Oxygen-induced lung injury in the pre-term guinea pig: the role of Leukotriene B₄

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Leukotriene B₄ (LTB₄) has been reported to promote the formation of lung oedema when infused into the pulmonary circulation of adult animals. The present study evaluated the hypothesis that LTB₄ was responsible, in part, for the oedema that develops during oxidative injury of the immature lung. Significant increases were found in LTB₄ concentration in bronchoalveolar lavage fluid obtained from pre-term guinea pig pups maintained in 95% oxygen for 48 h (P<0.05) and 72 h (P<0.05) compared to pups maintained in 21% oxygen. Cellular analysis of lavage fluid revealed a concurrent influx of neutrophils into the hyperoxic-injured lung at these times. The protein concentration of lavage fluid was also increased by 48-h hyperoxia exposure indicating elevated pulmonary microvascular permeability.

In a second series of experiments, pups exposed to 95% oxygen (and 21% oxygen controls) were treated with a specific LTB₄ antagonist (U-75302) at either 0.5, 1.5 or 3.0 mg 100 g body wt to ascertain if LTB₄ played a role in either neutrophil recruitment or oedema formation in the immature lung. The number of neutrophils recovered in bronchoalveolar lavage fluid was significantly reduced, compared to vehicle-treated pups, in pups treated with U-75302, at both 1.5 and 3.0 mg/100 g body wt but not 0.5 mg/100 g body wt. Histopathological analysis of pups treated with 1.5 mg U-75302/100 g body wt revealed fewer neutrophils in the pulmonary interstitium (198 vs. 218 mm⁻², P<0.05). The extent of lung microvascular permeability, elevated by hyperoxic exposure, was modulated by increasing concentrations of U-75302. Specifically, treatment with 0.5, 1.5 and 3.0 mg U-75302/100 g body wt reduced microvascular permeability by 17, 67 and 98%, respectively. In conclusion, LTB₄ plays an important role in oedema formation in acute oxidative injury of the immature lung and this is mediated, in part, through neutrophils.

Introduction

With improved survival of extremely premature infants, respiratory disorders in pre-term babies have become one of the major problems facing neonatologists (1). Clinically, two of the main features of both acute and chronic respiratory disease in infants are pulmonary inflammation (2) and oedema (3), and both correlate with the known action of Leukotriene B₄ (LTB₄) (5S, 12R-dihydroxy-6, 14-cis-8, 10-trans-eicosatetraenoic acid), a potent metabolite of arachidonate (4,5). Indeed, increased concentrations of this eicosanoid have been observed in bronchoalveolar lavage (BAL) fluid obtained from infants with chronic lung disease (CLD) (6). As the pathogenesis of both respiratory distress syndrome (RDS) and CLD have proved complex and difficult to manage, the control of pulmonary inflammation and oedema may represent useful therapeutic approaches.

It has previously been established that the pulmonary sequelae in the pre-term guinea pig following exposure to oxygen are similar to those seen in the human infant with RDS (7) and CLD (8). The main findings are pulmonary inflammation, particularly neutrophil infiltration of the lung, and increased microvascular permeability. Histological examination of the lungs of pre-term animals with respiratory distress has confirmed the presence of acute lung injury and hyaline membranes (7).

As part of ongoing studies of respiratory disorders of pre-term infants, this paper reports an investigation of the role of LTB₄ in acute oxidative lung injury of the pre-term guinea pig. Specifically, it was examined whether exposure to elevated concentrations of oxygen leads to an accumulation of LTB₄ in the alveoli of pre-term animals. Having established that
this was indeed the case, a specific LTB₄ antagonist (U-75302) was employed to determine whether interruption of the LTB₄ signalling pathway influenced either neutrophil accumulation or oedema formation in the immature lung.

Materials and Methods

All chemicals were obtained from Sigma Chemicals Co. (Poole, Dorset, U.K.) unless otherwise stated.

EXPERIMENTAL PROTOCOL

The pre-term guinea pig model has been described in detail previously (7). Briefly, guinea pig pups were delivered by caesarean section after 65 days gestation (full term is 68 days) and were immediately revived and dried gently in a stream of warm air. Up to five pre-term pups and a lactating surrogate female were housed in 25-l sealed Perspex cages supplied with dry gas mixtures. Pre-term pups were randomly allocated to receive either 21% or 95% oxygen for up to 72 h. In the first study, animals (6-8 pups group⁻¹) were anaesthetized after 24, 48 or 72 h and a BAL carried out as described below. Inflammatory cell influx, microvascular permeability and LTB₄ concentration were determined by examining the lavage fluid. In a second study, 3-day-old pre-term pups exposed to either 21% or 95% oxygen were randomly allocated to receive the LTB₄ antagonist (U-75302) or vehicle (5% ethanol; 1% Tween 80) at a dose of either 0.5, 1.5 or 3.0 mg/100 g body wt every 12 h over a 72-h period. At the end of this time, the animals were anaesthetized and BAL was performed (5-6 pups group⁻¹). In addition, in a further series of experiments in which pups received 1.5 mg U-75302/100 g body wt for 72 h, the lungs of the animals were perfused with fixative for histochemical analysis (2-3 pups group⁻¹).

BRONCHOALVEOLAR LAVAGE

Guinea pig pups were anaesthetized by intraperitoneal injection of pentobarbital (50 mg kg⁻¹). After exposure of the trachea, a tracheotomy was performed and a 14-gauge cannula inserted and secured. The animals were then exsanguinated by abdominal aortic section and the pulmonary circulation perfused with 5 ml of saline at 37°C. The lungs were lavaged in situ with five 2-ml aliquots of sterile saline (37°C). The lavage cells were then placed by centrifugation at 200 g for 10 min at 4°C. Supernatant (0.5 ml) was frozen at −70°C for subsequent analysis of total protein as an index of microvascular permeability, while the remainder of the lavage fluid was used for the determination of the concentration of LTB₄.

TOTAL AND DIFFERENTIAL LEUKOCYTE COUNTS

A total nucleated cell count in the BAL was performed using a Neubauer haemocytometer. Cytoplasm preparations were stained with May–Grunwald–Giemsa and differential cell counts performed on 300 cells. Results are expressed as the total number of cells per ml of lavage fluid recovered from each animal. No correction was made for the volume of lavage fluid recovered as this was uniformly 85–90% of the original volume used. Total and differential leukocyte counts were performed on blood taken by cardiac puncture prior to exsanguination.

ASSESSMENT OF MICROVASCULAR PERMEABILITY

Changes in vascular permeability were quantified by determining the protein content of the lavage fluid since it has previously been shown that approximately 50% of the total protein recovered in BAL from oxygen-exposed guinea pigs is plasma albumin (7). Total protein was measured in unconcentrated lavage fluid using the bicinchoninic acid assay (9). Absorbance was measured at 562 nm with a Dynatech MR 580 reader using bovine serum albumin standard. Results are expressed as mg of protein per ml of lavage fluid recovered.

EXTRACTION OF LTB₄

Leukotrienes were extracted from BAL fluid and purified as described by Taniguchi et al. (10). Bronchoalveolar lavage samples were immediately combined with two volumes of ethanol and kept on ice for up to 2 h. Water was then added to give a final concentration of 10% ethanol. The mixture was centrifuged at 450 g for 10 min. The acidified supernatant (pH 3-0) was then passed through an octadecylsilyl (ODS) silica cartridge, which was subsequently washed with 10% aqueous ethanol (15 ml), water (15 ml), petroleum ether (15 ml) and methylformate (10 ml). The final fraction was dried under nitrogen and the residue dissolved in 0.5 ml of radioimmunoassay (RIA) buffer (50 mM TRIS at pH 8.6 containing 0.1% gelatin). With this approach, recovery of added [³H]LTB₄ was greater than 90%.

ASSAY OF LTB₄

Leukotriene B₄ concentration in BAL fluid was measured in duplicate by radioimmunoassay (RIA) with antiserum selective for LTB₄. Aliquots (0.1 ml) of extracted BAL samples were placed in disposable glass tubes and [³H]LTB₄ in RIA buffer (0.1 ml
containing approximately 4000 dpm) was added to give a total incubation volume of 0.4 ml. The mixture was incubated at 4°C for 18 h. Free LTB₄ was adsorbed onto dextran-coated charcoal and after centrifugation at 2000 g for 15 min, the supernatant containing the antibody-bound LTB₄ was decanted into scintillation vials. Following the addition of scintillant, radioactivity was determined in a scintillation counter. Cross-reactivity of the LTB₄ antibody with 15-hydroxyeicosatetraenoic acid (HETE), di-HETE and thromboxane B₂ was less than 1%. Coefficient of variation was 8.9%.

HISTOPATHOLOGICAL ANALYSIS

The pulmonary histological sequelae of exposure to 95% O₂ in the presence or absence of U-75302 was assessed at 72 h. Animals were anaesthetized, the trachea cannulated and the pulmonary circulation flushed as described above. The lungs were inflated with air at a constant pressure of 10 cm H₂O and were simultaneously perfused via the right ventricle for 60 min with half-strength Karnovsky’s fixative. The heart–lung preparation was then dissected from the thorax and immersed in full-strength Karnovsky’s fixative (2.5% glutaraldehyde and 2.5% paraformaldehyde) for 24–48 h.

For light microscopy, 4 µm sections were stained with haematoxylin, and eosin and with Martius Scarlet Blue. The percentage airspace was determined by point counting from the section stained with Martius Scarlet Blue using an eyepiece graticule providing a grid with 122 points. A minimum of 600 points (five–six low-power fields) were examined for each section. Using the 100 x oil-immersion objective, the number of neutrophils in 30 random high-power fields was determined. The total granulocyte count was then adjusted for the percentage airspace to give the number of neutrophils per mm³ of tissue.

STATISTICAL ANALYSIS

The results are presented as means and standard deviations. The significance of differences between survival curves was assessed using the log rank test. The remaining data was analysed using a two-way analysis of variance (ANOVA). The unpaired Student's t-test was used when ANOVA indicated a within-group statistical significance.

Results

No difference was observed in the survival of pre-term pups maintained in 21% oxygen (six of seven pups) or 95% oxygen (seven of eight pups) for a 72-h period. Both fatalities were within 12 h of birth when the pups were in severe respiratory distress. Exposure to 95% oxygen increased the number of circulating inflammatory cells by 72 h. This was due mainly to an increase in the number of neutrophils (9.5 vs. 3.4*10⁵ ml⁻¹, P<0.05). Pulmonary microvascular permeability was significantly increased following 48 h in hyperoxic-exposed pups (P<0.05) as was the number of neutrophils recovered in BAL fluid (P<0.05; Fig. 1). These indices of lung injury were accompanied by a six-fold increase in the concentration of LTB₄ in BAL fluid (Fig. 1). Exposure to hyperoxia for a further 24 h did not result in any further increase in LTB₄ concentration, neutrophil numbers or microvascular permeability.

A second series of experiments was carried out to examine the effect of a LTB₄ antagonist, U-75302. In these experiments, in which three different concentrations of U-75302 were administered, three of 19 animals maintained in 21% oxygen died during...
Table 1 Neutrophil and eosinophil numbers and protein concentration in bronchoalveolar lavage fluid (BALF) from pre-term guinea pig pups exposed to 21% and 95% oxygen in the presence of varying concentrations of U-75302

<table>
<thead>
<tr>
<th>U-75302 (mg/100 g body wt)</th>
<th>Oxygen (%)</th>
<th>Neutrophils (10^6 ml^-1 BALF)</th>
<th>Eosinophils (10^6 ml^-1 BALF)</th>
<th>Protein (mg ml^-1 BALF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle 21</td>
<td>21</td>
<td>1.03 ± 0.61</td>
<td>0.32 ± 0.26</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Vehicle 95</td>
<td>95</td>
<td>6.21 ± 1.84*</td>
<td>0.45 ± 0.31</td>
<td>0.63 ± 0.11*</td>
</tr>
<tr>
<td>0.5 21</td>
<td>0.5 21</td>
<td>0.82 ± 0.58</td>
<td>0.76 ± 0.57</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>0.5 95</td>
<td>0.5 95</td>
<td>4.86 ± 1.78*</td>
<td>0.67 ± 0.45</td>
<td>0.53 ± 0.09*</td>
</tr>
<tr>
<td>1.5 21</td>
<td>1.5 21</td>
<td>1.52 ± 1.17</td>
<td>0.84 ± 0.28</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>1.5 95</td>
<td>1.5 95</td>
<td>1.93 ± 1.87†</td>
<td>0.79 ± 0.37</td>
<td>0.36 ± 0.10†</td>
</tr>
<tr>
<td>3.0 21</td>
<td>3.0 21</td>
<td>1.45 ± 1.56</td>
<td>0.94 ± 0.31†</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>3.0 95</td>
<td>3.0 95</td>
<td>1.85 ± 0.79†</td>
<td>0.88 ± 0.37</td>
<td>0.28 ± 0.12†</td>
</tr>
</tbody>
</table>

*Different from 21% O_2, P<0.005; values given as mean ± sd.
†Different from equivalent vehicle control, P<0.05.

Table 2 Percentage airspace and numbers of neutrophils in lung sections from pre-term guinea pig pups exposed to 21% and 95% oxygen in the presence of U-75302 or vehicle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxygen (%)</th>
<th>Airspace (%)</th>
<th>Neutrophils (No mm^-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>21</td>
<td>42.3 ± 7.6</td>
<td>110 ± 66</td>
</tr>
<tr>
<td>Vehicle</td>
<td>95</td>
<td>35.5 ± 4.7</td>
<td>218 ± 12.7*</td>
</tr>
<tr>
<td>U-75302</td>
<td>21</td>
<td>43.5 ± 3.5</td>
<td>108 ± 8.5</td>
</tr>
<tr>
<td>U-75302</td>
<td>95</td>
<td>37.0 ± 6.0</td>
<td>198 ± 10.9†</td>
</tr>
</tbody>
</table>

*Different from 21% O_2, control or U-75302, P<0.01; values given as mean ± sd.
†Different from 95% O_2, control, P<0.05.

of lung permeability in a concentration-related manner (Table 1). So much so, that pups treated with 3.0 mg U-75302/100 g body wt exhibited little increase in lung permeability at the end of 72 h in 95% oxygen.

Discussion

Neutrophils are present in large numbers in the lungs of infants who receive supplemental oxygen (11). Indeed, infants who subsequently develop CLD have persistently elevated neutrophil counts. Since activated neutrophils can generate oxygen radicals, release an array of proteolytic enzymes and generate arachidonate products (12), it has been proposed that they exacerbate the microvascular permeability and oedema associated with hypoxia-induced lung injury (13-15). To help elucidate the role of these immune cells in respiratory disease of pre-term neonates, an animal model of prematurity using the guinea pig has been developed (7).
Plate 1  (a) Section of lung from pre-term guinea pig given vehicle and exposed to 21% oxygen. The lung shows good expansion and minimal inflammatory infiltrate in the alveolar septa. (Haematoxylin and eosin; magnification × 200). (b) Section of lung from pre-term guinea pig given the LTB₄ antagonist, U-75302, and exposed to 21% oxygen. There is normal expansion and the septa are normal. (Haematoxylin and eosin; magnification × 200). (c) Section of lung from pre-term guinea pig given the LTB₄ antagonist, U-75302, and exposed to 95% oxygen. There is adequate alveolar expansion and a mild inflammatory infiltrate causing a widening of the septa. (Haematoxylin and eosin; magnification × 200). (d) Section of lung from pre-term guinea pig given vehicle and exposed to 95% oxygen. There is variable expansion with atelectasis and a moderate to severe inflammatory infiltrate within the septa. (Haematoxylin and eosin; magnification × 200.)
Maintenance of pre-term guinea pigs in a hyperoxic environment results in increased pulmonary microvascular permeability and oedema. Pulmonary oedema, an early event in this model occurring between 24–48 h, develops due to the disruption of the endothelial cell layer which allows protein-rich fluid to leak at an increased rate from the microcirculation into the pulmonary interstitium (7). Then, as the integrity of the epithelial cell layer is also compromised with Type 1 cell injury and less selective tight junctions, the proteinaceous fluid enters the alveoli and interferes with surfactant function and respiratory gas exchanges (16).

At present it is not clear what role, if any, neutrophil infiltration to the immature lung contributes to the increase in microvascular permeability. If, as suggested by some investigators, neutrophils are responsible for much of the lung injury seen following hyperoxic-exposure (13–15) then downregulating or preventing this immune response may represent a useful therapeutic approach. The goal of this study was to determine if LTB₄, a potent chemoattractant for neutrophils, was involved in their recruitment to the hyperoxia-injured lung and to examine the benefit of blocking the action of LTB₄ with a specific antagonist, U-75302.

The potential importance of LTB₄ as a neutrophil chemoattractant in the immature lung was confirmed in the first series of experiments in which 72 h of hyperoxic exposure resulted in a four-fold increase in microvascular permeability. This response was accompanied by a six-fold increase in the concentration of LTB₄ in BAL fluid, which in turn coincided with an accumulation of neutrophils in the pulmonary parenchyma and alveoli. Increased concentrations of LTB₄ in BAL fluid have been reported for infants with CLD who also have oedematous and inflammatory cell responses (6).

Leukotriene B₄ is a potent metabolite of arachidonate formed by the lipoxygenase pathway (17), and increased concentrations have been reported in several animal models of lung injury (10,18,19). Taniguchi et al. (10) reported elevated levels of LTB₄ in BAL fluid from adult rats exposed to 95% oxygen for 66 h, the increase correlating with the influx of neutrophils to the lung. Indeed, it is now widely accepted that LTB₄ is a potent chemotactic agent for attracting neutrophils to the lung (5,20) and causing their adherence to endothelial surfaces (21). The source of the LTB₄ was not investigated in the present study, although possible sources include damaged endothelial cells, Type 1 epithelial or inflammatory cells (22,23).

The second aim of the study was to determine what role LTB₄ plays in pulmonary inflammation and oedema formation. It has been demonstrated that hyperoxia primes cultured bovine pulmonary artery cells to release more prostaglandin (PGI₂) and LTB₄ when stimulated with calcium ionophores (24). Administration of exogenous LTB₄ to hyperoxia-exposed rats results in increased PGI₂ formation and increased capillary permeability compared to normoxic controls and it has been suggested that hyperoxic exposure primes lung tissue and capillaries to become more susceptible to the effects of LTB₄.

In the late 1980s it was shown that inhibition of leukotriene synthesis with diethylcarbamazine or antagonism of the sulfidopeptide leukotriene receptor with FPL 57231 prevented the acute pulmonary effects of endotoxin in cats (25), and also prevented death from an otherwise lethal dose of endotoxin in mice (18). In the present study, a structural analogue of LTB₄ (U-75302, 6-(6-(3-hydroxy-1E,5Z-undecadien-1-yl)-2-pyridinyl)-1,5-hexanediol) was used which competitively inhibits the binding of [³H]LTB₄ to receptors on human neutrophils (26) and is a selective antagonist of the myotropic activity of LTB₄ on isolated lung parenchymal strips from guinea pig (27). It was speculated that administration of this compound to pre-term guinea pigs, exposed to supplemental oxygen, would reduce or prevent the increase in microvascular permeability and the accumulation of neutrophils in the lung.

Accumulation of neutrophils into alveoli spaces was reduced dramatically after treatment with U-75302 at a dose of 1·5 mg/100 g body wt and this also occurred to a lesser extent in lung parenchyma, suggesting that LTB₄ is acting as a chemotactic factor for neutrophils in the immature lung. These results differ in this respect from those obtained by Richards et al. (28) who used U-75302 in a guinea pig model of antigen-induced bronchopulmonary eosinophilia. These workers found that the neutrophilia associated with their model was not prevented by treatment with U-75302. They did, however, find that the influx of eosinophils to the lung was reduced, something that was not observed in the oxygen-injury, pre-term model. Indeed, an increase in eosinophil influx was recorded in all pups treated with U-75302. These differences may have arisen due to the different route of treatment employed (intraperitoneal vs. oral), age of the guinea pigs used (pre-term vs. adult), or imposed stress (oxygen vs. albumin) utilized in the two studies.

The most important finding of the present study was that U-75302 prevented the increase in pulmonary microvascular permeability normally associated with exposure to an elevated concentra-
activation of neutrophils recruited to the lung and probably not solely responsible for the increased microvascular permeability seen in hyperoxia-exposed pups. However, it is conceivable that interruption of the LTB₄ signalling pathway prevented the activation of neutrophils recruited to the lung and thus may have indirectly prevented the increase in microvascular permeability. Irrespective of the precise mechanism involved, administration of an LTB₄ antagonist resulted in reduced microvascular permeability and oedematous injury of the immature lung.

Acknowledgements

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References