Bleomycin-induced lung injury in rats selectively abolishes hypoxic pulmonary vasoconstriction: evidence against a role for platelet-activating factor

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1. The role of platelet-activating factor in the attenuated hypoxic pulmonary vasoconstriction associated with lung injury was evaluated using specific platelet-activating factor antagonists and an isolated perfused lung preparation.

2. Intratracheal bleomycin was administered to rats to produce acute lung injury. Animals received intratracheal saline (control), intratracheal bleomycin or the platelet-activating factor antagonists BN 52021, WEB 2170 or WEB 2086 before and after bleomycin treatment. Forty-eight hours after intratracheal administration of bleomycin or saline the animals were killed.

3. The increases in pulmonary artery pressure during two periods of hypoxic ventilation and in response to 0.2 μg of angiotensin II were measured. Acetylcholine-induced vasodilatation after pre-constriction with prostaglandin F2α was also measured. To quantify lung injury, the wet/dry ratio of lung weight was determined.

4. Bleomycin treatment attenuated the first and second hypoxic pressor responses by 93% and 77%, respectively, but not the pressor response to angiotensin II nor the vasodilator response to acetylcholine. BN 52021 plus bleomycin augmented the first hypoxic pressor response compared with bleomycin treatment alone, but the structurally unrelated platelet-activating factor antagonists WEB 2170 and WEB 2086 had no significant effect on the bleomycin-induced attenuation of hypoxic pulmonary vasoconstriction. None of the platelet-activating factor antagonists blocked the increase in the wet/dry lung weight ratio induced by bleomycin.

5. Bleomycin-induced lung injury selectively attenuates hypoxic pulmonary vasoconstriction, an effect that does not appear to be mediated by platelet-activating factor. The mechanism remains to be elucidated, but may involve destruction of the hypoxic ‘sensor’ within the respiratory tract.

INTRODUCTION

The adult respiratory distress syndrome (ARDS) is characterized by pulmonary oedema due to increased vascular permeability [1]. The resultant hypoxaemia is exacerbated by attenuated hypoxic pulmonary vasoconstriction (HPV) which leads to mismatching of ventilation and perfusion [2], although the mechanism underlying this pulmonary vascular hyporeactivity is unknown.

Bleomycin, a polypeptide antibiotic, is a chemotherapeutic agent used extensively in the treatment of testicular tumours and lymphoma [3]. Unfortunately, use of the drug is limited by pulmonary toxicity, characterized in its extreme form by a pneumonitis clinically similar to that of ARDS [4-6]. Bleomycin administered intratracheally to rats is used as a model of acute lung injury with histological changes in the lungs indistinguishable from those of ARDS [7, 8] an effect that we have suggested previously may be mediated partially by the membrane phospholipid platelet-activating factor (PAF, acetyl glyceryl ether phosphorylcholine) [9].

PAF has been shown to have both pulmonary vasoconstrictor and vasodilator properties in a number of animal studies [10-12] and has been proposed as an endogenous vasodilator [13]. Additionally, it has been demonstrated that in a model of acute lung injury after injection of endotoxin, PAF is released and, by acting as a pulmonary vasodilator, attenuates HPV [14]. It is now possible, using selective PAF antagonists, to evaluate the role of PAF in various models of lung injury. New antagonists, such as WEB 2086, BN 52021 and the newly discovered WEB 2170, are potent and specific PAF receptor antagonists without intrinsic agonist activity [15-17]. Secondly, it has been proposed that cyclo-oxygenase products of arachidonic acid metabolism, such as the vasodilator prostacyclin, may be released as a result of acute lung injury and may attenuate HPV [18].

Key words: hypoxic pulmonary vasoconstriction, platelet-activating factor, pulmonary vascular reactivity.

Abbreviations: ARDS, adult respiratory distress syndrome; HPV, hypoxic pulmonary vasoconstriction; IPL, isolated blood-perfused lung; PAF, platelet-activating factor; \( Ppa \), pulmonary artery pressure.

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The aims of this study were therefore twofold: first, to evaluate HPV and pulmonary vascular reactivity in a model of acute lung injury caused by intratracheal bleomycin, and secondly, to investigate the possible role of PAF and cyclo-oxygenase products in this altered vasoreactivity.

**METHODS**

**Animals**

Male Wistar rats weighing 280–320 g were used throughout. They were anaesthetized with diazepam (0.6 mg/kg) intraperitoneally and Hypnorm (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml) intramuscularly. Intravenous catheters were placed into the internal jugular vein and were secured in place. The catheter was then tunneled to the back of the animal, exteriorized between the scapula and sutured into place. The catheter was fitted with an injection port allowing repeated injections. Animals were then allowed to recover for 48 h before any further procedure. Throughout the experiment all animals were allowed free access to food and water and were kept in room air.

**Treatment protocols**

Animals were randomly assigned to one of six treatment protocols all carried out over a 48 h period before they were killed, after which the lungs were removed for use in the isolated blood-perfused lung (IPL) preparation described below. The treatment protocols for the different groups were as follows:

**Group 1.** Animals \((n=12)\) were anaesthetized (diazepam and Hypnorm) and after surgically exposing the trachea, 0.3 ml of sterile saline (150 mmol/l NaCl) was administered (using a 25-gauge needle) intratracheally 48 h before the lungs were isolated from the IPL experiment.

**Group 2.** Animals \((n=14)\) were anaesthetized and 1.5 mg of bleomycin (dissolved in saline, 5 mg/ml) was administered by direct instillation into the trachea as previously described to cause acute lung injury [19]. Forty-eight hours later the lungs were removed for the IPL experiment.

**Group 3.** Animals \((n=8)\) were treated with BN 52021 (20 mg/kg intravenously) 12 h and 1 h before and q.12 h for 48 h after bleomycin (1.5 mg) was administered intratracheally as in group 2.

**Group 4.** WEB 2170 (dissolved in saline) \((n=6)\) was administered in doses of 10 mg/kg q.a.m. and 30 mg/kg q.p.m. by gavage. This treatment was given 12 h and 1 h before and q.12 h for 48 h after bleomycin (1.5 mg) was administered intratracheally as in group 2.

**Group 5.** Animals \((n=5)\) were given WEB 2086 (dissolved in a stock solution of 1 mg/ml of 10% ethanol in saline), 100 µg/kg intravenously, 12 h and 1 h before and q.12 h for 48 h after bleomycin (1.5 mg) was administered intratracheally as in group 2.

**Group 6.** Animals \((n=8)\) were given bleomycin intratracheally as in group 2 and left for 48 h. One hour before having their lungs isolated they were treated with meclofenamate (2 mg/kg intravenously).

**IPL experiments**

**Experimental preparation.** After anaesthesia with diazepam and Hypnorm, a donor rat was exsanguinated and the blood was heparinized and placed into a reservoir, maintained at 40°C by a surrounding water bath, and used to 'prime' the perfusion circuit. The perfusion circuit has been described previously [20] and consisted, in order, of a left atrial cannula, reservoir, roller pump (Watson Marlow MHRE 200, Falmouth, Cornwall, U.K.), connecting tubing, bubble trap, side arm pressure transducer (Bio Medical Systems Ltd, East Kilbride, Strathclyde, U.K.) and a pulmonary artery cannula. The pressure transducer was connected to a recorder (Multi-trace 2; Ormed, Welwyn Garden City, Herts., U.K.) to permit constant monitoring. The total blood volume of the circuit including the reservoir was 15–20 ml.

The experimental animal was anaesthetized and ventilated at a tidal volume of 4 ml and frequency of 15 breaths/min via a tracheostomy. The lungs were exposed through a median sternotomy and the animal was heparinized and exsanguinated via the abdominal aorta. A purse string suture was placed around the left atrium to secure a catheter inserted through an atriotomy, which was allowed to drain freely into a blood reservoir thereby ensuring ‘a left atrial pressure’ of zero. A cannula was inserted into the main pulmonary artery via a right ventriculotomy and was tied securely in place. A constant flow of 18 ml/min resulted in a mean initial pulmonary artery pressure \((P_{pa})\) of 18±0.3 \((n=58)\). For group 6 animals only (pretreated with meclofenamate), meclofenamate \((10 \mu g/ml)\) was added to the perfusing blood. Blood gases were monitored during each experiment by collecting blood anaerobically from the left atrial line and analysing pH using a Corning blood gas analyser (178 pH/blood gas analyser; Corning, Halstead, Essex, U.K.). Deviations in pH from normal \((7.35–7.45)\) were corrected with small volumes of NaHCO\(_3\) (1.0 mol/l) added to the reservoir.

**HPV.** The lungs were ventilated initially with a gas mixture of composition 21% O\(_2\), 5% CO\(_2\), and 74% N\(_2\) (normoxia). Pilot experiments revealed that the pressor response to hypoxia was slower in onset than normal in bleomycin-damaged lungs and therefore the period of hypoxic ventilation was extended to accommodate this. After the baseline \(P_{pa}\) was stable, the inspired gas mixture was changed to 3% O\(_2\), 5% CO\(_2\), and 92% N\(_2\) (hypoxia) for 24 min. The ventilating gas was then changed back to normoxia and the pressure was allowed to stabilize. After a stable baseline had been attained the preparation was again ventilated with hypoxic gas for 28 min before being returned to normoxic ventilation. The rise in \(P_{pa}\) during hypoxic ventilation was determined as the difference between the peak \(P_{pa}\) during hypoxic ventilation and the \(P_{pa}\) during the subsequent room air
Hypoxic pulmonary vasoconstriction and bleomycin-induced lung injury

Fig. 1. Trace demonstrating the pressor response to hypoxic ventilation in lungs from bleomycin-treated (A) and control (B) animals. The single solid arrow denotes time of start of hypoxic ventilation (fractional concentration of inspired oxygen = 0.08) and the open arrow indicates the time of resumption of room air ventilation. The double arrow indicates the time of administration of angiotensin II (0.2 µg).

Wet/dry ratios. The total perfusion time in each experiment was kept constant at 90 min, after which the heart and lungs were removed from the thorax of the animal. The right and left lungs were isolated and placed into plastic dishes and weighed. Additionally, a small (3–5 ml) aliquot of blood was collected from the circulating reservoir and weighed. The dishes were placed in a 70°C oven and were weighed daily until they attained a constant weight. The wet/dry ratio of the lungs were then calculated using the regression equation incorporating the wet/dry ratio of blood as described by Collins et al.[21].

Chemicals

The following chemicals were used: acetylcholine, angiotensin II, (Sigma, Poole, Dorset, U.K.), prostaglandin F₂α (Upjohn, Crawley, Sussex, U.K.), WEB 2086, WEB 2170 (gifts from Boehringer Ingelheim, Bracknell, Bucks., U.K.), BN 52021 (a gift from Dr P. Braquet, Paris), meclofenamate (a gift from Parke-Davis, Hounslow, Middx., U.K.) and bleomycin (Bristol Laboratories, Uxbridge, Middx., U.K.).

Statistics

Pressor responses to hypoxia and angiotensin II, acetylcholine-induced relaxation responses, and wet/dry ratios were compared between the groups by using one-way analysis of variance, and post hoc analysis utilized the Fisher protected least significant difference test [22]. Results are expressed as means±SEM, and a P value of <0.05 was considered significant.

RESULTS

Preparation stability

Baseline Ppa was not significantly different in any of the groups. For groups 1–6 respectively the baseline Ppa was 16±1, 17±1, 18±1, 19±1, 19±1 and 20±1 mmHg. In control (group 1) animals the baseline (room air) Ppa increased by 3.8±0.5 mmHg after two periods of hypoxic ventilation (more than 60 min later). Similarly, in the bleomycin-treated (group 2) animals the initial baseline Ppa had increased 5.9±1.4 mmHg (not significantly different from control after two periods of hypoxic ventilation).

HPV

Fig. 1 demonstrates the slower onset of the hypoxic pulmonary pressor response in bleomycin-treated animals. In the control (group 1) animals HPV resulted in a reproducible pressor response to hypoxic ventilation, the increase in Ppa during the first period of hypoxia being 14±2 mmHg and during the second 13±2 mmHg (Fig. 2). In the bleomycin-treated (group 2) animals HPV was markedly attenuated, such that during the two periods of hypoxia the increases in Ppa were 2±1 and 3±1 mmHg (P<0.05 compared with control). Fig. 2 shows that the PAF antagonists WEB 2086 and WEB 2170 had no effect on the attenuation of HPV compared with bleomycin alone. In group 3 animals (treated with BN 52021) the first hypoxic response was significantly greater than the first response in animals treated with
bleomycin alone (group 2) but the second hypoxic pressor response was not.

In animals treated with meclofenamate and bleomycin (group 6) the initial hypoxic pressor response was greater than the same response in animals treated with bleomycin alone (group 2) ($P<0.05$). The second hypoxic pressor response in the meclofenamate-treated group was not significantly different from group 2 animals (Fig. 2). For the second hypoxic episode, $^{**}$ denotes a significant difference from the control pressor response. Values are means $\pm$ SEM.

Response to angiotensin II

In Fig. 3 the pressor response to the bolus injection of angiotensin II (0.2 $\mu$g) at the end of the protocol is shown. Bleomycin treatment (group 2) did not attenuate this response compared with controls (group 1).

Acetylcholine-induced relaxation

Fig. 4 shows the effect of 1 $\mu$mol/l and 0.1 mmol/l acetylcholine on $P_{pa}$ after pre-contraction of the pulmonary vasculature with prostaglandin $F_{2\alpha}$ (30 $\mu$g). Administration of prostaglandin $F_{2\alpha}$ resulted in a contraction of 10 $\pm$ 1 and 7 $\pm$ 2 mmHg in bleomycin and control (intratracheal saline-treated) animals, respectively. In the control animals 1 $\mu$mol/l acetylcholine caused a 39 $\pm$ 8% relaxation and 0.1 mmol/l acetylcholine caused an 85 $\pm$ 8% relaxation. Similarly, in animals treated with intratracheal bleomycin the respective relaxations caused by acetylcholine were 47 $\pm$ 3% and 83 $\pm$ 10% (not significant compared with control).

Wet/dry ratios

To quantify the degree of pulmonary oedema and vascular leak, wet/dry ratios of lung weight were calculated. Fig. 5 shows that the wet/dry ratio in the control (group 1) animals (5.1 $\pm$ 0.1) was significantly less than the same ratio measured in the animals that received bleomycin alone (group 2, 6.8 $\pm$ 0.4). Additionally, treatment with WEB 2086, WEB 2170, BN 52021 or meclofenamate had no effect on the increase in the wet/dry ratio caused by bleomycin.

DISCUSSION

This study confirms that intratracheal bleomycin in the rat induces an acute lung injury associated with a marked attenuation in HPV. The latter is not due to general pulmonary vascular hyporeactivity as the pressor response to angiotensin II is preserved. Furthermore, this selective vascular hyporeactivity is not mediated by PAF, although cyclo-oxygenase products may play a minor role.

Attenuated or absent HPV has been noted in other models of lung injury or inflammation, specifically those of pulmonary oxygen toxicity [18], endotoxaemia [14, 23] and pneumonia due to Pseudomonas [24]. This phenomenon has significant clinical implications as HPV is important in the matching of pulmonary perfusion with ventilation. Attenuated HPV may therefore lead to perfusion of underventilated or damaged areas of lung, resulting in increased ventilation/perfusion mismatch and shunting [25, 26]. In addition, HPV may lessen the severity of non-hydrostatic pulmonary oedema after lung injury by reducing blood flow to areas of lung with increased capillary permeability [27].

Not all models of acute lung injury are associated with similar changes in pulmonary vascular reactivity. Thus, increased vascular responses to angiotensin II and hypoxia have been reported in rats treated with intraperitoneal $\alpha$-naphthylthioureia [28]. Why such differences occur remains unclear, although the liberation of varying vasoactive mediators in different models is possible.

To ensure PAF receptor blockade in the current study we administered somewhat larger doses of PAF antagonists than those used in previous studies. Thus WEB 2086 at 10 $\mu$g/kg intravenously [29], WEB 2170 at 1 mg/kg orally [15] and BN 52021 at 4 mg/kg subeutaneously have all been shown to be effective at blocking the effects of exogenously administered PAF.

It has been proposed that PAF may have a role in the pathogenesis of bleomycin-induced acute lung injury [9].
We questioned if increased PAF release might also account for the diminution of HPV seen in this model, as it is a potent vasodilator at low concentrations [12] and may act as an endogenous vasodilator in the normal [13] and diseased [14] pulmonary circulation. Previous studies investigating this problem have used the PAF receptor antagonists CV 3988 and SRI 63441 [14]. Unfortunately, both of these antagonists are structural analogues of PAF and may therefore act as partial agonists. However, in the current study, although the PAF antagonist BN 52021 slightly, but significantly, restored the first hypoxic pressor response in bleomycin-treated animals, subsequent experiments using the structurally unrelated PAF antagonists WEB 2086 and WEB 2170 had no such effect, suggesting that PAF is not important in this phenomenon.

In the current study meclofenamate also partially restored HPV in bleomycin-treated animals. This might suggest, as proposed previously [30], that vasodilator prostaglandins are released as a result of lung injury and in turn attenuate HPV. However, meclofenamate has been shown to augment HPV in normal lungs from a variety of species [31] and can itself induce non-flow-dependent pulmonary vasoconstriction [32]. The augmentation of HPV with meclofenamate may therefore not be specific to the setting of acute lung injury.

To confirm that the degree of lung injury was equivalent in animals treated with PAF antagonists and those not treated, wet/dry ratios of lung weight were measured. This confirmed that the degree of lung injury was not different in groups 2–5. This suggests that bleomycin-induced lung injury per se is not mediated through PAF or cyclo-oxygenase products.

The extent to which HPV is endothelially dependent in vivo is difficult to confirm or refute experimentally, as destruction of endothelial cells normally leads to the development of high permeability pulmonary oedema. Nevertheless, there is increasing evidence to suggest that HPV is modulated by the release of endothelially dependent relaxant factors [33]. However, in our study the endothelium-dependent vasodilatation in response to acetylcholine in vivo was prepared, a finding also reported after α-naphthylthiourea-induced lung injury [34]. It may be that in both models sufficient numbers of endothelial cells remain intact to respond to acetylcholine as studies in vitro suggest that comparatively few cells are necessary to preserve the vasodilator response [35].

Why selective attenuation of the pressor response to hypoxia should occur after bleomycin is unclear, although either the sensor or effector mechanisms of HPV may in some way be impaired. Damage to the latter seems unlikely in that normal pulmonary pressor responses to angiotensin II were demonstrated in vivo. Consequently, it seems more likely that the 'hypoxic sensor' may have been damaged by bleomycin, although the mechanism by which this occurs remains unknown. Although there are many hypotheses, many investigators feel that HPV is thought to be the result of stimulation of an unknown cell to liberate a mediator that in turn leads to pulmonary vascular smooth muscle constriction [36]. Intratracheal bleomycin might well cause direct damage to specific hypoxic "sensor" cell(s).

In summary, we have demonstrated that in bleomycin-induced acute lung injury there is a selective attenuation of HPV which is not mediated via the release of vasoactive mediators such as PAF. In addition, there is a retention of normal pressor responses to other stimuli. It seems more likely that the hypoxic sensor mechanism may be impaired in this model and further investigation into the mechanism underlying this phenomenon may lead to a better understanding of the mechanism of HPV, with important clinical implications for the management of patients with hypoxaemia secondary to acute lung injury.

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REFERENCES