), did not enhance the $PGF_{2\alpha}$-induced contraction of rings with endothelium intact. These results indicate that $PGF_{2\alpha}$ did not induce the release of EDRF or prostacyclin from the pulmonary artery and that the decrease of EDRF or prostacyclin was not the cause of the enhanced response to $PGF_{2\alpha}$ in pulmonary arteries in which the endothelial cells were injured.

We further tested whether the endothelium of the isolated pulmonary artery is capable of degrading $PGF_{2\alpha}$, using calf intra-pulmonary arteries. Two cut-open strips, 2 cm long, were made from the intra-pulmonary artery; one had intact endothelium, and the other was denuded of the endothelium. For the assay of $PGF_{2\alpha}$, other calf pulmonary artery strips denuded of endothelium were suspended in a small tube containing 1 ml Tyrode solution and its

isometric tension was recorded. Each pulmonary artery strip with or without endothelium was placed in 1.5 ml medium containing $PGF_{2\alpha}$ (5×10^{-6} M). After 5 min incubation, 1 ml of the medium was taken and added to the tube containing 1 ml medium and the artery for bioassay. Accordingly, $PGF_{2\alpha}$ in the original incubation medium was diluted by a factor of 2 in the assay medium. The medium with which the pulmonary artery without endothelium had been incubated, caused a contraction of $42 \pm 9\%$ ($n = 7$) of the maximal contraction due to 60 mM KCl, which corresponded to the response to 2.5×10^{-6} M $PGF_{2\alpha}$ if the agent was applied directly to the endothelium-denuded artery. On the other hand, the medium which had been incubated with the artery with endothelium, caused a contraction of $22 \pm 8\%$ ($n = 7$, $P < 0.05$), which corresponded to 1.3×10^{-6} M of directly applied $PGF_{2\alpha}$.

Conversion of angiotensin I to angiotensin II

Cumulative application of AI or AII induced a contraction of the pulmonary artery isolated from control rats. The contractile actions of AI and AII were approximately equal (Figure 5b). Pretreatment with captopril (2×10^{-4} M) suppressed the contraction due to AI (1×10^{-7} M) so that the contraction corresponded to the response induced by 1.5×10^{-9} M AII, whereas captopril did not affect the contraction due to AII. Thus it is clear that AI was converted to AII to induce a contraction in isolated pulmonary artery rings. Monocrotaline-treatment did not substantially affect the responses to AI and AII in pulmonary arteries at 3 or 4 weeks post-treatment (Figure 5a), although at 5 weeks post-treatment the response to AI was slightly depressed. Figure 5c shows the responses to AI and AII in pulmonary artery from control rats of which endothelium was removed by rubbing. Removal of endothelium did not modify the contractions due to AI and AII (Figure 5b and c).

The above results suggest that the conversion of AI to AII does not require the existence of the endothelium. In order to test whether this was specific for rat pulmonary artery, we applied AI (1×10^{-7} M) to various arterial strips with or without endothelium. In rat intra-pulmonary artery and aorta, guinea pig aorta, bovine intra-pulmonary artery, and canine intra-pulmonary artery the action of AI in inducing a contraction was comparable to that of AII even after the removal of endothelium (data not shown).

Discussion

The present results on isolated pulmonary artery rings are fully consistent with the results of experiments *in vivo* described in the accompanying paper (Ito *et al.*, 1988), since the response to prostaglandins was enhanced whereas the conversion of AI to AII was virtually unaffected by monocrotaline-treatment in both experiments. Mechanical removal of the endothelium of pulmonary arteries from control rats caused alterations of responses to vasoactive substances which are identical to those in monocrotaline-exposed arteries. These results suggest that the changes in blood pressure responses in intact rats or contractile responses in isolated preparations after monocrotaline-treatment were caused by changes in the pulmonary vascular endothelium. Histologically the tunica media, containing smooth muscle cells, of the pulmonary artery from monocrotaline-treated rats showed considerable lesions after monocrotaline-treatment. However, the contractile responses to high potassium, NA and AII of endothelium-injured (monocrotaline-treated or endothelium-denuded) arteries were not different from those of control artery. This means that the contractile function or the receptor system of smooth muscle cells was not impaired.

ACh-induced relaxation of precontracted vascular preparation is considered to be a functional index of the endothelium (Furchgott, 1984). In this study the response to ACh of pulmonary arteries precontracted by NA was significantly reduced after the injection of monocrotaline to a degree comparable to that of the artery with the endothelium removed mechanically. Reduction of ACh-induced relaxation developed progressively as a function of time following an injection of monocrotaline. The data indicate that the pulmonary endothelium was functionally impaired although a number of cells still survived after monocrotaline-treatment. Monocrotaline-induced lung injury is accompanied by a rise in pulmonary artery resistance (Meyrick *et al.*, 1980; Ghodsi & Will, 1981; Hilliker *et al.*, 1982; Kay *et al.*, 1982). If pulmonary diseases injure the endothelium which is capable of producing EDRF or prostacyclin, either spontaneously or in response to vasoactive substances, they would elevate the pulmonary artery resistance. Thus, the decreased release of EDRF following monocrotaline-treatment may explain at least one of the causes of the pulmonary hypertension well-known in this model.

The response to $PGF_{2\alpha}$ was significantly greater in the pulmonary artery affected by monocrotaline and also in control artery with the endothelium removed mechanically when compared to the pulmonary artery with the endothelium intact. It is unlikely that $PGF_{2\alpha}$ stimulates the release of EDRF or prostacyclin from the endothelium of the pulmonary artery in our experiments. This is supported by the observations that the relaxation due to PGE_1 and PGI_2 of canine pulmonary artery or the contraction due to

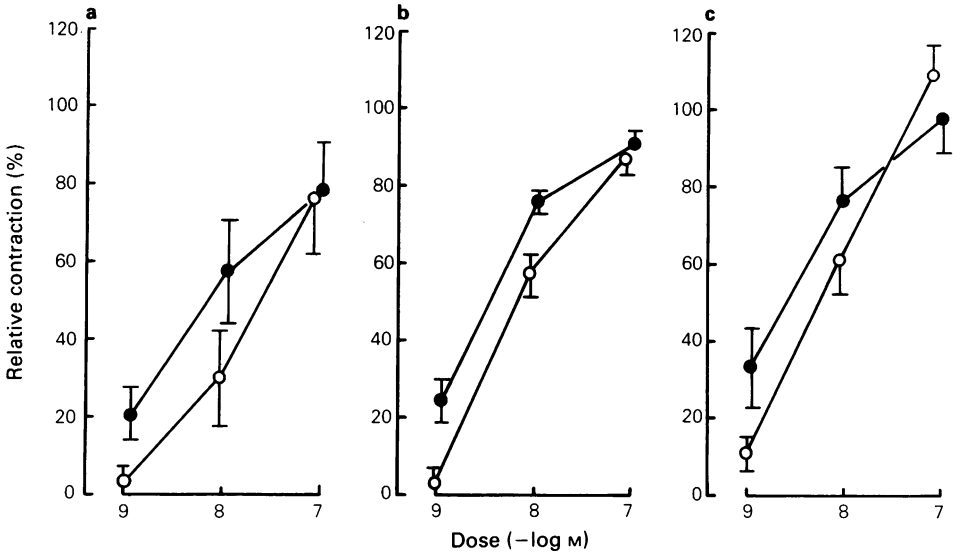

Figure 5 Dose-response relationship for angiotensin I (AI)- and AII-induced contractions of pulmonary artery rings: (○) response to AI; (●) response to AII. 100% on the ordinate scale represents the maximum contraction induced by 60 mM KCl. (a) Rings from rats at 4 weeks after an injection of monocrotaline; (b) rings from control rats, in which endothelium was preserved intact; (c) rings from control rats, from which endothelium was removed by rubbing. $n = 6$ in each panel.

$PGF_{2\alpha}$ of canine carotid artery was not affected by the removal of the endothelium (Chand & Altura, 1981; D'Orleans-Juste *et al.*, 1985). Another possible cause for the enhanced response to prostaglandins of endothelium-injured artery is removal of influence of basal (spontaneous) EDRF-release (Griffith *et al.*, 1984a, b; Martin *et al.*, 1986). However, if spontaneously released EDRF exerts a considerable relaxing action, it should alter the contractile responses to agonists such as high potassium or NA (Griffith *et al.*, 1984b; Martin *et al.*, 1986). Since there was no difference between the responses to 60 mM KCl or NA in endothelium-intact arteries and endothelium-damaged arteries, it is unlikely that the inhibition of spontaneous release of EDRF enhanced the contraction due to $PGF_{2\alpha}$ in arteries with endothelium injured.

Although it is generally accepted that a lung can inactivate PGE_2 or $PGF_{2\alpha}$ (Bakhle & Vane, 1974; Said, 1982), it is still not clear which cells in the lung are responsible for the inactivation. Ody *et al.* (1979) and Ali *et al.* (1980) reported that cultured endothelial cells of pig pulmonary artery and bovine aorta did not degrade $PGF_{2\alpha}$. In our experiments, incubation of $PGF_{2\alpha}$ with bovine pulmonary artery with intact endothelium decreased the activity of the substance to induce a contraction of another strip. This suggests that $PGF_{2\alpha}$ was degraded in the endothelium of the artery *in vitro* to a detectable degree. On the other hand, $PGF_{2\alpha}$-induced contraction of rat aortae was not affected by the removal of endothelium. Therefore, it is possible that endothelial cells can inactivate prostaglandins in certain vascular beds.

As for the sensitivity to AII of the monocrotaline-injured vascular bed, Hilliker & Roth (1985) and Gillespie *et al.* (1986) reported that the vascular responses to AII in isolated perfused lungs were enhanced for the first 1 or 2 weeks after administration of monocrotaline or monocrotaline pyrrole. Similarly, the latter group (Altiere *et al.*, 1986) found that contractions of isolated main pulmonary artery induced by AII, KCl and NA were enhanced at 4 days after monocrotaline treatment, and that at 14 days the contractile responses of segments between main pulmonary artery and intra-pulmonary artery to the agonists were eventually depressed. We do not know whether such an enhancement occurred at the initial stage in our experiments since we did not check the response before 3 weeks. We have never observed any depression of the response to AII, KCl or NA at the later times as observed by Altiere *et al.* (1986). This inconsistency may be due to the difference in severity of damage to vascular smooth muscle cells because Altiere *et al.* (1986) gave a larger dose of monocrotaline ($105\,mg\,kg^{-1}$) to Sprague-Dawley rats. In a preliminary experiment we found that $105\,mg\,kg^{-1}$ monocrotaline was lethal to Wistar

rats within 3 weeks. A slightly lower dose ($90\,mg\,kg^{-1}$) may be appropriate to produce endothelial injury without a significant alteration in contractility of vascular smooth muscle cells.

AI induced a captopril-sensitive contraction in isolated pulmonary artery. This suggests that AI was effectively converted to AII by converting enzyme present in the tissue. Severe endothelial damage due to monocrotaline or rubbing did not reduce the response to AI, suggesting that AI added was converted to AII in a way similar to control arteries. It is unlikely that the conversion was not due to the enzyme present in the surviving cells because we made sure by scanning electron microscopy that the removal was complete. Therefore we must consider the possibility that AI was converted to AII in sites other than the endothelium in endothelium-injured artery. The conversion of AI in the other site(s) is probably not specific for rat pulmonary artery but rather prevalent for arteries in general because all strips tested showed endothelium-independent contractions in response to AI. This is against the established notion that converting enzyme is localized in the endothelium of arteries and capillaries (Caldwell *et al.*, 1976; Ryan *et al.*, 1976). Supporting our view, however, Velletri & Bean (1982) found that converting enzyme is present not only in the intima but also in the adventitia and the media of rat aorta. Probably the converting enzyme in the media or adventitial layers is enough to convert AI to AII at concentrations used in this study.

The present results suggest that in some lung vascular diseases the function of pulmonary endothelium is impaired and as a result the injury may cause secondary effects on pulmonary and systemic haemodynamics through reduced EDRF and increased concentration of eicosanoids. Decreased production of EDRF in the pulmonary vascular bed may be at least partly involved in the development of pulmonary hypertension. The systemic influence of decreased metabolism of eicosanoids must be clarified in future studies.

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