Obesity and Asthma: Adiponectin Receptor 1 (Adipo R1) and Adiponectin Receptor 2 (Adipo R2) are expressed by normal human bronchial epithelial (NHBE) cells at air-liquid interface (ALI) and expression changes with IL-13 stimulation

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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List of Abbreviations

Acrp30:30-kDa adipocyte complement related protein
Adipo R1: Adiponectin Receptor 1
Adipo R2: Adiponectin Receptor 2
ALI: air-liquid-interface
AMPK: AMP-activated kinase
BMI: body mass index
BSA: bovine serum albumin
db/db: (mouse model of obesity) mouse that is homozygous for a point mutation in the gene for the leptin receptor
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
gAcrp30: globular portion of 30-kD adipocyte complement related protein
IL: Interleukin
MUC5AC: one of two secreted polymeric mucins that are the predominant glycoproteins in airway mucus
NHBE: normal human bronchial epithelial
rh: recombinant human
PPARα: peroxisome proliferator-activated receptor alpha
T2DM: Type 2 diabetes mellitus
TH2: helper t-cell type 2
Obesity is recognized as an important risk factor for the development of many chronic diseases such as hypertension, Type 2 diabetes mellitus (T2DM) cardiovascular disease, cancer, renal disease, neurologic dysfunction, metabolic syndrome and asthma (3, 4). Circulating serum adiponectin levels in obese asthmatics have been reported to be low. Therefore, we aimed to investigate the role of adiponectin in a mucus hypersecretion model and hypothesized that adiponectin would decrease IL-13 induced MUC5AC expression from differentiated NHBE cells and that increasing concentrations of IL-13 would cause a decrease in Adipo R1 and Adipo R2 expression. MUC5AC expression with exposure to adiponectin was not significant. However, mRNA expression of Adipo R1 and Adipo R2 was significantly decreased by stimulation of IL-13
for acute (24 hours) and chronic (14 days) exposure. Therefore, the obese state and specifically IL-13 concentration could play a role in Adipo R1 and Adipo R2 expression within NHBE cells.
Introduction

Obesity is considered a worldwide pandemic (1). The World Health Organization (WHO) estimates that in 2014, more than 1.9 billion adults, 18 years and older, were overweight. Of these over 600 million were obese. The estimated annual medical cost of obesity in the U.S. was $147 billion in 2008; the medical costs for people who are obese were $1,429 higher than those of normal weight (2). Obesity is recognized as an important risk factor for the development of a myriad of chronic diseases such as hypertension, Type 2 diabetes mellitus (T2DM) cardiovascular disease, cancer, renal disease, neurologic dysfunction, metabolic syndrome and asthma (3,4). The prevalence of an increase in asthma and obesity has paralleled each other in the U.S. over the last few decades. There is evidence that obesity increases the risk of asthma, atopic, and autoimmune diseases (5). Epidemiological data indicate that obesity is a risk factor for asthma, but the mechanistic basis for this relationship is not established (6). It is not known if adipocytes can directly affect airway epithelium. A large prospective study of obesity and incident asthma looked at nearly 86,000 women participating in the Nurses’ Health Study, and found that over a 4-year follow-up, the odds of developing asthma were 2.7 times higher in obese women (BMI≥30 kg/m²) compared with normal-weight women (BMI 20 kg/m²-24.9 kg/m²)(7).

Allergic diseases have reached epidemic proportions worldwide and their incidence is ever increasing (8). WHO estimates that 235 million people currently suffer from asthma. Asthma is an obstructive airway disease that involves chronic inflammation of the respiratory tract (9). It is the result of an allergic reaction or other form of hypersensitivity. It affects 5 to 10% of the population in developed countries and is associated with a large socioeconomic burden. The term asthma has evolved from a term describing a single disease to one
encompassing multiple subgroups (endotypes) (10). Asthma presentation is varied along with responsiveness to current treatments (11). Gibeon et al. (12) formed one of the largest cohorts of patients with severe asthma according to body mass index (BMI). Their data shows increasing BMI in severe asthma is associated with increasing corticosteroid resistance. This suggests patients who are obese and have severe asthma may represent a distinct clinical phenotype (12). Another study in which both BMI and percent body fat (using bioelectrical impedance) was measured found a significant association between body fat and asthma in women (p=0.04), but not men (p=0.75) (13). Bates states obesity is also having a major negative impact on asthma therapy and control. Individuals with obese asthma are almost fivefold more likely than lean patients with asthma to be hospitalized for an asthma exacerbation (14).

Adipose tissue is metabolically active and secretes a range of substances, including peptides, which are engaged in cell-to-cell signaling, termed adipose-derived hormones, or adipokines (15). A protein of interest within this study is adiponectin. This hormone, secreted by adipose tissue, is biologically active and receptors to this molecule are widely distributed throughout the body, including the lungs (16). Adiponectin (also known as 30-kDa adipocyte complement related protein ;Acrp30) generally acts as an anti-inflammatory hormone and is reduced by obesity, most likely due to macrophage release of TNFα and IL-6, which inhibits adipocyte production of adiponectin (15). It is uniquely expressed in adipose tissue and has high circulating (typically average 10µg/mL in men and 15µg/mL in women) serum levels (26). Adiponectin is secreted exclusively by adipocytes and aggregates in a range of forms, from low (trimer and hexamer) to high (12-18mer) molecular weight. In human plasma, the full-length adiponectin protein predominates. However, proteolytic cleavage of the full-length protein at
amino acid 110 generates a carboxyl-terminal globular domain (137aa), which circulates in low abundance (18).

Adiponectin exerts many of its cellular effects by binding to two receptor isoforms with seven transmembrane domains. These adiponectin receptor 1 (Adipo R1) and adiponectin receptor 2 (Adipo R2) isoforms have distinct distribution patterns within multiple tissues (19). Adipo R1 and Adipo R2 exert similar effects, but Adipo R1 has been more related to metabolic functions, whereas Adipo R2 has been mainly involved in anti-inflammatory and anti-oxidative mechanisms (20). Adipo R1 is a high-affinity receptor for gAcrp30 and a low affinity receptor for Acrp30, while Adipo R2 is an intermediate-affinity receptor for Acrp30 and gAcrp30 (19). Previous studies have suggested alteration of adiponectin concentrations may influence various diseases related to the development of asthma and atopy (21).

Asthma is characterized by chronic inflammation of the respiratory tract, which is mediated by increased expression of many inflammatory proteins, such as interleukin (IL) 8 (9,22). Production of IL-8 is one response of airway epithelial cells to IL-13, a helper t-cell type 2 (TH2) cytokine. IL-13 is the central mediator of allergic asthma, where it regulates eosinophilic inflammation, mucus secretion, and airway hyperresponsiveness (23). IL-13 overexpression is seen with increased mucus production and goblet cell hyperplasia, both of which play a role in asthma characterization (12,24). Tanabe et al (22) show normal human bronchial epithelial (NHBE) cells in air-liquid interface (ALI) exhibit a ciliated cell phenotype and express very low levels of IL-8 and MUC5AC, a mucin glycoprotein in the respiratory tract. After exposure with IL-13 for 14days, NHBE cells change to a goblet cell phenotype and express high levels of IL-8 and MUC5AC (25). Mucus hypersecretion is associated with morbidity and mortality in asthma (22).
Kwon et al. has reported that interleukin-13 (IL-13) is unexpectedly increased in adipose tissue of obese humans and high-fat diet (HFD)-fed mice, and the source of IL-13 is primarily the adipocyte (26). Adipose tissue may secrete pro-inflammatory and anti-inflammatory mediators (12,27). Nehete et al. has shown that TNF-α, IL-1β, IL-13, and IL-8 serum plasma levels were significantly higher in obese compared to lean chimpanzees (3). Surendar et al. noted a significant increase of TNF-α and IL-13 serum cytokines in metabolic syndrome subjects compared to non-metabolic syndrome subjects (28). Adipose tissue-derived hormones may prove to be an important factor in managing obese asthma, particularly in women (29). Adiponectin serum level is negatively correlated with obesity (30) Shore reported continuous infusion of adiponectin via subcutaneously implanted osmotic pumps to replenish decreased levels was found to attenuate ovalbumin-induced airway inflammation in mice through the attenuation of inflammatory cell influx, corresponding with a reduction in IL-13 and IL-5 (31). Yamauchi also reported in a db/db (mouse model of obesity) mouse that is homozygous for a point mutation in the gene for the leptin receptor had decreased expression levels of Adipo R1 and Adipo R2 in the liver. Threefold overexpression of Adipo R1 or fivefold overexpression of AdipoR2 in the liver of db/db mice ameliorated diabetes significantly (23). Therefore, we hypothesized that adiponectin would decrease IL-13 induced MUC5AC expression from differentiated NHBE cells and that increasing concentrations of IL-13 would cause a decrease in Adipo R1 and Adipo R2 expression.
Materials and Methods

Reagents

The following reagents were purchased from the indicated companies: recombinant human (rh) interleukin (IL)-13 (R&D Systems, Inc., Minneapolis, MN, USA.); rh-adiponectin/Acrp30 (R&D Systems); rh-gAcrp30/Adipolean (PeproTech Rocky Hill, NJ, USA.); DMEM, Ham’s F12 medium (Gibco, Grand Island, NY, USA); bronchial epithelial cell growth medium, SingleQuotR® kit and Hanks’ balanced salt solution (Lonza Walkersville Inc., Walkersville, MD, USA).

Normal human bronchial epithelial (NHBE) cell culture

NHBE cells (Lonza Walkersville Inc.) were cultured and differentiated into ciliated cells or goblet cells at ALI as we have previously described (32,33). NHBE cells from a total of three different donors (Table1) were seeded at 3500 cells/cm² and grown in bronchial epithelial cell growth medium supplemented with the SingleQuotR® kit at 37°C with 5% CO₂. The medium was changed every 48 h, and the cells were cultured until 70–80% confluence. At the second passage, the cells were seeded to polyester membrane transwell-clear inserts of 0.4 μm pore size, 6.5 mm diameter, 10 μm thickness (Corning, Lowell, MA, USA) at 2.0 × 10⁵ cells/cm². NHBE cells were then cultured in DMEM/Ham’s F12 medium with 1% insulin-transferrin-selenium A, recombinant epidermal growth factor (0.5 ng/mL), triiodothyronine (10 ng/mL), hydrocortisone (0.5 μg/mL), all-trans retinoic acid (1.0 × 10⁻⁷ M), bovine serum albumin (BSA) (2.0 μg/mL) and bovine pituitary extract (30 μg/mL). Culture medium was added to both the apical and basolateral side of the inserts, and the cells were cultured submerged in medium. After achieving 70–80% confluence, the apical side of the medium was removed and cells were cultured at an ALI. Culture medium (500μL) was changed from the basolateral side only every 48 h at 37°C with 5% CO₂ for 14 days (Figure 1)
Table 1. Normal Human Bronchial Epithelial (NHBE) cell (Lonza Walkersville Inc.) Donors

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Smoking-status</th>
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<tbody>
<tr>
<td>F</td>
<td>69</td>
<td>Hispanic</td>
<td>Non-smoking</td>
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<tr>
<td>F</td>
<td>49</td>
<td>Caucasian</td>
<td>Non-smoking</td>
</tr>
<tr>
<td>F</td>
<td>42</td>
<td>Hispanic</td>
<td>Non-smoking</td>
</tr>
</tbody>
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Figure 1. Method of Air Liquid Interface (ALI) used within the Rubin laboratory.

**Acute IL-13 and Adiponectin exposure**

NHBE cells grown with PBS for 14 days had medium changed every 48 hours. On day 14 NHBE cells were exposed from the basolateral side of the inserts to IL-13 (0, 1, or 5 ng/mL) with or without adiponectin (1µg/ml) for 24 hours before sample harvest (32) (Figure 2 and 3). The acute model is a basal 24 hour exposure of IL-13 ± adiponectin to fully differentiated ciliated NHBE cells.
Chronic IL-13 and Adiponectin exposure

NHBE cells grown with IL-13 (0, 1, or 5ng/mL) were stimulated with rh-adiponectin (0, 0.5, or 1μg/mL) for 14 days. The medium was changed every 48 h (Figure 3 and 4). The chronic model is a basal 14 day exposure of IL-13 ± adiponectin during NHBE cell differentiation.

Figure 2. Method of Acute (24hr) and Chronic (14d) full length Adiponectin Exposure
MUC5AC, IL8, AdipoR1, and Adipo R2 message from ciliated epithelial cells and IL-13-transformed goblet cells

Assay for MUC5AC

MUC5AC protein in supernatants was measured by ELISA (31). The 96-well plates were coated with a 50 μL sample that contained bicarbonate-carbonate buffer (50 μL) and incubated at 37°C overnight until samples dried. After washing with 0.05% Tween 20 PBS buffer (T-PBS), 2% BSA/T-PBS was added to each well at room temperature for 1 h. After washing wells with T-PBS, MUC5AC monoclonal antibody (45M1) in T-PBS was added to a concentration of 2 μg/mL and the plate was further incubated for 2 h. Anti-mouse-IgG HRP-linked whole antibody was added to each well, and the plate was incubated for 1 h. After washing with T-PBS, 3, 3’, 5, 5’-tetramethylbenzine peroxidase solution was added to the plate. The reaction was stopped with 2 N H₂SO₄, and absorbance was measured using an ELx808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).
Real-time quantitative PCR analysis of MUC5AC, IL-8 Adipo R1, and Adipo R2 mRNA

MUC5AC, IL-8, Adipo R1, and Adipo R2 mRNA expression was examined by real-time PCR (34). After acute exposure, the apical side of the cells was washed three times with PBS, and total RNA was extracted using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The total RNA was then used to synthesize the first-strand cDNA using the qScript™ cDNA synthesis kit (Quanta BioSciences, Inc., Gaithersburg, MD, USA). Quantitative PCR was performed on the C1000™ thermal cycler equipped with CFX96™ real-time PCR system (Bio-Rad). For the relative quantification of MUC5AC, IL-8, Adipo R1 and Adipo R2 mRNA expression, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Perfecta SYBR Green (Quanta) was used as a DNA intercalator dye to monitor amplified DNA quantification, and real-time quantitative PCR curves were analyzed by CFX Manager software (Bio-Rad) in order to obtain threshold cycle values for each sample. mRNA expression level was calculated based on comparative Ct method. The housekeeping gene GAPDH expression Cq values were comparable among all experiments. The following primers were used:

MUC5AC forward 5’- TACTCCACAGACTGCACCAACTG -3’;

MUC5AC reverse 5’- CGTGATTGCTTCCCGTCAA -3’;

IL-8 forward 5’- CTGCGCCAACACAGAAATTA -3’;

IL-8 reverse 5’- ACTTCTCCACAACCCTCTGC -3’;

Adipo R1 forward 5’- TTCTTCTCATGGCTGTGATGT -3’;

Adipo R1 reverse 5’- AAGAAGCGCTCAGGAATTTC -3’;
Adipo R2 forward 5’- ATAGGGCAGATAGGCTGGTTGA -3’;

Adipo R2 reverse 5’- GGATCCGGGCAGCATA -3’;

GAPDH forward 5’-TGAACGGAAGCTCAGTGG -3’;

GAPDH reverse 5’-TCCACCACCTGTGCTGTA -3’.

Statistical analysis

Data are expressed as mean values ± standard error of the mean (SEM). Statistical differences were examined by one-way analysis of variance (ANOVA) as appropriate with Tukey HSD comparison of means. A p value less than 0.05 was considered statistically significant. Statistical analysis was performed using JMP Pro12 for Windows (SAS, Cary, NC).
Results

Effect of Acute gAcrp 30 on MUC5AC, Adipo R1, and Adipo R2 mRNA expression

NHBE cells were cultured with PBS for 14 days at ALI to differentiate into ciliated cells. On day 14 these cells were stimulated from the basolateral side with IL-13 (0, 1, or 5 ng/mL) with or without gAdiponectin (1µg/ml) for 24 hours before extracting total RNA. MUC5AC (Figure 4), Adipo R1 (Figure 5), and Adipo R2 (Figure 6) expression with exposure to gAcrp is not significant compared to exposure with IL-13 only. However, IL-13 stimulation (5ng/ml) for 24 hours significantly decreased Adipo R1 (p=0.0055) and Adipo R2 (p=0.0101) mRNA expression compared with PBS (Figure 5 and 6). IL-13 stimulation (5ng/ml) for 24 hours also significantly decreased Adipo R1 (p=0.0100) mRNA expression compared with IL-13 stimulation (1ng/ml) for 24 hours. IL-13 stimulation for 24 hours significantly decreased Adipo R1 and Adipo R2 mRNA expression.
Figure 4. **MUC5AC mRNA expression with exposure to gAcrp 1µg/mL is not different compared to no exposure to gAcrp.** MUC5AC measured by real-time PCR. Data are expressed as the ratio of MUC5AC to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and shown as mean ± SEM of data from four experiments, 11 total wells in each group from three different donors.

Figure 5. **Basal IL-13 (5ng/mL) stimulation for 24 hours significantly decreased Adipo R1 mRNA compared to IL-13 (1ng/mL) and PBS.** Adipo R1 mRNA expression measured by real-time PCR. Data are expressed as the ratio of Adipo R1 to GAPDH mRNA and shown as mean ± SEM of data from four experiments, 11 total wells in each group from three different donors.
Figure 6. **Basal IL-13 (5ng/mL) stimulation on NHBE for 24 hours significantly decreased Adipo R2 mRNA compared to PBS.** Adipo R2 mRNA expression measured by real-time PCR. Data are expressed as the ratio of MUC5AC to GAPDH mRNA and shown as mean ± SEM of data from four experiments, 11 total wells in each group from three different donors.

**Effect of Chronic gAcrp 30 on MUC5AC, Adipo R1, and Adipo R2 mRNA expression**

We have previously reported that IL-13 transforms growing NHBE cells to a goblet cell morphology (32). NHBE cells were cultured with IL-13 (0, 1, or 5ng/mL) and gAcrp (0, 0.5, or 1μg/mL) for 14 days before extracting total RNA. MUC5AC mRNA expression is increased with IL-13 concentration as previously reported (25), but the presence of Acrp had no effect (Figure 7). Adipo R1 (Figure 8) and Adipo R2 mRNA (Figure 9) expression with chronic exposure to gAcrp is not different compared to chronic exposure with IL-13 only. However, IL-13 stimulation (5ng/mL) for 14 days significantly decreased Adipo R1 (p=0.0303) and Adipo R2 (p=0.0145) mRNA expression compared with PBS (Figure 8 and 9). IL-13 stimulation for 14 days significantly decreased Adipo R1 and Adipo R2 mRNA expression.
Figure 7. Effect of IL-13 on MUC5AC mRNA expression by NHBE with exposure to gAcrp 0.5 or 1µg/mL was not significantly different compared to no exposure to gAcrp. MUC5AC mRNA expression measured by real-time PCR. Data are expressed as the ratio of MUC5AC to GAPDH mRNA and shown as mean ± SEM of data from one experiment, 3 total wells in each group from one donor.
Figure 8. **IL-13 (5ng/mL) exposure for 14 days significantly decreased Adipo R1 mRNA compared to PBS.** Adipo R1 mRNA expression measured by real-time PCR. Data are expressed as the ratio of MUC5AC to GAPDH mRNA and shown as mean ± SEM of data from one experiment, 3 total wells in each group from one donor.
Figure 9. **IL-13 (5ng/mL) exposure for 14 days significantly decreased Adipo R2 mRNA compared to PBS.** Adipo R2 mRNA expression measured by real-time PCR. Data are expressed as the ratio of MUC5AC to GAPDH mRNA and shown as mean ± SEM of data from one experiment, 3 total wells in each group from one donor.
Discussion

Two seven-transmembrane-domain adiponectin receptors, designated Adipo R1 and Adipo R2, share 67% amino acid homology. Unlike G protein-coupled receptors, their C terminus is intracellular and does not couple with any known G protein. Adipo R1 binds with high affinity to globular adiponectin, while Adipo R2 binds to both full-length and globular adiponectin forms (35) (Figure 10). This study has shown that after exposure with IL-13 for 14 days, NHBE cells express low levels of Adipo R1 and Adipo R2.

![Candidate Indications of Adiponectin Receptor Activations](image)

Figure 10. Impaired Adiponectin action with obesity-linked diseases. Figure modified from (36)

In human plasma, the full-length adiponectin protein predominates. Proteolytic cleavage of the full-length protein at amino acid 110 generates a carboxyl-terminal globular domain (137aa), which circulates in low abundance (35). Both the full length and globular structures did not show significant results for MUC5AC, Adipo R1, or Adipo R2 mRNA expression.
However, both acute and chronic IL-13 concentrations decreased Adipo R1 and Adipo R2 mRNA expression. Adiponectin binding proteins in an in vivo model were expressed in lung tissue of mice and they were all reduced by OVA sensitization and challenge (31). This reduction in adiponectin binding protein expression suggests that allergen challenge might reduce the beneficial effects of adiponectin on the lung by attenuating components of adiponectin-signaling pathways (31). IL-13 might be another factor that reduces the beneficial effects of adiponectin, specifically on NHBE cells by attenuating components of adiponectin-signaling pathways. High serum concentrations of IL-13 in obese individuals would decrease Adipo R1 and Adipo R2 expression at the basal side of NHBE cells. Research clarifying the molecular mechanisms of adiponectin actions and the receptors (Adipo R1 and Adipo R2) expression within an obese-asthma endotype is still needed.

Adipo R1 and Adipo R2 serve as receptors for globular and full-length adiponectin and mediate increased AMP-activated kinase (AMPK), proliferator-activated receptor alpha PPARα ligand activities, fatty-acid oxidation, and glucose uptake by adiponectin (37). Immunofluorescence experiments have shown the presence of both receptors on the plasma-membrane of three different human lung cell lines (20). This experimentation has shown the presence of AdipoR1 and Adipo R2 on NHBE cells. Further determination of receptor location could be conducted by immunohistochemistry and protein production could be determined by western blot.

Lumeng et al. state that inflammation plays a crucial role in the many complications of obesity (38). The development of obesity increases the size of adipocytes, which secrete chemokines that attract leukocytes into adipose tissue. Classically activated macrophages produce pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and IL 1β (39).
Increased adipose tissue mass, which is a result of energy imbalance between intake and expenditure, is a characteristic of obesity (3,27). Another characteristic of the obese state is low-grade chronic systemic inflammation (3,5). This systemic inflammation provides a link between obesity and insulin resistance, type 2 diabetes, atherosclerosis, and asthma (28). Most patients with severe or difficult-to-control asthma in the United States are obese (29).

Further investigation is needed to determine if substances secreted from adipocytes play a role in asthma and could directly affect the bronchial epithelium. Adiponectin immunohistochemistry could be performed on lung tissue from obese asthmatics and compared to obese non-asthmatics and their lean counterparts. Scott et al reported a recent cluster analysis that identified a unique obese-asthma endotype (36). Future studies could also focus on a comparison of co-culture from mature white adipocytes and NHBE cells collected from obese asthmatics compared to obese non-asthmatics and their lean counterparts.
Conclusion

Obesity and asthma have been associated with each other in many clinical and population publications. However, the mechanism of how the association occurs is still controversial (Figure 1) (28). The NHBE cell culture from 3 female donors did not show significant results for MUC5AC, Adipo R1, or Adipo R2 mRNA expression compared with ciliated cells when stimulated with the full length or globular forms of adiponectin. Therefore, the hypothesis that adiponectin would decrease IL-13 induced MUC5AC expression from differentiated NHBE cells was not proven.

However, both acute and chronic IL-13 concentrations decreased Adipo R1 and Adipo R2 mRNA expression. The obese state could decrease Adipo R1 and Adipo R2 expression. Serum concentrations of IL-13 have been shown to increase in obese individuals, while adiponectin levels decrease (Figure 12). A direct link between IL-13 and the adiponectin receptors Adipo R1 and Adipo R2 within normal human bronchial epithelial cells would exacerbate the chronic state of inflammation.
Figure 11. Effect of Obese state with underlying allergic asthma (22)

Figure 12. Schematic of Experimental Results
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Vitae

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