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Hypoxia causes permeability oedema in the constant-pressure perfused rat lung

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ABSTRACT: Alveolar hypoxia causes pulmonary oedema associated with increased lung capillary pressure and decreased alveolar fluid reabsorption. However, the role of altered permeability is unclear. The aim of the present study was to test whether hypoxia affects alveolar permeability and induces pulmonary oedema in rat lungs, and whether terbutaline affects oedema formation.

Isolated lungs of normoxic rats were perfused at a constant pressure (12 cmH_2O) and exposed to different levels of oxygenation (1.5–35% O_2). Terbutaline (10^{-5} M) was applied as an aerosol or with the perfusate. Online measurements indicate an earlier onset of weight gain with an increasing degree of hypoxia and a shortened lung survival time (35% O_2: ~220 min; 1.5% O_2: ~120 min). Terbutaline did not prevent oedema formation in hypoxic lungs. The terbutaline-induced formation of cyclic adenosine monophosphate was decreased by 50% in hypoxia (1.5% O_2).

In experiments terminated after 75 min, bronchoalveolar lavage fluid of hypoxic lungs contained protein that originated from perfusate indicating alveolar leakage. Since lactate dehydrogenase in perfusate was not increased at the onset of oedema formation, cell damage does not explain the increased permeability.

In conclusion, these results indicate the formation of a leak for macromolecules of the isolated perfused rat lung, which is accelerated by hypoxia and causes alveolar flooding even at low perfusion pressure at a rate that exceeds absorption even after stimulation with terbutaline.

KEYWORDS: Beta-adrenergic agonists, capillary permeability, cyclic adenosine monophosphate, hypoxia, pulmonary oedema

Pulmonary oedema can occur in pathological situations that are directly or indirectly linked with alveolar hypoxia. High-altitude pulmonary oedema can be solely attributed to alveolar hypoxia. It occurs after rapid ascent to high altitude in a small percentage (~6%) of mountaineers [1]. In this situation, oedema formation is due to pulmonary hypertension and increased pulmonary capillary pressure [2]. Other possible mechanisms may include increased lung vascular endothelial and alveolar epithelial permeability [3, 4] and inhibition of alveolar fluid reabsorption [1]. Evidence for the latter comes from the following: 1) prevention of high-altitude pulmonary oedema by inhalation of β-adrenergic agonists [5]; 2) terbutaline-induced stimulation of reabsorption of fluid instilled into lungs of hypoxia-exposed rats [6]; and 3) β-adrenergic stimulation of transepithelial sodium transport in primary rat alveolar epithelial cells [7].

The significance of an increased capillary permeability in hypoxic oedema formation is not clear. Results from STELZNER *et al.* [3] on *in vivo* hypoxia of rats suggest an increase in permeability as indicated by an increase in pulmonary transvascular protein escape. Hypoxia, in combination with viral infection of the respiratory tract, increases the formation of pulmonary oedema and protein leak [8]. In humans exposed to high-altitude, permeability changes indicated by increased protein, inflammatory cytokines and leukocytes in bronchoalveolar lavage [4] appear to be a phenomenon secondary [9] to inhomogeneous vasoconstriction [10] and capillary stress failure [11]. However, *in vivo* experiments do not allow the discrimination of permeability changes from haemodynamic effects due to increased pulmonary artery pressure in hypoxia. An increased filtration coefficient has been found in isolated blood-perfused dog lung in hypoxia [12]. Increased endothelial permeability in hypoxia was not only found in the lung but also in endothelium from peripheral arteries [13, 14]. In cultured endothelial cells from the lung, this seems to be related to lowered cyclic adenosine monophosphate (cAMP) formation [15]. These results indicate that hypoxia *per se* increases the

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hydraulic conductance of vascular endothelium, without additional hypoxia-related changes, such as increased transmural pressure. The present study was designed to answer the question as to whether acute hypoxia causes the formation of pulmonary oedema at low lung perfusion pressure and the extravasation of large molecules, such as albumin. It was further tested as to whether the β-adrenoceptor agonist terbutaline prevents hypoxia-induced oedema formation by tightening the endothelial barrier [15, 16] or stimulating reabsorption [7] in the isolated perfused rat lung. Therefore, the effects of increased pulmonary artery pressure [3] were eliminated as it was observed in hypoxic rats *in vivo* by keeping perfusion pressure constant in an isolated rat lung model, which also allows precise control of alveolar and perfusate oxygenation [17].

MATERIALS AND METHODS

Isolated perfused rat lung

Lungs from male Sprague-Dawley rats (230–280 g; Charles-River-Wiga Laboratories, Sulzfeld, Germany) were prepared for isolated perfusion according to UHLIG and coworkers [17, 18]. Briefly, following anaesthesia by intraperitoneal injection of sodium thiopental (60 $mg \cdot kg^{-1}$; Trapanal®; Altana, Konstanz, Germany) and anticoagulation with heparin-Na (1,500 $IU \cdot kg^{-1}$), lungs were extracted and mounted in the humidified ''thorax chamber'' (37°C) of the isolated lung apparatus (model IL-2; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) [17, 18]. Lungs were perfused at a constant hydrostatic pressure (arterial: 12 cmH_2O; venous: 2 cmH_2O). The perfusate contained the following: 118 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 2.5 mM $CaCl_2$; 24.9 mM $NaHCO_3$; 3 mM HEPES; 5.5 mM glucose; and 3% bovine serum albumin (300 $mosmol \cdot kg^{-1}$ H_2O; pH 7.36 at 37°C). Lungs were ventilated (80 $breaths \cdot min^{-1}$) by negative pressure (end-inspiratory chamber pressure -8 cmH_2O; end-expiratory chamber pressure -2 cmH_2O) with pre-warmed humidified gas. Every 5 min, a timer-controlled hyperinflation was initiated by decreasing the end-inspiratory pressure to -16 cmH_2O to imitate physiological sighs and to avoid atelectasis. During preparation, initial equilibration (15 min) and experiments, lungs were ventilated and perfused. The perfusate was equilibrated with the respective gas using a counter-flow oxygenator. The gas used for ventilation and equilibration of the medium contained 14.5% O_2, 5.0% CO_2 and balance N_2 (normoxia). This concentration of O_2 resulted in an alveolar and perfusate partial pressure of O_2 (P_{O_2}) of ~13.3 kPa at a partial pressure of CO_2 of 4.78 kPa and a perfusate pH of 7.36.

Experimental conditions were initiated after the equilibration period. Oxygenation levels of the lungs were adjusted by switching to the desired gas mixture that was being used for equilibration of the perfusate and for lung ventilation. Terbutaline was applied as an aerosol (aerosol generator; Pari, Starnberg, Germany) or added to the perfusate (final concentration 10^{-5} M) at the end of the equilibration period. Long- and short-term experiments were performed to characterise the time course of oedema formation, type of oedema and terbutaline effects.

Long-term experiments

To evaluate the time course of the change in lung weight and to measure lung survival time in the isolated lung apparatus, individual lungs were exposed to different levels of oxygenation (1.5, 3, 6, 14.5 and 35% O_2, respectively; all with 5% CO_2 and balance N_2) with or without terbutaline applied as an aerosol. This resulted in perfusate P_{O_2} values of ~1.46, 2.79, 5.71, 13.69 and 33.25 kPa, respectively. Lung survival time was defined as time after the equilibration period until the tidal volume had decreased to zero. An arbitrarily chosen threshold of lung weight gain of 200 mg was defined as a marker of the onset of oedema formation.

Short-term experiments

After the equilibration period, normoxia (14.5% O_2) was continued or lungs were exposed to hypoxia (1.5% O_2). Terbutaline (aerosol or perfusate) was also added after the equilibration. Experiments were terminated after 75 min when ventilation was not yet affected by oedema formation. At the end of the experiment, lungs were removed and the right upper lobe was used to measure the wet-to-dry weight ratio after drying to a constant weight at 80°C. Remaining tissue from the right lung was frozen in liquid nitrogen and stored at -80°C for measurements of cAMP by radio immunoassay (Immunotech, Marseille, France). The left lung was lavaged with 4×3 mL of PBS. Bronchoalveolar lavage fluid (BALF) was pooled and centrifuged at $3{,}000 \times g$, 4°C for 10 min. The supernatant was stored at -80°C until further use.

Additional measurements

In all experiments, lung weight change, perfusate flow and ventilation parameters were continuously recorded. During the experiments, aliquots of perfusate were collected every 15 min and stored at -80°C for further analysis.

Lactate dehydrogenase (LDH) and alkaline phosphatase activity in perfusate were measured spectrophotometrically using test kits (Sigma Chemical Company, Deisenhofen, Germany) as indicators of cell damage.

In BALF from short-term experiments, albumin was identified by Western blot analysis using anti-bovine serum albumin antibodies (Sigma Chemical Company) and enhanced chemiluminescence (Amersham Pharmacia, Freiburg, Germany). The concentration of total protein in BALF was measured using a test kit from BioRad Laboratories (Hercules, CA, USA).

Statistical analysis

Results are expressed as mean±SD of the number of experiments as indicated in the figure legends. Oxygen dependence of changes was tested with one-way ANOVA and multiple pair wise comparisons with Tukey's test. Unpaired t-tests were used to determine differences between two group means. The level of significance was set to $p<0.05$ and calculated p-values are indicated in the text.

RESULTS

Hypoxic oedema formation

In long-term experiments, the time course of oedema formation was studied at different levels of oxygenation. Figure 1 shows that lung weight increased earlier when lungs were exposed to hypoxia rather than in normoxia or hyperoxia.

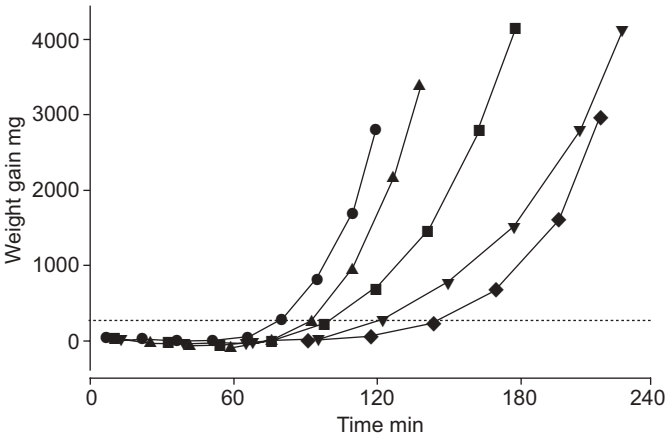

FIGURE 1. Time course of changes in lung weight of isolated rat lungs exposed to different levels of oxygenation. Isolated lungs were exposed to different levels of oxygenation after a 15-min equilibration period in normoxia. Curves show the means of weight gain from 5–8 lungs per experimental condition. ●: 1.5%; ▲: 3%; ■: 6%; ▼: 14%; ♦: 35%. ·····: lung weight gain of 200 mg, used as an indicator of onset of oedema formation.

Also, the duration of the experiment (''lung survival time'') decreased significantly with increasing degree of hypoxia (lung survival at 35% O_2 ~220 min and at 1.5% O_2 ~120 min; fig. 2a). Figure 2b demonstrates that the time at which lung weight had increased by 200 mg, a value chosen arbitrarily as an indicator of onset of oedema formation (threshold), was ~150 min at 35% O_2 and decreased significantly to ~85 min at 1.5% O_2.

When terbutaline was applied with an aerosol generator after the normoxic equilibration period, the survival time of hypoxic rat lungs in long-term experiments did not change, nor did terbutaline prevent or delay the hypoxia-induced increase in lung weight (fig. 2).

A series of short-term experiments, which were terminated after 75 min, was performed to characterise the nature of hypoxic oedema. Figure 3 shows significant oedema formation in hypoxic lungs (1.5% O_2) as indicated by an increased wet-to-dry weight ratio (fig. 3a, $p=0.038$) and an increase in lung weight of ~500 mg after 75 min of hypoxia (fig. 3b, $p=0.046$). Terbutaline applied in hypoxia significantly increased wet-to-dry weight ratios (fig. 3a) and accelerated lung weight gain (fig. 3b, $p=0.036$). The discrepancy between changes in the wet-to-dry weight ratio and lung weight gain in terbutaline-treated hypoxic lungs was probably due to entire filling of the lung lobe used for drying, whereas the whole lung was still able to take up water (fig. 3).

Protein content of BALF

Figure 4a shows that the total protein content was increased ~2.5-fold ($p=0.031$) in the BALF of lungs exposed to hypoxia (1.5%) for 75 min. Western blots (not shown) indicate that the protein contained in the BALF of oedematous lungs was comprised of perfusate-derived albumin.

Lung perfusion

The perfusate flow (mean of all experiments) at the end of the normoxic equilibration period was 10.5 ± 1.4 mL·min^{-1}·100 g^{-1}, which is approximately a third of reported *in vivo* values for rats at rest [19]. No change in perfusate flow was detected upon exposure to hypoxia. Application of terbutaline by aerosol increased perfusate flow significantly by ~1.4 mL·min^{-1} at levels of oxygenation above 3% O_2 (fig. 5). The small increase in perfusate flow after terbutaline application in lungs at 1.5% and 3% O_2 was not statistically significant. The increase in perfusate flow was more pronounced when terbutaline was applied with the perfusate than after aerosol application.

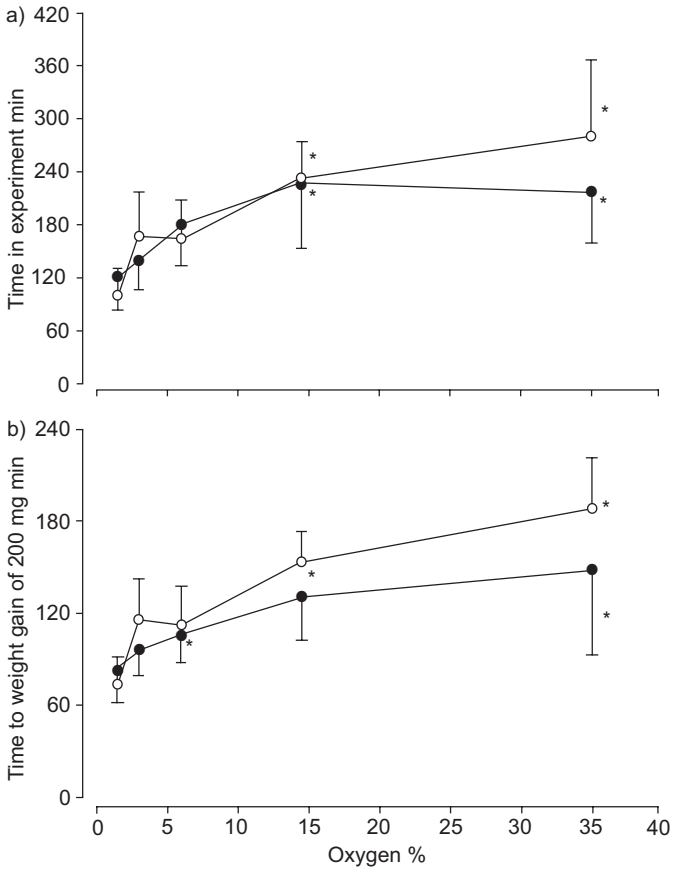

FIGURE 2. Oxygen dependence of the duration of long-term experiments and onset of oedema formation in isolated perfused lung. a) Time after the equilibration period for which the lungs maintained a tidal volume >0. b) Time after the equilibration period when the lungs had gained 200 mg of weight. This parameter was chosen arbitrarily as an indicator that defines the time of onset of oedema formation. Data are presented as mean±SD from 5–8 experiments per group. ●: control; ○: terbutaline. *: difference to lungs exposed to 1.5% O_2, $p<0.05$.

cAMP levels

To test whether hypoxia affected β-adrenergic signal transduction, cAMP was measured in perfusate and tissue. Figure 6a shows a small increase in cAMP over time in control lungs of long-term experiments that were not treated with terbutaline. cAMP increased significantly after terbutaline application, regardless of the level of oxygenation ($p=0.001$). The increase in cAMP in perfusate within the initial 15 min after terbutaline application, which might serve as an indicator of the rate of cAMP production and/or release, was increased approximately five-fold above baseline, but was not affected by the

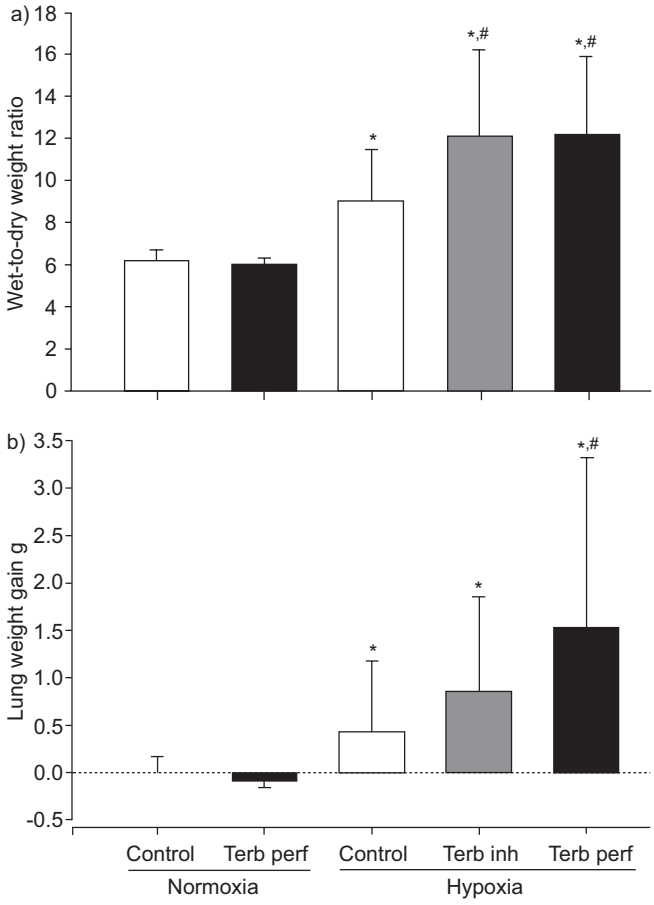

FIGURE 3. a) Lung wet-to-dry weight ratio measured in the right upper lobe after terminating the experiments, and b) weight gain in short-term experiments from online recordings. Experiments on normoxic (14.5%) and hypoxic (1.5% O_2) lungs were terminated 75 min after the equilibration period. Terbutaline was applied as an aerosol within the first 5 min after the equilibration period. In separate experiments, terbutaline was added to the perfusate (final concentration 10^{-5} mol·L^{-1}). Data are presented as mean±SD from 6–14 experiments. Terb perf: terbutaline perfusate; terb inh: terbutaline inhaled. *: indicates $p<0.05$ between normoxia and hypoxia; #: indicates $p<0.05$ control and terbutaline-treated lungs.

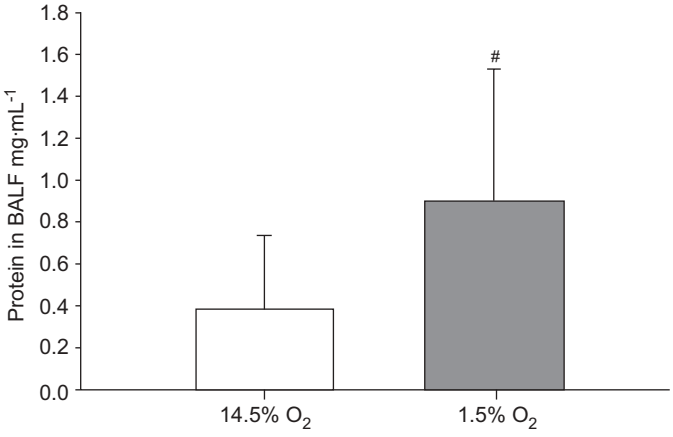

FIGURE 4. Protein content in bronchoalveolar lavage fluid (BALF) in short-term experiments. Experiments were terminated after 75 min at 14.5% (normoxia) and 1.5% O_2 (hypoxia) when lungs were lavaged for protein measurements. Data are presented as mean±SD from 5–8 experiments of total protein count of BALF. #: $p=0.031$.

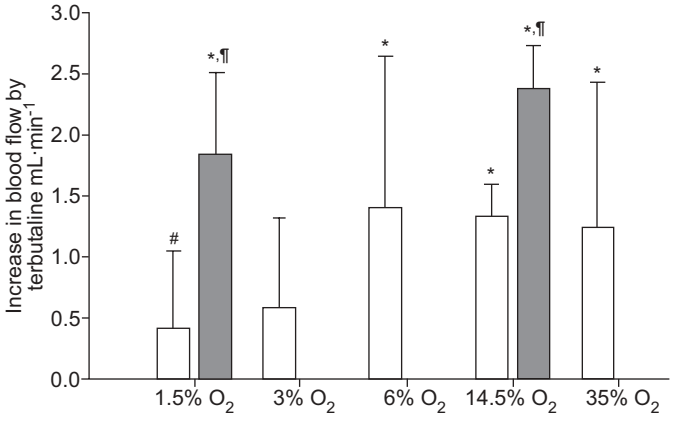

FIGURE 5. Effect of terbutaline on lung perfusate flow. After equilibration, lungs were exposed to the indicated levels of oxygenation. Terbutaline was applied as an aerosol and, in separate experiments, added to the perfusate (10^{-5} M). Data are presented as mean±SD from 6–12 lungs per experimental group. □: aerosol; ■: perfusate. *: significant increase in perfusate flow, $p<0.05$; #: significant difference to normoxia (14.5% O_2); ¶: significant difference between application modes at a given oxygenation level.

level of oxygenation ($p=0.42$). Maximal cAMP levels in the perfusate were significantly lower in lungs exposed to 1.5% O_2 than 14.5% and 35% O_2 ($p=0.005$).

In short-term experiments, cAMP was measured in lung homogenates. Figure 6b shows that cAMP in untreated lungs was the same in normoxia and hypoxia. While terbutaline caused a 2.5-fold increase in tissue cAMP levels ($p<0.002$) in normoxia, the increase was only 1.6-fold ($p=0.076$) in hypoxia. The difference in the terbutaline-induced increase in tissue cAMP between normoxic and hypoxic lungs was not statistically significant ($p=0.086$).

Measures of cell damage

The appearance of intracellular enzymes in the perfusate was measured to test whether oedema formation was associated with cell damage in the series of long-term experiments. No statistically significant increase in LDH activity was observed at the threshold for onset of oedema formation (weight gain of 200 mg; table 1). However, at termination of the experiments, LDH activity was elevated significantly in control lungs exposed to 6, 14.5 and 35% O_2, but not at lower oxygenation levels. Aerosolised terbutaline reduced the increase in LDH at 14.5% O_2 ($p=0.001$), but not at 6 and 35% ($p=0.09$). Changes in alkaline phosphatase followed the same pattern (data not shown).

DISCUSSION

The major finding of this study is that hypoxia induced formation of oedema in the isolated perfused rat lung at a constant, low perfusion pressure. Oedema formation was accelerated with decreasing oxygen concentrations. The concomitant appearance of protein in BALF indicates that, at least in severe hypoxia, oedema formation is accompanied by an increased permeability of the alveolar barrier. Oedema

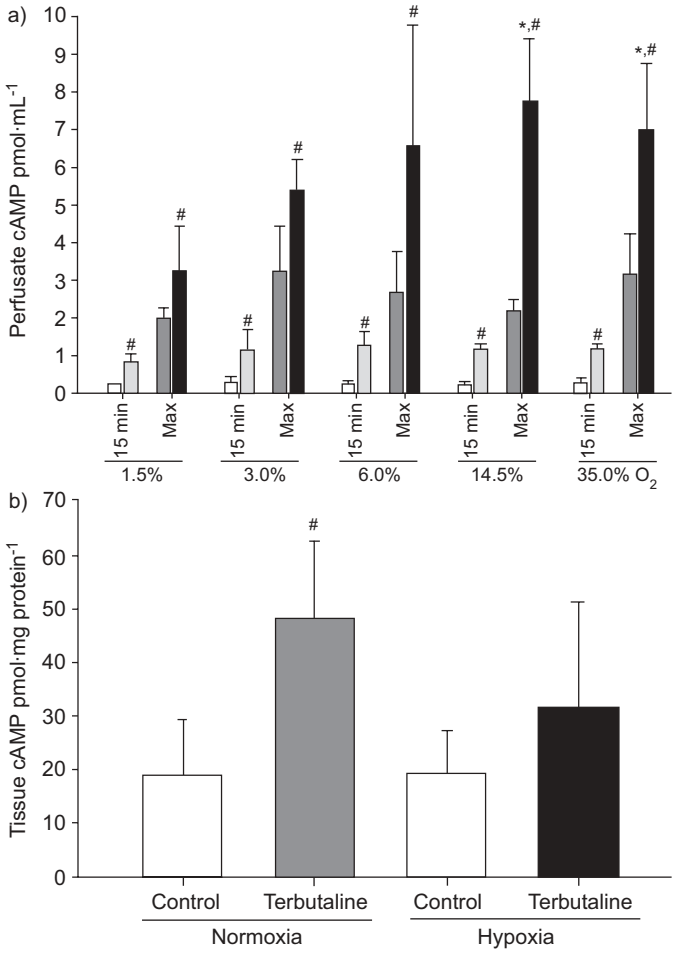

FIGURE 6. Terbutaline-induced formation of cyclic adenosine monophosphate (cAMP) in hypoxia. a) Perfusate cAMP during long-term experiments of control (□) and terbutaline-treated rat lungs (▩) in the first 15 min after equilibrium, and maximal values of cAMP in control (■) and terbutaline-treated rat lungs (■). Data are presented as mean±SD from 5–8 experiments. b) cAMP levels in tissue of lungs exposed to normoxia (14.5% O_2) and hypoxia (1.5% O_2). Data are presented as mean±SD from 4–7 experiments. 15 min: increase in cAMP if first 15 min after the equilibration period; Max: maximal values of cAMP. *: indicate difference to 1.5% O_2, $p<0.05$; #: indicate difference between control and terbutaline-treated lungs, $p<0.05$.

formation in hypoxia is not prevented by terbutaline, indicating that cAMP-dependent processes do not prevent endothelial leakage and that alveolar flooding overwhelms reabsorption capacity even when it is stimulated by terbutaline.

The isolated perfused lung was chosen as a model to study mechanisms of hypoxic oedema formation, because exaggerated pulmonary hypertension occurring in *in vivo* hypoxia can be prevented [3, 20]. This was achieved by perfusion at a constant pressure of 12 cmH_2O, which is slightly below the physiological range of rat pulmonary artery pressure [21] and which, in the worst case of hypoxic venous constriction, would expose capillaries to that pressure. Inhomogeneous vasoconstriction in hypoxia might cause regional overperfusion leading to oedema formation due to capillary stress failure [11]. In the current authors' system, maximal capillary pressures were lower than the pressures required causing

TABLE 1 Activity of lactate dehydrogenase (LDH) in perfusate

Oxygen %	Threshold	200 mg threshold	End of experiment
Control			
1.5	58.1±16.8	53.6±21.1	57.3±22.1*
3.0	46.9±15.4	47.3±15.3	53.5±17.7*
6.0	56.9±14.2	65.8±9.5	113.5±37.0#
14.5	72.2±10.5	85.0±14.5	162.5±32.0#
35.0	60.9±7.1	72.6±28.3	145.5±13.6#
Terbutaline treated lungs			
1.5	41.0±7.5	37.2±12.0	40.5±7.9*
3.0	32.4±3.7	34.0±9.3	54.7±16.0*
6.0	60.9±9.8	60.0±7.8	84.1±31.7#,+
14.5	62.3±10.9	68.0±9.4	101.2±16.5#
35.0	53.9±9.8	60.5±6.9	120.3±43.4#

Data are presented as mean±SD. LDH activity was measured in aliquots of perfusate during the long-term experiments. Threshold LDH activity was extrapolated to the time when the weight gained was 200 mg. End of experiment samples were taken at the termination of experiments. Data were obtained from 5–8 experiments. *: difference to 35% O_2, $p<0.05$; #: difference to begin of experiment, $p<0.05$; +: difference between control and terbutaline treated lungs, $p<0.05$.

alveolar damage [22]. Hypoxia did not cause significant pulmonary vasoconstriction, since no decrease in lung perfusion was found, which is in accordance with other results obtained in salt-perfused lungs [23, 24]. Nitric oxide (NO)-dependent vasodilation might account for this phenomenon, since the perfusate did not contain erythrocytes, which normally keep the NO concentration low [25].

As effects of altered haemodynamics appear minimal in this model, hypoxia-induced oedema formation of the isolated perfused lung must be due to an increase in the permeability of the alveolar endothelial and epithelial cell layer. The increased protein content of BALF of oedematous lungs supports this notion. Hypoxia-induced macromolecular leaks that were associated with extravasation of protein to the alveolar space have also been observed in rats *in vivo*. Exposure to hypobaria for up to 24 h resulted in alveolar haemorrhage, increased protein in lavage [26] and ruptured capillaries [27]. STELZNER *et al.* [3] reported an increased lung protein leak index and increased lung wet-to-dry weight ratios after exposure of rats to hypoxia for 24 h. Short-term exposure to hypoxia resulted in activation of the endothelium with oedematous disruption of the arteriolar wall, activation of intravascular leukocytes and platelets [28], and increased markers of inflammation [29]. However, the increased permeability found in those *in vivo* experiments was most likely associated with hypoxic pulmonary hypertension [3] and capillary stress failure [11, 21]. Therefore, those results allow no conclusion on the nature of the leak with regard to permeability and pressure. The present authors have demonstrated for the first time that alveolar permeability is increased solely by hypoxia, in the absence of pulmonary hypertension.

It is impossible to distinguish between interstitial and alveolar oedema in the isolated lung and in high-altitude pulmonary oedema. Subclinical oedema, which might be interstitial, has been implied to occur at high altitude [30]. This finding supports the notion that, when no large ruptures of the alveolar wall occur, hypoxia might loosen tight junctions of the alveolar vascular endothelium to cause fluid filtration into the interstitial space. Only after overwhelming the capacity of the interstitial compartment does the usually very tight alveolar epithelial cell layer break to allow alveolar flooding.

The current results indicate that terbutaline did not prevent, but rather increased, hypoxia-induced oedema formation in the isolated perfused rat lung. This finding contrasts with studies showing an improvement of lung fluid balance by β-adrenergics, but is supported by studies indicating adverse effects of these agents. Improvement of lung function by β-adrenergics manifests as a decrease in lung vascular resistance [31] and decreased alveolar permeability [32]. Stimulation of alveolar sodium transport and fluid clearance by β-adrenergics has been described extensively in normoxia [7] and in hypoxia [5, 6]. In contrast, in animal models, cAMP failed to stimulate alveolar fluid clearance in ventilator induced injury [33] and salbutamol dramatically increased lung water in acid-induced lung injury [34]. In the present experiments, terbutaline caused a pronounced increase in cAMP production in normoxia, whereas lung cAMP increased less in hypoxia. Therefore, it appears that in the isolated perfused lung, despite the observed increase in cAMP, terbutaline does not prevent the hypoxia-induced increase in alveolar permeability. The underlying mechanisms need further investigation. If alveolar sodium and water reabsorption were stimulated by terbutaline, the reabsorptive capacity must have been too low to balance alveolar flooding *via* the hypoxia-induced macromolecular leak.

One problem of studying isolated organ systems is the decay of organ function with time which, in the isolated perfused lung, is indicated by lung weight gain and impaired ventilation and perfusion. At high oxygenation levels, isolated lungs perform well for several hours [17, 35]. In contrast, in hypoxia, lung survival is decreased considerably in an oxygen-dependent manner where, at extreme hypoxia, lung fluid accumulation already occurs after ~60 min. Also, in rats, increased albumin was found after 1 h of *in vivo* hypoxia [29], whereas clinical signs of high-altitude pulmonary oedema were typically observed 24–48 h after arriving at high altitude [1]. In the present experimental set-up, albumin leakage (a clear sign of alveolar oedema and macromolecular leakage) has only been measured at the extreme hypoxia of 1.5% oxygen. Therefore, any statement on similar processes at less severe hypoxia appears speculative. However, at a lung weight gain in the range of grams (fig. 1), a restriction of accumulated fluid to the interstitial space (interstitial oedema) appears unlikely. The question arises whether oedema formation is initiated by lung cell damage. In none of the experimental conditions tested was oedema formation preceded by the release of intracellular enzymes, which are surrogate markers of cellular destruction. The large increase in perfusate LDH activity in long-term experiments at oxygenation levels >3% can be explained with the longer duration of these experiments. This indicates that cellular damage cannot explain the initiation of oedema formation, but that significant cell damage occurred only with extended duration of exposure to the experimental conditions.

In conclusion, the present results show that hypoxia causes an increase in both alveolar endothelial and epithelial permeability to water and macromolecules that is independent of increased pulmonary artery pressure and capillary stress failure. If this occurred *in vivo*, the hydrostatic driving force due to an increase in pulmonary capillary pressure in hypoxia [2] and the increased hydraulic conductance due to the permeability leak would add and favour the extravasation of water and blood constituents and, thus, oedema formation. The mechanisms which cause the hypoxia-induced increase in permeability and which blunt the potentially beneficial effects of terbutaline on oedema prevention in hypoxia remain to be clarified.

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