Cyclic Stretch of Human Lung Cells Induces an Acidification and Promotes Bacterial Growth

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The reasons for bacterial proliferation in the lungs of mechanically ventilated patients are poorly understood. We hypothesized that prolonged cyclic stretch of lung cells influenced bacterial growth. Human alveolar type II–like A549 cells were submitted *in vitro* to prolonged cyclic stretch. Bacteria were cultured in conditioned supernatants from cells submitted to stretch and from control static cells. *Escherichia coli* had a marked growth advantage in conditioned supernatants from stretched A549 cells, but also from stretched human bronchial BEAS-2B cells, human MRC-5 fibroblasts, and murine RAW 264.7 macrophages. Stretched cells compared with control static cells acidified the milieu by producing increased amounts of lactic acid. Alkalinization of supernatants from stretched cells blocked *E. coli* growth. In contrast, acidification of supernatants from control cells stimulated bacterial growth. The effect of various pharmacological inhibitors of metabolic pathways was tested in this system. Treatment of A549 cells and murine RAW 264.7 macrophages with the Na^+/K^+-ATPase pump inhibitor ouabain during cyclic stretch blocked both the acidification of the milieu and bacterial growth. Several pathogenic bacteria originating from lungs of patients with ventilator-associated pneumonia (VAP) also grow better *in vitro* at slightly acidic pH (pH 6–7.2), pH similar to those measured in the airways from ventilated patients. This novel metabolic pathway stimulated by cyclic stretch may represent an important pathogenic mechanism of VAP. Alkalinization of the airways may represent a promising preventive strategy in ventilated critically ill patients.

Keywords: ventilator-associated pneumonia; lactic acidosis; sodium pumps; pulmonary epithelial cells; cyclic stretch

Mechanical ventilation is a major risk factor for the development of nosocomial bacterial pneumonia (1). Colonization of the airways by bacteria originating from the gut and the oropharynx is key to the pathogenesis of ventilator-associated pneumonia (VAP) (1). It has also been appreciated over the years that injured lungs have a greater susceptibility to bacterial infections (2). Although still ill-defined, decreased innate immune defenses of the lung in ventilated patients have been proposed as a mechanism favoring bacterial growth and the subsequent development of VAP (3, 4).

The mechanical strain imposed on lungs of critically ill patients by positive pressure mechanical ventilation induces airway cell activation (5). The effects of mechanical ventilation on pulmonary cells can be modeled *in vitro* using cultured cells submitted to prolonged cyclic stretch (6–8). Cyclic stretch of airway cells activates various intracellular signaling pathways (9, 10). This leads to the production of inflammatory mediators, but also to the activation of ion channels and pumps (9, 11). Both cellular inflammation and damage participate in the pathogenesis of ventilator-induced lung injury (10, 12). Cyclic stretch of pulmonary epithelial cells also induces sublytic plasma membrane stress failure, with breaks in the membrane that can be resealed by increased lipid trafficking (12).

In this work, we show that airways from ventilated patients are more acidic than those of healthy subjects. We identify cyclic stretch as an inducer of lactic acidosis in a variety of cells *in vitro*, leading to a slight acidification of the milieu, via the activation of the Na^+/K^+-ATPase pump. H^+ ions produced by stretched cells account for a marked growth advantage of a wide variety of gram-negative and gram-positive pathogens. We thus describe a novel effect of cyclic stretch on lower airway lung cells, which may link the unusual mechanical strain imposed by mechanical ventilation, bacterial overgrowth and the development of VAP.

CLINICAL RELEVANCE

Cyclic stretch of human lung cells induces an acidification of the milieu, which promotes bacterial growth. This may represent an important pathophysiologic mechanism for the development of ventilator-associated pneumonia.

MATERIALS AND METHODS

Cyclic Stretch of Cells

A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM/F12 Glutamax medium (ref. 31331028, containing 14.3 mmol/L $NaHCO_3$ of and 17.5 mmol/L glucose; Invitrogen, Basel, Switzerland). Ten percent fetal bovine serum (FBS; Gibco, Basel, Switzerland) was added to the medium, but no antibiotics. The complete medium was then filtered (0.2 μm) and kept at 4°C. Three days before cell stretching, 2.5×10^5 A549 cells were seeded onto silastic coated with type I collagen in each well of 6-well Bioflex plates (Dunn Labortechnik, Asbach, Germany). Cells were grown in a 5%CO_2 incubator at 37°C for 72 hours in the BioFlex plates until a single-cell monolayer confluence was achieved. Plates were then transferred to the baseplate of the cell stretching device FX-3000 Flexercell strain unit (Flexcell International, Hillsborough, NC), and placed in a 37°C, 5% CO_2 incubator. Before stretching, the medium was changed with fresh culture medium. Cells were stretched using the following protocol: stretching rate of 20 cycles/min with a square signal, a 1:1 stretch: relaxation ratio, and a 20% maximal equibiaxial elongation. Control Bioflex plates were kept in static condition in the same incubator (8). In one experiment performed in duplicate, A549 cells were stretched with 10 and 20% elongation factor using two different baseplates (8). Similar experiments were performed with human bronchial BEAS-2B cells (ATCC), human fetal lung primary fibroblasts MRC-5 (ATCC), and murine macrophage RAW 264.7 cells (ATCC), using the same culture

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medium as that used with A549 cells. BEAS-2B and RAW 264.7 cells were seeded at the concentration of 2.5×10^5 cells/well, whereas MRC-5 cells were seeded at 4×10^5 cells/well.

In some experiments with A549 cells, 10 ng/ml IL-1β (Sigma-Aldrich, Buchs, Switzerland) were added to static plates. In other experiments, various pharmacologic inhibitors were added to static and stretched cells immediately before starting cyclic stretch. These include the NADPH oxidase inhibitor DPI (1 μM, diphenyleneiodonium chloride; Sigma); the anti-oxidants N-acetyl-cystein (5 mM; Sigma) and apocynin (50 μM; Sigma); the inhibitor of glycolysis, 2-deoxy-glucose (5 mM; Sigma); the lactate dehydrogenase inhibitor, oxamate sodium (50 mM; Fluka, Buchs, Switzerland); inhibitors of the cyclic AMP pathway: the cell permeable adenylate cyclase inhibitor 2′,5′ dideoxyadenosine (25 μM, 2′5′ dd-ADO; Sigma); the β-blocker propranolol (1 μM; GlaxoSmithKline, Middlesex, United Kingdom); the protein kinase A inhibitor H89 (10 μM; Sigma); the ENaC inhibitor 5-(N-Ethyl-N-isopropyl) amiloride (10 μM EIPA; Sigma); and the Na^+/K^+-ATPase inhibitor, ouabaïn (10^{-5}–10^{-10} M; Sigma). Ouabain was also tested in RAW 264.7 cells during cyclic stretch.

After 48 hours, cell-free conditioned supernatants were collected. The pH of conditioned supernatants was determined using a fine pH electrode (Orion Micro, réf. 9802-BN; Thermo Electron Corp., Waltham, MA) equilibrated between pH 4 and 9 using reference buffers. In some experiments, supernatants were read in a blood gas analyzer (ABL800 FLEX; Radiometer, Copenhagen, Denmark) to measure pH, lactate, and glucose concentrations. In other experiments HCl, NaOH or lactic acid (Sigma) was added to supernatants from static and stretched cells or to culture medium.

After harvesting of the supernatants, cells from BioFlex plates were trypsinized and resuspended in culture medium. They were counted by the cell counter-analyzer system Casy-1 (Schärfe System GmbH, Innovatis, Bielefeld, Germany). Cells were also analyzed by fluorescence-activated cell sorter (FACS) (FACScalibur; Becton Dickinson, Basel, Switzerland) after staining with To-Pro-3 iodide (Molecular Probes, Leyde, The Netherlands) and annexin V (Becton Dickinson) to determine the ratio of viable, apoptotic, and necrotic cells. In some experiments, the MTT assay was used to determine cell viability (13). Briefly, cells attached to the bottom of the 6-well plate were incubated with 2 ml of 0.5 mg/ml MTT dye (Sigma) diluted in culture medium for 3 hours. Two milliliters of isopropanol 100% was then added to the wells, and optical densities at 570 nm were determined.

Culture of Bacteria

Many experiments were performed with the *Escherichia coli* K12 strain. *E. coli* K12 were cultured on a Luria agar plate and kept at 4°C. From the agar plate, *E. coli* K12 was then collected and seeded into Luria broth (LB) at 37°C for 8 hours. The *E. coli* culture was then diluted with LB to obtain an optical density (OD) at 600 nm of 0.05, and further cultured at 37°C for 2 hours until an OD between 1.8 to 2.5 was obtained. Serial dilution experiments have shown that 1 OD at 600 nm corresponds to 5×10^8 bacteria/ml.

Conditioned supernatants from static and stretched cells (450 μl) were incubated with 2,500 colony-forming units (CFU) of *E.coli* K12 contained in 50 μl of sterile NaCl, diluted from the above-mentioned stock solution, and placed in a 37°C room on a circular shaker in 12-ml sterile Falcon tubes. After 13 to 15 hours (24 h for supernatants from BEAS-2B cells), the 600-nm OD from cultured supernatants were determined in a spectrophotometer. Three 600-nm OD corresponded to 1.5×10^9 bacteria (controlled by serial dilutions of conditioned supernatants with growth). In the absence of *E. coli,* conditioned supernatants did not grow.

In some experiments, *E. coli* K12 was substituted with gram-positive and gram-negative bacteria cultured in bronchoalveolar lavage (BAL) fluids ($> 10^4$ CFU/ml) from patients diagnosed with VAP. These included the following wild-type gram-negative strains: *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa*, *Serratia rubidaea*, and *Enterobacter aerogenes*. For gram-positive bacteria, we tested one clinical isolate of *Enterococcus feacalis*, seven clinical strains of *Staphylococcus aureus*, one *S. epidermidis*, and one lab strain of *Bacillus subtilis*. The culture technique for these bacteria was the same as that described above with *E. coli* K12. VAP was defined by the presence of a new or progressive pulmonary radiologic infiltrate or a new fever (> 38°C) in patients ventilated for more than 48 hours in whom pathogenic bacteria were cultured in BAL fluid at the concentration equal to or superior to 10^4 CFU/ml.

Fractionation of Supernatants

Supernatant from A549 cells stretched for 48 hours was fractionated by molecular size using Microcon centrifugal filter devices YM-3 and YM-30 (Millipore, Zug, Switzerland). *E. coli* K12 was seeded into fractions of supernatants corresponding to molecular sizes of < 3 kD; ≥ 3 but < 30 kD; and ≥ 30 kD and cultured as described earlier.

cAMP Measurement

A549 cells were stretched for various times ranging from 15 minutes to 48 hours. Cells were then lysed in 0.1 N HCl, and cAMP was determined in cell lysates using a commercial cAMP EIA kit according to the manufacturer's protocol (Biomol, Plymouth Meeting, PA). A positive control was cell lysates from cells treated with 10 μM forskolin (Fluka).

Exhaled Breath Condensate Sampling

Exhaled breath condensate (EBC) was collected using the ECoScreen apparatus (Viasys Healthcare, Conshohocken, PA) in a series of intubated and mechanically ventilated patients as described elsewhere (14). EBC pH has been previously shown to be a reliable marker of airway pH (15). A series of samples were also obtained in healthy volunteers using a mouth adapter and noseclips. In patients, the ECoScreen apparatus was connected at the level of the expiratory limb of the ventilator circuit for 15 to 20 minutes, yielding between 0.7 and 1.5 ml of EBC. EBCs were immediately frozen and kept at −70°C until the day of serial pH measurement. EBC pH was measured using a fine electrode and a pH meter as described above. In some cases, the pH was controlled using a blood gas analyzer (ABL, Radiometer). The Ethics Committee of our Institution approved the research protocol, and an informed consent was obtained from the patient or the next of kin.

RESULTS

Supernatant from Stretched Cells Promotes Bacterial Growth

Culture supernatants from A549 cells submitted to cyclic stretch for 48 hours markedly enhanced *E. coli* growth, whereas supernatants from cells in static condition did not (Figure 1A). When bacterial cultures were left for a longer period of time (36 h instead of 13–15 h), bacteria also grew in supernatants from unstretched cells. This suggested that stretched A549 cells produced a soluble factor enhancing bacterial growth. Both cyclic cell stretching and the pro-inflammatory cytokine IL-1β are capable of activating A549 cells resulting in the production of IL-8, for example (Figure 1A). The addition of IL-1β did not reproduce the bacterial growth enhancing effect observed with cell stretching (Figure 1A). This suggested that cyclic stretch induced an effect on bacterial growth different from that of pro-inflammatory cytokines, at least when IL-1β was used. To determine the physicochemical nature of this bacterial growth–enhancing factor, supernatant from stretched cells was fractionated according to molecular sizes. The enhancing factor was found to be a small molecule of less than 3 kD and resistant to heat (100°C), suggesting a nonproteinaceous nature of the factor (Figure 1B). This ruled out the possibility of cytokines promoting bacterial growth (16). Supernatants from static and stretched cells were then analyzed in a blood gas analyzer, and a remarkable difference appeared between the two types of supernatants. Stretched cells clearly and reproducibly produced increased amounts of lactate and acidified their milieu (Figure 1A). Glucose was also found to be used at a greater rate by stretched cells. Both the degree of acidification and the promotion of bacterial growth was found to be dependent on the degree of cell stretching: minimal in static condition, intermediate in supernatants from A549 cells stretched with a 10% elongation factor, and maximal in cells stretched with a 20% elongation factor (Figure 1C). Human bronchial BEAS-2B

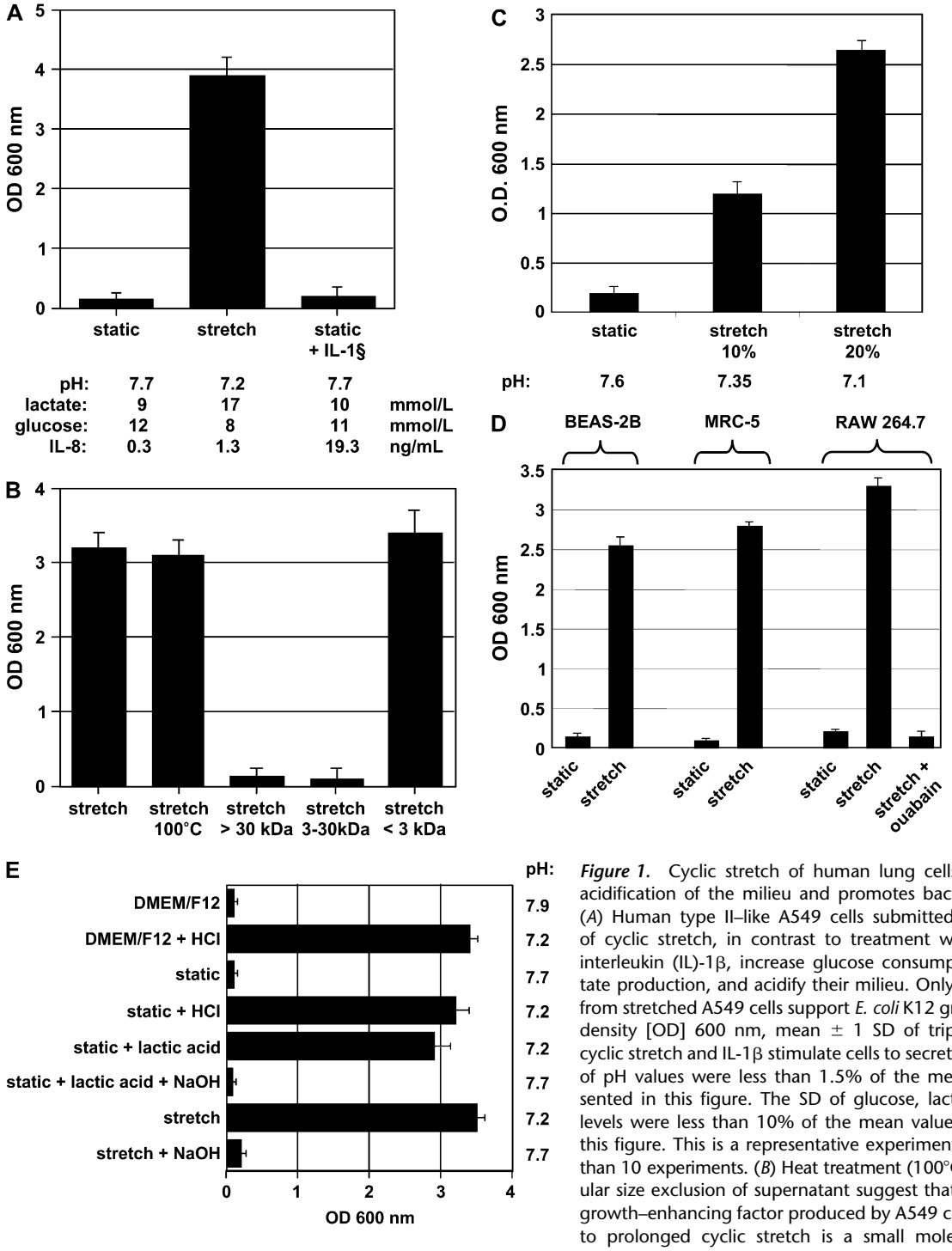

Figure 1. Cyclic stretch of human lung cells induces the acidification of the milieu and promotes bacterial growth. (*A*) Human type II–like A549 cells submitted to 48 hours of cyclic stretch, in contrast to treatment with 10 ng/ml interleukin (IL)-1β, increase glucose consumption and lactate production, and acidify their milieu. Only supernatants from stretched A549 cells support *E. coli* K12 growth (optical density [OD] 600 nm, mean ± 1 SD of triplicates). Both cyclic stretch and IL-1β stimulate cells to secrete IL-8. The SD of pH values were less than 1.5% of the mean value presented in this figure. The SD of glucose, lactate, and IL-8 levels were less than 10% of the mean value presented in this figure. This is a representative experiment out of more than 10 experiments. (*B*) Heat treatment (100°C) and molecular size exclusion of supernatant suggest that the bacterial growth–enhancing factor produced by A549 cells submitted to prolonged cyclic stretch is a small molecule of non-proteinaceous nature. The factor is heat stable and less than 3 kD, since *E. coli* K12 grew in boiled supernatants and in the filtrate of a size exclusion filter of 3 kD (OD 600 nm, mean ± 1 SD of triplicates). This is a representative experiment out of three different experiments showing the same results. (*C*) "Dose–response" of cyclic stretch in A549 cells kept in static condition, or stretched with elongation factors of 10 and 20% for 48 hours. Ten percent elongation factor induces intermediary acidification of the supernatants and intermediary bacterial growth. (*D*) *E.coli* growth (OD 600 nm, mean ± SD of duplicates) in supernatants from human bronchial epithelial BEAS-2B cells, human fetal lung MRC-5 fibroblasts, and murine RAW 264.7 macrophages submitted to cyclic stretch or kept in static condition. Ouabain at the concentration of 10^{-7} M blocked stretch-induced bacterial growth in RAW 264.7 macrophages. This is one representative experiment out of three different experiments showing similar results. (*E*) The proton H^+ is the factor produced by stretched A549 cells enhancing bacterial growth. Acidification of culture medium (DMEM/F12) with HCl to pH 7.2 is sufficient to promote *E. coli* K12 growth. Acidification of supernatants from A549 cells kept in static conditions with HCl or lactic acid to pH 7.2 promotes *E. coli* K12 growth. *E. coli* growth can be inhibited in supernatants from stretched cells with alkalinization with NaOH to pH 7.7. *E. coli* growth is also inhibited by alkalinization to pH 7.7 of supernatant from static A549 cells that was acidified with lactic acid. These experiments indicate that the factor enhancing bacterial growth is H^+ and not the molecule lactate. This is a representative experiment out of three different experiments showing the same results.

cells, human lung MRC-5 fibroblasts, and murine RAW 264.7 macrophages responded similarly to 48-hour cyclic stretch. Both an acidification of the milieu (not shown) and *E. coli* growth (Figure 1D) were observed in all cell types tested. In addition, ouabain blocked both acidification and bacterial growth in supernatants from stretched RAW 264.7 cells (Figure 1D).

The Factor Enhancing Bacterial Growth Is H^+

We next investigated whether the bacterial growth-enhancing factor could be H^+ or lactate. The simple acidification of the DMEM/F12 culture medium or supernatants from static cells with HCl to obtain a pH close to that measured after cell stretching (pH 7.2) induced *E. coli* growth (Figure 1E). Conversely, the alkalinization of supernatant from stretched cells with NaOH to pH measured in conditioned supernatant from static cells (pH 7.7) abrogated the bacterial growth–enhancing effect (Figure 1E). Lactic acid added to supernatants from static cells acidified the milieu to pH 7.2 and allowed *E. coli* growth (Figure 1E). However, buffering this milieu with NaOH to pH of 7.7 blocked bacterial growth (Figure 1E). Taken together, these experiments indicated that the heat-stable small molecule produced by stretched cells and enhancing bacterial growth was the proton H^+, and not the lactate molecule.

Bacteria from Patients with VAP Grow Better at Slightly Acidic pH

Various gram-negative and gram-positive bacterial strains obtained from BAL fluid in patients diagnosed with VAP were grown in culture medium (DMEM/F12 + 10% FCS) acidified with HCl to obtain a pH range from 8.0 to 4.0. With the exception of *S. aureus* growing at pH between 7.8 and 6, all bacteria had a marked growth advantage at slightly acidic pH (Figure 2). Almost invariably, when the pH dropped from 7.8 to 7.2 to 6.8 (i.e., the pH modification induced by cyclic stretch), the bacteria started to grow. In contrast to all other gram-negative and gram-positive bacterial strains tested, six different clinical strains of *S. aureus* and one of *S. epidermidis* grew equally well at pH between 7.8 and 6.0. The growth of these seven staphylococcal strains was, however, inhibited at pH 4.0, similar to all other bacterial strains tested (the pH-dependent growth one of these staphylococcal strains is shown in Figure 2). However, the growth of two other *S. aureus* strains was inhibited for pH ranging from 7.8 to 6.8 and at pH 4.0, but grew at pH 6.0. This indicated that not only *E. coli* K12 had a growth advantage at slightly acidic pH, but also a variety of clinically relevant bacterial strains causing VAP. Bacterial growth was also optimal at pH of about 6, but at more acidic pH (pH 4), bacterial growth was inhibited.

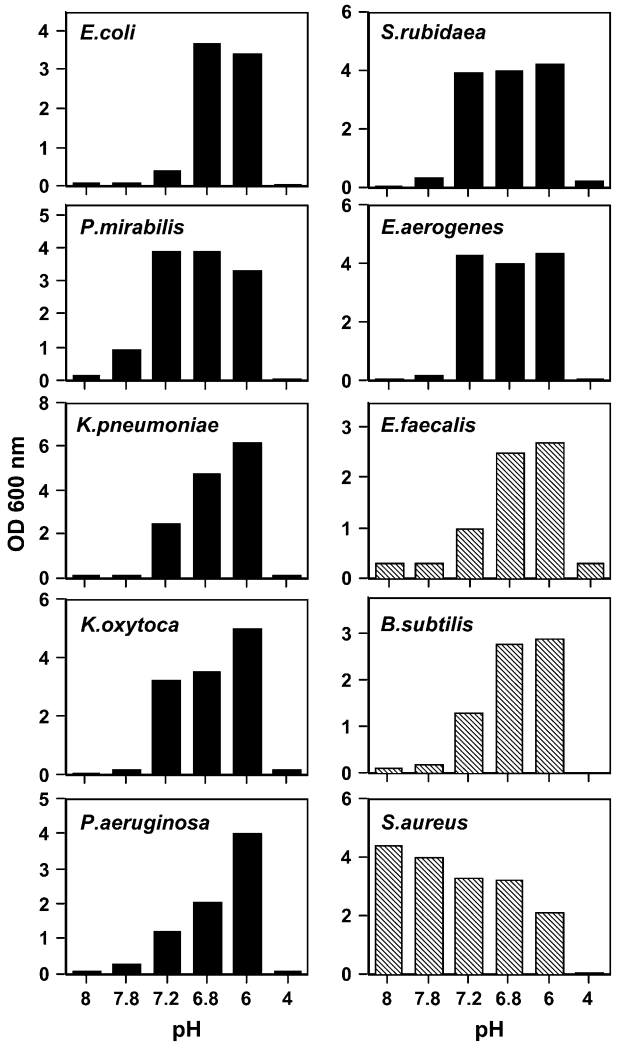

Figure 2. Various bacterial pathogens grow better at slightly acidic pH. The growth of various bacteria isolated in the lungs from patients with ventilator-associated pneumonia is markedly enhanced by a slight acidification of culture medium (DMEM/F12 medium). All bacterial strains, except for a methicillin-sensitive *S. aureus,* had a marked growth advantage in acidic pH (7.2–6). Further acidification of the milieu to pH 4 inhibited bacterial growth (assessed spectroscopically at OD 600 nm of the bacterial culture; mean ± 1 SD of triplicates). Gram-negative bacteria, *solid bars*; gram-positive bacteria, *hatched bars.*

Effect of Cell Treatment with Various Pharmacologic Inhibitors on Bacterial Growth

To investigate the metabolic pathway responsible for the observed acidosis caused by cyclic stretch, we added various pharmacologic inhibitors to stretched A549 cells. We first tested if NADPH oxidase, an enzymatic complex present in pulmonary epithelial cells that can be activated by cyclic stretch (17), could be responsible for the acidification of the milieu. DPI, a broad inhibitor of NADPH oxidases, did not show an effect on bacterial growth. Blocking the production of reactive oxygen species with N-acetylcysteine or apocynin did not show an effect, either (data not shown).

Since we showed that the increased lactic acid concentration in conditioned supernatants was accompanied by increased glucose consumption, we next tested whether a proximal inhibitor of glycolysis blocked the production of lactic acid. We found that 2-deoxyglucose abrogated glucose consumption by stretched cells, lactic acid production, and the subsequent bacterial growth (Figure 3). Not surprisingly, the lactic dehydrogenase (LDH) inhibitor oxamate also blocked lactic acid production and bacterial growth (Figure 3). Importantly, none of these inhibitors acted as "antibiotics" since bacteria grew well in Luria broth in the presence of the inhibitors. These pharmacologic inhibitors had no influence on cell viability at the dose tested, as assessed by the MTT assay and by FACS. The viability of stretched cells was consistently found to be above 95% in all conditions tested.

Cyclic stretch activates the Na^+/K^+-ATPase pump in alveolar type II cells (18), and the activation of this pump is associated with increased lactic acid production (19). We therefore tested the effect of the Na^+/K^+-ATPase pump inhibitor ouabain in A549 cells and in RAW 264.7 macrophages submitted to cyclic stretch. We found a dose-dependent inhibition of ouabain on both the acidification induced by cyclic stretch and the growth of bacteria in conditioned supernatants

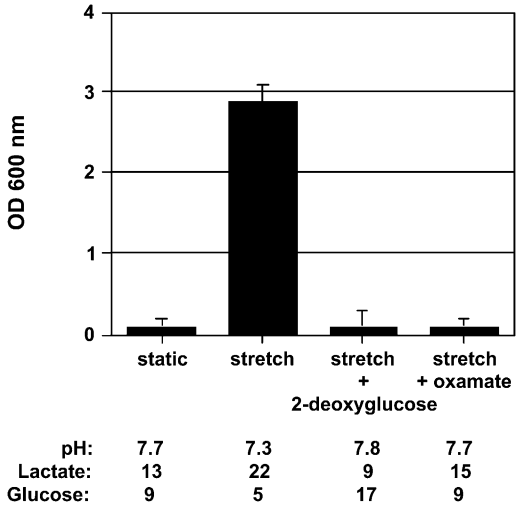

Figure 3. Effect of inhibitors of glycolysis on acidification and bacterial growth induced by cyclic stretch. *E. coli* K12 growth in supernatants from A549 cells submitted to 48 hours of cyclic stretch is inhibited by the treatment of cells with the glycolysis inhibitor 2-deoxyglucose (5 mM) and the lactate dehydrogenase inhibitor oxamate (100 mM). (*E. coli* K12 growth was measured by OD at 600 nm, mean ± 1SD of triplicates) 2-deoxyglucose and oxamate also blocked the acidification of the milieu, the stretched-induced glucose consumption, and the production of lactate. The SD of pH values were < 2% of the mean value presented in this figure. The SD of glucose, lactate, and IL-8 levels were less than 10% of the mean value presented in this figure. This is a representative experiment out of three different experiments showing the same results.

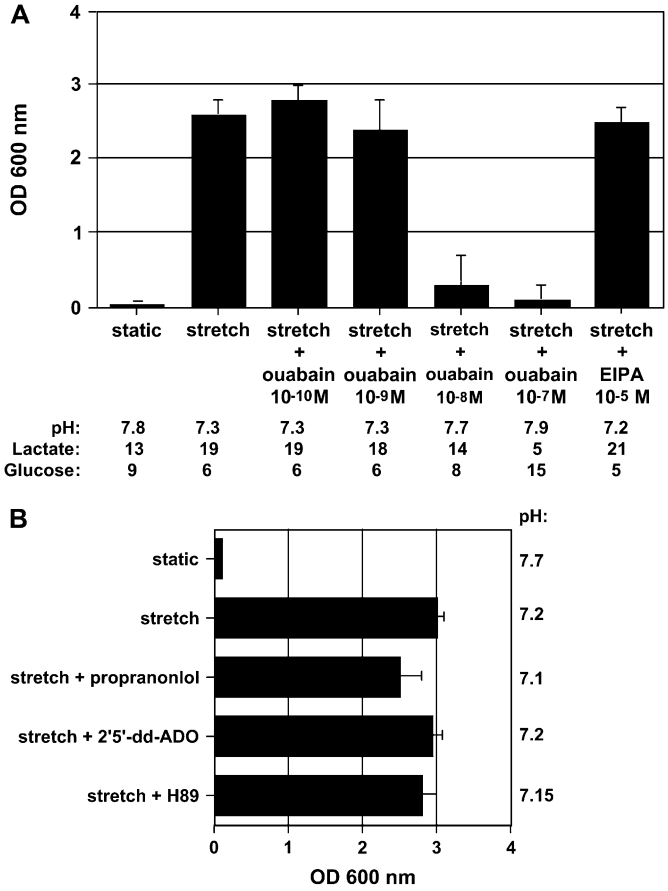

Figure 4. Effect of inhibitors of sodium pumps and cyclic AMP production on acidification and bacterial growth induced by cyclic stretch. (*A*) *E.coli* K12 growth in supernatants from A549 cells submitted to 48 hours of cyclic stretch is inhibited by the treatment of cells with the Na^+/K^+-ATPase inhibitor ouabain in a dose-dependent manner, but not by the amiloride-derivative EIPA blocking ENaC (*E. coli* K12 growth was measured by OD at 600 nm, mean ± 1 SD of triplicates). Ouabain also blocked the acidification of the milieu, the stretched-induced glucose consumption, and the production of lactate in a dose-dependent manner. The SD of pH values were less than 2% of the mean value presented in this figure. The SD of glucose, lactate, and IL-8 levels were less than 10% of the mean value presented in this figure. This is a representative experiment out of five different experiments showing the same results. (*B*) *E. coli* K12 growth in supernatants from A549 cells submitted to 48 hours of cyclic stretch is not inhibited by the treatment of cells with the β-blocker propranolol (1 μM), the adenylate cyclase inhibitor 2′,5′ dideoxyadenosine (2′,5′-dd-ADO, 1 μM) or the protein kinase A inhibitor H89 (10 μM). *E. coli* K12 growth was measured by OD at 600 nm, mean ± 1 SD of triplicates. This experiment has been repeated once with similar results.

(Figures 1D and 4A). The effect was maximal between 10 and 100 nM of ouabain, concentrations that were not associated with cell toxicity, as assessed by FACS. Ouabain concentrations greater than or equal to 1 μM induced significant cell death (apoptosis and necrosis > 40%, data not shown). EIPA, an inhibitor of the Na^+/H^+ antiporter ENaC, did not show any effect up to micromolar concentrations. These experiments indicated that the activation by stretch of the Na^+/K^+-ATPase pump was responsible for an increased activity of glycolysis necessary to feed the pump with ATP, and subsequently for the production of lactic acid.

In normoxic tissues, the activation of adenylate cyclase and protein kinase A (PKA) via β-agonists, for example, leads to an increased intracellular cAMP concentration (20). Cyclic AMP, in turn, activates the Na^+/K^+-ATPase pump, and stimulates the production of glycolysis-derived lactic acid (21). In contrast with forskolin treatment of A549 cells, we found that cyclic stretch did not induce cAMP compared with static cells at various time points (from 15 min up to 48 h, data not shown). Furthermore, inhibitors of this pathway, such as the β-blocker propranolol, the adenylate cyclase inhibitor 2′5′ dd-ADO, and the PKA inhibitor H89 neither blocked acidification nor had an effect on bacterial growth induced by cell stretching (Figure 4B). Taken together, these results suggested that the activation of the Na^+/K^+-ATPase pump was a direct mechanism through the recruitment of the pump into a glycolytic compartment at the plasma membrane induced by stretch, but not an indirect mechanism via the activation of the cAMP pathway. Although glycolysis was not directly measured, the observed glucose consumption in media and the effect of glycolysis inhibitors strongly suggest that the increased lactic acid production by stretched cells was in relation with an increased glycolytic activity.

pH in Exhaled Breath Condensate

EBCs were collected in seven intubated patients receiving positive pressure mechanical ventilation. The reasons for mechanical ventilation were the following: acute lung injury/ARDS (n = 3 patients), pneumonia (n = 2), post-cardiac surgery (n = 1), and cardiogenic shock (n = 1). The mean ± 1 SD duration of ventilation on the day of EBC sampling was 5 ± 6 days; the mean ± 1 SD arterial pH at the time of EBC sampling was 7.37 ± 0.06; Pa_{O_2}/FI_{O_2} ratio was 220 ± 89 mm Hg; and Pa_{CO_2} was 39 ± 7 mm Hg. EBCs were also collected in seven healthy volunteers. EBC pH values were significantly lower in

mechanically ventilated patients compared with healthy subjects (6.01 ± 0.8 versus 7.14 ± 0.2, respectively, $P = 0.0036$, Figure 5).

DISCUSSION

Herein, we show that human alveolar type II–like cells submitted to cyclic stretch *in vitro* acidify their milieu in a dose-dependent manner. This effect is dependent on the glycolytic pathway generating increased concentrations of lactic acid, due to the activation of the Na^+/K^+-ATPase pump. The growth of bacteria is markedly enhanced in conditioned supernatant from cells submitted to stretch compared with supernatants from cells cultured in static condition. This bacterial growth–enhancing effect is due to the secretion of H^+ by stretched cells. Interestingly, the airway pH in patients submitted to mechanical ventilation was found to be acidic compared with that of normal volunteers. A similar milieu of acidification and promotion of bacterial growth is also observed after prolonged cyclic stretch of other lung cells types such as bronchial cells, fibroblasts, and macrophages.

Intubated patients submitted to prolonged mechanical ventilation are at high risk of developing VAP (22). Colonization of their airways with commensal bacteria originating from the oropharynx and the gut most often precedes the development of VAP (23, 24). Among the various pathogenic mechanisms accounting for the propensity of these patients to acquire secondary and deadly airway bacterial superinfections are: (*1*) the presence of the endotracheal tube bypassing the protective upper airways; (*2*) a decreased cough reflex; (*3*) an impaired mucociliary clearance of bacteria; (*4*) dysfunctional local innate immune defenses; (*5*) an increased binding of bacteria to the airway epithelium (3); and (*6*) the putative stimulation of bacterial growth by proinflammatory cytokines (16). Unlike Meduri (16), we did not find that bacteria grew better when cultured with IL-1β. This may be due to different culture conditions or cytokine concentrations. Interestingly, animal models have clearly shown that mechanical ventilation favored both lung bacterial overgrowth, and that this correlated positively with the degree of airway stretching imposed by positive pressure mechanical ventilation (25, 26).

In this work, we show that airway pH may also be a critical factor promoting bacterial growth. Our data are in accordance with those from another group showing acidic pH in EBCs from mechanically ventilated patients, significantly lower than pH measured in normal subjects' airways (14). This is observed in patients without metabolic or respiratory acidosis. However, the reasons for such an airway acidification remain unclear. It may be due to the lung recruitment of acid-producing cells, such as phagocytes (27, 28). This is supported by numerous studies indicating that patients with acute lung inflammatory diseases, such as acute asthma bronchitis, COPD, and cystic fibrosis, have low airway pH measured in the EBC (reviewed in Ref. 29). We propose that, in addition to this mechanism, (more) acid is produced as a consequence of the unusual cyclic stretch imposed on the lung tissue by mechanical ventilation. Although not firmly established, our work suggests a link between cyclic stretch, acidification of the airways, and the generation of a slightly acidic epithelial lining fluid favoring bacterial growth. The origin of a low airway pH in ventilated patients may well be related to the stretch-induced production of acid by several airway cell types, such as alveolar and bronchial epithelial cells, fibroblasts, and macrophages. The consistent *in vitro* relationship found between acidification of the milieu and bacterial overgrowth suggests that stretch-induced acidification of the airways may participate in the process of lung superinfection in mechanically ventilated patients.

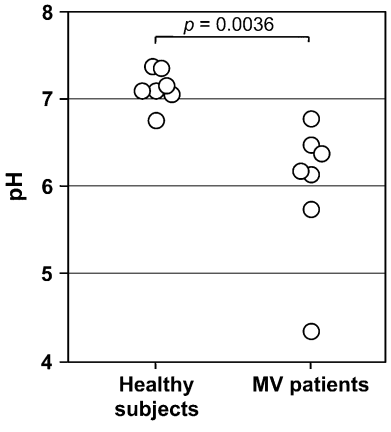

Figure 5. Airway pH in ventilated patients and healthy volunteers. pH in exhaled breath condensates obtained in critically ill patients submitted to mechanical ventilation (MV) and healthy subjects (6.01 ± 0.8 versus 7.14 ± 0.2, respectively; $P = 0.0036$).

Bacteria have developed an array of sensors for various extracellular physicochemical stimuli, such as osmolality, salt concentrations, temperature, and pH. Some of these stimuli, such as pH, significantly modify gene expression, and induce changes in numerous functions of the bacteria, including metabolism, growth, and virulence (30–32). pH sensors are not well characterized in bacteria that are pathogenic to humans, particularly for nonextreme variations of pH (33). According to our findings, it is likely that slightly acidic pH will stimulate growth programs in a number of pathogenic bacteria such as enterobacteria, nonfermentative gram-negative bacteria, but also gram-positive bacteria. The precise mechanisms of pH sensing and reprogramming in response to acidification of the milieu needs to be investigated in commensals and bacteria pathogenic for humans.

Among the various mechanisms responsible for the acidification of the milieu observed upon cell stretching, we considered and investigated three pathways. Pulmonary epithelial cells express NADPH oxidase and are therefore capable of generating a respiratory burst (17). The activation of NADPH oxidase is associated with an acidification of the milieu. In addition, it has recently been shown that cyclic stretch increased reactive oxygen species production by alveolar type II–like A549 cells (17). We found that the pharmacologic NADPH oxidase inhibitor DPI did not modify the pH of the milieu of stretched A549 cells, making it unlikely that this pathway played a role.

The second pathway studied was that of the activation of the Na^+/K^+-ATPase pump. Sodium pumps are in great part responsible for maintaining electrochemical membrane potential in mammalian cells. In particular, Na^+/K^+-ATPase plays a critical role as an engine powering vectorial sodium transport across the alveolar epithelium in pulmonary edema (34). At least two groups have reported that cyclic stretch of alveolar type II cells increased Na^+/K^+-ATPase activity (18, 35). Cyclic stretch increases the pump activity in a "dose-dependent" manner by targeting the enzyme to the plasma membrane from intracellular stores (18). Interestingly, the same sodium/potassium pump activity was also increased in lungs from rats ventilated with high tidal volume (36). It remains, however, unclear as to what triggers Na^+/K^+-ATPase activation by cyclic stretch. It has been shown that cyclic stretch of alveolar type II–like A549 cells, with elongation levels similar to those of the present study, created breaks in the membrane, without killing the cell (12). These breaks can be re-sealed by *de novo* plasma membrane synthesis. It is therefore likely that a sodium influx occurs in cells submitted to cyclic stretch, stimulating Na^+/K^+-ATPase activity as an attempt to move sodium out of the cell, and to maintain the electrochemical plasma membrane potential (19).

A major finding of our work is that the Na^+/K^+-ATPase inhibitor ouabain blocked the glycolysis-dependent production of lactic acid induced by cyclic stretch both in A549 cells and in RAW 264.7 macrophages. In contrast, an inhibitor of another sodium pump, ENaC, did not produce this effect. Na^+/K^+-ATPase derives its energy from glycolysis that is compartimentalized in association with the pump (37). In addition, it has also been reported that the activity of the Na^+/K^+-ATPase pump was one of the most energy-costly metabolic functions in mammalian cells after protein synthesis (38). Twenty percent of the ATP produced by humans is used to maintain a proper function of this pump, but can be as high as 50 to 60% in some tissues such as the brain and kidney (38). Data are lacking for the lung tissue. However, it is likely that the energy expenditure linked with the Na^+/K^+-ATPase activity is significant in the pulmonary epithelium, given its essential role in the Na^+/K^+-ATPase–dependent vectorial sodium transport necessary to maintain dry airways in normal and injured lungs (34). It is therefore not entirely surprising to observe a marked increase in glucose consumption with stretch-induced activation of this pump (18). The acidification of the milieu can be explained by the physical and functional association of the Na^+/K^+-ATPase pump with a glycolytic compartment producing lactic acid as a byproduct of glucose hydrolysis (37). Interestingly, Gessner and colleagues have reported that, compared with ventilated controls, patients ventilated for acute lung injury and acute respiratory distress syndrome airway had a more pronounced airway acidification, associated with an increased lactate concentration in exhaled breath condensates (14).

Another important pathway to investigate was that of adenylate cyclase–dependent production of cAMP. Indeed, increased cytoplasmic cAMP levels have been shown to activate Na^+/K^+-ATPase activity and the glycolysis-dependent production of lactic acid (21). We found that, in contrast with ouabain, pharmacologic inhibitors of adenylate cyclase and of the downstream effector PKA did not reverse the acidification induced by cyclic stretch. We therefore postulate that cyclic stretch, possibly by disrupting the plasma membrane resulting in an intracellular sodium influx, directly induces the membrane recruitment and the activation of the Na^+/K^+-ATPase, and this is not a secondary effect mediated by the cAMP pathway. Figure 6 summarizes our hypothesis of lung epithelial cell acidification induced by cyclic stretch and the role of Na^+/K^+-ATPase in this process.

The observation that many different lung cell types respond similarly to cyclic stretch by acidifying their milieu is somewhat surprising. However, all the cell types tested in the present study have previously been shown to be "stretch-responsive"; only the end products of cell activation may differ depending on the cell origin. Lung fibroblasts submitted to cyclic stretch have, for example, been shown to activate NF-κB, and to up-regulate COX-2, calcyclin, and proteoglycans (39–42). This common cellular response to cyclic stretch may be related to the protein that is targeted here, the Na^+/K^+ ATPase pump, which is a ubiquitous ion channel necessary for maintaining electrochemical polarization and therefore cell survival. We therefore postulate that the signaling pathway activated by cyclic stretch resulting in the acidification of the milieu is common to many different cell types. The activation of the Na^+/K^+ ATPase pump may be secondary to the activation of stretch-sensitive ion channels, the activation of integrins and related kinases of the focal adhesion plaque, or simply related to the sodium influx due to plasma membrane stress failure induced by stretch (10, 12).

Our study has some limitations. First, our study was performed essentially with cell lines. However, the cell lines used in the present study are representative of important cell types of distal airways, such as type II alveolar cells (43), bronchial epithelial cells, lung fibroblasts, and macrophages, all of which are submitted to stretch during mechanical ventilation. Human bronchial BEAS-2B cells have retained many features of bronchial cells and are sensitive to cyclic stretch (6–8). Macrophages were also shown to be major players in stretch-induced lung inflammation (5). We have tried to duplicate our results with small airway primary human epithelial cells, but these cells did not grow properly on collagen I–coated silastic membranes, as also reported by others (44). Alveolar type I and II epithelial cells can be isolated from resected lungs. However, the isolation procedure is tedious, yields a small number of fragile cells,

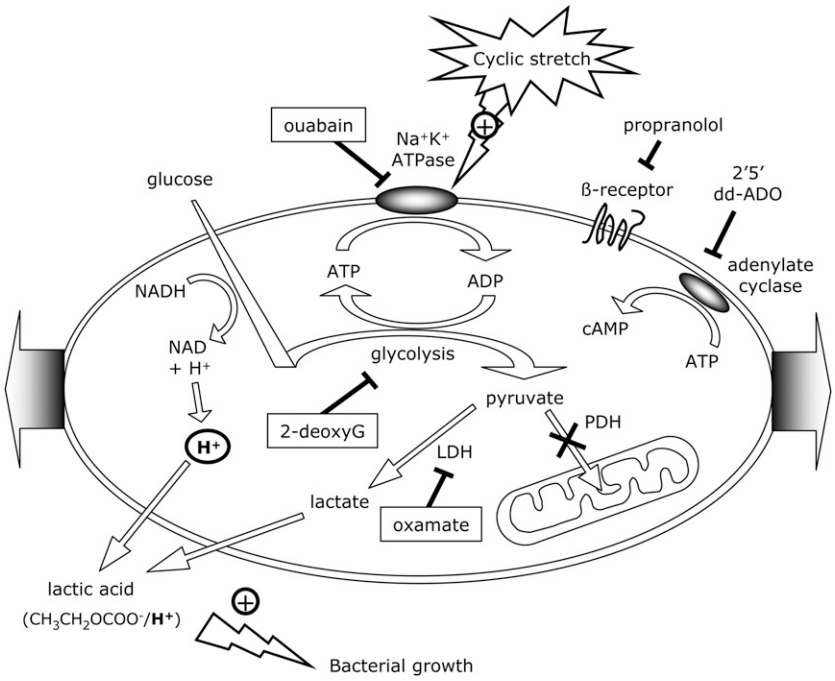

Figure 6. Pathogenesis of airway cell acidification and bacterial overgrowth induced by cyclic stretch. Schematic view of the proposed mechanism for the observed acidification of the milieu of human alveolar epithelial type II–like A549 cells submitted to cyclic stretch. Cyclic stretch activates Na^+/K^+-ATPase, stimulating glucose consumption and lactic acid production in a glycolytic compartment. The pharmacological inhibitors 2-deoxyglucose (2-deoxyG), oxamate, and the Na^+/K^+-ATPase inhibitor ouabain blocked acid lactic production induced by cyclic stretch. In contrast, propranolol, 2′,5′-dd-ADO blocking the cyclic AMP (cAMP) pathway had no effect on acid lactic production in response to cyclic stretch. In turn, increased H^+ concentration in the milieu induced by cyclic stretch stimulates the growth of bacteria. PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase.

which are not likely to adhere, grow, and resist to prolonged cyclic stretch in our system. Contamination with macrophages is also a common problem of the isolation procedure. Second, our study does not make a definite and causative link between cyclic epithelial distension and acidification of the airways in patients. The origin of airway acidity may well be multifactorial, and the respective role of positive pressure mechanical ventilation remains to be investigated. However, our data demonstrate in an *in vitro* model that this phenomenon occurs, that it is not related to cyclic stretch–induced cell activation by pro-inflammatory mediators such as IL-1β, but that it is rather dependent on the activation of the Na^+/K^+-ATPase pump. This seems to be a relevant problem since airway acidification is common in ventilated patients. The majority of VAP pathogens showed a growth advantage at acidic pH, although some pathogens also grew at pH measured in the airways from healthy subjects. The prevention of lung superinfection by means of airway alkalinization using sodium bicarbonate, for example, could be tested in an animal model or in mechanically ventilated patients.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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