

Original article

Adiponectin attenuates allergen-induced airway inflammation and hyperresponsiveness in mice

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Background: Epidemiologic data indicate an increased incidence of asthma in the obese.

Objective: Because serum levels of the insulin-sensitizing and anti-inflammatory adipokine adiponectin are reduced in obese individuals, we sought to determine whether exogenous adiponectin can attenuate allergic airway responses.

Methods: We sensitized and challenged BALB/cJ mice with ovalbumin (OVA). Alzet micro-osmotic pumps were implanted in the mice to deliver continuous infusions of buffer or adiponectin (1.0 µg/g/d), which resulted in an approximate 60% increase in serum adiponectin levels. Two days later, mice were challenged with aerosolized saline or OVA once per day for 3 days. Mice were examined 24 hours after the last challenge.

Results: OVA challenge increased airway responsiveness to intravenous methacholine, bronchoalveolar lavage fluid cells, and T_H2 cytokine levels. Importantly, each of these responses to OVA was reduced in adiponectin- versus buffer-treated mice. OVA challenge caused a 30% reduction in serum adiponectin levels and a corresponding decrease in adipose tissue adiponectin mRNA expression. OVA challenge also decreased pulmonary mRNA expression of each of 3 proposed adiponectin-binding proteins, adiponectin receptor 1, adiponectin receptor 2, and T-cadherin.

Conclusion: Our results indicate that serum adiponectin is reduced during pulmonary allergic reactions and that adiponectin attenuates allergic airway inflammation and airway hyperresponsiveness in mice.

Clinical implications: The data suggest that adiponectin might play a role in the relationship between obesity and asthma.

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Key words: Lung, airway responsiveness, IL-13, IL-5, eosinophil, IgE, adiponectin receptor 1, adiponectin receptor 2, T-cadherin, adipocyte

Epidemiologic data indicate that the prevalence of asthma is increased in obese adults and children.¹⁻⁵ The association between asthma and obesity has been confirmed by studies using objective measures of asthma, such as bronchodilator response, peak flow variability, or airway hyperresponsiveness.⁶⁻⁹ Innate airway hyperresponsiveness is also observed in obese mice.¹⁰⁻¹³

It is likely that obesity either causes or worsens asthma. Longitudinal studies indicate that obesity antedates asthma and that the relative risk of incident asthma increases with increasing obesity.³⁻⁵ Furthermore, morbidly obese asthmatic subjects studied after weight loss demonstrate decreased severity and symptoms of asthma.¹⁴⁻¹⁷ The importance of understanding the relationship between obesity and asthma is underscored by the extremely high prevalence of obesity in the US population, by observations indicating that obesity is a strong predictor of the persistence of childhood asthma into adolescence,¹⁸ and by the marked prevalence of obesity in subjects with severe asthma.^{8,19,20} Nevertheless, although a number of mechanisms have been postulated,^{2,21-23} the causality relating obesity and asthma remains to be established.

It is possible that adiponectin plays a role in the relationship between obesity and asthma. Adiponectin is an adipocyte-derived hormone (adipokine) that is abundant in plasma. In human subjects and in animals, adiponectin mRNA expression in adipocytes decreases in obesity and increases again with weight loss,²⁴⁻²⁸ and plasma adiponectin levels are inversely related to body mass index.^{29,30} In contrast, levels of most other adipokines, including leptin, resistin, and inflammatory cytokines, such as TNF-α, increase with obesity.³¹ Whereas the primary metabolic effects of adiponectin are on glucose regulation and fatty acid metabolism, adiponectin is also anti-inflammatory. Adiponectin inhibits inflammatory gene expression in a variety of cell types, inhibits or modulates nuclear factor κB (NF-κB) and extracellular signal-regulated kinase activation, and augments expression of anti-inflammatory genes, including the IL-1 receptor antagonist gene.³²⁻⁴³ The purpose of this study was to determine whether adiponectin can also inhibit allergic inflammation in the lung.

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Abbreviations used

AdipoR1: Adiponectin receptor 1
 AdipoR2: Adiponectin receptor 2
 BAL: Bronchoalveolar lavage
 BALF: Bronchoalveolar lavage fluid
 NF- κ B: Nuclear factor κ B
 OVA: Ovalbumin
 R_L: Pulmonary resistance
 TBS: Tris-buffered saline

METHODS**Allergen sensitization and challenge**

This study was approved by the Harvard Medical Area Standing Committee on Animals. Male and female 4-week-old BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Mice were sensitized to chicken egg albumin (ovalbumin [OVA]; Grade V, Sigma-Aldrich, St Louis, Mo) on day 0 by means of an intraperitoneal injection of 20 μ g of OVA and adjuvant and 2 mg of aluminum hydroxide (Al[OH]₃; J. T. Baker, Phillipsburg, NJ) dispersed in 0.2 mL of PBS, as previously described.⁴⁴ The mice were given a second injection of identical reagents on day 14. On days 28, 29, and 30, animals were challenged with an aerosol of either PBS or PBS containing 6% OVA (wt/vol), as previously described.⁴⁴

Implantation of alzet micro-osmotic pumps

On day 26, 2 days before the initiation of OVA or PBS aerosol challenges, Alzet micro-osmotic pumps (Model 1007D; DURECT Corp, Cupertino, Calif) were implanted subcutaneously in the intrascapular region of each mouse. The pumps infuse solutions at a rate of 0.5 μ L/h. The reservoir of each pump was preloaded with 96 μ L of either sterile Tris-buffered saline (TBS) or murine recombinant adiponectin (1.55 μ g/ μ L), resulting in an adiponectin infusion rate of just under 1 μ g/g/d. Full-length murine recombinant adiponectin was generated in eukaryotic cells, as previously described.^{45,46} During this procedure, mice were anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg). After implantation, mice were administered an analgesic, buprenorphine hydrochloride (0.1 mg/kg, Sigma-Aldrich), twice per day for 2 days.

Measurement of pulmonary mechanics

Twenty-four hours after the last exposure to OVA or PBS, mice were anesthetized with xylazine (7 mg/kg) and sodium pentobarbital (50 mg/kg). The trachea was cannulated with a tubing adaptor, and the tail vein was cannulated for the delivery of methacholine. The mice were ventilated at a tidal volume of 0.3 mL and a frequency of 150 breaths/min by using a specialized ventilator (flexiVent; SCIREQ, Montreal, Quebec, Canada) that was also used for delivering the forced oscillations used for measuring pulmonary resistance (R_L). Once ventilation was established, a wide incision was made in the chest wall bilaterally to expose the lungs to atmospheric pressure, and a positive end-expiratory pressure of 3 cm H₂O was applied by placing the expiratory line under water. These conditions were imposed to standardize end-expiratory lung volume because airway caliber depends on lung volume. Dose-response curves to intravenous methacholine were obtained as previously described.^{10,11} After each dose of PBS or methacholine dissolved in PBS (1 μ L/g), we measured R_L at a frequency of 2.5 Hz every eighth breath, until R_L peaked and started to decrease. For each dose, the 5 highest values of R_L were averaged and used to construct the dose-response curve.

Bronchoalveolar lavage

Twenty-four hours after the last aerosol challenge, mice were killed with an overdose of sodium pentobarbital. Blood was drawn, and the serum was stored at -20°C for subsequent assay of total serum adiponectin (Panomics, Inc, Redwood City, Calif) and IgE (BD Biosciences, San Diego, Calif) by means of ELISA. The lungs were lavaged twice with PBS (1 mL), which was instilled and then slowly withdrawn over 30 seconds. The recovered bronchoalveolar lavage (BAL) fluid (BALF) was centrifuged at 1200 rpm at 4°C for 10 minutes. BAL supernatant was stored at -80°C and subsequently analyzed by means of ELISA for IL-5 and IL-13 (R&D Systems, Minneapolis, Minn). BALF cells and differentials were counted as previously described.⁴⁴

RNA extraction and real-time PCR

Lungs and abdominal adipose tissue were harvested, frozen at -80°C , and subsequently homogenized with the PowerGen 125 (Fisher Scientific, Hampton, NH) at full speed in 3 mL of TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, Calif) for 5 minutes. Total RNA was then extracted in accordance with the manufacturer's instructions and stored at -80°C . In preparing RNA from adipose tissue, we pipetted off and discarded the fat layer overlying the homogenate after the initial spin after homogenization. After RNA extraction, samples were run through RNeasy RNA Cleanup (QIAGEN Inc, Valencia, Calif) to increase RNA purity. Reverse transcription was performed, and the reverse transcription reactions were stored at -80°C . Quantitative real-time RT-PCR was performed with an iCycler iQ Real Time Detection System and iQ SYBR Green Supermix in accordance with the manufacturer's instructions (BioRad, Hercules, Calif). Primer sets and product sizes for adiponectin, adiponectin receptor 1 (adipoR1), adiponectin receptor 2 (adipoR2), and T-cadherin were as follows: adiponectin forward 5'-TGT TGG AAT GAC AGG AGC TGA A -3' and reverse 5'-CAC ACT GAA CGC TGA GCG ATA C -3' (106 bp); adipoR1 forward 5'-CTT CTA CTG CTC CCC ACA GC -3' and reverse 5'-TCC CAG GAA CAC TCC TGC TC -3' (139 bp); adipoR2 forward 5'-CGG TGT ACT GCC ACT CAG AA -3' and reverse 5'-CAT GTC CCA CTG AGA GAC GA -3' (197 bp); and T-cadherin forward 5'-GCT TCG GAC AAG GAC CTT CA -3' and reverse 5'-TGG GCA GGT TGT AGT TTG C -3' (160 bp). TaqMan Ribosomal RNA primers and control (Applied Biosystems, Foster City, Calif) were used to measure 18S rRNA, according to the manufacturer's instructions. For adiponectin, a positive control used to construct standard curves for real-time PCR was generated by cloning the PCR product of cDNA from the adipose tissue of a carboxypeptidase E-deficient (obese) mouse into TOPO-TA vectors (Qiagen). Positive controls for adipoR1 and adipoR2 were cloned from the gastrocnemius muscle. cDNA prepared from mouse brain tissue was used to generate the T-cadherin control. For each set of primers, melting curve analysis yielded a single peak consistent with one PCR product. Changes in lung and adipose tissue mRNA transcript copy number were assessed relative to changes in 18S rRNA transcript copy number.

Statistical analysis

Data were analyzed by means of repeated-measures ANOVA (airway responsiveness and BALF cells) or factorial ANOVA (BALF cytokines, serum IgE and adiponectin levels, and mRNA expression) by using STATISTICA software (StatSoft, Tulsa, Okla). For IgE levels, BALF cell counts, and mRNA expression, data were logarithmically transformed before analysis because untransformed data were not normally distributed. A *P* value of less than .05 was considered statistically significant.

RESULTS

Body weight

There was a small reduction in body weight the day after implantation of the mini-Alzet pumps that averaged $2.3\% \pm 0.6\%$ of the initial body weight. Body weight recovered to baseline values by the day of the first OVA/PBS aerosol challenge in both TBS- and adiponectin-treated mice. There was no significant effect of either OVA/PBS challenge or adiponectin versus TBS treatment on body weight.

BAL cells and cytokines

Compared with PBS challenge, OVA challenge caused a significant increase in BAL macrophages ($P < .05$), neutrophils ($P < .01$), eosinophils ($P < .001$), and lymphocytes ($P < .001$, Fig 1). For macrophages and neutrophils, the effect of OVA was observed only in TBS-treated and not in adiponectin-treated mice. For eosinophils and lymphocytes, an effect of OVA was observed in adiponectin-treated, as well as TBS-treated, mice. The effect of OVA on BALF eosinophils was markedly reduced in the adiponectin-treated compared with the TBS-treated mice ($P < .001$). A similar trend was observed for lymphocytes but did not reach statistical significance ($P < .07$). Factorial ANOVA also indicated a significant effect of OVA versus PBS exposure on BALF IL-13 and IL-5 levels ($P < .05$ in each case, Fig 2). The effects of OVA challenge on BALF T_H2 cytokine levels were observed only in TBS-treated and not in adiponectin-treated mice.

Airway responsiveness

Neither adiponectin treatment nor OVA challenge had any effect on baseline R_L . In PBS-challenged mice there was no effect of adiponectin versus TBS treatment on airway responsiveness to intravenous methacholine (Fig 3). Compared with challenge with PBS, challenge with OVA caused an increase in airway responsiveness in mice treated with TBS buffer. In contrast, OVA challenge did not increase airway responsiveness in mice treated with adiponectin.

Serum IgE

In TBS-treated mice there was a significant increase in serum IgE levels, from 86 ± 11 ng/mL in PBS-challenged mice to 164 ± 36 ng/mL in OVA-challenged mice ($P < .05$), which is consistent with previous reports.⁴⁴ OVA challenge did not increase serum IgE levels in adiponectin-treated mice (184 ± 24 and 210 ± 45 ng/mL in PBS- and OVA-treated mice, respectively), although IgE levels were greater in adiponectin- versus TBS-treated mice, regardless of the aerosol challenge.

Serum adiponectin

To confirm that the delivery of adiponectin by the Alzet pumps resulted in an increase in circulating adiponectin levels, we measured serum adiponectin levels by means of ELISA (Fig 4, A). Factorial ANOVA indicated a significant, approximately 60%, increase in serum adiponectin

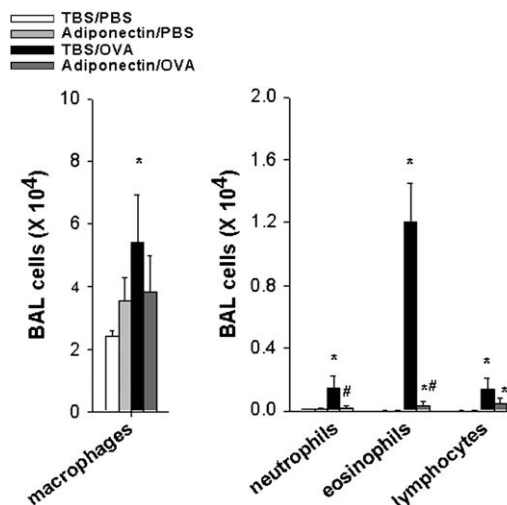


FIG 1. Effect of adiponectin on BAL leukocytes. Results are means $\pm$ SE of data from 4 to 20 mice in each group. * $P < .05$ versus PBS group with same treatment. # $P < .05$ versus OVA-challenged mice treated with TBS.

levels in mice that received adiponectin in the Alzet pumps compared with that seen in mice that received TBS buffer in the pumps ($P < .001$), as expected. However, we were surprised to find a substantial, approximately 30%, decrease in serum adiponectin in mice that were challenged with OVA versus mice that were challenged with PBS ($P < .001$). To determine whether this was the result of a decrease in adiponectin mRNA expression, we measured adiponectin mRNA expression by using real-time PCR in adipose tissue from these mice (Fig 4, B). Adipose tissue is the primary source of adiponectin.²⁴ Factorial ANOVA indicated a significant decrease ($P < .01$) in adipose adiponectin mRNA expression in OVA- versus PBS-challenged mice, demonstrating that allergen challenge in the lung suppresses adiponectin expression in adipocytes. Interestingly, there was also a significant effect of adiponectin versus TBS treatment ($P < .05$): mice treated with adiponectin had significantly greater adiponectin mRNA expression in adipose tissue compared with that seen in mice treated with TBS. Because glucocorticoids have been shown to inhibit adiponectin expression both *in vitro* and *in vivo*,⁴⁷⁻⁵⁰ we also examined serum corticosterone levels in these mice. No significant effect of either OVA challenge or adiponectin treatment on corticosterone concentrations was observed (data not shown).

Pulmonary gene expression

To examine the possibility that OVA challenge, adiponectin treatment, or both might also change pulmonary sensitivity to adiponectin, we measured pulmonary expression of adiponectin receptors, adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and T-cadherin by means of real-time PCR and normalized expression of each these genes by 18S rRNA transcript number. Each of these genes has been proposed to bind adiponectin.^{51,52} All 3 adiponectin-binding proteins were expressed in the lung, albeit at different levels. Factorial ANOVA indicated

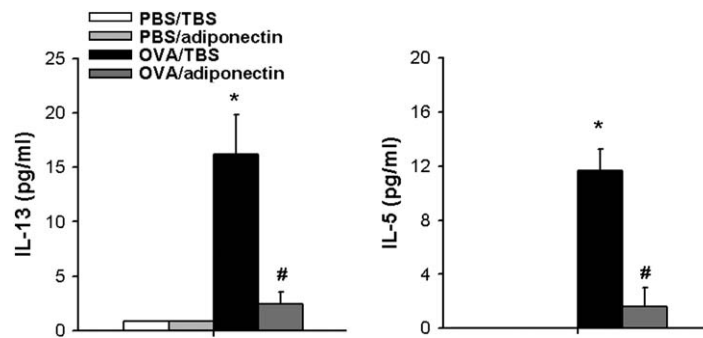


FIG 2. Effect of adiponectin on BALF T_H2 cytokines. Mice were treated with either buffer (TBS) or adiponectin and challenged with either PBS or OVA. Results are means $\pm$ SE of data from 4 to 11 mice in each group. * $P < .05$ versus PBS group with same treatment. # $P < .05$ versus OVA-challenged mice treated with TBS.

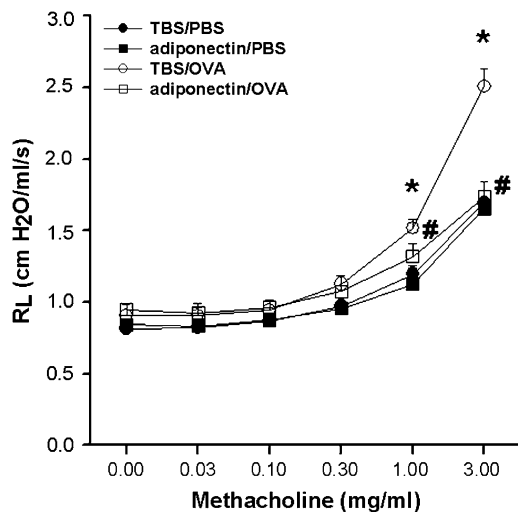


FIG 3. Effect of adiponectin on airway responsiveness to intravenous methacholine. Mice were treated with either buffer (TBS) or adiponectin and challenged with either PBS or OVA. R_L was measured by means of forced oscillation. Results are means $\pm$ SE of data from 4 to 8 mice in each group. * $P < .05$ versus PBS group with same treatment. # $P < .05$ versus OVA-challenged mice treated with TBS.

that pulmonary mRNA expression of all 3 adiponectin-binding proteins was reduced in OVA- versus PBS-treated mice ($P < .05$ in each case, Fig 5). In contrast, there was no significant effect of TBS versus adiponectin treatment.

DISCUSSION

Our results indicate for the first time that treatment of mice with full-length adiponectin at a dose sufficient to induce an approximate 60% increase in serum adiponectin levels virtually abolishes OVA-induced changes in inflammatory cell influx (Fig 1) and T_H2 cytokine expression in the lungs (Fig 2), as well as OVA-induced airway hyperresponsiveness (Fig 3). Obesity is associated with decreases in serum adiponectin levels that are on

the same order of magnitude as the increase in serum adiponectin levels that we brought about with our treatment.²⁴⁻²⁸ Hence it is possible that loss of these beneficial effects of adiponectin that occurs with obesity contributes to the increased incidence and severity of asthma observed in this population.^{1,2}

The ability of adiponectin to inhibit OVA-induced airway inflammation (Figs 1 and 2) is consistent with reports of anti-inflammatory effects of adiponectin in other cells and tissues, although an effect of adiponectin on T_H2 -mediated inflammation has not previously been reported. For example, in bone marrow adiponectin suppresses the formation of myelomonocytic lineage cells.³³ In macrophages adiponectin decreases the ability of LPS to elicit TNF- α and IL-6 production,³³⁻³⁶ apparently as a result of reduced NF- κ B signaling.³⁷ Adiponectin also inhibits Toll-like receptor-induced NF- κ B activation in macrophages.⁴³ In leukocytes and macrophages adiponectin induces the anti-inflammatory cytokine IL-10, the endogenous IL-1 receptor antagonist,^{37,38} or both, and in endothelial cells adiponectin reduces TNF- α -induced NF- κ B signaling and the resulting expression of the adhesion molecules.³⁹⁻⁴²

Adiponectin treatment also abolished OVA-induced increases in airway responsiveness in these mice (Fig 3). Adiponectin did not alter airway responsiveness in the absence of allergen challenge, and therefore it is likely that the ability of adiponectin to inhibit OVA-induced airway hyperresponsiveness is secondary to its ability to inhibit T_H2 expression in the lung. IL-13 in particular has been shown to be necessary for the airway hyperresponsiveness that is observed in this model.^{53,54}

Measurements of serum adiponectin were performed to confirm the efficacy of our pharmacologic intervention (Fig 4, A). Continuous infusion of adiponectin at a rate of about 1 μ g/g/d resulted in an approximate 60% increase in total serum adiponectin levels. The changes were significant and clearly capable of inducing physiologic responses (Fig 1-3). We also observed a substantial decrease in serum adiponectin levels in mice that were challenged with OVA versus mice that were challenged with PBS (Fig 4, A). The OVA-induced reductions in

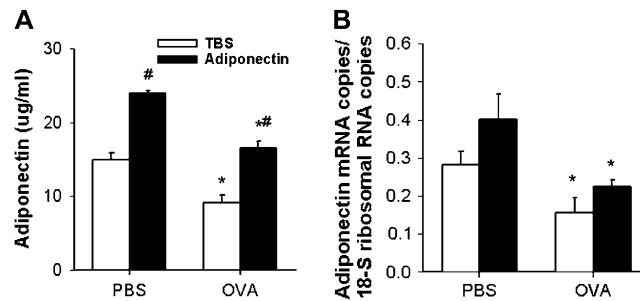


FIG 4. Effect of treatment with TBS buffer or adiponectin on serum adiponectin level (**A**) and adipose tissue adiponectin mRNA expression (**B**). Mice were challenged with either PBS or OVA. Results are means $\pm$ SE of data from 4 to 15 mice in each group. Adiponectin mRNA expression was normalized for expression of 18S rRNA. * $P < .05$ versus PBS group with same treatment. # $P < .05$ versus TBS treated mice with same aerosol challenge.

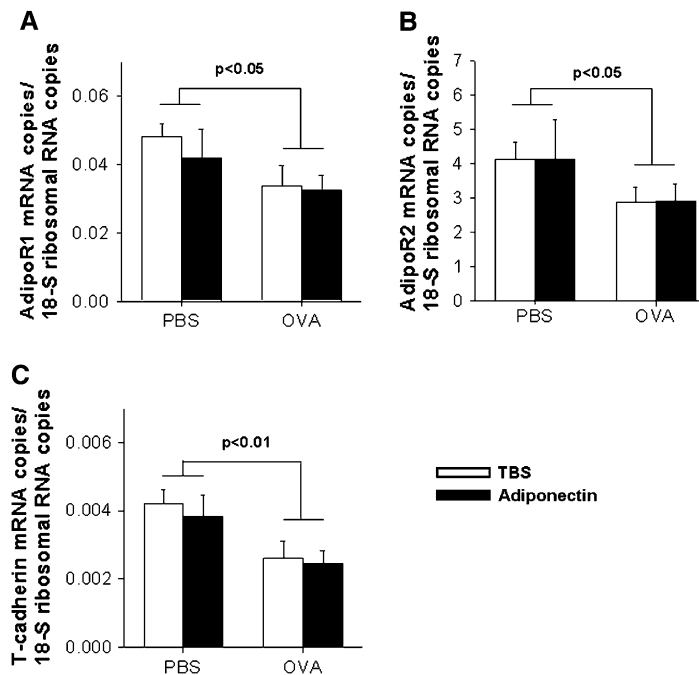


FIG 5. Effect of treatment with TBS buffer or adiponectin on pulmonary adiponectin (**A**), adiponectin (**B**), and T-cadherin (**C**) mRNA expression. Mice were challenged with either PBS or OVA. Results are means $\pm$ SE of data from 4 to 15 mice in each group. Gene expression was normalized for expression of 18S rRNA.

serum adiponectin levels were likely the result of decreases in adiponectin production by adipose tissue, the primary source of adiponectin,²⁴ because adiponectin mRNA expression in adipose tissue was also reduced in OVA- versus PBS-challenged mice (Fig 4, B). Thus allergic responses in the airways are capable of altering adipocyte function. We do not yet know how this occurs. Because glucocorticoids have been shown to inhibit adiponectin expression in cultured adipocytes^{47,48} and to reduce serum adiponectin levels after exogenous administration in human subjects,⁴⁹ we considered the possibility that alterations in endogenous glucocorticoids induced by allergen challenge might be involved in the

reduction in serum adiponectin levels observed after allergen challenge. However, our results indicated no effect on serum corticosterone levels of either OVA challenge or adiponectin administration. It is also possible that TNF- α might be involved in OVA-induced reductions in adiponectin expression. TNF- α has been shown to contribute to allergic airway responses in mice.⁵⁵ TNF- α inhibits adiponectin expression in adipocytes,^{28,47,56} and in human subjects serum TNF- α levels are negatively correlated with serum adiponectin level.²⁸ Regardless of how the allergen-induced decrease in adiponectin levels comes about, it has important functional implications. Given the important antiasthmatic effects of adiponectin in the lung

(Figs 1-3), it is possible that reductions in serum adiponectin levels induced by allergic reactions in the lung exacerbate allergic asthma, even in lean individuals.

Two proposed adiponectin receptors, adipoR1 and adipoR2, were recently cloned,⁵² although the mechanisms whereby they convey metabolic signals is not completely understood. T-cadherin, a member of the cadherin family of cell-surface proteins involved in calcium-mediated cell-cell interactions and signaling, has also been shown to bind specific isoforms of adiponectin.⁵¹ All 3 adiponectin-binding proteins were expressed in lung tissue, and all 3 were reduced by OVA sensitization and challenge (Fig 5). This reduction in adiponectin binding protein expression suggests that allergen challenge might reduce the beneficial effects of adiponectin on the lung, not only by attenuating the production of adiponectin by adipose tissue (Fig 4, B) but also by attenuating proposed components of adiponectin-signaling pathways (Fig 5).

In summary, our results indicate that treatment with exogenous adiponectin inhibits allergic responses in the airways. Our results also indicate that allergen challenge inhibits adipose tissue adiponectin expression and pulmonary adiponectin-binding protein expression. Taken together, the results indicate that alterations in adiponectin levels might play a role in the asthmatic diathesis not only in obese individuals but also in healthy lean subjects. The results also suggest a role for adiponectin in the treatment of asthma.

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