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*J Immunol* 2009; 182:684-691; doi: 10.4049/jimmunol.182.1.684
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Adiponectin and Functional Adiponectin Receptor 1 Are Expressed by Airway Epithelial Cells in Chronic Obstructive Pulmonary Disease

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We screened bronchoalveolar lavage (BAL) fluids from COPD-E (chronic obstructive pulmonary disease-Emphysema) and control subjects using a 120 Ab cytokine array and demonstrated that adiponectin was highly expressed in BAL in COPD-E. An adiponectin ELISA confirmed that adiponectin was highly expressed in BAL in COPD-E compared with smokers and healthy control subjects. Immunohistochemistry studies of lung sections from subjects with COPD-E demonstrated that airway epithelial cells expressed significant levels of adiponectin and adiponectin receptor (AdipoR) 1 but not AdipoR2. In vitro studies with purified populations of human lung A549 epithelial cells demonstrated that they expressed both adiponectin and AdipoR1 (but not AdipoR2) as assessed by RT-PCR, Western blot, and immunohistochemistry. Lung A549 epithelial AdipoR1 were functional as incubation with adiponectin induced release of IL-8, which was inhibited by small interfering RNA to AdipoR1. Using a mouse model of COPD, tobacco smoke exposure induced both evidence of COPD as well as increased levels of adiponectin in BAL fluid and increased adiponectin expression by airway epithelial cells. As adiponectin expression in adipocytes is dependent upon NF-κB, we determined levels of adiponectin in tobacco smoke exposed CC10-Cre/Rosa26/flox/flox mice (deficient in the ability to activate NF-κB in airway epithelium). These studies demonstrated that CC10-Cre/Rosa26/flox/flox and wild-type mice had similar levels of BAL adiponectin and airway epithelial adiponectin immunostaining. Overall, these studies demonstrate the novel observation that adiponectin and functional AdipoR1 are expressed by lung epithelial cells, suggesting a potential autocrine and/or paracrine pathway for adiponectin to activate epithelial cells in COPD-E.
mainly full length adiponectin (12). Adiponectin receptors are predicted to be seven transmembrane spanning receptors. However, the topology of AdipoR is opposite to all other G-protein coupled receptors with N terminus internal and the C-terminal external (11). Activation of AdipoR1 and/or AdipoR2 by adiponectin stimulates the activation of intracellular cytoplasmic signaling kinases (including AMP-activated protein kinase and p38 MAPK), the nuclear transcription factor peroxisome proliferators activated receptor-α, as well as NF-κB and AP-1 (6).

In this study, we have made several novel observations regarding adiponectin including the demonstration a) that adiponectin is not only expressed in adipocytes, but is also highly expressed in airway epithelium, b) that not only adiponectin but also adiponectin receptors are expressed by airway epithelial cells, c) that AdipoR1 on lung epithelial cells are functional and release IL-8 upon incubation with adiponectin, and d) using CC10CreER1/IIκκBΔ mouse (deficient in the ability to activate NF-κB in airway epithelium) we demonstrated that adiponectin expression in airway epithelium in vivo is NF-κB independent, which differs from results of studies in adipocytes in which expression of adiponectin is NF-κB dependent (13–15). Finally, we demonstrated that adiponectin is highly expressed in the lungs of human subjects with the emphysematous form of COPD (COPD-E). In contrast, exposure to tobacco smoke in subjects without COPD and control subjects were recruited as part of a National Institutes of Health Disease Research Institute or from subjects who underwent lung resection as part of their routine clinical care in protocols approved by the University of California-San Diego Human Subjects Protection Committee as previously described (16). Subjects with COPD had lung histology evidence of COPD-E, whereas control subjects without COPD had no lung histology evidence of COPD. Immunohistology was performed on paraffin embedded lung sections with primary Abs including anti-adiponectin (R&D Systems), anti-adiponectin-R1, and anti-adiponectin-R2 (Phoenix Pharmaceuticals) as well as species- and isotype-matched control Abs using the immunoperoxidase method as previously described in this laboratory (16).

Materials and Methods

COPD and control subjects

Study subjects with a chest computerized tomography (CT) scan diagnosis of COPD-E (n = 16) and fifteen non-COPD control subjects with a normal chest CT scan (nonsmokers n = 7 or current smokers n = 8) underwent bronchoscopy to determine their levels of biomarkers in bronchoalveolar lavage (BAL) fluid in a protocol approved by the University of California-San Diego Human Subjects Protection Committee. The COPD-E study and control subjects were recruited as part of a National Institutes of Health Biomarker study, and their clinical characteristics have previously been described in detail (16). In brief, subjects with COPD-E were recruited based on chest CT scan evidence of emphysema, which had to be present to be included in the study, while control subjects had to have a normal chest CT scan (16). Subjects also completed pulmonary function studies, the medical history questionnaires (history of chronic cough, sputum production, dyspnea), and a standardized COPD questionnaire (i.e., St. George Questionnaire) (16). The COPD-E cohort we enrolled were all subjects with GOLD Stage Iib moderate COPD (forced expiratory volume in 1 s 30–50%) with chest CT scan evidence of significant emphysema (16). As previously reported, COPD-E subjects had a significantly higher level in BAL of mediators noted to be elevated in COPD including myeloperoxidase, LTBA, and IL-8 when compared with either the non-smoking group or the current smoker group (16). Normal individuals were nonsmokers who had no evidence of disease on history and physical examination, and had a normal chest CT scan, as well as normal pulmonary function. Current smokers (≥20 pack year) who had no evidence of disease on history and physical examination, and had a normal chest CT scan, as well as normal pulmonary function were also recruited (16). All the study subjects had blood cotinine levels to verify their smoking status. None of the study subjects used oral or inhaled corticosteroids during the 6 mo before entering the study. Study subjects were allowed to use short-acting bronchodilators and were in a clinically stable condition.

Screening to detect cytokines in BAL in COPD using a cytokine array

We used a 120 human cytokine Ab array (RayBiotech) to screen BAL fluid from subjects with COPD-E and controls (16). The RayBio human 120 cytokine Ab array membranes were incubated with BAL samples overnight at 4°C. After washing, the cytokine Ab array membranes were incubated with biotin-conjugated Abs for 2 h at room temperature. The cytokine Ab array membranes were then washed and incubated with HRP-conjugated streptavidin and were exposed to x-ray film for detection. The cytokine Ab array membranes were scanned and the intensity of the positive cytokine spots on the membrane quantitated relative to positive and negative controls with ImageJ software (National Institutes of Health, Bethesda, MD).

Measurement of adiponectin in BAL in COPD and control study subjects by ELISA

Levels of human adiponectin were assayed in BAL fluid using an ELISA (R&D Systems) with a sensitivity of 15.6 pg/ml.

Immunohistochemistry to detect adiponectin and adiponectin receptor expression in lungs from COPD subjects

Lung tissue samples were obtained from a different cohort of subjects with COPD (n = 10) and without COPD (n = 5) from either the National Disease Research Institute or from subjects who underwent lung resection as part of their routine clinical care in protocols approved by the University of California-San Diego Human Subjects Protection Committee as previously described (16). Subjects with COPD had lung histology evidence of COPD-E, whereas control subjects without COPD had no lung histology evidence of COPD. Immunohistology was performed on paraffin embedded lung sections with primary Abs including anti-adiponectin (R&D Systems), anti-adiponectin-R1, and anti-adiponectin-R2 (Phoenix Pharmaceuticals) as well as species- and isotype-matched control Abs using the immunoperoxidase method as previously described in this laboratory (16).

RT-PCR, immunohistochemistry, and Western blot to detect adiponectin and adiponectin receptor expression in lung epithelial cells

Human A549 lung epithelial cells (ATCC) were used to determine whether purified populations of lung epithelial cells express adiponectin and/or adiponectin receptor mRNA (RT-PCR) and protein (immunohistochemistry, Western blot). Cultured A549 lung epithelial cells were incubated in 24-well plates with growth arrest media for 12 h. Growth arrested A549 lung epithelial cells were then stimulated for either 6 (for RNA detection) or 24 h (for protein detection) with 100 ng/ml of recombinant human TNF-α (R&D Systems). The epithelial cells were then used for either immunohistochemistry, Western blot, or RT-PCR to detect adiponectin and adiponectin receptor proteins. Lung epithelial cell supernatants were assayed for adiponectin by ELISA. Unstimulated A549 lung epithelial cells served as controls.

For RT-PCR studies, total cellular RNA was isolated from A549 lung epithelial cells using TRizol reagent (Invitrogen) and reverse transcribed into cDNA according to the manufacturer’s instructions. The expression of adiponectin, AdipoR1, and AdipoR2 was conducted using RT-PCR primers and conditions as previously described (17). Human adipose tissue cDNA (BioChain), which is known to express adiponectin, AdipoR1, and AdipoR2, was used as a control.

For Western blots, whole A549 cell lysates (TNF-stimulated or unstimulated) were prepared using a lysis buffer (Invitrogen). The cell lysates were separated by electrophoresis on a 10% SDS-PAGE. The gels were transferred to a polyvinylidene difluoride membrane (Invitrogen), which was blocked in 5% nonfat dry milk diluted in Tris-buffered saline. Blots were incubated with either anti-adiponectin (R&D Systems), anti-AdipoR1, or anti-AdipoR2 (both AdipoR Abs from Abcam) at 4°C overnight. Washed blots were incubated for 60 min with a secondary biotinylated Ab (Vector Laboratories), washed and incubated with HRP (1/200) (R&D Systems), followed by diaminobenzidine detection. Equivalent loading of lanes in gels was confirmed by anti-GAPDH (Sigma-Aldrich).

Effect of adiponectin on lung epithelial IL-8 expression

To determine whether the AdipoR1 receptors detected on lung epithelial cells were functional, A549 lung epithelial cells were incubated with or without adiponectin (0.01, 0.5, and 1 μg/ml) for 24 h and levels of IL-8 determined in supernatants by ELISA (R&D Systems) having a sensitivity of 32.5 pg/ml.

Small interfering RNA (siRNA) inhibition of AdipoR1 function in lung epithelial cells

To determine whether inhibiting AdipoR1 would inhibit adiponectin induced IL-8 release from lung epithelial cells, A549 cells were seeded at 4 × 10⁵ cells per well in 24-well plates and 24 h after subculture were transfected with 15 pmol AdipoR1 siRNA using Lithofectamine (Invitrogen) according to the manufacturer’s instructions. The AdipoR1 sense siRNA sequence was 5’-GGACAAGAGCUAUCCGUAT3’. Silencer negative control siRNA was used to demonstrate that the transfection does
not induce nonspecific effects on gene expression. Both siRNA were obtained from Ambion. Following transfection, A549 cells were cultured for 72 h and then serum starved for 12 h. A549 cells were then cultured in the presence or absence of adiponectin (1 µg/ml) for 24 h, and supernatants were assayed for IL-8 by Elisa.

**AP-1 activation assay**

As adiponectin induces activation of the transcription factor AP-1 (comprised of c-Fos and Jun protein families) (18), we investigated whether adiponectin induced activation of c-Fos, c-Jun, and JunB in the A549 human lung epithelial cell line. In these experiments, we incubated A549 epithelial cells with adiponectin (1 µg/ml) for 4 h and measured c-Fos, c-Jun, and JunB nuclear binding activity in nuclear extracts using a TransAM AP-1 ELISA kit (Active Motif) according to the manufacturer’s protocol.

**Mouse model of COPD demonstrating adiponectin expression in airway epithelium**

To induce COPD, different groups of mice were exposed for 6 mo to either tobacco smoke (n = 19 mice) or room air (n = 20 mice). Mice (C57BL/6 8-wk-old females) were subjected to chronic tobacco smoke exposure (main stream tobacco smoke from two cigarettes/day, 5 days/wk) generated by burning 28g of reference cigarettes (2.45 mg nicotine per cigarette; purchased from Tobacco Research Institute) using a smoking machine (Mchesney-Jaeger CSM-SSM Single Cigarette Machine; CH Technologies USA) regulated by programmable controls provided with JASPER Windows 9×2000 software over RS-232 communication ports (CH Technologies USA). The smoking machine settings delivered a puff volume of 35 ml, a frequency of one puff every 25 s, a total breathing time for one cigarette of 5 min, with no resting time between two cigarettes. The volume of the smoke chamber was 500 ml (3). The mice were exposed to the tobacco smoke in a 12 port nose-only directed flow inhalation exposure system. The number of neutrophils was quantitated in Wright-Giemsa stained lung sections with an image analysis system (Image-Pro Plus; Media Cybernetics). The extent of COPD was assessed by quantitating the linear intercept (Lm) in lung sections stained with H&E. The Lm, a measurement of the distance between the opposing walls of a single alveolus (21), was quantitated in µm using a light microscope (Leica DMLS; Leica Microsystems) attached to an image-analysis system (Image-Pro Plus; Media Cybernetics). In each mouse the Lm was quantitated in 10 alveoli/field, in each of 5 random lung fields, at ×20 magnification.

**Effect of NF-κB on adiponectin expression in airway epithelium**

As adiponectin expression in adipocytes is NF-κB dependent (13–15), we examined whether mutant mice deficient in their ability to activate NF-κB in airway epithelium would also be deficient in the ability to express adiponectin in airway epithelium. In these studies, we used CC10-Creβ/β Ikkβ+/−/− mice in which NF-κB signaling through IκB kinase β (IKKβ) is selectively ablated in the airway epithelium by conditional Cre-recombinase expression from the Clara cell (CC10) promoter (22). Such mice are unable to activate NF-κB in airway epithelial cells but are able to activate NF-κB in alveolar epithelial cells as well as in nonairway epithelial cells (i.e., peribronchial inflammatory cells) (22). In these experiments CC10-Creβ/β Ikkβ+/−/− littermate control mice (n = 12 mice/group) were exposed for 6 mo to tobacco smoke as described above. Levels of adiponectin were quantitated in BAL fluid by ELISA and the area of adiponectin immunostaining assessed in lung airway epithelial cells by immunohistochemistry and image analysis. Lungs from the different experimental groups were processed as a batch for immunostaining under identical conditions. Immunostained slides were all quantitated under identical light microscope conditions, including magnification (×20), gain, camera position, and background illumination. The area of epithelial adiponectin immunostaining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS) attached to an image analysis system (Image-Pro plus) as previously described (22). Results are expressed as the area of adiponectin staining per µm length of basement membrane of bronchioles 150–200 µm of internal diameter. FIGURE 1. Levels of adiponectin were quantitated in BAL fluid derived from subjects with COPD-E (n = 16), smokers without COPD (n = 8), and healthy controls (n = 7) by ELISA. COPD-E subjects had significantly higher levels of adiponectin compared with healthy normal controls (p = 0.03). Smokers without COPD had significantly reduced levels of BAL adiponectin (p = 0.003).

**Statistical analysis**

Results in the different groups were compared by ANOVA using the non-parametric Kruskal-Wallis test followed by post-testing using Dunn’s multiple comparison of means. All results are presented as mean ± SEM. A statistical software package (Graph Pad Prism) was used for the analysis. The p values of <0.05 were considered statistically significant.

**Results**

Detection of cytokines in BAL in COPD using a cytokine Ab array membrane

As anticipated the cytokine Ab array detected cytokines already known to be highly expressed in BAL in COPD (i.e., IL-8, MCP-1, TNF-α). However, interestingly, one of the most highly expressed BAL cytokines detected by the array was adiponectin, a cytokine not previously reported to be expressed in lung diseases or COPD. The level of BAL adiponectin expression detected by the cytokine array was 3.45 times higher in BAL fluid in COPD compared with levels noted in healthy nonsmokers.

**Measurement of adiponectin in BAL in COPD and control study subjects by ELISA**

To confirm the cytokine array data we used an ELISA to quantitate levels of adiponectin in BAL samples of COPD-E and control subjects (Fig. 1). COPD-E subjects had significantly higher levels of adiponectin in BAL compared with healthy normal controls who were nonsmokers (6.2 ± 1.8 vs 3.3 ± 0.6 ng/ml adiponectin, p = 0.03). Interestingly, in contrast to COPD-E subjects who had increased levels of BAL adiponectin, current smokers without COPD had significantly reduced levels of BAL adiponectin (6.2 ± 1.8 vs 0.2 ± 0.1 ng/ml adiponectin, p = 0.003).

**Immunohistochemistry demonstrating epithelial adiponectin expression in COPD subjects**

To address which lung cells express adiponectin, lung tissue samples were obtained from a different cohort of COPD-E patients and non-COPD control subjects. Immunostaining of lung sections from subjects with COPD-E demonstrated significant expression of adiponectin, which was predominantly noted in airway epithelial cells (Fig. 2A). Significant adiponectin expression was not noted in the lungs from non-COPD controls. We also examined the expression of AdipoR1 and AdipoR2 in lung sections of COPD-E patients and control subjects. Airway epithelial cells of COPD-E subjects had significant levels of expression of AdipoR1 compared...
with non-COPD control subjects (Fig. 2B). We did not detect significant levels of AdipoR2 expression in lung sections from either COPD-E or non-COPD control subjects (Fig. 2C). There was no nonspecific staining of lung tissue with either anti-adiponectin, anti-AdipoR1, or anti-AdipoR2 isotype control Abs (Fig. 2).

RT-PCR, immunohistochemistry, and Western blot demonstrating adiponectin and adiponectin receptor expression in pure populations of A549 lung epithelial cells

To determine whether purified populations of lung epithelial cells expressed adiponectin and adiponectin receptors, we used the A549 airway epithelial cell line. A549 lung epithelial cells stimulated with TNF expressed significant levels of adiponectin as assessed by immunohistochemistry (Fig. 3A), RT-PCR (Fig. 3D), and Western blot (Fig. 3E). Unstimulated A549 lung epithelial cells did not express significant adiponectin (Fig. 3A).

We also used immunohistochemistry, Western blots, and RT-PCR to determine whether adiponectin receptors (AdipoR1 and AdipoR2) were expressed by A549 lung epithelial cells. AdipoR1 was strongly expressed by stimulated A549 lung epithelial cells as assessed by immunohistochemistry (Fig. 3B), Western blot (Fig. 3E), and RT-PCR (Fig. 3D). Unstimulated A549 lung epithelial cells did not express AdipoR1 (Fig. 3B). In contrast to the expression of AdipoR1 by A549 lung epithelial cells, neither stimulated nor unstimulated A549 lung epithelial cells expressed AdipoR2 (Fig. 3, C–E).

Adiponectin receptors expressed on lung epithelial cells are functional

To determine whether adiponectin receptors expressed by airway epithelial cells are functional, we incubated A549 cells with different concentration of globular adiponectin and quantitated levels of the epithelial expressed chemokine IL-8. A549 cells had dose-dependent expression of IL-8 in response to stimulation with 0.01 µg/ml adiponectin ($p = 0.05$ vs control), 0.5 µg/ml adiponectin ($p = 0.04$ vs control), and 1 µg/ml adiponectin ($p < 0.001$ vs control) (Fig. 4A).

siRNA inhibition of AdipoR1 function in lung epithelial cells

To confirm the role of AdipoR1 in mediating IL-8 release from A549 lung epithelial cells, we inhibited AdipoR1 by using RNA interference. In the absence of siRNA, adiponectin induced significant release of IL-8 from A549 cells (397.1 ± 15.5 pg/ml vs 212.4 ± 6.3 pg/ml) (adiponectin vs no adiponectin) ($p = 0.05$). A549 cells transfected with AdipoR1 specific siRNA, and then stimulated with adiponectin, had significantly reduced levels of IL-8 (204.8 ± 7.3 pg/ml) compared with A549 cells stimulated with adiponectin alone in the absence of transfection (397.1 ± 15.5 pg/ml) ($p = 0.02$), or compared with A549 cells transfected with control siRNA and stimulated with adiponectin (400.2 ± 11.9 pg/ml) ($p = 0.02$) (Fig. 4B).

Mouse model of COPD demonstrating adiponectin expression in airway epithelium

To determine whether exposure to tobacco smoke modulated levels of airway adiponectin, we exposed wild-type (WT) mice to tobacco smoke for either 1 mo (a time period that is not sufficient to induce emphysema in mice) or 6 mo (a time period that induces emphysema). WT mice exposed to tobacco smoke for 1 mo did not have evidence of emphysema as assessed by measurement of the linear intercept ($L_m$) (data not shown). In contrast, WT mice exposed to
tobacco smoke for 6 mo had significant evidence of emphysema characterized histologically by destruction of the alveolus (Fig. 5A) as well as by measurement of a significantly increased Lm (533 ± 0.5 vs 28.7 ± 0.5 μm) (WT mice exposed to tobacco smoke for 6 mo vs WT mice no exposure to tobacco smoke for 6 mo) (p < 0.005) (Fig. 5B).

WT mice exposed to tobacco smoke for 6 mo had a significant increase in BAL neutrophils compared with WT mice that were not exposed to tobacco smoke (916.2 ± 0.6 pg/ml vs 0 ± 0 pg/ml) (p = 0.05) and MIP-2α (17.0 ± 1.5 pg/ml vs 1.3 ± 0.8 pg/ml) (p < 0.0001) were also significantly higher in WT mice exposed to 6 mo tobacco smoke compared with WT mice that were not exposed to tobacco smoke (Fig. 5, D and E, respectively).

WT mice exposed to tobacco smoke for 1 mo had reduced levels of BAL adiponectin compared with nontobacco smoke exposed control WT mice (0.6 ± 0.1 vs 1.1 ± 0.2 ng/ml BAL adiponectin) (p = 0.001) (Fig. 5F). In contrast, WT mice that were exposed to tobacco smoke for 6 mo had significantly higher levels of BAL adiponectin compared with nontobacco smoke exposed control WT mice (4.2 ± 0.9 vs 1.1 ± 0.2 ng/ml BAL adiponectin) (p < 0.0005) (Fig. 5F) and also demonstrated significantly increased levels of expression of adiponectin in lung epithelial cells as assessed by immunohistochemistry (Fig. 5G). In contrast, nontobacco smoke exposed WT mice or WT mice exposed to short-term tobacco smoke exposure for 1 mo did not have significant levels of expression of adiponectin in airway epithelium as assessed by immunohistochemistry (data not shown).

**FIGURE 3.** The human lung epithelial cell line A549 was stimulated with 100 ng/ml TNF-α for 24 h, and then immunostained with either an anti-adiponectin (A), anti-AdipoR1 (B), or anti-AdipoR2 (C) Ab. mRNA was isolated from the A549 cells stimulated with 100 ng/ml TNF-α for 6 h, and RT-PCR was performed with primer sets for adiponectin, AdipoR1, and AdipoR2 (D). Lane 1 is adipocyte tissue that expresses adiponectin, AdipoR1, and AdipoR2. Lane 2 is the unstimulated A549 cells, and lane 3 is the A549 cells stimulated with 100 ng/ml TNF-α for 6 h that express adiponectin and AdipoR1, but not AdipoR2. Western blot (E) of control and TNF-stimulated A549 cells detected with either anti-adiponectin, anti-AdipoR1, anti-AdipoR2, or control anti-GAPDH Ab.

**Adiponectin expression in airway epithelium in a mouse model of COPD-E is largely NF-κB independent**

Both WT mice (p = 0.03, WT tobacco smoke vs WT room air) and CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> mice (p = 0.01, CC10-Cre<sup>+/+</sup>/IκkB<sup>Δ/Δ</sup> tobacco smoke vs CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> room air) that were exposed to tobacco smoke for 6 mo had significantly increased levels of BAL adiponectin compared with nontobacco smoke exposed control mice (Fig. 6A). Although CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> mice exposed to tobacco smoke for 6 mo had a slight decrease in BAL adiponectin compared with WT mice this was not statistically significant (Fig. 6A). Similar results were noted with immunohistochemistry demonstrating significant adiponectin expression in both CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> and WT mice exposed to tobacco smoke for 6 mo (Fig. 6, B and C). Image analysis quantification of the area of adiponectin immunoreactivity in airway epithelium demonstrated that both WT mice (2.3 ± 0.2 vs 0.3 ± 0.1 μm<sup>2</sup>/μm area of adiponectin epithelium immunoreactivity) (WT tobacco smoke vs WT room air) (p = 0.001) and CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> (1.9 ± 0.1 vs 0.8 ± 0.1 μm<sup>2</sup>/μm area of adiponectin epithelium immunoreactivity) (CC10-Cre<sup>+</sup>/IκkB<sup>Δ/Δ</sup> tobacco smoke vs CC10-Cre<sup>+/+</sup>/IκkB<sup>Δ/Δ</sup> room air).
air) exposed for 6 mo to tobacco smoke had significantly increased levels of airway epithelial adiponectin immunoreactivity compared with nontobacco smoke exposed mice (Fig. 6C). Although tobacco smoke exposed WT mice had a slight increased level of airway epithelial adiponectin immunoreactivity compared with tobacco smoke exposed CC10-Cre<sup>β</sup>/Ikβ<sup>−/−</sup> mice, this was not statistically significant (Fig. 6C).

**Adiponectin activates AP-1 in lung epithelial cells**

To determine whether adiponectin activates the AP-1 (c-Fos, c-Jun, and JunB) signaling pathway in human lung epithelial cells we stimulated A549 cell line with globular adiponectin. Adiponectin significantly increased DNA binding activity for c-Fos in A549 cells (0.14 AU vs 0.08 AU; <i>p</i> = 0.04; adiponectin vs no adiponectin) (Fig. 7A). Adiponectin also induced c-Jun (0.10 AU vs 0.04 AU; <i>p</i> = 0.05; adiponectin vs no adiponectin) (Fig. 7B) and JunB (0.50 AU vs 0.26 AU; <i>p</i> = 0.05; adiponectin vs no adiponectin) (Fig. 7C).
Discussion

In this study, we have made the novel observation that adiponectin, a cytokine known to be expressed by adipocytes, is also significantly expressed by lung epithelial cells. In addition, we have noted that lung epithelial cells express functional AdipoR1, but not AdipoR2, suggesting that lung epithelial cell expression of adiponectin may result in autocrine or paracrine effects. The observations that lung epithelial cells express adiponectin was noted in lung epithelial cells derived from several different sources including human lung epithelial cells, a human lung epithelial cell line, and mouse lung epithelial cells. As in adipocytes, adiponectin and adiponectin receptor expression by lung epithelial cells was inducible, though preliminary studies suggest that the signaling mechanism for adiponectin expression may differ between adipocytes and lung epithelium. For example, in vitro studies with adipocytes have demonstrated that adiponectin expression is NF-κB dependent (13–15). In contrast, our in vivo studies demonstrate that CC10-CreER/lkβKO/mice (which are unable to activate NF-κB in airway epithelium) continue to express significant levels of adiponectin, suggesting that airway epithelial cells are not entirely dependent on NF-κB activation to express adiponectin. Finally, we demonstrated the potential disease relevance of adiponectin expression by lung epithelium by showing that adiponectin is highly expressed in the lungs of human subjects with the emphysematous form of COPD. In contrast, exposure to tobacco smoke in subjects without COPD down-regulates adiponectin expression. Overall these studies suggest that adiponectin expression by airway epithelium has the potential to modulate the inflammatory response in COPD through autocrine or paracrine pathways.

Adiponectin is known predominantly to exert anti-inflammatory effects (6), though recent studies have also provided evidence of potential proinflammatory effects (17, 23, 24). Anti-inflammatory effects of adiponectin may be mediated through several potential pathways including its capacity to suppress the synthesis of TNF as well as its ability to induce the production of anti-inflammatory cytokines such as IL-10 and the IL-1 receptor antagonist (6–8, 25). As macrophages are considered to be a key proinflammatory cell involved in the pathogenesis of COPD (1–3), the anti-inflammatory influence of adiponectin on macrophages may play an important role in inhibiting macrophage activation.

Another unexplored mechanism by which adiponectin might inhibit inflammation in COPD is through adiponectin’s known activation of peroxisome proliferator-activated receptor-α, which exert anti-inflammatory effects through inhibition of the transcriptional activation of proinflammatory response genes (26). TNF (6–8), oxidative stress (6–8), and tobacco smoke all reduce levels of adiponectin expression. Oxidative stress inhibits adiponectin expression in adipocytes (27), suggesting that a similar mechanism might account for the inhibitory effect of tobacco smoke on adiponectin expression by airway epithelial cells that we have noted. In adipocytes, reactive oxygen species reduced the transcriptional activity of the adiponectin gene, and the antioxidant N-acetyl cysteine reverses these effects (27).

In contrast to the aforementioned studies demonstrating an anti-inflammatory role for adiponectin, the ability of adiponectin to stimulate airway epithelial cells to express chemokines such as the neutrophil chemoattractant IL-8 also provides evidence that adiponectin receptors expressed by airway epithelial cells are functional and may exert potential proinflammatory effects in the airway. This observation is consistent with several studies indicating that adiponectin may also exert proinflammatory effects (17, 23).

Thus, adiponectin expression by airway epithelial cells could have both anti-inflammatory and proinflammatory effects in the airway in COPD. Which effect is dominant in COPD will require further in vivo studies in which adiponectin is neutralized. Interestingly, a recent study demonstrated that adiponectin-deficient mice spontaneously develop emphysema in the absence of tobacco smoke exposure (28), suggesting that adiponectin may play a role in alveolar development.

The potential disease relevance of adiponectin expression by airway epithelium is also suggested from our studies demonstrating that human subjects with COPD-E, as well as mice exposed for 6 mo to tobacco smoke to develop COPD, express increased levels of adiponectin. Interestingly, short-term exposure of mice to tobacco smoke suppresses adiponectin expression. We have noted similar observations in human smokers who have suppressed levels of adiponectin. In contrast, mice exposed to long-term tobacco smoke develop COPD and this is associated with increased adiponectin expression. Similar observations are noted in subjects with Gold Stage II COPD who have increased levels of BAL adiponectin. The molecular mechanism by which tobacco smoke exposure down-regulates adiponectin expression, and the development of COPD up-regulates adiponectin expression is unknown. We hypothesize that in subjects without COPD, one or several of the 4000 constituents of tobacco smoke induces an epithelial signaling pathway to inhibit transcription of adiponectin. Oxidative stress and reactive oxygen species are known to inhibit adiponectin expression in adipocytes (6–8, 27). With the development of
COPD, we hypothesize that an additional mediator (not adiponectin) is expressed by epithelial cells and/or nonepithelial cells (that is not induced in smokers alone). This mediator up-regulates adiponectin expression and counteracts the inhibitory effect of tobacco smoke on suppressing adiponectin expression. At present, there is no experimental data to support or refute this hypothesis.

Our immunohistology studies demonstrate that adiponectin is expressed by airway epithelial cells as opposed to alveolar cells the predominant site of disease in COPD-E. This suggests that adiponectin expressed by airway epithelium has alveolar effects by indirect mechanisms through binding to adiponectin receptors expressed by macrophages and/or airway epithelium. Thus, adiponectin released by airway epithelium may inhibit macrophage/epithelial production of proinflammatory mediators (matrix metalloprotease-9, TGF-β), as well as induce macrophages to express anti-inflammatory cytokines (IL-10, IL-1Ra), which consequently influence the extent of alveolar inflammation, alveolar injury, airspace enlargement, and repair. Interestingly, our studies using CC10-Cre/IKKβ/Δ/Δ mice in whom NF-κB is inactivated in airway epithelial cells demonstrate that NF-κB signaling in airway epithelial cells is not required for the expression of adiponectin by airway epithelial cells. This contrasts with the importance of NF-κB signaling to adiponectin expression by adipocytes (13–15).

As not all subjects exposed to tobacco smoke develop COPD, it is likely that COPD is an example of a disease that manifests because of gene environment interactions (29). One such gene polymorphism (i.e., adiponectin) interacting with an environmental stimulus (i.e., tobacco smoke) is potentially one of several gene environment interactions that may explain why only a subset of individuals exposed to tobacco smoke develop COPD. In the case of adiponectin, the SNP 276 adiponectin polymorphism is associated with significantly reduced adiponectin levels (8, 30). Thus, exposure to tobacco smoke in subjects with adiponectin gene polymorphisms may result in a significantly greater decrease in adiponectin levels, diminished anti-inflammatory responses, and permit the more rapid progression of tobacco smoke induced inflammation to COPD.

In summary, in this study we have made the novel observation that lung epithelial cells express adiponectin and functional AdipoR1. The potential disease relevance of adiponectin expression by lung epithelium is suggested from our studies demonstrating that adiponectin is highly expressed in the lungs of human subjects with the emphysematous form of COPD who have stopped smoking. In contrast, exposure to tobacco smoke in subjects without COPD down-regulates adiponectin expression. Further studies are needed to determine whether subjects with moderate severe COPD who continue to smoke also have increased levels of adiponectin. Overall, these studies suggest that adiponectin expression by airway epithelium may modulate the inflammatory response in COPD through autocrine or paracrine pathways.

Disclosures

The authors have no financial conflict of interest.

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