

# The Expression of Adiponectin Receptors and the Effects of Adiponectin and Leptin on Airway Smooth Muscle Cells

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**Purpose:** Obesity is a major risk factor for asthma and it influences airway smooth muscle function and responsiveness. Adiponectin is inversely associated with obesity and its action is mediated through at least 2 cell membrane receptors (AdipoR1 and AdipoR2). Leptin is positively associated with obesity. We investigated whether human airway smooth muscle (ASM) cells express adiponectin receptors and whether adiponectin and leptin regulate human ASM cell proliferation and vascular endothelial growth factor (VEGF) release. **Materials and Methods:** Human ASM cells were growth-arrested in serum-deprived medium for 48 hours and then stimulated with PDGF, adiponectin and leptin. After 48 hours of stimulation, proliferation was determined using a cell proliferation ELISA kit. Human AdipoR1 and -R2 mRNA expressions were determined by RT-PCR using human-specific AdipoR1 and -R2 primers. Concentrations of VEGF, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1 $\alpha$  in cell culture supernatant were determined by ELISA. **Results:** Both AdipoR1 and AdipoR2 mRNA were expressed in the cultured human ASM cells. However, adiponectin did not suppress PDGF-enhanced ASM cell proliferation, nor did leptin promote ASM cell proliferation. Leptin promoted VEGF release by human ASM cells, while adiponectin did not influence VEGF release. Neither leptin nor adiponectin influenced MCP-1 secretion from human ASM cells. Adiponectin and MIP-1 $\alpha$  were not secreted by human ASM cells. **Conclusion:** Human ASM cells expressed adiponectin receptors. However, adiponectin did not regulate human ASM cell proliferation or VEGF release, while leptin

stimulated VEGF release by human ASM cells.

**Key Words:** Smooth muscle cells, cell proliferation, vascular endothelial growth factor, leptin, adiponectin, receptors

## INTRODUCTION

Obesity has become a major health problem worldwide and its incidence has sharply increased. Prevalence of asthma has also been steadily increasing during the past 30 years. Obesity is a major risk factor for asthma, and it increases the prevalence and severity of asthma and also influences lung function. Girls who become obese between the ages of 6 and 11 years have an almost fivefold increased risk of developing new asthma symptoms.<sup>1</sup> Weight loss reduces airway obstruction as well as peak expiratory flow variability in obese patients with asthma.<sup>2</sup> Studies on mice have shown a causal relationship between obesity and asthma.<sup>3</sup> Obesity influences airway smooth muscle function and airway hyper-responsiveness (AHR). In epidemiological studies, the body mass index (BMI) has been shown to be associated with AHR,<sup>4</sup> and innate AHR is manifested in obese mice.<sup>5</sup>

Hyperplasia and hypertrophy of airway smooth muscle (ASM) are characteristic features of airway remodeling in asthma. There is limited information on how obesity influences smooth muscle function. Obesity may reduce lung growth in childhood, which leads to reduced functional residual capacity, and may accelerate airway remodeling when asthma is exacerbated.<sup>6</sup> Obesity induces systemic inflammation, which can affect the lung microenvironment by circulating cytokines

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and chemokines. Adiponectin, a fat tissue-derived hormone, is inversely associated with obesity. In children with obesity, serum adiponectin levels decrease,<sup>7</sup> and adiponectin mRNA expression from adipose tissue is negatively associated with obesity.<sup>8</sup> Adiponectin also demonstrates anti-inflammatory effects and down-regulation of vascular smooth muscle cell proliferation.<sup>9</sup> The activity of adiponectin is mediated by at least 2 cell membrane receptors (AdipoR1 and AdipoR2). However, there has been no report to date, on identification of adiponectin receptors in human ASM cells.

Leptin is a 16-kDa peptide released from fat cells and other tissues, and may exacerbate airway inflammation in asthma. It induces pulmonary inflammation after ozone exposure in mice,<sup>5</sup> and augments AHR in ovalbumin-sensitized mice.<sup>3</sup> Leptin induces cytokine production and cell proliferation in hematopoietic cells<sup>10</sup> and human umbilical vein endothelial cells.<sup>11</sup> However, it is unknown whether leptin influences ASM cell proliferation and cytokine production by ASM cells.

The goal of this study was to identify adiponectin receptors (AdipoR1 and AdipoR2) in human ASM cells and evaluate whether adiponectin and leptin regulate ASM cell proliferation. On the basis of the above, we hypothesized that leptin would enhance ASM cell proliferation, compared to no stimulation, and adiponectin would inhibit ASM cell proliferation enhanced by PDGF stimulation. In addition, we investigated whether adiponectin and leptin influence the release of vascular endothelial growth factor (VEGF), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , and adiponectin from ASM cells.

## MATERIALS AND METHODS

### Human airway smooth muscle cell culture

Primary human ASM cells and cell growth supplements were purchased from Clonetics (San Diego, CA, USA). Penicillin, streptomycin, fetal bovine serum (FBS), 10% Dulbecco's modified Eagle's medium (DMEM), and DMEM/F-12 medium were obtained from Gibco BRL. Bovine serum

albumin (BSA) and insulin-transferrin-selenium (ITS) were obtained from Sigma. Adiponectin, leptin and PDGF were purchased from R & D (R & D Systems, Minneapolis, MN, USA).

Human ASM cells were plated in 75 cm<sup>2</sup> culture flasks with 10% FBS/DMEM containing 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin, and 2 mM L-glutamine, and were incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. When the cells became confluent, they were passaged with the use of 0.025% trypsin in 0.01% EDTA. Cells at passages 3 to 6 were used for all experiments.

### Analysis of human ASM cell proliferation

Human ASM cells were seeded at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> in 96-well culture plates. When the cells reached 70% confluence, growth was arrested in serum-free DMEM/F-12 medium containing 0.1% BSA for 48 hours. Cells were then incubated with 20 ng/mL of platelet-derived growth factor (PDGF) for 48 hours as a positive control. ASM cell proliferations were compared after stimulation with 10, 50 and 100 ng/mL of leptin, and 10, 50, and 100 ng/mL of adiponectin with and without PDGF co-stimulation.

Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation ELISA kit (Roche Applied Science, Mannheim, Germany). The cells were cultured in 96-well plates under the same conditions as described above and incubated with 10  $\mu$ M BrdU for 24 hours. The BrdU incorporation into DNA was detected using a commercial kit. Briefly, cells were fixed and DNA was denatured. Anti-BrdU-POD monoclonal antibody was used to bind to the BrdU incorporated into the newly synthesized cellular DNA. The absorbance of immune complex was measured at 450 nm.

### Preparation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) for adiponectin receptors

The total cellular RNA was prepared using a Trizol reagent kit. After quantification by spectrophotometry, the RNA was reverse transcribed and cDNAs were produced. PCR was performed in a Takara PCR thermal cycler at a 50  $\mu$ L reaction

volume containing 100 ng of genomic DNA, 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.08% Triton X-100, 200 μM deoxyribonucleotides, 0.75 U Taq polymerase, and 1.0 μM oligonucleotide primers of each gene. The following primers were used: for human AdipoR1, the forward primer was 5'-TTCTTCCTCATGGCTGTGATGT-3', reverse: 5'-AAGAAGCGCTCAGGAATTCG-3' (GenBank accession number: NM015999); for human AdipoR2, the forward primer was 5'-ATAGGGCAGATAGGCTGGTTGA-3', and the reverse primer was 5'-GGATCCGGGCAGCATAACA-3' (GenBank accession number: nm024551); for β-actin, the forward primer was 5'-GATCTTGATCTTCATGGTGCTAGG-3', and the reverse primer was 5'-TTGTAACCAACTGGGACCATATGG-3' (GenBank accession number: AL451010).<sup>12,13</sup> After the initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C and extension for 1 minute at 72°C were carried out, and the PCR was terminated by a final extension at 72°C for 10 minutes. Reaction products were then separated on a 1% agarose gel in Tris acetate EDTA buffer. Sequence of the PCR products was confirmed by the National Center for Biotechnology Information (NCBI) nucleotide-nucleotide BLAST program.

### Cytokine assay

To evaluate the effects of adiponectin and leptin on the production of VEGF, MCP-1 and MIP-1α, by human ASM cells, the cells were cultured to confluence in 10% FCS/DMEM in humidified 5% CO<sub>2</sub> air at 37°C in 24-well culture plates and growth-arrested in serum-free DMEM/F-12 medium for 48 hours. Cells were stimulated with 20 ng/mL of PDGF, 10, 50, and 100 ng/mL of leptin and 10, 50, and 100 ng/mL of adiponectin. After 24-hours of incubation, the cell culture supernatant was harvested and stored at -80°C until ELISA for cytokines was performed.

### Measurement of VEGF, MCP-1 and MIP-1α by ELISA

VEGF, MCP-1 and MIP-1α in cell culture supernatants were analyzed by ELISA according to the manufacture's manuals (R & D Systems). The minimum detectable doses of cytokines were less

than 5 pg/mL for VEGF and MCP-1 and less than 10 pg/mL for MIP-1α.

### Statistics

Each experiment was repeated on multiple occasions with triplicate dishes. Data were evaluated by one-way ANOVA followed by the Bonferroni's multiple comparison test.

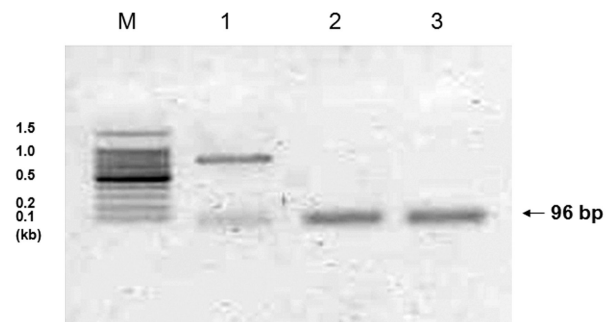
## RESULTS

### Expression of AdipoR1 and AdipoR2 mRNA on the human ASM cells

We found that the mRNAs of adiponectin receptors 1 and 2 were expressed in cultured human ASM cells. Sequencing of PCR products demonstrated that results were compatible with human adiponectin receptors 1 and 2 (according to the NCBI nucleotide-nucleotide BLAST program), thus confirming the presence of these genes in human ASM cells (Fig. 1).

### Effect of adiponectin and leptin on the proliferation of human ASM cells

To evaluate the effect of leptin on human ASM cell proliferation, ASM cells were stimulated with leptin (10, 50, and 100 ng/mL) and 20 ng/mL of



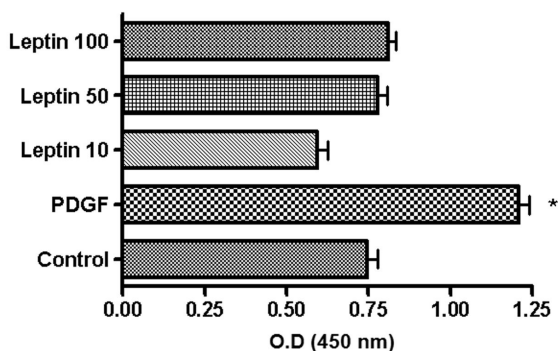
**Fig. 1.** Expression of adiponectin receptors AdipoR1 and AdipoR2 in the human airway smooth muscle cell. Expression of β-actin (lane 1), AdipoR1 (lane 2) and AdipoR2 (lane 3) mRNA is observed. Sequencing of these PCR products demonstrated that the products were compatible with human AdipoR1 and AdipoR2 (according to NCBI nucleotide-nucleotide BLAST); these findings confirmed the presence of these genes in the human airway smooth muscle cells.

PDGF, and the proliferation was then compared with unstimulated ASM cells. The stimulation with PDGF significantly enhanced ASM cell proliferation compared to control cells. However, leptin did not enhance nor suppress ASM cell proliferation at all concentrations studied (Fig. 2). Leptin also did not augment PDGF-enhanced ASM cell proliferation when co-stimulated with PDGF (data not shown).

Since the serum adiponectin level is inversely correlated with obesity, we investigated whether adiponectin had any effect on down-regulation of PDGF-enhanced ASM cell proliferation. Thus, human ASM cells were stimulated with 20 ng/mL of PDGF with and without adiponectin (10, 50, and 100 ng/mL), and the proliferation was compared to cells stimulated with PDGF only. The PDGF-stimulated ASM cells showed enhanced proliferation. However, adiponectin did not suppress PDGF-enhanced ASM cell proliferation at all doses studied (Fig. 3). We also stimulated ASM cells with adiponectin only, however, no difference in proliferation was observed compared to no stimulation (data not shown).

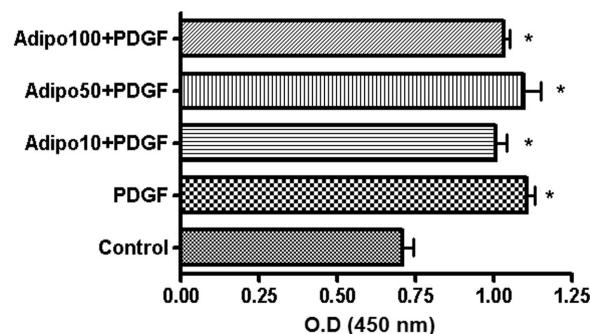
#### Effect of PDGF, adiponectin and leptin on VEGF release by human ASM cells

Human ASM cells were treated with PDGF (20 ng/mL), adiponectin (50 ng/mL), and leptin (50 ng/mL). VEGF release was significantly enhanced

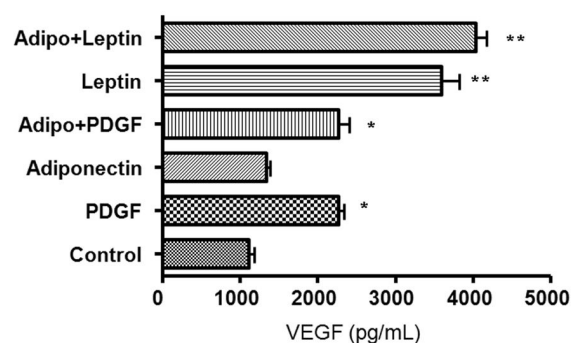


**Fig. 2.** Effect of leptin on the proliferation of human ASM cells. Human ASM cells were stimulated with leptin (10, 50 and 100 ng/mL) and 20 ng/mL of PDGF, and compared to unstimulated cells. Leptin did not enhance or suppress ASM cell proliferation, while PDGF significantly augmented cell stimulation. ASM, airway smooth muscle; PDGF, platelet-derived growth factor. \* $p < 0.01$  vs. Control.

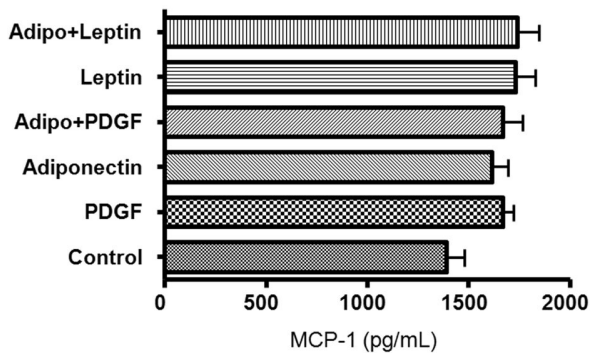
by both PDGF and leptin stimulation compared to controls. VEGF release by leptin stimulation was higher than PDGF stimulation. However, adiponectin did not show any additive effects on VEGF release when added to leptin. Adiponectin did not suppress PDGF-enhanced VEGF release nor did VEGF release by unstimulated ASM cells (Fig. 4).



**Fig. 3.** Effect of adiponectin on the proliferation of human ASM cells. Human ASM cells were stimulated with 20 ng/mL of PDGF in the presence or absence of adiponectin (10, 50, and 100 ng/mL), and proliferation was compared with the control. PDGF-stimulated ASM cells showed enhanced proliferation, however, adiponectin did not enhance or suppress PDGF-enhanced ASM cell proliferation. ASM, airway smooth muscle; PDGF, platelet-derived growth factor. \* $p < 0.01$  vs. Control.



**Fig. 4.** Effect of PDGF, adiponectin and leptin on VEGF release by human ASM cells. Human ASM cells were treated with 20 ng/mL of PDGF, 50 ng/mL of adiponectin and 50 ng/mL of leptin. VEGF release was significantly enhanced by PDGF stimulation compared to no stimulation. Leptin significantly augmented VEGF release compared to no stimulation or stimulation by PDGF. Adiponectin showed no stimulatory or inhibitory effects on VEGF release, when it stimulated cells with and without PDGF or leptin. PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor. \* $p < 0.05$  vs. Control, \*\* $p < 0.05$  vs. Control and PDGF.



**Fig. 5.** Effect of PDGF, adiponectin and leptin on MCP-1 release by human ASM cells. Human ASM cells were treated with PDGF (20 ng/mL), adiponectin (50 ng/mL), and leptin (50 ng/mL). MCP-1 was constitutively released from human ASM cells and was neither augmented by PDGF or leptin stimulation, nor suppressed by adiponectin. PDGF, platelet-derived growth factor; ASM, airway smooth muscle.

#### Effect of PDGF, adiponectin and leptin on MCP-1, MIP-1 $\alpha$ and adiponectin releases by human ASM cells

We evaluated the release of MCP-1 by human ASM cells treated with PDGF, adiponectin and leptin. MCP-1 was released by ASM cells, but was neither augmented, nor suppressed by PDGF, leptin or adiponectin stimulation. There were no differences in MCP-1 release when it was co-stimulated with adiponectin and leptin or with adiponectin and PDGF (Fig. 5). Human ASM cells did not secrete MIP-1 $\alpha$  or adiponectin after stimulation with PDGF, leptin or adiponectin (data not shown).

## DISCUSSION

Asthma and obesity are growing public health problems. Epidemiological data as well as data from animal models implicate obesity as an important risk factor for asthma and atopic disease. In this study, we studied the relationship between adiponectin, leptin, and airway smooth muscle cell proliferation. First, we demonstrated that adiponectin receptors (AdipoR1 and AdipoR2) were expressed in human ASM cells; to our knowledge, this is the first report on such finding. To evaluate the role of adiponectin and leptin in

the ASM cells, we stimulated ASM cells with adiponectin and leptin, and found that adiponectin and leptin did not directly influence human ASM cell proliferation. However, since leptin increased the release of VEGF from ASM cells, it must play some role in asthma. Although ASM cells expressed adiponectin receptors, they did not secrete adiponectin. It is, therefore, possible that adiponectin plays a role through its receptors. Since serum adiponectin declines in obesity and adiponectin has an anti-inflammatory effect, these anti-inflammatory effects might inhibit ASM cell proliferation. Although adiponectin did not suppress the proliferation of ASM cells, it appeared to act on adiponectin receptors and cause anti-allergic reactions. Indeed, some studies have shown that adiponectin attenuated allergen-induced airway inflammation and hyperresponsiveness in mice.<sup>6,14</sup>

Leptin acts on the hypothalamus to induce satiety and increase metabolism. In addition, leptin has proinflammatory effects.<sup>10</sup> It has been shown to protect T lymphocytes from apoptosis, regulate T-cell proliferation and activation, recruit and activate monocytes and macrophages, and promote angiogenesis.<sup>15</sup> Given the facts that serum leptin levels are increased in obesity and it has proinflammatory effects, it is reasonable to suggest that this hormone plays a role in asthma. Leptin enhanced pulmonary inflammation induced by acute ozone exposure and increased AHR and serum IgE levels in mice.<sup>3,5</sup> On the other hand, leptin has been shown to have no effect on IL-13, IL-4 and IL-5 mRNA expressions in lung tissue, and no direct effect on AHR in PBS-challenged mice, while it has AHR in OVA-challenged mice.<sup>3</sup> Leptin may be involved in airway remodeling by inducing angiogenesis. Leptin directly induces angiogenesis by activation of its functional receptor, Ob-Rb, which is expressed in endothelial cells.<sup>16</sup> In addition to its angiogenic activity, leptin is also able to induce vascular fenestrations and permeability.<sup>17</sup> Leptin could indirectly induce angiogenesis via upregulation of VEGF expression.<sup>11,18</sup> This fact is consistent with our results that showed that leptin induced VEGF release by ASM cells. VEGF is a multifunctional angiogenic regulator that stimulates subepithelial neovascularization and vascular

permeability, a key finding in asthma. It also promotes epithelial cell proliferation and endothelial cell survival.<sup>19,20</sup> Recent data have shown that VEGF also induces an asthma-like phenotype characterized by mucus hyperplasia, subepithelial fibrosis, smooth muscle hyperplasia, and AHR.<sup>21-24</sup>

There has been no prior report on the expression of leptin in ASM cells. Leptin mRNA was detected in the lungs of mice.<sup>3</sup> In addition, leptin expression has been found at sites of wound healing and in inflamed colonic epithelial cells.<sup>25,26</sup> Although we did not investigate the ASM cell leptin receptor in this study, we can assume that there are leptin receptors in ASM cells because leptin augmented VEGF release by the cultured ASM cells.

MCP-1 plays an important role in allergic inflammation. MCP-1 expression is up-regulated in asthmatic airways. Interestingly, it is also associated with obesity. In obese mice, its expression is augmented, and the adipose tissue inflammatory gene expression is reduced in mice without MCP-1 or MCP-1 receptors.<sup>27,28</sup> There is no prior report demonstrating that leptin or adiponectin affects MCP-1 release by ASM cells. In this study, the release of MCP-1 by human ASM cells was not influenced by adiponectin, leptin or PDGF. We also evaluated whether ASM cells released adiponectin and MIP-1 $\alpha$ . However, neither was detected in the cultured ASM cell supernatants (data not shown). Since we stimulated ASM cells only with leptin, adiponectin and PDGF and did not investigate mRNA expression of adiponectin and MIP-1 $\alpha$  by RT-PCR, we do not know whether adiponectin and MIP-1 $\alpha$  are produced by ASM cells. We did not investigate the effects of adiponectin and leptin on the release of inflammatory cytokines other than VEGF, MCP-1 and MIP-1 $\alpha$ . In conclusion, our results demonstrate for the first time that adiponectin receptors (AdipoR1 and R2) are expressed in human ASM cells. However, adiponectin did not influence human ASM cell proliferation or the release of VEGF and MCP-1. Leptin promoted VEGF release by human ASM cells, while it did not influence ASM cell proliferation. Taken together, leptin and adiponectin are not likely to be a therapeutic target for the prevention of human ASM cell proliferation, nevertheless leptin may modulate angiogenesis

and airway remodeling by promoting the release of VEGF from ASM cells, and thus being a potential therapeutic target in obese asthmatic patients.

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