β-adrenoceptor stimulation of alveolar fluid clearance is increased in rats with heart failure

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Running title: Heart failure and alveolar fluid clearance

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ABSTRACT

The alveolar epithelium plays a critical role in resolving pulmonary edema. We thus hypothesized that its function might be upregulated in rats with heart failure, a condition that severely challenges the lungs’ ability to maintain fluid balance. Heart failure was induced by left coronary artery ligation. Echocardiographic and cardiovascular hemodynamics confirmed its development at 16 weeks post-ligation. At that time, alveolar fluid clearance was measured by an increase in protein concentration over 1 h of a 5% albumin solution instilled into the lungs. Baseline alveolar fluid clearance was similar in heart failure and age-matched control rats. Terbutaline was added to the instillate to determine whether heart failure rats responded to β-adrenoceptor stimulation. Alveolar fluid clearance in heart failure rats was increased by 194% after terbutaline stimulation compared to 153% increase by terbutaline in control rats. To determine the mechanisms responsible for this accelerated alveolar fluid clearance, we measured ion transporter expression (ENaC, Na,K-ATPase, CFTR). No significant upregulation was observed for these ion transporters in the heart failure rats. Lung morphology showed significant alveolar epithelial type II cell hyperplasia in heart failure rats. Thus, alveolar epithelial type II cell hyperplasia is the likely explanation for the increased terbutaline-stimulated alveolar fluid clearance in heart failure rats. These data provide evidence for previously unrecognized mechanisms that can protect against or hasten resolution of alveolar edema in heart failure.

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INTRODUCTION

Approximately five million Americans are living with heart failure, with 400,000 new cases diagnosed annually (18). A major clinical problem in heart failure is the development of pulmonary venous (and consequently, pulmonary capillary) hypertension, which ultimately may lead to pulmonary edema and impaired blood oxygenation. Recent studies have demonstrated that excess pulmonary edema fluid is cleared from the alveoli secondary to active transepithelial Na transport (14, 31). The alveolar epithelium plays a critical role during pulmonary edema recovery by actively absorbing Na (14, 31). β-adrenoceptor and dopamine receptor agonists increase Na transport, and thus hasten the resolution of pulmonary edema (2, 6, 31). These observations have important clinical significance, because they suggest that β-adrenoceptor- or dopamine receptor-agonist therapy might help patients to recover more rapidly from pulmonary edema (2, 16).

Lungs of heart failure patients undergo extensive remodeling (18, 25, 26), resulting in architectural changes such as pulmonary artery and vein wall thickening, pulmonary arteriole muscularization, and capillary endothelial and alveolar epithelial basement membrane thickening (18). Collectively, such remodeling results in increased alveolocapillary barrier thickness, correlating with reduced microvascular fluid and protein permeabilities (11, 20, 45). In addition to the aforementioned changes, alveolar epithelial type II cell proliferation may occur in these lungs (25, 26). Hence, the hypothesis that alveolar epithelial type II cell hyperplasia increases Na absorption and accelerates alveolar fluid clearance (48) seems plausible because these cells contain the needed Na transport machinery. Furthermore, epithelial ion transport proteins (epithelial Na channel (ENaC), Na,K-ATPase, and cystic fibrosis transmembrane regulator (CFTR) expression and function can be altered by a variety of physiological and pathologic stimuli (31). Thus, the specific aims of this project were to determine: 1) whether
baseline and β-adrenoceptor agonist-stimulated alveolar fluid clearance rates were altered during heart failure and 2) the impact of heart failure on alveolar epithelial type II cell hyperplasia and changes in lung epithelial cell Na and Cl channel mRNA and protein expression. These aims were designed to test the general hypothesis that the ability to absorb airspace fluid is altered by alveolar epithelial type II cell hyperplasia and/or alterations in the ion transport machinery responsible for edema fluid reabsorption in lungs of rats suffering from heart failure.
MATERIALS AND METHODS

**Animals.** Male Sprague-Dawley rats (weight 300-350 g, \(N=45\); Charles River, Wilmington, MA) were used in this study. The rats were housed separately in a temperature- and humidity-controlled environment (20 ± 2 °C and 45 ± 15% relative humidity) and kept on a 12:12 h day-night cycle with access to standard rat chow (Purina, Copley Feed, Copley, OH) and tap water *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Northeastern Ohio Universities Colleges of Medicine and Pharmacy, Rootstown, OH.

**Solutions.** To measure alveolar fluid clearance, a 5% albumin instillate was prepared by dissolving 50 mg/ml bovine serum albumin (SeraCare Life Sciences, Oceanside, CA) in 0.9% NaCl. In studies designed to evaluate the capacity of \(\beta\)-adrenoceptor agonists to increase alveolar fluid clearance, \(10^{-4}\) M terbutaline (Sigma Chemicals Co., St. Louis, MO) was added to the instillate.

**Rat heart failure model.** The rats were injected with butorphanol (10 mg/kg body wt, im; Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA) and atropine (0.04 mg/kg body wt, im; Phoenix Scientific, St. Joseph, MO) 10 min before anesthesia induction. The rat was then placed in a flow-through chamber and exposed to 100% \(O_2/4\%\) isofluorane (Baxter, Deerfield, IL) with flow set at 2 l/min. After ~5 min, the anesthetized rat was removed from the chamber and intubated with a modified 14 g Venacath (Becton Dickinson, Sandy, UT). A laryngoscope equipped with a Miller 00 blade was used to visualize the trachea during intubation. After intubation, the rats were ventilated with a rodent ventilator (Harvard Apparatus, Nantucket, MA) at a tidal volume and frequency of 2.6 ml and ~70 breaths/min, respectively, and anesthesia was maintained with 0.5-1.5% isofluorane (\(O_2\) flow of 700 ml/min) using a Fortec isoflurane vaporizer (Cyprane, Keighly, U.K.). The heart was exposed via a left thoracotomy between the
5th and 6th ribs. Prolene® suture (6-0; Ethicon, Somerville, NJ) with a swedged on C-1 needle was passed through the apex of the heart and used as a handle to position the heart during left coronary artery ligation. The left coronary artery was ligated 1-2 mm ventral to the left atrial margin with the 6-0 Prolene® suture. After inflating the lungs with three breaths totaling 7-8 ml of air, the ribs were apposed with 2-0 cat gut suture (Ethicon). Xylocaine (0.2 ml, 2%; AstraZeneca, Wilmington, DE), was injected into the surgical site, and the chest was closed in layers with 4-0 Vicryl® suture with a FS-2 cutting needle (Ethicon). Immediately after surgery, the rats were given buprenorphine (0.03 mg/kg body wt; Buprenex®, Reckitt Benckines Healthcare, Hull, UK) for analgesia, a combination of benzathine and procaine penicillin subcutaneously at a dose of 44,000 U/kg body wt, and 0.9% NaCl (10 ml; Baxter) was given subcutaneously for fluid volume replacement. Buprenorphine was given as needed for analgesia during the first two post-operative days.

**Experimental time point and exclusion/inclusion criteria.** Based on our pilot work (29) and previous observations (22), we elected to study the rats 16 weeks post-coronary artery ligation. The choice of 16 weeks was done based on our pilot work (29) that showed hearts with large infarcts had extensive remodeling (heart weight approximately twice normal) and that the rats were in heart failure. Rats having small infarcts and unchanged heart/body wt ratios (<3 mg/kg body wt) did not show signs or symptoms of heart failure 16 weeks after surgery and were excluded from the study.

**Alveolar fluid clearance measurements.** Alveolar fluid clearance was measured as previously described (33, 34). Rats were anesthetized with pentobarbital sodium (60 mg/kg body wt ip; Nembutal®, Ovation Pharmaceuticals, Deerfield, IL), and anesthesia maintained with supplemental doses as needed. The carotid artery and jugular vein were cannulated (PE-50; Clay Adams, Becton Dickinson & Co., Sparks, MD) for hemodynamic measurements, blood gas
analyses, and blood collection for hormone analyses. Body temperature was monitored using a rectal temperature probe and maintained using a water-perfused heating pad. A tracheal cannula (PE-240; Clay Adams, Becton Dickinson & Co.) was placed in the rat’s airway via a tracheotomy and connected to a mechanical ventilator. The rat was ventilated with 100% O₂, at a respiratory rate of 40 breaths/min, and average tidal volume of 2.7 ml with a 2-3 cm H₂O positive end-expiratory pressure. The rat was placed at a 45° angle (head elevated), and a polyethylene catheter (PE-50; Clay Adams, Becton Dickinson & Co.) was inserted through a port in the tracheal cannula and into the lungs for fluid instillation. The rat was allowed to stabilize for 10 min after surgery. A 5% bovine serum albumin solution in Ringer’s lactate solution was then instilled into the left lung at a rate of 0.1 ml/min with a 1-ml syringe to a final volume of 3 ml/kg body wt. After 1 h, the rat was euthanized by exsanguination, the lungs were removed, and the remaining instilled fluid aspirated for analysis of albumin concentration by Lowry’s (28) technique modified for microtiterplates. Alveolar fluid clearance was determined using the mass balance equation: \(\text{AFC} = (1 – [\text{Alb}]_i / [\text{Alb}]_f) \times 100\), where alveolar fluid clearance (AFC) is expressed as the percent of the instilled fluid that left the airspaces during the experimental time period, and [Alb]ᵢ and [Alb]ᵢ are the initial and final albumin concentrations of the instillate, respectively.

**Hemodynamic measurements.** Immediately before the alveolar fluid clearance measurements, we measured heart rate, arterial blood pressure, central venous pressure, and left ventricular end-diastolic pressure. The pressures were obtained by catheterizing, respectively, a carotid artery and jugular vein using a 2.5 F Millar high fidelity transducer-tipped catheter (Houston, TX) and advancing the catheter into the left ventricle or right atrium. All pressures and derived variables were displayed on a Gould chart recorder.
**Echocardiographic assessment.** In vivo heart function was assessed using a Vevo 770 system (VisualSonics, Inc., Toronto, Ontario, Canada) with a 710B-075 transducer (20-30 MHz) designed specifically for small animal studies at a frame rate of 40-60 Hz. Rats were anesthetized using 2-2.5% sevoflurane administered via a nose cone, fur was shaved from the chest, and the rat was placed in the supine position on an adjustable platform equipped with ECG electrodes to monitor heart and respiration rates. A rectal temperature probe was placed and body temperature was maintained at 37.0-37.5 °C by placing the anesthetized rat between heating pads. Two-dimensional (2D) and M-mode, echocardiography images were obtained in parasternal short-, long-axis, apical four chamber views by previously validated techniques (5, 12, 39). M-mode and 2D images at the mid-papillary level were obtained from the parasternal short-axis view. Mitral valve inflow was obtained from apical four chamber view. A complete study with image acquisition was carried out in approximately 10 min. All measurements were averaged from at least three cardiac cycles in one age-matched control rat and one heart failure rat. Calculations and measurements were carried out offline using the Vevo 770/2.3 software.

**Plasma hormone analyses.** Plasma epinephrine and norepinephrine concentrations were measured by EIA (MP Chemicals, Aurora, OH) as described previously (36). The assays had sensitivities of 12 pg/ml and intra- and interassay variabilities of 5% and 12%, respectively.

**Specific protocol.** All rats that were surgically prepared for alveolar fluid clearance studies, were randomly divided into the following groups, and studied for one hour. Arterial blood was drawn for blood gas and hormone analyses at the end of the studies.

**Baseline alveolar fluid clearance studies.** Age-matched control rats (N=15) and the heart failure rats (N=11) were instilled with the 5% albumin solution 16 weeks after coronary artery ligation.
**Terbutaline-stimulated alveolar fluid clearance studies.** Age-matched control rats \((N=7)\) and heart failure rats \((N=6)\) were instilled with the 5% albumin solution containing \(10^{-4}\) M terbutaline 16 weeks after coronary artery ligation.

**Immunohistochemical evaluation of alveolar epithelial type II cell hyperplasia.** We evaluated the degree of alveolar epithelial type II cell hyperplasia using the following approach. We used a specific antibody directed against an alveolar epithelial type II cell surface antigen \((13)\) to determine cell number. We have previously used this technique to show alveolar epithelial type II cell hyperplasia after keratinocyte growth factor administration \((48)\). At the time of evaluation, rats \((N=3 \text{ age-matched controls and } N=3 \text{ heart failure})\) were anesthetized with 60 mg/kg body wt (ip) pentobarbital sodium (Ovation Pharmaceuticals), the chest opened, and the blood heparinized \((500 \text{ U/ml}; 10 \text{ ml}, \text{ right ventricular injection}; \text{ American Pharmaceutical Partners, Inc., Schaunburg, IL})\). Lungs were perfusion-fixed \((\text{perfusion pressure } 7-10 \text{ cm } \text{H}_2\text{O})\) via the pulmonary artery with 4% paraformaldehyde \((\text{Merck KGaA, Darmstadt, Germany})\), the lungs were removed, and 4% paraformaldehyde was also instilled intratracheally. The lungs were then cut into 1-cm\(^3\) cubes and immersed in 4% paraformaldehyde solution for 4 h followed by immersion in 30% sucrose. Sucrose-immersed cubes were embedded in O.C.T. compound \((\text{Ted Pella, Inc. and PELCO International, Redding, CA})\) and then snap-frozen in liquid nitrogen. After freezing, 10-\(\mu\)m sections were cut and mounted on 3-aminopropyl-triethoxysilane-coated slides \((\text{Thermo Fisher Scientific, Waltham, MA})\) and stored for later analysis at -80 °C.

Alveolar epithelial type II cell density was determined by using specific antibodies directed against a specific cell surface antigen \((\text{RTII-73; gift from Dr. L. Dobbs, UCSF, San Francisco, CA})\) \((13)\). After wash in 50 mM Tris buffered saline \((\text{TBS})\), slides were incubated in 3% horse serum \((\text{Invitrogen, Carlsbad, CA})\) in TBS for 30 min at room temperature. Sections then were exposed to the alveolar epithelial type II cell antibody, diluted 1:100 in 3% horse
serum for 20-40 min at room temperature and washed again in TBS. The sections were incubated with a secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN) or a FITC-conjugated goat-anti mouse IgG (Dakopatts, Copenhagen, Denmark), diluted 1:300 in 3% horse serum for 1 h at room temperature. After rinsing with TBS, the sections were incubated with a diaminobenzidine (DAB; Sigma Chemical Co.) solution (6 mg DAB in 10 ml 50 mM Tris-HCl buffer, pH 7.6, and 0.1 ml of 3% H₂O₂) and color development was monitored. When the desired intensity was reached (less than 1 min), slides were immediately washed in TBS.

For assessment of cell density, two random sections were selected. For each section, five random fields were selected. To control for potential changes in size of alveolar spaces in heart failure, which would change the number of cells per high power field, we used a measurement protocol whereby we counted the number of cells per unit membrane that stained positive for alveolar epithelial type II cell antigen. Observers were blinded to the identity of the samples.

*Transport protein analysis*

*RT-PCR of transporter mRNA expression.* Total RNA was extracted from lung tissue with a tissue RNA isolation kit from Gentra (Minneapolis, MN). RNA yield and purity were determined spectrophotometrically at 260/280 nm and RNA integrity was verified by agarose gel electrophoresis. A competitive reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using the One-Step RT-PCR kit from EMD Biosciences (San Diego, CA) in a volume of 25 µl system containing 50 ng of total RNA, 1 × PCR buffer, 0.2mM of each dNTP, 2.5 mM MgSO₄, 0.1 µM of each primer and 1.5 U rTth DNA polymerase, under optimized reaction conditions (27): 60 °C 30 min for reverse transcription, followed by 40 cycles at 94 °C for 45
sec, 60 °C for 2 min, and final extension for 7 min at 60 °C. Primer pairs (sense and antisense) were derived from GenBank sequences and synthesized for competitive RT-PCR. GAPDH was used as internal control. RT-PCR products were resolved in 1.5% agarose gels stained with ethidium bromide. Gels were scanned by a Typhoon 8610 Scanner (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

**Western blot.** Lung tissue from rats was homogenized in T-Per™ Reagent (Pierce, Rockford, IL) containing protease inhibitors, aprotinin (30 µg/ml; Sigma Chemical Co.) and leupeptin (1 µg/ml; Sigma Chemical Co.), with a homogenizer (Tissue Tearor; Thermo Fisher Scientific) on ice. The homogenate was centrifuged at 13,000 g for 5 min at 4 °C. Supernatant (membrane and cytosol) was collected, aliquoted in multiple vials, and snap-frozen in liquid N2. One vial was used for determining total protein concentration of the sample to ensure equal loading of the electrophoresis gel. Aliquots were stored at -80 °C until analyzed.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes (Pierce) were carried out using standard protocols. After the polyacrylamide gel electrophoresis and transfer, the nitrocellulose membranes were blocked (SuperBlock™ Dry Blend blocking buffer in tris buffered saline (TBS); Pierce) for 1 h at room temperature. After blocking, membranes were incubated with primary antibodies on an orbital shaker over night at +4 °C.

α-ENaC and β-ENaC antibodies were purchased from Alpha Diagnostics International (San Antonio, TX), directed against the N-termini of α-ENaC and β-ENaC. The antibodies recognize membrane proteins of appropriate sizes (85-95 kDa). Anti-Na,K-ATPase antibodies were obtained from Upstate Cell Signaling Solutions, Inc. (Waltham, MA) and directed against residues 338-518 of the α1-subunit. These antibodies specifically recognize membrane proteins of appropriate size (~95 kDa). Anti-CFTR polyclonal antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and directed against the N-terminus of the CFTR channel.
These antibodies specifically recognize a protein of appropriate size (~150 kDa). We used a rabbit monoclonal anti-GAPDH antibody from Cell Signaling Technology, Inc. (Danvers, MA) that detects endogenous levels of GAPDH as a control for loading and transfer. All primary antibodies were used at a 1:1,000 dilution. After incubation, membranes were washed 5 x 10 min with wash buffer (pH=7.5; TBS with 0.1% Tween-20). Membranes were incubated with HRP-conjugated secondary antibodies (1:1,000 dilution; goat-anti-rabbit IgG) for 1 h at room temperature. After incubation, membranes were washed again. Substrate solution (SuperSignal® West Femto; Pierce) was added and incubated for 5 min. The luminescence signal was detected using a Kodak image analyzer and densitometrically analyzed using TotalLab software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, U.K.).

**Statistics.** Data are presented as mean ± standard deviation (SD). Statistical analysis was carried out by Student’s *t* test or one-way analysis of variance with Dunkin’s test as *post hoc* test as appropriate. Differences were considered statistically significant when *P*<0.05.
RESULTS

Severity of infarct. Left coronary artery ligation is an established, well-accepted method for producing left ventricle dysfunction in rats (32) and has the advantage that it models the pathophysiological processes that result in heart failure from myocardial ischemia or infarction in humans. In these experiments, we studied rats 16 weeks after left coronary artery ligation and in age-matched controls. Only rats with large infarcts developed heart failure and underwent significant cardiac remodeling by 16 weeks. Variability in infarct size and consequently, the amount of cardiac remodeling has also previously been reported in this model (32). Thus, the high degree of variability was not an unexpected finding. Only coronary artery ligated rats with heart/body wt ratio >3 mg kg body wt exhibited signs of heart failure and were included in the study. Fig. 1 shows views of the anterior walls of representative hearts after coronary artery ligation (A) and in an age-matched control rat (B). The heart from the heart failure rat (A) had an elevated heart/body wt ratio (>3 mg/g body wt), showed extensive remodeling, and demonstrated severe left ventricle dysfunction. Left ventricular end-diastolic pressure and central venous pressure in the heart failure rats were significantly \( P<0.05 \) higher than that in age-matched control rats (Fig. 1C and 1D). Body weight gain was not different between the two groups.

Echocardiographic assessment. Echocardiographic parameters shown in Table 1 are consistent with our other observations in control and heart failure rats. Two-dimensional (2D) and M-mode images show large increases in left ventricle internal diameter (77% during systole and 36% during diastole), endocardial area (50% during systole and 19% during diastole), and left ventricle volume (102% during systole and 35% during diastole) suggesting significant chamber dilation in the heart failure rat (Table 1). Left ventricle mass was also greatly increased (62%) in the heart failure rat (Table 1), whereas relative wall thickness remained
unchanged. Ejection fraction and fractional area change were decreased by 30% and 33%, respectively, in the heart failure rat (Table 1). In addition, the heart failure heart had a significant decrease in wall motion (Fig. 2A) as compared to the healthy heart (Fig. 2B).

**Plasma hormone analyses.** Heart failure rats had higher ($P<0.05$) plasma epinephrine concentrations than age-matched control rats (Fig. 3A). Norepinephrine concentrations tended to be higher, but did not reach statistical significance in the heart failure rats (Fig. 3B).

**Alveolar fluid clearance determination.** We maximally stimulated alveolar fluid clearance with the $\beta_2$-adrenoceptor agonist terbutaline and compared alveolar fluid clearance rates between heart failure rats and age-matched controls. Alveolar fluid clearance was thus measured after terbutaline ($10^{-4}$ M) was instilled into the airspaces. Although baseline alveolar fluid clearance in normal control (14.9 ± 2.1% of the instilled fluid absorbed per h) and heart failure rats (17.6 ± 2.0%/h) did not differ, terbutaline-stimulated alveolar fluid clearance was greater in heart failure rats (49.4 ± 4.2%/h) compared to control rats (37.9 ± 1.8%) (Fig. 4). These data indicate that in heart failure rats $\beta_2$-adrenoceptor agonists were capable of stimulating alveolar fluid clearance to a greater degree than that which can be elicited in normal rats (Control: 153%, Heart failure: 194%).

**Alveolar epithelial type II cell hyperplasia.** A pronounced alveolar epithelial type II cell hyperplasia in heart failure rats was observed after both immunofluorescence (Fig. 5A and B) and immunoperoxidase staining (Fig. 5C and D). In addition, the interalveolar septum appeared thicker in heart failure rats than in age-matched control rats (Fig. 5), possibly reflecting deposition of collagen and other extracellular matrix proteins associated with lung fibrosis.
Molecular remodeling in heart failure lungs. We measured ion transporter mRNA (Fig. 6A-D) and protein expression (Fig. 7A-D) in whole lung homogenates from rats 16 weeks after coronary ligation and in age-matched controls. There were no significant differences in Na or Cl transport protein mRNA expression (Fig. 6A-D). α-ENaC protein expression was significantly increased by terbutaline administration in the age-matched control rats, but did not change in the heart failure rats (Fig. 7A). No changes in β-ENaC were observed between any of the treatment groups (Fig. 7B), and there were no differences in Na,K-ATPase (measured as α₁-Na,K-ATPase) (Fig. 7C) or CFTR (Fig. 7D) between any of the treatment groups.
DISCUSSION

Left ventricular and central venous pressures, echocardiographic assessment, and direct post-mortem cardiac examination were used to verify that heart failure had developed 16 weeks after left coronary arterial ligation in our test rats. Our findings showed an increased left ventricular end-diastolic pressure and an increased central venous pressure, both of which are signs of heart failure. Echocardiography is commonly used as a non-invasive method to assess cardiac function during heart disease (5, 7, 12, 39). Our 2D and 2D-guided M-mode images demonstrated an increased left ventricular internal diameter, endocardial area, and left ventricular volume in the heart failure rats compared to the control rats. Fractional shortening and ejection fraction were significantly lower in heart failure rats. Post-mortem measurement showed that left ventricular mass was increased in the heart failure rats. This occurred simultaneously with chamber enlargement, but with relatively normal wall thickness which suggests that the rats developed eccentric hypertrophy. Thus, rats with heart weight to body weight ratios >3 mg/kg displayed signs commonly associated with heart failure.

Although large increases in plasma epinephrine can increase alveolar fluid clearance (40), the relatively small increases in epinephrine observed in the heart failure group were not large enough to increase alveolar fluid clearance under baseline conditions because alveolar fluid clearance was not different between heart failure and control rats. This was also the case in an earlier study by Charron and colleagues (9) where epinephrine levels up to 185 pg/ml did not stimulate alveolar fluid clearance in rats. In contrast, the \( \beta_2 \)-adrenoceptor agonist, terbutaline, produced a greater increase in alveolar fluid clearance in the heart failure group. Potential mechanisms for the accentuated terbutaline-stimulated alveolar fluid clearance in heart failure rats may include 1) alveolar epithelial type II cell hyperplasia, 2) increased alveolar epithelial Na transport capacity (i.e., Na channel and/or Na,K-ATPase expression or function),
or 3) some combination of the two. In our study, the increased ability of terbutaline to stimulate alveolar fluid clearance occurred concomitantly with a marked alveolar epithelial type II cell hyperplasia. In contrast, there were no significant increases in any of the mRNA or expression of any of the transport proteins measured (α-ENaC, β-ENaC, α1-Na,K-ATPase, or CFTR) in the heart failure rats under baseline conditions. These data thus suggest that the predominant mechanism responsible for the enhanced terbutaline-stimulated alveolar fluid clearance observed in the heart failure rats was the development of alveolar epithelial type II cell hyperplasia.

The development of alveolar epithelial type II cell hyperplasia has previously been shown to also occur in experimental animals exposed to keratinocyte growth factor (KGF, a potent mitogen of alveolar epithelial type II cells), bleomycin, and hyperoxia (10, 15, 17, 19, 21, 35, 37, 38, 42, 46, 48, 49). Although all of these stimuli share the ability to induce a hyperplastic response, they appear to differ in the changes they produce in the cellular ion transport machinery and in the ability of the animal to clear fluid from the airspaces. For example, while KGF has been shown to increase ENaC mRNA expression by 120 h post-exposure and to produce an enhanced β-adrenoceptor-stimulated alveolar fluid clearance, baseline alveolar fluid clearance is also increased (48). In contrast, α- and β-ENaC mRNA and protein expression were not changed in the heart failure rats, nor was there a change in baseline alveolar fluid clearance. This comparative analysis raises the possibility that an increased epithelial membrane ENaC expression may be important for the development of an elevated baseline but not an enhanced β-adrenoceptor-stimulated alveolar fluid clearance. Paradoxically, alveolar epithelial type II cell hyperplasia produced by bleomycin is associated with a reduction in ENaC expression, although baseline alveolar fluid clearance is elevated (15). Finally, in hyperoxia, Na channel expression and changes in baseline and β-adrenoceptor-stimulated alveolar fluid clearance are variable and appear to depend on the duration of O₂
exposure and ambient PO₂ (10, 17, 42, 49). Such factors and possible differences in hormonal responses may also play a role in the differing responses observed in the heart failure rats and those treated with KGF and bleomycin. Thus, the effect of hyperplasia on alveolar fluid clearance is difficult to reconcile on the ion channel level from the information that is currently available.

Although not studied here, there may be time-related differences in lung remodeling as heart failure progresses. Thus, alveolar fluid clearance might be up- or downregulated depending on the stage of the disease progression. Clinically, many patients with the adult respiratory distress syndrome (ARDS) have radiographic (1) and morphologic evidence of less lung edema 7-14 days after onset (4), although they may still have respiratory failure from fibrosing alveolitis (30). A possible mechanism in the recovering patients, as well as in the rats in our study, could be that the proliferation of alveolar epithelial type II cells enables the alveolar epithelium to clear excess alveolar fluid, even in the presence of increased protein flux across the alveolar epithelium and unchanged or decreased ion transport protein expression. It was suggested in an earlier human study (47) that the intact-stimulated alveolar fluid clearance rate in the patients suffering from severe hydrostatic pulmonary edema could have been associated with a proliferative response of the alveolar epithelial type II cell. Although a pronounced alveolar epithelial type II cell hyperplasia was observed in the heart failure rats, the data do not exclude an effect on the alveolar epithelial type I cells. More recently, these cells have also been found to express the ion transport machinery necessary for transepithelial ion and fluid absorption (23). Also, since the alveolar epithelial type II cell is the progenitor cell for the alveolar epithelial type I cells (31), it is possible that the alveolar epithelial type I cells may also be involved in the pulmonary defenses against airspace flooding in heart failure through this mechanism.
A variety of experimental models have been used over a wide range of time frames to determine how the lungs respond to heart failure. These include acute elevation of left atrial pressure (8, 41), the creation of systemic left-to-right shunts (3), rapid electrical pacing of the heart (43-45), supracoronary aortic banding (24), as well as the coronary ligation technique used in this study. With respect to the lungs’ ability to clear alveolar fluid, acute increases in left atrial pressure has been shown to either depress baseline alveolar fluid clearance or inhibit the ability of β-adrenergic agonists to increase alveolar fluid clearance, whereas longer-term studies have shown either maintenance of or increased alveolar fluid clearance. With respect to the former, Campbell and colleagues (8) found that instilled β2-agonists failed to stimulate alveolar fluid clearance in sheep in which left atrial pressure had been increased for 4 h. Consistent results were observed in ex vivo isolated, perfused rat lungs (41), where lung fluid absorption was decreased following an hour elevation of left atrial pressure. In contrast, over a longer time frame of 7 days, Azzam and colleagues (3) observed that baseline alveolar fluid clearance was increased in rats with compensated heart failure produced by an aorto-caval fistula. These authors suggested that this response was mediated by an increased endogenous plasma norepinephrine upregulation of active Na transport. This interpretation would be consistent with the maintenance of baseline alveolar fluid clearance observed in our rats in the absence of any physiologically significant increase in plasma catecholamines, as well as the observed enhanced terbutaline-stimulated clearance. Recently, Kaestle and colleagues (24) have provided an interesting explanation for the differences observed in the acute and chronic studies by suggesting that the release of NO from the pulmonary vascular endothelium may inhibit alveolar fluid clearance during acute pulmonary hypertension, but that chronic pressure stress impairs lung microvascular NO production, thus allowing alveolar fluid clearance to be maintained or increased.
In summary, the capacity of the alveolar epithelium in heart failure to remove excess distal lung fluid was markedly upregulated in response to \( \beta_2 \)-adrenoceptor stimulation. The increased alveolar fluid clearance may be explained largely by an increase in the number of alveolar epithelial type II cells, resulting in a potentially greater transport capacity of the alveolar epithelium. However, these studies do not rule out other mechanisms that may upregulate the fluid transport capacity of the alveolar epithelium. Hyperplasia may be clinically relevant since sustained upregulation of alveolar fluid clearance by catecholamine-dependent and -independent mechanisms may be important recovery mechanism from pulmonary edema during heart failure mediated lung injury.
ACKNOWLEDGEMENTS

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**Tables**

Table 1. Two-dimensional (2D) echocardiographic measurements.

<table>
<thead>
<tr>
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<th>Control†</th>
<th>Heart failure†</th>
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<tbody>
<tr>
<td>Age (months)</td>
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<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>492</td>
<td>500</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>332</td>
<td>347</td>
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<tr>
<td>LV internal diameter (systole; mm)</td>
<td>5.0 ± 0.6</td>
<td>8.8 ± 0.3*</td>
</tr>
<tr>
<td>LV internal diameter (diastole; mm)</td>
<td>8.0 ± 0.5</td>
<td>10.8 ± 0.5*</td>
</tr>
<tr>
<td>LV length (systole; mm)</td>
<td>12.7 ± 0.3</td>
<td>14.2 ± 0.4*</td>
</tr>
<tr>
<td>LV length (diastole; mm)</td>
<td>15.8 ± 0.1</td>
<td>16.4 ± 0.1*</td>
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<tr>
<td>Endocardial area (systole; mm²)</td>
<td>55.7 ± 3.8</td>
<td>83.7 ± 3.0*</td>
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<tr>
<td>Endocardial area (diastole; mm²)</td>
<td>100.1 ± 6.7</td>
<td>118.9 ± 3.6*</td>
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<tr>
<td>Endo. LV volume (systole; μL)</td>
<td>207.6 ± 19.6</td>
<td>419.3 ± 19.2*</td>
</tr>
<tr>
<td>Endo. LV volume (diastole; μL)</td>
<td>541.2 ± 58.7</td>
<td>732.2 ± 33.7*</td>
</tr>
<tr>
<td>Endo. ejection fraction (%)</td>
<td>61.4 ± 3.5</td>
<td>42.7 ± 1.3*</td>
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<tr>
<td>Endo. fractional area change (%)</td>
<td>44.3 ± 2.5</td>
<td>29.7 ± 1.6*</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>1037 ± 50.8</td>
<td>1676 ± 31.2*</td>
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*P<0.05 compared to control (Student t test); bpm: beats per minute; LV: left ventricular; †All measurements were averaged from at least three cardiac cycles in one age-matched control rat and one heart failure rat 16 weeks after occlusion.
FIGURE LEGENDS

Figure 1. View of anterior wall of heart 16 weeks after coronary artery ligation in a rat with an increased heart/body wt ratio (i.e., heart failure) (A) and an age-matched control rat (B). Left ventricle end-diastolic (C; Control: N=16; heart failure: N=7) and central venous (D; Control: N=15; heart failure: N=9) pressures in heart failure- and age-matched control rats. Values are means ± SD. *P<0.05 compared to Control.

Figure 2. A representative echocardiography M-mode recording showing wall motion is shown for an age-matched control rat (A) and a heart failure rat 16 weeks after coronary artery occlusion (B).

Figure 3. Plasma epinephrine (A; Control: N=19; heart failure: N=18) and norepinephrine concentrations (B; Control: N=13; heart failure: N=14) in heart failure- and age-matched control rats.

Figure 4. Alveolar fluid clearance under baseline conditions and after terbutaline-stimulation in heart failure- and age-matched control rats. Values are means ± SD; Control: N=15; heart failure: N=11; terbutaline: N=7; heart failure + terbutaline: N=6. *P<0.05 compared to Baseline; †P<0.05 compared to terbutaline stimulation observed in controls.
Figure 5. Representative ten-µm frozen sections of normal lung tissue (heart/body wt ratio < 3 mg/g; AB) and lung tissue obtained from a heart failure rat (heart/body wt ratio > 3 mg/g; CD) reacted with RTII-73, an antibody specific for alveolar epithelial type II cells. Note the increase in RTII-73-positive cells (arrows) in the heart failure lung processed for immunofluorescence (A,C) or immunoperoxidase (B,D). Insets in B and D are higher magnification views of RTII-73-positive cells (arrows). N=3 animals at each condition.

Figure 6. $\alpha$-ENaC (A), $\beta$-ENaC (B), $\alpha_1$-Na,K-ATPase (C), and CFTR (D) mRNA under baseline conditions and after terbutaline-stimulation in heart failure- and age-matched control rats. Values are means ± SD; N=6 in each group.

Figure 7. $\alpha$-ENaC (A), $\beta$-ENaC (B), $\alpha_1$-Na,K-ATPase (C), and CFTR (D) under baseline conditions and after terbutaline-stimulation in heart failure- and age-matched control rats. Values are means ± SD; N=6 in each group. *P<0.05 compared to age-matched controls.
REFERENCES


Heart wt/Body wt:
4.9 mg/g  2.5 mg/g

Figure 1A-D
Normal

16 weeks after coronary artery occlusion
Figure 3AB

A

Plasma Epinephrine Levels (pg/ml)

Control  CHF

B

Plasma Norepinephrine Levels (pg/ml)

Control  CHF
Figure 7A-D

**A**

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<td>C</td>
<td>CHF</td>
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**αENaC**

- Control
- CHF

**GAPDH**

- Base
- Terb

**B**

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**βENaC**

- Control
- CHF

**GAPDH**

- Base
- Terb

**C**

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**α₁-Na,K-ATPase**

- Control
- CHF

**GAPDH**

- Base
- Terb

**D**

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**CFTR**

- Control
- CHF

**GAPDH**

- Base
- Terb

* *