Salbutamol Modulation of Ion Transport in Sheep Parietal Pleura Is Protein Dependent

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Received March 2, 2011; accepted September 27, 2011; published online September 28, 2011

The formation of pleural effusion during pulmonary edema is an important physiological mechanism of resolution of alveolar flooding. In cases of pulmonary edema resulting from acute respiratory distress syndrome (ARDS) these effusions are exudative, having high protein load. To this end, the effect of salbutamol in the presence of protein, on the ion transport properties of the sheep parietal pleura was investigated by Ussing chamber experiments. Our results show that salbutamol increases ion transport in the presence of protein in sheep parietal pleura by stimulation of $\beta_2$-adrenergic receptors since this effect was completely abolished by the specific $\beta_2$-adrenergic blocker, ICI-118551. This finding may be of importance regarding the acceleration of the resolution of protein-rich pleural effusions occurring in cases of ARDS.

Key words salbutamol; pleura; Ussing system

Acute respiratory distress syndrome (ARDS) is the severe form of acute lung injury (ALI) with an incidence of 59 cases/100000 inhabitants/year. These figures establish ARDS as a widespread disease with huge socio-economic impact. The mortality rate of ARDS estimates an average of more than 40% and only recently with the consensus on low tidal volume ventilation it has decreased to 31%. These data reveal that the search for new methods and therapeutic treatment options is imperative in order to achieve a much better therapeutical result.

Recently, Perkins and colleagues reported in the beta-agonist lung injury trial (BALT), that humans with ALI/ARDS receiving intravenous treatment with the $\beta_2$-agonist salbutamol, exhibited acceleration in the clearance of alveolar edema. It has been proposed that this treatment might be even more efficacious by aerosol administration due to the avoidance of cardiac side effects. In line with this, it has already been proven that using conventional delivery systems, aerosolized albuterol delivery in patients with acute lung injury results in concentrations of albuterol in the edema fluid in the range of 10—100 μM. These concentrations of $\beta_2$-adrenergic agonists have been proven efficacious in experimental animal models for the resolution of pulmonary edema. Additionally, one of the frequent radiological findings is pleural effusion. Leak of edema fluid into the pleural cavity in ARDS has been proposed to represent an important route of edema clearance from the interstitial space, acting as a safety factor to alveolar flooding. Interestingly, it has been already shown that pleural fluid clearance is affected by $\beta_2$-agonists, influencing solute coupled liquid absorption through the mesothelium. In physiological conditions solute coupled liquid absorption through the mesothelium is the main pathway of pleural fluid absorption and clearance. In pleural effusions lymphatic drainage is the main route of fluid absorption, while solute coupled liquid absorption is still a key mechanism of fluid removal.

The direct effect of salbutamol in the presence of protein on the parietal pleural membrane has never been studied. However, recent studies on the parietal pleura have revealed that the electrophysiological properties of the parietal pleura are influenced by adrenergic stimulation as it has been demonstrated by transmestothelial electrical resistance measurements. Furthermore, previous in vivo studies in rabbits have shown that $\beta_2$-adrenergic stimulation decreased the net rate liquid absorption of Ringer’s lactate hydrothoraces by 25%, while $\beta_2$-adrenergic stimulation in the setting of 1% albumin-Ringer’s lactate hydrothoraces increased it by 29%. These findings have been attributed to amiloride insensitive $Na^+$ transport alterations.

The objective of this study was to investigate the effect of salbutamol on the parietal pleura equivalent short circuit current ($I_{sc}$). Our experiments were oriented in mimicking the effect of salbutamol in conditions of exudative pleural effusions (high protein content) that are encountered in ARDS, by applying it apically on the parietal pleura. To our knowledge this is the first report on salbutamol effect on the parietal pleura.

MATERIALS AND METHODS

Specimen Collection and Preparation Intact sheets of parietal pleura were obtained from 14 adult sheep (males and females). The samples were collected from the slaughterhouse immediately after the death of the animal (time of warm ischemia close to 0) and transferred to the laboratory in oxygenated Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich Chemie GmbH, Munich, Germany) buffer at 4°C within 30 min of the death of the animal. The pieces of parietal pleura ones were carefully stripped from the chest wall and then examined for evidence of holes or adherent tissue by visual inspection. Care was taken to touch the surface as little as possible. Pieces of parietal pleura not likely to contain stomas were used, as suggested from anatomical studies in sheep. All experimental procedures were conducted in accordance with the guidelines of the Ethical Committee of the University of Thessaly.

Transmestothelial Electrical Resistance Measurements Parietal pleura specimens were carefully mounted in Ussing
chambers (Dipl.-Ing. K., Mussler Scientific Instruments, Aachen, Germany) with an opening surface area of 1 cm². Tissues were bathed with 4 ml of Krebs-Ringer bicarbonate (KRB) solution on each side of the membrane, continuously oxygenated with 95% O₂/5% CO₂ circulated by gas lift. The KRB solution was balanced at pH 7.4 and contained (in mM) 117.5 NaCl, 1.15 NaH₂PO₄, 24.99 NaHCO₃, 5.65 KCl, 1.18 MgSO₄, 2.52 CaCl₂, and 5.55 glucose. Two pairs of Ag/AgCl electrodes monitored the transmesothelial potential difference (Vₜₚₖ) in mVolts and electrical resistance (Rₜₚₖ) in ohms per square centimetre (Ω·cm²) under open-circuit conditions. The Rₜₚₖ were measured every 60 s. Experiments were conducted simultaneously in six computer-controlled chambers (Clamp version 2.14 software: AC Micro-Clamp, Aachen, Germany). Transmesothelial voltage and electrical resistance were measured in the basal state (that is, at the end of an equilibration time of 10–40 min), and after the addition of the treatment substances. By application of Ohms Law (I = V/R) we calculated the equivalent short circuit current (Iₑ) across the parietal pleura. The voltage responses to applied current pulses of given amplitude (50 μA) and duration (200 ms) were measured. The Rₜₚₖ was calculated by automatically deducing the initially measured resistance of the solution. Changes in Iₑ after the addition of the chemicals were determined as per cent (%) changes (ΔIₑ). Because active transport of ions is influenced by temperature, the Ussing chambers were held at 37°C.

**Experimental Procedure** All solutions were freshly prepared before each experiment, heated to 37°C, and bubbled continuously with a 95% O₂/5% CO₂ gas mixture. All chemicals (salbutamol, ICI 118551 and BSA) were purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany.

The mesothelial cell membrane side that in vivo faces the pleural fluid is referred to as apically. Measurements of Iₑ were made before and after exposure to substances for given time points (at minutes 1, 3, 5, 10, 15). Baseline equivalent short circuit current (Iₑ)—tissue with KRB solution—measurements were calculated after tissue equilibration period from all specimens before addition of substances. The control Iₑ remained stable during the period of 15 min which was the duration of the experiments.

In the initial set of control experiments, 1% BSA-KRB solution (final concentration) was added apically on the parietal (n=4) pleura, in different pleura specimens. In another set of experiments the β₂ adrenergic agonist salbutamol (final concentration of 10 μM) in 1% BSA-KRB solution and salbutamol (final concentration of 10 μM) plus the specific β₂ adrenergic blocker ICI 118551 (final concentration of 100 μM) in 1% BSA-KRB solution were added apically on the parietal pleura (n=5 in each case).

**Statistical Analysis** Statistical analyses were performed with GraphPad Prism v4 for Mac OSX (GraphPad Software Inc., San Diego, U.S.A.). All data are expressed as mean±S.E.M. The results presented in this study are the means of the stated number of experiments in each case. The probability of error for comparison of the mean values was calculated using one-way analysis of variance (ANOVA) with Dunnett post-test and two-way ANOVA with Bonferroni post-test where appropriate. Values of p<0.05 were regarded as significant.

**RESULTS**

The control equivalent short circuit current (Iₑ)—tissue with KRB solution-across the parietal pleura was 37.2±9.5 μA/cm² (n=14).

Addition of 1% BSA-KRB solution apically on the parietal pleura resulted in a gradual decrease of the Iₑ being statistically significant at 5 min (ΔIₑ = −33.5±9%; p<0.05; Fig. 1). Addition of salbutamol (10⁻³ M) in 1% BSA-KRB solution apically on the parietal pleura resulted in a gradual increase of the Iₑ being statistically significant after 10 min (ΔIₑ = 50.4±19%; p<0.05) an effect that was maintained for the whole experimental procedure (Fig. 1). Ending, addition of salbutamol (10 μM) in 1% BSA solution plus the specific β₂ adrenergic receptor blocker ICI 188551 (100 μM) apically resulted in a decrease of the Iₑ that was similar in size and pattern as the one in the experiments with 1% BSA KRB solution only (Fig. 1). More specifically the decrease was statistically significant after 1 min (ΔIₑ = −33.8±15.5%; p<0.05) and the effect was maintained for the whole experimental procedure. Salbutamol (10 μM) in 1% BSA-KRB ΔIₑ was significantly higher than 1% BSA-KRB as well as salbutamol (10 μM) in 1% BSA solution plus ICI 188551 (100 μM) after the 5th minute in both cases (p<0.05).

**DISCUSSION**

Our results demonstrate the potent effect of salbutamol on the parietal pleura in the presence of protein (1% BSA). Salbutamol induced a significant increase on the equivalent short circuit current (Iₑ) within 10 min after its addition apically on the parietal pleura. This effect was totally abolished under the same conditions when salbutamol was added along with the β₂ adrenergic blocker, ICI-118551, reducing the Iₑ to the levels that 1% BSA alone did. The above findings suggest that 1) salbutamol potently increases ionic permeability (transmesothelial and/or paracellular) in the parietal pleura in the presence of protein and 2) this effect is mediated through stimulation of β₂-adrenergic receptors that are present in the parietal pleura.

It has been shown by previous studies of our group that application of adrenaline in the sheep parietal pleura results
in changes in the electrophysiology. More specifically, adrenalin was found to increase the electrical resistance of the parietal pleura when applied apically in protein free medium, through stimulation of mainly \( \beta \)-adrenergic receptors as well as \( \alpha \)-adrenergic receptors depending on the origin of the pleura (costal or diaphragmatic).\(^{10,11,14}\) These findings were in accordance with in vivo experimental studies reproducing hydrothoraces. In these studies, \( \beta \)-adrenergic stimulation was found to either inhibit (in protein free) or accelerate (in the presence of protein) the resolution of the experimental hydrothoraces.\(^{35}\) In both isolated sheep pleura and in vivo rabbit studies these effects were mediated through changes in amiloride insensitive sodium transport. Moreover, in vivo studies conducted in rabbits comparing experimental hydrothoraces with and without protein content, have shown that protein content (1% BSA in Ringers) resulted in 2 times slower resolution than hydrothoraces with Ringers only.\(^{15}\)

This way they demonstrated that the presence of protein alters the profile of fluid clearance in the pleural cavity. These data are in accordance with our findings regarding the reduction of ionic transport in our experiments with 1% BSA alone or in the experiments with 1% BSA and salbutamol along with its blocker. The total abolishment of the effect of salbutamol in the latter case indicates that the increase in ionic transport due to salbutamol is mediated totally by \( \beta \)-adrenergic stimulation on the parietal pleura apically. The physiological basis of our findings is probably due to increase of amiloride insensitive sodium currents that have been proved to occur previously.\(^{5,10,11}\)

In ARDS patients that received intravenous salbutamol it has been shown that the reduction of lung water extravasation was not due to mitigation of neutrophil dependent inflammatory pathways, strengthening the notion that this effect is due to acceleration of water and solute transport out of the alveoli to the interstitium.\(^{16}\) Part of the interstitial edema fluid escapes alveoli and gathers in the pleural cavity in order to prevent alveolar flooding.\(^{6,7}\) In ARDS cases this fluid is rich in protein, so we used 1% BSA in our experiments to simulate and reproduce this condition.\(^{17,18}\) We hypothesized that if aerosolized salbutamol is used (in similar concentrations as in the study of Atabai et al. which is in the range of 10—100 \( \mu \)M) the drug will reach the pleural space in similar concentrations. In this context we tested the possible effect of the drug on the pleura under the appropriate conditions.\(^{39}\) We concluded that the salbutamol effect on the parietal pleura would further accelerate the resolution of the pleural effusion. Then, the pleural space would be available for more edematous fluid to influx from the flooded alveoli in cases of ARDS, promoting edema fluid clearance. In a recently published randomized, placebo-controlled clinical trial of aerosolized albuterol (in concentration that have previously been reported to reach a concentration of 1 \( \mu \)M in the pulmonary edematous fluid by Atabai et al.) for the treatment of acute lung injury it has been reported that albuterol did not improve clinical outcomes in patients with acute lung injury.\(^{5,19}\) These data contradict a previously published retrospective study that showed that similar doses of inhaled salbutamol in patients with Acute Lung Injury resulted in shorter duration and decreased severity of the Acute Lung Injury.\(^{29}\) Unfortunately in both studies the occurrence or the resolution of pleural effusions were not assessed.

Our finding that salbutamol increases the ionic transport on the parietal pleura in the presence of protein, may have implications in cases of ARDS complicated with pleural effusions. It is possible that the faster the pleural effusion resolves the more the lung water and edema content would reduce leading to increased survival or a better outcome in patients suffering ARDS.

Acknowledgements This research was supported by a Postdoctoral Grant of the National Scholarship Foundation of Greece (I. K. Y.) awarded to Dr. Sotirios G. Zarogiannis. The authors would like to thank Mr. Ioannis Makadasis for technical assistance. This work is dedicated to Georgios S. Zarogiannis, 27/04/1946—18/02/2010.

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