ONLINE DATA SUPPLEMENT

Leptin resistance protects mice from hyperoxia-induced acute lung injury

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Histology and calculation of lung injury scores. After tracheostomy and insertion of a 20-gauge angiocath was placed, the mice were bled by laceration of left renal artery and vein. The lungs and heart were removed en bloc and the lungs were inflated to total lung capacity with 1 ml of 4% paraformaldehyde (PFA). The lungs were then placed in 4% PFA at 4ºC for 24 hours. After the lungs were placed into PBS, they were paraffin embedded and 5μm sections were stained with hematoxylin and eosin for histologic evaluation.

The lung injury scores were calculated using the method described by Matute-Bello and colleagues (1). Blinded lung sections were scored using a 0-3 scale by an investigator for the presence of interstitial and alveolar inflammation, alveolar hemorrhage and edema where normal was scored as 0, 1 as mild, 2 as moderate and 3 as severe. The resulting three scores were averaged and presented as the lung injury score for that section. Two sections per animal were scored independently.

Total cell membrane isolation and western analysis. Membrane proteins were obtained by homogenizing lung tissue collected from the peripheral 1-2 mm of each lobe as previously described (2). For western analysis 10 μg of whole cell membrane protein was separated by 10% SDS-PAGE, electrophoretically transferred to nitrocellulose and probed with a rabbit anti-mouse leptin receptor antibody (1:2000 dilution, Abcam, Cambridge, MA).

***Immunohistochemistry*.** Paraffin-embedded, longitudinal sections (5μm) of mouse lungs were passed through a xylene and ethanol series and rehydrated into PBS. Antigen retrieval was then performed by heating (using microwave) of sections in 100mmol/L Tris (pH 10) and incubating for 5-minutes twice and then allowing the solution to slowly cool to room temperature. Sections were then rinsed through PBS and were treated with 3% H_2O_2 prior to blocking of non-specific immunoreactivity with non-immune blocking serum. A rabbit anti-mouse leptin receptor antibody (1:400 dilution, Abcam, Cambridge, MA) and Vectastain kit (Vector Laboratories, New Castle-upon-Tyne, UK) were used for immunodetection. This antibody is raised against the receptor itself and therefore is highly specific. It recognizes both the short and the long forms of leptin receptor.

Alveolar fluid clearance measurements in live mice (2, 3). The method of measurement was described before (2, 3). Briefly, mice were anesthetized with diazepam (5mg/kg, i.p. Abbott Laboratories, Chicago, IL) followed 10 minutes later by pentobarbital (50mg/kg, i.p. Abbott Laboratories, Chicago, IL). Body temperature was maintained at 37°C using a heating pad. After sedation and analgesia were achieved (assessed by lack of toe reflex), the trachea was exposed and cannulated with a 5 mm, 20-gauge plastic intravenous catheter (Angiocath, Becton-Dickenson, Sandy, UT). The catheter was connected to an animal ventilator (Harvard Apparatus, Holliston, MA) prior to paralysis with pancuronium bromide (2.0 mg/kg, i.p. Gensia Pharmaceuticals, Irvine, CA) and

ventilated with a tidal volume of 10 ml/kg at a frequency of 160 breaths per minute and 100% oxygen. Heart rate and rhythm were monitored continuously.

Three-hundred microliters of an iso-osmolar NaCl solution containing 5% acid-free bovine serum albumin (BSA, Sigma, St. Louis, MO) was instilled into the endotracheal catheter over 10 seconds followed by 200 μl of air to position the fluid in alveolar space. The animals were kept supine and inclined to 30-degrees during mechanical ventilation. Thirty-minutes after instillation the chest was opened to produce bilateral pneumothoraces and allow aspiration of fluid from the tracheal catheter. Protein concentration in the aspirate was measured using a modified Bradford assay (Bio-Rad laboratories, Hercules, CA). Alveolar fluid clearance was calculated using the following equation: AFC= $(1-C_0/C_{30})$. Where C_0, is the protein concentration of the instillate before instillation and C_{30}, is the protein concentration of the sample obtained at the end of 30 minutes of mechanical ventilation. Clearance is expressed as a percentage of total instilled volume cleared/30 minutes.

Na,K-ATPase function (P_i liberation from ATP) in the distal lung (2, 4)**.** Na,K-ATPase activity was quantified by comparing the amount of inorganic phosphate (P_i) liberated from ATP over 1 hour by 20 μg of basolateral cell membrane protein isolated from the peripheral lung in the presence and absence of the Na,K-ATPase inhibitor ouabain as previously described (2). Conditions used maximize Na,K-ATPase activity (Vmax) to produce an index of functional,

membrane-bound receptor number. Results represent triplicate measurements from three mice/group and are expressed as % of Na,K-ATPase activity in untreated wild-type mice.

References

E1. Matute-Bello G, Frevert CW, Kajikawa O, Skerrett SJ, Goodman RB, Park DR, and Martin TR. Septic shock and acute lung injury in rabbits with peritonitis: failure of the neutrophil response to localized infection. *Am J Respir Crit Care* Med 2001;163:234-243.

E2. Mutlu GM, Dumasius V, Burhop J, et al. Upregulation of alveolar epithelial active Na+ transport is dependent on beta2-adrenergic receptor signaling. *Circ Res* 2004;94:1091-1100.

E3. Mutlu GM, Adir Y, Jameel M, et al. Interdependency of beta-adrenergic receptors and CFTR in regulation of alveolar active Na+ transport. *Circ Res* 2005;96:999-1005.

E4. Dumasius V, Sznajder JI, Azzam ZS, Boja J, Mutlu GM, Maron MB, and Factor P. beta(2)-adrenergic receptor overexpression increases alveolar fluid clearance and responsiveness to endogenous catecholamines in rats. *Circ Res* 2001;89:907-914.

Figure Legends

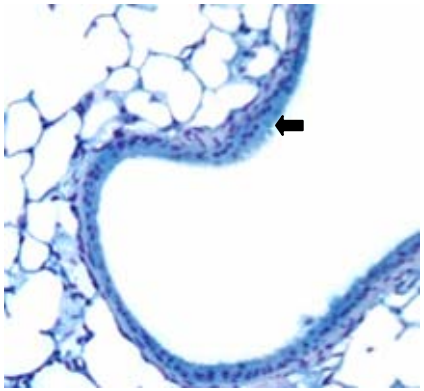
Figure E1. Leptin receptors in the lung. Immunostaining of whole lungs from wild-type mice using an anti-mouse leptin receptor antibody showing **(A)** the presence of leptin receptors (black arrows) in mouse bronchial epithelium (Magnification at 200x) and **(B)** circumferential pattern of immunostaining consistent with presence of the receptors in both alveolar type 1 and type 2 epithelial cells (Magnification at 1000x) Photomicrograph is 5 μm paraffin embedded section.

Figure E2. Effect of leptin receptor function on lung histology after 120 hours of exposure to hyperoxia. Photomicrograph of hematoxylin and eosin stained lungs from db/db mice exposed to hyperoxia for 120 hours. (Magnification at 200x)

Figure E3. Changes in weight during hyperoxia. Weights of wild-type and db/db mice at baseline and after exposure to hyperoxia for 84 hours.

Figure E1

A



B

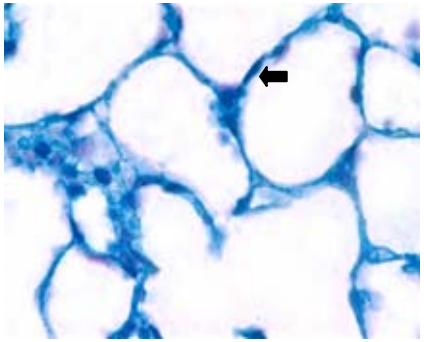


Figure E2

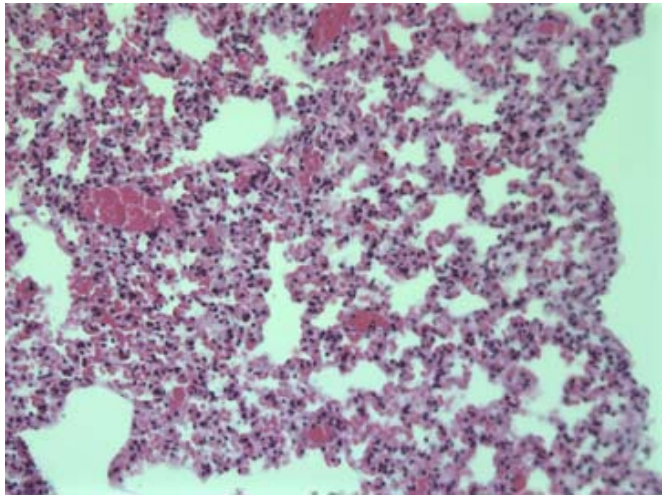
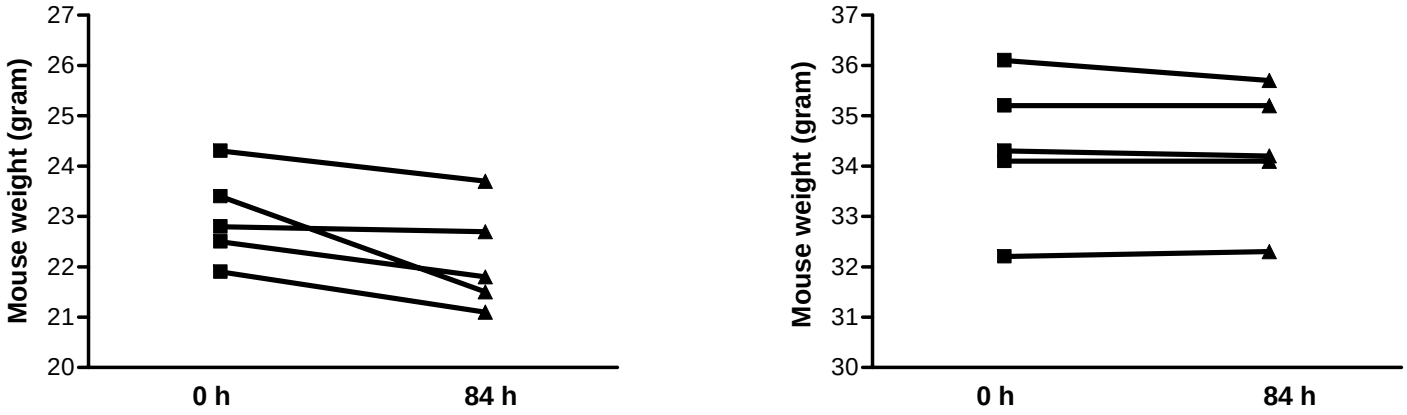


Figure E3


Mouse weight (gram)
27
26
25
24
23
22
21
20
0 h
84 h
Mouse weight (gram)
37
36
35
34
33
32
31
30
0 h
84 h