Preservation solution supplemented with biliverdin prevents lung cold ischaemia/reperfusion injury

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Abstract

OBJECTIVES: Biliverdin (BV), one of the byproducts of heme catalysis through the heme oxygenase system, is a known scavenger of the reactive oxygen species. We hypothesized that adding BV to the perfusate and cold storage solution could protect rat lung grafts from oxidative injuries via its antioxidant efficacies.

METHODS: Orthotopic left lung transplantation was performed in a syngenic Lewis-to-Lewis rat combination under 100% oxygen. Grafts were preserved in low-potassium dextran (LPD; Perfadex) at 4°C for 6 h with or without supplementation of 1 or 10 μM of BV into LPD.

RESULTS: Prolonged cold storage and reperfusion resulted in a considerable deterioration of graft functions associated with massive apoptosis in the grafts after reperfusion. The untreated grafts exhibited the early up-regulations of mRNA for inflammatory mediators and an increase in a marker of lipid peroxidation, showing oxidative injuries. Although BV supplementation of LPD at a lower concentration (1 μM) did not improve the graft gas exchange, the grafts treated with BV (10 μM) showed a significant improvement of oxygenation and less inflammatory responses as well as reduced lipid peroxidation and apoptosis. Although the rapid activations of mitogen-activated protein kinases (MAPKs) were seen 30 min after reperfusion in the grafts stored in control LPD, BV treatment significantly reduced phosphorylated-MAPK protein expression.

CONCLUSIONS: This study demonstrates that the exposure of the lung grafts to BV during cold storage can impart potent cytoprotective effects to lung cold ischaemia/reperfusion injury and significantly improve the lung graft function following extended cold preservation and transplantation by the mechanism of a reduction in oxidative injury and following inflammatory events.

Keywords: Lung transplantation • Ischaemia reperfusion injury • Cold preservation • Biliverdin

INTRODUCTION

Lung transplantation is the ultimate treatment for patients with end-stage lung disease. However, primary graft dysfunction due to ischaemia/reperfusion (I/R) injury, occurring with an incidence of ~10–20%, remains a major complication of lung transplantation and significantly contributes to the 30-day mortality risk [1]. Furthermore, I/R injury results in an increased risk of an acute rejection of the lung allograft, as well as a chronic deterioration of the lung allografts such as bronchiolitis obliterans syndrome, contributing to survival declines at a linear rate of 1-year post-transplant [2]. I/R injury is unpredictable, occurring in even allografts from younger or non-extended criteria donors [1, 3]. Therefore, the prevention of cold I/R injury could have significant beneficial effects in improving the short- and long-term lung allograft outcomes. Although all the underlying mechanisms that may be involved in the lung cold I/R process are not known, the generation of reactive oxygen species (ROS) due to aerobic metabolism during I/R injury is considered one of the mechanisms of cellular injury by peroxidizing membrane lipids, oxidizing DNA and denaturing enzyme proteins, followed by the activation of inflammatory responses.

Biliverdin (BV) is a bile pigment formed as a byproduct of heme breakdown through the heme oxygenase (HO) system [4]. BV, a water-soluble bile pigment, is immediately reduced to bilirubin by BV reductase, which is present in large functional excess in all mammalian tissue [5]. This reaction can be seen when a bruise changes colour from purple (heme) to green (BV), and yellow (bilirubin). Although these bile pigments are associated with the risk of a developing neurological dysfunction
due to a preferential deposition of bilirubin and its toxic effects on cellular functions, these byproducts of heme degradation such as BV and bilirubin play beneficial roles as powerful antioxidants and contribute to the cellular and tissue protection against oxidative injuries [6–10].

The beneficial effects of these bile pigments can be partially explained by their potent capacity of scavenging ROS. The oxidation of bilirubin by ROS results in the conversion of bilirubin to BV, which is a precursor of bilirubin in the heme degradation and is recycled to bilirubin by BV reductase in mammalians. This recycling between bilirubin and BV is believed to be one explanation for bilirubin’s powerful antioxidant effects on the redox cycle [5]. In fact, bilirubin is biochemically known as the most abundant endogenous antioxidant in mammalian tissues, accounting for the large part of the antioxidant activity of human serum [11]. Since BV is a physiological agent and can be safely used at an appropriate concentration, we hypothesized that an addition of BV into the perfusate and preservation solution could protect rat lung grafts from cold I/R injuries via its antioxidant efficacies. In this study, we tested this hypothesis using a rat orthotopic lung transplant model and showed that BV ameliorated the lung graft cold I/R injury associated with the improvement of gas exchange in the graft lung. To our knowledge, this is the first report to describe the preventative effects of BV delivered in the preservation solution, and as such represents a potentially novel and easily applicable solution to a difficult clinical scenario including primary graft dysfunction.

MATERIALS AND METHODS

Reagents

BV hydrochloride (Frontier Scientific, Logan, UT, USA) was dissolved in 0.2 N NaOH and adjusted to a final pH of 7.4 with hydrogen chloride, subsequently diluted with saline (0.9% sodium chloride) to the final concentrations. In the control group, the same amount of saline was added to the low-sodium dextran (LPD).

Animals

Inbred male LEW (RT1J) rats weighing 220–250 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh. Animals were fed a standard diet and provided water ad libitum. All procedures were performed in accordance with the guidelines of the University of Pittsburgh’s Institutional Animal Care and Use Committee and the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals (protocol #0707795).

Rat lung transplantation (LTX)

Orthotopic left lung transplantation was performed (syngenic: LEW to LEW) utilizing a previously described cuff technique [12, 13]. Described briefly, donor rats underwent tracheotomy and were mechanically ventilated with a mixture of 100% oxygen and isoflurane, positive end-expiratory pressure (PEEP) of 2 cm H2O, tidal volume of 10 ml/kg and respiratory rate of 70 breaths/minute. Donor animals were heparinized (300 units) and a laparosternotomy was performed. The lungs were flushed through the main pulmonary artery with 20 ml of cold (4°C) LPD (Perfadex®) and excised. Recipient animals were intubated orotracheally and were ventilated at the same settings as the donors. A left thoracotomy was performed, and after 6 h of cold preservation in LPD, the lung graft was implanted using the cuff technique. Sham-operated animals underwent anaesthesia and a thoracotomy. The recipients were extubated within 10 min after surgery when adequate recovery from anaesthesia and spontaneous breathing were observed. The recipients were evaluated 30 min, 2 h or 6 h after reperfusion.

Experimental groups

Four groups of animals were analysed, including sham-operated animals, the lung transplant recipients with control grafts and the recipients with BV-treated grafts. In the BV treatment group, the lung grafts were perfused and stored in LPD containing 1 or 10 μM BV for 6 h.

Lung graft function

Lung graft function was assessed by determining the oxygen partial pressure (PO2) and carbon dioxide partial pressure (PCO2) (iSTAT Portable Clinical Analyzer; iSTAT Corporation, East Windsor, NJ, USA) of blood drawn from the pulmonary vein of the transplanted lung on an FiO2 of 1.0. Two hours after reperfusion, the recipient rats underwent a tracheostomy and were ventilated for 5 min at a tidal volume of 1.5 ml, a respiratory rate of 100 breaths/min and 3.0 cmH2O of PEEP (n = 6 for each group). Before the blood gas analysis, the right hilar structures were clamped to isolate the left lung graft.

SYBR green real-time reverse transcription-polymerase chain reaction

RNA was isolated from the rat lung tissues 2 h after reperfusion using Tri-Reagent (Sigma) according to the manufacturer’s protocol. One microgram of RNA from each sample was used for reverse transcription (RT) with an oligo-dT (Life Technologies, Grand Island, NY, USA) and a Superscript II (Life Technologies) to generate first-strand cDNA. The polymerase chain reaction (PCR) mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Using the manufacturer’s software, the real-time PCR data were plotted as the ΔRn fluorescence signal vs the cycle number. The threshold cycle was defined as the cycle number at which the ΔRn crosses this threshold. The mRNA for tumour necrosis factor-α (TNFα), interleukin (IL)-6, inducible nitric oxide synthase (iNOS), cyclo-oxygenase (COX)-2, IL-8, HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in duplicate using SYBR Green two-step, real-time RT-PCR (n = 6 for
Table 1: Nucleotide sequences of oligonucleotide primers (RT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>TNFα</td>
<td>5'-GGTGGACGCTCCCAACAGGA-3'</td>
<td>5'-CAATGCTGGGCTGACTAC-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-TTGGGACAACTGATCTCAAGC-3'</td>
<td>5’-GTCCTGCCAAGCTTCTCTG-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’-GGAGAGATTTTTCACACACAC-3'</td>
<td>5’-CCATGCTAAATTGGACTTGCA-3'</td>
</tr>
<tr>
<td>COX-2</td>
<td>5’-CTCTGGATCTCGTCTTCCAG-3'</td>
<td>5’-AAAGGATTGCTGCTGCGTCG-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’-TAAAGTTGCTAATTCGCTACC-3'</td>
<td>5’-AACTCCGACTCCACGGT-3'</td>
</tr>
<tr>
<td>HO-1</td>
<td>5’-CATCAACAGGATGCTAAAGG-3'</td>
<td>5’-ATGCCACTCGCAAGGT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-ATGGCACAGTCAAGGCTGAGA-3'</td>
<td>5’-ATGGCACAGTCAAGGCTGAGA-3'</td>
</tr>
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Western blot analysis

Cytoplasmic protein was isolated from the lungs 30 min after reperfusion, and western blot was performed using primary rabbit polyclonal or mouse monoclonal antibodies and secondary goat anti-rabbit or anti-mouse antibody (1:10,000, Pierce Chemical, Rockford, IL, USA). The following primary antibodies were used: anti-phosphorylated (p)-extracellular signal-regulated protein kinase (ERK)1/2, anti-total (t)-ERK1/2, anti-p-p38 mitogen-activated protein kinases (MAPKs), anti-p-c-Jun N-terminal kinase (JNK), anti-t-p38 MAPK and anti-t-JNK (all from Cell Signaling Technology, Beverly, MA, USA; n = 3 for each group).

Stain for infiltrating neutrophils

Lung graft tissues taken 2 h after reperfusion were fixed in 10% formalin, embedded in paraffin and sectioned into 6-µm thickness. Neutrophils in transplanted lungs were stained using a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St Louis, MO, USA). Positively stained cells were counted in 20 high-power fields (HPF, ×400) per section in a blind manner and expressed as the number of cells per HPF (n = 4 for each group).

Measurement of malondialdehyde

Lung graft tissues were harvested 30 min after reperfusion, snap frozen and kept at -80°C until analysis (n = 6 for each group). The tissue was homogenized and tissue malondialdehyde (MDA) concentration, a marker of lipid peroxidation, was determined using the manufacturer’s kit direction (Kit MDA-586; Oxidresearch, Portland, OR, USA).

Immunohistochemical analysis for 4-hydroxynonenal

Lung graft tissues taken 2 h after reperfusion were fixed in 10% formalin, embedded in paraffin and sectioned into 6-µm thickness. After blocking non-specific reaction by bovine serum albumin, the slides were incubated with mouse monoclonal anti-4-hydroxynonenal (4-HNE) antibody (MHN-020; JAICA, clone: HNEJ-2, Shizuoka, Japan). The slides were washed with phosphate-buffered saline and then incubated with a biotinylated anti-mouse IgG antibody (Vector Laboratories) at 1:200 for 1 h at room temperature. They were subsequently incubated with the avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min and then developed using diaminobenzidine as a peroxidase substrate. In each study, a set of sections was stained in a similar way without the primary antibody as a negative control.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay

Lung grafts taken 6 h after reperfusion were fixed with 10% formalin, paraffin-embedded sections (6 µm) and investigated using the ApopTag Peroxidase Kit (Intergen Co., Purchase, NY, USA). The peroxidase activity was visualized with a 3-amin-9-ethyl-carbazole substrate. Haematoxylin was used as a counterstain. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-positive cells were counted and expressed as the number of TUNEL-positive cells per HPF (×400; n = 4 for each group).

Data analysis

Results are expressed as the mean ± SEM. Statistical analysis was done using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were determined with a one-way ANOVA followed by the Student–Newman–Keuls tests. P-values of <0.05 were considered statistically significant.

RESULTS

Biliverdin improved syngenic lung graft function

Prolonged cold storage and reperfusion impaired pulmonary graft functions and resulted in the significant decrease in oxygen partial pressure (PO2) levels of the graft pulmonary vein to 157.9 ± 46.1 mmHg 2 h after reperfusion in the control-LTx group, from 297.0 ± 17.7 mmHg of sham-operated animals. Although BV at 1 µM did not improve lung graft function, the perfusion and storage in BV-supplemented LPD (10 µM) significantly improved graft gas exchange to the comparable levels of 270.3 ± 26.8 mmHg with sham-operated animals. The carbon dioxide partial pressure (PCO2) levels were not significantly different among groups (Fig. 1).
Biliverdin ameliorates the expression of proinflammatory mediators in syngenic lung grafts

The effects of BV were also evaluated by mRNA levels for proinflammatory cytokines 2 h after reperfusion utilizing the real-time RT-PCR. The real-time RT-PCR revealed the significant elevation of mRNA levels for TNFα, IL-6, iNOS, COX-2, IL-8 and HO-1 compared with those of sham-operated controls. BV (10 µM) markedly inhibited the increase in these inflammatory mediators and the stress-induced molecules such as iNOS and COX-2, whereas a lower dose of BV did not alter these inflammatory responses (Fig. 2). There was no significant difference in mRNA expression for GAPDH.

Based on these results, including graft function and inflammatory markers, we used a BV concentration of 10 µM throughout the remaining experiments.

Biliverdin treatment reduced mitogen-activated protein kinase phosphorylation in lung grafts

Since ROSs are known to serve as intracellular signalling molecules controlling inflammation, we investigated the phosphorylation of three MAPK sub-types, including p38 MAPK, ERK and JNK, to determine the effects of BV on the inhibition of the activation of MAPK’s pathway and the amelioration of the inflammatory pathways. Western blot demonstrated that all three MAPK sub-types, known to be potent inflammatory signalling pathways, were rapidly up-regulated within 30 min after reperfusion. The BV treatment of the lung grafts markedly reduced the expression of p-p38 MAPK, p-ERK and p-JNK (Fig. 3).

Biliverdin treatment reduced neutrophil infiltration in syngenic graft lungs

As the recruitment and sequestration of neutrophils is a critical component of the inflammatory response of lung I/R injury, the number of infiltrating neutrophils was assessed by naphthol AS-D

Figure 1: Gas exchange function of the graft lung. Blood samples were drawn from the graft pulmonary vein 2 h after reperfusion (FiO2 1.0). (A) Untreated LTx recipients showed markedly reduced PO2 levels, which was significantly improved by BV (10 µM) treatment. (B) PCO2 levels were comparable without statistical difference among the groups (n = 6 for each group; LTx: lung transplant; BV: biliverdin; *P < 0.05 vs LTx, #P < 0.05 vs LTx with 1 µM BV).

Figure 2: Graft mRNA levels for inflammatory mediators. Six-hour cold ischaemia and 2 h reperfusion resulted in an increase in mRNA for inflammatory mediators such as TNFα, IL-6, iNOS, COX-2, IL-8 and HO-1. These increases in mRNA expression were significantly attenuated in the grafts treated with BV (10 µM; n = 6 for each group; LTx: lung transplant; BV: biliverdin; *P < 0.05 vs LTx, #P < 0.05 vs LTx with 1 µM BV).

Figure 3: Phosphorylation of MAPKs in the lung graft after I/R. p-p38, p-ERK1/2 and p-JNK in the lung graft at 30 min after reperfusion were analyzed by western blot using lung cytoplasmic protein. Phosphorylation of the three MAPK sub-families after I/R was reduced in the BV treatment group. Results are representative of three independent experiments (BV: biliverdin at 10 µM).
chloroacetate esterase staining. There was a remarkable increase in the number of neutrophils (103.6 ± 20.4/HPF) in the lung grafts stored in control LPD compared with sham-operated animals (22.3 ± 5.9/HPF). BV treatment significantly reduced neutrophil sequestration into the lung grafts 2 h after reperfusion (Fig. 4).

Biliverdin treatment reduced graft oxidative injuries

After I/R, the untreated lung grafts showed a significantly increased MDA level, a marker of lipid peroxidation. BV treatment significantly reduced tissue MDA levels 30 min after reperfusion, showing its antioxidant effects (Fig. 5A). Graft oxidative injuries were also assessed by immunostain for 4-HNE, which is generated in the oxidation of lipids containing polyunsaturated omega-6 acyl groups. Cells positive for 4-HNE in the sham-operated lung section were scarce. However, a number of 4-HNE positive cells were noted in the lung grafts treated with the control-LTx group. BV treatment significantly decreased the number of stained cells for these oxidative injuries 2 h after reperfusion (Fig. 5B).

Biliverdin-supplemented cold preservation solution resulted in decrease in apoptosis

Apoptosis can be initiated by a wide variety of stimuli, including I/R injury, and is considered to play a critical role during the I/R injury process. The apoptosis of the graft tissue taken 6 h after reperfusion was determined by the TUNEL assay. There were few apoptotic cells in the lung of sham-operated animals. Although a number of I/R-induced apoptotic cells were noted in the grafts, apoptotic graft cells were reduced when the lung grafts were perfused with, and stored in, the LPD containing BV (Fig. 6).

DISCUSSION

The prevention of cold I/R injury associated with lung transplantation is of the utmost importance. Our data clearly show that this objective can be achieved by simply adding BV to the preservation solution as presented in this study. Although BV and bilirubin share antioxidant activities, the conversion of BV to bilirubin through BV reductase explains a powerful redox cycle that results in the augmentation of bilirubin, as bilirubin is most likely involved in antioxidant activity via H-donation to an incipient radical, such as lipid peroxyl radical (LOO·), to form lipid hydroperoxide (LOOH) and bilirubin radical. Of note, BV’s antioxidant activity is due to the formation of a resonance-stabilized, carbon-centred radical resulting from the addition of radicals such as LOO· to BV. The amplification afforded by the BV-bilirubin cycle can readily explain the ability of low concentrations of bilirubin to overcome 10 000-fold higher concentrations of oxidants. However, it is worth noting that BV is not always produced upon the oxidation of bilirubin.
Bilirubin administration is shown to prevent I/R injury \cite{15, 16} oxidative injuries. There have been accumulating reports in pigments were shown in experimental and/or clinical models of \cite{17} or anti and also results in anti-inflammation. JNK1/2 activation, which are important cascades of proinflammatory effects were followed by the suppression of proinflammatory cytokines. As seen in our study, these anti-inflammatory effects were followed by the suppression of proinflammatory cytokine mRNA up-regulation such as TNFα and IL-8, and the reduction in neutrophil recruitment. BV treatment might show protective effects through resident or inflammatory macrophages.

The potential toxic actions of bile pigments are dose-dependent and range from itching in jaundiced patients to severe neuronal damage, primarily of basal ganglia, as observed in kernicterus. Although neuronal tissue seems to be particularly susceptible to toxic actions of bile pigments, a more general toxic action of bile pigments is assumed to result from the damage to the lipid bilayers of the biological membranes \cite{25}. An experimental study of hepatic perfusion by a bilirubin-containing perfusate demonstrated that the long-term extended supplementation of bilirubin seemed to be harmful because of the intrahepatic accumulation of the substance, causing cytotoxic effects on the graft, although a short-term rinse treatment with micromolar levels of bilirubin attenuated biliary dysfunction and cell injury of the grafts associated with I/R injury after liver transplantation \cite{15}. Thus, it is undoubted that more rigorous investigations are needed to determine whether bile pigments can be used as therapies for human disease, given the potential toxicities. In this regard, an ex vivo approach targeting the lung grafts during cold preservation, as presented in this study, is an ideal strategy to avoid the systemic exposure of the bile pigment to the recipients.

We acknowledge that this experimental model, like most animal models, does not recapitulate all aspects of clinical lung transplantation. We are aware that the transplantation of an isograft is artificial and it does not happen in the clinical transplantation setting, except in the rare case of identical twins. However, syngeneic transplantation is considered an ideal experimental model to study I/R injury because it allows the isolation of factors related to I/R injury from factors involved in alloimmune reactions.

In conclusion, LPD containing BV significantly prevented lipid peroxidation and apoptosis due to oxidative DNA damage as well as inhibiting inflammatory cascades during the lung cold I/R injury process. The use of BV may be a feasible approach in the clinical setting and may assist in reducing primary graft dysfunction or rejecting lung allografts. Further studies are nevertheless warranted to assess the efficacy of this approach in the transplantation outcome in allogeneic lung grafts.

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Conflict of interest: none declared.

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