Lung injury following pulmonary resection in the isolated, blood-perfused rat lung

E.A. Williams*, G.J. Quinlan*, P.B. Anning*, P. Goldstraw†, T.W. Evans*

Abstract

Lung resection may be complicated by postpneumonectomy pulmonary oedema. Oxidant generation following surgery-induced ischaemia-reperfusion may be responsible. This hypothesis was tested utilizing isolated, in situ, blood perfused rodent lungs subjected to continuous perfusion (control subjects); one lung ventilation followed by pneumonectomy (group 1); or one lung ventilation followed by reinflation of the collapsed lung (group 2).

In control subjects, no significant changes in markers of oxidant damage, oxygenation, pulmonary artery pressure or extravascular albumin extravasation were detected. In group 1 lungs, hydroxyl radical-like damage was detected in association with impaired oxygenation (p<0.05), and increased pulmonary artery pressure and extravascular albumin accumulation in both lungs. In group 2, there was evidence of hydroxyl radical-like damage, and a fall in oxygenation (p<0.05) occurred during one lung ventilation. There was a transient rise in pulmonary artery pressure following lung reinflation and extra vascular albumin accumulation was significantly increased in both lungs (right<left, p<0.05). Both changes were attenuated (p<0.05) following treatment with the reactive oxygen species (ROS) scavenger superoxide dismutase (group 2a) and the nitric oxide synthase inhibitor Nω-nitro-arginine methyl ester (group 2b). Hydroxyl radical-like damage was undetectable following nitric oxide synthase inhibition.

Oxidant stress may contribute to the pathologies seen in this model of lung injury.


Methods and materials

Protocols were designed to investigate the hypothesis that the clinical techniques of one lung ventilation (OLV) followed either by pneumonectomy or reinflation might result in lung injury, quantified by measuring changes in pulmonary artery pressure (PAP), extravascular albumin accumulation (EAA) and oxygenation index. Specific evidence of ROS generation was sought by measuring levels of d-ortho- and d-meta-tyrosine formed from added d-phenylalanine, indicating evidence of hydroxyl (-OH)-like damage.

Preparation

All experimental protocols were carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK). An in situ ventilated, blood-perfused rat lung preparation described in full elsewhere [16] was used throughout. Briefly, male Wistar rats (175–225 g) were
anaesthetized (pentobarbitone 60 mg·kg⁻¹ i.p.), the trachea cannulated and ventilation commenced (gas 21% O₂, 5% CO₂, balance nitrogen N₂; tidal volume 2.5 mL, frequency 60 cycles·min⁻¹ with positive end-expiratory pressure (PEEP) 2 cmH₂O).

Exsanguination via the abdominal aorta was then performed following heparinization (30 mg·kg⁻¹ body weight), the blood placed in a reservoir at 40 °C, and supplemented with 4% bovine serum albumin (BSA) in 0.9% normal saline to a volume corresponding to the in vivo circulating blood volume. The lungs were exposed and a loose ligature placed around the right main bronchus in experiments requiring OLV, and a snapper applied to allow reversible isolation of the right bronchial tree when required. Cannulae were inserted into the pulmonary artery (PA) and left atrial (LA) appendage. The completed circuit consisted of the LA cannula, a pressure transducer, 20 mL reservoir, roller pump, PAP transducer and PA cannula. The lungs were perfused at 18 mL·min⁻¹ via the PA cannula, a pressure transducer, 20 mL reservoir, roller pump, PAP transducer and PA cannula. The lungs were perfused at 18 mL·min⁻¹ via the PA cannula, a pressure transducer, 20 mL reservoir, roller pump, PAP transducer and PA cannula. Free drainage of blood from the LA to blood reservoir ensured that changes in PAP were directly proportional to, and therefore representative of changes in pulmonary vascular resistance. Blood gas tensions were analysed at three time points during each experiment and pH maintained in the physiological range by addition of small aliquots of sodium bicarbonate as appropriate.

Experimental protocols

Stabilization period: an initial period of 30 min was included in all protocols to stabilize the preparation. During this time 15 mg D-phenylalanine and 15 mL of iodinated human serum albumin were added to the perfusate.

Control Group: after stabilization, lungs were subjected to 120 min of continuous ventilation and perfusion with no intervention. PAP was analysed for changes from baseline at 60 and 120 min. Baseline perfusate oxygen tension was measured at 0 (baseline), 50 and 110 min. Paired 0.5 mL samples of perfusate were collected at 0, 50, 70 and 120 min for later assay of markers of ROS production. Circuit volume was maintained at each sampling by replacement with equivalent volumes of BSA. At 120 min (end-point), tissue samples were resected from each lung for gamma activity estimation (concurrent with 1 mL samples of circuit perfusate) and calculation of EAA.

Group 1: effect of one lung ventilation and right pneumonectomy on lung injury. After 30 min stabilization, the right lung was collapsed but the pulmonary vessels were not ligated and OLV performed for 60 min. The pulmonary artery, vein and bronchus were ligated at the hilum and the right lung resected. Ventilation and perfusion of the left lung continued for a further 60 min. EAA was measured in both the resected and contralateral lungs. To control for the effects of volotrauma, tidal volume was reduced during OLV to maintain constant Paw. The indices of lung injury that were measured were identical in timing and nature to those performed in control subjects.

Group 2: effect of collapse-reinfation of the right lung on lung injury. Preliminary studies revealed that reinflation of right lung tissue following partial resection could not be reliably undertaken whilst controlling for the variable effects of volotrauma and reinflation. Thus, following 30 min stabilization the collapsed right lung was reinflated (without resection of tissue) and ventilation and perfusion of both lungs continued for a further 60 min. The indices of lung injury that were measured were identical in timing and nature to those performed in control subjects and group 1 animals.

Protocol 2a: effect of superoxide dismutase on pulmonary artery pressure and lung injury following collapse-reinflation of the right lung. During the stabilization period, 4,400 IU of freshly prepared SOD dissolved in 200 μL 0.9% NaCl was added to the perfusate reservoir, prior to the collapse-reinflation procedure. The indices of lung injury that were measured were identical in timing and nature to those performed in the control subjects, and in groups 1 and 2 animals. The protocol for group 2 (right lung reinflation) was chosen for this aspect of the study as such animals had shown the most marked degree of lung injury.

Group 2b: effect of L-NAME on pulmonary artery pressure and lung injury following collapse-reinflation of the right lung. During the stabilization period, 1.5 mg·L⁻¹ L-NAME dissolved in 200 μL 0.9% NaCl was added to the perfusate to produce a final circulating concentration of 1 mM·L⁻¹ (chosen as pilot studies using lower concentrations failed to show a consistent effect). Collapse-reinflation of the right lung was then performed as in Group 2 animals and identical parameters to those in control subjects, groups 1, 2 and 2a were measured.

Experimental methods

Extravascular albumin accumulation. ¹²⁵I-human serum albumin (HSA) (15 mL, ~18.5 MBq) was added to the perfusate during the equilibration period (see Experimental protocols). At the end of each experiment (120 min) 1 mL of blood perfusate was collected for the estimation of radioactivity. Residual radioactivity was flushed out by circuit perfusion with 75 mL of 4% BSA in 0.9% normal saline at a perfusion pressure two thirds of the final PAP, and confirmed by direct comparison of counts in 1 mL of the final perfusate with background levels of activity, measured using an empty cuvette. Paired tissue samples from both lungs were weighed after gentle blotting, placed in cuvettes and together with the perfusate samples counted for a period of 10 min per cuvette. EAA was calculated as the ratio of counts per min (cpm) per g (lung) to cpm per mL (blood). For studies in which a right pneumonectomy was carried out comparisons of EAA were made between the resected lung and the left (i.e. remaining) lung.

Measurement of reactive oxygen species production. Evidence for production of the hydroxyl radical was sought by utilizing its ability to hydroxylate phenylalanine [8]. Low molecular mass constituents were separated from plasma samples by size exclusion, freeze-dried and derivatized [11]. d-meta- and d-ortho-tyrosine were detected based on their retention times and characteristic mass spectra, by positive ion electron impact gas chromatography mass spectrometry. d- and l-isomers were resolved by use of a chiral gas chromatography (GC) column.
lateral lungs compared to corresponding control subjects (EAA for resected right lung 0.42±0.02 cpm·g tissue⁻¹·mL blood⁻¹·min⁻¹ versus 0.21±0.03 for left lung, p<0.05; control EAA 0.054±0.01 and 0.055±0.00 for right and left lung respectively, (fig. 3a)). Elevated levels of d-ortho- and d-meta-tyrosine were detected at 50, 70 and 120 min (which were significantly increased for d-ortho-tyrosine after 70 min, p<0.05) following pneumonectomy (n=3) providing evidence of -OH-like mediated damage (fig. 4).

Group 2 (n=5), one lung ventilation followed by right lung reinflation. There was a significant, progressive and acute rise in PAP following reinflation of the right lung (mean elevation 3.9±0.5 mmHg, p<0.05). PAP returned to baseline within 10 min and remained unchanged from baseline at 120 min (mean elevation 0.8±0.1 mmHg, p>0.05, (fig. 1a)). Oxygenation fell during OLV (25.68±0.96 kPa baseline versus 20.37±1.60 kPa, p<0.05), and showed a small rise on return to dual lung ventilation to 21.8±1.1 kPa although this did not reach baseline levels (fig. 2). EAA was significantly increased in both lungs (right lung 0.56±0.06, left lung 0.38±0.05) compared to control values (right lung 0.054±0.01, left lung 0.055±0.00, p<0.01, fig. 3a). The increase in EAA was significantly greater in the left lung following right lung reinflation compared to the left lung following pneumonectomy (0.36±0.05 versus 0.21±0.03, p<0.05, (fig. 3a)). Evidence of -OH-like damage was detectable at 50, 70 and 120 min; peak concentrations for d-ortho-tyrosine (p<0.05) being measured at the 70 min timepoint (10 min following reinflation/reperfusion). Mean concentrations following correction for dilution are shown in figure 4.

Group 2a (n=7), effect of superoxide dismutase on lung injury following collapse-reinflation of the right lung. Baseline PAP (9.3±0.4 mmHg, n=7) was unaffected by the addition of SOD, and following reinflation, at 60 and 120 min, no significant elevations in PAP were observed (9.9±0.3 and 9.4±0.4 mmHg respectively, (fig. 1b). EAA
was significantly lower for both lungs (right lung 0.15±0.02, left lung 0.10±0.01) compared to the values obtained for right lung re-inflation alone (group 2 animals, right lung 0.56±0.06, left lung 0.38±0.05, p<0.01, fig. 3b). The addition of 4,400 IU SOD and 120 min perfusion only caused no significant change in EAA (right lung 0.13±0.07, left lung 0.11±0.05, p=0.4 mmHg and 10.3±0.5 mmHg at 60 and 120 min respectively) compared to 120 min continuous perfusion alone (right lung 0.054±0.00 and left lung 0.055±0.01, p>0.05). No n-orth- or n-meta-tyrosine were detectable in perfusate samples, suggesting that -OH-like damage was inhibited by SOD administration (differences almost reached significance at 70 min for n-ortho-tyrosine, p=0.052 and n-meta-tyrosine, p=0.082, (fig. 4)). Protocol 2b (n=7, effects of N^G-nitro-l-arginine methyl ester on lung injury following collapse-reinflation of the right lung). Addition of l-NAME had no discernable effect on baseline PAP (9.8±0.02 mmHg, n=7). Following re-inflation there was no significant elevation in PAP (10.0±0.15 mmHg and 10.3±0.02 mmHg at 70 min continuous perfusion (C) (control subjects, n=5) compared to the values obtained from the resected right lung was compared with the left lung 0.055±0.01, p>0.05). No n-ortho- or n-meta-tyrosine were detectable in perfusate samples, again suggesting that -OH-like damage was inhibited by SOD administration (differences almost reached significance at 70 min for n-ortho-tyrosine, p=0.052, and for n-meta-tyrosine, p=0.082, (fig. 4)).

Discussion

In this study, OLV followed by pneumonectomy caused a sustained rise in PAP, a fall in oxygenation index, a rise in EAA, and evidence of -OH-like damage. By contrast, re-inflation of the right lung following OLV induced transient increases in PAP and oxygenation. EAA was again increased, but to a significantly greater extent in the contralateral lung. Evidence of -OH-like damage was also detectable, peaking at 70 min. Both SOD and l-NAME prevented the increases in PAP and EAA seen after OLV, and appeared to inhibit -OH-like damage.

As in previous studies published by others [17], PAP remained stable in control animals. By contrast, group 1 (pneumonectomy) animals displayed an acute rise in PAP at the time of ligation of the pulmonary vessels which was sustained, and continued to increase gradually over the following 60 min. Early experimental work on the immediate effects of lung excision in anaesthetized dogs showed PAP increased transiently, with variable changes in cardiac output [18]. Clinically, no increase in PAP is observed, at rest following pneumonectomy in young adults [19]. The current study also revealed an acute, but transient rise in PAP following re-inflation of the right lung (group 2), presumably due to vasoactive effects of ROS or other inflammatory mediators generated during OLV. The influence of these changes in PAP and therefore hydrostatic forces on EAA is difficult to assess, but the rise in PAP alone was probably insufficient in itself to

![Fig. 3. Changes in extravascular albumin accumulation (EAA). a) EAA measured in the right (□) and left (■) lungs of rats subjected to 120 min continuous perfusion (C) (control subjects, n=12); right pneumonectomy (RP) (group 1, n=10); or one lung ventilation (OLV) following re-inflation (RR) (group 2, n=5). #: denotes p<0.05 cf controls. *: denotes p<0.05 cf RR corresponding RP. EAA data obtained from the resected right lung was compared with the left lung at 120 min. b) EAA measured in the right (□) and left (■) lungs of rats subjected to OLV and re-inflation (RR) (group 2); OLV and re-inflation following the addition of superoxide dismutase (SOD) 4.400 IU (SODRR) (group 2a); or OLV and re-inflation following the addition of N^G-nitro-l-arginine methyl ester (l-NAME) (l-NAMERR) (to final concentration 1 mmol·L^-1 group 2b). #: denotes p<0.05 c.f. corresponding RR side. AEI: albumin escape index; cpm: counts per minute. Data are presented as mean and SEM.]

![Fig. 4. Changes in concentration of n-ortho-tyrosine. Changes measured at sequential timepoints (0, 50, 70, and 120 min) for: O: right pneumonectomy (RP) (group 1); and V: right re-inflation (RR) (group 2 protocols, together with d-meta-tyrosine concentrations for; ●: RP; and ▲: RR at the same timepoints (n=3 in each case). Markers were not detectable in control subjects, or in the protocols in which superoxide dismutase or N^G-nitro-l-arginine methyl ester were added and are therefore not represented here. *: denotes p<0.05 when compared with controls. d-o-t/d-m-t: n-ortho-tyrosine/d-meta-tyrosine. Data are presented a mean±SEM.]

ortho- and D-meta-tyrosine which are specific markers of ischaemia), did not significantly inhibit the reperfusion of the isolated lung [20, 21], although few investigated any effect on the contralateral lung. Impaired oxygenation under the former circumstances has been shown to correlate with measurable changes in lung permeability, neutrophil infiltration and production of ROS: all of which are implicated in the pathophysiology of clinical ARDS [22].

Compared to time-matched controls, a marked increase in EAA was noted in both lungs following collapse-resection (group 1) and after collapse re-inflation (group 2) of the right lung (fig. 3). In both cases, EAA was greater in the right lung. This implies firstly, that hyperperfusion (if not frank ischaemia) alone may induce vascular injury. Secondly, as EAA rose further in both lungs (right-left) following right lung re-inflation, reperfusion injury may have exacerbated any injury attributable to hyperperfusion alone. Importantly, in the ventilated rat lung reperfusion is unnecessary for lipid peroxidation to occur during an ischaemic insult [23], and experimental studies concerning optimization of lung preservation have demonstrated that injury occurs during the storage/ischaemic phase as well as on reperfusion [24].

ROS are formed during reperfusion injury, although their precise source remains unknown. This study sought evidence of OH-like mediated damage by measuring D-ortho- and D-meta-tyrosine which are specific markers formed when D-phenylalanine becomes hydroxylated. They were identified in both the collapse-resection (group 1) and collapse-re-inflation (group 2) animals, but not in control subjects, implicating a role for ROS in the rise in EAA. Interestingly, these metabolites were identified at 50 min, prior to re-inflation/resection of the lung, indicating that ROS production also occurs during the hyperperfusion of OLV in this model. However, why levels of D-ortho- and D-meta-tyrosine were greatest in group 1 animals, whereas EAA was highest in group 2 is unclear.

Addition of the ROS scavenger SOD and the NOS inhibitor L-NAME in separate protocols (groups 2a and 2b) prevented the acute rise in PAP that occurred on re-inflation/reperfusion of the collapsed right lung. However, addition of either SOD or L-NAME immediately prior to reperfusion (i.e. after the period of hyperperfusion/ischaemia), did not significantly inhibit the reperfusion-induced rise in PAP, further supporting the authors’ contention that ROS are produced during hyperperfusion/ischaemia (data not shown). Neither agent influenced resting PAP. In previous studies using this preparation, increased pulmonary artery pressure and no change in PAP have been reported in response to NOS inhibition [25, 26]. Similar findings in the rabbit lung suggest that NO modulates pulmonary vascular reactivity in the presence of blood. Further, SOD has been shown previously to prevent the augmentation in hypoxic pulmonary vasodilation observed following I-R in the same model [13, 27]. Lastly, both SOD and L-NAME were independently associated with a decrease in lung reperfusion injury as assessed by EAA. Protection by SOD in this study implicates superoxide as a key intermediate in causing the damage detected. Superoxide is known to rapidly interact with NO to produce peroxynitrite, which degenerates to form an -OH-like species under acid conditions. Peroxynitrite itself displays many cytotoxic effects, including lipid peroxidation of biological membranes, sulphhydril oxidation of proteins and nitration of specific amino acids [28]. The rate of reaction of superoxide with NO is approximately three times faster than its reaction with SOD and thirty times faster than the reaction of NO with oxyhaemoglobin [29]. Under pathological conditions where high levels of superoxide are present, the reaction may be so rapid that endogenous SOM may be insufficient to prevent peroxynitrite formation. Thus, superoxide generation may decrease NO concentrations, favouring an increase in arterial tone and formation of peroxynitrite accounting for the resultant tissue injury. Decreased NO concentration favours the Fenton reaction if redox active iron is present, thereby producing hydroxyl radicals and hydroxylations via a second pathway. A previous in vivo study in which inhaled NO given at the time of reperfusion increased injury that could be prevented by the addition of SOD lends partial credence to this hypothesis [30]. The protective effect afforded by inhaled NO against pulmonary oedema formation in various models, including the isolated perfused rat lung, suggests a different mechanism of action for NO at this site [31]. These opposing findings illustrate the diversity of action and interaction of NO, under differing conditions.

In the current study, OH-like oxidative modification of marker molecules was found following reperfusion, as well as during ischaemia. However, no trace of these markers could be found in preparations treated with SOD and L-NAME, implying the protection that they afforded was mediated via the inhibition of a common intermediate, the effects of which resulted in hydroxylation of D-phenylalanine.

In summary, these experiments show that both NO and N^G-nitro-L-arginine methyl ester and superoxide dismutase are protective against lung injury occurring following collapse-reperfusion of the lung. It is speculated that peroxynitrite may be the source of hydroxyl-like species in this model of lung injury.

References


