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

(Blue-201009-1409C_R1)

(Marked up version)

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Support: ES015024 (GMM), American Lung Association (GMM), ES013995 (GRSB) HL071643 (GMM, GRSB, and NSC), Northwestern University Clinical and Translational Sciences Institute (NUCATS) CTI Pilot Award (UL1 RR025741 (NCCR), (GMM) and Northwestern Memorial Foundation Dixon Young Investigator Grant (GMM) and Scleroderma Research Foundation Grant (JV).

Running title: Leptin promotes lung fibrosis

Descriptor number: 4.1 ALI/ARDS: Biological Mechanisms

Word count (for text): 3561

At a Glance Commentary:

Scientific Knowledge on the Subject. Patients with diabetes mellitus have an approximately 50% lower incidence of the ARDS and those that develop ARDS are less likely to die. However, the mechanisms that underlie this protection are unknown. The development of TGF-β1-mediated fibroproliferation after lung injury is associated with poor clinical outcomes in patients with ARDS.

What This Study Adds to the Field. We found that leptin augments TGF-β1 signaling in lung fibroblasts and consequently promotes fibroproliferative ARDS by inhibiting PPARγ. These findings provide a mechanism for the observed protection against ARDS observed in patients with diabetes.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: Diabetic patients have a lower incidence of ARDS and those that develop ARDS are less likely to die. The mechanisms that underlie this protection are unknown.

Objectives: To determine whether leptin resistance, a feature of diabetes, prevents fibroproliferation after lung injury.

Methods: We examined lung injury and fibroproliferation after the intratracheal instillation of bleomycin in wild-type and leptin resistant (db/db) diabetic mice. We examined the effect of leptin on TGF-β1-mediated transcription in primary normal human lung fibroblasts. Bronchoalveolar lavage fluid (BAL) samples from patients with ARDS and ventilated controls were obtained for measurement of leptin and active TGF-β1 levels.

Results: Diabetic mice (db/db) were resistant to lung fibrosis. The db/db mice had higher levels of PPARγ, an inhibitor of the transcriptional response to TGF-β1, a cytokine critical in the pathogenesis of fibroproliferative ARDS. In normal human lung fibroblasts, leptin augmented the transcription of profibrotic genes in response to TGF-β1 through a mechanism that required PPARγ. In patients with ARDS, BAL leptin levels were elevated and correlated with TGF-β1 levels. Overall, there was no significant relationship between BAL leptin levels and clinical outcomes; however in non-obese patients, higher BAL leptin levels were associated with fewer intensive care unit- and ventilator-free days and a higher mortality.

Conclusions: Leptin signaling is required for bleomycin induced lung fibrosis. Leptin augments TGF-β1 signaling in lung fibroblasts by inhibiting PPARγ. These findings provide a mechanism for the observed protection against ARDS observed in diabetic patients.

Abstract word count: 240. **Keywords:** ARDS, ALI, adipokine, fibrosis, lung

INTRODUCTION

Acute lung injury (ALI) and the Acute Respiratory Distress Syndrome (ARDS) are common clinical syndromes affecting almost 200,000 people per year in the United States (1). Although progress has been made in the supportive care of these patients, the mortality remains unacceptably high (~40%). Patients with diabetes mellitus (DM) are ~50% (up to 62%) less likely to develop ARDS and those with DM who develop ARDS have lower mortality rates than non-diabetic patients (2-5). The hypothesis that this protection is conferred by the immunosuppressive effects of hyperglycemia has been challenged by the results of recent studies suggesting that hyperglycemia exacerbates inflammation and worsens ALI (2-3, 6).

Leptin is a 16-kDa non-glycosylated protein encoded by the obese gene located on human chromosome 7 and on mouse chromosome 6 (7). While classically considered a hormone because it regulates the balance between food intake and energy expenditure, leptin is also a member of the type I cytokine family. Type II diabetes is associated with hyperleptinemia and an acquired resistance to signaling through the leptin receptor (8-9). Serum levels of leptin correlate with body mass index (BMI) and are increased in patients with sepsis, the most common cause of ARDS, suggesting that leptin may play a role in the pathogenesis of ARDS (10-12).

In some patients with ALI/ARDS, the activation of Transforming Growth Factor-β1 (TGF-β1) contributes to an exuberant and persistent fibroproliferative response characterized by collagen deposition and a prolonged impairment in gas exchange (13). We and others have reported that patients with ARDS who develop an early fibroproliferative response in the lung are at increased risk for poor clinical outcomes (14-17). Recently, investigators have reported that leptin plays an important role in the development of

cirrhosis and renal fibrosis, although the mechanisms that underlie this protection are poorly understood (18-22). We sought to determine whether leptin plays a role in the pathogenesis of fibroproliferative stage of ARDS and whether this effect is mediated via TGF-β1 signaling. Some of the results of these studies have been previously reported in the form of an abstract (23).

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). Leptin receptor deficient (db/db) mice are obese, hyperleptinemic, hyperglycemic, hyperinsulinemic and exhibit insulin resistance. As all of these features are observed in patients suffering from Type II DM, these mice are used as a model for this disease (24).

Lung histology, collection of bronchoalveolar lavage (BAL) fluid and measurement of leptin and active TGF-β1 levels. These assays were performed as previously described using commercially available assays (25). Details are included in the Online Data Supplement.

Lung homogenates and immunoblotting. These assays were performed as previously described (26). Details are included in the Online Data Supplement. 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 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 (27). Details are included in the Online Data Supplement.

Human lung fibroblasts. Normal human lung fibroblasts (NHLF) (Cambrex, Charles City, IA) were grown in FGM-2 Fibroblast Growth Medium-2 (Lonza, Inc, Allendale, NJ) supplemented with SingleQuots (Cambrex, Charles City, IA) in a humidified incubator (5% CO_2) at 37°C. Quantitative RNA experiments were performed on cells at passage <

5 and with >70% confluence. The cells were incubated in serum-free media for 24 hours prior to treatment with TGF-β1 and/or leptin.

Quantitative real-time reverse transcription PCR (qRT-PCR). These assays were performed as previously described (28). All values were normalized to mitochondrial ribosomal protein RPL19. Specific primer sequences, RNA protocols and normalization procedures are described in the Online Data Supplement.

Human Study Population. Subjects were recruited from the medical intensive care unit at Northwestern Memorial Hospital. The protocol was approved by the Institutional Review Board of Northwestern University and has been previously described (28). Details are provided in the Online Data Supplement.

Collection of BAL fluid. BAL fluid was collected within 48 hours of intubation and stored as previously described (28). Details are provided in the Online Data Supplement.

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 $p<0.05$.

RESULTS

Leptin resistant mice are protected from bleomycin-induced pulmonary fibrosis. The intratracheal instillation of bleomycin in mice results in lung injury, which peaks 3 to 5 days later and is followed by fibroblast proliferation, collagen deposition and pulmonary fibrosis, which is evident at 21 days (25, 29). To address the role of leptin in the regulation of fibroproliferative ARDS, we treated db/db and wild-type control mice with intratracheal bleomycin (0.075 units/mouse) in sterile saline or saline (control). We found that BAL fluid levels of leptin were increased 6-fold in bleomycin compared with PBS-treated wild-type mice (Figure 1A). Twenty-one days after the instillation of bleomycin, we observed severe fibrosis in wild-type mice as assessed by Masson-Trichrome staining (Figure 1B and Figure E1). By contrast, db/db mice did not exhibit fibrosis. Total lung collagen, as evaluated by immunoblotting whole lung homogenates using an antibody that recognizes collagen I (Figure 1C) and picrosirius red collagen precipitation (Figure 1D) was significantly higher in wild-type mice than db/db mice.

The protection against bleomycin-induced pulmonary fibrosis in mice is independent of bleomycin induced lung injury. We evaluated the severity of bleomycin-induced lung injury in wild-type and db/db mice 5 days after the intratracheal administration of bleomycin. Examination of hematoxylin and eosin stained lung sections from wild-type and db/db mice did not reveal differences in lung injury severity (Figure 2A). The bleomycin-induced increase in the numbers of inflammatory cells (Figure 2B) and levels of pro-inflammatory cytokines (Figure 2C) in the BAL fluid were similar in wild-type and db/db mice.

Leptin signaling affects bleomycin-induced TGF-β1 activation downstream of upregulation of the integrin αvβ6. The activation of TGF-β1 requires increased

epithelial expression of the integrin αvβ6, which interacts with the latent TGF-β1 complex in the lung interstitium to release active TGF-β1 (30). We observed a similar induction in the levels of β6 mRNA in lung homogenates of wild-type and db/db mice 5 days after treatment with saline or bleomycin by quantitative real-time reverse transcription PCR (qRT-PCR) (Figure 3A). We then measured active TGF-β1 levels in freshly isolated BAL fluid samples from wild-type and db/db mice 5 days after the intratracheal instillation of bleomycin or PBS. The intratracheal administration of bleomycin led to a 5-fold rise in the BAL fluid level of active TGF-β1 in wild-type mice, whereas the bleomycin-induced increase in active TGF-β1 in db/db mice was significantly attenuated (Figure 3B).

Increased expression of the integrin αvβ6 spatially restricts the activation of TGF-β1 to regions of epithelial injury. However, active TGF-β1 induces its own transcription and release from resident lung fibroblasts in an autocrine loop that can amplify the fibrotic response (31-32). To test the hypothesis that signaling through the leptin receptor might be involved in this amplification, we treated normal human lung fibroblasts (NHLFs) with TGF-β1 and measured TGF-β1 transcription in the presence or absence of leptin. Leptin alone did not alter TGF-β1 mRNA but it augmented the induction of TGF-β1 in response to active TGF-β1 (Figure 3C).

Leptin augments the TGF-β1-induced transcription of profibrotic genes in normal human lung fibroblasts. The finding that leptin augmented the autocrine release of TGF-β1 in NHLF suggested that leptin may positively regulate the TGF-β1-induced transcription of other pro-fibrotic genes. To test this hypothesis, we treated NHLFs with vehicle or TGF-β1 in the absence or presence of different concentrations of leptin *in vitro* and 24 hours later measured the induction of profibrotic genes including α-smooth

muscle actin (αSMA), collagen I and collagen III using qRT-PCR. While leptin treatment by itself did not induce the transcription of profibrotic genes, the addition of leptin to TGF-β1 augmented their TGF-β1-mediated transcription (Figure 3D-F). The augmentation mediated by leptin at the highest dose was on average 2-fold higher than that induced by TGF-β1 alone. Treatment with SB431542 (10 μM), an inhibitor of the ubiquitously expressed TGF-β1-receptor ALK5 (33), completely inhibited the stimulatory effects of TGF-β1 on profibrotic genes in the presence and absence of leptin (Figure E2).

Leptin decreases the expression and activity of the TGF-β1 suppressor PPARγ in normal human lung fibroblasts. Activation of PPARγ has been shown to provide protection against organ fibrosis in the lung, kidney and skin by acting as a co-repressor of Smad-dependent gene transcription (34-35). We found lower levels of PPARγ mRNA and protein in NHLFs treated with leptin (100 ng/ml) compared with vehicle 24 hours after their administration (Figure 4A and B) and treatment with the PPARγ agonist rosiglitazone suppressed the TGF-β1 induced transcription of αSMA (Figure 4C). To verify the importance of leptin signaling on PPARγ expression, we immunoblotted total lung homogenates from wild-type and db/db mice using an antibody that recognizes PPARγ. The protein abundance of PPARγ was increased almost 2-fold in db/db mice compared to wild-type mice (Figure 4D).

To determine how leptin affects PPARγ response, we treated NHLFs with rosiglitazone, a PPARγ agonist or control vehicle and treated with PBS (control), GW9662, a selective inhibitor of PPARγ or leptin (100 ng/ml) and performed mRNA levels of fatty acid-binding protein 4 (FABP4), a transcriptional target gene of PPARγ (qRT-PCR) (36).

Rosiglitazone-induced increase in FABP4 mRNA was suppressed by both GW9662 and leptin (Figure 4E).

PPARγ is required and sufficient for the leptin-mediated augmentation of TGF-β1 transcription in normal human lung fibroblasts. To determine whether PPARγ is required for the leptin-induced augmentation of TGF-β1 transcriptional activity, we used a lentiviral shRNA to generate NHLFs harboring a stable knockdown of PPARγ. In these cells, the levels of PPARγ were about 50% of those observed in control transfected cells (Figure 5A). We treated control and PPARγ knockdown NHLFs with TGF-β1 in the absence or presence of leptin (100 ng/ml) and 24 hours later measured mRNA levels of CTGF, a transcriptional target of TGF-β1 (qRT-PCR). Compared with control transfected cells, NHLFs in which PPARγ expression was knocked down showed an enhanced increase in CTGF mRNA in response to TGF-β1. The leptin-induced augmentation of TGF-β1 transcriptional activity in control transfected cells was prevented in the PPARγ knockdown cells (Figure 5A). To determine whether PPARγ was sufficient to prevent the leptin mediated augmentation of TGF-β1, we treated wild-type NHLFs with rosiglitazone in the presence or absence of leptin and measured the expression of plasminogen activator inhibitor-1, another transcriptional target of TGF-β1. Treatment with rosiglitazone completely prevented the leptin-induced augmentation of PAI-1 transcription (Figure 5B).

Increased levels of BAL fluid leptin and TGF-β1 levels are associated with adverse outcomes in patients with ARDS. To evaluate the role of leptin signaling in human ARDS, we measured leptin and TGF-β1levels in BAL fluid obtained from ALI/ARDS patients or ventilated control patients without lung disease within 72 hours of intubation.

Table 1 summarizes the demographics and physiology of these patients. The BAL fluid levels of leptin were 6-fold higher in non-obese patients with ALI/ARDS compared to the control patients (Figure 6A). There was a significant correlation between BAL fluid levels of leptin and TGF-β1 ($r=0.522$, $p<0.001$) when all patients with ALI/ARDS were included in the analysis. This association was stronger in the cohort of ARDS patients with normal BMI ($r=0.637$, $p<0.0001$). None of the control patients had leptin levels greater than 100 pg/ml, which was used as the cut-off value to define patients with high leptin (≥100 pg/ml) and low leptin (<100 pg/ml) levels. Patients with ALI/ARDS and high leptin levels had higher BAL fluid levels of active TGF-β1 (Figure 6B).

Overall, there was no difference in clinical outcomes between ARDS patients with low and high lung leptin levels (Figure 6C and 6D). Obesity correlates with serum leptin levels in humans and animals and is associated with hyperleptinemia secondary to an acquired leptin resistance (9, 37). To exclude the effects of BMI on leptin levels, we evaluated the relationship of BAL leptin levels with clinical outcomes in patients with a normal BMI (BMI<30 kg/m^2) (38). In the subgroup of patients with ARDS and a normal BMI, higher BAL levels of leptin were associated with fewer ventilator- and intensive care unit (ICU)-free days (Figure 6C) and a higher mortality (Figure 6D).

DISCUSSION

Leptin is a peptide hormone that acts in the brain to reduce hunger and increase energy expenditure (37). However, the functional long form of the leptin receptor, Ob-Rb is ubiquitously distributed in almost all tissues including the lung, where its functions have been less well studied (39). The majority of obese patients with type II diabetes exhibit chronic elevations of leptin and demonstrate resistance to leptin signaling (8-9). This population is resistant to the development ALI/ARDS and has a lower mortality when it develops (1-3). We and others have shown that markers of fibrosis in the BAL fluid are predictors of outcome in patients with ARDS and the resolution of fibrosis coincides with clinical improvement (14, 16-17, 28). As leptin plays an essential role in murine models of liver (18, 40) and kidney (22) fibrosis, we hypothesized that leptin might contribute to the development of fibroproliferative response during ALI/ARDS. Consistent with this hypothesis, we observed that leptin receptor deficient mice are resistant to bleomycin-induced fibrosis and found a positive correlation between BAL levels of leptin and active TGF-β1 in patients with ARDS. In non-obese patients with ARDS, higher levels of leptin in the BAL fluid were associated with fewer ventilator- and ICU-free days and a higher mortality.

The long form of the leptin receptor resembles the gp130 family of cytokine receptors and leptin has been reported to function as an immunomodulator (41-43). Leptin enhances phagocytosis in macrophages and induces the transcription and secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6) from inflammatory cells (dentritic cells and monocytes), adipocytes, microglia, endometrial and gastrointestinal epithelial cells (44-48). The stimulatory effect of leptin on IL-6 release appears to be NF-κB mediated (47-48). Consistent with these findings, we and others have shown that mice

with leptin resistance (db/db) and leptin deficiency (ob/ob) have less lung injury and improved survival in a murine model of ALI/ARDS induced by exposure to hyperoxia, (11-12). Using the same methods employed in those reports, we observed a similar degree of acute inflammation in the lungs of wild-type and db/db mice after the instillation of bleomycin. Furthermore the bleomycin-induced transcription of the β6 integrin was similar in wild-type and db/db mice. As this integrin has been shown to be required for the activation of TGF-β1 and the development of bleomycin-induced fibrosis downstream of the acute inflammatory response, these results suggest that the protection conferred against fibrosis by the loss of the leptin receptor is independent of an effect on bleomycin induced lung injury (25, 30). This is further supported by our in vitro finding that leptin augments TGF-β1-mediated transcription in normal human lung fibroblasts. However, we cannot exclude the possibility that we failed to detect a small but important difference in bleomycin-induced lung inflammation, which may have contributed to the observed protection.

PPARγ is a nuclear hormone receptor and transcription factor that is essential for normal adipogenesis and glucose homeostasis (49). Activation of PPARγ can be induced by endogenous lipids and eicosanoids or by the thiazolidinedione class of anti-diabetic drugs such as rosiglitazone (49). The activation of PPARγ has been shown to attenuate fibrosis in the bleomycin model of lung fibrosis and other murine models of tissue fibrosis, where it inhibits TGF-β1 signaling by interfering with the binding of activated Smads to their genomic DNA consensus sequences (35, 50-54). We found that the administration of leptin to normal human lung fibroblasts reduced both the protein abundance and activity of PPARγ. In cells harboring a stable knockdown of PPARγ and in cells treated with rosiglitazone to augment PPARγ, the leptin-induced augmentation of

TGF-β1-mediated transcription was lost. These results suggest that leptin augments TGF-β1-mediated transcription by reducing the abundance and activity of PPARγ. Consistent with this mechanism, the levels of PPARγ were higher in leptin receptor deficient mice.

BAL levels of leptin were significantly higher in patients with ARDS than in control ventilated patients and positively correlated with the levels of active TGF-β1. These levels were not predictive of clinical outcomes in unselected patients with ARDS. Patients with type II DM develop an acquired leptin resistance and elevated leptin levels, which correlate with BMI. We therefore performed a separate analysis of the relationship between BAL fluid leptin levels and clinical outcomes in patients without obesity as defined by the World Health Organization (BMI > 30). In these patients, higher levels of leptin correlated with poor clinical outcomes. While our study was not designed to evaluate leptin as a prognostic indicator, these findings support our hypothesis that signaling through the leptin receptor might play a pathophysiologic role in the development and progression of acute lung injury.

As obesity is associated with leptin resistance even in the absence of type II DM, our results suggest that the risk reduction seen in patients with DM may also be present in obese patients. Unfortunately, BMI has not been prospectively evaluated as a potential risk factor in many of the large cohorts examining ARDS outcomes (1, 55). Investigators who have examined this association retrospectively have reported conflicting results. In 1291 patients with ARDS, Dossett et al reported that the 30% who were obese had a lower severity-adjusted rate of ARDS (odds ratio=0.36) but similar mortality and longer lengths of stay when compared with non-obese patients (56). In contrast, in two

retrospective analyses of prospectively collected cohorts of patients at risk for ARDS, Gong et al and Anzueto et al found that an elevated BMI was positively associated with the development of ARDS but was not associated with an increased mortality or other adverse outcomes (57-58). In 902 mechanically ventilated patients with ALI, O'Brien et al found that BMI was not independently associated with improved or worsened outcomes (59). Our results and these highlight the need for further prospective epidemiologic studies examining the influence of obesity, insulin and leptin levels and insulin and leptin resistance on the development of and outcomes after ALI and ARDS.

In this investigation and our previous report, we have focused on the role played by leptin resistance in the protection observed in patients with Type II DM against the development of lung injury and fibroproliferation. Type II DM is a complex disease that is primarily characterized by hyperglycemia and hyperinsulinemia (insulin resistance), which develop spontaneously in leptin receptor deficient mice. There is evidence that leptin and insulin modulate each other's action in target organs and both have a direct and independent role in the regulation of blood glucose levels (60-66). It is therefore likely that hyperinsulinemia, insulin resistance or other hormonal and metabolic consequences of type II DM play a role in the protection we observe in leptin receptor deficient mice.

The experimental model used to study fibroproliferative response during ARDS in our studies has some limitations. Pathologically, ARDS in humans has three phases including (a) an early exudative phase of edema and inflammation, (b) a proliferative phase with alveolar epithelial cell hyperplasia and myofibroblast proliferation and (iii) a fibrotic phase with collagen deposition and progressive lung fibrosis (25). The fibroproliferative response, if excessive, impairs gas exchange and is associated with

increased morbidity and mortality. While the intratracheal instillation of bleomycin mimics the pathology of human ARDS as it first causes lung injury followed by fibroblast proliferation, collagen deposition and pulmonary fibrosis, (25, 29), it results in severe fibrosis, which is not seen in most cases of ARDS. Therefore, the clinical implication of our findings may be limited to patients with severe ARDS with excessive fibroproliferative response and fibrosis.

In conclusion, we report that lung leptin levels are increased in patients and mice with acute lung injury. Signaling through the leptin receptor is required for bleomycin-induced lung fibrosis in mice. Leptin exerts a profibrogenic effect in primary human lung fibroblasts by augmenting the transcriptional activity of TGF-β1 via suppression of anti-fibrotic activity of PPARγ. The loss of leptin signaling inhibits the bleomycin-induced activation of TGF-β1 and the TGF-β1-mediated transcription of profibrotic genes in part by augmenting the expression and activity of PPARγ. In non-obese patients with ARDS, elevated levels of leptin in the lung are associated with increased levels of active TGF-β1 and the development of adverse clinical outcomes. Given that variable leptin resistance is observed in patients with type II DM, these results provide a potential mechanism explaining the unexpected protection against ALI/ARDS observed in this population and suggest that therapeutic strategies that inhibit leptin signaling might be effective in selected patients with ALI/ARDS.

ACKNOWLEDGEMENTS

None.

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FIGURE LEGENDS

Figure 1. Mice with leptin resistance are protected against bleomycin-induced pulmonary fibrosis. (A) Bronchoalveolar lavage (BAL) fluid levels of leptin in wild-type mice 5 days after intratracheal instillation of bleomycin (0.075 units) or PBS. **(B)** Masson's Trichrome staining for collagen in lungs from mice (wild-type and db/db) 21 days after intratracheal instillation of bleomycin or PBS. Both low power images captured using MBF Neurolucida (x5) and high-power field views (x200) are shown. Total collagen content in lungs at 21 days after bleomycin treatment was assessed by **(C)** collagen I immunoblotting and **(D)** picrosirius red collagen precipitation ($p<0.05$ *bleomycin vs. PBS treatment, n=8 in each treatment group from 2 independent experiments).

Figure 2. The protection against bleomycin-induced pulmonary fibrosis is independent of bleomycin induced lung injury in mice. (A) Hematoxylin and eosin stained lungs from mice (wild-type and db/db) 5 days after the intratracheal administration of bleomycin. Both low power images captured using MBF Neurolucida (x5) and high-power field views (x200) are shown. **(B)** Cell count and **(C)** levels of proinflammatory cytokines/chemokines in the BAL fluid from mice (wild-type and db/db) 5 days after the intratracheal administration of bleomycin. ($p<0.05$ *bleomycin vs. PBS treatment, n=5 in each treatment group).

Figure 3. Leptin signaling affects bleomycin-induced TGF-β1 activation downstream of upregulation of integrin αvβ6. (A) Real-time quantitative mRNA levels of integrin $\alpha v\beta 6$ (corrected to keratin mRNA) ($p<0.05$ *bleomycin vs. PBS). **(B)** BAL fluid levels of TGF-$\beta 1$ in wild-type and db/db mice 5 days after intratracheal instillation of bleomycin or PBS ($p<0.05$ *bleomycin vs. PBS, †db/db+bleomycin vs. wild-

type+bleomycin). **(C)** Normal human lung fibroblasts were treated with either vehicle or TGF-β1 (5 ng/ml) in the presence of different concentrations of leptin and TGFβ1 mRNA was measured 24 hours later (qRT-PCR). **(D-F)** Normal human lung fibroblasts were treated with either vehicle or TGF-β1 (5 ng/ml) in the presence of different concentrations of leptin and α-smooth muscle actin (αSMA), collagen I and collagen III mRNA were measured 24 hours later (qRT-PCR). ($p<0.05$ *leptin vs. PBS) ($n\geq4$ in each treatment group from 3 independent experiments).

Figure 4. Leptin decreases the expression and activity of TGF-β1 suppressor, PPARγ. (A) mRNA (qRT-PCR) and **(B)** protein (immunoblot) levels of PPARγ in normal human lung fibroblasts 24 hours after treatment with leptin (100 ng/ml) or vehicle ($p<0.05$ * leptin vs. control treatment). **(C)** α-smooth muscle actin (αSMA) mRNA (qRT-PCR) in normal human lung fibroblasts 24 hours after treatment with TGF-β1 (5 ng/ml) or vehicle (control) in the presence or absence of rosiglitazone (50 μM) (a PPARγ agonist) ($p<0.05$ *TGF-β1 vs. control, † TGF-β1+vehicle vs. TGF-β1+rosiglitazone). **(D)** PPARγ protein levels (immunoblot) in mouse lungs from untreated wild-type and db/db mice (*wild-type vs. db/db). **(E)** mRNA (qRT-PCR) for fatty acid binding protein 4 (FABP4) in NHLFs treated with rosiglitazone (50 μM) in the presence or absence of GW9662 (a PPARγ antagonist) (10 μM) and leptin (100 ng/ml) ($p<0.05$ *GW9662 vs. vehicle, *leptin vs. vehicle). ($n\geq4$ in each treatment group from 3 independent experiments)

Figure 5. Leptin mediated augmentation of TGF-β1 transcription in lung fibroblasts requires PPARγ. (A) NHLFs were stably transfected (lentivirus) with control shRNA or a shRNA against PPARγ and cell lysates were immunoblotted for PPARγ (top). These

cells were treated with media and TGF-β1 (5 ng/ml) in the absence or presence of leptin (100 ng/ml) and 24 hours later CTGF mRNA expression was measured (qRT-PCR) (p<0.05 *leptin vs. vehicle, †wild-type+TGF-β1+vehicle vs. PPARγ-knock down+ TGF-β1+vehicle). **(B)** NHLFs were treated with TGF-β1 (5 ng/ml), leptin (100 ng/ml) and/or rosiglitazone (50 μM) and 24 hours later plasminogen activator inhibitor-1 mRNA was measured (qRT-PCR) (p<0.05 *TGF-β1 vs. TGFβ1+leptin). (n=4 in each treatment group from 2 independent experiments)

Figure 6. Alveolar levels of leptin and TGF-β1 correlate in patients with ARDS. (A) BAL fluid levels of leptin in healthy intubated control patients and all patients with ARDS. **(B)** BAL fluid levels of TGFβ1 (all patients) and **(C)** clinical outcomes (ventilator-free days, ICU-free days) and **(D)** survival in patients ARDS with low (<100 pg/ml) and high (>100 pg/ml) levels of leptin. (p<0.05 †ARDS vs. healthy control, *high leptin vs. low leptin) (n=36 patients with ARDS and n=15 healthy intubated patients)

TABLES

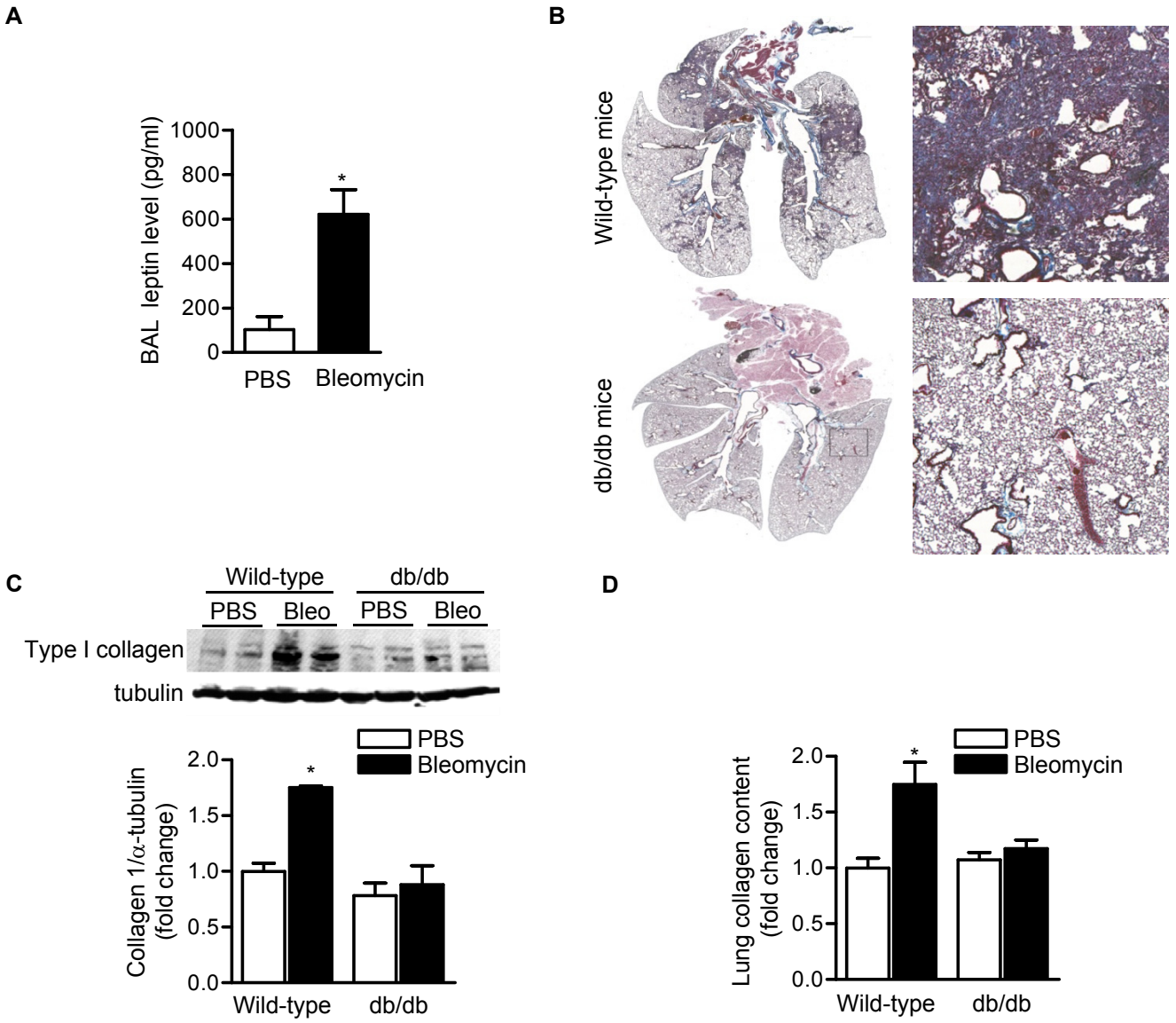
Table 1. Patient demographics and physiology

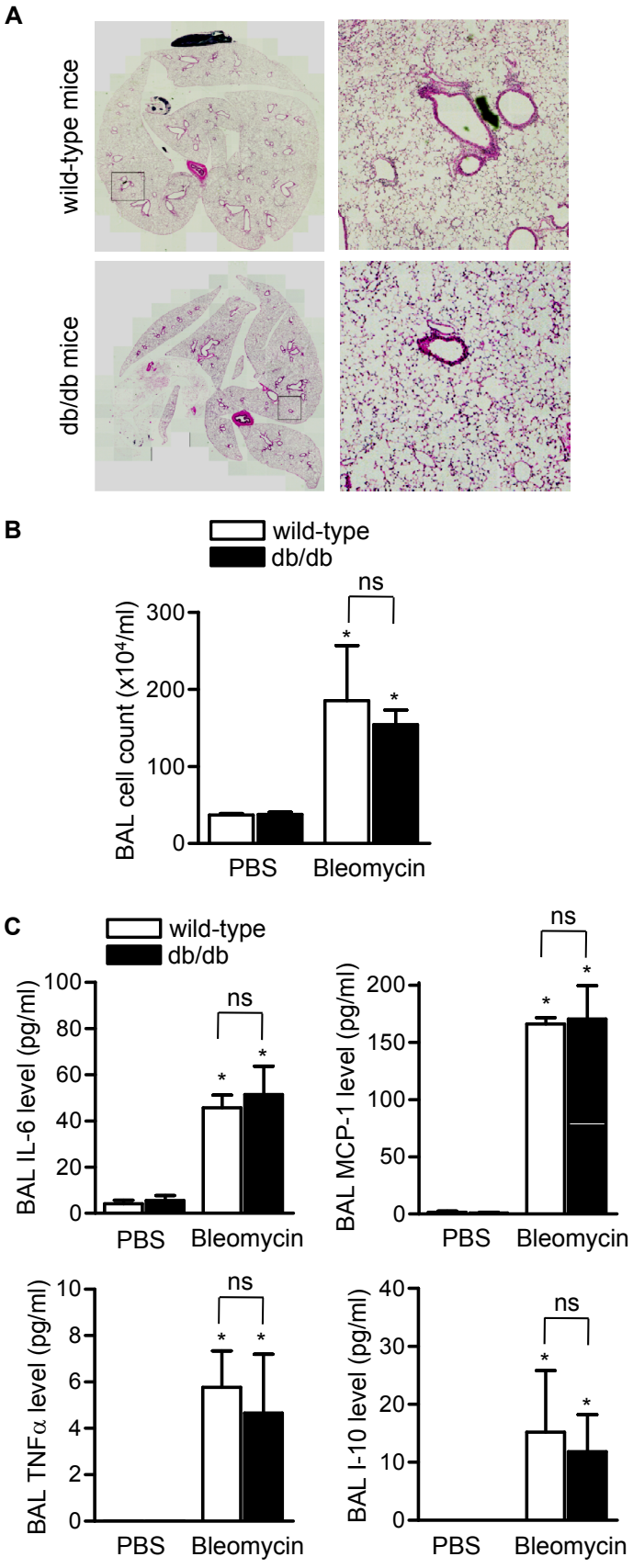
Characteristic	ALI/ARDS Subjects (n=36)
Age (years)	53±18
Gender	
Male	21 (58%)
Female	15 (42%)
BMI* (kg/m^2)	25.9±6.2
Patients with BMI≥30 kg/m^2	10 (28%)
Patients with BMI<30 kg/m^2	26 (72%)
Diabetes mellitus	6 (16.7%)
PaO_2/FiO_2 ratio	127±53
$PaCO_2$ (mmHg)	44±14
APACHE† II score	25±9
Risk factors for ALI/ARDS	
Pneumonia	15 (42%)
Extrapulmonary sepsis	14 (39%)
Other	7 (19%)

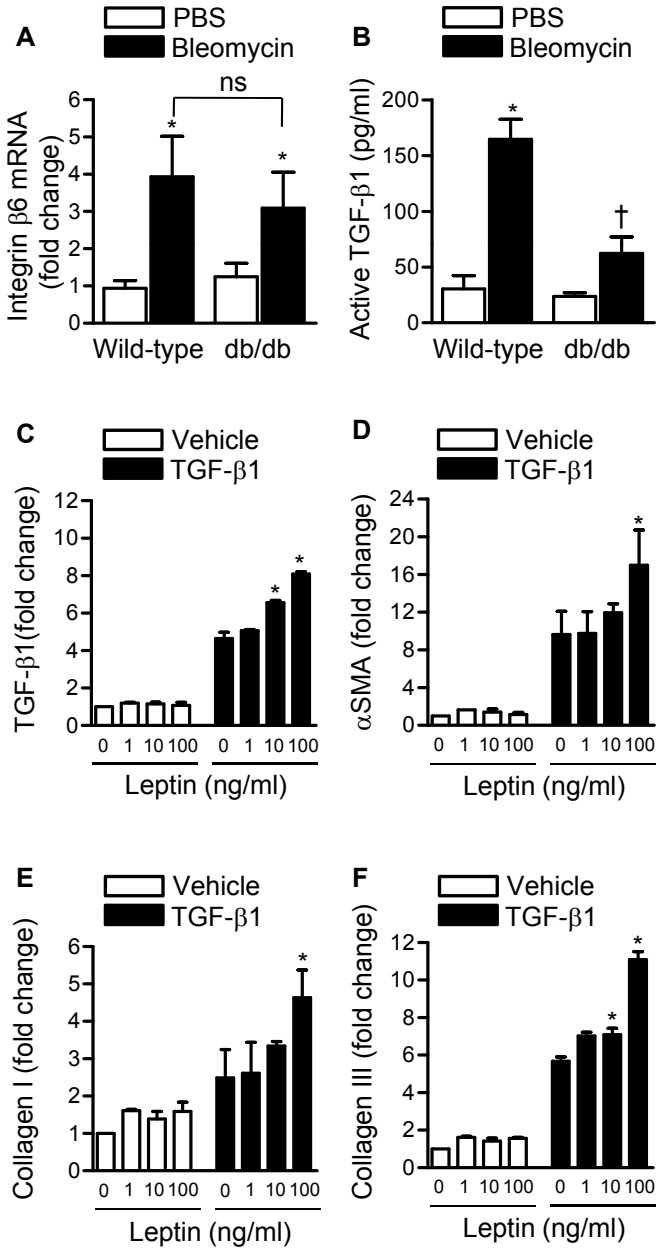
Values are presented as mean ± SD

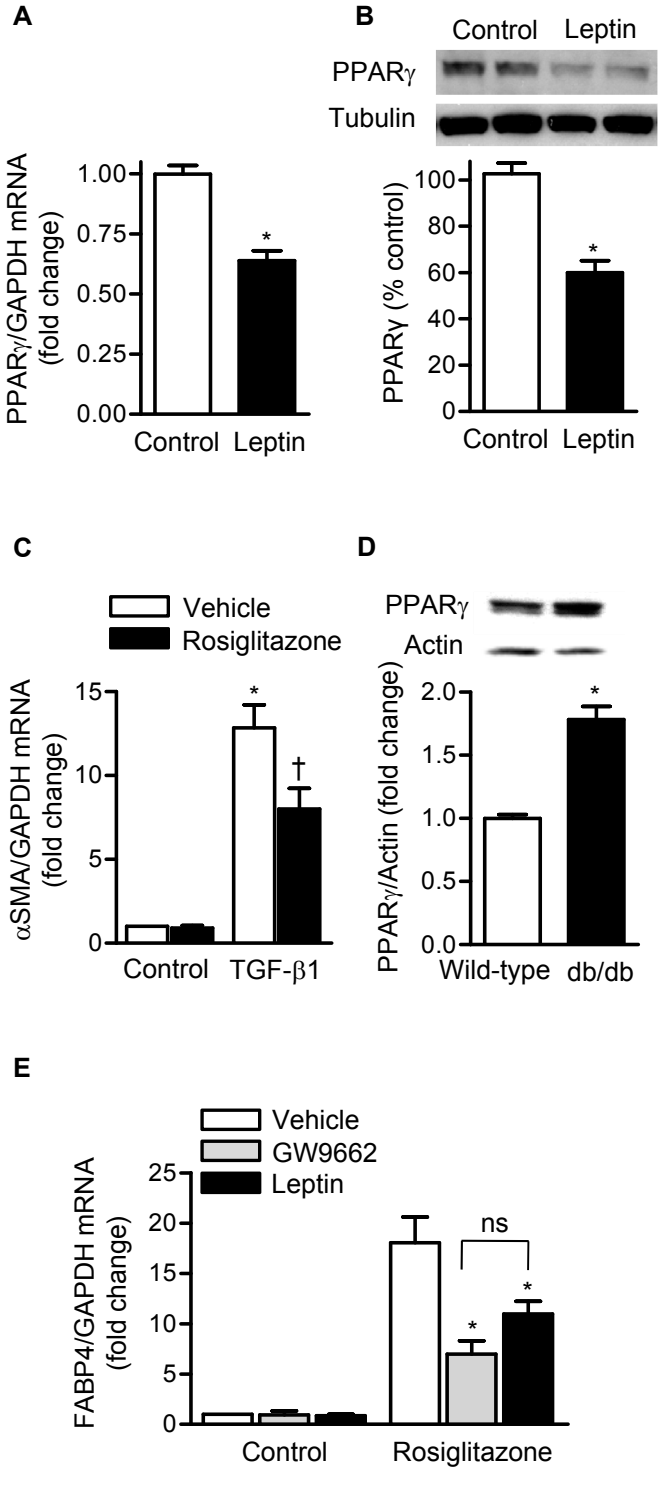
*Body Mass Index

†Acute physiology and chronic health evaluation

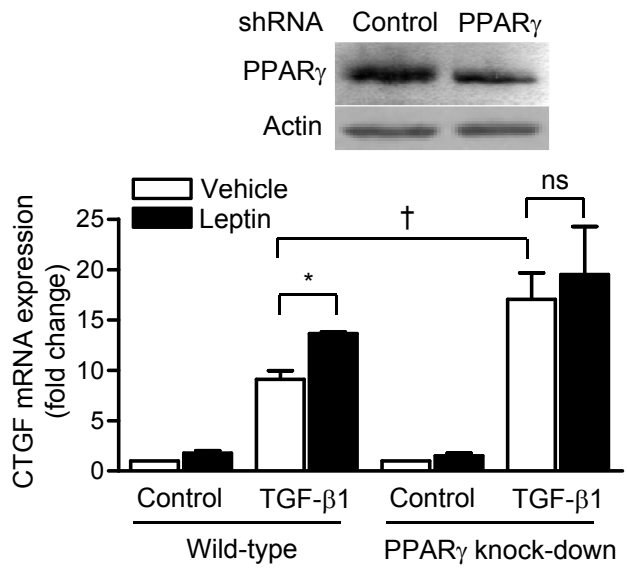

A
BAL leptin level (pg/ml)
1000
800
600
400
200
0
*
PBS
Bleomycin
B
Wild-type mice
db/db mice
C
Wild-type
db/db
PBS
Bleo
PBS
Bleo
Type I collagen
tubulin
Collagen 1/α-tubulin (fold change)
2.0
1.5
1.0
0.5
0.0
*
PBS
Bleomycin
Wild-type
db/db
D
Lung collagen content (fold change)
2.0
1.5
1.0
0.5
0.0
*
PBS
Bleomycin
Wild-type
db/db


A
wild-type mice
db/db mice
B
wild-type
db/db
ns
BAL cell count (x10⁴/ml)
300
200
100
0
PBS
Bleomycin
C
wild-type
db/db
BAL IL-6 level (pg/ml)
100
80
60
40
20
0
BAL MCP-1 level (pg/ml)
200
150
100
50
0
BAL TNFα level (pg/ml)
10
8
6
4
2
0
BAL I-10 level (pg/ml)
40
30
20
10
0
PBS
Bleomycin
ns

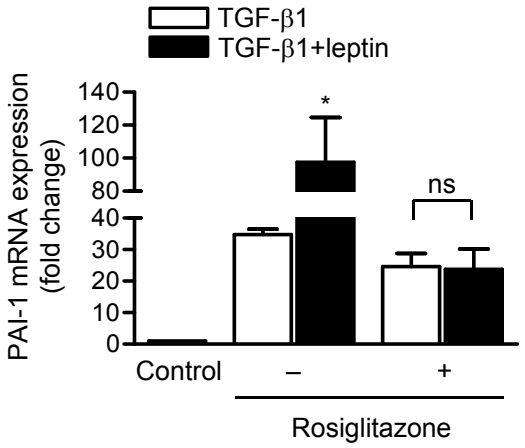

A
PBS
Bleomycin
ns
Integrin β6 mRNA (fold change)
Wild-type
db/db
B
PBS
Bleomycin
Active TGF-β1 (pg/ml)
Wild-type
db/db
C
Vehicle
TGF-β1
TGF-β1(fold change)
0 1 10 100 0 1 10 100
Leptin (ng/ml)
D
Vehicle
TGF-β1
αSMA (fold change)
0 1 10 100 0 1 10 100
Leptin (ng/ml)
E
Vehicle
TGF-β1
Collagen I (fold change)
0 1 10 100 0 1 10 100
Leptin (ng/ml)
F
Vehicle
TGF-β1
Collagen III (fold change)
0 1 10 100 0 1 10 100
Leptin (ng/ml)

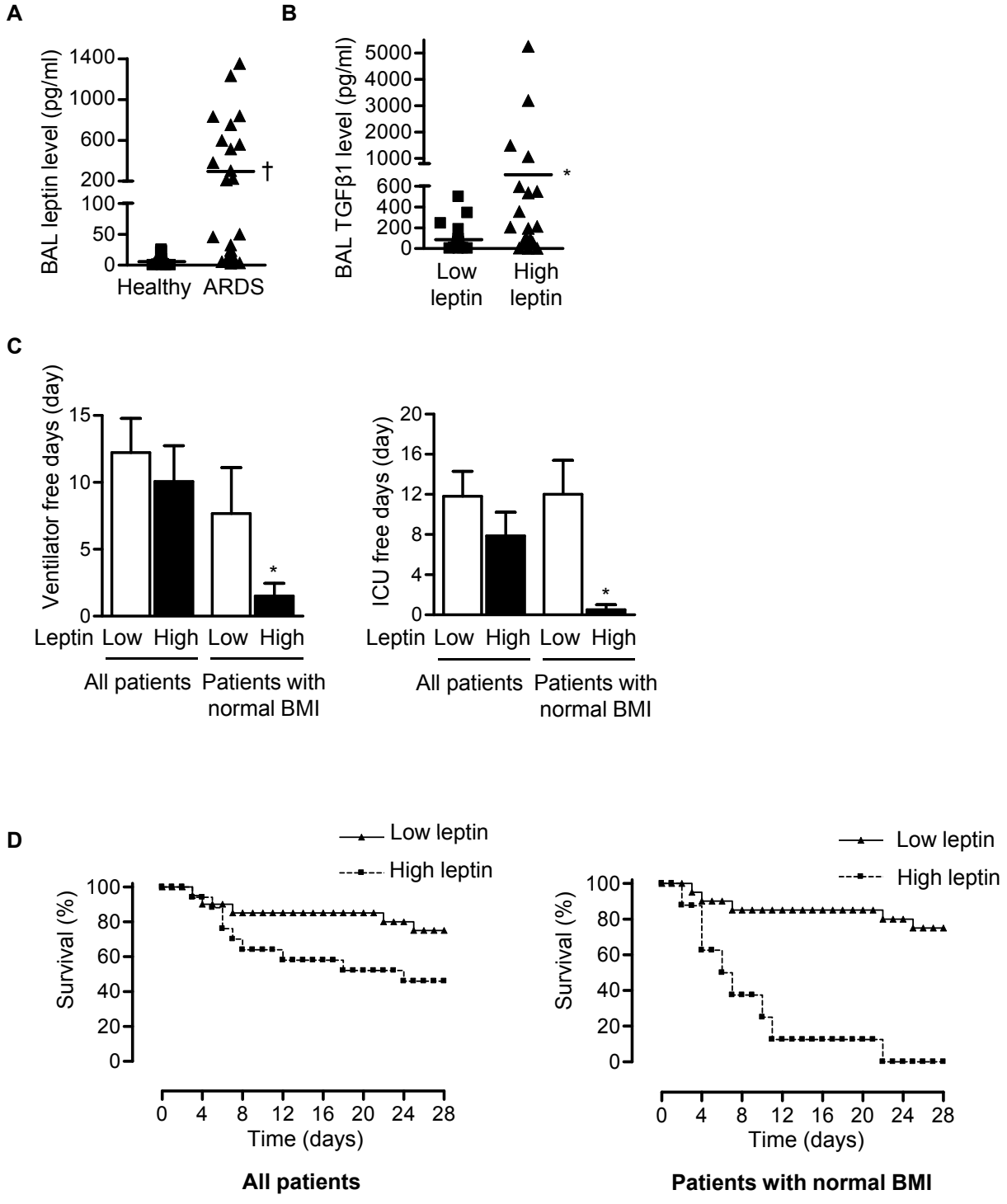

A
PPARγ/GAPDH mRNA (fold change)
1.00
0.75
0.50
0.25
0.00
*
Control
Leptin
B
Control
Leptin
PPARγ
Tubulin
PPARγ (% control)
100
80
60
40
20
0
*
Control
Leptin
C
Vehicle
Rosiglitazone
αSMA/GAPDH mRNA (fold change)
15
10
5
0
*
†
Control
TGF-β1
D
PPARγ
Actin
PPARγ/Actin (fold change)
2.0
1.5
1.0
0.5
0.0
*
Wild-type
db/db
E
Vehicle
GW9662
Leptin
FABP4/GAPDH mRNA (fold change)
25
20
15
10
5
0
ns
*
*
Control
Rosiglitazone

A


shRNA
Control
PPARγ
PPARγ
Actin
Vehicle
Leptin
CTGF mRNA expression (fold change)
25
20
15
10
5
0
*
†
ns
Control
TGF-β1
Control
TGF-β1
Wild-type
PPARγ knock-down

B


TGF-β1
TGF-β1+leptin
PAI-1 mRNA expression (fold change)
140
120
100
80
40
30
20
10
0
*
ns
Control
–
+
Rosiglitazone


A
BAL leptin level (pg/ml)
1400
1000
600
200
100
50
0
†
Healthy
ARDS
B
BAL TGFβ1 level (pg/ml)
5000
4000
3000
2000
1000
600
400
200
0
*
Low leptin
High leptin
C
Ventilator free days (day)
15
10
5
0
*
Leptin Low High Low High
All patients
Patients with normal BMI
ICU free days (day)
20
16
12
8
4
0
*
Leptin Low High Low High
All patients
Patients with normal BMI
D
Low leptin
High leptin
Survival (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 28
Time (days)
All patients
Low leptin
High leptin
Survival (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 28
Time (days)
Patients with normal BMI

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

(Blue-201009-1409C_R1)

(Marked up 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).

Figure E1

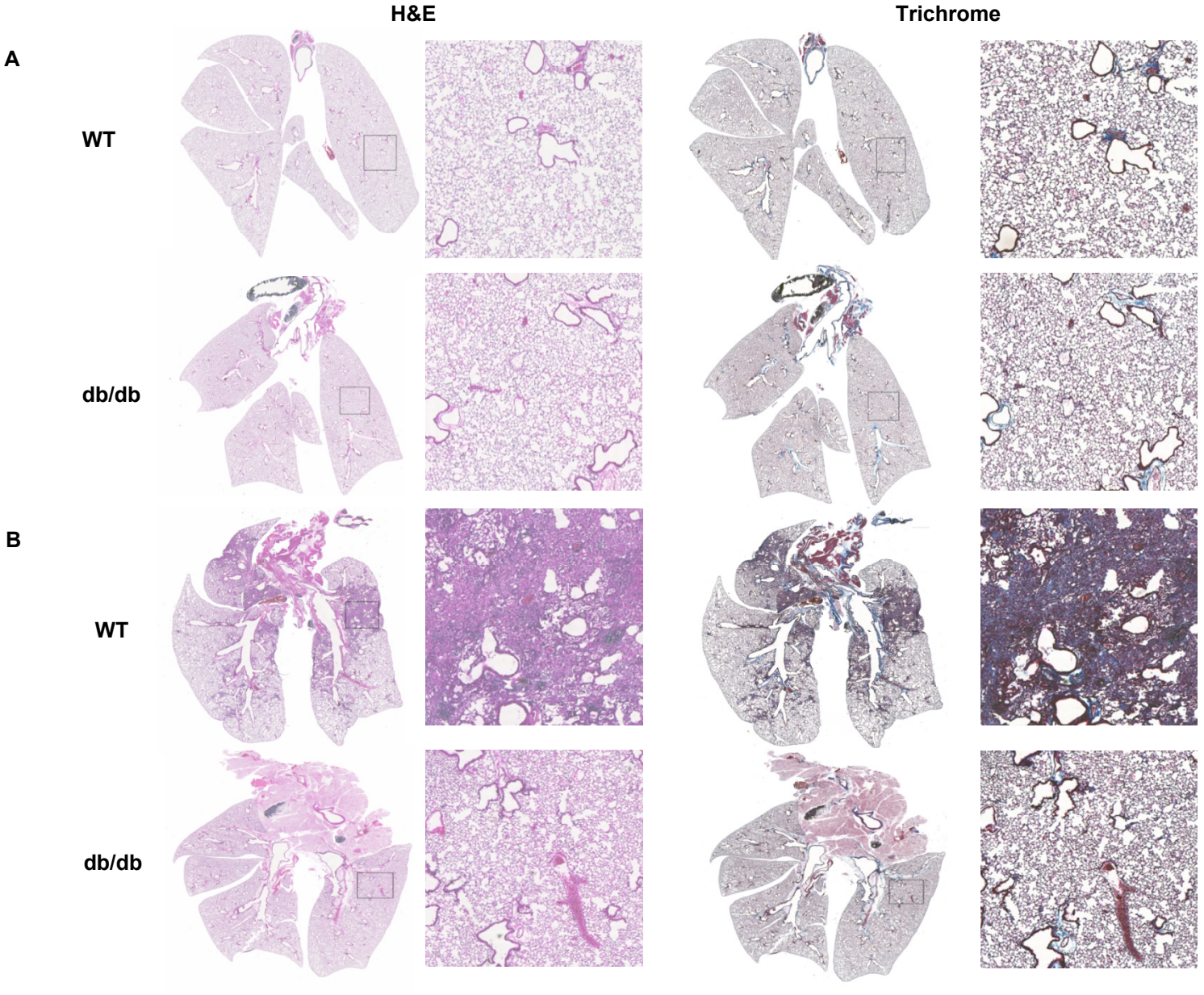
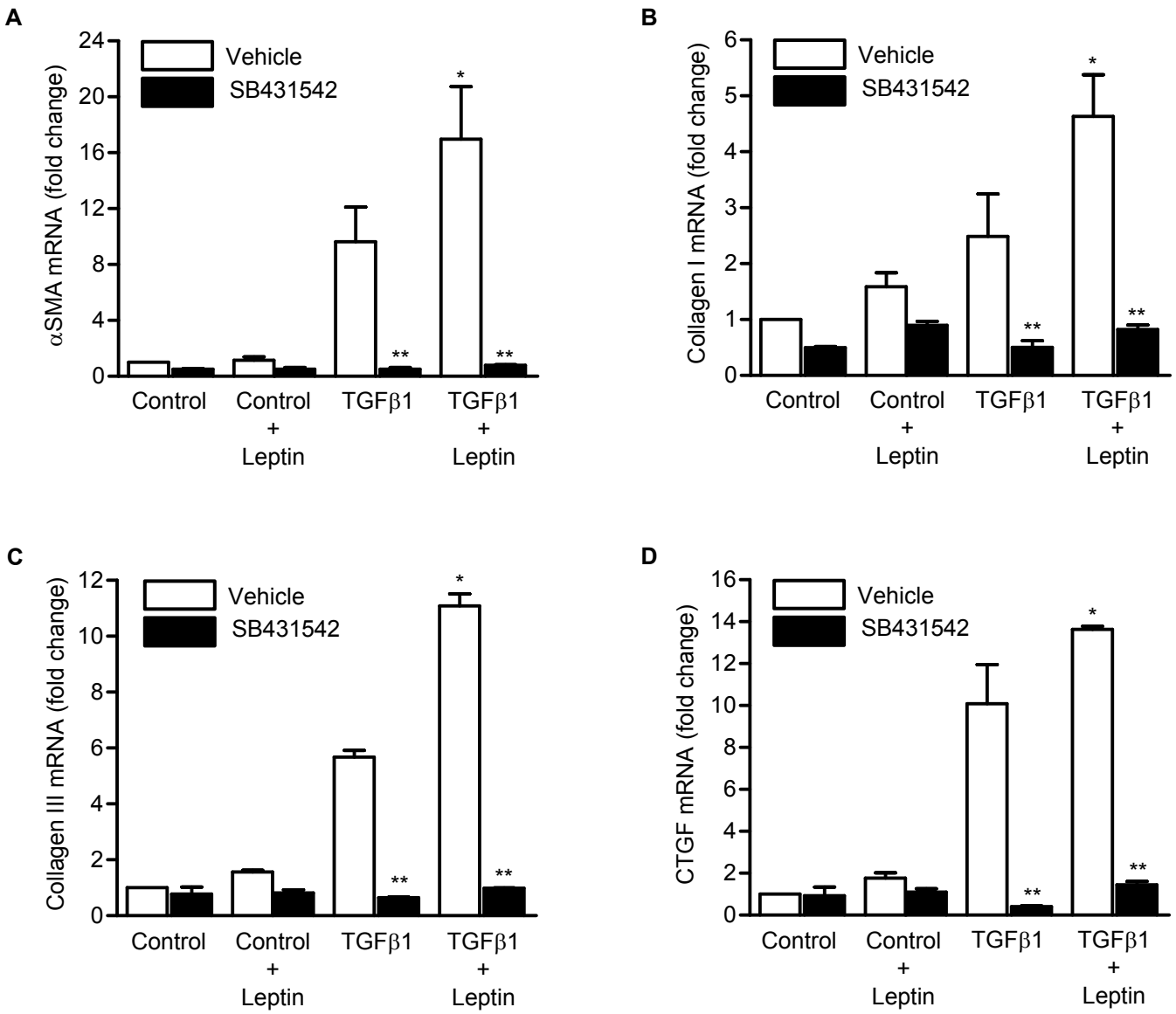


Figure E2


A
αSMA mRNA (fold change)
Vehicle
SB431542
Control
Control + Leptin
TGFβ1
TGFβ1 + Leptin
B
Collagen I mRNA (fold change)
C
Collagen III mRNA (fold change)
D
CTGF mRNA (fold change)

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

(Blue-201009-1409C_R1)

(Clean version)

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Support: ES015024 (GMM), American Lung Association (GMM), ES013995 (GRSB) HL071643 (GMM, GRSB, and NSC), Northwestern University Clinical and Translational Sciences Institute (NUCATS) CTI Pilot Award (UL1 RR025741 (NCCR), (GMM) and Northwestern Memorial Foundation Dixon Young Investigator Grant (GMM) and Scleroderma Research Foundation Grant (JV).

Running title: Leptin promotes lung fibrosis

Descriptor number: 4.1 ALI/ARDS: Biological Mechanisms

Word count (for text): 3561

At a Glance Commentary:

Scientific Knowledge on the Subject. Patients with diabetes mellitus have an approximately 50% lower incidence of the ARDS and those that develop ARDS are less likely to die. However, the mechanisms that underlie this protection are unknown. The development of TGF-β1-mediated fibroproliferation after lung injury is associated with poor clinical outcomes in patients with ARDS.

What This Study Adds to the Field. We found that leptin augments TGF-β1 signaling in lung fibroblasts and consequently promotes fibroproliferative ARDS by inhibiting PPARγ. These findings provide a mechanism for the observed protection against ARDS observed in patients with diabetes.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: Diabetic patients have a lower incidence of ARDS and those that develop ARDS are less likely to die. The mechanisms that underlie this protection are unknown.

Objectives: To determine whether leptin resistance, a feature of diabetes, prevents fibroproliferation after lung injury.

Methods: We examined lung injury and fibroproliferation after the intratracheal instillation of bleomycin in wild-type and leptin resistant (db/db) diabetic mice. We examined the effect of leptin on TGF-β1-mediated transcription in primary normal human lung fibroblasts. Bronchoalveolar lavage fluid (BAL) samples from patients with ARDS and ventilated controls were obtained for measurement of leptin and active TGF-β1 levels.

Results: Diabetic mice (db/db) were resistant to lung fibrosis. The db/db mice had higher levels of PPARγ, an inhibitor of the transcriptional response to TGF-β1, a cytokine critical in the pathogenesis of fibroproliferative ARDS. In normal human lung fibroblasts, leptin augmented the transcription of profibrotic genes in response to TGF-β1 through a mechanism that required PPARγ. In patients with ARDS, BAL leptin levels were elevated and correlated with TGF-β1 levels. Overall, there was no significant relationship between BAL leptin levels and clinical outcomes; however in non-obese patients, higher BAL leptin levels were associated with fewer intensive care unit- and ventilator-free days and a higher mortality.

Conclusions: Leptin signaling is required for bleomycin induced lung fibrosis. Leptin augments TGF-β1 signaling in lung fibroblasts by inhibiting PPARγ. These findings provide a mechanism for the observed protection against ARDS observed in diabetic patients.

Abstract word count: 240. **Keywords:** ARDS, ALI, adipokine, fibrosis, lung

INTRODUCTION

Acute lung injury (ALI) and the Acute Respiratory Distress Syndrome (ARDS) are common clinical syndromes affecting almost 200,000 people per year in the United States (1). Although progress has been made in the supportive care of these patients, the mortality remains unacceptably high (~40%). Patients with diabetes mellitus (DM) are ~50% (up to 62%) less likely to develop ARDS and those with DM who develop ARDS have lower mortality rates than non-diabetic patients (2-5). The hypothesis that this protection is conferred by the immunosuppressive effects of hyperglycemia has been challenged by the results of recent studies suggesting that hyperglycemia exacerbates inflammation and worsens ALI (2-3, 6).

Leptin is a 16-kDa non-glycosylated protein encoded by the obese gene located on human chromosome 7 and on mouse chromosome 6 (7). While classically considered a hormone because it regulates the balance between food intake and energy expenditure, leptin is also a member of the type I cytokine family. Type II diabetes is associated with hyperleptinemia and an acquired resistance to signaling through the leptin receptor (8-9). Serum levels of leptin correlate with body mass index (BMI) and are increased in patients with sepsis, the most common cause of ARDS, suggesting that leptin may play a role in the pathogenesis of ARDS (10-12).

In some patients with ALI/ARDS, the activation of Transforming Growth Factor-β1 (TGF-β1) contributes to an exuberant and persistent fibroproliferative response characterized by collagen deposition and a prolonged impairment in gas exchange (13). We and others have reported that patients with ARDS who develop an early fibroproliferative response in the lung are at increased risk for poor clinical outcomes (14-17). Recently, investigators have reported that leptin plays an important role in the development of

cirrhosis and renal fibrosis, although the mechanisms that underlie this protection are poorly understood (18-22). We sought to determine whether leptin plays a role in the pathogenesis of fibroproliferative stage of ARDS and whether this effect is mediated via TGF-β1 signaling. Some of the results of these studies have been previously reported in the form of an abstract (23).

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). Leptin receptor deficient (db/db) mice are obese, hyperleptinemic, hyperglycemic, hyperinsulinemic and exhibit insulin resistance. As all of these features are observed in patients suffering from Type II DM, these mice are used as a model for this disease (24).

Lung histology, collection of bronchoalveolar lavage (BAL) fluid and measurement of leptin and active TGF-β1 levels. These assays were performed as previously described using commercially available assays (25). Details are included in the Online Data Supplement.

Lung homogenates and immunoblotting. These assays were performed as previously described (26). Details are included in the Online Data Supplement. 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 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 (27). Details are included in the Online Data Supplement.

Human lung fibroblasts. Normal human lung fibroblasts (NHLF) (Cambrex, Charles City, IA) were grown in FGM-2 Fibroblast Growth Medium-2 (Lonza, Inc, Allendale, NJ) supplemented with SingleQuots (Cambrex, Charles City, IA) in a humidified incubator (5% CO_2) at 37°C. Quantitative RNA experiments were performed on cells at passage <

5 and with >70% confluence. The cells were incubated in serum-free media for 24 hours prior to treatment with TGF-β1 and/or leptin.

Quantitative real-time reverse transcription PCR (qRT-PCR). These assays were performed as previously described (28). All values were normalized to mitochondrial ribosomal protein RPL19. Specific primer sequences, RNA protocols and normalization procedures are described in the Online Data Supplement.

Human Study Population. Subjects were recruited from the medical intensive care unit at Northwestern Memorial Hospital. The protocol was approved by the Institutional Review Board of Northwestern University and has been previously described (28). Details are provided in the Online Data Supplement.

Collection of BAL fluid. BAL fluid was collected within 48 hours of intubation and stored as previously described (28). Details are provided in the Online Data Supplement.

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 $p<0.05$.

RESULTS

Leptin resistant mice are protected from bleomycin-induced pulmonary fibrosis. The intratracheal instillation of bleomycin in mice results in lung injury, which peaks 3 to 5 days later and is followed by fibroblast proliferation, collagen deposition and pulmonary fibrosis, which is evident at 21 days (25, 29). To address the role of leptin in the regulation of fibroproliferative ARDS, we treated db/db and wild-type control mice with intratracheal bleomycin (0.075 units/mouse) in sterile saline or saline (control). We found that BAL fluid levels of leptin were increased 6-fold in bleomycin compared with PBS-treated wild-type mice (Figure 1A). Twenty-one days after the instillation of bleomycin, we observed severe fibrosis in wild-type mice as assessed by Masson-Trichrome staining (Figure 1B and Figure E1). By contrast, db/db mice did not exhibit fibrosis. Total lung collagen, as evaluated by immunoblotting whole lung homogenates using an antibody that recognizes collagen I (Figure 1C) and picrosirius red collagen precipitation (Figure 1D) was significantly higher in wild-type mice than db/db mice.

The protection against bleomycin-induced pulmonary fibrosis in mice is independent of bleomycin induced lung injury. We evaluated the severity of bleomycin-induced lung injury in wild-type and db/db mice 5 days after the intratracheal administration of bleomycin. Examination of hematoxylin and eosin stained lung sections from wild-type and db/db mice did not reveal differences in lung injury severity (Figure 2A). The bleomycin-induced increase in the numbers of inflammatory cells (Figure 2B) and levels of pro-inflammatory cytokines (Figure 2C) in the BAL fluid were similar in wild-type and db/db mice.

Leptin signaling affects bleomycin-induced TGF-β1 activation downstream of upregulation of the integrin αvβ6. The activation of TGF-β1 requires increased

epithelial expression of the integrin αvβ6, which interacts with the latent TGF-β1 complex in the lung interstitium to release active TGF-β1 (30). We observed a similar induction in the levels of β6 mRNA in lung homogenates of wild-type and db/db mice 5 days after treatment with saline or bleomycin by quantitative real-time reverse transcription PCR (qRT-PCR) (Figure 3A). We then measured active TGF-β1 levels in freshly isolated BAL fluid samples from wild-type and db/db mice 5 days after the intratracheal instillation of bleomycin or PBS. The intratracheal administration of bleomycin led to a 5-fold rise in the BAL fluid level of active TGF-β1 in wild-type mice, whereas the bleomycin-induced increase in active TGF-β1 in db/db mice was significantly attenuated (Figure 3B).

Increased expression of the integrin αvβ6 spatially restricts the activation of TGF-β1 to regions of epithelial injury. However, active TGF-β1 induces its own transcription and release from resident lung fibroblasts in an autocrine loop that can amplify the fibrotic response (31-32). To test the hypothesis that signaling through the leptin receptor might be involved in this amplification, we treated normal human lung fibroblasts (NHLFs) with TGF-β1 and measured TGF-β1 transcription in the presence or absence of leptin. Leptin alone did not alter TGF-β1 mRNA but it augmented the induction of TGF-β1 in response to active TGF-β1 (Figure 3C).

Leptin augments the TGF-β1-induced transcription of profibrotic genes in normal human lung fibroblasts. The finding that leptin augmented the autocrine release of TGF-β1 in NHLF suggested that leptin may positively regulate the TGF-β1-induced transcription of other pro-fibrotic genes. To test this hypothesis, we treated NHLFs with vehicle or TGF-β1 in the absence or presence of different concentrations of leptin *in vitro* and 24 hours later measured the induction of profibrotic genes including α-smooth

muscle actin (αSMA), collagen I and collagen III using qRT-PCR. While leptin treatment by itself did not induce the transcription of profibrotic genes, the addition of leptin to TGF-β1 augmented their TGF-β1-mediated transcription (Figure 3D-F). The augmentation mediated by leptin at the highest dose was on average 2-fold higher than that induced by TGF-β1 alone. Treatment with SB431542 (10 μM), an inhibitor of the ubiquitously expressed TGF-β1-receptor ALK5 (33), completely inhibited the stimulatory effects of TGF-β1 on profibrotic genes in the presence and absence of leptin (Figure E2).

Leptin decreases the expression and activity of the TGF-β1 suppressor PPARγ in normal human lung fibroblasts. Activation of PPARγ has been shown to provide protection against organ fibrosis in the lung, kidney and skin by acting as a co-repressor of Smad-dependent gene transcription (34-35). We found lower levels of PPARγ mRNA and protein in NHLFs treated with leptin (100 ng/ml) compared with vehicle 24 hours after their administration (Figure 4A and B) and treatment with the PPARγ agonist rosiglitazone suppressed the TGF-β1 induced transcription of αSMA (Figure 4C). To verify the importance of leptin signaling on PPARγ expression, we immunoblotted total lung homogenates from wild-type and db/db mice using an antibody that recognizes PPARγ. The protein abundance of PPARγ was increased almost 2-fold in db/db mice compared to wild-type mice (Figure 4D).

To determine how leptin affects PPARγ response, we treated NHLFs with rosiglitazone, a PPARγ agonist or control vehicle and treated with PBS (control), GW9662, a selective inhibitor of PPARγ or leptin (100 ng/ml) and performed mRNA levels of fatty acid-binding protein 4 (FABP4), a transcriptional target gene of PPARγ (qRT-PCR) (36).

Rosiglitazone-induced increase in FABP4 mRNA was suppressed by both GW9662 and leptin (Figure 4E).

PPARγ is required and sufficient for the leptin-mediated augmentation of TGF-β1 transcription in normal human lung fibroblasts. To determine whether PPARγ is required for the leptin-induced augmentation of TGF-β1 transcriptional activity, we used a lentiviral shRNA to generate NHLFs harboring a stable knockdown of PPARγ. In these cells, the levels of PPARγ were about 50% of those observed in control transfected cells (Figure 5A). We treated control and PPARγ knockdown NHLFs with TGF-β1 in the absence or presence of leptin (100 ng/ml) and 24 hours later measured mRNA levels of CTGF, a transcriptional target of TGF-β1 (qRT-PCR). Compared with control transfected cells, NHLFs in which PPARγ expression was knocked down showed an enhanced increase in CTGF mRNA in response to TGF-β1. The leptin-induced augmentation of TGF-β1 transcriptional activity in control transfected cells was prevented in the PPARγ knockdown cells (Figure 5A). To determine whether PPARγ was sufficient to prevent the leptin mediated augmentation of TGF-β1, we treated wild-type NHLFs with rosiglitazone in the presence or absence of leptin and measured the expression of plasminogen activator inhibitor-1, another transcriptional target of TGF-β1. Treatment with rosiglitazone completely prevented the leptin-induced augmentation of PAI-1 transcription (Figure 5B).

Increased levels of BAL fluid leptin and TGF-β1 levels are associated with adverse outcomes in patients with ARDS. To evaluate the role of leptin signaling in human ARDS, we measured leptin and TGF-β1levels in BAL fluid obtained from ALI/ARDS patients or ventilated control patients without lung disease within 72 hours of intubation.

Table 1 summarizes the demographics and physiology of these patients. The BAL fluid levels of leptin were 6-fold higher in non-obese patients with ALI/ARDS compared to the control patients (Figure 6A). There was a significant correlation between BAL fluid levels of leptin and TGF-β1 ($r=0.522$, $p<0.001$) when all patients with ALI/ARDS were included in the analysis. This association was stronger in the cohort of ARDS patients with normal BMI ($r=0.637$, $p<0.0001$). None of the control patients had leptin levels greater than 100 pg/ml, which was used as the cut-off value to define patients with high leptin (≥100 pg/ml) and low leptin (<100 pg/ml) levels. Patients with ALI/ARDS and high leptin levels had higher BAL fluid levels of active TGF-β1 (Figure 6B).

Overall, there was no difference in clinical outcomes between ARDS patients with low and high lung leptin levels (Figure 6C and 6D). Obesity correlates with serum leptin levels in humans and animals and is associated with hyperleptinemia secondary to an acquired leptin resistance (9, 37). To exclude the effects of BMI on leptin levels, we evaluated the relationship of BAL leptin levels with clinical outcomes in patients with a normal BMI (BMI<30 kg/m^2) (38). In the subgroup of patients with ARDS and a normal BMI, higher BAL levels of leptin were associated with fewer ventilator- and intensive care unit (ICU)-free days (Figure 6C) and a higher mortality (Figure 6D).

DISCUSSION

Leptin is a peptide hormone that acts in the brain to reduce hunger and increase energy expenditure (37). However, the functional long form of the leptin receptor, Ob-Rb is ubiquitously distributed in almost all tissues including the lung, where its functions have been less well studied (39). The majority of obese patients with type II diabetes exhibit chronic elevations of leptin and demonstrate resistance to leptin signaling (8-9). This population is resistant to the development ALI/ARDS and has a lower mortality when it develops (1-3). We and others have shown that markers of fibrosis in the BAL fluid are predictors of outcome in patients with ARDS and the resolution of fibrosis coincides with clinical improvement (14, 16-17, 28). As leptin plays an essential role in murine models of liver (18, 40) and kidney (22) fibrosis, we hypothesized that leptin might contribute to the development of fibroproliferative response during ALI/ARDS. Consistent with this hypothesis, we observed that leptin receptor deficient mice are resistant to bleomycin-induced fibrosis and found a positive correlation between BAL levels of leptin and active TGF-β1 in patients with ARDS. In non-obese patients with ARDS, higher levels of leptin in the BAL fluid were associated with fewer ventilator- and ICU-free days and a higher mortality.

The long form of the leptin receptor resembles the gp130 family of cytokine receptors and leptin has been reported to function as an immunomodulator (41-43). Leptin enhances phagocytosis in macrophages and induces the transcription and secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6) from inflammatory cells (dentritic cells and monocytes), adipocytes, microglia, endometrial and gastrointestinal epithelial cells (44-48). The stimulatory effect of leptin on IL-6 release appears to be NF-κB mediated (47-48). Consistent with these findings, we and others have shown that mice

with leptin resistance (db/db) and leptin deficiency (ob/ob) have less lung injury and improved survival in a murine model of ALI/ARDS induced by exposure to hyperoxia, (11-12). Using the same methods employed in those reports, we observed a similar degree of acute inflammation in the lungs of wild-type and db/db mice after the instillation of bleomycin. Furthermore the bleomycin-induced transcription of the β6 integrin was similar in wild-type and db/db mice. As this integrin has been shown to be required for the activation of TGF-β1 and the development of bleomycin-induced fibrosis downstream of the acute inflammatory response, these results suggest that the protection conferred against fibrosis by the loss of the leptin receptor is independent of an effect on bleomycin induced lung injury (25, 30). This is further supported by our in vitro finding that leptin augments TGF-β1-mediated transcription in normal human lung fibroblasts. However, we cannot exclude the possibility that we failed to detect a small but important difference in bleomycin-induced lung inflammation, which may have contributed to the observed protection.

PPARγ is a nuclear hormone receptor and transcription factor that is essential for normal adipogenesis and glucose homeostasis (49). Activation of PPARγ can be induced by endogenous lipids and eicosanoids or by the thiazolidinedione class of anti-diabetic drugs such as rosiglitazone (49). The activation of PPARγ has been shown to attenuate fibrosis in the bleomycin model of lung fibrosis and other murine models of tissue fibrosis, where it inhibits TGF-β1 signaling by interfering with the binding of activated Smads to their genomic DNA consensus sequences (35, 50-54). We found that the administration of leptin to normal human lung fibroblasts reduced both the protein abundance and activity of PPARγ. In cells harboring a stable knockdown of PPARγ and in cells treated with rosiglitazone to augment PPARγ, the leptin-induced augmentation of

TGF-β1-mediated transcription was lost. These results suggest that leptin augments TGF-β1-mediated transcription by reducing the abundance and activity of PPARγ. Consistent with this mechanism, the levels of PPARγ were higher in leptin receptor deficient mice.

BAL levels of leptin were significantly higher in patients with ARDS than in control ventilated patients and positively correlated with the levels of active TGF-β1. These levels were not predictive of clinical outcomes in unselected patients with ARDS. Patients with type II DM develop an acquired leptin resistance and elevated leptin levels, which correlate with BMI. We therefore performed a separate analysis of the relationship between BAL fluid leptin levels and clinical outcomes in patients without obesity as defined by the World Health Organization (BMI > 30). In these patients, higher levels of leptin correlated with poor clinical outcomes. While our study was not designed to evaluate leptin as a prognostic indicator, these findings support our hypothesis that signaling through the leptin receptor might play a pathophysiologic role in the development and progression of acute lung injury.

As obesity is associated with leptin resistance even in the absence of type II DM, our results suggest that the risk reduction seen in patients with DM may also be present in obese patients. Unfortunately, BMI has not been prospectively evaluated as a potential risk factor in many of the large cohorts examining ARDS outcomes (1, 55). Investigators who have examined this association retrospectively have reported conflicting results. In 1291 patients with ARDS, Dossett et al reported that the 30% who were obese had a lower severity-adjusted rate of ARDS (odds ratio=0.36) but similar mortality and longer lengths of stay when compared with non-obese patients (56). In contrast, in two

retrospective analyses of prospectively collected cohorts of patients at risk for ARDS, Gong et al and Anzueto et al found that an elevated BMI was positively associated with the development of ARDS but was not associated with an increased mortality or other adverse outcomes (57-58). In 902 mechanically ventilated patients with ALI, O'Brien et al found that BMI was not independently associated with improved or worsened outcomes (59). Our results and these highlight the need for further prospective epidemiologic studies examining the influence of obesity, insulin and leptin levels and insulin and leptin resistance on the development of and outcomes after ALI and ARDS.

In this investigation and our previous report, we have focused on the role played by leptin resistance in the protection observed in patients with Type II DM against the development of lung injury and fibroproliferation. Type II DM is a complex disease that is primarily characterized by hyperglycemia and hyperinsulinemia (insulin resistance), which develop spontaneously in leptin receptor deficient mice. There is evidence that leptin and insulin modulate each other's action in target organs and both have a direct and independent role in the regulation of blood glucose levels (60-66). It is therefore likely that hyperinsulinemia, insulin resistance or other hormonal and metabolic consequences of type II DM play a role in the protection we observe in leptin receptor deficient mice.

The experimental model used to study fibroproliferative response during ARDS in our studies has some limitations. Pathologically, ARDS in humans has three phases including (a) an early exudative phase of edema and inflammation, (b) a proliferative phase with alveolar epithelial cell hyperplasia and myofibroblast proliferation and (iii) a fibrotic phase with collagen deposition and progressive lung fibrosis (25). The fibroproliferative response, if excessive, impairs gas exchange and is associated with

increased morbidity and mortality. While the intratracheal instillation of bleomycin mimics the pathology of human ARDS as it first causes lung injury followed by fibroblast proliferation, collagen deposition and pulmonary fibrosis, (25, 29), it results in severe fibrosis, which is not seen in most cases of ARDS. Therefore, the clinical implication of our findings may be limited to patients with severe ARDS with excessive fibroproliferative response and fibrosis.

In conclusion, we report that lung leptin levels are increased in patients and mice with acute lung injury. Signaling through the leptin receptor is required for bleomycin-induced lung fibrosis in mice. Leptin exerts a profibrogenic effect in primary human lung fibroblasts by augmenting the transcriptional activity of TGF-β1 via suppression of anti-fibrotic activity of PPARγ. The loss of leptin signaling inhibits the bleomycin-induced activation of TGF-β1 and the TGF-β1-mediated transcription of profibrotic genes in part by augmenting the expression and activity of PPARγ. In non-obese patients with ARDS, elevated levels of leptin in the lung are associated with increased levels of active TGF-β1 and the development of adverse clinical outcomes. Given that variable leptin resistance is observed in patients with type II DM, these results provide a potential mechanism explaining the unexpected protection against ALI/ARDS observed in this population and suggest that therapeutic strategies that inhibit leptin signaling might be effective in selected patients with ALI/ARDS.

ACKNOWLEDGEMENTS

None.

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FIGURE LEGENDS

Figure 1. Mice with leptin resistance are protected against bleomycin-induced pulmonary fibrosis. (A) Bronchoalveolar lavage (BAL) fluid levels of leptin in wild-type mice 5 days after intratracheal instillation of bleomycin (0.075 units) or PBS. **(B)** Masson's Trichrome staining for collagen in lungs from mice (wild-type and db/db) 21 days after intratracheal instillation of bleomycin or PBS. Both low power images captured using MBF Neurolucida (x5) and high-power field views (x200) are shown. Total collagen content in lungs at 21 days after bleomycin treatment was assessed by **(C)** collagen I immunoblotting and **(D)** picrosirius red collagen precipitation ($p<0.05$ *bleomycin vs. PBS treatment, n=8 in each treatment group from 2 independent experiments).

Figure 2. The protection against bleomycin-induced pulmonary fibrosis is independent of bleomycin induced lung injury in mice. (A) Hematoxylin and eosin stained lungs from mice (wild-type and db/db) 5 days after the intratracheal administration of bleomycin. Both low power images captured using MBF Neurolucida (x5) and high-power field views (x200) are shown. **(B)** Cell count and **(C)** levels of proinflammatory cytokines/chemokines in the BAL fluid from mice (wild-type and db/db) 5 days after the intratracheal administration of bleomycin. ($p<0.05$ *bleomycin vs. PBS treatment, n=5 in each treatment group).

Figure 3. Leptin signaling affects bleomycin-induced TGF-β1 activation downstream of upregulation of integrin αvβ6. (A) Real-time quantitative mRNA levels of integrin $\alpha v\beta 6$ (corrected to keratin mRNA) ($p<0.05$ *bleomycin vs. PBS). **(B)** BAL fluid levels of TGF-$\beta 1$ in wild-type and db/db mice 5 days after intratracheal instillation of bleomycin or PBS ($p<0.05$ *bleomycin vs. PBS, †db/db+bleomycin vs. wild-

type+bleomycin). **(C)** Normal human lung fibroblasts were treated with either vehicle or TGF-β1 (5 ng/ml) in the presence of different concentrations of leptin and TGFβ1 mRNA was measured 24 hours later (qRT-PCR). **(D-F)** Normal human lung fibroblasts were treated with either vehicle or TGF-β1 (5 ng/ml) in the presence of different concentrations of leptin and α-smooth muscle actin (αSMA), collagen I and collagen III mRNA were measured 24 hours later (qRT-PCR). ($p<0.05$ *leptin vs. PBS) ($n\geq4$ in each treatment group from 3 independent experiments).

Figure 4. Leptin decreases the expression and activity of TGF-β1 suppressor, PPARγ. (A) mRNA (qRT-PCR) and **(B)** protein (immunoblot) levels of PPARγ in normal human lung fibroblasts 24 hours after treatment with leptin (100 ng/ml) or vehicle ($p<0.05$ * leptin vs. control treatment). **(C)** α-smooth muscle actin (αSMA) mRNA (qRT-PCR) in normal human lung fibroblasts 24 hours after treatment with TGF-β1 (5 ng/ml) or vehicle (control) in the presence or absence of rosiglitazone (50 μM) (a PPARγ agonist) ($p<0.05$ *TGF-β1 vs. control, † TGF-β1+vehicle vs. TGF-β1+rosiglitazone). **(D)** PPARγ protein levels (immunoblot) in mouse lungs from untreated wild-type and db/db mice (*wild-type vs. db/db). **(E)** mRNA (qRT-PCR) for fatty acid binding protein 4 (FABP4) in NHLFs treated with rosiglitazone (50 μM) in the presence or absence of GW9662 (a PPARγ antagonist) (10 μM) and leptin (100 ng/ml) ($p<0.05$ *GW9662 vs. vehicle, *leptin vs. vehicle). ($n\geq4$ in each treatment group from 3 independent experiments)

Figure 5. Leptin mediated augmentation of TGF-β1 transcription in lung fibroblasts requires PPARγ. (A) NHLFs were stably transfected (lentivirus) with control shRNA or a shRNA against PPARγ and cell lysates were immunoblotted for PPARγ (top). These

cells were treated with media and TGF-β1 (5 ng/ml) in the absence or presence of leptin (100 ng/ml) and 24 hours later CTGF mRNA expression was measured (qRT-PCR) ($p<0.05$ *leptin vs. vehicle, †wild-type+TGF-β1+vehicle vs. PPARγ-knock down+ TGF-β1+vehicle). **(B)** NHLFs were treated with TGF-β1 (5 ng/ml), leptin (100 ng/ml) and/or rosiglitazone (50 μM) and 24 hours later plasminogen activator inhibitor-1 mRNA was measured (qRT-PCR) ($p<0.05$ *TGF-β1 vs. TGFβ1+leptin). (n=4 in each treatment group from 2 independent experiments)

Figure 6. Alveolar levels of leptin and TGF-β1 correlate in patients with ARDS. (A) BAL fluid levels of leptin in healthy intubated control patients and all patients with ARDS. **(B)** BAL fluid levels of TGFβ1 (all patients) and **(C)** clinical outcomes (ventilator-free days, ICU-free days) and **(D)** survival in patients ARDS with low (<100 pg/ml) and high (>100 pg/ml) levels of leptin. ($p<0.05$ †ARDS vs. healthy control, *high leptin vs. low leptin) (n=36 patients with ARDS and n=15 healthy intubated patients)

TABLES

Table 1. Patient demographics and physiology

Characteristic	ALI/ARDS Subjects (n=36)
Age (years)	53±18
Gender	
Male	21 (58%)
Female	15 (42%)
BMI* (kg/m^2)	25.9±6.2
Patients with BMI≥30 kg/m^2	10 (28%)
Patients with BMI<30 kg/m^2	26 (72%)
Diabetes mellitus	6 (16.7%)
PaO_2/FiO_2 ratio	127±53
$PaCO_2$ (mmHg)	44±14
APACHE† II score	25±9
Risk factors for ALI/ARDS	
Pneumonia	15 (42%)
Extrapulmonary sepsis	14 (39%)
Other	7 (19%)

Values are presented as mean ± SD

*Body Mass Index

†Acute physiology and chronic health evaluation

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

(Blue-201009-1409C_R1)

(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).