The effect of β-2 adrenoreceptor agonist inhalation on lungs donated after cardiac death in a canine lung transplantation model

Jin Sakamoto, MD, Fengshi Chen, MD, PhD, Daisuke Nakajima, MD, Tetsu Yamada, MD,
Akihiro Ohsumi, MD, Xiangdong Zhao, MD, PhD, Hiroaki Sakai, MD, PhD,
Toru Bando, MD, PhD, and Hiroshi Date, MD, PhD

From the Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

KEYWORDS:
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BACKGROUND: It is a matter of great importance in a donation after cardiac death to attenuate ischemia–reperfusion injury (IRI) related to the inevitable warm ischemic time.
METHODS: Donor dogs were rendered cardiac-dead and left at room temperature. The dogs were allocated into 2 groups: the β-2 group ($n = 5$) received an aerosolized β-2 adrenoreceptor agonist (procaterol, 350 μg) and ventilation with 100% oxygen for 60 minutes starting at 240 minutes after cardiac arrest, and the control group ($n = 6$) received an aerosolized control solvent with the ventilation. Lungs were recovered 300 minutes after cardiac arrest. Recipient dogs underwent left single-lung transplantation to evaluate the functions of the left transplanted lung for 240 minutes after the reperfusion.
RESULTS: Oxygenation and dynamic compliance were significantly higher in the β-2 group than in the control group. The β-2 group revealed significantly higher levels of cyclic adenosine monophosphate and high-energy phosphates in the donor lung after the inhalation than before it. Histologic findings revealed that the β-2 group had less edema and fewer inflammatory cells.
CONCLUSION: Our results suggest that β-2 adrenoreceptor agonist inhalation during the pre-procurement period may ameliorate IRI.
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One method of countering chronic lung donor shortages is the practice of donation after cardiac death (DCD). This technique inevitably leads to ischemia–reperfusion injury (IRI) related to warm ischemia. However, DCD is now increasingly accepted in some countries.

In humans, β-2 agonists injected intravenously or aerosolized and administered through the airway were reported to have protective effects against high-altitude lung edema,[1] acute respiratory distress syndrome,[2] pulmonary edema after lung resection,[3] and pulmonary edema of discarded lungs from brain-dead donors in an ex vivo model.[4] Moreover, β-2 agonists have already been reported to have pro-

tective effects against IRI in rat models, including a study by our group.[5] We therefore hypothesized that a β-2 adrenoreceptor agonist would ameliorate IRI in our canine model.

Materials and methods

The study used 11 pairs of weight-matched TOYO Beagles (8.55–10.65 kg, Kitayama Labs Co Ltd, Hongo Farm, Yamaguchi, Japan). All animals 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 Institute of Laboratory Animal Resources (National Institutes of Health 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.

Reprint requests: Hiroshi Date, MD, PhD, Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Telephone: +81-75-751-4975. Fax: +81-75-751-4974.
E-mail address: hdate@kuhp.kyoto-u.ac.jp

Donor preparation

The donor dogs were anesthetized via 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 mechanically ventilated at a tidal volume of 25 ml/kg, a rate of 15 breaths/min, a positive end-expiratory pressure of 5.0 cm H_2O, and an inspired fraction of oxygen of 1.0. The dogs were euthanized via the intravenous administration of potassium chloride (0.5 mEq/kg), without heparinization. The ventilators were removed, and the tracheal tubes were left open to room air.

The donor dogs were left at room temperature (21°C) for 300 minutes. The β-2 group ($n = 5$) received inhalation of the β-2 adrenoreceptor agonist (0.01% procaterol [3.5 ml] inhalation solution [350 μg procaterol]) during ventilation with 100% oxygen for 60 minutes starting at 240 minutes after cardiac arrest. The control group ($n = 6$) received inhalation of the control solvent (3.5 ml) with the same ventilation setting as the β-2 group.

The inhalation solution and the control solvent were aerosolized by a nebulizer (Aeroneb Professional Nebulizer System, Aerogen, Ireland), which was inserted into the inspiratory loop of a ventilator. A pulmonary arterial flush was performed on all donors at 300 minutes after the cardiac arrest, using ET-Kyoto solution (Otsuka Pharmaceutical Co Ltd, Tokyo, Japan), an extracellular preservation solution that we originally developed for clinical lung transplantation.[6] Before use, the ET-Kyoto solution was stored at 4°C without heparin. For the antegrade flush, we used 100 ml/kg, with 50 ml/kg used for the retrograde flush. Both were performed from 30 cm above the donors' chests. Ventilation was performed during the flush. The recovered organs, semi-inflated (within 20 cm H_2O) with 100% oxygen, were preserved at 4°C for 120 minutes.

Recipient preparation and transplantation

Recipient dogs were anesthetized, maintained, and ventilated in the same manner as the donors. Peak inspiratory pressure was monitored by a pressure transducer that was attached to the tracheal tube. For each recipient, a 5F thermodilution catheter (151F7, Edwards Lifesciences, Irvine, CA) was placed in the main pulmonary artery from the right femoral vein to measure pulmonary artery pressure, central venous pressure, and cardiac output. A femoral arterial line was inserted for measuring aortic pressure and for arterial blood gas analysis. After thoracotomy, a 2.0-mm catheter was inserted directly into the left atrium for monitoring left arterial pressure.

We then performed a left pneumonectomy and left lung transplantation. The right pulmonary artery was encircled with vascular tape and clamped with a tourniquet continuously from 60 to 240

Table 1 Assessment of Lung Function in Surviving Recipients in the Control ($n = 5$) and β-2 ($n = 5$) Groups

Variables[a]	Pre-op	Time after transplantation, min				p-value[b]
		75	120	180	240	
Mean AoP, mm Hg						
Control	123.2 ± 27.2	98.0 ± 27.2	110.8 ± 26.6	86.6 ± 24.0	65.0 ± 38.8	
β-2	114.6 ± 17.2	102.0 ± 12.3	124.0 ± 13.4	118.2 ± 26.8	94.8 ± 36.5	0.17
Mean PAP, mm Hg						
Control	17.6 ± 2.0	40.0 ± 11.7	41.8 ± 7.8	40.8 ± 6.7	27.0 ± 12.3	
β-2	19.8 ± 2.1	39.2 ± 14.4	38.2 ± 16.1	38.8 ± 16.2	37.6 ± 16.2	0.87
Mean CVP, mm Hg						
Control	5.6 ± 2.0	6.4 ± 2.0	6.0 ± 2.5	6.8 ± 1.6	8.6 ± 4.7	
β-2	5.4 ± 2.3	9.0 ± 3.6	8.2 ± 3.4	8.0 ± 2.5	7.8 ± 3.5	0.47
Mean LAP, mm Hg						
Control	11.8 ± 1.9	6.0 ± 1.8	5.2 ± 1.9	7.4 ± 2.3	7.4 ± 1.1	
β-2	11.2 ± 2.7	13.6 ± 7.4	10.8 ± 5.0	11.0 ± 6.5	10.2 ± 5.0	0.10
Cardiac output, liters/min						
Control	0.8 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.3	
β-2	0.8 ± 0.2	1.1 ± 0.4	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.087
PVR, dyn · s · cm^{-5} × 10^3						
Control	569.4 ± 76.3	4292.4 ± 1915.0	3844.8 ± 1212.7	3415.1 ± 1160.8	2717.3 ± 844.1	
β-2	491.1 ± 169.4	1939.6 ± 939.6	2174.0 ± 1151.2	2366.0 ± 942.7	2522.8 ± 1196.3	0.086
Pa_{O_2}, mm Hg						
Control	569.8 ± 28.3	349.4 ± 102.0	213.0 ± 160.6	139.2 ± 133.0	112.8 ± 87.3	
β-2	623.6 ± 42.3	625.2 ± 55.1	516.6 ± 161.1	537.0 ± 131.7	526.8 ± 130.2	<0.001
Pa_{CO_2}, mm Hg						
Control	17.2 ± 1.9	31.9 ± 6.3	39.1 ± 4.5	48.5 ± 9.1	47.3 ± 6.0	
β-2	20.9 ± 2.3	32.1 ± 1.3	39.3 ± 6.3	39.5 ± 9.1	39.0 ± 8.6	0.24
Dynamic compliance, ml/cm H_2O						
Control	19.4 ± 1.1	12.9 ± 1.3	13.8 ± 1.6	13.8 ± 1.6	13.6 ± 1.0	
β-2	18.4 ± 1.8	19.9 ± 2.6	18.7 ± 3.6	17.3 ± 3.1	16.9 ± 3.1	0.013

AoP, aortic pressure; CVP, central venous pressure; LAP, left atrial pressure; PAP, pulmonary artery pressure; Pa_{CO_2}, partial pressure of arterial carbon dioxide; Pa_{O_2}, partial pressure of arterial oxygen; PVR, pulmonary vascular resistance.

[a]The data are shown as the means ± standard deviation.

[b]p-values are for the differences between groups determined by a repeated measures analysis of variance between 75 and 240 min.

minutes after reperfusion to evaluate the function of the transplanted left lung.

Definitions

The warm ischemic time (300 minutes) was defined as the time from cardiac arrest to the pulmonary arterial flush. The cold ischemic time (120 minutes) was defined as the time from the initiation of the flush of the donor lung until the lung was removed from cold storage for transplantation. The transplantation time (60 minutes) was defined as the time from the removal of the lung from storage to the reperfusion after we performed transplantation. The total ischemic time was therefore defined as the sum (480 minutes).

Assessment of the lungs and their function

Arterial and pulmonary arterial blood samples were used to analyze the blood gases with an iSTAT Portable Clinical Analyzer (iSTAT Corp, East Windsor, NJ).

Lung wet weight–to–dry weight ratio

Left lung tissues (100–300 mg) were used to calculate the wet weight–to–dry weight ratio (WDR) at 240 minutes after

reperfusion or at death. The tissues were excised from the tip of the lung, and the cut end was closed by ligation. Wet weight (mg) was measured first, and the dry weight (mg) was measured after the tissue had been dried overnight at 180°C. The WDR was calculated by dividing the wet weight by the dry weight.

Measurement of adenine nucleotide levels

We used pieces of peripheral lung tissues collected from the left lungs of recipients (normal lungs) just after the induction of anesthesia, samples from donors before and after ventilation, and samples from recipients at 240 minutes after reperfusion. 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, Japan). Total adenosine nucleotides (TAN) and the energy charge were calculated as previously reported.[5]

Measurement of cyclic AMP levels

Pieces of the peripheral lung tissues from donors before and after ventilation were analyzed to determine cyclic AMP (cAMP) lev-
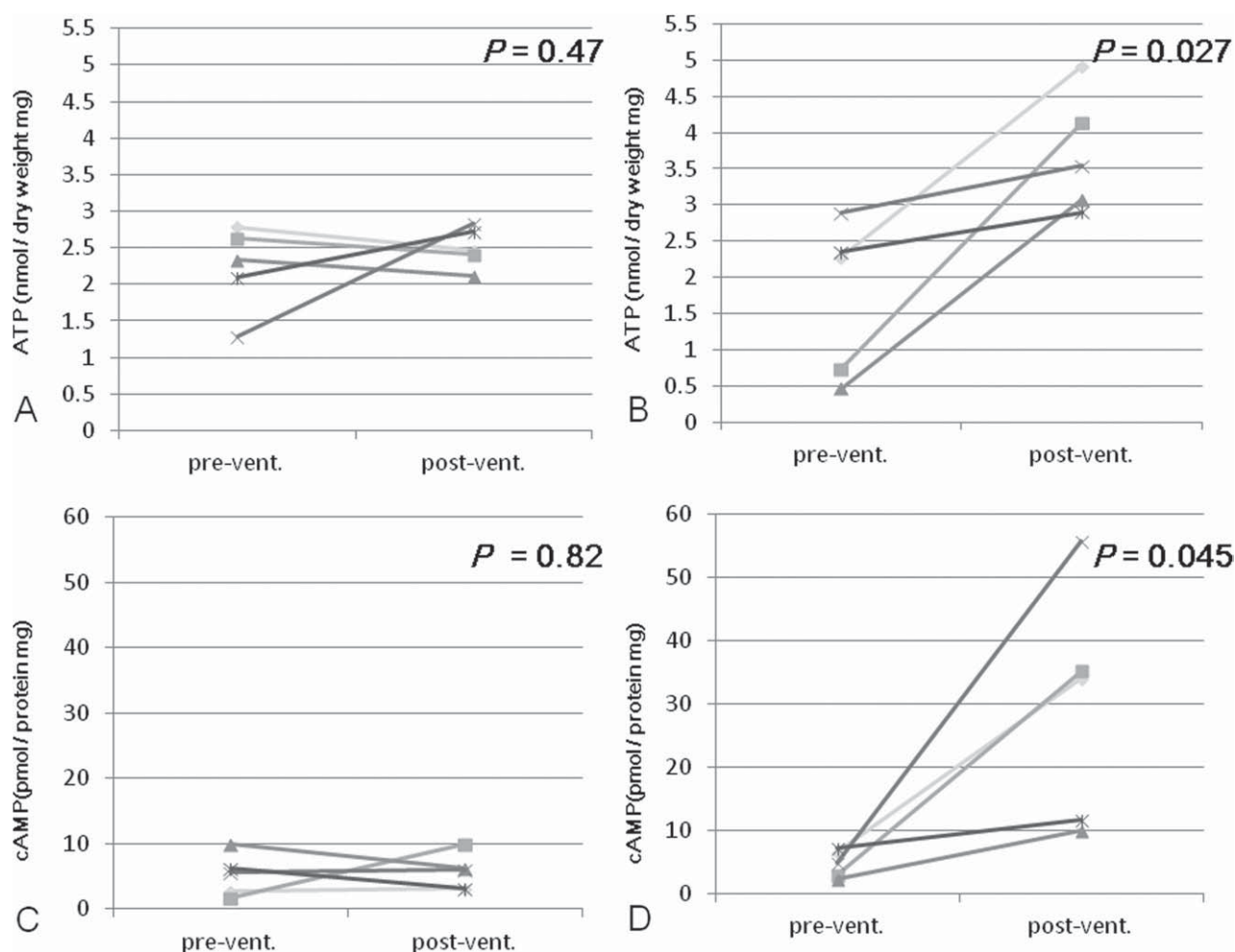

Figure 1 (A and B) Adenosine triphosphate (ATP) and (C and D) cyclic adenosine monophosphate (cAMP) levels are shown before (pre-vent.) and after (post-vent.) the inhalation in donor lungs. The ATP and cAMP levels did not change significantly in the control group (A, C), but increased significantly in the β-2 group (B, D), as determined using a paired t-test.

els. The levels of cAMP were measured with a cAMP radioimmunoassay kit (Yamasa, Chiba, Japan), as previously reported.[5] Protein levels were measured at the same time according to the method described by Lowry et al.[7]

Macroscopic findings and histology

The macroscopic appearances of the left lungs were recorded 240 minutes after reperfusion. Left lung specimens collected 240 minutes after reperfusion were also used for the histologic analysis. Each lung was immersed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Naphthol AS-D chloroacetate esterase staining

The accumulation of activated neutrophils in paraffin-embedded sections of left lungs harvested at 240 minutes after reperfusion was assessed by staining for chloroacetate esterase, a neutrophil-specific marker, according to the method described by Leder.[8] Polymorphonuclear cells, identified by their staining and morphology, were counted in 10 randomly chosen high-power fields per section at original magnification ×400. Three investigators (J.S., F.C., and D.N.) evaluated, interpreted, and reached a consensus, without any knowledge of the experimental groups.

Myeloperoxidase activity

The level of myeloperoxidase (MPO) was measured in the peripheral lung tissues collected in recipients at 240 minutes after reperfusion. We followed the instructions in the manufacturer's manuals from the MPO Assay Kit (BioVision Research Products, Mountain View, CA).

Serum cytokine levels

We used enzyme-linked immunosorbent assays (ELISA) to measure the serum interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α levels at 240 minutes after reperfusion in surviving animals. In all cases, we followed the instructions in the manufacturer's manuals from the IL-6, IL-8, and TNF-α ELISA kits (Quantikine, R&D Systems Inc, Minneapolis, MN).

Statistical analyses

All data were analyzed using JMP 9 software (SAS Institute Inc. Cary, NC). A paired *t*-test was used to compare the samples before and after ventilation with or without the β-2 agonist inhalation. An unpaired *t*-test or a repeated-measures analysis of variance (ANOVA) was used to explore the differences between and within the groups. The ANOVA was performed for data between 75 and 240 minutes to evaluate only the transplanted left lungs after the clamp of right pulmonary artery. Significance was defined as $p < 0.05$.

Results

There were no significant differences between the treatment groups in donor or recipient weights in the β-2 vs the controls (donor weight: 10.3 ± 0.2 vs 10.1 ± 0.3 kg;

recipient weight: 9.2 ± 0.4 vs. 9.3 ± 0.2 kg). We set 60 minutes for the implantation time in our protocol, and were able to perform all transplants within 60 minutes. The 5 animals in the β-2 group survived until the end-point of the study (240 minutes after reperfusion). In the control group, 5 of 6 animals survived, with 1 dog dying of graft dysfunction between 75 and 120 minutes after reperfusion.

Assessment of lung function

The partial pressure of arterial oxygen (Pao$_2$) at 240 minutes after reperfusion was 526.8 ± 130.2 mm Hg for the β-2 group vs 112.8 ± 87.3 mm Hg for the control group. A repeated ANOVA showed the Pao$_2$ and dynamic compliance were significantly higher in the β-2 group ($p < 0.001$) than in the control group ($p = 0.013$; Table 1). Cardiac output was lower and pulmonary vascular resistance was higher in the control group, but this difference did not reach significance ($p = 0.087$ and $p = 0.086$, respectively Table 1).

Lung WDR

The lung WDR in the control group was generally higher than that in the β-2 group at 240 minutes after reperfusion (7.0 ± 2.0 vs 9.23 ± 1.95, respectively), but the difference was not significant ($p = 0.11$).

Measurement of adenine nucleotide and cAMP levels

The control solvent inhalation did not increase the ATP levels in the control group (Figure 1A), whereas ATP levels in the β-2 group were significantly improved after the 60-minute inhalation compared with before the inhalation ($p =$

Table 2 Energy Levels in (A) Normal Lungs and in (B) Recipients 240 Minutes After Reperfusion

(A) Normal Lungs

Energy levels	Normal lung
ATP, nmol/mg · dw	4.20 ± 0.57[a]
Energy charge[b]	0.79 ± 0.02
TAN,[c] nmol/mg · dw	6.21 ± 0.59

(B) Recipient Lungs

Energy levels	Control group	Beta-2 group	*p*-value
ATP	2.21 ± 0.71	4.42 ± 1.35	0.012
Energy charge	0.85 ± 0.05	0.87 ± 0.03	0.48
TAN	2.83 ± 0.86	5.43 ± 1.51	0.027

ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; TAN, total adenosine nucleotides.

[a]The data are presented as the means ± standard deviation and were analyzed with unpaired *t*-tests.

[b]Energy charge = (ATP + 0.5 ATP)/(ATP + ADP + AMP).
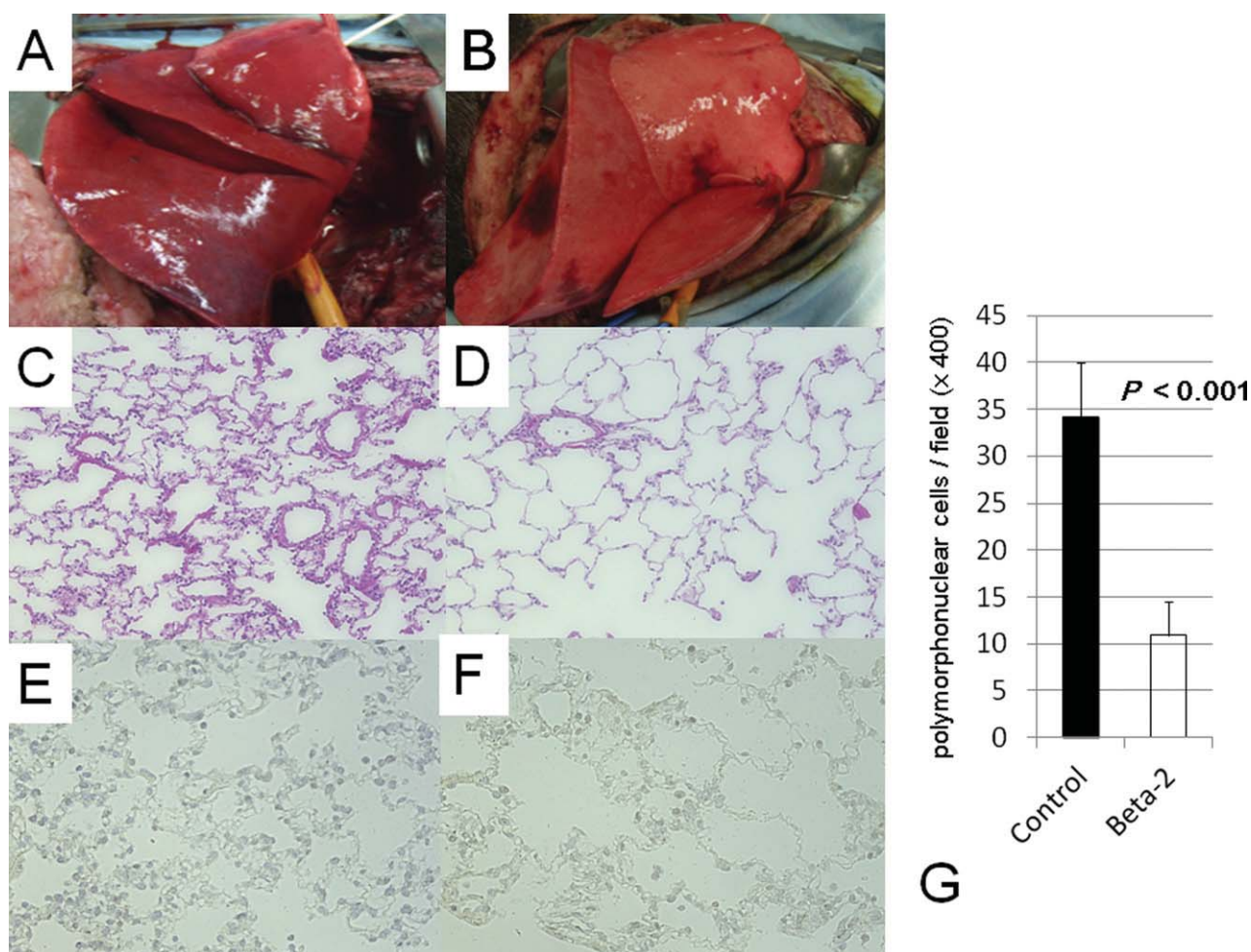
[c]TAN = ATP + ADP + AMP.

Figure 2   The results of the (A, B) macroscopic and (C-F) histologic analyses conducted 240 minutes after reperfusion are shown. Panels C and D show hematoxylin-eosin staining at original magnification ×100. Panels E and F show naphthol AS-D chloroacetate esterase staining at original magnification ×400. In the control group, we observed many dark-red spots (A) and alveolar congestion, hemorrhage, infiltration, aggregation of inflammatory cells, and edema (C). However, the lungs in the β-2 group looked relatively normal (B, D). Naphthol AS-D chloroacetate esterase staining showed significantly higher numbers of polymorphonuclear cells were present in the control group dogs (E) than in the ventilation group (F). (G) The data are presented as the means ± standard deviation and were analyzed using an unpaired t-test.

0.027; Figure 1B), Moreover, at 240 minutes after reperfusion, the ATP and TAN (TAN = ATP + ADP +AMP) levels in recipient lungs were significantly higher in the β-2 group ($p = 0.012$) than in the control group ($p = 0.027$; Table 2B), and the ATP and TAN levels improved similarly to those in the normal lungs (Table 2A). The control solvent inhalation also did not increase the cAMP levels in the control group (Figure 1C). In the β-2 group, the cAMP levels were significantly improved after the 60-minute inhalation compared with the levels before the inhalation ($p = 0.045$; Figure 1D).

Macroscopic findings and histology

At 240 minutes after reperfusion, the lungs from the control group macroscopically had dark red areas exhibiting more patchiness than was observed in the β-2 group (Figures 2A and B). The histologic study indicated severe intraparenchymal hemorrhaging and inflammation only in the control group (Figure 2C), in contrast to the β-2 group (Figure 2D). Staining with naphthol AS-D chloroacetate esterase showed significantly more polymorphonuclear cells were present in the lungs of the control group (Figure 2E) than in the β-2 group ($p < 0.001$; Figure 2F and G).

MPO activity and serum cytokine levels

At 240 minutes after reperfusion, the MPO activity levels in lung tissues were significantly higher in the control group than in the β-2 group ($p = 0.0058$; Figure 3A). Serum TNF-α levels were significantly higher in the control group than in the β-2 group ($p = 0.011$; Figure 3B), whereas no significant difference was found for IL-6 and IL-8 (Figures 3C and D).

Discussion

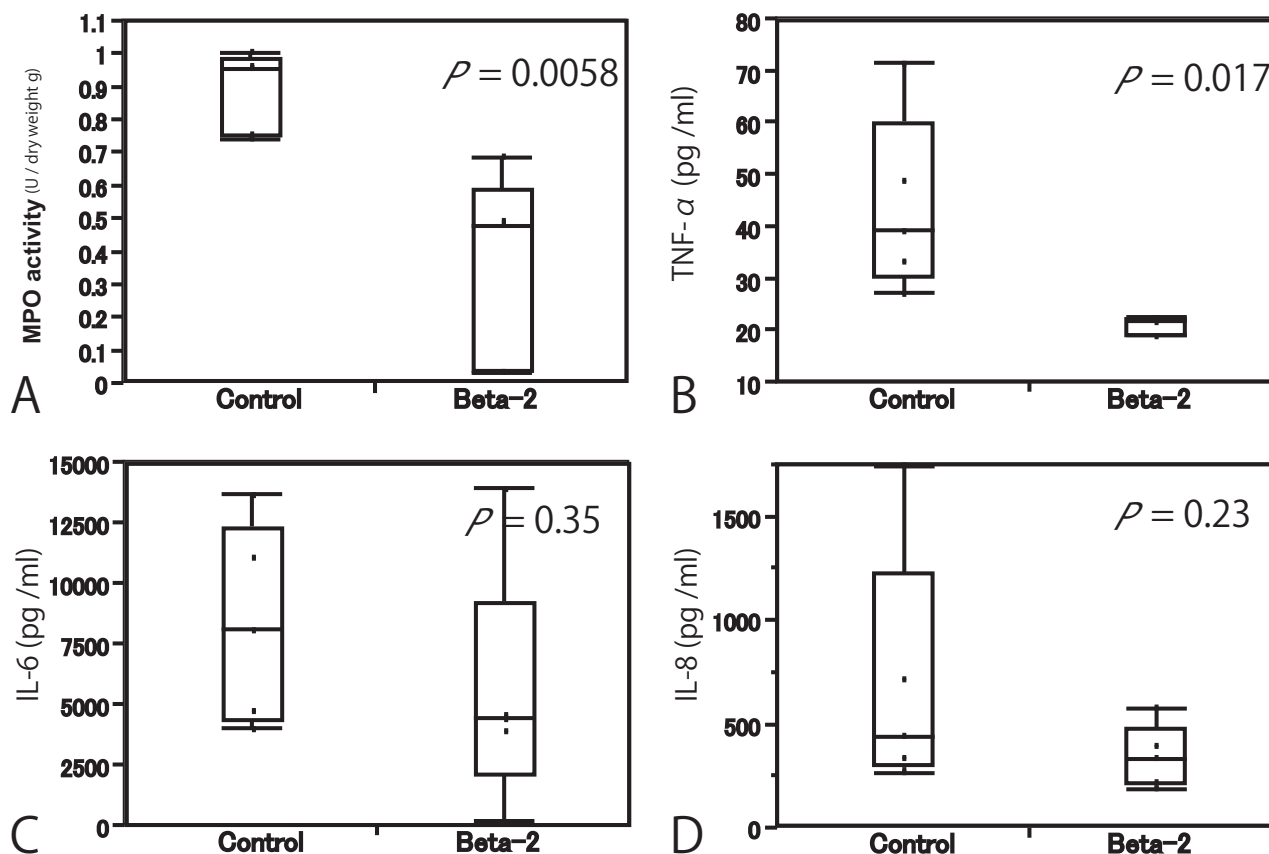In this study, we found that β-2 adrenoreceptor agonist inhalation ameliorated IRI in our canine model, confirming

Figure 3 (A) The myeloperoxidase (MPO) activity of lung tissues and the serum cytokine levels of (B) tumor necrosis factor (TNF)-α, (C) interleukin (IL)-6, and (D) IL-8 are shown in the control and β-2 groups 240 minutes after reperfusion. (A) MPO activity and (B) TNF-α concentrations were significantly higher in the control group. The data were analyzed using an unpaired t-test. The horizontal line in the middle of each box indicates the median; the top and bottom borders of the box mark the 75th and 25th percentiles, respectively; and the whiskers mark the 90th and 10th percentiles.

and expanding our previous study in rats.[5] β-2 Adrenoreceptors were reported to be distributed in alveolar cells, airway epithelium, airway smooth muscle, and pulmonary vessels in humans,[9] which suggested that inhaled β-2 agonists might have actions on various pulmonary tissues, especially with regard to relaxing the airway and vessels. Moreover, β-2 agonist administration was reported to cause vasodilation and increase the bronchial blood flow, which arises from the aorta and forms a peribronchial plexus of vessels that is part of the systemic circulation.[10] In addition, the β-2 inotropic effect of the agonist significantly increased cardiac output and pulmonary artery pressure while decreasing pulmonary vascular resistance.[11] These reports may coincide with our findings, wherein we detected significantly higher dynamic compliance, a relatively higher level of cardiac output ($p = 0.087$), and a relatively lower level of pulmonary vascular resistance ($p = 0.086$) in the β-2 group, although we did not evaluate the compliance of only the transplanted left lungs because we did not clamp the right bronchus.

The mechanism by which the inhalation provides protection can be inferred from our measurements of the cAMP levels, adenine nucleotide levels, and inflammatory cells and cytokines: First, cAMP levels were significantly increased between the measurements taken before and after the inhalation. The β-2 adrenoreceptor agonists were reported to decrease capil-

lary permeability by increasing cAMP[12] as a result of tighter intercellular junctions and actomyosin myofibril relaxation,[13] thereby decreasing the lung edema.

Second, the adenosine nucleotide levels recorded suggest that the β-2 agonist inhalation increased the energy TAN and ATP levels in the donor lungs as well as the ATP and TAN levels in the recipients. A previous report revealed that increased TAN levels have been significantly associated with lung viability.[14]

Third, infiltrating leukocytes are a major factor involved in IRI.[15] Polymorphonuclear cells with AS-D staining were less prevalent in the current study, and MPO activity was lower in the β-2 group. These findings coincide with reports that the systemic administration and inhalation of β-2 adrenoreceptor agonists diminished neutrophil recruitment to the lungs of experimental animals. In terms of the mechanism, diminished intercellular cAMP in the endothelial cells during hypoxia is known to increase leukocyte adhesion,[16] and cAMP negatively regulates the migratory capacity of neutrophils.[17] Our findings of higher cAMP levels in the β-2 group may therefore coincide with these mechanisms.

Fourth, we observed significant decreases in the serum levels of the inflammatory cytokine TNF-α. In previous reports, the systemic administration of several β adrenoreceptor

agonists potently inhibited TNF-α during endotoxemia,[18] and inhaled salmeterol reduced TNF-α in the bronchoalveolar lavage fluid after inhalation of lipopolysaccharide in humans.[19] Our finding showing a reduced serum TNF-α level suggests that β-2 adrenoreceptor agonist inhalation may reduce the inflammatory change in uncontrolled DCD donors.

One limitation of our study is that we did not examine an adequate dose of procaterol in our model. Our dose of 350 μg was likely too high, because 30 to 50 μg is clinically prescribed for one inhalation of an adult. However, frequent inhalation (∼500 μg/day) of procaterol in younger and older adults was reported to be safe.[20] We also used high doses of medication in the DCD donors 240 minutes after cardiac arrest, which meant that there was a long enough time after the declaration of death from an ethical aspect. Therefore, in our experiments, we selected the dose of procaterol based on the likely maximum dose that could be used for the 60 minutes of ventilation.

Inhalation of β-2 adrenoreceptor agonist has not yet been routinely performed in brain-dead donors in Japan. One reason is that it is difficult to change the law concerning the management of brain-dead donors in Japan. Now, our group has revealed the effect of β-2 agonist inhalation in the experimental DCD in rat[5] and this canine model. Therefore, these results would strongly encourage clinicians to conduct β-2 adrenoreceptor agonist inhalation in brain-dead donors and, in the near future, DCD donors.

In conclusion, the protective effect of β-2 adrenoreceptor agonist inhalation during the last 60 minutes of a 300 minutes of warm ischemic time preserves the lung function of DCD donors. Therefore, the use of the β-2 adrenoreceptor agonist inhalation during this period should be conducted in a clinical setting of DCD.

Disclosure statement

The procaterol inhalation solutions were purchased from Otsuka Pharmaceutical Co Ltd, and the control solvents were provided by the company free of charge without any restrictions on the nature of the research.

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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