We have tested if inhaled nitric oxide (NO) is beneficial in ischaemia–reperfusion (IR) lung injury using an isolated perfused rabbit lung model. Ischaemia for 60 min was followed by reperfusion and ventilation with nitric oxide 40 ppm \( (n=6) \) or without nitric oxide ventilation \( (n=6) \) for 60 min. In the control group \( (n=6) \), the lungs were perfused continuously for 120 min. Permeability coefficient \( (K_{fc}) \) and vascular resistance \( (PVR) \) were measured serially for 60 min after reperfusion. We also determined the left lung \( W/D \) ratio and measured nitric oxide metabolites \( (NOx) \) and \( cGMP \) concentrations in bronchoalveolar lavage (BAL) fluid from the right lung. IR increased \( K_{fc} \), \( PVR \) and \( W/D \) followed by decreased \( cGMP \). Ventilation with nitric oxide restored these changes by preventing the decrease in \( cGMP \). Differences in \( NOx \) concentrations in BAL fluid between the control and IR groups were not statistically significant.

Our results indicate that IR impaired pulmonary vascular function and resulted in microvascular constriction and leakage. Ventilation with nitric oxide from the beginning of the reperfusion period improved pulmonary dysfunction such as vasoconstriction and capillary leak by restoring \( cGMP \) concentrations.

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ventilated mechanically (Harvard 681, Harvard Apparatus, Natick, MA, USA) using room air at a rate of 40 strokes per minutes, tidal volume of 6 ml kg⁻¹ and a positive end-expiratory pressure (PEEP) of 3 cm H₂O. Whole blood (70 ml) was obtained via the right carotid artery. The pulmonary artery and left atrium were cannulated via the right and left ventricles, respectively. The lung and heart tissues were extirpated en bloc from the chest cavity. The lungs were then ventilated with a warm humidified gas mixture (21% oxygen, 5% carbon dioxide, balance nitrogen) and perfused under a constant pressure using a recirculation closed system. Packed cell volume of the perfusate was adjusted to approximately 15% by mixing autologous whole blood with physiological salt solution (PSS). PSS contained (in mmol litre⁻¹): NaCl 119, KCl 4.7, MgSO₄ 1.17, NaHCO₃ 22.61, KH₂PO₄ 1.18 and CaCl₂ 3.2. To each 100 ml of this stock solution, we added dextrose 100 mg, insulin 20 mU and bovine serum albumin 3 g (Sigma, Chemical Co., St Louis, MO, USA). The pH of PSS was adjusted to 7.35–7.45 by addition of sodium bicarbonate. The heart–lung preparation was removed en bloc and enclosed in a warm (38°C), humid chamber and suspended from the counter-balanced force-displacement transducer (T1-200-240, Orientec, Tokyo, Japan) connected to the transducer indicator (San-emi, Tokyo, Japan). Pulmonary arterial (Ppa) and venous (Ppv) pressures were monitored continuously by transducers connected to amplifiers (model 2238, San-emi, Tokyo). The flow probe (model, Tokyo) connected to an electromagnetic flowmeter (Nihon Kohden, Tokyo) was placed in the perfusion circuit for continuous monitoring of blood flow (Q). At the beginning of the experiment, a constant Ppa was determined to maintain flow at 30 ml min⁻¹ kg⁻¹ by adjusting the arterial reservoir level. Ppv was also maintained at 8 mm Hg to maintain the lungs in a zone 3 condition. The driving pressure was maintained constant throughout the experiment. Zero level referred to the bottom of the lung. All signals were digitized every 200 µs (5 kHz) using an analogue-to-digital converter (DigiData 1200, Axon instruments, Foster City, CA, USA) and analysed using commercially available software (Axogragh ver. 3.0, Axon instruments).

Measurements of segmental vascular resistance
Arterial and venous catheters were occluded independently by rapid pinch valves (PK 0305-NO, Takasago, Tokyo) for 5 s and arterial and venous occlusion pressures (Pao and Pvo) were measured using the method of Hakim.⁸ During arterial and venous occlusion, ventilation was changed to a continuous positive airway pressure mode of 3 cm H₂O (CPAP=3 cm H₂O). Total PVR (Rt) and its longitudinal distribution was calculated using the following equations:

\[ R_t = \frac{(P_{pa} - P_{pv})}{Q} \]

\[ R_a = \frac{(P_{pa} - P_{ao})}{Q} \]

\[ R_m = \frac{(P_{ao} - P_{vo})}{Q} \]

where \( R_a \), \( R_m \) and \( R_v \) represent pulmonary vascular arterial, middle (or microvascular), and venous resistances, respectively.

Coefficient of filtration
The coefficient constant of fluid filtration (Kfc) was determined using the method of Drake, Gaar and Taylor.⁹ After stabilization for 30 min, an isogravimetric state was attained. Capillary pressure was changed by increasing both arterial and venous reservoirs by 6 cm H₂O for 7 min. To determine capillary pressure, both arterial and venous lines were occluded simultaneously for 5 s with rapid pinch valves (Takasago), and \( P_{pa} \) and \( P_{pv} \) converged to a given level, which was defined as double occlusion pressure (Pdo). During measurement of Kfc, ventilation was changed to CPAP 3 cm H₂O.

An increase in lung weight represented two distinct components: a rapid component caused by blood volume shifting into the pulmonary vascular system, and a slow component thought to be caused by fluid filtering across the pulmonary exchange vessels into the interstitial spaces. The initial rate of fluid filtration (\( (\Delta W/t)/(\Delta t)_{t=0} \)) can be estimated by extrapolating the slow component to zero time. As the only force acting across the capillary membrane was the change in capillary pressure (\( \Delta P_{do} \)), Kfc was calculated as:

\[ K_{fc} = \frac{(\Delta W/t)/(\Delta t)_{t=0}}{\Delta P_{do}} \]

To exclude the effect of the first phase, the second phase was selected from 3 to 6 min after zero time. Double occlusion was performed before and 7 min after the reservoir level was increased. Baseline wet lung weight was estimated at the end of the experiment by measuring the weight of the lungs and extrapulmonary tissues before perfusion and subtracting the weight of the extrapulmonary tissues.

Blood cell counts and packed cell volume measurements
Counts of white blood cells (WBC) and measurement of packed cell volume (PCV) were performed on a Celltac Counter (model ME-5158, Nihon Kohden).

Wet and dry lung weights
The left lung was excised at the end of the experiment and weighed wet. It was then dried in an oven at 60°C for 2 weeks and weighed again to determine lung water weight compared with pulmonary tissue weight (W/D), using the formula:

\[ W/D = \frac{(\text{wet} - \text{dry})}{\text{dry}} \]

Preparation of BAL fluid and analysis
At the end of the experiment, the right lung was used for bronchoalveolar lavage (BAL) fluid preparation. For this purpose, normal saline 5 ml was instilled through the trachea and then drained. The procedure was repeated four times. Fluid was centrifuged at 250 g at 4°C for 10 min. The supernatant was divided into several aliquots and stored
at –80°C until analysis. cGMP was analysed using an enzyme immunoassay (ELISA) system (code RPN 226, Amersham International, Buckinghamshire, UK). Concentrations of nitrate and nitrite were measured using a calorimetric assay kit (Cat No.7 80001, Cayman Chemical Company, Ann Arbor, MI, USA). Myeloperoxidase (MPO) was measured by enzyme assay, as described by Henson and colleagues.10

Ventilation using nitric oxide
Nitric oxide was supplied as a mixture of nitric oxide 3550 parts per million (ppm) in nitrogen (Taiyo Sanso, Osaka, Japan) and was added to the breathing circuit to produce an inspired concentration of 39–41 ppm. This concentration was determined according to our previous report; nitric oxide 40 ppm is the supramaximum concentration which completely inhibited the pulmonary hypoxic vasoconstriction response in the same model. Concentrations of nitric oxide were monitored using a nitric oxide monitor (model SC-90, TaiyoSanso) in the inspiratory limb of the circuit. Soda lime (Wako-Junyaku, Osaka) was installed in the nitric oxide delivery system to absorb nitrogen dioxide11 and the concentration of nitrogen dioxide was measured by the pilot indicator method (No. 10, Gastec, Kanagawa, Japan) to ensure that concentrations remained less than 0.2 ppm.

Experimental study
The lungs were allowed to equilibrate for 30 min to achieve an isogravimetric state and then baseline values were obtained. One of the following procedures was used. In the control group (n=6), the lungs were perfused continuously for 120 min. Measurements were performed at the same times as in the IR group. In the IR (n=6) and IR+NO (n=6) groups, after baseline measurements, ventilation and perfusion were interrupted (ischaemia), and the lungs were maintained in the humidified chamber at a temperature of 38°C for 60 min. After ischaemia, the lungs were reperfused and re-ventilated for 60 min using nitric oxide 39–41 ppm in the IR+NO group and without nitric oxide administration in the IR group. Measurements were performed at baseline, and 5, 30 and 60 min after reperfusion.

Statistical analysis
Data are expressed as mean (SEM). Within-group differences were analysed by ANOVA with repeated measures. When statistical significance was observed by ANOVA, a contrast test was performed for multiple comparisons (Super ANOVA, Abacus Concepts, Berkeley, CA, USA). Intergroup differences at the same times were analysed using ANOVA and subsequent post hoc analysis by Bonferroni/Dunn. Significance was determined when P<0.05.

Results
Under baseline conditions, vascular resistance (Fig. 1), Kfc (Fig. 2) and Pdo (Fig. 3) in the study groups were similar to those of controls. In the control group, Kfc remained stable throughout the experiment. However, control Rt, Rm and Pdo changed slightly but significantly at the end of the experiment; they were similar to those in the IR group at 60 min after reperfusion. These changes reflect the effect of the experimental set-up at 60 min.

Effect of IR on PVR, Kfc and Pdo
IR resulted in a significant increase in total PVR (Rt in Fig. 1), which consisted mostly of an increase in microvascular tone (Rm in Fig. 1). A significant increase in Ra at end of the experiment contributed partially to this, but Rv did not change with IR. Kfc also increased immediately after ischaemia, followed by a temporary decline to baseline values at 30 min, and then increased to significant levels at 60 min after reperfusion (Fig. 2). Final values of Kfc were significantly larger than those in the control or IR+NO group. However, Pdo was significantly lower at 5 and 60 min after reperfusion compared with baseline (Fig. 3).

Effect of inhaled nitric oxide on PVR, Kfc and Pdo
Reperfusion with nitric oxide ventilation inhibited the increases in Rt and Rm from 5 to 60 min after reperfusion (Fig.1). But Rt and Rm in the IR+NO group at 5 and 60 min after reperfusion were still significantly higher than after 5 min of reperfusion. This implies that inhaled nitric oxide 40 ppm partially but not completely inhibited pulmonary vasoconstriction induced by IR. Reperfusion with nitric oxide ventilation also inhibited the increase in Kfc at 30 and 60 min but not at 5 min after reperfusion (Fig. 2). Furthermore, the decrease in Pdo after reperfusion at 60 min but not at 5 min was inhibited by nitric oxide ventilation (Fig. 3).

W/D ratio
W/D ratio in the IR group increased significantly compared with controls from 5.34 (0.15) to 5.78 (0.12) g g⁻¹. W/D ratio in the IR+NO inhalation group was 5.57 (0.08) g g⁻¹ and there were no significant differences between the IR and IR+NO groups.

Circulating WBC counts
WBC count in the IR group increased significantly after 5 min of reperfusion but was lower than baseline at 60 min after reperfusion. These changes were not observed in the control or IR+NO groups (Fig. 4).

BAL fluid analysis
There were no significant differences in MPO among the three groups and in NO concentrations between the control and IR group, whereas cGMP concentrations in the IR group were significantly lower than those in the control and IR+NO groups (Table 1).
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Fig 1 Changes in pulmonary vascular resistance (PVR) in the control, IR and IR+NO groups. \( R_t \)=total PVR, \( R_a \)=arterial resistance, \( R_m \)=middle segmental resistance and \( R_v \)=venous resistance. Most changes in \( R_t \) consisted of changes in \( R_m \). \( R_t \) and \( R_m \) increased significantly during IR and were inhibited partially by inhalation of nitric oxide. There were no significant changes in \( R_a \) and \( R_v \) with IR. Data are mean (SEM) (\( n=6 \)). *\( P<0.05 \) vs baseline, †*\( P<0.05 \) vs 5 min, ‡*\( P<0.05 \) vs 30 min.

Fig 2 Changes in \( K_{fc} \) in the control, IR and IR+NO groups. \( K_{fc} \) increased significantly at 5 and 60 min after reperfusion in the IR group. Inhalation of nitric oxide inhibited the increase in \( K_{fc} \) at 60 min after reperfusion, although it did not cause such inhibition at 5 min after reperfusion. Data are mean (SEM) (\( n=6 \)). *\( P<0.05 \) vs baseline, †*\( P<0.05 \) vs 5 min, ‡*\( P<0.05 \) vs 30 min.

Fig 3 Changes in \( P_{do} \) in the control, IR and IR+NO groups. \( P_{do} \) decreased at 5 and 60 min after reperfusion in the IR group and at 120 min in the control group. Inhaled nitric oxide inhibited the decrease in \( P_{do} \) at 60 min after reperfusion. Data are mean (SEM) (\( n=6 \)). *\( P<0.05 \) vs baseline, †*\( P<0.05 \) vs 5 min.
**Discussion**

The main findings of the present study were that IR increased pulmonary microvascular resistance and capillary leakage by decreasing cGMP concentrations in BAL fluid, and that inhalation of nitric oxide during the reperfusion period prevented these impairments.

IR caused two forms of physiological impairment: increased vascular resistance and permeability. PVR increased immediately after reperfusion and remained at this level for 60 min. One of the mechanisms of vasoconstriction immediately after reperfusion is release of vasoactive substances (e.g. thromboxane A$_2$) and diminished intracellular adenosine 3',5'-cyclic phosphate (cAMP) during the ischaemic period. However, the most likely explanation of vasoconstriction is impairment of nitric oxide availability and inhibition of nitric oxide-dependent vasorelaxation after IR. Diminished nitric oxide availability, which decreases soluble guanylate cyclase activity, inhibits conversion of guanosine 5'-triphosphate (GTP) to cGMP, thereby inducing constriction of vascular smooth muscle. In the IR group, cGMP concentrations in BAL fluid were significantly lower than those in the controls. Diminished cGMP concentrations has been postulated as the fundamental mechanism of vascular dysfunction in IR lung injury, and caused endothelial dysfunction with subsequent vasoconstriction.

In a study using isolated pulmonary arterial rings, cGMP-mediated relaxation was not impaired even after cold ischaemia and reperfusion. If impairment of nitric oxide availability confers vasoconstriction by a decline in cGMP activity in pulmonary vascular smooth muscle, inhalation of nitric oxide may be beneficial to relax vascular resistance. However, in our study, we could not find direct evidence of decreased nitric oxide availability. There were no significant differences in NOx concentrations in BAL fluid between the control and IR groups. This can be explained by the sensitivity of NOx measurements from BAL fluid as an indicator of IR injury. Higher NOx concentrations may be expected in samples from lung tissues or perfusate rather than from BAL fluid. Furthermore, cGMP in vascular smooth muscle is regulated not only by nitric oxide; ischaemia itself decreases cGMP activity by inactivating cell metabolism. Therefore, decreased nitric oxide is not an essential factor of decreased cGMP activity.

cGMP concentrations in BAL fluid decreased by approximately 27% during IR. Although it is unclear if this amount is sufficient to inactivate the cGMP pathway and increase vascular resistance, approximately 50% decline in cGMP concentrations in the lung is reported after reperfusion.

However, conflicting results have been reported regarding the involvement of nitric oxide in IR-induced lung injury. Increased metabolism of nitric oxide in IR and subsequent excess production of peroxynitrite is considered the main cause of vascular endothelial injury. Based on these theoretical concerns, inhaled nitric oxide is detrimental for IR-induced lung injury. Instead of stimulation of the cGMP-dependent pathway by inhaled nitric oxide, distal stimulation of the nitric oxide–cGMP pathway by both administration of cGMP analogues to the preservation solution or inhalation of an inhibitor of cGMP-selective phosphodiesterase, have been used successfully for lung transplantation to overcome these complications.

Based on these considerations, assuming that cGMP concentrations in BAL fluid reflect the events occurring in vascular smooth muscle, it is possible that the low cGMP concentrations in vascular smooth muscle cause vasoconstriction. When these findings are considered together with increased $R_m$ and decreased $P_d$, compression of capillaries induced by accumulation of interstitial fluid could also act as another mechanism for increased resistance.

Hyperpermeability is an additional major feature of IR lung injury. The IR-induced increase in $K_f$ indicates microvascular capillary leak, which was also confirmed by the change in W/D ratio. One of the explanations of a change in $K_f$ by IR is decreased cGMP concentrations. Activation of the cAMP–protein kinase A (PKA) pathway has been shown to reverse rat lung hyperpermeability. In addition to cAMP, cGMP can also act as a potent regulator of endothelial permeability in human pulmonary artery endothelial cell monolayers. If cGMP activates the PKA pathway in pulmonary vessels, as shown by Kelley, Al
Nakkash and Drumm, it is possible that reduced cGMP concentrations in endothelial cells would increase microvascular permeability in our rabbit lung model. Inhaled nitric oxide may act as a regulator of capillary permeability. Fluid filtration is regulated not only by Kf but also by hydrostatic and oncotic pressure differences across the membrane. As a vasodilator, nitric oxide decreases filtration volume by directly decreasing capillary pressure. However, this was not the mechanism in our preparation because Pdo did not increase after IR, and did not decrease after inhalation of nitric oxide.

It is obvious that an initial key factor of IR lung injury is activation of circulating neutrophils. However, it is not clear if neutrophils were sequestered in our rabbit lungs because the appearance of activated neutrophils in the tissue was not analysed histologically, and MPO concentrations in BAL fluid did not increase during IR. WBC counts in the perfusate, however, decreased gradually after reperfusion, and this decline was prevented by inhalation of nitric oxide. These findings may indirectly indicate that inhaled nitric oxide plays a role in the regulation of neutrophil adhesion in pulmonary capillaries.

In summary, we have shown that ischaemia–reperfusion of the perfused lung impaired pulmonary vascular function and resulted in microvascular constriction and leakage. Ventilation with nitric oxide from the beginning of the reperfusion period improved pulmonary dysfunction, such as vasoconstriction and capillary leak, by restoring cGMP concentrations.

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