INCREASED HYPEROXIA-INDUCED LUNG INJURY IN NITRIC OXIDE SYNTHASE 2 NULL MICE IS MEDIATED VIA ANGIOPOIETIN 2

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ONLINE DATA SUPPLEMENT

Supplemental Methods

Histology

Young adult mice were sacrificed 60h after oxygen exposure. Tissues were fixed overnight in 10% buffered formalin. After washing in fresh PBS, fixed tissues were dehydrated, cleared, and embedded in paraffin by routine methods. Coronal sections (5 μm) were collected on Superfrost Plus positively charged microscope slides (Fisher Scientific Co., Houston, Texas, USA), deparaffinized, and stained with hematoxylin & eosin.

TUNEL staining

End labeling of exposed 3'-OH ends of DNA fragments was undertaken with the TUNEL *in situ* cell death detection kit AP (Roche Diagnostics, CA) as described by the manufacturer. We used the Nitro-Blue Tetrazolium Chloride (NBT)/(5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) (BCIP) blue stain and a nuclear red counterstain. The TUNEL Index was calculated by randomly selecting multiple high powered fields on each slide, counting 200 cells in each area and expressing the number of TUNEL +ve cells as a percentage. All cells were counted in the high power fields.

Ang2 protein (catalog#AB3121, Millipore, Billerica, MA) and surfactant protein-C (SP-C) (catalog#Ab28744-50, Abcam, Cambridge, MA) double immunostaining with TUNEL staining was performed as described previously (23), with slight modifications. In brief, deparaffinized and rehydrated sections were incubated with Cytonin (Trevigen, Gaithersburg, MD) for 60 minutes at room temperature, and after blocking 10 minutes at room temperature, incubated with anti-Ang2 or anti-SP-C antibodies diluted with cytonin at 4°C overnight. After washing with Tris buffer, incubating with AP conjugate diluted in Cytonin at room temperature for 30 minutes, and immersing slides in Quenching Solution for 5 minutes at room temperature for blocking endogenous peroxidase activity, the slides were subsequently used for TUNEL assay using TACS In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD), as instructed in the manual. Finally, the sections were reacted with EXPOSE Mouse and Rabbit specific AP (red) reagents (Abcam, Cambridge, MA) for color development.

Analysis of mRNA

Mice were anesthetized 60h after oxygen exposure, and the lungs were rapidly removed and frozen on liquid nitrogen. RNA was isolated from frozen lungs using TRIzol Reagent (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's instructions. RNA samples were then DNase treated and subjected to semiquantitative RT-PCR. The primers used for semiquantitative RT-PCR: Caspase 3, 5'-AGTCTGACTGGAAAGCCGAA-3', 5'-AAATTCTAGCTTGTGCGCGT-3', Caspase 6, 5'-TTCAGACGTTGACTGGCTTG-3', 5'-TTTCTGTTCACCAGCGTGAG-3', Caspase 8, 5'-GCTGGAAGATGACTTGAGCC-3', 5'-CGTTCCATAGACGACACCCT-3', Caspase 9, 5'-CCTGCTTAGAGGACACAGGC-

3’, 5’-TGGTCTGAGAACCTCTGGCT-3’, FAS, 5’-ATGCACACTCTGCGATGAAG-3’, 5’-TTCAGGGTCATCCTGTCTCC-3’, FAS-L, 5’-CATCACAACCACTCCCACTG-3’, 5’-GTTCTGCCAGTTCCTTCTGC-3’, Bcl-2, 5’-CTGGCATCTTCTCCTTCCAG-3’, 5’-GACGGTAGCGACGAGAGAAG-3’, Bcl-XL, 5’-TTCGGGATGGAGTAAACTGG-3’, 5’-TGGATCCAAGGCTCTAGGTG-3’, PKC-δ, 5’-TACCGGGCTACGTTTTATGC-3’, 5’-CCAGGAGGGACCAGTTGATA-3’, A1, 5’-CAGGGAAGATGGCTGAGTCT-3’, 5’-TTCTGCCGTATCCATTCTCC-3’, BIM, 5’-GCCAAGCAACCTTCTGATGT-3’, 5’-CATTTGCAAACACCCTCCTT-3’, BID 5’-TCCACAACATTGCCAGACTA-3’, 5’-CACTCAAGCTGAACGCAGAG-3’, BAX, 5’-CTGCAGAGGATGATTGCTGA-3’, 5’-GAGGAAGTCCAGTGTCCAGC-3’, BAK, 5’-CCAACATTGCATGGTGCTAC-3’, 5’-AGGAGTGTTGGGAACACAGG-3’, Ang1, 5’-AGGCTTGGTTTCTCGTCAGA-3’, 5’-TCTGCACAGTCTCGAAATGG-3’, Ang2, 5’-GAACCAGACAGCAGCACAAA-3’, 5’-AGTTGGGGAAGGTCAGTGTG-3’, Ang3/4, 5’-CCAGCTTAACAGCCTCCAAG-3’, 5’-CTCTGCACAGTCCTGGAACA-3’,
ß-actin, 5’-GTGGGCCGCTCTAGGCACCA-3’, 5’-TGGCCTTAGGGTTCAGGGGG-3’

Western Blotting.

Pro-caspase 3 (catalog#9662, Cell Signaling, Danvers, MA), cleaved caspase 3 (catalog #9661, Cell Signaling, Danvers, MA), and β-actin (Santa Cruz, Santa Cruz, CA) was detected in the lung tissues by Western Blotting.

Supplemental Figure Legends

Figure E1. Role of NOS2 on pulmonary injury in hyperoxia. *NOS2* $^{+/+}$ and *NOS2* $^{-/-}$ young adult mice were exposed to 100% O_2 for 60h. Figure **E1A** (H&E stain;10x) shows that the injury was evident in the *NOS2* $^{-/-}$ lungs in hyperoxia with septal polymorphonuclear infiltrate, slightly thickened alveoli, proteinaceous alveolar exudates and scattered areas of hemorrhage**.** The figure is illustrative of assessments in a minimum of 4 animals in each group. Figure **E2A** (H&E stain; 40x) highlights the specific aspects of lung injury. Top Panel: black arrows: septal polymorphonuclear septal infiltrate; #: slightly thickened alveoli; *: proteinaceous exudate. Bottom Panel: black arrows: scattered areas of hemorrhage. HYP: hyperoxia.

Figure E2. Role of NOS2 in hyperoxia-induced alterations in cell death regulators and angiopoietins. Figure **E2A** (10x) and **E2B** (20x) shows low-power magnifications localization of Ang2 protein with TUNEL staining, and the localization of SP-C protein with TUNEL staining in hyperoxia-exposed *NOS2* $^{+/+}$ and *NOS2* $^{-/-}$ young adult mice lung samples. The black arrows point to the localization of Type II pneumocytes. HYP: hyperoxia.

Supplemental Figures

Figure E1A.

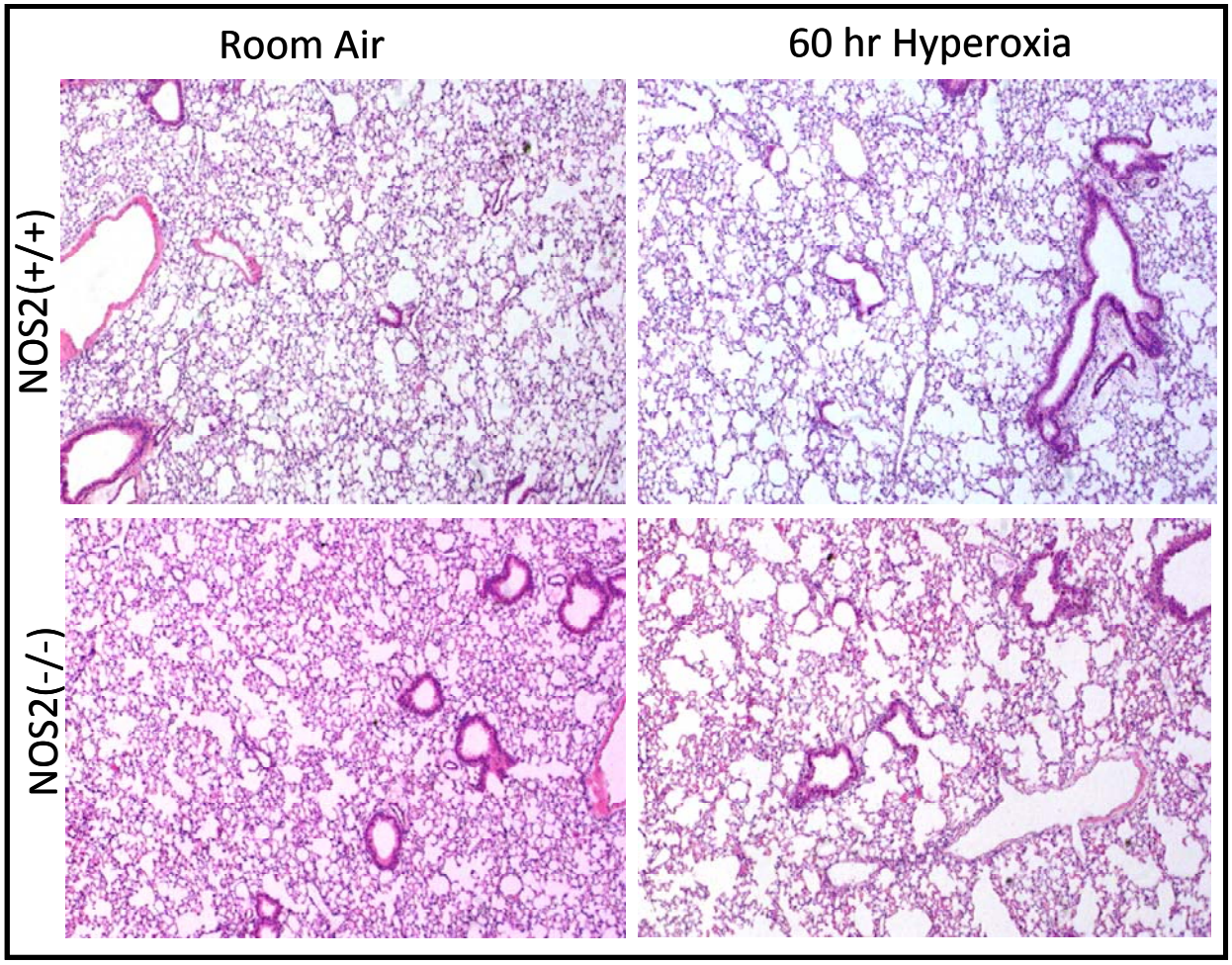

Figure E1B

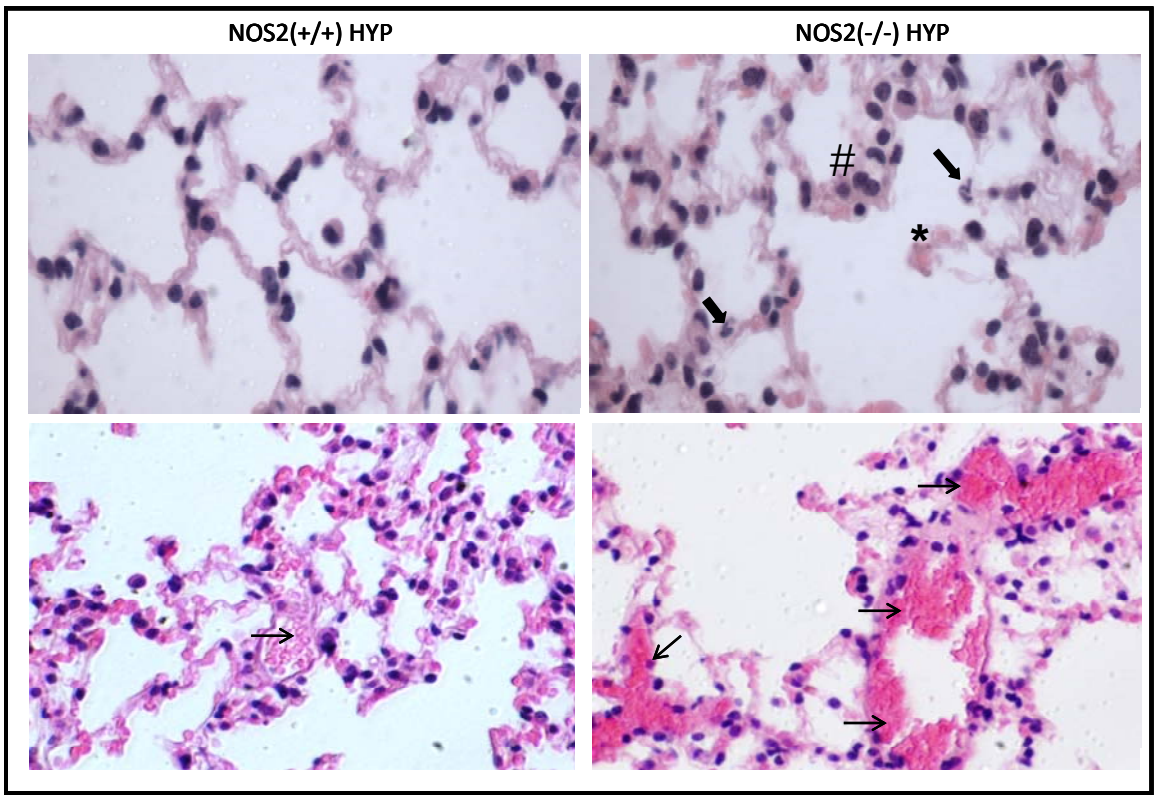

Figure E2A

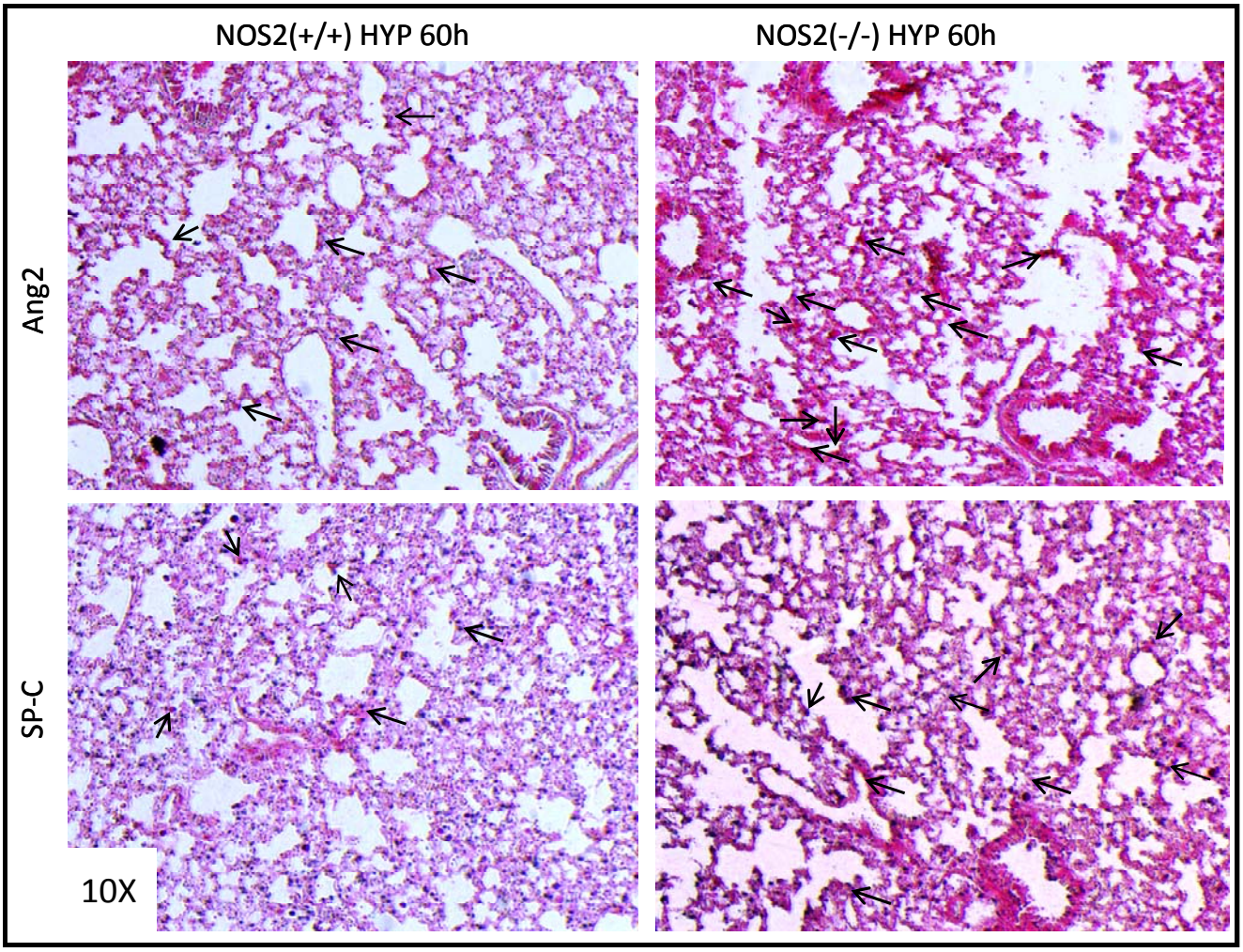

Figure E2B