ONLINE DATA SUPPLEMENT

Inhaled Ethyl Nitrite Prevents Hyperoxia-Impaired Postnatal Alveolar Development in Newborn Rats

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Methods

*Animal exposure*s:

Procedures were approved by the Institutional Animal Care and Use Committee. Details of animal care and oxygen exposure are as previously described in detail(E1). Nursing dams were exchanged daily, and pups placed into fresh cages during cage changes that took ~ 2 minutes per cage. Medical grade oxygen, nitrogen, and ethyl nitrite were supplied by National Specialty Gases, Durham NC. Ethyl nitrite gas was supplied in 1,000 ppm cylinders with nitrogen as the balance gas. Medical grade nitric oxide (INO Therapeutics, Clinton NJ) was supplied in 800 ppm cylinders (nitrogen balance gas). Gas flows to cages were regulated by electronic mass flow controllers (oxygen, nitrogen, ethyl nitrite: model GFC, Aalborg, Orangeburg, NY; nitric oxide: Aeronox™, PulmonNOx, Edmonton, AL, Canada). Gas lines (polytetrafluoroethylene, Cole Parmer, Vernon Hills, IL) from the flow controllers were connected to a 30 cm length of Tygon™ tubing connected to each cage, and complete mixing was verified by gas analysis described below. Total gas flow was 2.5 liters/min/cage in standard vivarium rat cages (20 x 24 x 46 cm) sealed with Lucite™ lids that were equipped with fittings to supply gases, exhaust, and gas sample lines. Gases were sampled at multiple zones within the cage and the ethyl nitrite concentration was verified to be equal to the calculated concentration at the using Fourier transform infrared spectroscopy. Similarly nitric oxide and nitrogen dioxide were monitored with electrochemical cells (Aeronox™) calibrated with gas standards (INO Therapeutics, Clinton NJ), and the measured nitric oxide concentrations were equal to the calculated concentrations. NO_2 concentrations were < 0.1 ppm for all treatment groups. Oxygen was analyzed with an electrochemical cell

(MAXO2, Ceramatec, Salt Lake City, UT). At the end of exposures, pups were euthanized with sodium pentobarbital 150 mg/kg given intraperitoneally.

SNO Measurements:

Bronchoalveolar lavages, 0.5 ml × 4 for each pup, were pooled, subjected to centrifugation for five minutes at room temperature, then snap frozen on liquid nitrogen. Samples were thawed on ice immediately before analysis by UV photolysis/chemiluminescense. All samples were done in duplicate, and were analyzed with and without reduction by $HgCl_2$. SNO signal was reported as the difference between total and reduced signal. A standard curve using duplicate S-nitrosoglutathione standards, 31-500 nM was analyzed alongside experimental samples, as previously described in detail (E2).

Tissue homogenization:

For myeloperoxidase studies, tissues were homogenized in 0.5% hexadecyl trimethylammonium bromide, 50 mM KCl as previously described in detail (E3). For cytokine and nitrotyrosine measurements, tissues were homogenized in 100 mM Tris-HCl, NaCl with protease inhibitor cocktail (CompleteOne, Roche, Indianapolis, IN), and centrifuged at 10,000 × *g* × 10 min. at 4° C. Supernatant protein concentrations were measured by the Bradford method according to the manufacturer (Protein Assay, Bio-Rad, Hercules CA).

CINC-1 expression

Quantitative real-time reverse transcriptase PCR was performed using the Applied Biosystems 7300 apparatus(Foster City, CA). Reverse transcription was performed on 0.5 μg total lung RNA extracted using TriZol (Invitrogen, Carlsbad, CA) using SuperScript III reverse transcriptase (Invitrogen) as directed by the manufacturer to generate cDNA. Real-time PCR was formed on 25% of the cDNA reaction mix in triplicate using the Applied Biosystems SYBR-Green Master Mix as directed by the manufacturer. Primer concentrations were 80 nM. Primers sequences were:

CINC-1 F	CCA AAA GAT GCT AAA GGG TGT CC
CINC-1R	CAG AAG CCA GCG TTC ACC A
L32-F	CAT CTC CTT CTC GGC ATC A
L32-R	AAA CCT GTT GTC AAT GCC TC

The crossing point absorbances for both CINC-1 and L32 cyclin RT-PCR products were compared. Values for CINC-1 were normalized to L32 cyclin values (loading control) in each sample. Mean CINC-1/L32 crossing-point ratios from 95% O_2 ± ENO 10 ppm–exposed groups were expressed a proportion of the mean CINC-1/L32 ratio in the air exposed group.

CINC-1 Immunohistochemistry

Sections were dewaxed in xylene, rehydrated in graded ethanols, treated with 0.3% H_2O_2 in methanol x 30 minutes to quench peroxidases, then blocked 1 hour in 3% goat serum in PBS. Sections were incubated overnight in 1 μg/ml rabbit anti-rat CINC-1 (Cedar Lane, Burlington, ON, Canada) in PBS+Tween-20 0.1% (PBS-T), washed in PBS-T, then

incubated with biotinylated goat anti-rabbit 1:1,000 (Vector Labs, Burlingame, CA), followed by detection with avidin:biotin complex (ABC *Elite*™ , Vector Labs) and peroxidase chromagen (NovaRed™ , Vector Labs).

Antioxidant Enzyme Expression: Activity

Lungs were homogenized with a Tek-Miser™ (Tekmar Company, Franklin Lakes, NJ) in 4 volumes of homogenization buffer consisting of Tris-HCl 0.1M, dithiothreitol 10 mM, and protease inhibitor cocktail, one table/10 ml (CompleteOne, Roche, Indianapolis IN). Lysates were centrifuged at 10,000 × *g* for 10 minutes at 4° C, and supernatant protein concentration was determined by the Bradford method (Bio-Rad, Hercules CA). Superoxide dismutase activity was determined by the ability to inhibit superoxide formation using a commercial kit according to the manufacturer's directions (WST-1, Dojindo, Gaithersburg, MD). Catalase activity was determined by the ability to inhibit hydrogen peroxide reaction with AmplexRed (Invitrogen, Carlsbad CA) according to the manufacturer's directions. Catalase from bovine blood cells was used to prepare a standard curve. Measurements were made in duplicate or triplicate and standards were in triplicate. Both SOD and catalase activities were normalized to protein content.

Antioxidant Enzyme Expression: immunoblotting

Equal protein masses were reduced in DTT 10mM, heat-denatured in SDS, and loaded onto 4-12% gradient polyacrylamide gels (EasyGel, Bio-Rad, Hercules CA), followed by eletrophoresis for 1 h. Gels were transferred overnight onto PVDF (Hybond-P, GE Healthcare, Piscataway NJ), blocked (Superblock, Pierce, Rockford IL), and detected

with rabbit anti-SOD1, SOD2 (Stressgen, Victoria, BC, Canada) 0.1 μg/ml, anti-SOD3 (gift of E. Nozik-Grayck) 1:5,000, or anti-catalase (Calbiochem, San Diego CA)1:10,000, followed by goat anti-rabbit:peroxidase (Vector) 1:20,000 and detection with enhanced chemiluminescense(SuperSignal, Pierce) exposure of film, ~10 second exposures. Blots were re-detected with rabbit anti-β-actin (Abcam, Cambridge MA) 0.01 μg/ml as a loading control. Bands were quantified by scanning using a cooled-chip camera gel documentation system (UVP, Upland CA) and signals for each lane were normalized to the corresponding β-actin control.

NF-κB Activation

Whole lung assay. Frozen lungs were pulverized under liquid nitrogen and rapidly homogenized using a Dounce homogenizer as previously described in detail(4) with the following modification. Pulverized tissues were incubated in hypotonic lysis buffer (HEPES 10mM pH 7.7, KCl 10mM, Nonidet™-P40 0.5%, EDTA 0.5 mM, MgCl 1.5 mM, protease inhibitor as directed (PI Set III, Chemicon)) × 10 min before homogenizing. Extracted nuclear proteins were quantified using the Bradford assay (Bio-Rad, Hercules CA). Nuclear extracts from lungs from three pups per treatment group were analyzed (10 μg/well), along with extracts from lipopolysaccharide (LPS, *E. coli* serotype 055, 6 mg/kg intraperitoneally) or vehicle-treated adult rats (positive and negative controls). A positive assay control (stimulated Jurkat cells, 2.5 μg/well) was provided in the kit (Trans AM, NF-κB, Active Motif, Carlsbad CA). After binding samples to NF-κB consensus binding motif oligonucleotides, samples were washed and detected with anti-p65-peroxidase conjugate as described in the instruction manual.

Immunohistochemical localization of activated NF-κB. Random paraffin sections from each neonatal treatment group (4 pups/group), and from LPS-treated rat (positive control) were dewaxed, rehydrated, subjected to antigen retrieval (Antigen Unmasking, Vector Labs) as directed by the manufacturer, then treated with 0.1% hydrogen peroxide in methanol for 30 minutes. Sections were rinsed in PBS and blocked for 2 h in PBS-T with 3% horse serum. Sections were incubated overnight in PBS-T, 3% horse serum, and anti-p65 2 μg/ml (Chemicon, MAB 3026), which recognizes the nuclear localization epitope exposed during NF-κB activation. After washing in PBS-T, sections were incubated in biotinylated horse-anti-mouse IgG 1:1,000, washed, and incubated in avidin:biotin complex as above, followed by chromogenic detection with diaminobenzidine (DAB, Vector).

Pulmonary Morphometry

After euthanasia, tracheas were cannulated and lungs inflation fixed at 25 cmH_2O with buffered formalin overnight, then embedded, and sectioned at 4-6 μm. Three random sections per animal (N=6-7/treatment group) were stained with malachite green according to the manufacturer's directions (Sigma) and counterstained with Hart's elastin as previously described(E5). Three random, non-overlapping 400× images per section were acquired from each section using a Nikon E400 microscope and digital camera (DP11, Olympus, NY) to measure alveolar volume density (proportional to alveolar

number) and alveolar surface density (proportional to surface area) as previously described in detail (E1). Digital color images were converted to grayscale and then to binary black-white images using digital thresholding to delineate alveolar septal tissue (Metamorph v6.3, Molecular Devices, Downingtown PA). Large vascular and bronchial structures were omitted. A 10 × 10 point array was overlain atop the black and white binary images, and points overlying alveolar tissue were automatically calculated using the image analysis software. Alveolar tissue volume density was calculated from $P_{alvoli}/P_{parenchyma}$. To measure alveolar surface density, a digital array of parallel lines of known length (calibrated with a stage micrometer image) were overlain on the same images. The number of line intersections (I) by alveolar tissue were determined automatically. Alveolar surface density was calculated: 2 × I alveoli/L_T, where L_T is the test line length. The color images described above were used to determine secondary septal crest/tissue ratios. The 10 × 10 test point array was overlain atop the images and the number of test points overlying elastin-positive septal tips were normalized to the number of points overlying alveolar tissue in order to assess alveolar development independent of lung inflation differences, as previously described (E5).

References

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