HHS Public Access

Author manuscript

Curr Respir Med Rev. Author manuscript; available in PMC 2018 January 29.

Published in final edited form as:

Curr Respir Med Rev. 2016 ; 12(2): 167–174. doi:10.2174/1573398X12999160506104327.

Choline Triggers Exacerbations of Chronic Obstructive Pulmonary Disease in Patients Infected with *Pseudomonas aeruginosa*

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Abstract

Background—Although exacerbations of chronic obstructive pulmonary disease produced by *Pseudomonas aeruginosa* infections are a major cause of death, the molecular mechanism that produces them is not well known. Here we focused on the energetic basis of dyspnoea, hypercapnia and acidosis symptoms.

Methods and Findings—We used an in vivo exacerbation model exposing mice to cigarette smoke and LPS, to mimic emphysema and infections, and choline challenges to trigger exacerbations, that showed 31% increased in the airway resistance for naïve mice and 250% for smoke/LPS treatment. Tissue resistance was increased 32%, in naïve mice, and 169% for smoke/LPS treatment. A decreased tissue elastance, was confirmed by decreased collagen content and increased alveoli chord length. Consequently, the O_2 demanded was 260% greater for smoke/LPS treated mice, to provide the energy required to pump the same volume of air then for naïve mice. The extra CO_2 produced per ml of air pumped caused hypercapnia and acidosis by 4% decrease in pH.

In addition, the bacteria grown with choline had a decrease of 67% in phosphate, 23% ATP and 85% phospholipids with an increase of 57% in polyphosphates, 50% carbohydrates, 100% LPS, consuming 45% less energy relative to the bacteria grown with succinate.

Conclusion—choline, released by *P. aeruginosa*, triggers exacerbation symptoms by increasing lung resistance, O_2 consumption and producing more pCO_2 in blood with dyspnea, hypercapnia and acidosis. The energetic shift of decreased O_2 bacterial demand and increased lung demand benefits the infection, thus restoring the energetic balance on the host will favor *P. aeruginosa* eradication.

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AUTHORS CONTRIBUTION

SMG, conceived, design, drafted the manuscript, executed, analyzed and/or interpretated data.

Keywords

Bacterial infections; COPD exacerbations; LPS; emphysema; animal model; respiratory infections

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) develops alone in 18% of all patients and associated with several disorders in 62% of patients [1], with infection by *Pseudomonas aeruginosa* being comorbid in 20% of patients [2], Infection by *P. aeruginosa* causes a steeper loss of pulmonary function and triggers exacerbations with increased risk of death [3], Exacerbations of COPD are characterized by increased sputum production, cough, dyspnea, respiratory rate and work of breathing (WOB). In a normal resting state WOB accounts for 5% of the total oxygen consumed by the body but it increases dramatically during exacerbation, due to the inability to ventilate CO_2, which produces hypercapnia with a pCO_2 in blood above 50 mmHg, pH bellow 7.35 and CO_3H^- above 26 mM [4].

Upon infection *P. aeruginosa* delivers to the host cell membrane, lipopolysaccharide (LPS) shielded vesicles [5] loaded with phospholipase C (PLC) [6] and cholinesterase (PChP) [7] decreasing the surfactant levels in the lung by degradation of dipalmitoylphosphatidylcholine to choline [8], a cholinergic agonist, thereby activating K^+ [9] and Ca^{++} efflux [10] which induces bronchoconstriction and vascular smooth muscle relaxation [11].

LPS from *P. aeruginosa* binds to toll like receptors on T- cells [12] and macrophages, the activated macrophages release macrophage metallo elastase 12 (MMP12), destroys the extracellular matrix (ECM) and accelerates the development of emphysema promoting an elastolytic environment [13].

Choline binds the muscarinic and nicotinic receptors on monocytes [14] and in T cells [15] regulating inflammation, and the lymphoid cholinergic system in an autocrine and/or paracrine manner that may trigger exacerbations of COPD.

Since choline challenges the lung to produce bronchoconstriction and it is used as a means of detecting asthma and differentiating it from COPD, many different choline derivatives are used, such as β-methyl-acetylcholine, acetyl-choline, or choline [16–17], thus we hypothesized that *P. aeruginosa* may be triggering exacerbation by choline release. In this study we test if choline induces exacerbations symptoms in mice by increasing the WOB in a model of *P. aeruginosa* infection in cigarette smoke-induced emphysema, combining the action of smoke with LPS.

METHODS

All animal experiments were perfonned according to protocols approved by the School of Public Health at Harvard Medical School due to availability of animal care facilities in compliance with international regulations and adequate equipment, while experiments with bacteria were performed at the University of Rio Cuarto according to institutional protocols.

Animals

Cigarette smoke exposure—Age and sex matched C57/BL6 mice of 8–12 weeks (Jackson Laboratories. Bar Harbor, ME, USA), were exposed to main stream smoke of four unfiltered cigarettes (from University of Kentucky, USA) per day, six days a week for six months according to standard procedures [18]. Designated mice were treated for 2 months with a weekly dose of LPS (100 ng) delivered intranasally (*Pseudomonas* Serotype 10, Sigma Chemical Co., St. Louis, MO, USA).

Broncho-Alveolar Lavage: (BAL)—*Broncho-Alveolar Lavage* (BAL) was performed by the standard method as previously described [18]. At the set time point, mice designated for BAL analysis were sacrificed and BAL was performed *via* a 22-g i.v. catheter inserted into the trachea. The lungs were lavaged 4 times with 1 ml of phosphate-buffered saline (PBS), red blood cells were lysed, and the BAL fluid was centrifuged 3 min at 3000 rpm. Cell pellets were resuspended in 1 ml of PBS and used to determine the total cell number and differential cell counts using a hemocytometer and cytospins stained with Hema3 (Biochemical Sciences In, NJ, USA).

Alveoli Chord Length (CL)—*Alveoli Chord Length* (CL) was determined in lungs inflated with 10% formalin through an intratracheal catheter under a pressure of 25 cmH_2O and fixated for 48hr at 25°C, rinsed with PBS, dehydrated in ethanol series, and embedded in paraffin [18]. Mid-sagittal lung sections of 5 pm were stained with Gill's hematoxylin and used to calculate the CL [19]. Ten photographs per slide were taken at randomly selected 200x fields, using MetaMorph image analysis software (Molecular Devices, Downingtown, PA) and analyzed with Scion Image Software (Scion Corporation), airway and vascular structures were excluded from the analysis [18]. Collagen deposition was quantified using trichrome staining, performed by the Brigham and Women's Hospital immune-staining core, according to standard procedures, and quantified as described above [20].

MMP12 assay—*MMP12 assay* was performed on macrophages induced using 4% thioglycollate in PBS introduced peritoneally in wild type (wt) mice, plated in Hank's Balanced Salt Solution (HBSS) medium (GIBCO BRL, Gaithersburg, Maryland, USA), and stimulated with 200 ng/ml LPS (*Pseudomonas* Serotype 10, Sigma Chemical Co., St. Louis, MO, USA) and INFγ, in a dose dependent manner (5, 50 and 500 ng/ml). Supernatant was used to determine MMP12 by western blot with anti-MMP12 chicken antibody (Genway Biotech, San Diego, CA, USA).

Lung Function Resistance—*Lung Function Resistance* was measured in mice divided in four groups for naïve, smoke exposed, LPS or LPS/smoke treatment. Methacholine (Sigma–Aldrich Inc., St. Louis, MO, USA) was used as agonist, to produce broncho constriction by inhalation using a computer-controlled ventilator (flexiVent, SCIREQ, Montreal, QC, Canada). Each mouse was anesthetized with 60mg/kg pentobarbital sodium i.p. (Ovation Pharmaceuticals Inc., Deerfield, IL, USA), performed a tracheotomy, and then attached the mouse to the ventilator. Each animal then received deep lung inflation of 30 cmH_2O, distending pressure, after which we took baseline readings. Then we administered saline solution, sequentially increasing concentrations of methacholine (1, 3, 10, and 30

ml/kg) *via* nebulizer, for 10 seconds through the tracheotomy. To determine airway resistance, we fitted a constant phase model to the data obtained from the multiple frequencies simultaneously applied at the airway opening. After each of the 5 challenges, we recorded airway resistance (Raw) in 12 cycles of 30–60s. Raw, tissue resistance (G) and dynamic elastance (H) values were obtained by fitting the impedance data measured to the constant phase impedance model {ΔP/ΔV= Raw + (G-Hj/freq)} and adjusting the model parameters (which are Raw, G and H) to optimize the fit between the data and the model [21,45], We calculated the work of breathing (WOB) considering that the lung volume of a mouse is 1 ml, and the surface of air exchange is constant within the same mouse at the beginning and end of the PFT, at isothermic condition, T=37 °C, the heat transferred is cero, Q=0, and work done is the same then the energy used, W=E, $\{\Delta W_{Total} = [\Delta P_{airway} + \Delta P_{tissue}] V\}$. Then, we fitted data from other researchers [22], to further convert their Raw and G values, for control and LPS, to calculate energy and pCO_2 values, to double check for possible missed labeled results from unrecoverable samples, and we found the same values for LPS treated (43.39 mmHg pCO2) for Reis Gonçalvcs [22], as compared with our results (42.95 mmHg) (Table 1) and for pH values measured by Dixon [23].

Microorganism

Growth condition—*Growth condition* colonies of *P. aeruginosa*, Fildes III, 1924, NCTC, UK, were isolated from Luria-Bertani (LB) agar [24] at 37°C and cultured into LB broth overnight and then $1/10^{th}$ dilution was made in the basal salt medium with 20mM choline, as the sole carbon and nitrogen source, or with 20mM succinate plus 18.7mM NH_4Cl with high Phosphate (Pi) (39mM), or low-Pi (2mM) was used to increase the specific activity of ^{32}Pi. All experiments in this study were carried out with bacteria grown to an OD_{660} between 0.6 and 0.8, harvested by centrifugation at 10,000g for 10min, and washed twice with 155mM NaCl.

Metabolites quantification—Protein concentration was measured by Bradford [25], total phosphate (Pi) quantified by Mc Clare [26] after mineralization with perchloric acid for 30min. For ATP quantification we used Molecular Probes luciferine luciferase Kit (A-6608), according to the manufacturer's instructions. Briefly, 10ml of bacterial culture was harvested, and the intracellular metabolites extracted with 70% ethanol. Then, a 100μl of supernatant was added with 600μl of 25mM Tricine buffer pH 7.8, plus 2.5μl of 0.5mM luciferin and 5mg/ml luciferase. The light emission was detected in a Spex Fluoromax, detecting fluorescence at 560nm without excitation for 30 seconds, to establish a baseline prior addition of 100μl of the metabolite extracted. Then, fluorescence was recorded for 300 seconds and the total emission calculated by subtraction of the background emission to the average of the final emission. Polyphosphates were extracted [27] and quantified hydrolyzing a sample with HCl (1:1 v/v) to detect Pi. The quantity of polyphosphate was the difference between Pi in basal, non-hydrolyzed, and hydrolyzed samples.

LPS extraction and characterization—Triton X-100 was used to isolate LPS [28] and quantified by 2-keto-3-deoxyoctonate (KDO) [29]; carbohydrate [30] and Pi contents were measured as above. The fatty acid composition, in the Lipid A of the LPS, was determined [31]. Methyl esters from fatty acids were produced from the LPS with chloroform: methanol

(1:2, v/v) followed by derivatization with 2% H_2SO_4 in methanol:benzene (9:1 v/v) at 80°C for 2hr [32], Hydroxyl acids were treated with bis-trimethyl-silil-fluoroacetamide at 60°C for 30min and identified by gas chromatograph with flame detector (GC-MS Hewlett-Packard 5890) and mass spectrometer (HP 5972), operated at 70eV, with a column of phenyl methyl silicone of 30m × 0.25mm; helium was run through the column at 18 ml/min at 180°C for 3min and then increased at 25°C/min up to 280°C and maintained at this temperature for 28min.

For radioactive labeling 1 ml of bacteria was cultured with 1 μCi of ^{32}Pi (specific radioactivity 3.15 nCi/nmol Pi) and extracted by the Bligh and Dyer procedure [33], The chloroform phase was dried out with N_2 flow, redissolved in chloroform:methanol (4:1, v/v) and separated on a silica gel thin layer chromatography plate, run with chloroform:pyridine: 88% formic acid:water (40:60:16:5, v/v) and developed with iodine vapor and autoradiography. Compounds containing ^{32}Pi were scraped off the plate and measured by scintillation counting and Cernekov radiation.

RESULTS

Based on the concept that choline could be released by *P. aeruginosa* (Fig. 1A) we tested if choline could be triggering exacerbations in patients with emphysema and infected by *P. aeruginosa*, simulated in animal model by LPS delivered intranasally to the lung of the mice exposed to cigarette smoke.

LPS Synergizes with Cigarette Smoke to Increase Lung Damage

LPS induced *in vitro* greater amounts of MMP12 from macrophages synergizing with INFγ in a dose dependent manner (Fig. 1B) and significantly increased inflammation *in vivo* (Fig. 1C) duplicating the total number of inflammatory cells and macrophages and triplicating the number of lymphocytes in LPS/smoke treated mice relative to smoke exposed mice BAL, causing loss of elastic recoil (H). This was *in vivo* demonstrated by increased lung injury and longer CL from 23.8 ±1.8μm (average±SD), for control mice, to 29.8 ±0.9μm p=0.05 for smoke exposed mice, and 33.9 ±5.7μm, p=0.14 for smoke/LPS treated mice, but not for LPS treatment 25.62 ±0.9μm.

Choline Reproduces Exacerbation in Mice

Since choline is released by *P. aeruginosa* and this is used to produce bronchoconstriction, we hypothesized that it may be triggering exacerbations in infected patients. To that end we studied mice lung mechanics, using a flexi vent ventilator, challenging the lung with methacholine, a synthetic ester of choline. Mice exposed to cigarette smoke incur a significant increase in central airway resistance (209%) (Fig. 1D, Raw), as do mice exposed to both smoke/LPS or LPS alone (250% and 210% respectively) compared to smoke naïve mice (31%). Resistance of the lung parenchyma (Fig. 1D, G), was increased in smoke exposed mice (163%), upon choline challenge, in smoke/LPS (169%) or LPS treated (254%), compared to naïve mice (32%). Changes in tissue elasticity (H) reflect lung derecruitment or airway closure [34], the decreased elasticity represented as dynamic elastance (17%) (Fig. 1D, H) of mice treated with smoke and smoke/LPS had an inflexion

point above 10 mg/ml of choline and it was reversed to 80 and 59 % increased elasticity at the highest dose, compared to LPS treated mice (116%), similar to those reported by others [45].

Recessive Spiral of O_2 Starvation During Exacerbations

We quantified the WOB (Table 1) from tissue and airways resistance, to displace the same volume of air in the lung, after choline challenge was increased from basal to the maximal dose. The WOB increases from 5 to 10, 12 to 19% initiating a recessive spiral of O_2 available for cellular subsistence, CO_2 accumulation and rised pCO_2 (Table 1, Fig. 2A). The O_2 required to produce this WOB was 8 times and 12 times higher for mice exposed to smoke or smoke/LPS, respectively, compared to naïve mice. Thus, LPS produce at least 1.6 times greater O_2 consumption then smoke exposure (Fig. 2B). Those mice with increased lung damage (CL) have more oxygen depleted (Table 1) from their arterial blood by the lung producing air grasping, an exacerbation symptom [4].

Increased Lung Resistance Produces Hypercapnia

Since pressure is an intensive property that permits comparison from a small lung, like the mice, to a larger lung, like the human; we compared values from mice lung to those values measured in exacerbated patients. Therefore, if during acute exacerbations in humans pCO_2 rises between 56 to 90mmHg [4], with pH bellow 7.35, and 26 mmol/1 of HCO_3^-, compared to 46mmHg and 29mmol/l of HCO_3^- in stable COPD [4] and normal arterial pCO_2 of 35–45 mmHg in normal individuals. Then, the pCO_2values (Table 1) in naïve mice (47mmHg) and smoke/LPS exposed (52mmHg), after challenge with choline, demonstrate the animal model fitted the physiologic parameters of exacerbated and normal patients, departing in only 7% from the lower limit of exacerbators humans.

Considering the protons produced by the CO_2 dissolved in blood we calculated the WOB done by the lung (Table 1) to exhale the same volume of air overcoming the increased resistance produced by choline, the loss of alveoli units (CL) and elastic recoil. We demonstrated a pH drop, in choline treated mice, that is similar to the one seen in human exacerbations (Table 1).

P. aeruginosa is Metabolically Modified by Choline

Lung infections with *P. aeruginosa* are not easily eradicated by standard antibiotics treatments, resulting from increased bacterial polysaccharides secretion, such as LPS and alginate that inhibit immune clearance thereby promoting colonization [35], Hence, we hypothesized that choline may possibly act in an autocrine/paracrine manner on the bacteria producing changes in its LPS or glycosides, shifting to a mucoid variant for permanent colonization (Fig. 1A). To that end, we determined the cell components like LPS, glycosides, phospholipids, phosphate, ATP and polyphosphates when grown with choline, which is the nutrient obtained upon lung infection, and compared it with its favored carbon and nitrogen source. Results summarized in Table 2 show that cells grown only with choline contains less total Pi, ATP, and phospholipids than bacteria grown with succinate and ammonium. Conversely, the bacteria grown with choline had more polyphosphates (Table 2, Fig. 3), carbohydrates and LPS than those grown with succinate.

LPS from *P. aeruginosa* is Enhanced by Choline

There was a significant increase of carbohydrate length in the LPS from bacterium grown with choline, as only carbon and nitrogen source, compare to the one grown with the favorite carbon and nitrogen source (Table 3). Unlike the total Pi content in the bacteria the LPS phosphorylation was similar in both mediums. Moreover, the incorporation of ^{32}Pi from the beginning of the growth did not show any appreciable difference in the Lipid A phosphorylation of LPS from bacteria grown with choline or succinate (data not shown). We further inspected the Lipid A from LPS, using mass spectrometry, showing that the lipid profile was not modified by choline (Table 3).

CONCLUSION

In this study we replicated the exacerbations of COPD in a murine model using cigarette smoke and LPS treatment followed by choline challenges. Three major findings emerge from using this model.

First, treatment of mice with LPS replicates major aspects of *P. aeruginosa* infections in patients with severe COPD [1,4]. Similar to COPD, there was increased inflammatory infiltration of T cells, macrophages and neutrophils and lung damage by MMP12 that resulted in increased airway and tissue resistance caused by the loss of alveolar units; shown by the significant increase of the CL in mice treated with LPS and cigarette smoke. The loss of elastic recoil caused by LPS is reflected in a decreased dynamic elastance (H) in smoke/LPS treated mice, which also had decreased collagen content in their lung tissue (data not shown). This indicates increased elastolitic environment caused by synergistic effect of LPS and INFγ on macrophages inducing MMP12 and ECM destruction, that coincided with previous descriptions of the inflammation in which macrophages are activated by cigarette smoke [13] and LPS binding to CD 14 [36] and toll like receptors on T cells [12]. We also found increased T cells infiltration in BAL due to LPS only, indicating that T cells are selectively recruited and activated through TLR4 [37], further recruiting and activating more macrophages that increased the CL and decreased the elastic recoil (H) by ECM degradation which are the causes of steeper loss of lung function in COPD patients with *P. aeruginosa* infections [36,38,43].

Second, choline challenges replicated the exacerbations, which were quantified as WOB considering the thermodynamic aspect of the physiological process behind the increased breathing difficulty. Since the temperature of the body is constant, the WOB done by the lung can be calculated from airway and tissue resistance data (Raw and G) to exhale the same volume of air. The energy for this work is provided as ATP with concomitant O_2 consumption and CO_2 production, reaching higher pCO_2 in blood and lower physiological pH, in addition pCO_2 increase impedes ventilation on other organs; this constitutes a recessive spiral of O_2 starvation that produces hypercapnia Although we did not measure directly the pO_2 or pCO_2 in mice, the semi empirical values obtained for pCO_2 had less than 10% error, which indicates that the animal model fitted the physiological values shown by COPD patients during exacerbations [4] and the arterial pCO_2 or pH values measured in rats by Dixon [39], showing an accurate assumption of the body mass independence for physiological values.

Third, we demonstrate that choline also plays an autocrine/paracrine role on the bacteria equipping *P. aeruginosa* with more LPS and longer glycosidic chain feeding forward the infection loop by increasing the damage on the host tissue manifested as more inflammation, larger chord length (CL) and labored breathing (higher WOB).

Choline and LPS are bacterial products that have a profound effect on the host, as well as the bacteria. *P. aeruginosa* was metabolically modified by choline, decreasing total Pi, ATP, and phospholipids while increasing glycosides, LPS, and polyphosphates, over all decreasing its energetic demand becoming equipped to secrete more glycosides for permanent colonization.

Of note is that an energetic shift from the bacteria reduces its O_2 demand while forces the lung to use more energy to maintain its function, entering in a recessive spiral of O_2 starvation that triggers exacerbations, giving an advantage to the opportunistic bacteria survival. Herein we suggest that delivery of succinate to the lung of COPD patients infected with *P. aeruginosa* may help to reduce its effectiveness for colonization and help restore the energetic balance of the lung.

Acknowledgments

This study was founded by the NHLBI of NIH USA (ROlHL08254104), and from Argentina CONICOR and SeCyT of UNRC.

Jandhyala Dakshina for his invaluable critical review of the manuscript. To Marcelo Ciani for his great help with the figures. To Edward Ingenito for his assistance with Flexivent.

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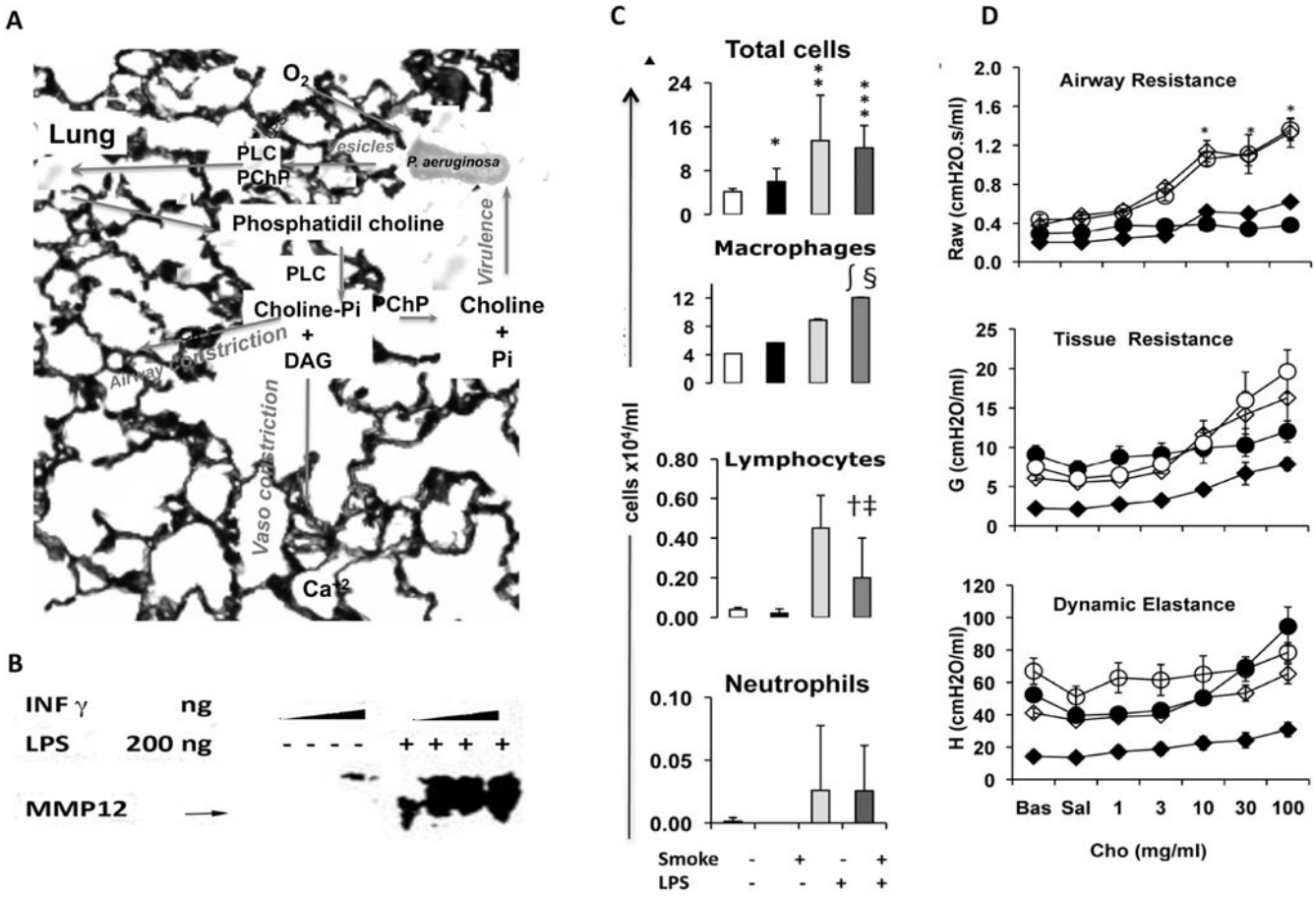

Fig. (1). Exacerbations in mice

(A) Representative scheme of the host-pathogen interaction in mice lung during exacerbations of COPD. As an extracellular pathogen *P. aeruginosa* releases to the medium phospholipase C (PLC) (6) and phosphoryl-choline phosphatase (PChP) (7) within vesicles (5) that degrades the membranes of lung epithelial cells from phosphatidyl choline to phosphoryl-choline and Diacylglycerol (DAG) [40], that causes Ca^{2+} mediated vaso-constriction (10). Choline and Pi released by PChP produces airway constriction in the lung tissue, and LPS and PolyP accumulation in *P. aeruginosa*. **(B)** Western blot of MMP12 from supernatant of peritoneal macrophages stimulated with or without LPS (200 ng) and incremental doses of INFγ of 5, 50 or 500 ng. **(C)** Representative experiment of inflammatory cells present in BAL of naïve mice (n=5), mice treated with of LPS (n= 4), smoke exposed (n=8) and smoke plus 100 ng/weekly of LPS (n= 3) from *P. aeruginosa*. *P= 0.01 relative to naïve mice ⁂P= 0.04 relative to naïve mice, ⁝P= 0.01 relative to smoke exposed, §P=0.01 relative to naïve mice, †P=0.05 relative to smoke exposed, ∫P=0.05 relative to naïve mice, ‡P=0.01 relative to smoke exposed. **(D)** Plots show the pressure offered by the lung of mice smoke naïve (black circles, n=7), LPS (black squares, n=4), smokes exposed (open circles, n=4) and smoke plus LPS exposed (open squares, n=5) due to airway resistance (top plot) tissue resistance (middle plot) and dynamic elasticity (bottom plot). Data is expressed as mean+s.e.m, *P≤0.0001 determined by t-student test.

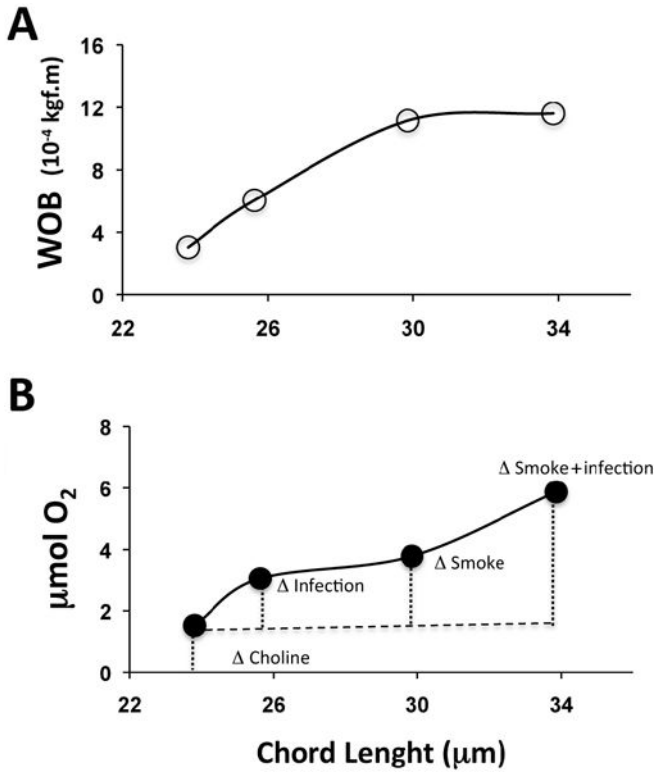

Fig. (2). Impact of inflammation on the lung mechanics

(A) Plot showing the energy consumed by the extra work of breathing (WOB) done by the lung to exhale the same volume of air (1 ml) versus the alveoli chord length (CL) determined by morphometry, quantified in mice treated with cigarette smoke (n=8) and smoke plus LPS (n=3) compared to naïve control (n=8) or the value for LPS obtained from [44], **(B)** Oxygen consumed to exhale the same volume of air; dotted line separates the O_2 due to obstruction produced by inflammation from smoke exposure, LPS or combination of both after choline induced resistance, dots represent average±SD.

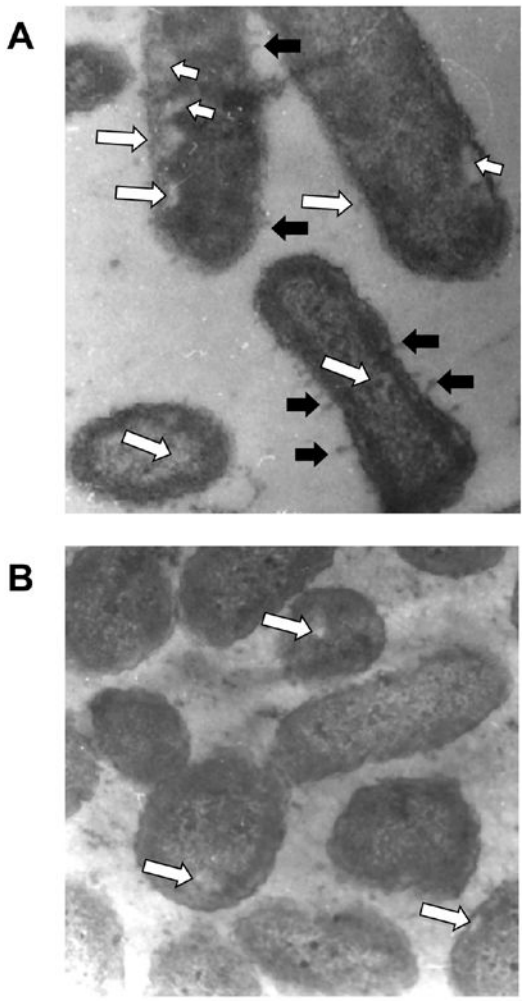

Fig. (3). Morphology of *P. aeruginosa*

Electron microscopy from *P. aeruginosa* grown with choline **(A)** or succinate **(B)** showing vesicles (black arrow) and zones of high refringency (white arrows). The inclusions of high electronic density are due to polyphosphate accumulation [41], Vesicle formation [5] 52,000×.

Table 1

Semi-empirical physiologic values in mice exacerbations induced by choline challenges.

Treatment (n)	WOB (10^{-6} Kgf.m)[a]	Energy ATP (nmol/ml)[b]	CO_2 (nmol/ml)	H^{+}[c] (μmol/ml)[b]	pH	pCO_2 (mmHg)[d]
Naïve (7)	30.4 (5%)	10	1.9	25.7	7.6	47
LPS (4)	60.5 (10%)	19.4	3.8	36.3	7.4	49
Smoke (4)	76(13%)	24	4.8	40.4	7.4	50
Smoke+LPS (5)	116(19%)	37	7.4	50.3	7.3	52

Formulas used to calculate the values in this table.

[a] ΔW total= $(\Delta P_{airway}+\Delta P_{tissue})V=(\Delta P=Raw+G)$, V_{lung}=1ml, T=37°C, Q=0 and W=E.

[b] Glucose + 6 O_2+ 38 ADP + Pi→ 6 CO_2 + 6 H_2O + 38 ATP.

[c] $pH= (K_a . [CO_2])^{1/2}$; $K_a=[H^+] . [CO_3H^-]/[CO_2]=4.3\times10^{-7}$.

[d] $pCO_2=K_{Henry} . [CO_2]$; $K_{Hmry}=1.25 \times 10^6$ mmHg/mol/1 at 25 °C.

Table 2

Total phosphate, ATP, polyphosphates, carbohydrates, and LPS in *P. aeruginosa* grown with succinate and ammonium chloride or choline[a].

Composition	Succinate plus NH_4Cl		Choline			
	µg. (mg Protein)$^{-1}$	µmol. (mg Protein)$^{-1}$	µg. (mg Protein)$^{-1}$	µmol. (mg Protein)$^{-1}$	%	p[b]
Phosphate	1400±100	14.7 ±0.7	460 ± 90	4.8 ±0.7	33	0.001
ATP	1650 ±330	3.0 ± 0.6	1270±165	2.3 ± 0.3	−23	0.32
Polyphosphates	4.0 ± 1.8	0.042 ±0.01	6.3 ± 1.4	0.066 ± 0.008	57	0.004
Carbohydrates[d]	210 ±40	1.2 ±0.2	330 ± 50	1.8 ±0.2	50	0.03
LPS[e]	19 ± 4	0.08 ±0.02	41 ±9	0.16 ±0.03	100	0.02
Phospholipids[c]	114 ± 7	0.65 ±0.04	71 ±4	0.1 ±0.02	−85	
Total Biosyinthetic cost						
Energy (ATP)[f]	–	1675	–	924	45	

[a] Bacteria were grown in a HPi-BSM with 20 mM succinate plus 18.7 mM NH_4C1 or 20 mM choline chloride. All chemical determinations were done on whole bacteria, harvested at the end of the exponential growth phase at $OD_{660} \cong 0.7$. Total cellular contents were 1.05±0.16 and 1.00±0.20 mg.ml^{-1}, respectively. Results are the average of four independent experiments ± SD.

[b] Values obtained by ANOVA analysis.

[c] Total phospholipids from bacteria grown with succinate/NH_4Cl or choline were calculated from [42].

[d] Total carbohydrates were measured by the phenol method (30).

[e] Measured as the content of KDO according to the determination of formylpyruvic with thiobarbituric acid (29). More details related to the extraction and determinations of these compounds are found in material and method.

[f] Value obtained by calculation of the biosynthetic cost of LPS 470 µmol ATP/gr of cells, 1 µmol ATP/gr Polyphosphate, 470 (µmol ATP/gr of glycoside and 2578 µmol ATP/gr of Phospholipids.

Table 3

Total phosphate, carbohydrates and lipids in LPS of *P. aeruginosa* grown with succinate or choline[a].

Composition	Succinate plus NH_4C1 (μmol/μmol KDO[b])	Choline (μmol/μmol KDO)	%	P
LPS				
Total Pi	27 ± 5	33 ± 8	22	NS[e]
Carbohydrates[c]	0.09 ± 0.01	0.15 ±0.02	67	<0.05
Lipid A				
% of Palmtic acid[d]	34 ±2	39 ± 5	15	NS
% 12 carbon-hydroxyl-acid[d]	32±14	45±20	41	NS

[a]Bacteria were grown in a HPi-BSM with 20 mM succinate plus 18.7 mM NH_4C1 or 20 mM choline chloride. All chemical determinations were carried out on LPS isolated with Triton X-100 (28) from whole bacteria harvested at OD_{660}≅0.7. Total cellular contents were 1.05+0.16 and 1.00+0.20 mg/ml respectively. Results are the average of four independent experiments ± SD. P values obtained by ANOVA analysis.

[b]KDO quantified by (29).

[c]Carbohydrates quantified by phenol method (30).

[d]Lipids were hydrolyzed from Lipid A, identified by mass spectrometry. Results are expressed relative to stearic acid and averaged of three independent experiments ± SD.

[e]No significative.