Protective agents used as additives in University of Wisconsin solution to promote protection against ischaemia–reperfusion injury in rat lung

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Abstract

1. An intervention to reduce ischaemia–reperfusion lung injury will be an important advance in transplant medicine. Although the mechanisms associated with producing ischaemia–reperfusion endothelial injury have not been completely elucidated, many of the injury mediators have been studied in detail. While no single pharmacological therapy is likely to be totally effective in eliminating this complex injury, we have developed a mixture of agents that are known to block pathways involved in producing ischaemia–reperfusion-associated lung vascular injury.

2. The present study modified University of Wisconsin solution (UW) by adding one of the protective agents prostaglandin E₁ (PGE₁), dexamethasone (Dex) or dibutyryl cAMP (Bt₂-cAMP), or a combination of these, to the perfusate of rat lungs exposed to 4 h of cold ischaemia followed by 1 h of reperfusion. Nine modified UW solutions were studied: (1) UW ± Dex, (2) UW ± PGE₁, (3) UW ± Bt₂-cAMP, (4) UW ± Dex × 3, (5) UW ± PGE₁ × 3, (6) UW ± Bt₂-cAMP × 3, (7) UW ± Dex + PGE₁, (8) UW ± Dex + Bt₂-cAMP, (9) UW ± PGE₁ + Bt₂-cAMP. These solutions were utilized in individual experiments to assess haemodynamic changes, lung weight gain, the capillary filtration coefficient ($K_{fc}$) and pathology in all lungs.

3. The results indicate that lung weight gain and $K_{fc}$ values were significantly lower than with UW alone in groups 1, 2 and 3, which contained only one additional protective agent. In groups 4, 5 and 6, which contain three times the concentration of each protective agent, both $K_{fc}$ and lung weight gain were similar to those measured in groups 1, 2 and 3, i.e. lungs were protected but the protection was not dose dependent. In groups 7, 8 and 9, which contained two protective agents, lung weight gain and $K_{fc}$ were greatly reduced compared with UW alone. Histopathological studies showed similar decreases in the injury profiles of lungs.

4. Although UW contains several antioxidant protective agents such as allopurinol and glutathione, it did not provide effective protection in our ischaemia–reperfusion lung injury model. UW modified with an additive of PGE₁, Dex or Bt₂-cAMP attenuated ischaemia–reperfusion injury. Furthermore, UW containing two of these protective agents augmented the protection. Among

Key words: dibutyryl cAMP, dexamethasone, ischaemia–reperfusion lung injury, prostaglandin E₁, University of Wisconsin solution.

Abbreviations: Bt₂-cAMP, dibutyryl cAMP; Dex, dexamethasone; I/R, ischaemia–reperfusion; LWG, lung weight gain; PGE₁, prostaglandin E₁; TNF, tumour necrosis factor; UW, University of Wisconsin solution.

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INTRODUCTION

None of the clinical lung preservation solutions now used in transplant laboratories provides reliable preservation of human lung allografts for longer than 6 h [1,2]. The susceptibility of the lung tissue to ischaemia–reperfusion (I/R) injury obviously makes lung preservation difficult since reperfusion must occur after the implant [3,4]. Since reduction in I/R injury would greatly improve early lung function after transplantation, we have designed an I/R injury study using perfused rat lungs to evaluate the protective effects of various other antioxidants and substances known to both protect and reverse endothelial damage associated with different experimental I/R models. The University of Wisconsin solution (UW) was developed by Wahlberg and colleagues in 1986 [5], and has been successfully used to preserve the function of the pancreas, kidney, liver and even the heart before transplantation. The protective effects of UW are at least equivalent to the modified Euro-Collins solution used by the lung transplantation group at Pittsburgh [6].

Our previous studies have shown that UW partially attenuates our model of normothermic I/R lung injury and reduces the release of tumour necrosis factor (TNF)-α [7]. In the present study, we found that prostaglandin E₁ (PGE₁), dexamethasone (Dex) or dibutyryl cAMP (Bt₂-cAMP) in combination with UW solution provided additional protection against a hypothermic I/R lung injury compared with UW alone. Our basic hypothesis is that a larger number of protective agents will interfere with more injurious pathways and be more protective in preventing I/R injury associated with organ storage and transplantation. In another well-established cold I/R rat lung model [8,9], either PGE₁, Bt₂-cAMP, Dex or a combination of these compounds, when added to UW, attenuated the degree of lung injury.

METHODS

Preparation of isolated and perfused rat lungs

Our isolated perfused lung in situ I/R model has been described previously [8,9]. Briefly, male Sprague–Dawley rats (250–350 g body weight) were anaesthetized with sodium pentobarbital (20–25 mg intraperitoneally). A tracheotomy was performed to permit ventilation with a Harvard rodent ventilator (Model 683) at 55 breaths/min, at a tidal volume of 2.5 ml, and a positive end-expiratory pressure of 2 cmH₂O. The inspired gas mixture contained 5% CO₂ and 95% air. After median sternotomy was performed, heparin (1 unit/g) was injected into the right ventricle. Blood was drawn from the right ventricle and preserved until needed for use with the preservation solution. Ten millilitres of blood was mixed with 15 ml of the selected perfusate in the venous reservoir and used for lung perfusion. A cannula was placed into the pulmonary artery through a puncture in the right ventricle, and a tight ligature was placed around the main trunk of the pulmonary artery. A large catheter was inserted into the left atrium through the left ventricle and mitral valve, fixed by ligature at the apex of the heart, and used to divert the pulmonary venous outflow into a reservoir. A third ligature was placed above the atrioventricular junction to prevent perfusate flowing back into the ventricles. The lungs were perfused with the chosen perfusate using a peristaltic pump (Minipulse 2, Gilson Medical Electronics, Meddleton, WI, U.S.A.) at a constant flow of 0.03 ml·min⁻¹·g⁻¹ body weight. An initial 75 ml of lactate Ringer solution perfusate, which contained residual blood cells and plasma, were discarded and not recirculated. An additional 25 ml of the chosen perfusate were recirculated in the lung with the temperature held at 4 °C. Pulmonary artery (Ppa) and pulmonary venous (Ppv) pressures were continuously monitored with pressure transducers (Statham P23 ID) from a side-arm of the inflow and outflow cannulas and continuously recorded on a polygraph recorder (Gould Instruments, Cleveland, OH, U.S.A.). The Ppa was set at 2.5 mmHg by adjusting the height of the venous outflow reservoir and zone III flow conditions (arterial > venous > alveolar pressures) were maintained in all experiments.

The isolated perfused lung remained in situ, and the weight of the whole rat was monitored on an electronic balance and recorded on an oscillograph after digital-to-analog conversion. Any change in the preparation weight (body weight) was considered a result of changes in lung weight [9]. The isolated lung preparation described above had to meet three criteria before continuing the experiment: (1) no leakage was observed at the sites of cannula insertion, (2) no evidence of oedema was present, and (3) the lung attained an isogravimetric state, i.e. the lung was neither gaining nor losing weight.

Perfusates

Several perfusates were used: UW (DuPont-Merck Pharmaceuticals, Wilmington, DE, U.S.A.), which is composed of 50 g/l pentastarch, 35.83 g/l lactobionic acid, 3.4 g/l monobasic potassium phosphate, 1.23 g/l mag-
nesium sulphate heptahydrate, 17.83 g/l raffinose pentahydrate, 1.34 g/l adenosine, 0.136 g/l allopurinol and 0.922 g/l glutathione. Perfusate osmolarity was 320 mosmol, sodium concentration was 29 mmol/l, potassium concentration was 125 mmol/l and pH was 7.4 (Table 1). Nine types of modified UW solution were prepared as described below by the addition of one or a triple concentration of the same protective agents or a combination of any two as follows: (1) UW + Dex (0.04 g/l), (2) UW + PGE₃ (20 µg/l), (3) UW + Bt-cAMP (1 mg/l), (4) UW + Dex × 3 (0.12 g/l), (5) UW + PGE₃ × 3 (60 µg/l), (6) UW + Bt-cAMP × 3 (3 mg/l), (7) UW + Dex + PGE₃, (8) UW + Dex + Bt-cAMP and (9) UW + PGE₃ + Bt-cAMP.

### Measurement of microvascular permeability

The pulmonary capillary filtration coefficient ($K_{pc}$) was used as an index of the microvascular permeability to solvent. The $K_{pc}$ was measured using a method described previously and has been used as an index of permeability in many published studies [11]. Briefly, after an isogravimetric state was attained in the lung, $P_{pa}$ was rapidly elevated to 6–8 cmH₂O for 15 min. The increase in lung weight was recorded. The recording shows a characteristic rapid weight gain (vascular filling) which is followed by a slower rate of weight gain. The rate of weight change ($\Delta W/\Delta t$) occurring in the 6- to 14-min interval was analysed using linear regression of the log-transformed $\Delta W/\Delta t$ calculated at each minute. The initial rate of weight gain was then determined by extrapolation of $\Delta W/\Delta t$ to zero time. $K_{pc}$ was then calculated by dividing $\Delta W/\Delta t$ at time 0 by the change in $P_{pa}$ that was imposed after venous outflow pressure was increased. The $K_{pc}$ value was normalized using the baseline wet lung weight and expressed as ml min⁻¹ cmH₂O⁻¹ 100 g⁻¹ lung tissue.

### Experimental protocols

The rats were divided into 10 groups with UW as control ($n = 4$) and the following nine different perfusates: (1) UW + Dex ($n = 4$), (2) UW + PGE₃ ($n = 4$), (3) UW + Bt-cAMP ($n = 4$), (4) UW + Dex × 3 ($n = 4$), (5) UW + PGE₃ × 3 ($n = 5$), (6) UW + Bt-cAMP × 3 ($n = 4$), (7) UW + Dex + PGE₃ ($n = 4$), (8) UW + Dex + Bt-cAMP ($n = 4$), and (9) UW + PGE₃ + Bt-cAMP ($n = 5$). The isolated lungs were perfused with one of the perfusates described above. The closed system of circulation was maintained at constant flow, volume and temperature. The experiment began after a haemodynamic stability period of 15 min. The protocol used to produce I/R lung injury was as follows: the isolated lung was neither ventilated nor perfused for 4 h and during this period the entire rat was kept in a room at 4 °C (cold ischaemia). This hypothermic ischaemia was then followed by the reinstitution of ventilation and perfusion (reperfusion) for 60 min at room temperature.

### Lung histopathology

After termination of each experiment, the whole lungs were dissected and immediately fixed in 10% neutral buffered formalin. After fixation the right middle lobes were dehydrated through a graded series of alcohol, cleared in xylene and embedded in paraffin. All sections were cut at 5 µm and stained with haematoxylin and eosin.

### Statistical analysis

Values are expressed as means ± S.D. Comparisons among all groups for a given variable were carried out using a one-way analysis of variance and Dunnett’s
method. Comparisons between baseline and post-reperfusion values within each group for given variables were made by using paired Student’s $t$-test and $P < 0.05$ was considered a statistically significant difference.

**RESULTS**

**Lung weight gains**

In the modified UW groups there was a reduction in post-reperfusion lung weight gain (LWG) compared with the UW perfusate group. In groups 4, 5 and 6, a 3-fold concentration of the protective agent also caused a reduction of LWG similar to that seen with a lower concentration. However, in groups 7, 8 and 9, in which UW was combined with two additives in the perfusate, LWG was significantly less than for UW containing one protective agent (groups 1–6) (Table 2 and Figure 1).

**Capillary filtration coefficient ($K_{fc}$)**

In comparison with the UW group, all modified UW perfusates produced a significant reduction in microvascular permeability at 60 min after reperfusion, as shown by a smaller $K_{fc}$. The $K_{fc}$ of group 9 (UW + PGE$_1$-Bt$_2$-cAMP) was significantly less than those of groups 2, 4 and 5 (Table 2).

**Haemodynamics**

After I/R challenge, a significant increase in $P_{pa}$, $P_{pc}$, $R_a$ and $K_r$ occurred in UW perfused groups, but there were no significant changes in other groups (Table 3).

**Histological findings**

In the UW group, marked perivascular oedema, focal intra-alveolar haemorrhage, interstitial infiltrate, proteinous exudate and intra-alveolar debris were identified (Figure 2A–C). In the other groups, all these histological findings were alleviated remarkably (Figure 2D and 2E), especially in groups 8, 9 and 10, where lung tissue appeared near normal except for slight perivascular oedema (Figure 2E).

**DISCUSSION**

In the present study it is evident that the addition of PGE$_1$, dexamethasone or Bt$_2$-cAMP to UW solution produced significantly less endothelial damage and oedema formation compared with UW alone in lungs subjected to cold ischaemia (4°C) for 4 h and then reperfused. These results are similar to recent findings in which the same three anti-inflammatory compounds were added to UW in our I/R models, but at 1 h ischaemia at room temperature [7]. It also appears from the present study that UW + PGE$_1$-Bt$_2$-cAMP is the best preservation solution to use for lungs exposed to I/R injury.

Hypothermic I/R produces endothelial damage similar to that seen in other I/R rat lung models [7,12], and the $K_{fc}$, LWG and histopathology are consistent with inflammatory lung damage. Similarly to previous reports [7,12], an increased vascular permeability, reflected by the increased $K_{fc}$, pulmonary hypertension and oedema, occurred in cold I/R injury. In addition, leucocyte infiltration was observed in the lung tissue.

Hypoxia induces tissues macrophages to release pro-inflammatory cytokines [7,13–18]. Cytokines mediate polymorphonuclear neutrophil adherence to endothelial cells which release oxygen radicals, beginning a complex phenomenon that results in vascular injury [19–22] and infiltration of polymorphonuclear neutrophils into both the interstitium and alveoli. This sequence is followed by additional inflammatory cells being recruited into interstitial spaces and alveoli. Although it is clear that neutrophil–endothelial interactions regulated by both humoral and local mediators are necessary for the I/R injury [23], it is well known that oxygen-derived free radicals, proteases, cytokines, eicosanoids, complement activation products, platelet-activating factor and nitric oxide are involved as signalling and effector molecules related to the lung endothelial damage [24]. Therefore, the aim of all storage perfusates is to inhibit
multiple steps in the development of I/R injury [24]. Theoretically, a combination of several known protective agents should act synergistically and greatly lessen or even prevent I/R injury. UW solution contains several protective substances which attenuate I/R endothelial injury, as shown in previous studies [7]. Glutathione and allopurinol contained in UW prevent and reduce cytotoxic injury from oxygen free radicals [25–27]. The mechanisms by which these compounds attenuate I/R injury remain unclear. The mechanisms of action of Dex, PGE, and Bt₂-cAMP were not evaluated in our study, but it is
known that: (1) Dex, a phospholipase $A_2$ inhibitor, suppresses neutrophil function and cytokine production and reduces the formation of leukotrienes [28,29]; (2) PGE$_1$ causes vasodilatation [30,31], bronchodilation [32], less aggregation of platelets and leucocytes [31], the suppression of TNF [7,33], immunosuppression [33–35], and produces a variety of ‘cyto-protective’ effects [36–38]. In an orthotopic rat lung transplant model, Naka et al. [39] showed that vasodilatation alone is insufficient to enhance lung preservation and that PGE$_1$ promotes lung preservation by stimulating cAMP-dependent protein kinase and promoting non-vasodilatory mechanisms of pulmonary protection. Importantly, previous I/R lung studies have shown that the increased microvascular permeability associated with I/R can actually be reversed by cAMP [40,41]. A possible explanation for the cAMP effect is that the endothelial cytoskeleton retains its normal state, acting through a phospholipase, which results in the cell junctional sizes retaining their integrity in microvascular vessels [42]. Goodman et al. [43] have shown an up-regulation of transport from air spaces to interstitium which could also contribute to the beneficial effect of Bt$_2$-cAMP on total LWG but not $K_{F}$. In previous studies we also found that Dex, PGE$_1$ or Bt$_2$-cAMP

**Figure 2** Histological findings

Left lungs of rats in the UW group showed (A) marked perivascular oedema ($\times 80$), (B) focally marked intra-alveolar haemorrhage and proteinous exudate ($\times 80$), and (C) focally interstitial leukocytic infiltrate and intra-alveolar debris including macrophages and pneumonocytes ($\times 160$). (D) Left lungs of rats in the UW + Dex group demonstrated mild perivascular oedema and slight intra-alveolar haemorrhage. The interstitial leukocytic infiltrate, intra-alveolar debris and proteinous exudate are all inconspicuous ($\times 80$). (E) Left lungs of rats in the UW + PGE$_1$ + Bt$_2$-cAMP group had a normal appearance except for mild perivascular oedema ($\times 80$).
reduced TNFα release, and a significant correlation existed between TNFα and $K_r$, and TNFα and LWG [7].

Our results demonstrate that UW containing two protective agents gave greater protection against I/R injury than UW with one additive. Among the modified solutions, it appeared that UW + PGE, + Br- cAMP protected the lungs to a greater extent than all other solutions used in our study. We hypothesize that inactivation of pathways associated with I/R injury is more effective in attenuating the endothelial damage which occurs in reperfusion injury, and the modified perfusate should prove useful in preventing the inflammatory response associated with the storage of organs for long periods of time and subsequent reperfusion.

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