Leptin Promotes Fibroproliferative Acute Respiratory Distress Syndrome by Inhibiting Peroxisome Proliferator–activated Receptor-γ

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Rationale: **Diabetic patients have a lower incidence of acute respiratory distress syndrome (ARDS), and those who 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 transforming growth factor (TGF)-β_1–mediated transcription in primary normal human lung fibroblasts. Bronchoalveolar lavage fluid (BAL) samples from patients with ARDS and ventilated control subjects were obtained for measurement of leptin and active TGF-β_1 levels.**

Measurements and Main Results: **Diabetic mice (*db*/*db*) were resistant to lung fibrosis. The *db*/*db* mice had higher levels of peroxisome proliferator–activated receptor-γ (PPARγ), an inhibitor of the transcriptional response to TGF-β_1, 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-β_1 through a mechanism that required PPARγ. In patients with ARDS, BAL leptin levels were elevated and correlated with TGF-β_1 levels. Overall, there was no significant relationship between BAL leptin levels and clinical outcomes; however, in nonobese patients, higher BAL leptin levels were associated with fewer intensive care unit– and ventilator-free days and higher mortality.**

Conclusions: **Leptin signaling is required for bleomycin-induced lung fibrosis. Leptin augments TGF-β_1 signaling in lung fibroblasts by inhibiting PPARγ. These findings provide a mechanism for the observed protection against ARDS observed in diabetic patients.**

Keywords: acute lung injury; fibrosis; lung; diabetes mellitus

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Patients with diabetes mellitus have an approximately 50% lower incidence of acute respiratory distress syndrome (ARDS) and those that develop ARDS are less likely to die. However, the mechanisms that underlie this protection are unknown. The development of TGF-β_1–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-β_1 signaling in lung fibroblasts and consequently promotes fibroproliferative ARDS by inhibiting peroxisome proliferator–activated receptor-γ. These findings suggest a mechanism for the observed protection against ARDS observed in patients with diabetes.

Acute lung injury (ALI) and 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 approximately 50% (up to 62%) less likely to develop ARDS, and those with DM who develop ARDS have lower mortality rates than nondiabetic patients (2–5). The hypothesis that this protection is conferred by the immunosuppressive effects of hyperglycemia has been challenged by the results of studies suggesting that hyperglycemia exacerbates inflammation and worsens ALI (2, 3, 6).

Leptin is a 16-kD nonglycosylated protein encoded by the *obese* gene located on human chromosome 7 and on mouse chromosome 6 (7). Although 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-β_1 (TGF-β_1) 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

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ARDS who develop an early fibroproliferative response in the lung are at increased risk for poor clinical outcomes (14–17). Investigators have reported that leptin plays an important role in the development of cirrhosis and renal fibrosis, although the mechanisms that underlie the protection seen with leptin resistance 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-β_1 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 (Chicago, IL). We used 12-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 Laboratory (Bar Harbor, ME). Leptin receptor–deficient (*db*/*db*) mice are obese, hyperleptinemic, hyperglycemic, and 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 Fluid, and Measurement of Leptin and Active TGF-β_1 Levels

Lung histology, collection of bronchoalveolar lavage fluid, and measurement of leptin and active TGF-β_1 levels were performed as previously described, using commercially available assays (25). Details are included in the online supplement.

Lung Homogenates and Immunoblotting

Lung homogenization and immunoblotting were performed as previously described (26). Details are included in the online supplement. Membranes were probed with antibodies to type I collagen (1 μg/ml) (Southern Biotechnology, Birmingham, AL), peroxisome proliferator–activated receptor-γ (PPARγ, 1 μg/ml), and actin (0.5 μg/ml) (Santa Cruz Biotech, Santa Cruz, CA).

Quantitative Assessment of Lung Collagen Content

Lung collagen was measured by a modification of a previously described method for the precipitation of lung collagen, using picrosirius red (27). Details are included in the online supplement.

Human Lung Fibroblasts

Normal human lung fibroblasts (NHLFs) (Cambrex, Charles City, IA) were grown in fibroblast growth medium-2 (FGM-2; Lonza, Inc, Allendale, NJ) supplemented with SingleQuots (Cambrex) in a humidified incubator (5% CO_2) at 37°C. Quantitative RNA experiments were performed on cells before passage 5 and more than 70% confluent. The cells were incubated in serum-free medium for 24 hours before treatment with TGF-β_1 and/or leptin.

Quantitative Real-time Reverse Transcription PCR

Quantitative real-time reverse transcription PCR 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 supplement.

Human Study Population

Subjects were recruited from the medical intensive care unit at Northwestern Memorial Hospital (Chicago, IL). The protocol was approved by the Institutional Review Board of Northwestern University and has been previously described (28). Details are provided in the online supplement.

Collection of Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage (BAL) fluid was collected within 48 hours of intubation and stored as previously described (28). Details are provided in the online supplement.

Statistical Analysis

Data are expressed as means ± SEM unless otherwise specified. Differences between groups were analyzed by one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference, we explored individual differences with the Student *t* test, using the Bonferroni correction for multiple comparisons. Direct comparisons between two treatment groups were performed with the unpaired Student *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-β_1 levels were not normally distributed, we used the Spearman 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 unit/mouse) in sterile saline or with saline (control). We found that BAL fluid levels of leptin were increased sixfold in bleomycin-treated compared with phosphate-buffered saline (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 *see* Figure E1 in the online supplement). By contrast, *db*/*db* mice did not exhibit fibrosis. Total lung collagen, as evaluated by immunoblotting whole lung homogenates with 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.

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 numbers of inflammatory cells (Figure 2B) and levels of proinflammatory cytokines (Figure 2C) in the BAL fluid were similar in wild-type and *db*/*db* mice.

Leptin Signaling Affects Bleomycin-induced TGF-β_1 Activation Downstream of Up-regulation of Integrin $\alpha_v\beta_6$

Activation of TGF-β_1 requires increased epithelial expression of integrin $\alpha_v\beta_6$, which interacts with latent TGF-β_1 complex in the lung interstitium to release active TGF-β_1 (30). We observed by quantitative real-time reverse transcription PCR (qRT-PCR) a similar induction in the levels of integrin β_6 mRNA in lung homogenates of wild-type and *db*/*db* mice 5 days after treatment with saline or bleomycin (Figure 3A). We then measured active TGF-β_1 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

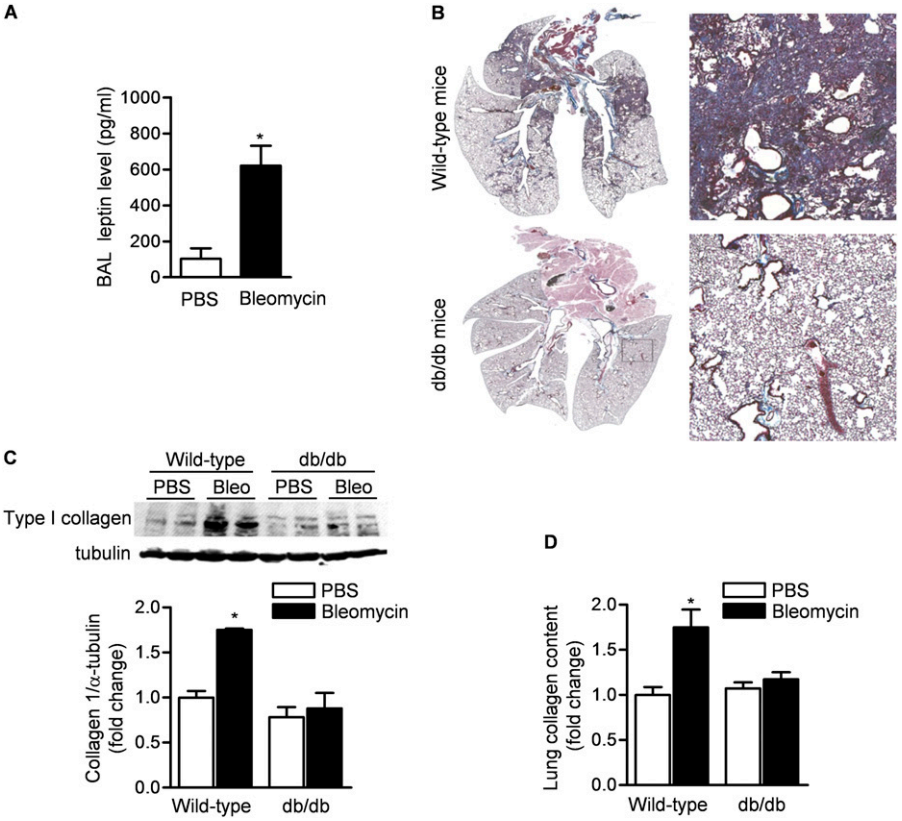

Figure 1. Mice with leptin resistance are protected against bleomycin-induced pulmonary fibrosis. (*A*) Bronchoalveolar lavage (BAL) fluid levels of leptin in wild-type mice 5 days after intratracheal instillation of bleomycin (0.075 unit) or phosphate-buffered saline (PBS). (*B*) Masson's trichrome 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 were captured with Neurolucida software (MBF Bioscience, Williston, VT) (*original magnification*: ×5) and high-power field views (original magnification, ×200) 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 two independent experiments).

administration of bleomycin led to a fivefold rise in the BAL fluid level of active TGF-β_1 in wild-type mice, whereas the bleomycin-induced increase in active TGF-β_1 in *db*/*db* mice was significantly attenuated (Figure 3B).

Increased expression of integrin $\alpha_v\beta_6$ spatially restricts the activation of TGF-β_1 to regions of epithelial injury. However, active TGF-β_1 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-β_1 and measured TGF-β_1 transcription in the presence or absence of leptin. Leptin alone did not alter TGF-β_1 mRNA but it augmented the induction of TGF-β_1 in response to active TGF-β_1 (Figure 3C).

Leptin Augments TGF-β_1–induced Transcription of Profibrotic Genes in Normal Human Lung Fibroblasts

The finding that leptin augmented the autocrine release of TGF-β_1 in NHLFs suggested that leptin may positively regulate the TGF-β_1–induced transcription of other profibrotic genes. To test this hypothesis, we treated NHLFs with vehicle or TGF-β_1 in the absence or presence of various concentrations of leptin *in vitro* and 24 hours later measured the induction of profibrotic genes, including α-smooth muscle actin (α-SMA), collagen I, and collagen III, by qRT-PCR. Whereas leptin treatment by itself did not induce the transcription of profibrotic genes, the addition of leptin to TGF-β_1 augmented their TGF-β_1–mediated transcription (Figures 3D−3F). The augmentation mediated by leptin at the highest dose was on average twofold higher than that induced by TGF-β_1 alone. Treatment with SB431542 (10 μM), an inhibitor of the ubiquitously expressed TGF-β_1 receptor ALK5 (activin receptor–like kinase-5) (33), completely inhibited the stimulatory effects of TGF-β_1 on profibrotic genes in the presence and absence of leptin (Figure E2).

Leptin Decreases Expression and Activity of TGF-β_1 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 corepressor 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 (Figures 4A and 4B) and treatment with the PPARγ agonist rosiglitazone suppressed the TGF-β_1–induced transcription of α-SMA (Figure 4C). To verify the importance of leptin signaling in 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 twofold in *db*/*db* mice compared with wild-type mice (Figure 4D).

To determine how leptin affects the PPARγ response, we treated NHLFs with rosiglitazone (a PPARγ agonist) or control vehicle and then treated them with PBS (control), GW9662 (a selective inhibitor of PPARγ), or leptin (100 ng/ml) and determined mRNA levels of fatty acid–binding protein-4 (FABP4), a transcriptional target gene of PPARγ (qRT-PCR) (36). The rosiglitazone-induced increase in FABP4 mRNA was suppressed by both GW9662 and leptin (Figure 4E).

PPARγ Is Required and Sufficient for Leptin-mediated Augmentation of TGF-β_1 Transcription in Normal Human Lung Fibroblasts

To determine whether PPARγ is required for the leptin-induced augmentation of TGF-β_1 transcriptional activity, we used a lentiviral short hairpin RNA (shRNA) to generate NHLFs harboring a stable knockdown of PPARγ. In these cells, the levels of

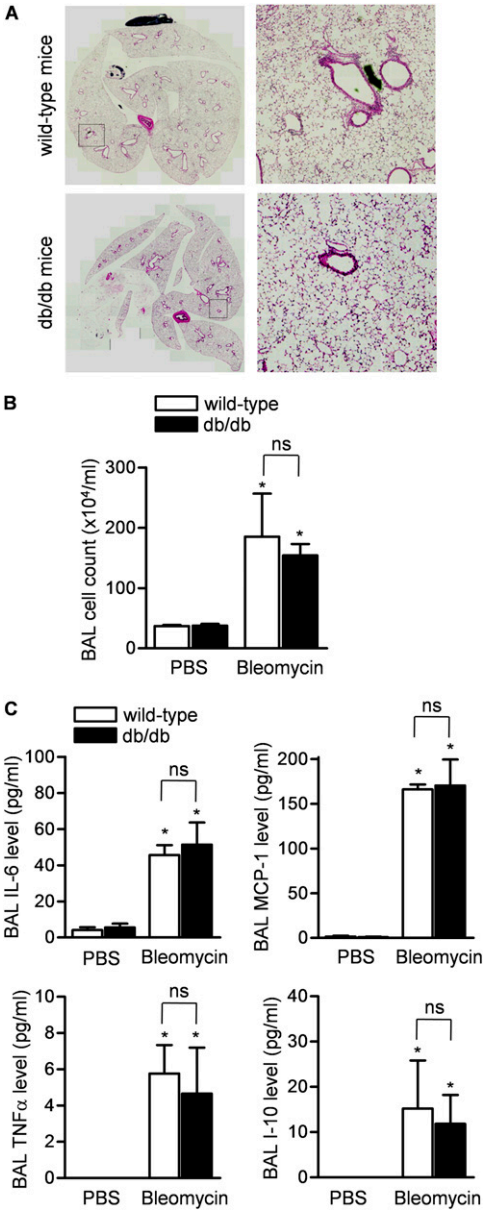

Figure 2. Protection against bleomycin-induced pulmonary fibrosis is independent of bleomycin-induced lung injury in mice. (*A*) Hematoxylin and eosin–stained lungs from mice (wild-type and *db*/*db*) 5 days after the intratracheal administration of bleomycin. Both low-power images were captured with MBF Neurolucida (*original magnification*: ×5) and high-power field views (*original magnification*: ×200) are shown. (*B*) Cell count and (C) levels of proinflammatory cytokines/chemokines in the bronchoalveolar lavage (BAL) fluid from mice (wild-type and *db*/*db*) 5 days after the intratracheal administration of bleomycin. (*$P < 0.05$, bleomycin vs. phosphate-buffered saline [PBS] treatment; n = 5 in each treatment group). MCP-1 = monocyte chemotactic protein-1; TNF-α = tumor necrosis factor-α; ns = not significant.

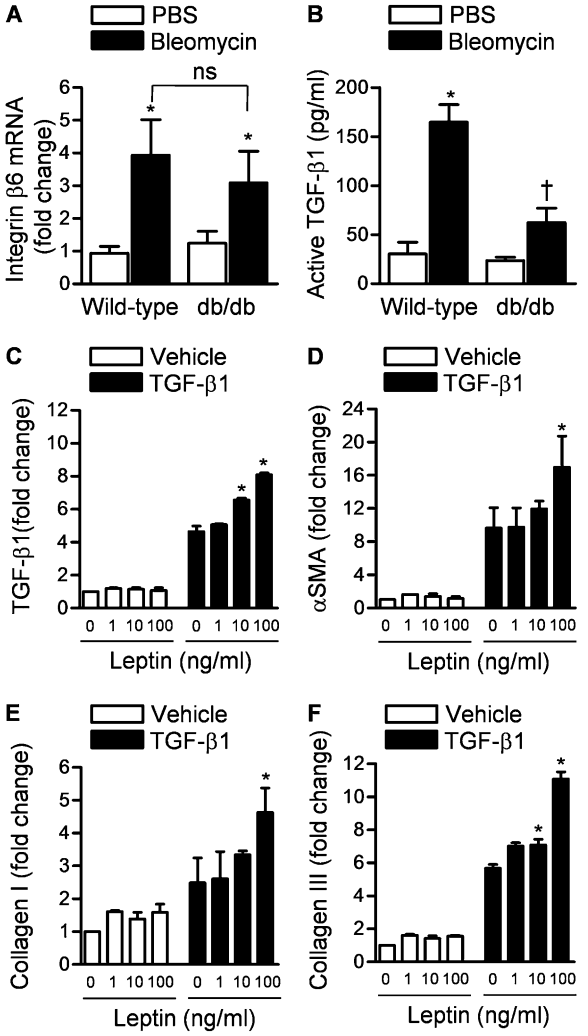

Figure 3. Leptin signaling affects bleomycin-induced transforming growth factor (TGF)-β_1 activation downstream of up-regulation of integrin $\alpha_v\beta_6$. (*A*) Real-time quantitative mRNA levels of integrin $\alpha_v\beta_6$ (corrected to keratin mRNA) (*$P < 0.05$, bleomycin vs. phosphate-buffered saline [PBS]; ns = not significant). (*B*) Bronchoalveolar lavage (BAL) fluid levels of TGF-β_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* plus bleomycin vs. wild-type plus bleomycin). (C) Normal human lung fibroblasts were treated with either vehicle or TGF-β_1 (5 ng/ml) in the presence of various concentrations of leptin and TGF-β_1 mRNA was measured 24 hours later by quantitative real-time reverse transcription PCR (qRT-PCR). (*D–F*) Normal human lung fibroblasts were treated with either vehicle or TGF-β_1 (5 ng/ml) in the presence of various concentrations of leptin, and α-smooth muscle actin (α-SMA), collagen I, and collagen III mRNAs were measured 24 hours later (qRT-PCR). (*$P < 0.05$ leptin vs. PBS) (n ⩾ 4 in each treatment group from three independent experiments).

PPARγ were about 50% of those observed in control transfected cells (Figure 5A). We treated control and PPARγ knockdown NHLFs with TGF-β_1 in the absence or presence of leptin (100 ng/ml) and 24 hours later measured mRNA levels of CTGF, a transcriptional target of TGF-β_1 (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-β_1. The leptin-induced augmentation of TGF-β_1 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-β_1, we treated wild-type NHLFs with rosiglitazone in the presence or absence of leptin and measured the expression of plasminogen activator inhibitor (PAI)-1, another transcriptional target of TGF-β_1. Treatment with rosiglitazone completely prevented the leptin-induced augmentation of PAI-1 transcription (Figure 5B).

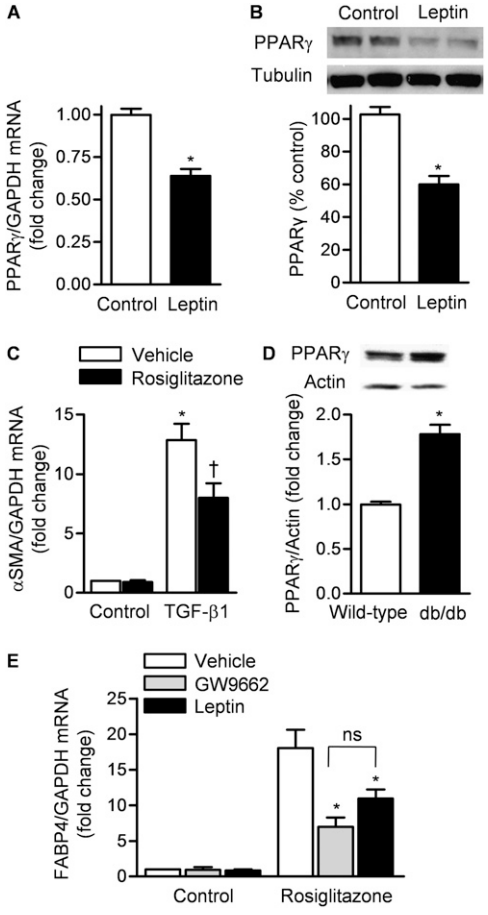

Figure 4. Leptin decreases the expression and activity of the transforming growth factor (TGF)-β_1 suppressor, peroxisome proliferator–activated receptor-γ (PPARγ). (*A*) mRNA (quantitative real-time reverse transcription PCR [qRT-PCR]) and (*B*) protein (immunoblot) levels of PPARγ in normal human lung fibroblasts 24 hours after treatment with leptin (100 ng/ml) or vehicle (*$P < 0.05$, leptin vs. control treatment). GAPDH = glyceraldehyde-3-phosphate dehydrogenase. (*C*) α-Smooth muscle actin (α-SMA) mRNA (qRT-PCR) in normal human lung fibroblasts 24 hours after treatment with TGF-β_1 (5 ng/ml) or vehicle (control) in the presence or absence of rosiglitazone (50 μM) (a PPARγ agonist) ($P < 0.05$: *TGF-β_1 vs. control, †TGF-β_1 plus vehicle vs. TGF-β_1 plus rosiglitazone). (*D*) PPARγ protein levels (immunoblot) in mouse lungs from untreated wild-type and *db*/*db* mice ($P < 0.05$: *wild-type vs. *db*/*db*). (*E*) mRNA (qRT-PCR) for fatty acid–binding protein-4 (FABP4) in normal human lung fibroblasts (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; ns = not significant) (n ⩾ 4 in each treatment group from three independent experiments).

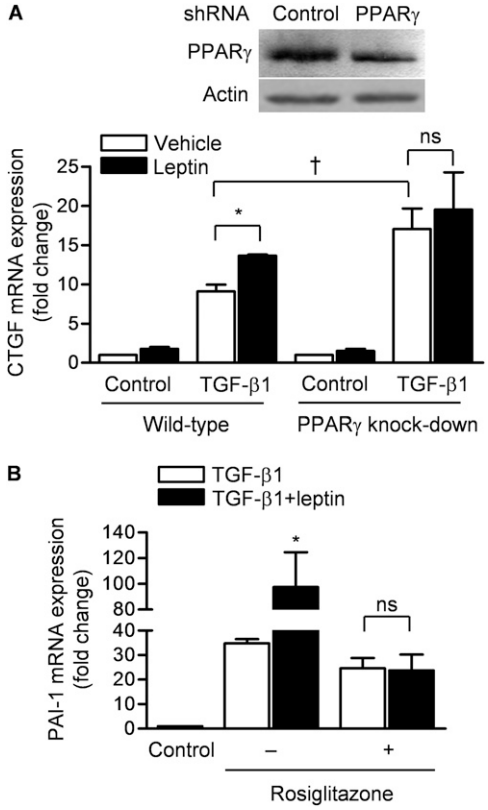

Figure 5. Leptin-mediated augmentation of transforming growth factor (TGF)-β_1 transcription in lung fibroblasts requires peroxisome proliferator–activated receptor-γ (PPARγ). (*A*) Normal human lung fibroblasts (NHLFs) were stably transfected (lentivirus) with control short hairpin RNA (shRNA) or an shRNA against PPARγ and cell lysates were immunoblotted for PPARγ (*top*). These cells were treated with medium and TGF-β_1 (5 ng/ml) in the absence or presence of leptin (100 ng/ml) and 24 hours later connective tissue growth factor (CTGF) mRNA expression was measured (quantitative real-time reverse transcription PCR [qRT-PCR]) ($P < 0.05$: *leptin vs. vehicle, †wild-type plus TGF-β_1 plus vehicle vs. PPARγ knockdown plus TGF-β_1 plus vehicle; ns = not significant). (*B*) NHLFs were treated with TGF-β_1 (5 ng/ml), leptin (100 ng/ml), and/or rosiglitazone (50 μM) and 24 hours later plasminogen activator inhibitor (PAI)-1 mRNA was measured (qRT-PCR) (*$P < 0.05$, TGF-β_1 vs. TGF-β_1 plus leptin) (n = 4 in each treatment group from two independent experiments).

Increased Levels of BAL Fluid Leptin and TGF-β_1 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-β_1 levels in BAL fluid obtained from patients with ALI/ARDS 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 sixfold higher in nonobese patients with ALI/ARDS compared with the control patients (Figure 6A). There was a significant correlation between BAL fluid levels of leptin and TGF-β_1 ($r = 0.522$, $P < 0.001$) when all patients with ALI/ARDS were included in the analysis. This association was stronger in the cohort of patients with ARDS 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 cutoff 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-β_1 (Figure 6B).

Overall, there was no difference in clinical outcomes between patients with ARDS with low and high lung leptin levels (Figures 6C and 6D). Obesity correlates with serum leptin levels in humans and animals and is associated with hyperleptinemia secondary to acquired leptin resistance (8, 9). 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) (37). 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–free days (Figure 6C) and a higher mortality (Figure 6D).

TABLE 1. PATIENT DEMOGRAPHICS AND PHYSIOLOGY

Characteristic	Subjects with ALI/ARDS (*n* = 36)
Age, yr	**53 ± 18**
Sex	
Male	**21 (58%)**
Female	**15 (42%)**
BMI, kg/m²	**25.9 ± 6.2**
Patients with BMI ⩾ 30 kg/m²	**10 (28%)**
Patients with BMI < 30 kg/m²	**26 (72%)**
Diabetes mellitus	**6 (16.7%)**
Pa_{O_2}/FI_{O_2} ratio	**127 ± 53**
Pa_{CO_2} (mm Hg)	**44 ± 14**
APACHE II score	**25 ± 9**
Risk factors for ALI/ARDS	
Pneumonia	**15 (42%)**
Extrapulmonary sepsis	**14 (39%)**
Other	**7 (19%)**

Definition of abbreviations: ALI/ARDS = acute lung injury/acute respiratory distress syndrome; APACHE = Acute Physiology and Chronic Health Evaluation; BMI = body mass index; FI_{O_2} = fraction of inspired oxygen.

Values are presented as means ± SD.

DISCUSSION

Leptin is a peptide hormone that acts in the brain to reduce hunger and increase energy expenditure (8). 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 (38). 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 of ALI/ARDS and has 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, 39) 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-β_1 in patients with ARDS. In nonobese patients with ARDS, higher levels of leptin in the BAL fluid were associated with fewer ventilator- and intensive care unit–free days and 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 (40–42). Leptin enhances phagocytosis in macrophages and induces the transcription and secretion of proinflammatory cytokines such as IL-6 from inflammatory cells (dendritic cells and monocytes), adipocytes, microglia, and endometrial and gastrointestinal epithelial cells (43–47). The stimulatory effect of leptin on IL-6 release appears to be mediated by nuclear factor-κB (46, 47). 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

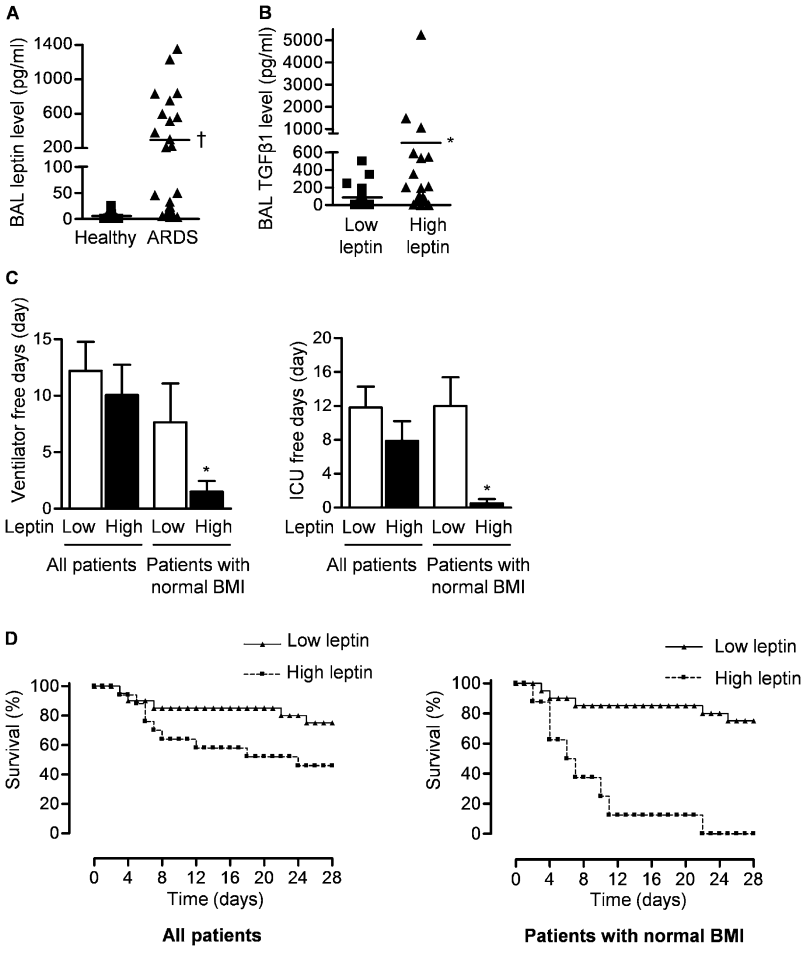

Figure 6. Alveolar levels of leptin and transforming growth factor (TGF)-β_1 correlate in patients with acute respiratory distress syndrome (ARDS). (*A*) Bronchoalveolar lavage (BAL) fluid levels of leptin in healthy intubated control patients and all patients with ARDS. (*B*) BAL fluid levels of TGF-β_1 (all patients) and (*C*) clinical outcomes (ventilator-free days, intensive care unit [ICU]–free days) and (*D*) survival of patients with ARDS with low (<100 pg/ml) and high (>100 pg/ml) levels of leptin. ($P < 0.05$: †ARDS vs. healthy control subjects, *high leptin vs. low leptin) (n = 36 patients with ARDS and n = 15 healthy intubated patients). BMI = body mass index.

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 integrin β_6 was similar in wild-type and *db*/*db* mice. As this integrin has been shown to be required for the activation of TGF-β_1 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-β_1–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 (48). Activation of PPARγ can be induced by endogenous lipids and eicosanoids or by the thiazolidinedione class of antidiabetic drugs such as rosiglitazone (48). 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-β_1 signaling by interfering with the binding of activated Smads to their genomic DNA consensus sequences (35, 49–53). 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-β_1–mediated transcription was lost. These results suggest that leptin augments TGF-β_1–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-β_1. These levels were not predictive of clinical outcomes in unselected patients with ARDS. Patients with type II DM develop 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. Although 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 pathophysiological 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, 54). Investigators who have examined this association retrospectively have reported conflicting results. In 1,291 patients with ARDS, Dossett and colleagues 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 nonobese patients (55). In contrast, in two retrospective analyses of prospectively collected cohorts of patients at risk for ARDS, Gong and colleagues and Anzueto and colleagues found that an elevated BMI was positively associated with the development of ARDS but was not associated with increased mortality or other adverse outcomes (56, 57). In 902 mechanically ventilated patients with ALI, O'Brien and colleagues found that BMI was not independently associated with improved or worsened outcomes (58). Our results and these highlight the need for further prospective epidemiological 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 (59–65). 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 we used to study fibroproliferative response during ARDS has some limitations. Pathologically, ARDS in humans has three phases including (*1*) an early exudative phase of edema and inflammation, (*2*) a proliferative phase with alveolar epithelial cell hyperplasia and myofibroblast proliferation, and (*3*) 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. Although the intratracheal instillation of bleomycin model mimics the pathology of human ARDS, as it initially causes lung injury followed by fibroblast proliferation, collagen deposition, and pulmonary fibrosis (25, 29), it leads to severe lung 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 complicated by 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-β_1 via suppression of the antifibrotic activity of PPARγ. The loss of leptin signaling inhibits the bleomycin-induced activation of TGF-β_1 and the TGF-β_1–mediated transcription of profibrotic genes in part by augmenting the expression and activity of PPARγ. In nonobese patients with ARDS, elevated levels of leptin in the lung are associated with increased levels of active TGF-β_1 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.

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