β_2-Adrenoreceptor Agonist Inhalation During Ex Vivo Lung Perfusion Attenuates Lung Injury

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Background. **Attenuation of ischemia reperfusion injury (IRI) is important in lung transplantation. Our group previously reported that β_2-adrenoreceptor agonist inhalation during the period before procurement successfully attenuated IRI in donated lungs after cardiac death. We therefore hypothesized that β_2-adrenoreceptor agonist inhalation during ex vivo lung perfusion (EVLP) after procurement might also have a protective effect.**

Methods. **Cardiac-dead beagles were left at room temperature for 210 minutes, and all lungs were subsequently procured and subjected to EVLP for 240 minutes. The beagles were allocated to 2 groups: the β_2 group (receiving an aerosolized β_2-adrenoreceptor agonist 20 minutes after initiation of EVLP; n = 7) and the control group (receiving an aerosolized control solvent at the same time point; n = 6). Physiologic data, including lung function, were evaluated during EVLP.**

Results. **The β_2 group showed significantly lower peak airway pressure and pulmonary artery pressure than the control group. Dynamic pulmonary compliance was higher, pulmonary vascular resistance (PVR) was lower, and the wet-to-dry lung weight ratio was lower in the β_2 group than in the control group. Cyclic adenosine monophosphate (cAMP) and total adenosine nucleotide (TAN) levels in lung tissue after EVLP were higher in the β_2 group than in the control group. The β_2 group also showed more cystic fibrosis transmembrane conductance regulator (*CFTR*) gene expression.**

Conclusions. **After procurement, β_2-adrenoreceptor agonist inhalation during EVLP attenuates lung injury in a canine model of organ donation after cardiac death.**

(Ann Thorac Surg 2015;100:480–6)

The chronic shortage of brain-dead donors is a problem in clinical lung transplantation. A practical method to resolve this problem is to transplant lungs acquired by donation after cardiac death (DCD). Warm ischemia inevitably occurs in DCD donors and may cause ischemia reperfusion injury (IRI) after lung transplantation. Severe IRI leads to primary graft dysfunction and remains a significant cause of early morbidity and mortality after lung transplantation [1]. Therefore, it is important to attenuate IRI caused by warm ischemia in DCD donors. Many groups have studied IRI caused by warm ischemia. Our group previously reported that β_2-adrenoreceptor agonist inhalation before procurement during donation successfully attenuated IRI in a DCD model [2, 3]. Steen and colleagues [4] introduced ex vivo lung perfusion (EVLP) to evaluate the function of DCD donor lungs before transplantation, and this system was subsequently developed for clinical use by a Toronto group [5]. EVLP has since been used not only for the assessment but also for the reconditioning of marginal and DCD donor lungs [5–7]. To date, several studies have been conducted using EVLP as a drug delivery system [8–12].

Accepted for publication Feb 10, 2015.

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We previously demonstrated that β_2-adrenoreceptor agonist inhalation during the period before procurement attenuated IRI, but it is unknown if β_2-adrenoreceptor agonist inhalation after procurement could affect the injured lungs. We hypothesized that β_2-adrenoreceptor agonist inhalation during EVLP after procurement would also have a protective effect.

Material and Methods

Animals

All animals used in this study received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals, prepared by the National Institutes of Health (NIH publication No. 86-23, revised 1996). The study protocol was approved by the Ethics Committee of the Graduate School of Medicine at Kyoto University, Japan (Medkyo 13257).

Study Design

The study time line is shown in Figure 1. The donor dogs were anesthetized through an intramuscular injection of midazolam (0.5 mg/kg), xylazine (0.25 mg/kg), and atropine sulfate (0.05 mg/kg). They were intubated and

0003-4975/$36.00
http://dx.doi.org/10.1016/j.athoracsur.2015.02.136

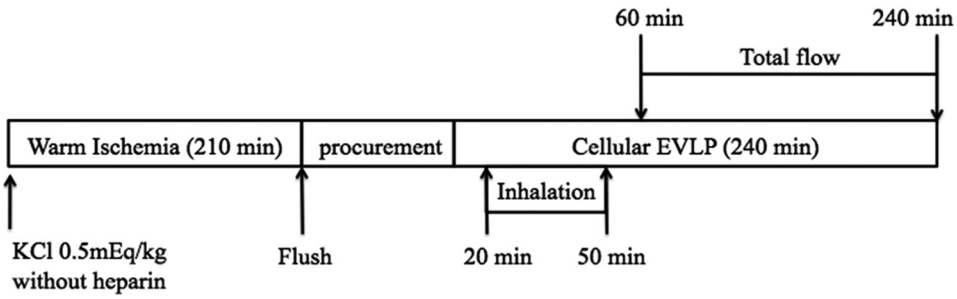

Fig 1. Time line of the study. (EVLP, ex vivo lung perfusion; KCl, potassium chloride.)

mechanically ventilated with a tidal volume of 25 mL/kg, a respiration rate of 15 breaths/min, a positive end-expiratory pressure (PEEP) of 5.0 cm H_2O, and a fraction of inspired oxygen (Fio_2) of 1.0. The dogs were euthanized by intravenous administration of potassium chloride (0.5 mEq/kg), without heparinization. The ventilators were then removed and the tracheal tubes exposed to room atmosphere.

The donor dogs were left at room temperature (23°C) for 210 minutes. A median sternotomy was performed, and a pulmonary artery flush was performed on all donors 210 minutes after cardiac arrest using ET-Kyoto solution (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan), an extracellular preservation solution we developed for clinical lung transplantation [13]. Before use, the ET-Kyoto solution was stored at 4°C without heparin. The flush was performed with ventilation, 30 cm above the donor's chest. Just after flushing, the donor lungs were retrieved and subjected to EVLP.

Before EVLP, we collected blood from the other dogs. We administered 5,000 IU heparin and collected blood from the incised left atrial appendage, superior vena cava, and inferior vena cava. The collected blood was centrifuged with a cell-saving device (Cell Saver 5; Haemonetics Corp, Braintree, MA) through a leukocyte filter, stored with citrate phosphate dextrose adenine (Sepacell Integra CA; Asahi Kasei Medical Co Ltd, Tokyo, Japan), and used to prime the EVLP circuit.

The lungs were placed in an XVIVO chamber (Vitrolife, Denver, CO). The pulmonary artery was directly cannulated and connected to the perfusion circuit. The left atrium was open so that the left atrial pressure was always 0 mm Hg. The EVLP perfusate consisted of STEEN solution (XVIVO perfusion, Englewood, CO) (1,500 mL) with approximately 650 mL of concentrated red blood cells, methylprednisolone (500 mg), heparin (10,000 IU), cefazolin (500 mg), and THAM SET (Otsuka Pharmaceutical Factory, Inc, Tokushima, Japan). A centrifugal pump at a constant flow rate drove the perfusate, and the flow was started at 10% of the full flow rate at room temperature. The temperature of the perfusate was gradually increased to 37°C. Twenty minutes after initiation of EVLP (when the temperature of the perfusate reached 32°C), ventilation was initiated with an Fio_2 of 0.25, a tidal volume of 15 mL/kg, a frequency of 12 breaths/min, and a PEEP of 5 cm H_2O. Deoxygenation of the lung perfusate was initiated with a gas mixture of nitrogen (86%), carbon dioxide (8%), and oxygen (6%). The perfusate flow rate was gradually increased to the full flow rate, which was 40% of the estimated cardiac output (100 mL/kg), 60 minutes after initiation of EVLP. The canine lungs were subjected to EVLP for a total of 240 minutes.

Study Groups

The donor lungs were divided into 2 groups: (1) the β_2 group (n = 7), which inhaled the β_2-adrenoreceptor agonist (0.01% procaterol [3.5 mL] inhalation solution [350 μg procaterol]) for 30 minutes, starting at the same time as ventilation (20 minutes after initiation of EVLP) and (2) the control group (n = 6), which inhaled the control solvent (3.5 mL) in the same setting.

Functional Assessment

Physiologic data were obtained 20, 70, 120, 180, and 240 minutes after initiation of EVLP. Dynamic pulmonary compliance was defined as the tidal volume/(peak airway pressure–PEEP) mL/cm H_2O. Pulmonary vascular resistance (PVR) was calculated as (pulmonary artery pressure × 80)/pulmonary artery flow (dynes/s/cm^5). Blood gas analysis of the perfusate from the lung was performed after 10 minutes of exposure to an Fio_2 of 1.0.

Lung Wet Weight–to–Dry Weight Ratio

The entire right lung was used to calculate the wet weight–to–dry weight ratio (WDR) 240 minutes after initiation of EVLP. Wet weight (in milligrams) was measured first, and the dry weight (in milligrams) was measured after the lung had been dried overnight at 180°C. We measured dry weight multiple times at intervals and found that the weights were the same at all time points. The WDR was calculated by dividing the wet weight by the dry weight.

Adenine Nucleotide Levels

Peripheral lung tissue samples were collected from the left lungs 240 minutes after initiation of EVLP. The concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were measured using high-performance liquid chromatography (Shim-pack CLC-ODS column; 15 cm × 6.0 mm; Shimadzu Corp, Kyoto, Japan). Total adenosine nucleotide (TAN) values were calculated as previously reported [3]. We were unable to collect data from two cases in the β-2 group and one case in the control group

due to trouble with the lyophilization machine. Therefore, in total we evaluated 5 cases in the β_2 group and 5 cases in the control group.

Cyclic Adenosine Monophosphate Levels

We evaluated cyclic AMP (cAMP) levels in addition to ATP levels in all 5 cases in both groups. Peripheral lung tissue samples taken 70 minutes and 240 minutes after initiation of EVLP were analyzed to determine cAMP levels. We collected 2 $\times$ 2 cm samples from the left upper lobe in every case. We also closed the wound using a running suture to avoid air leaks in every case. The cAMP levels were measured with a cAMP radioimmunoassay kit (Yamasa, Chiba, Japan), as previously reported [3]. Protein levels were measured at the same time points described in the method by Lowry and colleagues [14].

Perfusate Cytokine Levels

Enzyme-linked immunosorbent assay was used to measure perfusate interleukin (IL)-8 levels 70 and 240 minutes after initiation of EVLP. In all cases, the manufacturer's instructions provided in the IL-8 enzyme-linked immunosorbent assay kits were followed (Quantikine, R&D Systems Inc, Minneapolis, MN).

Macroscopic and Histologic Findings

Macroscopic appearance of the lungs was recorded 240 minutes after initiation of EVLP. Left lung specimens collected 240 minutes after initiation of EVLP were used for histologic analysis. Each lung was immersed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Cystic Fibrosis Transmembrane Conductance Regulator

We performed immunofluorescence staining for cystic fibrosis transmembrane conductance regulator (*CFTR*) on lung samples collected 240 minutes after initiation of EVLP. We used *CFTR*-specific goat polyclonal IgG (sc-8909; Santa Cruz Biotechnology, Inc, Dallas, TX) as the primary antibody and donkey antigoat IgG (Alexa 555; Molecular Probes/Life Technologies, Frederick, MD) as the secondary antibody, as previously described [15].

Statistical Analysis

All statistical analyses were performed using StatView, version 5.0, software (Abacus Concepts, Berkeley, CA). All values are presented as the mean $\pm$ standard deviation. Data were evaluated using repeated measures analysis of variance, Scheffe's post hoc multiple comparison test, and the Mann-Whitney *U* test. A p value of less than 0.05 was considered statistically significant.

Results

Beagles weighing 9 to 11.6 kg (Kitayama Labes Co, Ltd, Hongo Farm, Yamaguchi, Japan) were used in this study. There were no significant differences in beagle body weights between the control group and the β_2 group (10.6 $\pm$ 0.8 kg versus 10.8 $\pm$ 0.9 kg).

Assessment of Lung Function

The β_2-treated group showed significantly lower peak airway pressure and higher dynamic pulmonary compliance than the control group ($p < 0.001$ and $p = 0.026$, respectively) (Figs 2A, 2B). In addition, the pulmonary arterial pressure and PVR in the β_2 group was lower than that in the control group ($p < 0.001$ and $p = 0.0068$, respectively) (Figs 2C, 2D). Both groups showed similar PO_2 ($p = 0.12$).

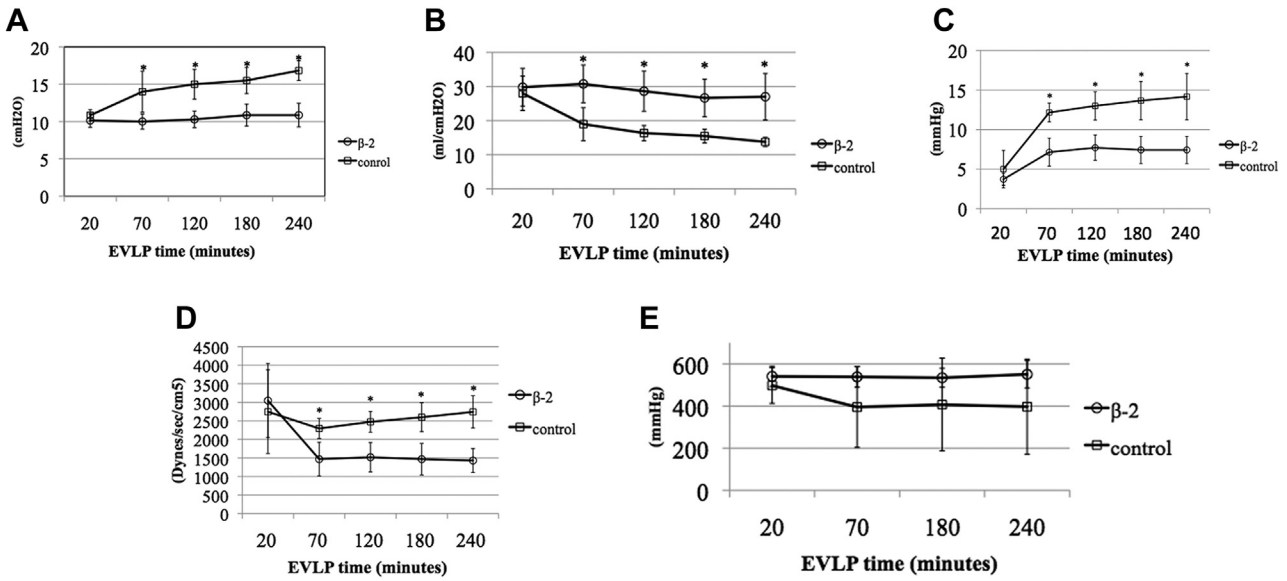

*Fig 2. Physiologic lung functions during ex vivo lung perfusion (EVLP). (A) Peak airway pressure. (B) Dynamic pulmonary compliance. (C) Pulmonary artery pressure. (D) Pulmonary vascular resistance. (E) PO_2 of the perfusate from the lung. *$p < 0.05$ between the β_2 group (open circles) and the control group (open boxes) in Scheffe's post hoc multiple comparison test.*

Lung WDR

The lung WDR in the β_2 group was significantly lower than that in the control group (8.78 ± 0.61 versus 5.72 ± 0.88; $p = 0.0027$) (Fig 3). This result indicated that the lungs in the control group were more edematous.

Adenine Nucleotide and cAMP Levels

The β_2 group showed higher ATP, ADP, and AMP levels than the control group ($p = 0.02$, $p = 0.03$, and $p = 0.03$, respectively). Consequently, the β_2 group showed higher TAN (TAN = ATP + ADP + AMP) levels than in the control group (3.50 ± 0.58 nmol/mg versus 2.17 ± 0.57 nmol/mg; $p = 0.02$) (Fig 4).

The cAMP levels 70 minutes after EVLP initiation in the β_2 group were significantly higher than those in the control group (4.09 ± 3.42 pmol/mg versus 0.58 ± 0.29 pmol/mg; $p = 0.03$). Although the cAMP levels decreased 240 minutes after EVLP initiation in the β_2 group, they were still higher than those measured in the control group (1.23 ± 0.98 pmol/mg versus 0.30 ± 0.19 pmol/mg; $p = 0.03$) (Fig 5).

Cytokine Levels in the Perfusate

Seventy minutes after EVLP initiation, IL-8 levels in the perfusate were very low in both groups. At 240 minutes after EVLP initiation, IL-8 levels increased equally in both groups (Fig 6).

Macroscopic and Histologic Findings

At 240 minutes after EVLP initiation, the lungs in the control group had more areas of macroscopic, broad, dark red tissue than those in the β_2 group (Figs 7A, 7B). On histologic examination, more edematous changes were noted in the control group than in the β_2 group (Figs 7C, 7D).

CFTR Immunofluorescence Staining

CFTR was expressed in the lungs at a higher level in the β_2 group than in the control group (Figs 8A, 8B).

Comment

In this study, we used a canine model of DCD to investigate the effect of β_2-adrenoreceptor agonist inhalation during EVLP. In our study, the β_2 group showed better lung function (including lower peak airway pressure, higher dynamic pulmonary compliance, lower pulmonary arterial pressure, and lower PVR), lower WDR, higher cAMP levels, higher TAN levels, and more *CFTR* expression than in the control group. Thus our study indicated that β_2-adrenoreceptor agonist inhalation during EVLP after lung procurement had a protective effect. Our group had previously reported that β_2-adrenoreceptor agonist inhalation during the period before procurement attenuated IRI [2, 3]. In this study, we demonstrated a protective effect of β_2-adrenoreceptor agonist inhalation after procurement. This might be useful in clinical practice, because inhalation after procurement does not affect any other organs.

According to Yeung and colleagues [16], compliance and airway pressure during EVLP are important for evaluating donor graft function. In our study, the β_2 group showed significantly lower peak airway pressure and higher dynamic pulmonary compliance than did the control group. These results indicate a protective effect of β_2-adrenoreceptor agonist inhalation during EVLP. Furthermore, lower PVR was confirmed in the β_2 group. As pointed out by Mak and colleagues [17], β_2-adrenoreceptors are distributed in pulmonary blood vessels as well as airway epithelial cells, airway smooth muscle, and alveolar walls. Leblais and associates [18] also mentioned

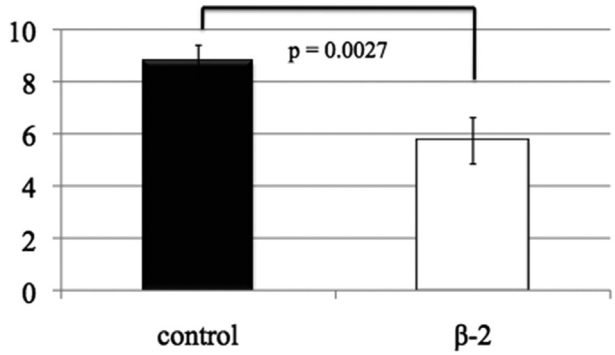

Fig 3. Lung wet-to-dry weight ratio.

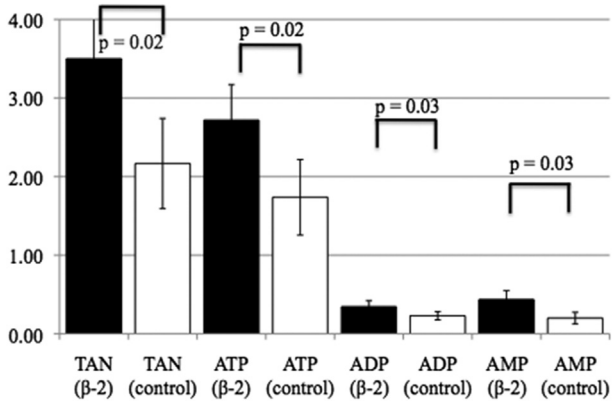

Fig 4. Adenosine nucleotide levels (nmol/mg dry weight) measured 240 minutes after ex vivo lung perfusion (EVLP) (TAN = ATP + ADP + AMP). ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; TAN, total adenosine nucleotide.

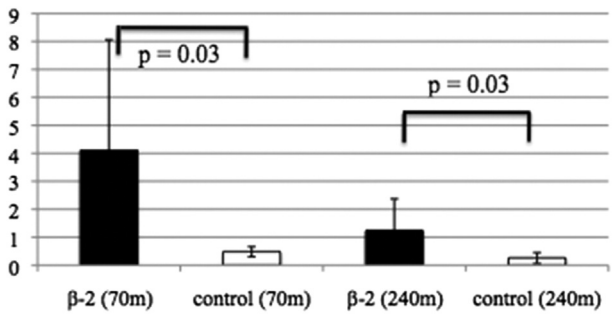

Fig 5. Peripheral lung tissue cyclic AMP (cAMP) levels measured at 70 and 240 minutes after ex vivo lung perfusion (EVLP) initiation.

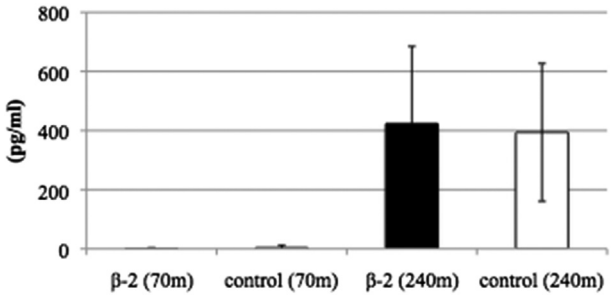

Fig 6. Perfusate interleukin-8 (IL-8) levels measured at 70 and 240 minutes after ex vivo lung perfusion (EVLP) initiation.

that β_2-adrenoreceptors mediate relaxation of the pulmonary artery. Our results were consistent with those of these previous studies. The PO_2 levels in the control group were higher than we expected for at least 2 reasons. First, the perfusion was not a total flow in the current EVLP setting. The perfusate flow was gradually increased to a maximum of 40% of the estimated cardiac output (100 mL/kg) according to the Toronto protocol [16]. As Yeung and colleagues [16] also reported, PO_2 levels fall with increasing perfusion flow in the cellular EVLP. Second, lung injury occurred heterogeneously, and lung injury areas might be underperfused. Hypoxic vasoconstriction might occur in the damaged area; therefore, there might be a low perfusion/ventilation mismatch in the control group. As a result, the control group had high PO_2 levels. One might wonder if this is a good example, supporting the idea that PO_2 levels are not a good marker of lung function when used alone.

β_2-Adrenoreceptor agonists have been reported to enhance alveolar fluid clearance (AFC) through a cAMP-dependent mechanism [19]. In our study, the lung WDR in the β_2 group was significantly lower than that in the control group. In addition, cAMP levels in the β_2 group were significantly higher than those in the control group. These results support the possibility that β_2-adrenoreceptor agonist inhalation stimulated AFC in our model. However, the cAMP levels in the β_2 group decreased 240 minutes after EVLP initiation, which may be because procaterol is a short-acting β_2 agonist. Further investigation using longer-acting β_2 agonists will be required.

The β_2 group showed higher ATP and TAN levels. The β_2 agonists did not increase ATP and TAN directly. High ATP and TAN levels were associated with lung viability [2]. The β_2 agonists attenuated lung injury; therefore, β_2 agonists enhanced lung viability, which resulted in high ATP and TAN levels.

Recent studies have demonstrated that β_2-adrenoreceptor agonists enhance AFC through the upregulation of *CFTR* [20–22]. Here we showed that *CFTR* expression was higher in the β_2-treated group. In addition, cAMP was reported to play an important role in activating the *CFTR* promoter [23], and our results were consistent with this reported mechanism. The previous clinical data suggested that the recovery from acute lung injury required the removal of fluid from the alveolar space. Patients with acute lung injury who retained maximal AFC had better clinical outcomes [24]. The upregulation of AFC through the *CFTR* attenuated lung injury in our study as well. In the previous report, fluid and chloride transport from the airspace was accelerated in the presence of the β agonist

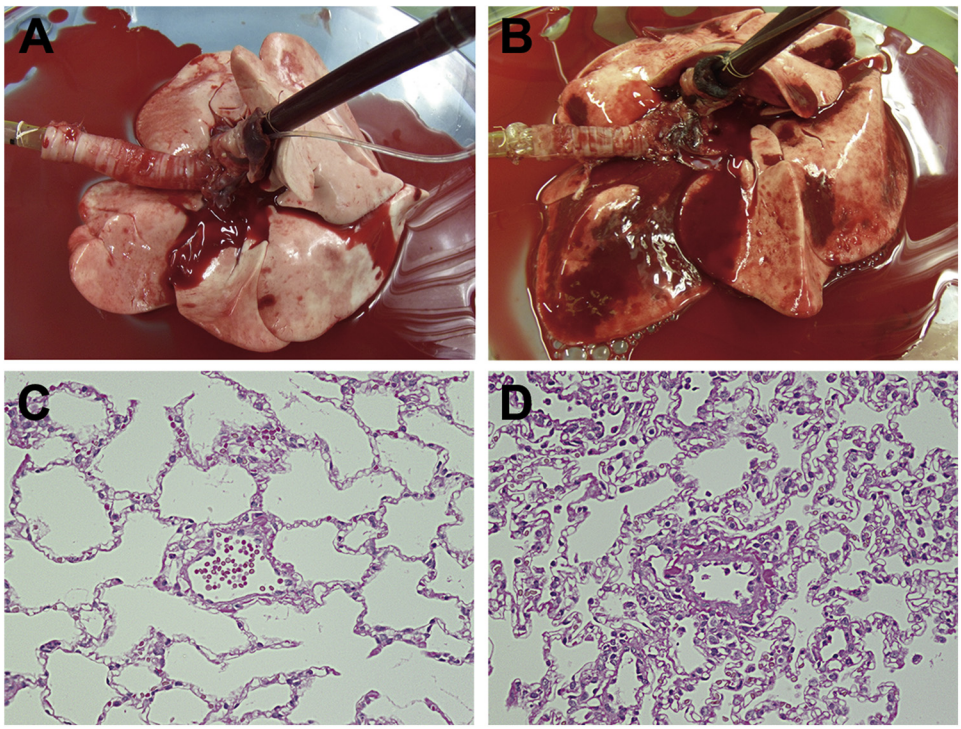

Fig 7. (A and B) Macroscopic and (C and D) histologic findings after 240 minutes of ex vivo lung perfusion (EVLP). C and D show hematoxylin and eosin staining at ×400 magnification. (B) The lungs in the control group have more areas of broad dark red tissues macroscopically than do those in the β_2 group (A). (D) The control group showed more edematous changes histologically than did the β_2 group (C).

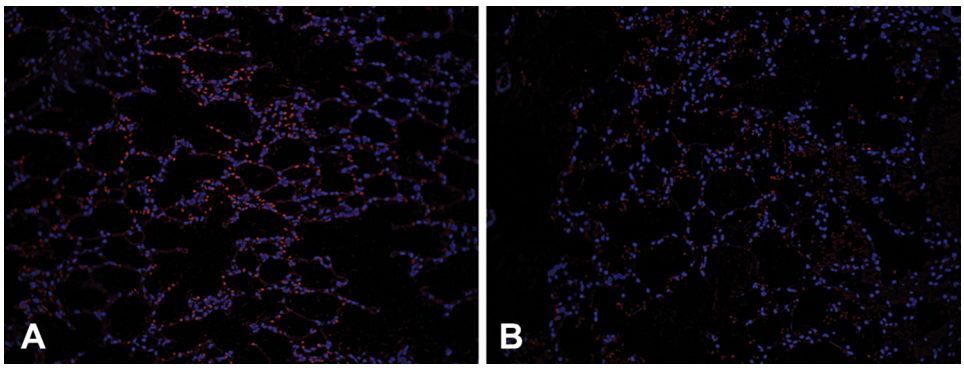

Fig 8. CFTR-expressing cells were visualized by using fluorescent microscopy with Alexa 555 (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) at ×200 magnification. (A) The β_2 group showed higher CFTR expression in the lungs than did the (B) control group. (CFTR, cystic fibrosis transmembrane conductance regulator.)

isoproterenol within 30 minutes [21]. Additionally, the isoproterenol-induced increase in chloride transport was inhibited by the *CFTR* inhibitor glibenclamide, providing evidence that the cAMP-stimulated uptake of chloride was mediated by *CFTR*. In our study, we showed high *CFTR* expression 3 hours after inhalation of a β_2-adrenoreceptor agonist. These immediate benefits were consistent with the previous report, although the mechanism was not investigated in our study.

IL-8 inhibits cAMP-stimulated alveolar epithelial fluid transport. Roux and colleagues [24] showed that IL-8 decreases the net fluid transport across alveolar epithelial cells through a reduction in *CFTR* activity and biosynthesis. This reduction was mediated by heterologous β_2-adrenergic receptor desensitization through the G-protein–coupled receptor kinase 2 (GRK2)/phosphatidylinositol-3-kinase (PI3K) signaling pathway. In our study, IL-8 levels were very low at 70 minutes after EVLP initiation, which means that the β_2 agonists worked properly under these circumstances. Roman and colleagues [6] stated that IL-8 in the EVLP perfusate rose mainly during the first 6 hours of EVLP. We also do not think that β_2 agonists suppress this rise of IL-8, and IL-8 levels in the perfusate rose in both groups in our study. In addition, de Perrot and coworkers [25] mentioned that IL-8 levels increase after reperfusion in human lung transplantation. If IL-8 levels were high, β_2-adrenoreceptor agonists would fail to enhance AFC. Thus, the very low IL-8 levels in the early phase of EVLP were significant in our study.

There were several limitations to our study. First, we assessed lungs only during EVLP. Future studies should include assessment of the entire lung transplantation model. Second, cardiac arrest in this study was induced by intravenous injection of potassium chloride, which is not performed in clinical practice. In this study, we selected this procedure for the uniform induction of cardiac arrest. Third, we mentioned the limitations regarding *CFTR* immunofluorescence staining. *CFTR* is expressed in type I and type II alveolar epithelial cells and distal airway epithelium [21]. Thus, we interpreted that *CFTR* was expressed in alveolar epithelial cells in our study, but we cannot deny that our results were influenced by the viable alveolar macrophages.

In conclusion, β_2-adrenoreceptor agonist inhalation after procurement during EVLP attenuates lung injury in a canine model of lung donation after cardiac death.

We would like to thank Mr. Katsuyoshi Okawa for his expert technical support.

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INVITED COMMENTARY

Lung transplantation is a lifesaving treatment for patients with end-stage pulmonary disease; however, its success is limited by significant donor organ shortages because the majority of donor lungs are currently deemed unsuitable. Ex vivo lung perfusion (EVLP) is a technique of normothermic acellular perfusion recently developed to expand the donor lung pool by enabling functional assessment of high-risk donor lungs such as those obtained through donation after cardiac death (DCD). The first clinical application of EVLP was reported by Steen and colleagues [1]; this has been followed by numerous supportive studies, both experimental and clinical. In August 2014, the US Food and Drug Administration approved the XVIVO Perfusion System with Steen solution (XVIVO Perfusion, Göteborg, Sweden) for use in preserving donor lungs that do not initially meet the standard criteria for transplantation. Moreover, the use of EVLP as a platform to therapeutically recondition injured donor lungs has gained rapid attention, which adds yet another strategy for the expansion of the donor lung pool.

In this issue of *The Annals of Thoracic Surgery*, Kondo and colleagues [2] investigate the antiinflammatory aspects of a β_2-adrenoreceptor agonist, procaterol, as a novel strategy to protect DCD donor lungs during EVLP. Although widely used in clinical practice to treat asthma and chronic obstructive pulmonary disease, β_2-adrenoreceptor agonists, including procaterol, have also been reported to have antiinflammatory effects both in vivo and in vitro by modulating the activities of a range of immune and inflammatory cells [3, 4]. The authors performed a simple study in which DCD donor lungs from beagles underwent EVLP with aerosolized procaterol versus a vehicle preparation. After 240 minutes of EVLP with procaterol treatment, the authors noted significantly improved lung function (reduced peak airway pressure, pulmonary artery pressure, and pulmonary vascular resistance along with increased pulmonary compliance) and reduced pulmonary edema. This was accompanied by elevated cyclic adenosine monophosphate and total adenosine nucleotide levels in treated lungs. Thus the authors concluded that inhalation of a β_2-adrenoreceptor agonist during EVLP attenuates injury in DCD donor lungs.

Despite the limitation that these lungs were not transplanted, these results add to the body of mounting evidence that marginal donor lungs can, in many cases, be reconditioned during EVLP through therapeutic strategies. With future advances in these methodologies, the clinical application of EVLP-mediated therapeutic treatments could soon make a significant impact on the severe shortage of acceptable donor lungs that severely limits the survival of patients on the lung transplant waiting list.

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0003-4975/$36.00
http://dx.doi.org/10.1016/j.athoracsur.2015.02.137