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Alveolar liquid clearance in lung injury: Evaluation of the impairment of the β_2-adrenergic agonist response in an ischemia-reperfusion lung injury model

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ABSTRACT

While alveolar liquid clearance (ALC) mediated by the β_2-adrenergic receptor (β_2-AR) plays an important role in lung edema resolution in certain models of lung injury, in more severe lung injury models, this response might disappear. Indeed, we have shown that in an ischemia-reperfusion-induced lung injury model, β_2-agonists do not enhance ALC. The objective of this study was to determine if downregulation of the β_2-AR could explain the lack of response to β_2-agonists in this lung injury model. In an *in vivo* canine model of lung transplantation, we observed no change in β_2-AR concentration or affinity in the injured transplanted lungs compared to the native lungs. Furthermore, we could not enhance ALC in transplanted lungs with dcAMP + aminophylline, a treatment that bypasses the β_2-adrenergic receptor and is known to stimulate ALC in normal lungs. However, transplantation decreased αENaC expression in the lungs by 50%. We conclude that the lack of response to β_2-agonists in ischemia-reperfusion-induced lung injury is not associated with significant downregulation of the β_2-adrenergic receptors but is attributable to decreased expression of the ENaC channel, which is essential for sodium transport and alveolar liquid clearance in the lung.

1. Introduction

The capacity of the lung to clear fluid has been shown to be one of the factors that can influence the prognosis of cardiogenic and non-cardiogenic pulmonary edema (Matthay and Wiener-Kronish, 1990; Verghese et al., 1999; Ware and Matthay, 2001). Active Na^+ transport is the main driving force in the physiological process of edema clearance from the alveolar space. The epithelial sodium channel (ENaC) at the apical surface and the sodium–potassium–adenosine triphosphatase (Na^+-K^+-ATPase) at the basolateral surface of the alveolar epithelial cells are two essential drivers of this alveolar liquid clearance (ALC) as they create an osmotic gradient that leads to transalveolar liquid transport (Dagenais et al., 2009; Matalon et al., 2015; Matthay et al., 2002). In normal lungs, several compounds can stimulate ALC, but the best-studied agents are β-adrenoceptor agonists (β-AR agonists) (Berthiaume and Matthay, 2007; Berthiaume et al., 1987; Mutlu et al., 2004b). Overexpression of the β-adrenergic receptor in rats has also been shown to increase edema clearance (Dumasius et al., 2001).

Although β-AR agonists, as well as endogenous catecholamine response, can enhance ALC in models of lung injury (Modelska et al., 1997; Pittet et al., 1994; Saldias et al., 1999), the response to β-AR agonists might depend on the severity of injury (Berthiaume and Matthay, 2007). Indeed, we previously demonstrated in a canine single lung transplant model with significant lung injury induced by ischemia-reperfusion that ALC is significantly lower in transplanted versus native lungs after 4 h of reperfusion and that the transplanted lung did not respond to β-AR agonist stimulation (Sugita et al., 2003). The objective of the present study was to explore the cause of this dysfunction and, in particular, the lack of response of ALC to β-adrenergic agonist stimulation in a lung injury induced by ischemia-reperfusion following lung transplantation.

2. Methods

2.1. Animals

All animal experiments were approved by the institutional animal care committee of the research center of the Centre hospitalier de

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l'université de Montréal (CHUM) and are in accordance with the Principles of Laboratory Animal Care formulated by the Institute of Laboratory Animal Resources and the Guide for the Care and use of Laboratory (National Institutes of Health Publication No. 86-23, revised 1985). We used a single lung transplantation model in dogs, as we have previously shown that we can induce significant lung injury with this model (Sugita et al., 2003) and that dog lungs have a rate of alveolar liquid-clearance similar to that of humans (Berthiaume et al., 1988; Grimme et al., 1997). Thus, we used 18 dogs of both sexes weighing 19–30 kg. Nine left orthotopic lung transplants were performed using size-matched animals. Four of 9 animals were treated with cAMP and aminophylline, and 5 served as controls.

2.2. Surgical procedures

2.2.1. Surgical procedure for harvesting donor lungs

This surgical procedure is similar to one described previously (Sugita et al., 2003). Each donor animal was anesthetized with an intravenous (IV) injection of Propofol (4 mg/kg; Sigma-Aldrich, Oakville, ON) after intramuscular injection of glycopyrrolate (0.01 mg/kg; Sabex Inc., Quebec, Canada), Atravet (0.04 mg/kg; Ayerst Laboratories, Quebec, Canada), and meperidine (4 mg/kg; Sabex Inc., Quebec, Canada) as premedication. Following endotracheal intubation, dogs were ventilated with a volume-cycled ventilator (Harvard Apparatus, Dover, MA) at a tidal volume of 18 ml/kg, a respiratory rate of 15 breaths/minute, an inspiratory oxygen concentration of 1.0, and a positive end-expiratory pressure of 5 cmH_2O. We did not use a protective ventilation strategy since the purpose of this model was to induce lung injury to study ALC under conditions of significant lung injury. However, tidal volume was decreased to 9 ml/kg during pneumonectomy or during transplantation to avoid barotrauma. Anesthesia was maintained with 1.5–2.0% isoflurane and pancuronium (0.1 mg/kg; GlaxoWellcom Inc., Quebec, Canada) IV. Following a median sternotomy, the heart-lung block was harvested following a standard procedure. Heparin sodium (400 U/kg, Leo Pharma Inc.) was given intravenously, and a catheter was inserted into the trunk of the pulmonary artery (PA). The donor pulmonary vasculature was flushed with cold modified Euro-Collins solution (50 ml/kg; Baxter Corporation, Ontario, Canada) through the PA catheter at a height of 30 cm. Topical cooling was achieved with cold Ringer's lactate solution. At the completion of flushing, lungs were inflated with room air, clamped at the trachea, excised, and placed in a plastic bag containing Ringer's lactate solution. The bag was sealed and placed in crushed ice.

2.2.2. Surgical procedure for recipients

Animals were anesthetized using the same procedures as for donor animals. Before performing left-sided thoracotomy, a 16-gauge catheter was inserted in the femoral artery for arterial pressure measurements and blood sampling, and an 8 Fr. Swan-Ganz catheter was introduced into the PA for pressure measurements. The native left lung was excised by left-sided thoracotomy. Transplantation of the left lungs was performed as previously described (Sugita et al., 2003). Total ischemic time was 200 ± 4 min and 220 ± 15 min for treated and untreated groups, respectively. Mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and heart rate (HR) were monitored during anesthesia and for another 8 h following reperfusion.

2.3. Alveolar liquid clearance

Because measurements of ALC in nonperfused lung can be altered by the presence of interstitial edema (Fukuda et al., 2000), we measured ALC in an *in vivo* preparation wherein pulmonary circulation remains intact. Four hours after transplantation, left-sided thoracotomy was performed. The left lower lobe bronchus was cannulated with a catheter (Tygon® Tubing; Cole-Parmer Instrument Company, Vernon Hills, IL). Through the catheter, we instilled 30 ml of a solution (130 mM Na^+, 4 mM K^+, 1.5 mM Ca^{++}, 109 mM Cl^-, 28 mM lactate, 11.1 mM glucose, 5.0 g/dl bovine serum albumin (Sigma Chemical Co., St. Louis, MO)) prewarmed to 37 °C. The instilled lobe was kept inflated with continuous positive air pressure of 6–7 cm H2O (FiO2 = 0.21) but not ventilated. Approximately 1 ml of alveolar liquid was aspirated one minute after instillation as the initial alveolar liquid, and approximately 700 µl of alveolar liquid was aspirated hourly for 4 h to measure ALC. To determine if cAMP could stimulate ALC, we added 10^{-3} M dcAMP and 10^{-3} M aminophylline to the instillate solution in the treatment group as it has been previously shown to stimulate alveolar and lung liquid clearance (Berthiaume, 1991). After 4 h, instillate solution was aspirated and used as the final alveolar liquid to measure total protein concentration using the Biuret method (Doumas et al., 1981). Eight hours post transplantation, animals were euthanized by cross clamping the pulmonary artery and aortic stump, and the heart-lung block was removed. The lung was divided into lobes that were snap-frozen in liquid nitrogen and stored at −40 °C for further study (wet-to-dry lung weight ratio, β-AR, mRNA analyses).

ALC was calculated using measurements of the initial (1 min after instillation) and final alveolar protein concentration using the following formula (Berthiaume et al., 1987).

$$ALC\ (\%) = (Vi \times Fwi - Vf \times Fwf) / (Vi \times Fwi) \times 100.$$

where Fw is the water fraction of the initial (*i*) and the final (*f*) alveolar liquid. The water fraction is the volume of water per volume of solution measured by the gravimetric method. V represents the volume of the initial liquid (*i*) and the final alveolar liquid (*f*), and V*f* is calculated by the following equation:

$$Vf = (Vi \times TPi) / TPf,$$

where TP represents protein concentration.

Because we wanted to study the expression of β_1 and β_2 receptors in native and transplanted lungs using radiolabeled ligands, we were unable to use radiolabeled albumin to study protein permeability in these experiments. Although, in theory, ongoing protein leak could influence our measurement of ALC, we previously demonstrated that ALC measurement obtained using this technique is similar, if not identical, to ALC measured by a gravimetric method by which the results would not be influenced by protein leak (Sugita et al., 2003).

2.4. Gravimetric measurements

The middle lobe of native lungs and the lingula of transplanted lungs were used for this measurement. After homogenizing 3–4 g of lung parenchyma, we measured the wet-to-dry lung weight ratio (g water/g dry lung) adjusted with lung hemoglobin concentration, according to a previously described method (Pearce et al., 1965).

2.5. Quantitative and affinity analysis of β-AR

Two 3 g aliquots of frozen lung parenchyma were homogenized with 5 ml lysis buffer (20 mM $NaHCO_3$) two times for 10 s in a polytron. The homogenate was then centrifuged at 40,000 g for 30 min at 4 °C two times, and the pellet was vortexed in a buffer solution (TRIS 10 mM (pH 7.4), 154 mM NaCL and 0.55 mM ascorbic acid) for determination of membrane protein concentration using the Bradford method (Bradford, 1976).

To compare the proportion of β_1 and β_2 receptors in native and transplanted lungs, we performed competition binding curves using the specific radiolabeled antagonist of β-AR, iodo cyano pindolol-iodure^{125} (ICYP-I^{125}, 2200 Ci/mmol, PerkinElmer, Woodbridge, ON), which has the same affinity for the 2 populations of studied receptors. Before performing the experimental protocol, we assessed optimal incubation time as well as protein and radiolabeled concentration for construction of competition curves (results not shown). The optimal amount of

radiolabeled tracer was determined in the presence of propanolol, an unspecific β-AR antagonist. For the competition binding curves, we used a membrane protein concentration of 10 µg and 20,000 cpm of ICYP I^{125} in 200 µL of incubation buffer. Incubation was performed for 2 h at ambient temperature on a stirring plate in the presence of 16 different concentrations (10^{-12} to 10^{-3} M) of specific antagonist, CGP 20712 A and ICI 118551, for β_1 and β_2-AR, respectively. Determination of the inhibition constant (Ki) allowed us to calculate affinity. We used the *Allfit* program for Windows 2.13 (C. and A. de Léan,1993–1997) with a 2-binding site analysis for our results.

For data analysis, we used only the inhibition constant for ICI 118 551 because the curve generated with the CGP 20712 A antagonist was too variable to provide meaningful data. We believe this variability and our difficulty in obtaining an appropriate curve is most likely because the quantity of β_1-adrenergic receptors in the lung is relatively low compared to the expression of β_2-adrenergic receptors (less than 20%) (Barnes et al., 1983). However, ICI 118 551, which is a specific antagonist of β_2-adrenergic receptors at low concentration (K_{iH}), can bind to β_1-adrenergic receptors at high concentration (K_{iL}) (Bilski et al., 1983). We used this value to estimate the quantity of β_1-adrenergic receptors. Therefore, for determination of β-AR affinity, we compared Ki values obtained with ICI 118551 from native and transplanted lungs. K_{iH} indicates high affinity Ki of ICI 118551 for β_2-AR, and K_{iL} indicates low affinity Ki for β_2-AR.

2.6. Northern blot hybridization

Study of ENaC expression was performed by northern blot hybridization as previously described (Sugita et al., 2003). Total RNA was prepared from lung tissues by homogenization and acid phenol/chloroform extraction with TRIZOL® reagents (Life Technologies, Grand Island, NY) following the manufacturer's directions. For northern blotting, 10 µg/lane of total RNA was fractionated by agarose-formaldehyde gel electrophoresis, transferred to GeneScreen nylon membranes (NEM, Boston, MA) and hybridized with a ^{32}P-labeled full-length cDNA probe for αENaC (a gift from Dr. B.C. Rossier, Institut de Pharmacologie et de Toxicologie, Université de Lausanne, Lausanne, Switzerland) as previously described (Sugita et al., 2003). Expression of ENaC in transplanted lungs was compared to the level of expression in the control lung of each animal.

2.7. Statistical analysis

All results are expressed as the mean ± standard error of the mean. Student's paired and unpaired *t*-tests were used to compare means between two groups. Physiological parameters between control and transplanted groups, intracellular cAMP levels and ALC were compared with repeated measurements ANOVA. Phosphorimager readings of the mRNAs of interest were normalized to corresponding 18S rRNA readings to correct for RNA yield and gel loading. A Mann-Whitney test was used to compare northern blot results, and differences with a *p*-value < 0.05 were considered significant.

3. Results

3.1. Physiological parameters

Hemodynamics parameters were monitored before surgery until 8 h after reperfusion and are shown in Table 1. There were no clinically significant differences in MAP, HR or pH between control and treated (dcAMP and aminophylline) dogs. Interestingly, MAP decreased progressively during anesthesia in both groups. Similarly, arterial PaO_2 to inspired O_2 fraction ratio (PaO_2/FiO_2) decreased throughout the 8 h of reperfusion but to the same extent in both group (Fig. 1). Six hours after experiments, MPAP of treated dogs (dcAMP and aminophylline) was reduced (12 ± 2 vs 19 ± 2). This decrease did not reach statistical

Table 1
Physiological parameters of control (n = 5) and treatment groups (n = 4, dcAMP + aminophylline) before and during reperfusion. MAP: mean arterial pressure, MPAP: mean pulmonary arterial pressure, T: transplantation, H: hours after transplantation. Values are means ± SE.

Parameters	Groups	Before T.	Immediately after T.	1H	2H	3H	4H	5H	6H	7H	8H
Arterial pH	Control	7,37 ± 0,03	7,40 ± 0,06	7,44 ± 0,02	7,35 ± 0,03	7,33 ± 0,02	7,29 ± 0,04	7,35 ± 0,04	7,38 ± 0,01	7,40 ± 0,03	7,35 ± 0,02
	Treated	7,40 ± 0,03	7,49 ± 0,04	7,38 ± 0,05	7,39 ± 0,07	7,41 ± 0,04	7,40 ± 0,04	7,36 ± 0,04	7,41 ± 0,07	7,39 ± 0,06	7,32 ± 0,01
MAP (mmHg)	Control	91 ± 13	72 ± 9	73 ± 8	63 ± 2	61 ± 2	68 ± 4	68 ± 5	68 ± 6	66 ± 6	64 ± 4
	Treated	86 ± 4	87 ± 10	77 ± 9	69 ± 5	72 ± 6	65 ± 4	69 ± 6	65 ± 4	65 ± 5	65 ± 3
MPAP (mmHg)	Control	14 ± 2	18 ± 3	20 ± 2	18 ± 3	18 ± 2	18 ± 2	20 ± 3	19 ± 2	19 ± 2	17 ± 2
	Treated	16 ± 5	14 ± 2	18 ± 2	17 ± 2	16 ± 2	15 ± 2	14 ± 2	12 ± 2	11 ± 2	12 ± 2

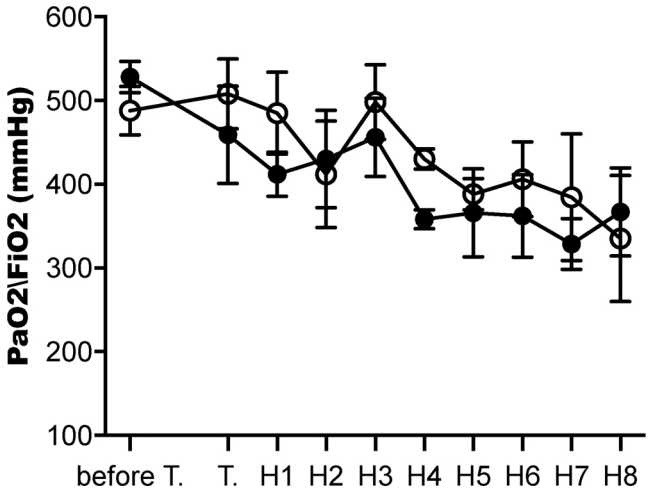

Fig. 1. Changes in PaO_2/FiO_2 before surgery and during 8 h of monitoring. Although there is a progressive decrease in oxygenation during the procedure, there is no difference between the treated (open circle, n = 4) and control groups (closed circle, n = 5). Values are means ± SE. T: transplantation, H: hours after transplantation.

significance due to the lack of power in the experimental protocol. Although it did not have a major impact on the hemodynamic behavior of these animals, it might explain by the decreased resistance of the pulmonary vasculature and increased perfusion in the area where the solution containing cAMP was instilled to study ALC (Berthiaume et al., 1987).

3.2. Effects of transplantation on the numbers and affinity of lung β-AR

To explain the ineffectiveness of the β_2-agonist previously reported (Sugita et al., 2003), we assessed whether reperfusion might decrease numbers of β-ARs. There was no statistically significant difference in β_1-AR (150 ± 29 vs 127 ± 39 fmol/mg of protein respectively) or β_2-AR (1172 ± 157 vs 1031 ± 123 fmol/mg of protein respectively) between native and transplanted lungs (Fig. 2). Furthermore, we found no statistically significant differences of K_{iH} or K_{iL} for ICI 118 551 between native and transplanted lungs (1.6 ± 0.2 vs 1.9 ± 0.4 nM and 1603.0 ± 601.5 vs 1963.7 ± 782.3 nM, respectively, Fig. 3).

3.3. Effects of intra-alveolar dcAMP and aminophylline instillation on ALC after lung transplantation

ALC progressively decreased over time in the control group as previously reported (Sugita et al., 2003) (Fig. 4, white bars). In the presence of dcAMP and aminophylline, ALC also progressively decreased during the reperfusion period (Fig. 4, black bars). Surprisingly, the impairment was more pronounced in the treatment group. Differences between control (transplanted but untreated) and treatment groups were statistically significant at 60, 180 and 240 min.

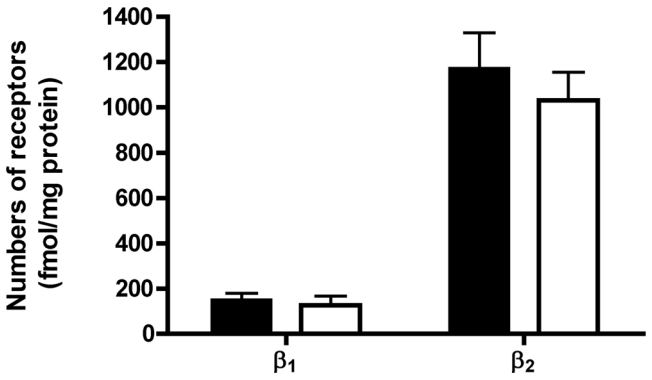

Fig. 2. Comparison of β_1 and β_2-adrenergic receptor concentrations in distal lung of native (black bars, n = 6) and transplanted lungs (white bars, n = 6). No significant differences in β_1 or β_2 in β-AR concentration were found for either subtypes. Data are means ± SE.

3.4. Effects of intra-alveolar dcAMP and aminophylline instillation on wet-to-dry lung weight ratio (WtD) after lung transplantation

As expected, we found a significant increase in the WtD between native and transplanted lungs for both control and treatment groups (Fig. 5). However, there was no difference in the magnitude of the increase in WtD induced by transplantation between control and treatment groups, suggesting that lung injury was comparable between both groups.

3.5. Effect of ischemia-reperfusion on αENaC mRNA expression (Fig. 6)

After 8 h reperfusion, αENaC expression decreased in transplanted lungs compared to native lungs (Fig. 6).

4. Discussion

The objective of this study was to determine if the lack of ALC stimulation by β-agonists following transplantation-induced I–R lung injury could be secondary to a decrease in expression or affinity of β-AR. Our results showed that, following lung injury, there was no decrease in the quantity or affinity of β-AR in the lungs. Furthermore, we showed that administration of an analog of cAMP, the intracellular signaling pathway induced by β-agonist that stimulates ALC (Mutlu and Sznajder, 2005), did not enhance ALC in injured lungs. These data suggest that the primary explanation for impaired β-agonist stimulation of ALC in injured lung is distal to the β-AR-induced increase in intracellular cAMP and could be explained by the observed decrease in ENaC expression in these lungs.

Despite 50 years of research, there is still no disease-modifying treatment for lung injury or ARDS (Laffey and Matthay, 2017). β-agonist stimulation has been proposed as a pharmacological approach to enhance edema resolution in injured lungs. This strategy originated from experimental observations that in some models of lung injury induced by hemorrhagic shock or sepsis, β-agonists stimulate ALC (Berthiaume and Matthay, 2007). Based on these observations, multiple clinical trials were conducted (Perkins et al., 2006) (Gao Smith et al., 2012; Matthay et al., 2011). The results of these trials were inconclusive. The failure of either inhaled or intravenous β-adrenergic agonists as a therapeutic strategy could be explained by several mechanisms, including loss of an intact alveolar epithelial barrier (Albertine, 1998; Matthay, 2014), defective beta-agonist receptor function (Mutlu and Factor, 2008; Davis et al., 2004; Modelska et al., 1999) or dysfunction in active Na^+ transport (Berthiaume and Matthay, 2007). However, we cannot exclude the possibility that the failure of beta-agonist therapy might be related to dysfunction or decreased expression of β_2-adrenergic receptors in injured lungs. In this study, we specifically explored the possibility that the lack of stimulation of ALC by β-agonists we observed previously in ischemia-reperfusion injury associated with lung transplantation (Sugita et al., 2003) is secondary to decreased expression or affinity of β-receptors. Our results excluded this hypothesis. Indeed, both the quantity and affinity of β-AR in native and transplanted lungs were found to be similar (Fig. 3). We would have liked to confirm these results by evaluating expression of β_2-AR by western blot. We were, however, unable to identify a reliable antibody that works in dog tissue. Nevertheless, we can be relatively confident of our results since the total concentration of β-AR binding sites, as well as K_{iH} for β_2-AR measured in our tissues, are in the same range as those that have been previously reported in lung tissue (Barnes et al., 1983;

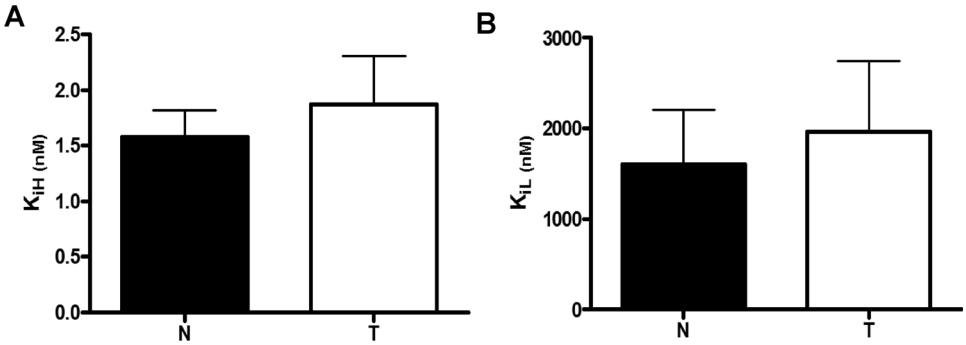

Fig. 3. Comparison of affinity constant ICI 118 551 between native (black bars, n = 6) and transplanted lungs (white bars, n = 5) for β_1 and β_2-AR. A) There was no significant difference in K_{iH} between native (1.6 ± 0.2 nM) and transplanted lungs (1.9 ± 0.4 nM). B) There was no significant difference in K_{iL} between native (1603.0 ± 601.5 nM) and transplanted lungs (1963.7 ± 782.3 nM). K_{iH}: High affinity inhibitory constant of ICI 118 551 for β_2-AR. K_{iL}: low affinity inhibitory constant of ICI 118 551 for β_1-AR. N: native lung, T: transplanted lung. Data are means ± SE.

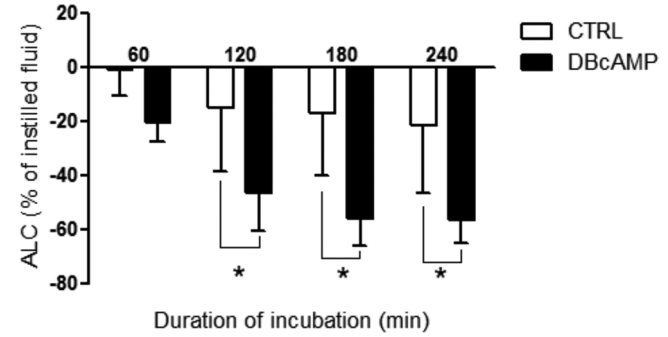

Fig. 4. Representation of the course of ALC during the reperfusion period in transplanted lungs, unstimulated (white bar, n = 5) and stimulated by dcAMP + amynophylline (black bar, n = 4). ALC significantly decreased during the reperfusion period in both groups. There was a trend for a stronger decrease of ALC in the treatment group, which reached significance at 120, 180 and 240 min (*p < 0.05) compared to the untreated group. Data are means ± SE.

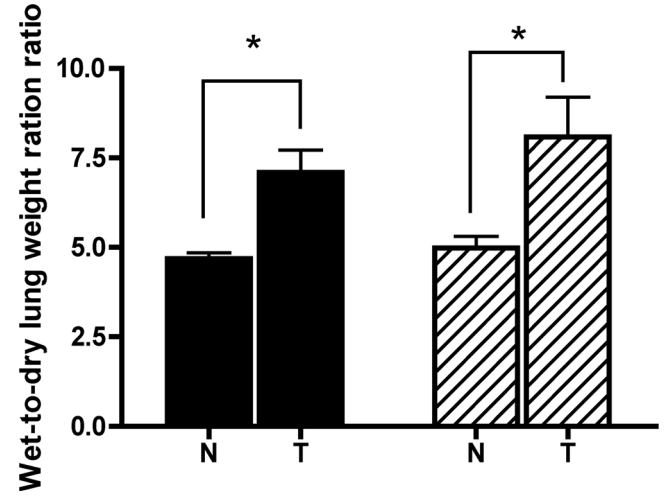

Fig. 5. Comparison of wet-to-dry lung weight ratios (WtD). Ischemia-reperfusion caused a significant increase of WtD when comparing transplanted (T) to native (N) lungs (*p < 0.05). The increase in lung WtD after transplantation was similar in the group treated with dcAMP + aminophylline (hatched bar) and in the control group that was not treated with dcAMP + aminophylline (black bar). Data are means ± SE.

Emala et al., 1996). We were also unable to specifically measure the quantity of β_1-AR in the lung tissue. This is probably not critical to the interpretation of our data since data from knockout mice suggest that it is β_2-AR that is responsible for the bulk of β-AR-sensitive ALC (Mutlu et al., 2004a). Few studies have explored the expression of beta-adrenergic receptor in injured lungs. Kabir et al. (Kabir et al., 2009) have shown in lungs injured with 2-choloroethyl ethyl sulfide, a mustard gas analog, that there is no statistically significant change in β_2 or β_1-AR expression at the mRNA or protein level. However, researchers did observe decreased β_2-AR affinity, as well as slight desensitization of the receptor.

Showing that the quantity of β-AR is not decreased does not exclude the possibility that there is dysfunction of the β-AR. Indeed, Kabir et al. (2009) reported that, in injured lungs, desensitization of β_2-AR leads to a decrease in lung tissue cAMP. To test whether a similar phenomenon might occur post ischemia-reperfusion injury, we administered dcAMP and aminophylline directly into the lungs, thereby allowing us to bypass β-AR and to directly increase cAMP in the lung tissue. We have previously shown that this experimental strategy leads to increased ALC in uninjured lungs (Berthiaume, 1991). However, this treatment failed to improve ALC in the ischemia-reperfusion injured lung model. Rather, we observed a significant deterioration of ALC in the injured lungs. This result is somewhat surprising since cAMP has been shown to improve endothelial barrier integrity (Sayner, 2011) and to stimulate ALC in an animal model of ventilator-induced lung injury (Frank et al., 2003). However, ischemia-reperfusion associated with lung transplantation induces overproduction of reactive oxygen species and nitric oxide that lead to endothelial injury (Chatterjee et al., 2014) or ion transport dysfunction (Guidot et al., 2006; Modelska et al., 1999). The severity of the injury, as shown by the relatively high wet-to-dry ratio, might have been too great to be overcome by dcAMP treatment. Indeed, beyond injury induced by ischemia-reperfusion, we cannot exclude possible amplification of injury by volutrauma induced by the ventilatory strategy used (Beitler et al., 2016; de Perrot et al., 2002). We chose not to apply a protective ventilation strategy since our principle objective was to determine if ALC in the presence of severe injury can be modulated by dcAMP. Furthermore, we cannot exclude the possibility that dcAMP did permeate more deeply into the endothelial or epithelial cell cytosol, leading to reorganization of microtubules and barrier disruption (Sayner, 2011) that would decrease ALC (Frank et al., 2002; Lin et al., 2016). Finally, it is possible that mechanical factors might have slowed ALC. Indeed, the presence of dcAMP in the alveolar space and in the circulation might increase pulmonary blood flow to the area where the liquid was instilled to study ALC, as shown previously when β-adrenergic agonists are present in the instilled liquid (Berthiaume et al., 1987). This increase in pulmonary blood flow in the presence of endothelial injury increases fluid filtration and interstitial edema formation, subsequently slowing ALC (Fukuda et al., 2000) and the accumulation of liquid in the alveolar space. Ventilation with high tidal volume might have also contributed significantly to the decrease in ALC, as shown previously (Frank et al., 2003).

In this series of experiments, we did not perform control experiments in which ALC was measured in nontransplanted lungs. We believe this is justified since we have reported in three previous manuscripts the results of experiments performed in nontransplanted lungs with or without treatment of a β-adrenergic agonist (Berthiaume et al., 1988; Sugita et al., 2009, 2003). In all these experiments, ALC in the normal lung after 4 h is approximately 15–20% of liquid instilled. Furthermore, liquid clearance measured after 4 h in control

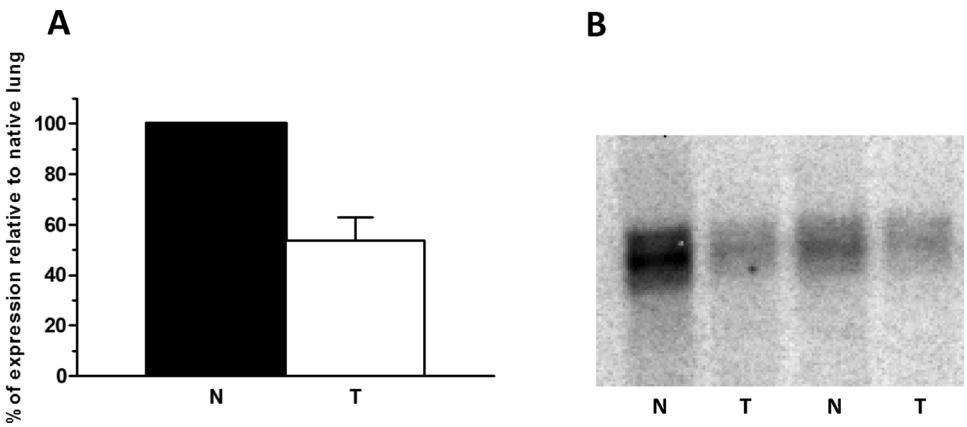

Fig. 6. Modulation of αENaC expression after 8 h of reperfusion in native and transplanted lungs. (A) Densitometric analyses reveal that ischemia-reperfusion reduces αENaC mRNA expression in transplanted lungs (white bars) compared to native lungs (black bars; arbitrarily set at 100%) by approximately 50% (*$p < 0.05$; control lungs n = 5, treated lungs n = 4). Data are means ± SE. (B) Representative northern blots. Lanes identified as N represent αENaC mRNA from native lungs, whereas lanes identified as T were from transplanted lungs.

transplanted lungs (in the absence of dcAMP and aminophylline) in the present experimental protocol was almost identical (−21%) to that measured previously (Sugita et al., 2003). Overall, these results demonstrate that the lack of efficacy of the β-adrenergic agonists as a therapy for injured lungs and ARDS patients is not due to a decreased expression or dysfunction of β-AR.

Since β-AR dysfunction does not seem to be the principal mechanism associated with decreased ALC in our injury model, it is most likely that dysfunction of ion transport mechanisms could be responsible for abnormal ALC. ENaC is one of the constituents of the Na^+ transport system in the lungs and has been shown to play an essential role in ALC (Dagenais et al., 2009; Matalon et al., 2015; Matthay et al., 2002). Mice with targeted inactivation of the ENaC gene (Hummler et al., 1996) were unable to clear liquid from their lungs after birth. Furthermore, in a transgenic mouse model in which αENaC expression was reduced, there was a delay in the resolution of pulmonary edema following thiourea-induced lung injury (Egli et al., 2004). Finally, it has been shown that engineering a decrease of ENaC expression in the lung by administration of ENaC siRNA leads to decreased stimulation of amiloride-sensitive ALC and inhibition of terbutaline stimulation of ALC (Li and Folkesson, 2006). We have also reported previously that the observed decrease in ALC in transplanted lungs is paralleled by a decrease of both ENaC mRNA and protein expression (Sugita et al., 2003). We have confirmed this observation in the present study, in which we report an approximately 50% decrease in αENaC mRNA in transplanted lungs. Although these results suggest that a decrease in expression of αENaC mRNA contributes significantly to decreased ALC seen in injured lungs and to the lack of response by β-agonists, further investigation is needed to demonstrate that indeed the changes in αENaC mRNA are also associated with a decrease in protein expression and function.

Other components of the ion transport system could also be affected. Indeed, it has been shown that Na-K-ATPase is an essential component for effective transepithelial sodium transport and for ALC (Sznajder et al., 2002). Although we did not examine its expression or activity in this series of experiments, we have previously shown that there were no significant changes in Na-K-ATPase expression in transplanted lung injury (Sugita et al., 2003). However, decreased ENaC expression leading to decreased absorption of Na^+ at the apical membrane could result in decreased activity of Na-K-ATPase. A modulation of CFTR expression might also be important to maintain lung liquid clearance in response to β-adrenergic stimulation (Fang et al., 2002, 2006; Mutlu et al., 2005). Data from CFTR-deficient mice (ΔF508 transgenics) indicate that CFTR is not required for alveolar homeostasis in uninjured lung but is essential in the presence of excess airspace fluid and β-AR-mediated enhancement of ALC (Fang et al., 2002). Thus, we cannot exclude that a decrease in expression of CFTR could, in the presence of decreased expression of ENaC in transplanted lungs, contribute to the lack of stimulation of ALC by β-adrenergic agonists.

We cannot exclude the possibility that alternative mechanisms could also explain the lack of stimulation of ALC by β-agonists. Obviously, if the alveolar epithelium is severely injured, it would be difficult to maintain a vectorial gradient and stimulate lung liquid clearance (Berthiaume and Matthay, 2007). However, we have previously shown in our model that ischemia-reperfusion injury following lung transplantation without protective ventilation was not associated with significant histological changes in the alveolar epithelium (Sugita et al., 2003). Circulating factors could also limit the action of β-adrenergic agonists. For example, in the presence of left atrial hypertension, atrial natriuretic peptide can inhibit the stimulatory effect of β-adrenergic agonists on ALC (Campbell et al., 1999).

In conclusion, we propose that the failure of ALC to increase in response to β-agonist stimulation in the present model of ischemia-reperfusion injury secondary to lung transplantation is explained, at least in part, by decreased expression of ENaC and not by decreased expression or dysfunction of β-AR. These results might also explain the failure of beta-agonists to improve the outcome of edema resolution in acute lung injury patients.

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