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Journal of Microbiological Methods 62 (2005) 293–302
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Feasibility of assessment of regulatory lipids in breath condensate as potential presymptomatic harbingers of pulmonary pathobiology☆

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Received 28 February 2005; accepted 4 April 2005
Available online 15 June 2005

Abstract

Regulatory lipids from the airway surface readily form aerosols that can be recovered non-invasively by cooling expired breath to form breath condensate (BC). Regulatory lipids have been detected previously utilizing enzyme-linked-immunosorbent serologic assay (ELISA). Here we test the feasibility of assessment of regulatory lipids in BC by mass spectrometry so presently unknown lipid regulatory components can be detected without addition of specific antibodies as in the ELISA procedure. Baseline regulatory lipids were detected in >pg/mL BC in control animals or human lung tissue culture cells. In nearly every case animals exposed to toxins or infectious bacteria showed increases in the BC regulatory components.

Lipids were recovered from BC by solid phase extraction. Phosphatidylcholine (PC) based lipids were detected as the progenitor (parent) ions of isomers that fragmented in producing product positive ions at m/z 184 (of phosphocholine) in tandem MS using capillary HPLC and electrospray ionization. BC eicosanoids such as prostaglandins, thromboxane, and isoprostanes require capillary gas chromatography for separation and detection that necessitates methoximation, pentafluorobenzyl (PFB) ester formation, and trimethyl silylation of hydroxyls prior to gas chromatography/ion trap tandem mass spectrometry of negative ions after chemical ionization (NICI). Tetradeuterated internal standards were utilized for quantitation with the GC/NICI/MS.

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

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0167-7012/$ - see front matter
doi:10.1016/j.mimet.2005.04.013

Changes in concentrations of lipids and eicosanoids were observed in piglets, and rats exposed to aerosolized 100 μg/kg lipopolysaccharide (LPS), or 50 μg/kg and 150 μg/kg aerosolized Staphylococcal enterotoxin B (SEB) in BC as well as in human THP-1 cell culture cell supernatants and bronchoalveolar lavage (BAL) samples in rats. Responses of the molecular species of phosphatidylcholines (PCs), platelet activating factors (PAFs) and specific eicosanoids correlated to the toxin and bacterial infections suggesting that patterns of differential responses could be detected with further experimentation. Initial targets included prostaglandins (PGE_2, $PGF_{2\alpha}$), thromboxane (TXB_2), and prostacyclin (as 6-Keto $PGF_{1\alpha}$) that show differential responses to inflammation, the leukotriene (LTB_4) and PGD_2 for allergic responses, isoprostanes (8-*iso*-$PGF_{2\alpha}$) for free radical oxidative stress responses, and HETEs for differential lipoxygenase activities. PAFs and lysoPAFs have been shown to increase with inflammation and in the feasibility experiments reported here. Preliminary studies show pulmonary responses of piglets to intrathecal exposure of toxicants (LPS and SEB) or infections with *Actinobacillus pleuropneumoniae* induce increased levels of lipids and two eicosanoids with the suggestion that differential patterns might be detected with expanded testing. Preliminary experience indicates numerous other eicosanoids were available for assay in BC. This suggests an important potential application of BC to observe a wide array of factors to establish comprehensive profiles for physiological and pathophysiological states. Ultimately this technique could be used as a non-invasive possibly presymptomatic assessment of pulmonary pathobiology.

Keywords: Breath condensate; Eicosanoids; Non-invasive assessment; Phosphatidylcholine; Platelet activating

1. Introduction

The non-invasive recovery of regulatory lipids and eicosanoids in exhaled breath condensate (BC) could provide a means of monitoring critical processes in human responses to a multitude of factors inducing stress. The aim of this study is to determine the feasibility of utilizing mass spectrometry of BC to detect a multitude of eicosanoids and regulatory lipids and attempt to establish they can signal stress and possibly even show that patterns of these regulatory lipid components possibly correlate with specific stresses. The eicosanoids 8-isoprostane $PGF_{2\alpha}$ and leukotriene B_4 have been detected in BC and shown to increase in asthma utilizing enzyme-linked-immunosorbent serologic assay (ELISA) (Antczak et al., 2002; Kostikas et al., 2003; Montuschi et al., 1999; Csoma et al., 2002). The problem with ELISA is that the specific component(s) to be assayed needs to be preselected. However mass spectrometry allows unselected components to be detected. Differential patterns of a large number of these regulatory lipids could possibly then be detected in response to different provocations. These lipid regulatory agents can differentially interact with the complex inflammatory cascade. Eicosanoids are generated by a host of factors acting on phospholipase A_2 in response to stress, which liberates arachidonic acid from the lipid Sn_2 site (Funk, 2001). Arachidonic acid is converted to eicosanoids whose stereochemical precision in formation and recognition and nanomolar ranges of activities bespeak of powerful regulatory effects. Eicosanoids are not stored but released and act locally when cells are stimulated by cytokines, collagen, ADP, bradykinin, thrombin or mechanical trauma. Transformation of arachidonic acid to eicosanoid effectors is exquisitely orchestrated in specific cells. Thromboxanes (assayed herein as TXB_2) in platelets and macrophages are potent platelet aggregators and vasoconstrictors. Prostacyclin (assayed as 6-keto $PGF_{1\alpha}$) is formed in the endothelium and acts as a powerful vasodilator and coagulation inhibitor. The PGF series of prostaglandins (assayed herein as $PGF_{2\alpha}$) are essential for parturition and may be involved in smooth muscle contraction. The PGD series (assayed as PGD_2) are formed in both mast cells and brain and are involved in atopic asthma, allergies, and leukocyte chemotaxis. The PGE prostaglandins (assayed as PGE_2) are responsible for the inflammatory response, fever generation, pain, and osteoclastic bone resorption. In contrast to prostaglandins, the leukotrienes (assayed herein as LTB_4) are formed by lipoxygenases primarily in inflammatory cells such as polymorphonuclear neutrophils (PMNs), macrophages, and mast cells. LTB_4 is a potent attractor of neutrophils and an inducer of bronchoconstriction in asthma. Gene targeting has disrupted virtually all the genes encoding enzymes and receptors in the prostaglandin and leukotriene pathways. The consequences

of these gene disruptions have established that eicosanoids are implicated in the functions of every organ, tissue, and cell. BC could be an excellent recovery medium for the non-invasive recovery of these regulatory lipids. BC is much less complex than blood, plasma, or urine and can be recovered non-invasively by condensation on a cooled surface.

Eicosanoids attract and activate white blood cells, redistribute blood, alter capillary permeability, generate fever and pain, modulate the immune response, and influence atopic allergy. The eicosanoids also offer quantitative assessment of oxidative stress (Morrow and Roberts, 2002). 8-Isoprostane $PGF_{2\alpha}$ is the major isoprostane isomer. It is increased in infection and asthma (Antczak et al., 2002; Kostikas et al., 2003; Montuschi et al., 1999, 2000). Like the eicosanoids, platelet activating (PAFs) have proinflammatory activities (Longphre et al., 1999) with atopic allergic response activation, vascular hyperpermeability induction, PMN sequestration, and increased bronchoconstriction. The PAFs can offer the significant advantage that they are found at trace levels in normal subjects but can rapidly increase in the inflammatory response.

2. Materials and methods

2.1. Materials

Authentic PAFs and lysoPAFs, and eicosanoids as well as tetradeuterated internal standards were purchased from Cayman Chemical Corp., Ann Arbor, MI. Solvents and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO. HPLC columns were purchased from ThermoHypersil-Keystone, Bellefonte, PA. Solid phase absorbent tubes and gas chromatographic columns were acquired from Phenomenex Corp., Torrance, CA. Other reagents and equipment are listed below.

2.2. Sample recovery

2.2.1. Samples from human tissue culture cell exposures

The THP-1 cell line was obtained from the American Type Culture Collections (Manassas, VA). The cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) with 0.05 mM 2-mercaptoethanol containing 10% (v/v) heat inactivated fetal calf serum (FCS, Summit Biotechnology, Ft. Collins, CO). The cells were grown in suspension (100 mL) in 75-cm^2 T-flasks at 37 °C in an atmosphere of 5% CO_2. Cells were spilt periodically to maintain the cellular density between 1×10^5 and 1×10^6/mL. One milliliter of physiological saline with 100 μg/kg LPS (Sigma St. Louis, MO), 10^6 *Staphylococcus aureus* (ATCC S13) or 10^6 *Erwinia herbicola* (ATCC 21933) was added and samples of the suspending fluid were recovered after 1.5, 3, and 6 h of exposure. The samples were then centrifuged at $2000\times g$ for 3 min and suspended in 2 mL medium with FCS. The fluid was frozen and sent to CBA.

2.2.2. BC recovered from piglets infected with Actinobacillus pleuropneumoniae

A. pleuropneumoniae serotype 6 (ATCC 33590) was obtained from American Type Culture Collection (ATCC) [Manassas, VA]. Stock cultures of *Actinobacillus* were prepared by growing them in GC medium containing 1% (v/v) Hemoglobin and 1% (v/v) Isovitalex at 37 °C in 5% CO_2. Overnight cultures of *Actinobacillus* were washed, quantified and suspended in saline for piglet infections. Piglets (21-day-old, 4 kg) were briefly anesthetized by masking with isofluorane 2–3% v/v (Abbott Labs, North Chicago, IL), intubated using a 3.5-mm ID endotracheal tube (Rusch Inc., Duluth, GA) and administered either 1 mL of physiological saline (Sham group—piglets #260, #263) or 1 mL of 1×10^8 CFU of *A. pleuropneumoniae*, serotype 6, (Infected group—piglets #261, #262, #264, #265) via a 5-french nasogastric feeding tube (Global Veterinary Products Inc., New Buffalo, MI). The piglets were revived and exhaled breath was sampled at periodic intervals. Exhaled breath was collected and condensed over dry ice using a homemade collection device. A modified nose cone type device with a one-way inlet valve for air was placed over the snout of the piglet. This nose cone was connected to a series of two 50-mL condensing tubes placed on dry ice. The collected exhaled breath condensates (EBC) were shipped to the Center for Biomarker Analysis (CBA, UTK, Knoxville, TN) for analysis on dry ice. Breath was sampled every hour for durations of 30 min starting immediately upon *Actinobacillus* infection ($T=0$), for a total of 9 sampling time points. All animal proce-

dures were performed as per protocol #PO 03-02, which was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Walter Reed Army Institute of Research (WRAIR).

2.3. Analysis of BC samples

2.3.1. Concentration of BC samples

While the frozen samples were thawing, the Phenomenex, Strata-X 33 μm polymeric sorbent columns, (60 mg/3 mL) were conditioned by a flush-through of 1 mL methanol and 1 mL of water by gravity and the columns were kept moist until BC samples were loaded. Prior to loading on the Strata-X columns, tetradeuterated internal standards were added to a disposable test tube (VWR 60818-618) to which 5 μL BHT (10 mg/mL MeOH; 2,6-Di-*tert*-butyl-4-methylphenol, Aldrich part #: D47404) was added via pipetman, and 70 μL of internal standard mix containing d4-$PGF_{2\alpha}$, d4-PGE_2, d4-PGD_2, d4-TxB_2, d4-6-Keto $PGF_{1\alpha}$, d4-lysoPAF(16), and d4-PAF(16) (Cayman Chemical Co., Ann Arbor, MI) each at 0.1 ng/μL in methanol was added and mixed with a vortex mixer. A known volume of thawed BC or BAL sample (usually 3 mL) was added to each tube containing the tetradeuterated standards via pipetman, and the samples were vortexed again. The samples with internal standards and BHT were loaded onto the moist Strata-X columns with a pipetman and drained by gravity through the column slowly.

2.3.2. On column methoximation

After loading the sample, the Strata-X columns were washed with 1 mL of water containing 20% methanol. The sample in the Strata-X column was then treated with 300 μL of MOX solution after the water wash has passed through the Strata-X column. The MOX solution consists of 100 mg methoxylamine HCl (stored in a desiccator) dissolved in 3.33 mL water prepared freshly every day. The Strata-X column tops were wrapped in aluminum foil and the tube tips capped with syringe caps. The capped tubes were heated for 1 h at 37 °C. Following the methoximation reaction, the Strata-X columns were washed with 1 mL air equilibrated water and dried with a vacuum applied to the tubes while flushing with nitrogen to remove any remaining water for an additional 15−20 min. The samples and standards were then eluted with 1 mL of 1:1 methanol (MeOH)/ethyl acetate (EtOAc) into a glass V-Vial (reactivial) by allowing the elution solvent to contact the sorbent for about 30 s and then to elute dropwise. This elution was followed by 1 mL of MeOH into the same vial. The samples were then dried under nitrogen.

2.3.3. Analysis for PAFs and lysoPAFs

PAFs and lysoPAFs are detected from 10% of the BC sample recovered just after methoximation (which destroys a small fraction of the lysoPAFs) utilizing the LC-Tandem-MS API365 (Applied Biosystems/MDS SCIEX, Concord, Ontario) in the positive ion mode for precursor ions at *m*/*z* [M+H] that gave rise to fragment ions of *m*/*z* 184 and *m*/*z* 104 in Q3, respectively. The dried samples were dissolved with 100 μL MeOH, and 10 μL was injected for PAF analysis (on the LC-MS). The remaining 90 μL was dried under nitrogen for further eicosanoid analysis. The PAF aliquots were fractionated with a solvent gradient and analyzed using an electrospray MS/MS method. The 10 μL methanol was applied to a DASH-8 20×2.1 5 μm HPLC column (Thermo Hypersil-Keystone, Bellefonte, PA). The PAFs were eluted with a gradient with sequential elution. Solvent A is formed from MeOH/H_2O 95:5 (containing piperidine 0.002%), solvent B from MeOH/H_2O 5:95 (containing piperidine 0.002%), and solvent C from acetonitrile (ACN)/MeOH (60:30), for column wash, with a flow of 250 μL/min and a run time of 20 min. The components elute in the order lysoPAF(16) that overlaps with d4-lysoPAF(16) internal standard, PAF(16), and its d4 internal standard, lysoPAF(18) and PAF(18). Each component was detected by single reaction monitoring (SRM) with 6 transitions as precursor ions at *m*/*z* 524.5 for PAF(16), *m*/*z* 552.5 for PAF(18), and *m*/*z* 528.5 for d4-PAF(16) of product ion m/z^+ 184 and *m*/*z* 482.5 for lysoPAF(16), *m*/*z* 486.5 for d4-lysoPAF(16), and *m*/*z* 510.5 for lysoPAF(18) of product ion *m*/*z* 104 for lyso forms as the signal intensity of *m*/*z* 104 was two times higher than of *m*/*z* 184 for lyso forms. ES/MS/MS parameters were set as follows: 200 ms dwell time for each mass transition; declustering potential, focusing potential, and collision energy were 54, 180, and 45 for PAFs and

30, 180, and 35 for the lyso forms, respectively. Ionspray voltage was −4500 V. Ion source temperature was 400 °C; and nitrogen was utilized as the CAD gas. Reproducibility estimated from the internal standard, 16:0-PAF-d4 (d4-PAF(16)), was 9130 (953) pg/mL or 10.4% for $n=9$.

2.3.4. Detection of PC isomers from lung cell tissue culture fluid

Tissue culture fluid was extracted with the Strata-X solid phase extractor tubes, eluted and analyzed by HPLC/ESI/MS/MS under the conditions described above. Results represent the precursor positive ion isomers yielding product ion *m*/*z* 184 which is specific for lipid with a phosphocholine head group.

2.3.5. Derivatization of eicosanoids

The eicosanoids present in the 90% of the eluent recovered after methoximation were dried under nitrogen. Then they were dissolved in 20 μL of 10% diisopropyl ethylamine (DIPE, part number D-3887) by volume in anhydrous acetonitrile (ACN) and 40 μL of 10% pentafluorobenzyl bromide (PFBBr, Aldrich part number: 10105-2) for esterification to form pentafluorobenzyl esters. These volumes were added using a pipetman. All samples were mixed by vortex briefly, and heated for 20 min at 37 °C. This is done in the hood as PFBBr is a strong lachrimator. After heating, the solvents were evaporated to dryness under nitrogen. Unreacted PFBBr and other byproducts were removed with a silica cartridge (Phenomenex, Strata Si-1, 55 μm, 70Å, 100 mg/mL, part no. 8B-S012-EAK) that was first conditioned with 1 mL methanol and then with 1 mL ethyl acetate. V-vials for collection were placed under each column prior to loading with 3 transfers of approximately 50 μL EtOAc each. The methoximated PFB esters of the eicosanoids were then eluted with 1.5 mL EtOAc/MeOH (95:5, v/v). The eluate was then dried under nitrogen. Trimethylsilyl esters of the analytes were formed by adding 50 μL BSTFA (Sigma-Aldrich part no. 3-3084), mixing, and heating for 1 h at 37 °C. The samples were dried under a nitrogen blowdown. Each sample was reconstituted in 20 μL undecane (VWR part no. IC15823180), dried over calcium hydride, (Fisher part no. AC196791000), and transferred to a GC sample vial with a 300-μL insert.

2.3.6. Analysis of derivatized eicosanoids by GC/ECNCI/ITMS

Eicosanoids were detected in the electron capture negative ion chemical ionization mode (ECNICI). The autosampler of the ThermoFinnigan PolarisQ was set to inject 2 μL onto a Restek RTX-5 30 m × 0.25 ID × 0.25 μm capillary gas chromatography column at a constant flow of 1.1 mL/min, 6.8 psi at 190 °C. The initial GC oven temperature was 190 °C for 0.5 min and was then increased at 20 °C/min to 300 °C and held for 0.75 min. The eicosanoids were assessed utilizing negative ion/chemical ionization gas chromatography/ion trap tandem mass spectrometry (GC/ECNCI/ITMS). In this system the 8-*iso*-$PGF_{2\alpha}$ elutes in 8.6 min and $PGF_{2\alpha}$ elutes in 8.9 min. They are detected at *m*/*z* 569 and 573(d-4). LTB_4 coelutes with $PGF_{2\alpha}$ but can be detected at *m*/*z* 479. PGD_2 elutes at 9.2 min, and PGE_2 elutes at 9.51 min. These two are assessed at *m*/*z* 524 and 528(d-4). TxB_2 elutes at 9.7 min. 6-Keto $PGF_{1\alpha}$ elutes at 9.9 min and is assessed at *m*/*z* 614 and 618(d-4).

2.3.7. Exploratory statistics

Data were compiled and entered in Microsoft Excel. The Hierarchical Cluster Analysis and associated graph were generated using Statistica for Windows, '99 Edition, release 5 (Statsoft, 1999). Multisource regression models (Littell et al., 1996) were fit using time as the explanatory variable, and slopes were compared between treated and control groups. For PAF variables, this approach was used to compare slopes between two replicates and were found to be similar. The slopes were pooled by fitting a simple linear regression model. Total PAF, as the sum of the four PAF measurements, was tested in a similar manner. A 5% significance level was used for all tests.

3. Results

3.1. Exposure to lung cell tissue culture

The supernatant of a strain of THP-1 human lung cell tissue culture was exposed to 100 ng/mL LPS (Fig. 1, upper panel), 10^8 *Staphylococcus aureus* (Fig. 1, middle panel), and 10^8 *E. herbicola* (Fig.

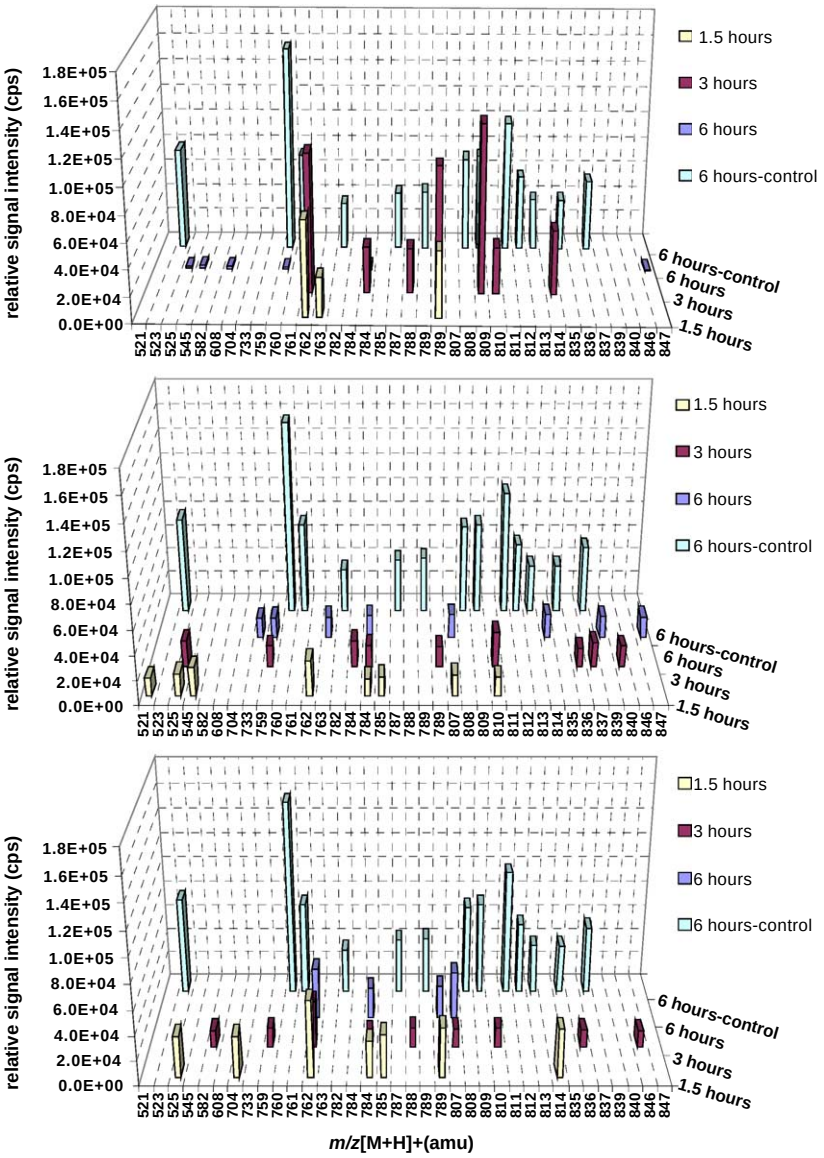

Fig. 1. Differences (from control at 6 h) of Intensities and m/z of M+H of progenitor positive ion isomers of PC (that yield m/z 184^+ on fragmentation) from human lung tissue culture cell THP-1, after exposures of 1.5, 3.0, and 6.0 h. Top panel: exposure to 50 μg/mL LPS aerosol, middle panel: exposure to 10^8 *Staphylococcus aureus*, and lower panel: exposure to 10^8 *Erwinia herbicola*.

1, lower panel) Samples were recovered from the tissue culture supernatant after exposures of 1.5, 3, and 6 h. The relative intensities of the PC molecule isomers defined as the progenitor ions of the product ion at m/z 184^+ found in the treated cultures but not found in the unexposed controls are illustrated in Fig. 1.

The intensity patterns of isomers of PC molecule precursors of phosphocholine containing lipids induced in the treated cell cultures were shown to be different from the unexposed controls for the same exposure times. They showed differences between times and between the three exposures. These differences are illustrated in the hierarchical cluster ana-

lyses (HCL) (Fig. 2). The first primary cluster contained the 6-h control intensity pattern. The second primary cluster contained all of the exposed intensity patterns. This single experiment encouraged experiments with animals and better-defined exposure experiments.

3.2. Detection of PCs in BC

The HPLC/ES/MS/MS technique allowed detection of PC molecules in BC from experimentally infected piglets. Clusters of progenitor positive ions representing isomers between *m*/*z* 516 and 859 were detected. Each PC progenitor ion formed the *m*/*z* 184, product ion fragment. Component progenitor ions in BC for the PC isomers in the *m*/*z* 516–526 range with product ions of *m*/*z* 184^+ appeared especially prominent and became the focus of further experimentation. These molecular species are in the ranges of lysoPCs, PAFs, and lysoPAFs. Preliminary experimentation which showed exposure of piglets to Staphylococcal enterotoxin (SEB) at 50 μg/kg induced increases in PC progenitor ion intensities between *m*/*z* 516 and 526 within 1 h of exposure.

3.3. Presymptomatic elevation in PAFs and lysoPAFs in A. pleuropneumoniae infection prodrome

Inter-tracheal exposure of piglets to 10^8 *A. pleuropneumoniae* induced increases in PAFs and lysoPAFs that were not detected in the control animals (Fig. 3, upper and lower panels). The combined BC of piglets #264 and #265 showed that PAF(16) and PAF(18), appeared at 4 h and peaked at 7 h at 0.52 and 0.58 ng/mL and were decreasing at 8 h which was the end of BC sampling. Almost no lysoPAFs were detected. Combined BC from sham inoculated control piglets 260 and 263 showed no PAFs or lysoPAFs (<0.01 ng/mL BC). In a repeated experiment combined BC of piglets #261 and #262 showed maximal PAF(16) at 7 and 8 h with an increasing level of lysoPAF(18) at the 8-h termination of BC collection. Symptoms of the infection (vomiting) began 10–12 h after inoculation for both sets of inoculated piglets. The control sham inoculated piglets showed no symptoms of pneumonia. Regression analysis of PAF(16) and the sum of all PAFs showed no significant differences between the replicate piglet groups ($P=0.2808$ and $P=0.4136$, respectively). There were however significant differences in the

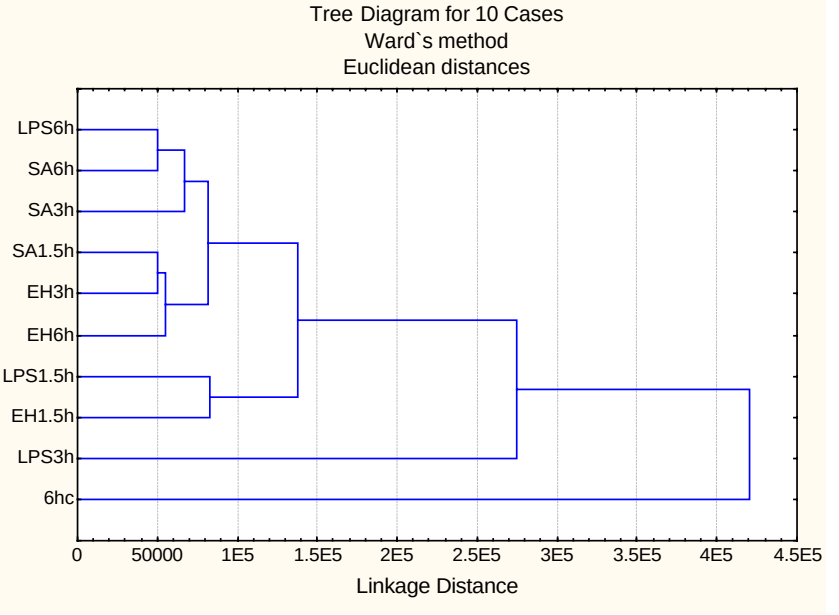

Fig. 2. Hierarchical Cluster Analysis from the data of Fig. 1 show progenitor positive ions isomers of PC formed after exposure human lung tissue culture cell THP-1, after exposures of 1.5, 3.0, and 6.0 h, (exposure 100 ng/mL LPS (LPS), exposure to 10^8 *Staphylococcus aureus* (SA), and exposure to 10^8 *Erwinia herbicola* (EH)) from the patterns found in unexposed controls at 6 h (6hc).

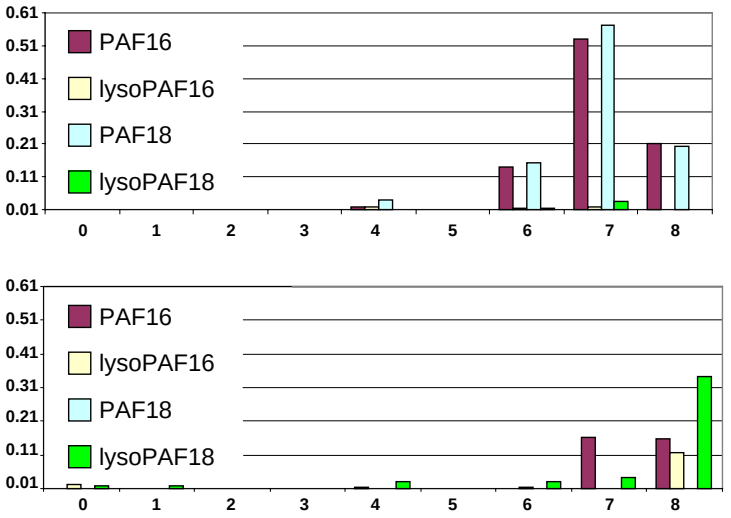

Fig. 3. PAFs and lysoPAFs in exhaled breath condensate after exposure to 10^8 *Actinobacillus pleuropneumoniae*. Upper panel: responses BC combined of piglets #264 and #265. Lower panel: responses of combined BC from piglets #261 and #262. Sham inoculated control piglets #260 and #263 were <0.01 ng/mL BC. First symptoms of pneumonia appeared in 10–12 h. No symptoms detected in the controls.

slopes from the sham inoculated control piglets and the infected piglets for PAF(16) ($P=0.0271$) and sum of all PAFs ($P=0.0289$).

3.4. Detection of eicosanoids in BC in the in A. pleuropneumoniae infection prodrome

Preliminary experiments performed with BC recovered from piglets #264 and #265 exposed intratracheally to infection with the bacteria *A. pleuropneumoniae* showed that $PGF_{2\alpha}$ steadily increased from 5 to 120 pg/mL over 8 h at each of the 6 time points during the experiment (Fig. 4, upper panel). Exploratory linear regression analysis of $PGF_{2\alpha}$ revealed a trend and difference in slope between the infected and control piglets. The slope for the infected piglets was 10.03 pg/mL/h with an intercept of 1.32 and the slope for the control piglets was 4.80 pg/mL/h with an intercept of −0.87 (slopes differ, $P=0.1633$). The slope for 8-*iso*-$PGF_{2\alpha}$ in the infected piglets was higher than in the uninfected controls in 4 of 6 assessments (Fig. 4, lower panel). The 8-*iso*-$PGF_{2\alpha}$ started at 5 pg/mL, peaked at 60 pg/mL at 4 h, and then remained at 10–20 pg/mL for 8 h in the infected piglets. Exploratory quadratic regression showed a possible difference in the response of the infected versus the sham inoculated control

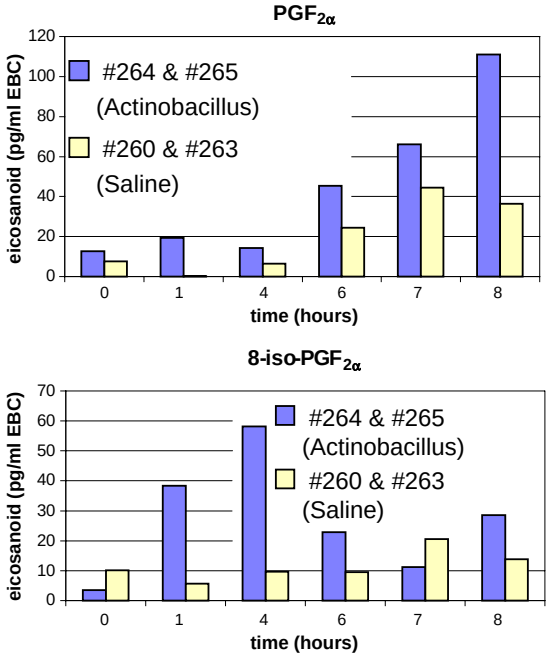

Fig. 4. Recovery of $PGF_{2\alpha}$ from combined breath condensates of piglets #264 and #265 infected with 10^8 *Actinobacillus pleuropneumoniae* compared to control in breath condensate from piglets #260 and #263 (upper panel). Lower panel: recovery of 8-*iso*-$PGF_{2\alpha}$ in the same experiment.

piglets ($P=0.15$ with slopes of −2.03 and +0.21). The infected group showed a strong downward curve whereas the control was basically a straight line.

3.5. Preliminary evidence for differential response to LPS and SEB in piglets

Exposure of piglets to aerosolized lipopolysaccharide (LPS) at 100 μg/kg induced a small peak initially in phosphatidylcholine, no detectable PGE_2 (<20 pg/mL) and the sudden appearance of a peak in $PGF_{2\alpha}$ at 8 h of 1600 pg/mL whereas exposure to aerosolized Staphylococcus enterotoxin B (SEB) at 150 μg/kg induced a steady increase in amount and number of phosphatidylcholine molecular species, a steady elevated level 330 (200.2) pg/mL PGE_2 over 8 h, and a constant low level of 20–90 pg/mL $PGF_{2\alpha}$ over the 8-h experiment (data not shown).

4. Discussion

4.1. BC phospholipid structure with HPLC/ES/MS/MS

Cole and Enke (1991) showed that the structure of intact phospholipids could be characterized in the negative ions produced by fast atom bombardment with the structures of polar head group and the localization and structure of the fatty acids. Fang and Barcelona (1998) improved the sensitivity of intact phospholipid analysis with electrospray with in-source collision induced dissociation, with proper adjustment of the cone voltage in a single quadrupole instrument. Tandem MS/MS provided more control over the fragmentation and increased the sensitivity of structural analysis of negatively charged phospholipids (Lytle et al., 2000). PC, with its quaternary nitrogen positive charge, is most sensitively analyzed as positive ions after acidification (Pulfer and Murphy, 2003). The PC molecules in the sample are identified as the m/z $[M+H]^+$ of each progenitor that yields a product m/z 184 $[M+H]^+$ of the phosphocholine polar head group. For example, the PC molecules in BC with a progenitor at m/z 783 (positive ion) suggest PC isomers with 36 carbon atoms and 4 double bonds that could be 1-palmityl-2-arachidonyl PC, 1-arachidonyl-2-palmityl PC, or a number of other isomers.

4.2. Feasibility of detecting PC based lipids; PAFs and eicosanoids in BC by mass spectrometry

This study shows that it is feasible to detect PC-based and eicosanoid lipid regulatory molecules in BC. This sample recovery system is non-invasive. The feasibility of measuring the molecular structure of these regulatory molecules with mass spectrometry for a large number of components non-selectively was also strongly indicated. Multiple components can be measured from each breath condensate sample so patterns associated with specific syndromes could be established. In contrast to immunologically based assessment methods, unexpected components would be detectable with the mass spectrometry based detection methods.

Experiments reported herein establish the feasibility of expanded programs to test whether changes in the breath condensate lipid regulatory molecule concentrations appear many hours prior to symptoms in infected or intoxicated animals exposed to bacteria or toxins. Further preliminary evidence is presented that suggests that the patterns of lipid regulatory components change with time after exposure to an agent and that exposures to different agents or toxins could possibly induce different patterns as suggested by the data in Fig. 1.

BC can be sampled non-invasively and is potentially a much cleaner matrix from which recovery of lipid regulatory biomarkers should be easier and the interpretation more straightforward than recovery from blood, plasma, or urine.

Herein we demonstrate that it is feasible to detect lipid regulatory molecules in BC with mass spectrometry so presently unknown components might be detected. The sampling of regulatory molecules from breath condensate provides feasibility that these compounds can be tested thoroughly in expanded studies. This could lead to incorporating new entities from the vast panoply of BC eicosanoid and PC lipid palette from which to explore patterns of presymptomatic detection, etiologic differentiation and harbingers of ultimate responses.

Acknowledgements

We wish to thank Dr. Joany Jackman of the Johns Hopkins University, Applied Physics Laboratory, Laurel, MD, for supplying the supernatants of the human lung tissue culture experiments and for helping in the recovery of the piglet breath samples. This research was supported by DARPA/DSO, NBCH1020006, "Rapid identification of biochemical signatures of infection with semi-volatile expired breath condensate analysis" and Institute for Science and Health, 03-1000-01RFA02 seed project: "Feasibility of Breath condensate lipids and eicosanoids as biomarker predictors of exposure and harm".

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