Leptin promotes fibroproliferative ARDS by inhibiting peroxisome proliferator-activated receptor-γ

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(Clean version)

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ONLINE DATA SUPPLEMENT

SUPPLEMENTARY METHODS

Animals. The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. We used twelve week old, male, BKS.Cg-m +/+ Lepr^{db}/J (db/db) mice (mice with leptin resistance due to defective leptin receptor) and age and sex matched wild-type controls from Jackson laboratories (Bar Harbor, Maine). The db/db mice have a mutation on the chromosome 4 that inhibits the expression of the leptin receptor (long isoform) (1). These mice have type 2 diabetes mellitus, which is similar to type 2 diabetes mellitus in adult humans characterized by obesity, hyperglycemia, and insulin resistance/hyperinsulinemia (2). All mice had free access to food and water during experiments.

Intratracheal administration of bleomycin. Was performed as previously described (3). Mice were anesthetized with pentobarbital (50-75 mg/kg i.p.) and intubated orally with a 20-gauge angiocath (Becton-Dickenson, Sandy, UT) as previously described (3-6). Mice were then treated with intratracheal injection of 50 µl sterile PBS (control) or bleomycin (0.075 units in 50 µl sterile PBS, Bristol-Myers Squibb, New York, NY) administered in two equal aliquots, 3 minutes apart as previously described (3-6). After each aliquot the mice were placed in the right and then the left lateral decubitus position for 10-15 seconds.

Administration of PPARγ antagonist. To determine the effect of leptin on PPARγ, a group of mice treated intracheally with PBS or bleomycin were also treated with a PPARγ antagonist, GW9662 (0.3 mg/kg i.p.) (Biomol International, Plymouth Meeting, PA) or vehicle (50% DMSO) daily until BAL fluid was obtained at day 5 or lungs were harvested for histologic evaluation or determination of collagen at day 14 (7).

Lung histology. A 20-gauge angiocath was sutured into the trachea and the lungs and heart were removed en bloc at day 21 after instillation of bleomycin or PBS. The lungs were inflated to 20 cm H_2O with PBS and then fixed paraformaldehyde (4%) as

previously described (3-6). The lungs were fixed in paraffin and 5-μm sections were stained with hematoxylin/eosin and Masson's Trichrome stain (for detection of collagen fibers) for histologic evaluation. Low power field images of whole mouse lungs (50x) were obtained using Neurolucida Software (MBF Biosciences, Williston, VT) (8).

Collection of bronchoalveolar lavage (BAL) fluid and measurement of cell count, cytokines, leptin and active TGF-β1 levels. Collection of BAL fluid was performed through a 20-gauge angiocath ligated into the trachea. One milliliter of sterile PBS was instilled into the lungs and then carefully removed three times. A 200-μl aliquot of the BAL fluid was placed in a cytospin and centrifuged at 500 g for 5 minutes. The glass slides were Wright stained and subjected to a manual cell count. The remaining BAL fluid was centrifuged at 200 g for 5 minutes and the supernatant was used for the measurement of cytokines/chemokines, TGF-β1 and leptin levels in freshly isolated samples. Levels of TGF-β1 (Promega, Madison, WI) and leptin (R&D Systems, Minneapolis, MN) were measured using commercially available ELISA kits according to the instructions provided (3, 6). We used BD Cytometric Bead Array (BD Biosciences, San Diego, CA) to measure systemic and BAL levels of cytokines/chemokines. Samples were analyzed in triplicate using the Mouse Inflammation Kit (BD Biosciences), which detects IL-6, IL-10, monocyte chemoattractant protein 1 (MCP-1) and TNF-α according to the instructions provided.

Lung homogenates and immunoblotting. Whole mouse lungs were homogenized as previously described (3-6). Equal amounts of lung proteins derived from control and treated mice were resolved by electrophoresis in 4-20% Tris-Glycine gradient gels (BIORAD, Hercules, CA), transferred to PVDF membranes and subjected to immunoblot analysis as described (9). Membranes were probed with antibodies to Type I collagen (1

μg/ml) (Southern Biotechnology, Birmingham, AL), PPARγ (1 μg/ml), actin (0.5 μg/ml) (Santa Cruz Biotech, Santa Cruz, CA).

Quantitative real-time reverse transcription PCR (qRT-PCR). Connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), collagen 1 and 3 and TGF-β1 mRNA expression was determined in NHLF by qRT-PCR using SYBR green chemistry in response to saline (negative control), recombinant TGF-β1 (5ng/ml) (positive control) with or without different concentrations of human recombinant leptin (R&D Systems, Minneapolis, MN). The following primer sequences were used: for CTGF, GGCTTACCGACTGGAAGAC and AGGAGGCGTTGTCATTGG; for α-SMA, GGCGGTGCTGTCTCTCTAT and CCAGATCCAGACGCATGATG; for collagen 1, GCAGAGATGGTGAAGATGGT and GCCTCTAGGTCCCATTAAGC, for collagen 3, ATGATGAGCTTT GTGCAAAA and TCCTGTTGTGCCAGAATAAT; for PPARγ, TTCAAGACAACCTGCTACAAG and GTGTTCCGTGACAATCTG; for TGF-β1, GCAACAATTCCTGGCGATACC and CTCCAGGGCTCAACCACTG; for plasminogen activator inhibitor-1 (PAI-1), TGCTGGTGAATGCCCTCTACT and CGGTCATTCCCAGG TTCTCTA and for fatty acid binding protein 4 (FABP4), TCAAGAGCACCATAACCTTAG and GTGGAAGTGACGCCTTTC. Total RNA was isolated after 24 hours of incubation using the Aurum Total RNA Mini Kit (Bio-Rad, Life Science, Hercules, CA). The cDNA was synthesized from 1μg of total RNA using the RNAqueous 4-PCR kit (Applied Biosystem/Ambion, Austin, TX) with random decamer primers. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein RPL19 (10-11).

Quantitative assessment of lung collagen content. Lung collagen was measured using a modification of a previously described method for the precipitation of lung collagen using picrosirius red (12). Mouse lungs were harvested and suspended in 0.5 N acetic acid and then homogenized first with a tissue homogenizer (30 seconds on ice)

and then using 12 strokes in a Dounce homogenizer (on ice). The resulting homogenate was spun (10,000 x g) for 10 minutes and the supernatant was used for subsequent analysis. Collagen standards were prepared in 0.5 N acetic acid using rat tail collagen (Sigma-Aldrich). Picrosirius red dye was prepared by mixing 0.2 g of Sirius Red F3B (Sigma-Aldrich) with 200 ml of saturated picric acid in water (solid picric acid maintained at the bottom of the flask to insure saturation). 1 ml of the picrosirius red dye was added to 50μL of the collagen standard or the lung homogenates and then mixed continuously at room temperature on an orbital shaker for 30 minutes. The precipitated collagen was then pelleted and washed once with 0.5 N acetic acid (10,000 x g for 10 minutes each). The resulting pellet was resuspended in 500 μL of 0.5 M NaOH and Sirius red staining was quantified spectrophotometrically (540 nm) using a colorimetric plate reader (BioRad).

Human Study Population. Subjects were recruited from the medical intensive care unit at Northwestern Memorial Hospital between 2004 and 2006. The protocol was approved by the Institutional Review Board of Northwestern University. Patients with respiratory failure, bilateral infiltrates, absence of left atrial hypertension or (when available) a pulmonary artery wedge pressure or a central venous pressure less than 18 mm Hg and a PaO_2/F_IO_2 ratio less than 300 were eligible for the study in the first 48 hours after intubation. Healthy subjects with normal lung parenchyma who were intubated for other reasons included as control. Control BALs were obtained from healthy subjects with normal lung parenchyma who underwent elective outpatient bronchoscopy. None of the healthy controls had known history of DM. Patients with ALI/ARDS were followed for 28 days, until hospital discharge or death. Informed consent was obtained from subjects or surrogates.

Collection of BAL fluid. Each mechanically ventilated patient had a fiberoptic bronchoscope or a BAL catheter wedged into position of a distal bronchus and sterile

saline was instilled in 60 cc aliquots and then aspirated and collected within 48 hours of intubation. This was repeated up to 3 times. There was no significant difference in the volume of lavage saline instilled between the two cohorts. The fluid was centrifuged at 1500 rpm within 30 minutes of collection for 10 minutes, aliquotted and frozen at -80°C (11).

Statistical analysis. Statistical analysis. Data are expressed as mean ± SEM unless otherwise specified. Differences between groups were analyzed using one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference, we explored individual differences with the Student's *t* test using Bonferroni correction for multiple comparisons. Direct comparisons between two treatment groups were performed with the unpaired Student's *t* test or the nonparametric Mann-Whitney test when the data sets were not normally distributed. (Prism 4, Graphpad Software, Inc., San Diego, CA). As human BAL fluid leptin and TGF-β1 levels were not normally distributed, we used Spearman's coefficient for the correlation analysis (SPSS for Windows 11.5 (SPSS Inc., Chicago IL). Statistical significance in all experiments was defined as $p<0.05$.

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SUPPLEMENTARY FIGURE LEGENDS

Figure E1. **Mice with leptin resistance are protected against bleomycin-induced pulmonary fibrosis.** Shown are whole tissue (5x) and high power field images (x200) of lungs from mice (wild-type and db/db) 21 days after intratracheal instillation of bleomycin or PBS, stained with **(A)** hematoxylin/eosin (H&E) and **(B)** Masson's Trichrome. Low power images of whole lungs were captured using MBF Neurolucida software (MBF Biosciences, Williston, VT).

Figure E2. Leptin induced augmentation of the transcriptional activity of TGF-β_1 is mediated via TGF-β receptor type I (ALK5). Normal human lung fibroblasts were treated with TGF-β1 and/or leptin (100 ng/ml) with the addition of ALK5 specific inhibitor, SB431542 or vehicle in vitro and 24 hours later using qRT-PCR, we measured the effect of SB431542 on leptin and/or TGF-β1 mediated induction of profibrotic genes including **(A)** α-smooth muscle actin (α-SMA), **(B)** collagen I, **(C)** collagen III and **(D)** connective tissue growth factor (CTGF). ($p<0.05$ *TGF- β1+leptin vs. TGF- β1 treatment, **SB431542 vs. vehicle, $n\geq4$ in each treatment group)**.**