Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers


Department of Respiratory Medicine, University Hospital Maastricht, Maastricht, The Netherlands; Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, Maastricht, The Netherlands; Department of Pathology, University Hospital Maastricht, Maastricht, The Netherlands; Department of Anatomy & Embryology, Maastricht University, Maastricht, The Netherlands; Department of Respiratory Diseases, University Hospital Ghent, Ghent, Belgium; Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands; CIRO Horn, Horn, The Netherlands.

Author for correspondence and reprint requests:
Dr. Juanita H.J. Vernooy
Department of Respiratory Medicine
University Hospital Maastricht
P.O. Box 5800
6202 AZ Maastricht, The Netherlands
Tel + 31-43-3884245
Fax + 31-43-3875051
E-mail: j.vernooy@pul.unimaas.nl

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ABSTRACT
Background: COPD is characterized by an abnormal inflammatory reaction of the lungs, involving activation of epithelial cells. Leptin is a pleiotropic cytokine important in the regulation of immune responses via its functional receptor Ob-Rb. This study was undertaken to test the hypothesis that severe COPD is associated with increased leptin expression in epithelial cells.

Methods: Immunohistochemistry for leptin was performed on peripheral lung specimens from 20 COPD patients (GOLD 4), 14 asymptomatic (ex-)smokers and 13 never smokers. Leptin and Ob-Rb mRNA expression was determined by rtPCR in cultured primary bronchial epithelial cells (BEC) and primary type II pneumocytes. NCI-H292 and A549 cell lines were used to study functional activation of leptin signalling.

Results: Leptin immunoreactivity in lung tissue was observed in BEC, type II pneumocytes, macrophages (tissue/alveolar), and interstitial lymphocytic infiltrates. rtPCR analysis confirmed pulmonary leptin and Ob-Rb mRNA expression in primary BEC and pneumocytes. Leptin-expressing BEC and alveolar macrophages were markedly higher in severe COPD patients and (ex-) smokers versus never smokers (p<0.02). Exposure of cultured primary BEC to smoke resulted in increased expression of both leptin and Ob-Rb (p<0.05). Leptin induced phosphorylation of STAT3 in both NCI-H292 and A549 cells.

Conclusions: Leptin expression is increased in BEC and alveolar macrophages of (ex-) smokers with or without severe COPD versus never smokers. A functional leptin signalling pathway is present in lung epithelial cells.
INTRODUCTION
Chronic obstructive pulmonary disease (COPD) is a leading and increasing cause of morbidity and mortality worldwide. The airflow limitation in COPD is associated with both structural changes and an abnormal pulmonary inflammatory response to noxious particles or gases, including tobacco smoke.[1] Different kinds of inflammatory cells (macrophages, neutrophils, CD8+ T-lymphocytes) have been implicated in this chronic inflammation, and a distinct inflammatory pattern has been described in each lung compartment.[2] To date, the mechanisms of both recruitment and survival of inflammatory cells in response to noxious particle exposure, which eventually results in a persistent pulmonary inflammation without need of the primary stimulus, remain incompletely understood.

A novel candidate to regulate pulmonary immune function is leptin, a 16 kDa non-glycosylated type I cytokine encoded by the obese (ob) gene.[3] Leptin was originally described as an adipocyte-derived hormone involved in balancing food intake and energy expenditure by activating the full-length functional isoform of the leptin receptor (Ob-Rb) in the hypothalamus.[4] Recent studies reported leptin secretion not only by adipocytes, but also by various epithelial cell types, as shown for intestinal and gastric epithelial cells,[5,6] mammary epithelial cells,[7] and bronchial epithelial cells.[8] The almost universal distribution of leptin receptors reflects the multiplicity of biological effects in extraneural tissues. Recently published studies demonstrate that leptin has a potentiating role in the function of both innate and adaptive immunity.[9] On neutrophils and macrophages, leptin stimulates chemotaxis,[10] and enhances functional capacities like oxidative burst[10] phagocytosis[11] and cytokine secretion.[12] In addition, leptin exerts proliferative[13] and anti-apoptotic effects[14] on T-lymphocytes and promotes Th1 cell differentiation.[15] Furthermore, leptin enhances host response to inflammation and infection by stimulating tissue repair via its mitogenic and angiogenic properties on epithelium and endothelium.[16,17]

Normal lung tissue displays particularly high levels of Ob-Rb,[18,19] and specific leptin-binding sites have been identified in airway and alveolar epithelial cells.[20,21] This designates the lung as peripheral site of action for leptin in pulmonary diseases. As we and others recently demonstrated that leptin protein is actually present in induced sputum[22] and proximal airway biopsies[8] of COPD patients, we hypothesize that COPD is associated with increased leptin expression in lung epithelial cells. To this end, we investigated leptin protein in peripheral lung tissue from patients with severe COPD, (ex-)smokers with normal lung function and never smokers. In addition, we analyzed the effect of smoke on leptin and Ob-Rb mRNA expression using primary bronchial epithelial cell (PBEC) cultures. NCI-H292 and A549 cell lines were used to study functional activation of leptin signalling.
MATERIALS AND METHODS
For full details see supplementary material online.

Selection of subjects
Human lung tissue was obtained from: 1a. Twenty stable COPD patients (GOLD 4) admitted to the pulmonary rehabilitation centre CIRO Horn (Horn, The Netherlands) for six weeks before undergoing lung volume reduction surgery (LVRS) and 1b. Twenty-seven control subjects (6 current smokers, 8 ex-smokers, 13 never-smokers) with normal lung function, who underwent a lobectomy or pneumectomy for lung cancer in the University Hospital Maastricht and University Hospital Ghent, to obtain tissue blocks for immunohistochemistry;[23] 2. Nine anonymous patients who underwent a lobectomy or pneumectomy for lung cancer at the Leiden University Medical Center, Leiden, The Netherlands to isolate PBEC for in vitro experiments; 3. Three lung transplant recipients to isolate primary alveolar type II pneumocytes (AT-II) for in vitro experiments (see online supplement). The study was approved by the medical ethical committees of the University Hospital in Maastricht, Ghent and Leuven. All subjects gave their informed consent in writing.

Immunohistochemistry
Paraffin sections (4 µm) were processed for immunohistochemical analysis of leptin, CD68 and Thyroid transcription factor-1 (TTF-1). A validated protocol for sequential double staining was used to identify leptin+ macrophages. Sections were incubated with anti-leptin (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using Blue substrate kit III (Vector Laboratories, Burlingame, CA). Consecutively, sections were incubated with anti-CD68 (Dako Cytomation) and developed using Red substrate kit I (Vector Laboratories). A similar protocol was used to identify leptin+ AT-II using anti-TTF-1 (Lab Vision, Fremont USA) and DAB substrate (Dako Cytomation).

Cytoplasmic immunostaining for leptin was examined in bronchial epithelial cells (BEC), tissue macrophages (TM), and alveolar macrophages (AM). At least 500 cells were counted by one independent observer unaware of the clinical data, and expressed per cell type as percentage leptin+ cells of total counted cells.

Cell culture
Human PBEC. Subcultures of PBEC were cultured either submerged (n=4 subjects) or at an air-liquid interface (ALI; n=5 subjects). Cigarette smoke condensate (CSC) was prepared using commercial cigarettes (Caballero, British American Tobacco Group). Cultures were stimulated with 2 AU/ml CSC for 24h (submerged) or 1-5-10 AU/ml CSC for 48h (ALI; apically) and medium was stored at -20°C. RNA was isolated from submerged and ALI cultures and stored at -80°C until further use.

A549 and NCI-H292 cell lines. A549 and NCI-H292 cells were serum-deprived for 18h-24h followed by stimulation for 0.5h-1h-2h in 0.5% FBS containing medium: a) without additions; b) 20-100-500 ng/ml recombinant human leptin (R&D systems); c) 50 ng/ml recombinant human IL-6 (PeproTech). Cell lysates were stored at –80°C until rtPCR or western blotting analyses.

Quantitative real-time PCR
Total RNA was isolated using the RNeasy Microkit/Minikit (QIAGen Inc. CA) and reverse transcribed into cDNA (Reverse-iT 1st strand Synthesis Kit; Abgene, Epsom, UK). Primers for leptin, Ob-Rb, PPIA, HPRT-1 and HSP-90B are listed in the online supplement. PCR reactions were performed on an iCycler iQ Real-Time PCR system (BioRad, Hercules,
California) using SYBRgreen dye (Biorad). The standardcurve method was used to calculate the relative quantity of the respective genes. The geNorm applet (http://medgen.ugent.be/~jvdesomp/genorm/) was used to calculate a normalization factor based on expression of three reference genes (PPIA, HPRT-1, HSP-90B).

**Leptin ELISA**
Leptin levels in basal medium of PBEC cultured at ALI were determined using a commercially available human leptin immunoassay (Quantikine DLP00; R&D systems, Minneapolis, USA) with a lower detection limit of 7.8 pg/ml.

**Western blotting**
Whole cell lysates were separated by SDS-PAGE (reducing conditions) and transferred to nitrocellulose. Blots were incubated with antibodies raised against phospho-STAT-3 or total-STAT-3 (Cell Signaling, Beverly, MA). Immunoreactive proteins were visualized using chemoluminescence (Pierce Biotechnology, Rockford, IL).

**Statistical analysis**
Results are presented as mean ± standard deviation (SD) for normally distributed variables and median (range) otherwise. Non-parametric data were compared by Kruskal-Wallis test followed by Mann-Whitney U test as appropriate. The Chi-square test was used to compare categorical variables. Correlations between parameters were evaluated using Pearson’s rank correlation analysis (SPSS 13.0, SPSS Inc., Chicago, IL). A p-value <0.05 denotes the presence of a significant statistical difference.
RESULTS
Subject characteristics
The clinical and lung function characteristics are summarized in Table 1. COPD patients had decreased FEV\textsubscript{1} values (%pred), FEV\textsubscript{1}/FVC ratios and DL\textsubscript{CO} (%pred), and increased RV (%pred). The COPD group and (ex-)smoking control group were similar with regard to age, gender and BMI. The ratio current versus ex-smokers was significantly different in the COPD group compared to asymptomatic smokers group, whereas the smoking history (pack-years) was not. The never smokers group was matched for age and BMI, but consisted of considerably more females.

| TABLE 1 |
|------------------|------------------|------------------|
| **Clinical characteristics of study subjects*** | **Severe COPD** (n=20) | **Asymptomatic Smokers** (n=14) | **Never Smokers** (n=13) |
| **Age, years** | 61 ± 7 | 63 ± 6 | 60 ± 10 |
| **Gender, M/F** | 13/7 ‡ | 9/5 ‡ | 1/12 |
| **FEV\textsubscript{1}, % predicted** | 22 ± 4 †‡ | 107 ± 15 | 108 ± 17 |
| **FEV\textsubscript{1}/FVC** | 25 ± 6 †‡ | 78 ± 6 | 80 ± 6 |
| **RV, % predicted** § | 255 (141) †‡ | 104 (164) | 102 (101) |
| **DL\textsubscript{CO}, % predicted** † | 39 (47) †‡ | 103 (79) | 93 (39) |
| **Pack-years** | 40 (132) | 34 (48) | 0 (0) |
| **Smoking status, current /ex** | 0/20 † | 6/8 | 0/0 |
| **BMI** | 24 ± 3 ‡ | 25 ± 4 | 27 ± 5 |
| **Surgery** | LVRS | TR | TR |

* Lung tissue was used for immunohistochemical analysis of leptin. Values are means ± SD for normally distributed variables and median (range) otherwise; † p<0.05 versus AS; ‡ p<0.05 versus NS; § Values from 1 COPD patient, 4 asymptomatic smokers and 3 never smokers are missing; † Values from 8 COPD patients, 1 asymptomatic smoker and 3 never smokers are missing.

COPD = chronic obstructive pulmonary disease; FEV\textsubscript{1} = forced expiratory volume in 1 second; FVC = forced vital capacity; RV = residual volume; DL\textsubscript{CO} = diffusing capacity of the lung for CO; pack-year = smoking 1 pack per day for 1 year; ex-smoker = quitting smoking for at least 6 months before the start of the study; BMI = body mass index; LVRS = lung volume reduction surgery; TR = tumor resection.

Leptin immunostaining in peripheral lung
Leptin immunostaining was observed in lung tissue of every subject examined. Leptin staining in severe COPD patients was most pronounced in lung epithelium, especially in the cytoplasm of BEC at the apical (luminal) side (Figure 1A). Consistent staining was noted in the cytoplasm of AT-II (TTF-1+) in peribronchiolar and perivascular areas, and in alveolar walls with interstitial fibrosis. Leptin staining was also noted in (infiltrating) inflammatory cells. Intense staining was detected in anthracosis pigment-containing TM (Figure 1D) and a subset of CD68+ AM (Figure 1C). Lymphocytes situated in interstitial lymphocytic infiltrates (Figure 1E) showed weaker and more diffuse staining for leptin. Pulmonary vessels showed no specific staining (Figure 1F). Comparable staining pattern and intensity was observed in current smokers, ex-smokers and never smokers with normal lung function, with one
exception: AM from never smokers mostly showed a membranous staining for leptin, and a less intense cytoplasmic staining (Figure 1G).

Subsequent quantitative analysis of leptin+ cells was performed in BEC, AM and TM on sections sequentially double stained for leptin and CD68, hereby allowing distinguishing between AM and AT-II in alveolar walls. Due to the small amount of cytoplasm of TTF-1+ AT-II (especially in control subjects) combined with the sometimes weak staining in these subjects, TTF-1+/leptin+ double-stained AT-II could not be quantified. As shown in Figure 2, the counts for leptin+ BEC were significantly higher in COPD patients (median 89.3%) and asymptomatic smokers (median 90.7%) as compared to never-smokers (median 80.7%; p<0.05). In addition, severe COPD patients had significantly more leptin-expressing AM (median 77.2%) versus asymptomatic smokers (median 66.3%; p=0.01). The counts for leptin+ AM were even lower in never smokers (median 11.4%; p<0.001). TM containing anthracosis pigment showed 100% positivity for leptin in all subjects examined (including never-smokers). No significant differences were noted between ex-smokers and current smokers or between males and females.

**Leptin and Ob-Rb mRNA expression in cultured primary epithelial cells and cell lines**

To confirm expression of leptin and its functional receptor Ob-Rb in human lung epithelial cells, rtPCR analysis was performed on cultured PBEC, primary AT-II, and the NCI-H292 and A549 cell lines. First, PBEC cultures were isolated from 9 donors and cultured submerged (n=4) or at an ALI (n=5). Basal leptin and Ob-Rb mRNA expression was detected in all PBEC cultures. As the immunodata strongly suggest that smoking itself may induce leptin expression, PBEC cultures were stimulated with increasing doses of CSC. Stimulation of submerged PBEC cultures with 2 AU/ml CSC resulted in increased leptin mRNA expression in 3 out of 4 donors (mean increase 128% of untreated cultures). A dose-response experiment performed in the ALI cultures showed a significant induction of leptin mRNA expression after 24h stimulation with 5-10 AU/ml CSC (Figure 3A); Ob-Rb mRNA expression showed a similar pattern (Figure 3B). Measurements of leptin protein in basal medium by ELISA strengthened the mRNA data, demonstrating constitutive leptin protein production (14.4 pg/ml) and a significant, dose-dependent increase after stimulation with CSC (Figure 3C). Leptin mRNA expression and protein production in ALI cultures showed a strong correlation in the individual samples examined (R=0.941, p<0.001). Second, primary AT-II purified from lungs of lung transplant recipients showed mRNA expression for leptin and Ob-Rb (see online data supplement). Third, both NCI-H292 and A549 cells expressed leptin and Ob-Rb mRNA (data not shown), which is in line with the results of the primary cultures (Figures 3 and E1). Constitutive mRNA expression levels of both leptin and Ob-Rb were approximately 5-fold higher in A549 cells versus NCI-H292 cells. These data suggest that both BEC and AT-II are a source of leptin in the human lung and can potentially respond to leptin due to expression of the functional leptin receptor isoform Ob-Rb.

**Leptin induced STAT-3 phosphorylation in lung epithelial cell lines**

We evaluated functionality of Ob-Rb in NCI-H292 and A549 cells by stimulating cells with leptin and determining the phosphorylation status on tyrosine 705 of STAT-3 by Western blot analysis. Figure 4A shows that STAT-3 is constitutively phosphorylated in NCI-H292 cells, but not in A549 cells. Leptin stimulation induced phosphorylation of STAT-3 in both cell lines, with IL-6 stimulation as positive control and heat-inactivated leptin as negative control. Leptin stimulation induced phosphorylation of STAT-3 in a dose-dependent (Figure 4B) and a time-dependent (Figure 4C) manner in both cell lines. Together, these results show that bronchial epithelial cells and alveolar type II cells can respond to leptin, resulting in activation of leptin’s classical signal transduction pathway JAK/STAT.
DISCUSSION
Leptin is increasingly recognized as a pleiotropic cytokine important in regulating food intake and energy expenditure, reproduction, haematopoiesis, angiogenesis and immune responses. In this light, we previously reported increased circulating leptin levels in emphysema patients related to the systemic pro-inflammatory status and dietary intake.[24] We also demonstrated that leptin protein levels in induced sputum are highly correlated with inflammatory markers (TNF-α and C-reactive protein) in mild-to-moderate COPD patients.[22] More recently, Bruno and colleagues[8] reported leptin expression in proximal bronchial biopsies of smokers with or without COPD. Our present study extends the latter results to the peripheral compartment and investigated expression and localization of leptin on protein and mRNA level in peripheral lung tissue from (ex-)smoking subjects with and without severe COPD, and never smokers. In addition, smoke-induced expression of leptin was investigated using ALI cultures of PBEC. Functionality of leptin signalling was investigated using in vitro cell culture models.

This study provides the first evidence that BEC, AT-II, and macrophages are a source of leptin in human peripheral lung. Immunohistochemical analysis demonstrated a strong cytoplasmic staining for leptin in BEC at the apical site and quantification showed significant differences between never-smokers versus smokers, whereas differences between asymptomatic smokers and severe COPD patients were less obvious. These data strongly suggest that smoking itself induces leptin expression in BEC. Using ALI cultures of PBEC we were able to confirm this hypothesis, showing that increasing doses of CSC induced mRNA expression of leptin and its functional receptor Ob-Rb, and secretion of leptin protein to the basal medium. Our observation that distal bronchiolar epithelium is capable of producing leptin is in line with data from Bruno et al[8], who noted positive immunostaining for leptin protein in central airway epithelium of bronchial biopsies. In contrast, Bruno et al[8] showed decreased leptin+ epithelial cells in smokers and COPD patients versus never smokers, which may be explained by different tissue specimens used in the two studies. As recently demonstrated for TLR4 and beta-2-defensin, central airway epithelium may behave differently from distal airway epithelium in COPD patients.[25] Epithelial leptin expression may be further affected by the inflammatory pattern that is known to be clearly different in central versus peripheral airways.[26] Future studies are necessary to study differences in epithelial cell behaviour throughout the bronchial tree.

In addition to BEC, we noted clear staining for leptin protein in TTF-1+ AT-II. Additionally, leptin mRNA expression was demonstrated in freshly isolated and cultured AT-II from severe COPD patients (see online supplement). Taken together, these data strongly suggest that both BEC and AT-II are a source of leptin in the human lung.

Next to lung epithelium, leptin protein was demonstrated in AM and in interstitial TM. Quantification of leptin+ AM showed an increase in (ex-)smokers versus never-smokers, and a further increase in severe COPD patients. The difference in ratio current versus ex-smokers in the study groups makes it difficult to evaluate whether the differences between groups are to be attributed to presence/absence of COPD or to presence/absence of a subgroup of current smokers. Leptin expression in macrophages was previously described in lymphnodes and acute/active inflammatory lesions in brain and spinal cord in murine experimental autoimmune encephalomyelitis.[27] Whether macrophages are indeed capable of expressing leptin or that immunoreactivity of these macrophages is due to leptin uptake, remains to be studied. Preliminary data indicate however that leptin is detectable in AM in BAL (unpublished results).

Leptin exerts its functions through Ob-Rb, and this study confirms abundant Ob-Rb expression in human total lung, PBEC, primary AT-II, NCI-H292 cells and A549 cells. Moreover, functionality of Ob-Rb signalling in lung epithelial cells was demonstrated by a
time- and dose-dependent increase in phosphorylation of the transcription factor STAT-3 in both A549 and NCI-H292 cells after stimulation with leptin. Our observation that pulmonary leptin expression differs between severe COPD patients, asymptomatic smokers and never smokers, whereas BMI does not, lends further support for local production of leptin in the lung independent of metabolic parameters. The question as to which role leptin fulfils in the lung is therefore of great interest. Many studies indicate potent immunomodulating effects of leptin, which may also be true for pulmonary leptin. Systemic leptin was shown to protect against TNF-α induced toxicity[28], suggesting that leptin is involved in protective mechanisms that allow an organism to cope with potentially autoaggressive effects of its immune system. Herewith in line, studies from Mancuso et al showed that leptin-deficient mice exhibit impaired host defence in bacterial pneumonia.[11,29,30] In contrast, leptin is also known as neutrophil survival factor[8] and intratracheal instillation of leptin caused lung oedema and injury.[21] Besides its possible role in immune-modulation, leptin may also act as growth factor in the lung[19], contributing to epithelial repair and regeneration in COPD.[31] In any case, presence of Ob-Rb on epithelium and immune cells[32] indicates that pulmonary leptin is a potentially important mediator allowing cross-talk between resident lung epithelial cells and immune cells in response to noxious particles.

A limitation to our study is the control groups used. The study groups were carefully matched for gender, age, body mass index and smoking-status (pack-years smoked), which have been recognized as potential confounders[33-35]. In contrast to severe COPD patients, whose lung tissue was obtained during LVRS, all control subjects had primary lung cancer adjacent to the macroscopic normal lung tissue studied, raising the concern that leptin expression may have been directly affected by the adjacent tumor. Although only those specimens without any histological abnormalities (apart from different stages of emphysema and alveolar macrophages with smoker’s pigment) were selected, we cannot exclude that our data are biased by the presence of the tumor. We addressed this limitation by checking gene expression for leptin and Ob-Rb in (cultured) primary lung epithelial cells, in which the influence of a nearby tumor can be excluded, and by performing additional in vitro stimulation experiments using well-characterized bronchial and alveolar epithelial-like cell lines.

In summary, our study provides the first evidence that BEC, AT-II, and macrophages are a source of leptin in human peripheral lung tissue. We demonstrated increased numbers of leptin+ BEC and leptin+ AM in smokers with and without severe COPD as compared to never smokers. In addition, leptin expression in primary BEC could be induced by stimulation with smoke, indicating that leptin may be important in smoking-induced pathology. Furthermore, bronchial and alveolar epithelial cells were shown to express the functional leptin receptor isoform Ob-Rb and to respond to leptin, resulting in activation of the JAK/STAT signalling pathway. Detailed studies are currently in progress to determine functional target genes of leptin signalling in lung epithelial cells, and to elucidate the action of leptin in lung, which may add to understanding the role of lung epithelial cells in the pathogenesis of COPD.
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COMPETING INTERESTS

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FIGURE LEGENDS

Figure 1. Immunohistochemical localization of leptin in peripheral lung specimen. Anti-human leptin (blue) and CD68 antibodies (red) were used to distinguish interstitial macrophages from AT-II in the alveolar walls. (A) Leptin staining in bronchiolar epithelium at the apical side. (B) Strong staining for leptin in AT-II in areas with interstitial fibrosis. (C) Positive leptin staining in a subset of CD68+ alveolar macrophages. (D) Intense leptin staining in anthracosis pigment containing tissue macrophages. (E) Weak, diffuse leptin staining in lymphocytes situated in interstitial lymphocytic infiltrates. (F) Endothelial cells and smooth muscle cells of pulmonary vessels show no leptin staining. (G) Membrane staining for leptin in CD68+ alveolar macrophages of never smokers. (H) Absence of staining for leptin and CD68 when both primary antibodies were omitted. Photographs A-F and H represent immunostaining in very severe COPD patients; comparable staining pattern was observed in controls with normal lung function. Photograph G represents characteristic immunostaining in never smokers. Original magnification ×400.

Figure 2. Quantification of A) leptin+ bronchial epithelial cells (BEC) and B) leptin+ alveolar macrophages (AM) in patients with very severe COPD, asymptomatic (ex-) smokers and never smokers on leptin / CD68 double stained sections. At least 500 cells were counted, and data are expressed per cell type as percentage leptin+ cells of total counted cells. The Mann-Whitney U test was used for statistical analysis.

Figure 3. Leptin and Ob-Rb expression after cigarette smoke condensate (CSC) stimulation of cultured PBEC. PBEC from 5 different donors were cultured at an ALI and stimulated for 48h with CSC (in arbitrary units/ml; AU/ml). Leptin and OB-Rb mRNA expression was determined by rtPCR and levels were corrected using a GeNorm calculated normalization factor based on mRNA expression of three stable reference genes (PPIA, HPRT-1, HSP-90B). CSC stimulation induced A) leptin mRNA expression and B) Ob-Rb mRNA expression in a dose-dependent manner. C) Leptin protein production in supernatants PBEC determined by a commercially available human leptin ELISA responded dose-dependently to CSC stimulation. Values are means ± SD; n = 5. Asterisk represents a statistical significance (p<0.05 versus unstimulated cells; Mann-Whitney U test).

Figure 4. Leptin stimulation induces rapid phosphorylation of STAT-3 in lung epithelial cells. A549 and NCI-H292 cells were stimulated with leptin or interleukin (IL)-6 and whole cell lysates were prepared. Equal amounts of protein were separated by SDS-PAGE and subjected to western blot analysis for determination of phosphorylated (P-) STAT-3 and total STAT-3 protein. A) Illustration of western blot analysis for P-STAT-3 and STAT-3 protein. A549 and NCI-H292 cells were stimulated for 1h with medium alone (Control), 500 ng/ml leptin, 50 ng/ml IL-6 or 500 ng/ml heat-inactivated leptin (leptin<sup>100</sup>; 100°C for 15 min). B) Leptin dose-response curves. A549 and NCI-H292 cells were stimulated for 1h with 20-100-500 ng/ml leptin. C) Leptin time-response curves. A549 and NCI-H292 cells were stimulated with 500 ng/ml leptin for 0.5h, 1h, 2h. Band intensities were determined using Quantity One software (BioRad). Data are expressed as ratios of P-STAT-3 to STAT-3 protein. Values are means ± SD; n = 3. Data shown are representative of 3 independent experiments.
REFERENCES


Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers


Online Data Supplement (1):
Extended Material & Methods
MATERIALS AND METHODS

Selection of subjects

Human lung tissue was obtained from three groups of patients:

1. Lung tissue was obtained from 20 stable COPD patients who underwent lung volume reduction surgery (LVRS). All patients met the GOLD criteria[1] for the diagnosis of very severe COPD (GOLD 4), and were admitted to the pulmonary rehabilitation centre CIRO Horn (Horn, The Netherlands) for six weeks before undergoing surgery. All COPD patients were ex-smokers (quitting smoking for at least 6 months before the start of the study) with a previous history of at least 20 pack-years of smoking. All patients were prescribed combination therapy of inhaled corticosteroids and long-acting β2-agonists, tiotropium, and salbutamol on demand. A history of respiratory diseases other than COPD as well as increased respiratory complaints or respiratory tract infection during 4 weeks preceding the study, were considered as criteria for exclusion.

2. Lung tissue from 27 control subjects with normal lung function (without cough and/or sputum production) was obtained from lung lobes resected for a solitary peripheral tumor in the University Hospital Maastricht and University Hospital Ghent. 6 control subjects were current smokers, 8 were ex-smokers and 13 were never smokers at the time of surgery. For the (ex-) smokers, a smoking history of at least 10 pack-years was used as criterion for inclusion. A history of respiratory diseases (other than lung cancer) was considered as criterion for exclusion.

3. Anonymized lung tissue from 9 patients who underwent a lobectomy or pneumectomy for lung cancer at the Leiden University Medical Center, Leiden, The Netherlands was collected for isolation of human primary bronchial epithelial cells (PBEC).
The study was approved by the medical ethical committees of the University Hospital Maastricht, and the University Hospital Ghent. All subjects gave their informed consent in writing.

**Immunohistochemistry**

Tissue blocks were taken from the subpleural area in the upper lobes (LVRS; n=20 subjects) or from macroscopic normal lung tissue in the subpleural area at appropriate distance from the tumor (tumor resection; n=27 subjects). Paraffin sections (4 µm) were cut and processed for immunohistochemical analysis of leptin, CD68 and Thyroid transcription factor-1 (TTF-1).

Slides were stained for leptin and CD68 (M0876, Dako Cytomation) to identify leptin+ macrophages using a validated protocol for sequential double staining. Immunohistochemical staining for leptin was performed according to Löffler et al.[2] with slight modifications. Immunoreactive epitopes of leptin were exposed by treatment with 0.05% Pronase E in 0.5M Tris/HCl, pH 7.6 and non-specific binding was blocked with 5% BSA in TBS. Leptin was detected using a rabbit polyclonal antibody (SC-842, Santa Cruz Biotechnology, Santa Cruz, CA) followed by biotin-conjugated goat anti-rabbit IgG antibody (E-0431, Dako Cytomation, Glostrup, Denmark). After applying alkaline-phosphatase labelled avidin-biotin complex (ABC-AP, K-0376, Dako Cytomation), enzymatic reactivity was visualized using Blue substrate kit III (SK-5300, Vector Laboratories, Burlingame, CA). After immunostaining for leptin, sections were again blocked, incubated with anti-CD68 followed by biotin-conjugated rabbit anti-mouse IgG antibody (E-0413, Dako Cytomation) and ABC-AP. Enzymatic reactivity was visualized using Red substrate kit I (SK-5100, Vector Laboratories).

For identification of leptin+ type II pneumocytes, slides were stained for leptin and TTF-1 (MS-699, Lab Vision, Fremont USA), which is specific for epithelial cells of the lung and
thyroid gland and gives rise to an intense nuclear staining. TTF-1 immunoreactive epitopes were unmasked by microwave heating in Tris-EDTA buffer (Klinipath, Duiven, The Netherlands). After applying the primary antibody, biotin-conjugated rabbit anti-mouse IgG antibody (E-0413, Dako Cytomation) and horse radish peroxidase labelled avidin-biotin complex (ABC-HRP, K-0376, Dako Cytomation), the enzymatic reactivity was visualized using Liquid DAB+ substrate (K3467, Dako Cytomation). After TTF-1 immunostaining, sections were stained for leptin as described and enzymatic reactivity was visualized using Blue substrate kit III.

To obtain a clear interpretation and to check for non-crossreactivity after consecutive staining, the results of single immunostaining were evaluated and compared with those from double labeling. Staining controls were performed by omitting the primary antibody of either the first or the second incubation, or both, whereas secondary antibodies and both substrates were always applied. Double labelling was only visible when both primary antibodies were applied. Furthermore, specificity of leptin staining was confirmed by preincubating the primary antibody with recombinant human leptin (R&D systems, Minneapolis, USA), which resulted in complete blocking of the staining.

Cytoplasmic immunostaining for leptin was examined in bronchial epithelial cells (BEC), tissue macrophages (anthracosis pigment containing mononuclear cells with well represented cytoplasm located in the lung interstitium) and alveolar macrophages (AM) using a light microscope (Leica DMRB, Leica Microsystems, Rijswijk, The Netherlands) at a 20x magnification in blinded fashion. To quantify leptin expression in BEC, CD68+ tissue macrophages and CD68+ AM, only cells with cytoplasmic immunostaining for leptin were counted as leptin+. Membranous-stained cells with no cytoplasmic staining are likely to have solely bound extracellular leptin, and were therefore scored as leptin negative. Five to ten fields were randomly selected for each section and at least 500 cells were counted by one
independent observer unaware of the clinical data. Leptin counts are expressed per cell type as percentage positive cells of total counted cells.

**Cell culture**

*Human Primary Bronchial Epithelial Cells.* Subcultures of primary bronchial epithelial cells (PBEC) were obtained from resected lung tissue from 9 subjects by enzymatic digestion, passaged by trypsinization and cultured either submerged (n=4) or at an air-liquid interface (ALI; n=5) as previously described.[3,4] To establish an ALI, cells were grown submerged for 4-7 days on semi-permeable Transwell membranes precoated with a mixture of 10 µg/ml BSA, 30 µg/ml Vitrogen (30 µg/ml Celtrix Laboratories, Palo Alto, CA) and 10 µg/ml fibronectin in phosphate buffered saline (PBS), after which they were exposed to an ALI for another 2 weeks prior to stimulation. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cigarette smoke condensate (CSC) was prepared using commercial cigarettes (Caballero, British American Tobacco Group) as described previously.[5] The concentration was calculated by measuring the OD value of the 100-fold diluted solution at a wavelength at which the maximal absorbance was detected, and expressed as arbitrary units (AU) per ml (maximal absorbance (ODmax) x 2 x 100 (dilution factor)). Submerged cultures were stimulated with 2 AU/ml CSC for 24h. ALI cultures were stimulated with various concentrations of CSC (1-5-10 AU/ml) at the apical side for 48h and basal medium was stored at -20°C until further analysis. From both submerged and ALI cultures RNA was isolated and stored at -80°C until further use.

*A549 and NCI-H292 cell lines.* The human lung adenocarcinoma cell line A549 and the human mucoepidermoid carcinoma cell line NCI-H292 were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 (Gibco)
supplemented with 10% fetal bovine serum (FBS, Biochrome), 2 mM L-glutamine (Invitrogen), 50U/ml penicillin and 50µg/ml streptomycin at 37 °C with 5% CO2 in humidified air.[6] Cells were plated at 5.65 x 10^4/cm² and 18-24 hours prior to stimulation the concentration of serum was reduced to 0.5% FBS. Cells were stimulated for 0.5h-1h-2h in 0.5% FBS containing medium: a) without any additions; b) 20-100-500 ng/ml recombinant human leptin (R&D systems, Minneapolis, USA); c) 50 ng/ml recombinant human IL-6 (PeproTech). Cell lysates were prepared and stored at –80°C until rtPCR or western blotting analyses.

**Quantitative real-time PCR**

RNA was isolated from cultured primary cells using the RNeasy Microkit/Minikit including a DNase I treatment (QIAGen Inc. CA). Total RNA (0.5 µg) was reverse transcribed into complementary DNA using the Reverse-iT 1st strand Synthesis Kit and oligo dT primers (Abgene, Epsom, UK). The primer pairs (designed with Primer Express 2.0 Software; Applied Biosystems, Foster City, USA) are listed in Table 1. Primers for the functional leptin receptor Ob-Rb were designed in the 3’ side of the transcript, hereby excluding amplification of the other leptin receptor isoforms. Real-time PCR reactions were performed in duplicate using diluted cDNA template (dilution 1:5 for leptin and 1:25 for other genes), 0.6 pmol of each primer (Table 1) and IQ™ SYBR® Green Supermix I dye (Biorad, Hercules, California) in a total volume of 20 µl. A standard curve derived from the serial dilutions of a mixture of all samples was included on each plate, allowing data processing using the standard curve based method. Quantitation and real time detection of the PCR products was followed on an iCycler iQ Real-Time PCR system (BioRad) with the following cycling conditions: 3 min at 95°C for Platinum Taq activation and 40 cycles for the melting (15 sec, 95°C) and
annealing/extension (45 sec, 60°) steps. These conditions generate specific PCR products of the desired length, which was verified by gel electrophoresis on an ethidium bromide-stained 2% agarose gel and by nucleotide sequencing. Expression of target genes (leptin, Ob-Rb) was corrected by a normalization factor that was calculated based on expression of three reference genes (PPIA, HPRT-1, HSP-90B) using the geNorm applet ([http://medgen.ugent.be/~jvdesomp/genorm/](http://medgen.ugent.be/~jvdesomp/genorm/)) according to the guidelines and theoretical framework previously described.[7]

**TABLE 1**

Accession numbers* and oligonucleotide sequences of primers used for real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sequences</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>NM_000230</td>
<td>Forward: 5’-CGGAGAGTACAGTGAGCCAAGA-3’</td>
<td>67 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CGGAATCTCGCTCTGTCATCA-3’</td>
<td></td>
</tr>
<tr>
<td>Ob-Rb</td>
<td>NM_0002303</td>
<td>Forward: 5’-ACAAGTCAAGGGTATCGTGCC-3’</td>
<td>299 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-TTCTTTTCTCTCTCCCCACCCA-3’</td>
<td></td>
</tr>
<tr>
<td>PPIA</td>
<td>NM_021130</td>
<td>Forward: 5’-CTCGAATAAGTTTGACTTGTGTT-3’</td>
<td>164 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CTAGGCATGGGAGGGAACA-3’</td>
<td></td>
</tr>
<tr>
<td>HPRT-1</td>
<td>NM_000194</td>
<td>Forward: 5’-AGAATGTCTTGGATTGGAAGA-3’</td>
<td>96 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-ACCTTGACCATCTTTGGATTA-3’</td>
<td></td>
</tr>
<tr>
<td>HSP-90B</td>
<td>NM_007355.2</td>
<td>Forward: 5’-CCCCTGCTGGTGATGTAGTAGT-3’</td>
<td>106 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CCAATCTGCTGTAAGATGAG-3’</td>
<td></td>
</tr>
</tbody>
</table>

* According to GenBank

Ob-Rb = leptin receptor functional b isoform; PPIA = cyclophilin A / peptidylprolyl isomerase A; HPRT-1 = hypoxanthine phosphoribosyltransferase 1; HSP-90B = heat shock 90 kDa protein 1 beta.
Western blotting

Adherent cells were washed in PBS, and whole cell lysates were prepared as described.[8] In brief, cells were scraped in lysis buffer (20mM Tris pH 7.4, 150mM NaCl, 1% (v/v) Nonidet P-40, 1mM DTT, 1mM Na3VO4, 1mM PMSF, 10mg/ml leupeptin and 1% (v/v) aprotinin). Lysates were cleared by centrifugation at 16,000 g for 30 min. Protein contents in cell lysates were determined using Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA). Twenty-five µg (Phospho-STAT-3) or 2.5 µg (Total STAT-3) protein/lane was separated by SDS-PAGE under reducing conditions. After transfer to nitrocellulose (Bio-Rad), blots were blocked with 5% (w/v) non-fat dry milk in TBS Tween 20 (0.05% v/v), followed by overnight incubation at 4°C with a polyclonal antibody specific for STAT-3 (#9132, Cell Signaling, Beverly, MA) or a monoclonal antibody specific for phospho-STAT3 (#9138, Cell Signaling). Immunoreactive proteins were visualized using HRP-conjugated secondary antibodies (Vector Laboratories) and visualized by Supersignal WestPico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The data were digitalised and quantified by densitometric analysis.

Statistical analysis

Results are presented as mean ± standard deviation (SD) for normally distributed variables and median (range) otherwise. Non-parametric data were compared by Kruskal-Wallis test followed by Mann-Whitney U test as appropriate. The Chi-square test was used to compare categorical variables. Correlations between parameters were evaluated using Pearson’s rank correlation analysis (Statistical Package for the Social Sciences, version 13.0 for Windows, SPSS Inc., Chicago, IL). A p-value <0.05 denotes the presence of a significant statistical difference.
References


Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers


Online Data Supplement (2):

Leptin and Ob-Rb mRNA expression in primary type II pneumocytes
Lung tissue from 3 lung transplant recipients who underwent lung transplantation at the University Hospital Gasthuisberg Leuven (2 males, 1 female) was used to isolate and purify primary type II pneumocytes (AT-II) for *in vitro* cell stimulation experiments. The 3 lung transplant patients (age 61 ± 4 years) were diagnosed with severe COPD with a mean FEV$_1$ of 32 ± 7% predicted, RV of 177 ± 52% predicted and DLCO of 28 ± 6% predicted. All lung transplant patients were ex-smokers with 37 ± 6 pack-years. The study was approved by the medical ethical committee of the Faculty of Medicine of K.U. Leuven / University Hospital Gasthuisberg Leuven. All subjects gave their informed consent in writing.

AT-II were isolated from explant lung tissue (dissected free of pleura, visible vessels and bronchi; n=3 subjects) as described previously.[1-3] RNA was isolated from freshly isolated AT-II and unstimulated primary cells cultured for 48h and stored at -80°C until further use. rtPCR analysis was performed on primary AT-II to confirm expression of leptin and its functional receptor Ob-Rb. Freshly isolated primary AT-II showed constitutive mRNA expression for leptin (Figure E1A) that tended to decrease in primary cells that were cultured for 48 hours. This may suggest loss of typical type II cell characteristics due to culture, which is also known for the xenobiotic-metabolising activity of AT-II.[4] On the other hand, the constitutive Ob-Rb mRNA expression in freshly isolated primary AT-II (Figure E1B) tended to increase after *in vitro* culture.

*Figure E1.* A) Leptin and B) OB-Rb mRNA expression determined by rtPCR in freshly isolated (F) and cultured for 48h (C) type II pneumocytes (biological duplicates) from 3 different severe COPD patients undergoing lung transplantation. mRNA expression levels for leptin and Ob-Rb were corrected using a GeNorm calculated normalization factor based on mRNA expression of three stable reference genes (PPIA, HPRT-1, HSP-90B).
References


Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers

Juanita HJ Vernooy, Nadja EA Drummen, Robert Jan Van Suyle, Roy HE Cloots, Gertrude M Moller, Ken R Bracke, Suzanne Zuyderduyn, Mieke A Dentener, Guy G Brusselle, Pieter S Hiemstra and Emiel FM Wouters

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