α-Tocopherol Transfer Protein Mediates Protective Hypercapnia in Murine Ventilator-Induced Lung Injury

Authors: Otulakowski, Engelberts, Arima, Hirate, Bayir, Post, Kavanagh

ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Animal Model: C57BL/6J male mice (20-25 g, Charles River, St. Constant, QC, Canada) were anesthetized (ketamine 150 mg/kg, xylazine 15 mg/kg, IP), and ventilated *via* tracheotomy using a computer controlled small animal ventilator (SCIREQ, Flexivent, Montreal, Canada) as previously described [1]. Baseline (low stretch, protective) ventilation was with tidal volume (V_T) 10 mL/kg, PEEP 2.0 cmH_2O, frequency 135/min, and FiO_2 0.21. Lung compliance was measured at baseline and hourly thereafter. Murine VILI models typically require high tidal volume (far higher than used clinically) and low PEEP in order to develop measurable injury within hours [2]. We used two VILI models: Model-1: Severe Injury (Peak Inspiratory Pressure 27 cmH_2O, V_T 35-40 mL/kg, PEEP 0 cmH_2O, frequency 30-35/min, for 3 h), and Model-2: Moderate Injury (V_T 20 mL/kg, PEEP 0 cmH_2O, frequency 45/min, FiO_2, for 4 h).

For microarray analysis, animals were randomized to one of four groups following baseline ventilation: to continue baseline (low stretch) or receive 'severe injury' (*i.e.*, high stretch, Model 1) ventilation in the setting of normocapnia (FiO_2 0.75, $FiCO_2$ 0, balance N_2) or hypercapnia (FiO_2 0.75, $FiCO_2$ 0.12, balance N_2). After completion of the experiment, mice were killed by exsanguination under anesthesia, bronchoalveolar lavage was performed for protein analysis, and lungs removed and snap frozen.

A second series of C57BL/6J were ventilated with our moderate injury protocol (Model 2) parameters after establishment of baseline and randomized to normocapnia (room air) or hypercapnia ($FiCO_2$ 0.12, balance room air) (n=4-8 / group) to verify induction of αTTP in another model.

Genetically Modified Mice: *Ttpa*$^{+/-}$ (strain B6.129S4-Ttpa^{tm1Far}/J) breeders were obtained from Jackson Laboratories (Sacramento, CA), and a colony was established to generate *Ttpa*$^{-/-}$ mice. Mice were genotyped using primers recommended by Jackson Laboratories. Male knockout (KO, α-TTP$^{-/-}$) and wild type sibling (WT, α-TTP$^{+/+}$) controls (20-25 g, > 5 weeks age) were subjected to our severe injury protocol (Model 1) and randomized to normocapnia or hypercapnia as described above.

Microarray Analysis: We performed an mRNA array using lung samples from mice included in our previous study [3], ventilated according to the severe injury protocol. Non-ventilated control mice (n=5) were included for microarray analysis. RNA was isolated from lung tissue using Trizol (Life Technologies, Burlington, ON, Canada) and further purified using RNeasy mini spin columns (QIAGEN, Mississauga, ON, Canada). RNA from five groups: 1) non-ventilated, 2) low stretch normocapnia, 3) low stretch hypercapnia, 4) high stretch normocapnia, and 5) high stretch hypercapnia groups (n = 5/group non-ventilated and low stretch groups; n=10/group high stretch groups) was used to prepare biotinylated cRNAs, which were hybridized to Affymetrix (Santa Clara, CA) mouse gene 1.0 ST microarrays (total 35 chips) in the Microarray Facility of the Centre for Applied Genomics at the Hospital for Sick Children. Primary data sets are accessible through NCBI's GEO Series accession number GSE86229.

Analysis of changes in gene expression in microarray data was performed using Partek Genomics Suite (Partek Incorporated, St. Louis, MI). Data were subjected to robust multichip analysis (RMA) normalization, and preliminary analysis by principal components analysis identified two outliers from within high-stretch normocapnia group (identified as #72 and #49, ***Figure 1-A***) that exhibited gene expression patterns more similar to the high-stretch hypercapnia and the low-stretch normocapnia groups; these two samples were eliminated from further analysis to maximize detection of genes specifically associated with protection by hypercapnia. To identify changes in RNA expression, 2-way ANOVA using ventilation (non-ventilated, high stretch, low stretch) and CO_2 (normocapnia, hypercapnia) as factors was used, with Benjamini and Hochberg false discovery rate 5%, and fold-change cutoff for contrasts at 1.5 fold. Overrepresented canonical pathways associated with gene expression changes induced by stretch and by hypercapnia were identified using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA).

RT-PCR: Changes in gene expression were measured using relative quantitative realtime PCR was performed in duplicate on reverse transcribed cDNAs using an AB 7900HT Detection System (Applied Biosystems, Foster City, CA) and Power SYBR Green (Applied Biosystems) reaction mix. Gene expression was calculated relative to 18S rRNA. Primers used are shown in ***Supplementary Table S1***.

Lung Tissue MPO: Lung tissue was homogenized in 5 volumes HTAB buffer (50 mM potassium phosphate pH6.0; 5 mg/mL hexadecyltrimethylammonium bromide), subjected to repeated freeze-thaw and brief sonication. Following clarification by centrifugation for 15 min at 14,000 x g, MPO activity was measured as the rate of increase in OD at 460 nm following addition of 50 µL lung extract to 1.95 mL reaction buffer (0.00167 % H_2O_2, 0.167 mg/mL *o*-dianisidine dihydrochloride).

Quantification of Bronchoalveolar Cytokines and Eicosanoids: IL-6, KC, MCP-1 in bronchoalveolar (BAL) fluid were quantitated using a Milliplex mouse Cytokine Immunoassay Kit (Millipore, Billerica, MA) and processed with Luminex® xMAP™ Technology (Luminex, Austin, TX) according to manufacturer's directions. BAL samples for eicosanoid analysis were spiked with deuterated standards before extraction of total lipid. BAL lipid mediators of the arachidonic acid pathway were quantitated by liquid chromatography-tandem mass spectrometry using a QTRAP 5500 triple-quadruple mass spectrometer (Sciex: Framingham, Massachusetts, USA) in negative electrospray ionization mode by MRM data acquisition with an Agilent 1290 HPLC (Agilent Technologies: Santa Clara, California, USA). Chromatography was performed on a Zorbax SB-phenyl column (3x50 mm) (Chromatographic Specialties, Brockville, Ontario, Canada). The HPLC flow was maintained at 600 µL/minute with a gradient consisting of: A= Water + 0.0004% Formic Acid and B = Acetonitrile + 0.0004% Formic Acid, returning to initial conditions at 10 minutes. Quantification was done on Analyst 1.6.1 software (ABSciex : Framingham, Massachusetts, USA) by plotting the sample peak area ratios (Analyte peak area/Internal Standard peak area) of individual lipid mediators against their corresponding standard curves generated from calibration mixes of lipid mediators from 0.01 ng to 10 ng. After ethanol precipitation of proteins and acidification, BAL samples for CysLT measures were purified on C18 Sep-Pak cartridges, eluted in ethyl acetate, dried under nitrogen, and resuspended in assay buffer. The cysteinyl leukotriene ELISA kit (ADI-900-070, Enzo Life Sciences, Farmingdale, NY) was used to measure LTC4, LTD4 and LTE4.

Quantification of Lung Tissue α-Tocopherol: Lung tissue α-tocopherol was quantitated from lung homogenates prepared by acetone extraction [4], spiked with internal standard δ-tocopherol (Sigma-Aldrich Canada Co., Oakville, Ontario Canada). Tocopherols were measured by HPLC using a Dionex Ultimate-3000 system and a Dionex RF 2000 fluorescence detector (Exc: 292 nm, Em: 325 nm). Chromatography was performed on a Waters 3.9x75 mm Nova-Pack C18 column, 4 µm particle size. The HPLC flow was maintained at 1.0 mL/minute, isocratically with a solvent consisting of methanol/water (94/6). Quantification was performed using Chromeleon software (version6.80 SR11d Build 3302) by plotting the sample peak area ratios (Analyte peak area/Internal Standard peak area) against α-tocopherol standard from 5 ng to 1000 ng.

Superoxide Radical Detection: KO or WT mice were subjected to the high stretch ventilation protocol as above under normo- or hypercapnia. After 2.5 hr of ventilation, hydroethidine (10 mg/kg) was injected i.p. and the experiment was terminated 30 min later. Lungs were saline perfused *via* the pulmonary artery, snap frozen and stored at -80 °C until analysis. Superoxide radical in ventilated mouse lungs was detected through quantification of 2-hydroxyethidium in ethanol extracts of lung tissue using LC-MS-MS analysis [5].

Statistics

Data are presented as dot plots with mean (for normally distributed data) or median (non-normal distributions) indicated by a horizontal bar. RT-PCR data was normalized using log transformation. Statistical differences were calculated with Sigmaplot v12.3 (Systat software Inc.) using ANOVA for multigroup comparisons. Two group comparisons used t-tests on normally distributed data; Mann Whitney was used with non-normally distributed datasets. P values <0.05 were considered significant.

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