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Survival and graft function in a large animal lung transplant model after 30 h preservation and substitution of the nitric oxide pathway ☆

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Abstract

Objective: Substitution of the nitric oxide- (NO-) pathway improves early graft function following lung transplantation. We previously demonstrated that 8-Br-cGMP (second messenger of NO) to the flush solution and tetrahydrobiopterin (BH4, coenzyme of NO synthase) given as additive during reperfusion improve post-transplant graft function. In the present study, the combined treatment with 8-Br-cGMP and BH4 was evaluated. **Methods**: Unilateral left lung transplantation was performed in weight matched outbred pigs (24–31 kg). In group I, grafts were preserved for 30 h ($n = 5$). 8-Br-cGMP (1 mg/kg) was added to the flush solution (Perfadex™, 1.5 l, 1°C) and BH4 (10 mg/kg/h) was given to the recipient for 5 h after reperfusion. In group II, lungs were transplanted after a preservation time of 30 h ($n = 3$) and prostaglandin E_1 (250 g) was given into the pulmonary artery (PA) prior to flush. In all recipients 1 h after reperfusion the contralateral right PA and bronchus were ligated to assess graft function only. Survival time after reperfusion, extravascular lung water index (EVLWI), hemodynamic variables, and gas exchange (PaO_2) were assessed during a 12 h observation period. **Results**: All recipients in group I survived the 12 h assessment, whereas none of the group II animals survived more than 4 h after reperfusion with a rapid increase of EVLWI up to 24.8 ± 6.7 ml/kg. In contrast, in group I EVLWI reached up to 8.9 ± 1.5 ml/kg and returned to nearly normal levels at 12 h (6.1 ± 0.8 ml/kg). In two animals of group I the gas exchange deteriorated slightly. The other three animals showed normal arterial oxygenation over the entire observation time. **Conclusion**: Our data indicate that the combined substitution of the NO pathway during preservation and reperfusion reduces ischemia/reperfusion injury substantially and that this treatment even allows lung transplantation after 30 h preservation in this model.

Keywords: Nitric oxide; Lung transplantation; Ischemia reperfusion injury

1. Introduction

Endothelial dysfunction plays a key role in ischemia/reperfusion injury following organ transplantation and results in reduced production of nitric oxide (NO). NO protects the endothelium and antagonizes vasoconstrictive and thrombogenic substances as endothelin-1, platelet-activating factor, oxygen free radicals, or hypoxia [1]. The balance of protective and proinflammatory agents determines endothelial cell integrity during reperfusion after pulmonary ischemia. NO is essential for numerous endothelium-dependent mechanisms such as vasodilation, modulation of neutrophil adhesion, and platelet aggregation, as well as for the maintenance of endothelial barrier properties [2]. During reperfusion, the NO availability is reduced and the lack of NO accelerates graft dysfunction. It may be substituted by the application of NO donors, by NO inhalation, or by the substitution of other steps of the NO synthesis (Fig. 1). However, most of these methods are not suitable for clinical application.

In previous studies, we demonstrated that the 8-Br-cGMP, a membrane permeable analogue of cGMP, or tetrahydrobiopterin (BH4), an essential coenzyme of the NO synthases (NOSs), either given in the flush or during reperfusion improves post-transplant graft function. Addition of the 8-Br-cGMP to the flush solution [3] was superior to continuous infusion of the analogue during reperfusion [4]. Furthermore, continuous treatment of the recipient

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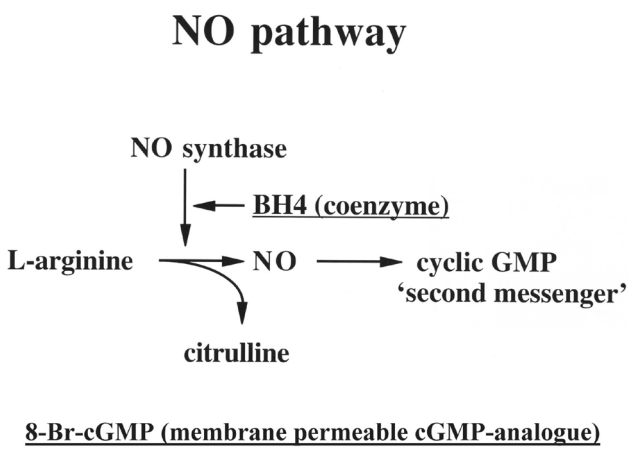

Fig. 1. NO pathway [22]. BH4 is an essential coenzyme of the NOSs, 8-Br-cGMP a non-toxic membrane permeable cGMP analogue.

with BH4 during the first hours after reperfusion increased pulmonary venous cGMP up to ten-fold and improved graft function impressively [5].

As a consequence of these experiments, we evaluated the effect of combined treatment with the 8-Br-cGMP as additive to the flush solution and continuous infusion of BH4 to the recipient during reperfusion on early graft function in a large animal model of unilateral lung transplantation.

2. Materials and methods

Eleven weight matched pairs of outbred pigs served as donors and recipients. Harvest and left lung transplantation were performed as previously reported [3–6]. Lungs were flushed with 1.5 l of low potassium dextran (LPD) solution (Perfadex, generously provided by XVIVO AB, Uppsala, Sweden) and stored at a temperature of 1°C for 30 or 6 h, respectively. One hour after reperfusion, the right contralateral lung was excluded from perfusion and ventilation. All animals received humane care in compliance with the European Convention on Animal Care. The protocol was approved by the local animals study commitee.

2.1. Study groups

Three groups were studied. In group I ($n = 5$), 8-Br-cGMP (1 mg/kg, Sigma Chemicals, Switzerland) was added to the flush solution. In addition, BH4 (10 mg/kg/h, intravenously (i.v.)) was given to the recipient as continuous infusion for 5 h after reperfusion, starting 15 min before reperfusion. In group II ($n = 3$), 8 μg/kg prostaglandin E_1 (PGE_1) (Prostin VR Pediatric, The Upjohn Company, Kalamazoo, MI, USA) was injected directly into the main pulmonary artery (PA) before the flush as in clinical use. Preservation time of groups I and II donor lungs was 30 h. As additional control group (group III), three animals were transplanted after 6 h of cold ischemic preservation, and PGE_1 (8 μg/kg) was given prior to flush as in group II.

2.2. Assessment

One hour after reperfusion of the transplanted lung the right pulmonary arteries and the right main bronchus were ligated to assess allograft function for the first 12 h after reperfusion. During the assessment period, anesthesia was maintained with fluothane 1.5%. FiO_2 was 100%, the ventilation volume 5.5 l at a respiratory rate of 20 per min and a positive endexspiratory pressure (PEEP) of 5 mm H_2O. Systemic arterial, PA, central venous, and left atrial pressure were recorded continuously. Arterial and mixed venous blood were collected for gas analysis every 60 min.

At the end of the observation period, 12 h after reperfusion, the animals were sacrificed. In group I and group III animals that survived the entire assessment, the upper lobe allograft samples were submitted to histologic examination, tissue myeloperoxidase (MPO), and thiobarbituric acid-reactive substance (TBARS) assay. Normal lung tissue was excised from the flushed upper lobe of each donor, snap frozen and assessed as the allograft samples.

All values are given as the mean ± standard deviation (in the figures standard error) of the mean (SEM).

2.3. Extravascular lung water (EVLW)

EVLW as direct assessment of reperfusion edema was measured as previously described [6]. A fiberoptic catheter (System Cold Z-021, Pulsion, Munich, Germany) is advanced via the external carotid artery into the descending aorta. The indicator bolus consists of two components: indocyanine green serves as intravascular marker and ice cold 5% glucose as a thermal intra- and extravascular indicator. The bolus is injected via the external jugular vein with a temperature-controlled injector. The dilution curves for dye and temperature are recorded simultaneously in the descending aorta with the thermistor tipped fiberoptic catheter. Thoracic intra- and extravascular fluid volumes are determined based on the measurement of the mean transit times for thermal and dye indicators and of the decay time volumes calculated from the indicator dilution curves. The lung water computer (System Cold Z-021, Pulsion, Munich, Germany) determines the mean transit time for the thermal indicator and for the dye indicator and calculates total thermal volume (ITTV), intrathoracic blood volume (ITBV), and extravascular thermal volume (ETV). The ETV is calculated as follows: ETV = ITTV − ITBV. All measurements were made in triplicate. The mean value was used for analysis.

2.4. MPO assay

Donor and recipient lung samples were frozen immediately and stored at −80°C until assay. Quantitative MPO activity was determined using routine methods as previously described [6]. The frozen lung tissue (100 mg) was homogenized in 1 ml of 0.5% hexadecyltrimethylammonium bromide, 5 mmol/l EDTA, and 50 mmol/l

potassium phosphate buffer (pH 6.2) with a tissue grinder. The homogenate was centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was assayed for total soluble protein and MPO activity. Enzyme activity was measured spectrophotometrically. Five-fold supernatant (10 mg) was combined with 0.6 ml Hank's bovine serum albumin (BSA), 0.5 ml of 100 mmol/l potassium phosphate buffer (pH 6.2), 0.1 ml 0.05% H_2O_2, and 0.1 ml of 1.25 mg/ml *o*-dianisidine. The reaction was stopped by the addition of 1% NaN_3 after 5 and 20 min, respectively, at room temperature. The optical density was measured at 460 nm with a spectrophotometer (Kadas 100, Dr Lange AG, Zurich, Switzerland). Color development from 5 to 20 min was linear. Enzyme activity is expressed as change in optical density units per milligram of tissue protein per minute (ΔOD/mg/min).

2.5. TBARS assay

TBARS levels in lung tissue were measured with 10% wet weight per volume homogenate [6]. Aliquots (0.2 ml) of this homogenate were added to tubes containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% solution of thiobarbituric acid. The mixture was brought to a volume of 4 ml by the addition of distilled water, heated at 95°C for 60 min, and then cooled with tap water. Distilled water (1 ml) and 5 ml of butanol/pyridine (15:1) were added (all chemicals by Fluka AG, Switzerland). The solution was centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm with a spectrophotometer (Kadas 100, Dr Lange AG, Zurich, Switzerland). The TBARS levels were determined by reference to a standard curve of 1,1,3,3-tetramethoxypropane (Sigma Chemicals, Buchs, Switzerland), and the results were expressed as picomoles of malondialdehyde (MDA) per gram of wet lung.

2.6. Hemodynamic parameters and gas exchange

Systemic arterial, PA, central venous, and left atrial pressure were recorded continuously with a hemodynamic monitor system (Hellige, Freiburg, Germany). Measurement of cardiac output is necessary for the lung water assessment (System Cold Z-021, Pulsion, Munich, Germany). Pulmonary vascular resistance (PVR) was calculated according to the formula $PVR = 80 \times (MPAP - PCWP)/CO$ (MPAP, mean pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output). Arterial and mixed venous blood were collected for gas analysis every 60 min. For assessment of PaO_2, an automatic blood gas analyzer (AVL 993, AVL List GmbH, Graz, Austria) was used.

3. Results

3.1. Characteristics of experimental groups

No differences between the groups were noted in donor weight (group I 29.3 ± 2.5 vs. group II 27.7 ± 3.1 vs. group III 28.6 ± 1.6 kg), recipient weight (group I 28.7 ± 2.7 vs. group II 27 ± 2.8 vs. group III 26.5 ± 0.5 kg), and warm ischemic time (group I 70 ± 7 vs. group II 67 ± 5; group III 66 ± 7 min). Total preservation time did not differ in group I and group II (group I 30.01 ± 0.3 vs. group II 29.98 ± 0.43; group III 5.86 ± 0.07 h).

3.2. Survival

In treatment group I, all five recipients survived the 12 h assessment period with good pulmonary function. In control group II, two animals died within 2 h, and one 3 h after occlusion of the right native lung developing severe pulmonary edema and subsequent right heart failure. In control group III all recipients survived the entire procedure.

3.3. Reperfusion edema

Extravascular lung water index (EVLWI) in the allografts of group I animals rose to 8.9 ± 3.4 ml/kg and returned to nearly normal levels at the end of the assessment (6.1 ± 1.8 ml/kg). In group II animals, EVLWI showed a rapid increase up to 24.8 ± 11.6 ml/kg. In group III, normal levels of EVLWI (5 ± 1.7 ml/kg) were measured over the entire observation period (Fig. 2).

3.4. Gas exchange and hemodynamic parameters

In two of the treated recipients the gas exchange deteriorated slightly. The other three animals showed normal arterial oxygenation over the entire observation time (Fig. 3).

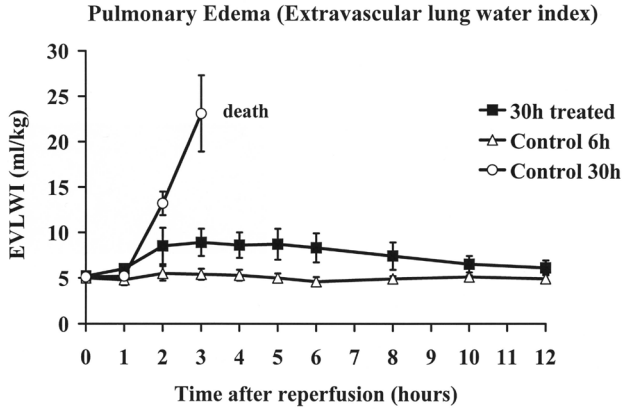

Fig. 2. EVLW as measurement for pulmonary edema. EVLW in the treated pulmonary allografts (group I) rose to 8.9 ± 1.5 ml/kg and returned to nearly normal levels at the end of the assessment (6.1 ± 0.8 ml/kg). None of the untreated animals with grafts preserved for 30 h (group II) survived more than 4 h after reperfusion with a rapid increase of EVLWI up to 24.8 ± 6.7 ml/kg (mean ± SEM).

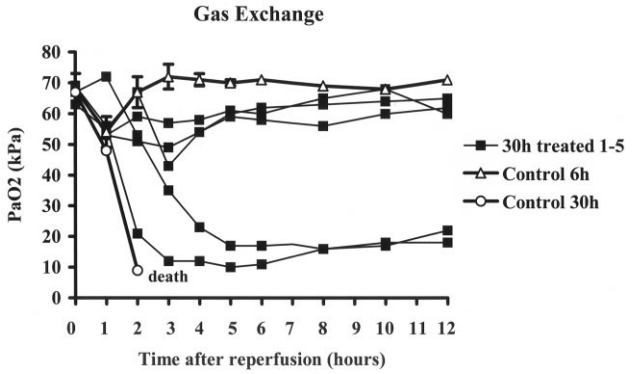

Fig. 3. Arterial oxygenation (PaO_2) over the first 12 h. In two of the treated recipients the gas exchange deteriorated slightly. The other three animals showed normal arterial oxygenation over the entire observation time (group I) (mean ± SEM).

The PVR increased at the time of exclusion of the right native lung in all groups. In groups I and III, the PVR remained constant during the assessment at levels around 700–800 dyn/s/cm^{-5} in contrast to group II animals, in which the PVR rose to values over 1000 dyn/s/cm^{-5} (Fig. 4).

3.5. Neutrophil migration

Allograft MPO activity 12 h after reperfusion was comparable in groups I and III (group I 2.2 ± 0.4 vs. group III 2.3 ± 0.7 ΔOD/mg/min). The MPO activity in normal flushed lung tissue (snap frozen without storage) was 0.5 ± 0.2 ΔOD/mg/min (Fig. 5).

3.6. Lipid peroxidation

Free radical tissue damage expressed as MDA concentration per gram wet lung was not different between groups I and III (group I 69.2 ± 37.8 vs. group III 50.2 ± 27.9 pmol/g). The MDA concentration in normal lung tissue (snap frozen without storage) was 43 ± 10.1 pmol/g (Fig. 6).

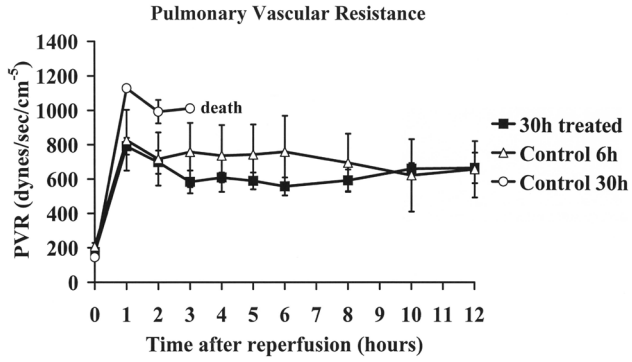

Fig. 4. PVR increased at the time of exclusion of the right native lung, but the changes were similar between treated animals (group I) and 6 h controls (group III) (mean ± SEM).

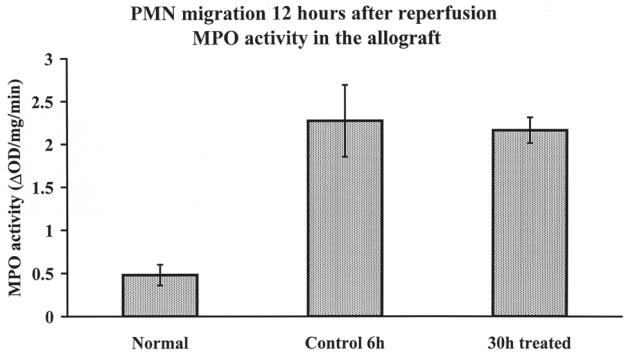

Fig. 5. Polymorphnuclear lymphocytes (PMN) migration measured as MPO activity in the graft tissue of surviving recipients shows similar levels in the treated animals (group I) and 6 h controls (group III) at the end of the assessment. Normal, normal flushed lung tissue (mean ± SEM).

4. Discussion

NO substitution with 8-Br-cGMP in the flush solution and BH4 given during reperfusion allows lung transplantation after 30 h of graft preservation in this model. The injury in the treated grafts was comparable to that of the control group with a preservation time of 6 h. None of the untreated animals with 30 h preservation time survived more than 4 h after reperfusion and all showed rapid development of severe reperfusion injury.

Ischemia and reperfusion are accompanied by an intracellular decrease of cAMP and cGMP. cAMP is the second messenger of PG and cGMP of NO. Both mediate vasodilation and play a major role in the modulation of the vascular tone in the lung, as well as in the regulation of the endothelial permeability and neutrophil adhesivity [2]. Our previous study [3], in accordance with the study of Bhabra et al. [7], suggests that the substitution of the NO/cGMP pathway is more important than substitution of the PG/cAMP pathway.

A series of experiments shows that the enhancement of local NO levels improves organ function after lung transplantation [1,2,8,9]. NO has been substituted in experimental or clinical studies by the administration of exogenous L-

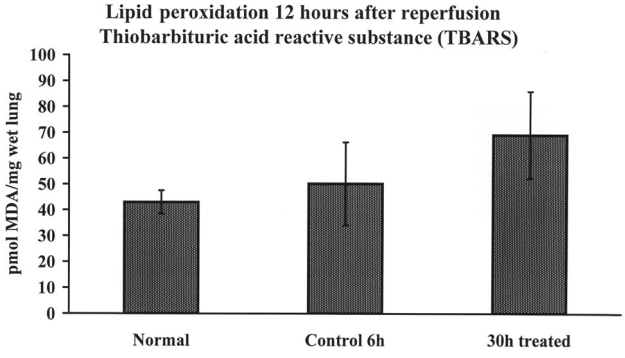

Fig. 6. Lipid peroxidation in the graft tissue of surviving recipients as measurement for the free radical injury. TBARS levels in the allograft 12 h after reperfusion were comparable between the survivors (groups I and III) (mean ± SEM).

arginine [10], NO donors [11], or NO inhalation [12,13]. Application of the NO donors is mainly limited by systemic side effects (e.g. hypotension). NO inhalation is used in a wide area of clinical settings to reduce ischemia reperfusion injury; however, the application in clinical practice is technically cumbersome and expensive. Therefore, we developed new strategies to substitute the NO pathway in lung transplantation.

BH4 has specific properties to qualify as the limiting step for NO synthesis in dysfunctional endothelium following ischemia and reperfusion. It increases the affinity of NOS to L-arginine [14], it stabilizes NOSs [15], and it inhibits the negative feedback of NO on NOSs [16]. In the absence of BH4, NOSs no longer produce NO, but catalyze reactions leading to oxygen free radical production [17]. In addition, BH4 has a very low toxicity [18] and can be administered even in high doses simply by the i.v. route.

8-Br-cGMP is a non-toxic membrane permeable and enzyme resistant analogue of cGMP with equal biological effects and has been used previously to reduce ischemia/reperfusion injury [2,3,19–21].

The two substances, 8-Br-cGMP and BH4, were applied based on the following theoretical reflections. During perfusion with the cold preservation solution, temperature in the graft decreases rapidly and therefore stimulation of NO synthesis with substrates of NOS seems to be useless, but the administration of cGMP as 'second messenger' of NO can be applied in the flush solution and is effective in the very early phase of reperfusion. Supplementation of the BH4 during reperfusion increases the NO synthesis; however, this accelerated NO synthesis is still regulated endogenously, and side effects of exogenous NO may be avoided. The BH4 even seems to be suitable for clinical application as it has a long half-life and an extremely low toxicity [18].

Pinsky et al. showed that intracellular cGMP levels decrease sharply at the time of reperfusion. The constitutional NO synthase (cNOS) itself remains essentially unaffected by either preservation and reperfusion; however, NO production is decreased and consumption during reperfusion is increased due to rapid reaction of NO with oxygen free radicals [2]. A 'saturation' with cGMP during the flush and enhancement of NO production in the first phase of reperfusion would be the logical conclusion.

Survival of the animals after reperfusion demonstrates clearly that a preservation time of 30 h in the control group without treatment did not result in sufficient graft function. We therefore decided to limit the number of animals in the control groups.

The intragroup variety in arterial oxygenation in the treated recipients of group I (Fig. 3) confirms our hypothesis from former studies that the gas exchange is not a good parameter to assess lung injury in this model. In spite of the fact that PaO_2 in many studies is used as the main parameter for reperfusion injury, the lung water computer employed in this model allows us to assess hemodynamic parameters and the dynamic changes of the lung edema very precisely.

We employed an observation period of 12 h after reperfusion to observe changes after the 5 h treatment with BH4. No deterioration of lung function after cessation of the BH4 infusion was noted, which underlines the important role of NO in the early phase after reperfusion. Lung edema in animals with NO substitution increased slowly over the first 5 h after reperfusion. In contrast to untreated animals, after only 20 h preservation, the peak of pulmonary edema was already reached 2–3 h after reperfusion [3–5]. From the present study and from previous experiments with the aim of optimizing strategies to enhance NO in lung transplantation, we could conclude that substitution of the NO pathway during both preservation and reperfusion is important.

Our data confirm that combined substitution of the NO pathway during preservation and reperfusion reduces ischemia/reperfusion injury, and indicates that the treatment with 8-Br-cGMP and BH4 seems to allow prolongation of the preservation time to 30 h in this model. This strategy may open new perspectives for clinical lung transplantation.

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