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Adiponectin-AdipoR1/2-APPL1 Signaling Axis Suppresses Human Foam Cell Formation; Differential Ability of AdipoR1 and AdipoR2 to Regulate Inflammatory Cytokine Responses

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Abstract

Objective—Adiponectin is an adipokine that exerts anti-inflammatory and anti-atherogenic effects during macrophage transformation into foam cells. To further understand the signaling pathways of adiponectin involved in macrophage foam cell transformation, we investigated the roles of two adiponectin receptors (AdipoR1 and AdipoR2) and their downstream adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL1) in mediating adiponectin action on foam cell transformation.

Methods and Results—Transfections were performed to overexpress or knockdown AdipoR1 or AdipoR2 genes in human THP-1 monocytes. Lentiviral-shRNAs were also used to knockdown APPL1 gene in these cells. Foam cell transformation was induced via exposure to oxidized low-density lipoprotein (oxLDL). Our results showed that both AdipoR1 and AdipoR2 were critical for transducing the adiponectin signal that suppresses lipid accumulation and inhibits transformation from macrophage to foam cell. However, AdipoR1 and AdipoR2 were found to have differential effects in diminishing proinflammatory responses. While AdipoR1 was required by adiponectin to suppress tumor necrosis factor alpha (TNF) and monocyte chemoattractant protein 1 (MCP-1) gene expression, AdipoR2 served as the dominant receptor for adiponectin suppression of scavenger receptor A type 1 (SR-A1) and upregulation of interleukin-1 receptor antagonist (IL-1Ra). Knockdown of APPL1 significantly abrogated the ability of adiponectin to inhibit lipid accumulation, SR-A1 and nuclear factor- κ B (NF- κ B) gene expression, and Akt phosphorylation in macrophage foam cells.

Conclusions—In current studies, we have demonstrated that adiponectin's ability to suppress macrophage lipid accumulation and foam cell formation is mediated through AdipoR1 and AdipoR2 and the APPL1 docking protein. However, AdipoR1 and AdipoR2 exhibited a differential ability to regulate inflammatory cytokines and SR-A1. These novel data support the

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Conflict of Interest: none declared

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idea that the adiponectin-AdipoR1/2-APPL1 axis may serve as a potential therapeutic target for preventing macrophage foam cell formation and atherosclerosis.

Keywords

adiponectin; adiponectin receptor; APPL1; macrophages; foam cells; atherosclerosis; inflammation

Introduction

Atherosclerosis is a chronic disease characterized by cholesterol plaque formation within the blood vessel wall, and is one of the leading causes of mortality in developed countries ¹. The pathogenesis of atherosclerosis involves a network of vascular wall cells and mediators ², in which macrophages play critical roles by producing proinflammatory factors and via transition to lipid-laden foam cells that initiate the formation of atherosclerotic lesion ^{3, 4}. Therefore, intensive efforts are warranted to identify therapeutic targets that prevent cholesterol accumulation and inflammation in macrophage foam cells during atherogenesis.

Adiponectin (also known as ACRP30) is expressed and secreted by adipocytes ⁵, and a plethora of epidemiological data links low circulating levels with insulin resistance, Metabolic Syndrome, obesity, coronary artery disease, and Type 2 Diabetes ⁶⁻¹⁰. We have previously studied mechanisms that could explain these epidemiological relationships and demonstrated that adiponectin, particularly the high molecular weight multimeric form of adiponectin, is negatively correlated with traits that comprise the Metabolic Syndrome trait cluster ¹¹, acts as an autocrine/paracrine factor to regulate adipocyte biology in adipose tissue ¹², and inhibits foam cell formation in macrophages in addition to facilitating HDL-mediated cholesterol efflux from these cells ¹³. However, the mechanisms by which adiponectin exerts these effects in target cells has not been clear. Two cell-surface cognate receptors have been identified for adiponectin, Adiponectin Receptor 1 (AdipoR1) and Adiponectin Receptor 2 (AdipoR2), and expression of these receptors has been reported in a wide variety of tissues ¹⁴⁻¹⁶. Recently, an adapter protein has been identified as a facilitator of signaling through AdipoR1/2, namely, the adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper-containing protein 1 (APPL1). Overexpression of APPL1 increases, while suppression of APPL1 reduces, adiponectin signaling and adiponectin-mediated downstream events through adiponectin receptors ^{17, 18}.

Adiponectin exerts anti-inflammatory and anti-atherogenic effects by down-regulating the expression of inflammatory factors on endothelial cells ¹⁹, reducing the proliferation of vascular smooth muscle cells ²⁰ and suppressing the migration of monocytes and their transformation into macrophage foam cells in the vascular wall ^{19, 21, 22} during atherosclerosis. We have recently reported that overexpressing adiponectin in human THP-1 cells can reduce lipid accumulation in foam cells through regulating genes involved in lipid uptake, efflux, and metabolism, and by reducing production of inflammatory cytokines ¹³. However, the mechanisms and signaling pathways of adiponectin action on regulating macrophage foam cell transformation remain to be explored.

In the present studies, we have further investigated the roles of adiponectin receptors and their downstream transducer APPL1 in mediating adiponectin action in macrophage foam cells. We demonstrated that modulation of AdipoR1 and/or AdipoR2 can alter adiponectin's ability to regulate macrophage lipid accumulation and foam cell formation, and that APPL1 acts as a downstream transducer for adiponectin receptor signaling. Importantly, however, AdipoR1 and AdipoR2 were found to have a differential ability to regulate cytokine and scavenger receptor expression. Our observations indicate that adiponectin-adiponectin

receptors-APPL1 is a key signaling axis in suppressing macrophage lipid accumulation and inflammation.

Methods

Experimental materials

THP-1 human monocytic cells were purchased from American Type Culture Collection (ATCC). Tissue culture medium, fetal bovine serum (FBS), 2-mercaptoethanol, penicillin/streptomycin and phosphate-buffered saline (PBS) were all purchased from Invitrogen. Phorbol myristate acetate (PMA) was purchased from Sigma-Aldrich. Human adiponectin derived from HEK293 cells were purchased from Biovendor. SR-AI, phosphorylated-Akt, total-Akt, -Actin specific antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz. NF- κ B p65 specific antibody was purchased from BD biosciences.

Cell culture

THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 0.05mM 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C with 5% CO₂. The cell culture medium was replaced every 2-3 days until experiments were performed. THP-1 monocytes were treated with 100 nM PMA for 24 h to stimulate differentiation into macrophages, and then the adherent macrophages were washed three times with PBS and rested in fresh complete cell culture medium for 24 h. After that, the macrophages were treated with and without adiponectin for 24 h followed by exposure to 100 μ g/ml oxidized low-density lipoprotein (oxLDL) for another 24 h to transform them into foam cells.

LDL isolation and modification

Human plasma from healthy donors was obtained from American Red Cross, and the density was adjusted to 1.21 g/ml by adding solid KBr. Native LDL was isolated from the plasma by sequential ultracentrifugation at 45,000 rpm for 36 h at 10 °C in a 60 Ti rotor (Beckman Coulter). The separated LDL was dialyzed against PBS at 4 °C for 36 h. Oxidation of LDL was conducted by incubating LDL with 40 μ M CuCl₂ in PBS overnight at 37°C followed by intensive dialysis against PBS at 4 °C for 36 h as we previously described¹³.

Molecular cloning, cell transfection, and stable cell line selection

cDNAs containing full-length human coding sequences for adiponectin receptors were subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). THP-1 cells were transfected with the expression vector or the pcDNA3.1-LacZ control plasmid (Invitrogen) by nucleofection method (Amaxa) according to the manufacturer's instructions. Stable THP-1 cell lines, which overexpress adiponectin receptors or LacZ protein, were generated by geneticin (Invitrogen) selection for 2 weeks. Lentiviral-shRNA constructs for specific knockdown of APPL1 expression in THP-1 cells and the scramble control non-specific lentiviral-shRNA plasmid were obtained from Sigma-Aldrich. To generate lentivirus, 7.5 μ g of each lentiviral-shRNA plasmid plus 5 μ g VSV-G (Vesicular Stomatitis Virus-G protein) and 7.5 μ g pGag/Pol packaging plasmids were co-transfected in HEK293T cells in 10cm plates with calcium precipitation method. Viral particles were harvested from the supernatant 48 h after transfection, and then THP-1 cells were transduced with virus at MOI~50 with 8 μ g/ml Polybrene (Sigma-Aldrich). Stable cell lines expressing lentiviral-shRNA were generated by puromycin selection for 2 weeks. Transient knockdown of AdipoR1 or AdipoR2 in THP-1 cells were conducted by transfecting siRNA using

nucleofection method (Amaxa) as the manufacturer instructed. siRNA sequences used are: human AdipoR1, AAGGACAACGACUAUCUGCUACAtt (generated by Sigma-Aldrich); human AdipoR2 (Ambion Silencer Select Validated siRNA); siRNA negative control (Sigma-Aldrich universal negative control 1).

Cholesterol Measurement

Macrophage foam cells were harvested with cell lysis buffer (Sigma-Aldrich) containing protease inhibitors (Roche). The concentrations of cholesterol in the cell lysates were quantitatively measured by enzymatic colorimetric assays with Wako cholesterol E kit (Wako Chemicals) as the manufacturer instructed. The sample volume and measuring conditions were as we previously described¹³. The concentrations of cellular proteins from the cell lysates were measured with Bio-Rad protein assay kit.

Quantitative PCR analysis

Total RNA was extracted from THP-1 cells using RNeasy mini kit (Qiagen) and treated with DNase (Qiagen) according to the manufacturer's instructions. cDNA was reverse transcribed using SuperScriptIII reverse transcription kit and random hexamer primers (Invitrogen). Real time quantitative PCR was performed on a Stratagene Mx3000p (Stratagene) using SYBR Green Supermix (Invitrogen). House keeping gene GAPDH was used as internal control. Relative quantification of gene transcripts was calculated in comparison to GAPDH transcripts by using 2^{-Ct} method²³. Primer sets used in quantitative PCR analysis are listed in Table 1.

Western blot analysis

Macrophage foam cells were harvested with cell lysis buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Roche). Protein concentrations in cell lysates were measured by protein assay kit (Bio-Rad). Total proteins (50 ug/lane) were separated by SDS-polyacrylamide gel electrophoresis followed by transfer onto PVDF membranes, and were then incubated 1 h at room temperature in 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20). The membranes were subsequently incubated with specific primary antibodies in 1% nonfat milk in TBST overnight at 4°C, and washed with TBST buffer. Horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz) against the primary antibody were added onto the membrane in 1% nonfat milk in TBST for 1 h at room temperature, and washed three times for 10 minutes each with TBST. Immunodetection analyses were conducted using Amersham ECL detection system (GE).

Statistics

Experimental data are quoted as mean±range. Comparisons between treated and control macrophage foam cells were made using unpaired Student's t-test. Except for analysis in figure 4 and figure 6E-H, one-way Anova was used, followed by Student's t-test to further identify differences between each experimental group and control group. Significance was defined as $p < 0.05$.

Results

1) Adiponectin receptor expression in THP-1 cells

We have previously reported that adiponectin inhibits foam cell formation¹³. In order to define the roles of AdipoR1 and AdipoR2, the expression levels of the two adiponectin receptors in THP-1 cells were examined by quantitative PCR analysis during monocyte to macrophage differentiation (following exposure to PMA), and macrophage to foam cell transformation (following treatment with oxLDL). As shown in Figure 1A, the AdipoR1

expression level was unchanged throughout all stages of the differentiation and transformation process, while AdipoR2 expression levels were progressively decreased as monocytes became macrophages and then foam cells (Figure 1B). However, comparison of absolute AdipoR1 and AdipoR2 gene expression levels revealed that AdipoR1 gene was the predominant species at all three cell stages with mRNA levels that were 6-fold, 11-fold, and 16-fold higher in monocytes, macrophages, and foam cells, respectively, compared with AdipoR2 mRNA (Figure 1C). Another putative adiponectin receptor, T-cadherin, was minimally expressed in THP-1 cells as compared to both AdipoR1 and AdipoR2 (Figure 1C). Since T-cadherin also lacks the cytoplasmic domain to transduce adiponectin signals²⁴, we only focused on investigating the roles of AdipoR1 and AdipoR2 in subsequent experiments.

2) Effects of AdipoR1 and AdipoR2 regulation on adiponectin lipid suppression function

To study the role of adiponectin receptors in mediating adiponectin's effect to suppress lipid accumulation during the THP-1 macrophage foam cell transformation, RNA interference was used to suppress expression of AdipoR1 and AdipoR2 in THP-1 cells, both separately (Figure 2A and 2C) and simultaneously (Figure 2E). siRNA sets for AdipoR1 or AdipoR2 were transfected into THP-1 cells, and specific knockdown of the receptors was confirmed by quantitative PCR analysis (Figure 2A, 2C and 2E). Macrophages were then pretreated with or without adiponectin for 24h followed with another 24h treatment with oxLDL to generate foam cells. To analyze the lipid accumulation response, cholesterol concentrations in AdipoR1, AdipoR2, and AdipoR1+2 siRNA transfected foam cells, as well as in scramble RNA controls, were measured. In Figure 2A, AdipoR1 siRNA dramatically reduced AdipoR1 expression without affecting AdipoR2, and this led to a significant reduction (48%; $p<0.05$) in the ability of adiponectin to inhibit cholesterol accumulation (Figure 2B). On the other hand siRNA for AdipoR2 led to a marked reduction in AdipoR2 mRNA without affecting AdipoR1 (Figure 2C), and, while there was a tendency to impair the adiponectin effect to restrain lipid accumulation (34%; $p=NS$), the differences did not achieve statistical significance (Figure 2D). Double knockdown of both AdipoR1 and AdipoR2 (Figure 2E), as would be predicted, was associated with a significant diminution (41%; $p<0.05$) in this adiponectin action (Figure 2F). Thus, it was clear that the ability of adiponectin to fully inhibit foam cell formation was dependent upon AdipoR1; however, we were not able to make the same claim for AdipoR2 given that differences were not statistically different. This might be explained by the predominance of AdipoR1 expression over that for AdipoR2. To further address the role of AdipoR1 and AdipoR2, we stably overexpressed AdipoR1 or AdipoR2 in THP-1 cells to determine if this would enhance the adiponectin effect. AdipoR1 was hyperexpressed seven-fold without affecting AdipoR2 (Figure 3A) and its hyperexpression was associated with a significant enhancement (14%; $p<0.05$) in adiponectin action on suppressing cholesterol accumulation (Figure 3B). As shown in Figure 3C, three-fold hyperexpression of AdipoR2 did not alter AdipoR1 expression but did significantly accentuate (33%, $p<0.05$) the ability of adiponectin to further inhibit cholesterol accumulation (Figure 3D). This observation indicates that the adiponectin signal can also be transduced by AdipoR2.

3) AdipoR1 and AdipoR2 exert differential roles in gene regulation

Yamauchi *et al* first reported differences in receptor-mediated adiponectin signal transduction comparing AdipoR1 and AdipoR2 in mice liver²⁵. Here we studied whether AdipoR1 and AdipoR2 play differential roles in regulating key genes involved in macrophage lipid loading and the foam cell transition. Again, AdipoR1 and AdipoR2 in THP-1 cells were separately or simultaneously suppressed using siRNA transfection. mRNA expression levels for each receptor in single, double, or control siRNA transfected foam cells are shown in Figure 4A.

These siRNA transfected THP-1 macrophages were then pretreated with different adiponectin concentrations (0, 5, or 10 µg/ml) followed by exposure to oxLDL. Expression of genes involved in lipid metabolism and inflammation were examined by quantitative PCR (Figure 4B-E). The mRNA expression levels in 5 µg/ml or 10 µg/ml adiponectin pre-treated foam cells were examined relative to their control cells in each experiment treated in the absence of adiponectin. The data show that expression of scavenger receptor A type 1 (SR-AI), which facilitates oxidized lipid uptake, was suppressed by adiponectin in a dose-dependent manner in control, AdipoR1 knockdown, AdipoR2 knockdown, and AdipoR1+2 double knockdown foam cells (Figure 4B). However, in AdipoR2 knockdown cells, the suppression of SR-AI expression by adiponectin was significantly less than that in control cells (34% vs. 70% with 5 µg/ml adiponectin, $p<0.01$; or 39% vs. 80% with 10 µg/ml adiponectin, $p<0.05$) (Figure 4B). Similarly, expression of interleukin-1 receptor antagonist (IL-1Ra), which is defined as an anti-inflammatory cytokine, was increased by adiponectin in a dose-dependent manner; however, in AdipoR2 knockdown cells, the induction of IL-1Ra was less pronounced than after knockdown of adipoR1 (Figure 4C). Thus, the adiponectin-mediated changes in SR-AI and IL-1Ra gene expression are more sensitive to the level changes of AdipoR2 than with adipoR1 in macrophage foam cells.

In contrast, adiponectin suppression of inflammatory cytokines, namely TNF and MCP-1, was found to be more sensitive to AdipoR1 knockdown compared with that for adipoR2 (Figure 4D and E). Adiponectin suppressed TNF expression in a dose-dependent manner, and these effects were similar in magnitude in both control and AdipoR2 knockdown foam cells. However, AdipoR1 knockdown cells exhibited a reduced degree of TNF suppression when compared to that in controls (4% vs. 22%, $p=NS$) with 5 µg/ml of adiponectin treatment and (7% vs. 29%, $p<0.05$) with 10 µg/ml adiponectin treatment (Figure 4D). Similar to the results obtained for TNF, MCP-1 was suppressed by adiponectin in control and AdipoR2 knockdown foam cells; however, suppression of MCP-1 was effectively abrogated following AdipoR1 knockdown at the intermediate adiponectin concentration (5 µg/ml), but not at higher adiponectin concentration (Figure 4E).

4) APPL1 is required for Adiponectin suppression of lipid accumulation in foam cells

Since APPL1 had been reported as a downstream signal for adiponectin receptors in skeletal muscle¹⁷ and endothelial cells¹⁸, we further investigated the regulatory signaling pathway for adiponectin action by assessing the role of APPL1 in human THP-1 macrophages. THP-1 cells were transduced with APPL1 lentiviral shRNA and stable APPL1 knockdown cell lines were generated. APPL1 knockdown resulted in a ~60% ($p<0.01$) reduction of APPL1 protein in these cells when compared to control cells transduced with a scramble control lentiviral shRNA construct (Figure 5A). In light of a recent report that APPL2, an isoform of APPL1, is alternatively regulated in mediating the adiponectin signal in muscle cells²⁶, we measured APPL2 protein levels in the APPL1 knockdown cell lines, and observed an increase in APPL2 expression. This is consistent with the purported competition between APPL1 and APPL2 for responding to the adiponectin signals in THP-1 cells (Figure 5B). The expression levels of both adiponectin receptors were unaffected in APPL1 knockdown cells and we observed no changes of APPL1 expression levels in AdipoR1 and/or AdipoR2 knockdown cells, either (data not shown).

Control and APPL1 knockdown macrophages were then pre-treated with 5 µg/ml adiponectin or PBS for 24hr, followed by transformation into foam cells via addition of oxLDL for another 24hr. Cholesterol content in scramble RNA control and APPL1 shRNA transduced foam cells were examined relative to levels for these cell lines treated in the absence of adiponectin. APPL1 knockdown led to a significant reduction (69%; $p<0.05$) in the ability of adiponectin to inhibit cholesterol accumulation (Figure 5C).

5) APPL1 mediates adiponectin signaling and regulation of gene expression during the macrophage foam cell transformation

As shown in Figure 6A, adiponectin decreased expression of SR-AI in control foam cells, but had no effect on altering SR-AI expression in APPL1 knockdown cells. Similarly, NF- κ B p65 expression levels and the phospho-Akt to total Akt ratio were also decreased in adiponectin-treated control foam cells, but not in APPL1 knockdown cells. APPL1 knockdown also attenuated the suppression effect of adiponectin on TNF, MCP-1 and SR-AI gene expressions levels and the upregulation effect of adiponectin on IL-1Ra was abrogated (Figure 6E-H).

Discussion

We¹³ and others^{21, 22} had previously shown that adiponectin suppresses lipid accumulation and inflammation in macrophage. Here, we have further investigated the mechanisms underlining the reductions of lipid accumulation and inflammation in macrophages during the transition to foam cells by exploring the role of adiponectin receptors and APPL1 in adiponectin signaling.

AdipoR1 and AdipoR2 were first cloned from a skeletal muscle cDNA library¹⁴, and expression was detected in various tissues, including macrophages^{27, 28}. We first examined mRNA expression for both receptors as THP-1 cells transitioned from monocytes to macrophages then to foam cells. We also tried to examine the protein expression levels of AdipoR1 and 2 by using multiple commercial antibodies, however, none of these antibodies yielded results that gave us confidence in western blot analyses. Therefore, we have only reported mRNA expression levels. As the results shown, while AdipoR1 was consistently expressed at a higher level than AdipoR2, only the latter exhibited significant changes during cell differentiation and transformation to the foam cell stage with a progressive diminution. Our study is the first to examine differential regulation of adiponectin receptors during foam cell formation. The data indicate that any beneficial effects of adiponectin mediated specifically by adipoR2 may be diminished once macrophages transition to foam cells during atherogenesis.

The current data indicate that both AdipoR1 and AdipoR2 participate in adiponectin's action to reduce lipid accumulation and inhibit foam cell formation, as manifest by the significant decrease in cholesterol content observed in AdipoR1 or AdipoR2 overexpressing macrophages, and by the cholesterol increments in AdipoR1 and/or AdipoR2 knockdown cells in response to oxLDL. There was no further impairment of adiponectin-mediated lipid suppression functions, however, in AdipoR1 and AdipoR2 double knockdown foam cells compared to that in AdipoR1 single knockdown cells. We speculate that it is probably due to the differences between knockdown efficiency in these cell lines (i.e., AdipoR1 knockdown efficiency is less in double knockdown cells than that in single knockdown cells; also, siRNA-induced knockdown is more robust for adipoR1 than adipoR2). In addition, it might probably be explained by the fact that AdipoR1 is the dominantly expressed receptor, thus, no further impairment would be observed in double knockdown cells compared to AdipoR1 single knockdown, since this dominant receptor was reduced efficiently in both cell lines.

Interestingly, however, the adiponectin receptors were found to have a differential ability to regulate gene expression involved in inflammation and lipid uptake. In response to adiponectin, AdipoR2 predominates in the suppression of SR-AI which imports lipid, and in the induction of IL-1Ra which exerts anti-inflammatory functions, while profound suppression of adipoR1 had no significant effect on these biological functions. On the other hand, AdipoR1 was more potent in the suppression of inflammatory cytokines, TNF and MCP-1. As for TNF, knockdown of adipoR2 had no observable impact on adiponectin's

regulatory effect, and, in the case of MCP-1, both receptors had activity at the high adiponectin concentration but only AdipoR1 knockdown influenced MCP-1 expression at the lower adiponectin concentration (i.e., the sensitivity of adiponectin action). It is important to keep in mind that AdipoR1 mRNA levels exceeded that for AdipoR2 by 11-fold in macrophages and 16-fold in foam cells. This must be taken into account when interpreting the data regarding different roles for AdipoR1 and AdipoR2. The higher level of AdipoR1 expression constitutes bias against the data showing greater potency for suppression of SR-A1 and induction of IL-1Ra by AdipoR2, and strengthens our conclusion. This question is less clear pertaining to the apparent ability of AdipoR1 to exert the more potent effect for suppression of TNF and MCP-1. However, knockdown of AdipoR2 expression by more than 66% had no effect on adiponectin's ability to suppress TNF, nor affect suppression of MCP-1 at the lower adiponectin concentration, in contrast to the clear loss of adiponectin action upon knockdown of AdipoR1. On balance, the data are consistent with the conclusion that AdipoR1 and AdipoR2 play differential roles in regulating genes related to lipid metabolism and inflammation in human macrophages during the foam cell transformation. These results are consistent with the previous reports that AdipoR1 and AdipoR2 regulated different signaling pathways following adiponectin treatment in mice liver²⁵.

In the current studies, we investigated whether the adaptor protein APPL1 played a role in transducing adiponectin's downstream action and receptor signaling. Knockdown of APPL1 abrogated adiponectin action to reduce lipid accumulation upon exposure to oxLDL in THP-1 human macrophages. Furthermore, APPL1 knockdown was associated with the loss of SR-A1 reduction in foam cells treated with adiponectin. These results revealed that APPL1 is an important signaling molecule for mediating anti-atherogenic effects of adiponectin in macrophage foam cells. Since NF- κ B activation by the PI3-kinase/Akt pathway is well recognized as a proinflammatory response mechanism for upregulating inflammatory cytokines^{29, 30}, and APPL1 has been reported to bind with Akt and regulate Akt activity¹⁷. We then speculated that knockdown of the APPL1 can diminish the ability of adiponectin to suppress Akt phosphorylation and NF- κ B expression levels in macrophage foam cells. Therefore, our data demonstrated that APPL1 is also required for transducing adiponectin's anti-inflammatory actions in THP-1 macrophage foam cells possibly through regulating the PI3K/Akt/NF- κ B genes. Interestingly, we observed that in APPL1 knockdown cells, APPL2, an isoform of APPL1 that forms a dimer with APPL1, is elevated. It is reported that APPL2 can associate with both AdipoR1 and AdipoR2 and negatively regulate adiponectin signaling in muscle cells²⁶. We speculate that of the elevated level of APPL2 might compete with APPL1 to bind with AdipoR1 and AdipoR2, and down-regulate adiponectin signaling.

In conclusion, our present results indicate that the adiponectin-AdipoR1/2-APPL1 axis in human macrophages can play a key role in regulating lipid metabolism, inflammation, and foam cell transformation. In addition, AdipoR1 and AdipoR2 exhibited a differential ability to regulate proinflammatory cytokines and SR-A1. Enhancing adiponectin receptors and APPL1 function could serve as a therapeutic target for the treatment and prevention of atherosclerosis.

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- Adiponectin suppresses foam cell transformation through AdipoR1, AdipoR2 and APPL1.
- AdipoR1 is required by adiponectin to suppress TNF and MCP-1 gene expression.
- AdipoR2 is the dominant receptor to suppress SR-AI and upregulate IL-1Ra.
- APPL1 is required to inhibit SR-AI & NF- κ B gene expression and Akt phosphorylation.

Figure 1. AdipoR1 and AdipoR2 expression in THP-1 cells during macrophage differentiation and transformation

The THP-1 monocytes were stimulated with PMA for 24h followed with another 24h rest to differentiate into macrophages. Then the macrophages were exposed to 100 μ g/ml of oxLDL to transform into foam cells. **(A)** AdipoR1 or **(B)** AdipoR2 mRNA relative expression levels were measured by quantitative PCR in monocytes, macrophages and foam cells. **(C)** AdipoR1, AdipoR2 and T-cadherin mRNA relative expression levels were measured by quantitative PCR in monocytes, macrophages and foam cells. Experimental data were presented as mean $\pm$ range from three separate experiments (n=3). *, $p < 0.05$.

Figure 2. Effects of reducing expression levels of AdipoR1 and AdipoR2 on adiponectin functions to suppress lipid accumulation

siRNA for specifically knocking down of AdipoR1 and/or AdipoR2 gene expressions were transfected into THP-1 monocytes, followed with PMA treatment and oxLDL stimulation.

AdipoR1 knockdown: (A) AdipoR1 (open bar) and AdipoR2 (closed bar) mRNA relative expression levels in control or AdipoR1 siRNA transfected foam cells. (B) Cholesterol accumulations in control siRNA (gray bar) or AdipoR1 siRNA (slashed bar) transfected foam cells pre-treated with or without adiponectin. **AdipoR2 knockdown:** (C) AdipoR1 (open bar) and AdipoR2 (closed bar) mRNA relative expression levels in control or AdipoR2 siRNA transfected foam cells. (D) Cholesterol accumulations in control siRNA (gray bar) or AdipoR2 siRNA (slashed bar) transfected foam cells pre-treated with or without adiponectin. **AdipoR1+2 double knockdown:** (E) AdipoR1 (open bar) and AdipoR2 (closed bar) mRNA relative expression levels in control siRNA (Control) or AdipoR1 and AdipoR2 double siRNAs (AdipoR1+2) transfected foam cells. (F) Cholesterol accumulations in control siRNA (gray bar) or AdipoR1+2 siRNA (slashed bar) transfected

foam cells pre-treated with or without adiponectin. Experimental data were presented as mean $\pm$ range from three separate experiments (n=3). *, $p<0.05$.

Figure 3. Effects of increasing expression levels of AdipoR1 and AdipoR2 on adiponectin functions to suppress lipid accumulation

THP-1 cells stably overexpressing AdipoR1 or AdipoR2 were differentiated and transformed into macrophage foam cells followed with PMA treatment and oxLDL stimulation. **AdipoR1 overexpression:** (A) AdipoR1 (open bar) and AdipoR2 (closed bar) mRNA expression levels in foam cells stably overexpressing LacZ protein (LacZ) or human AdipoR1 protein (AdipoR1). (B) Cholesterol accumulations in LacZ overexpressing (gray bar) or AdipoR1 overexpressing (slashed bar) foam cells pre-treated with or without adiponectin. **AdipoR2 overexpression:** (C) AdipoR1 (open bar) and AdipoR2 (closed bar) mRNA expression levels in foam cells stably overexpressing LacZ protein (LacZ) or human AdipoR2 protein (AdipoR2). (D) Cholesterol accumulations in LacZ overexpressing (gray bar) or AdipoR2 overexpressing (slashed bar) foam cells pre-treated with or without adiponectin. Experimental data were presented as mean±range from three separate experiments (n=3). *, $p<0.05$

Figure 4. Gene expression responses to adiponectin treatment in THP-1 macrophage foam cells with regulated levels of AdipoR1 and AdipoR2

THP-1 cells were separately or simultaneously transfected with AdipoR1 or AdipoR2 siRNA. Foam cell transformations were induced with oxLDL stimulation following with different concentrations of adiponectin pre-treatment (0, 5, 10 μg/ml). Gene expressions in the foam cells were measured by quantitative PCR normalized to control housekeeping GAPDH gene levels. **(A)** AdipoR1 (right panel) and AdipoR2 (left panel) mRNA relative expression levels in four different siRNA pairs transfected cell groups: control siRNA (open bar), AdipoR1 siRNA (closed bar), AdipoR2 siRNA (hatched bar) or AdipoR1+2 siRNAs (slashed bar). **(B)** SR-AI **(C)** IL-1Ra **(D)** TNF **(E)** MCP-1 mRNA relative expression levels in 0, 5 or 10 μg/ml adiponectin pre-treated foam cells transfected with four different groups of siRNAs: control siRNA (square), AdipoR1 siRNA (triangle), AdipoR2 siRNA (upside-down triangle) or AdipoR1+2 siRNAs (diamond). mRNA relative expression levels in 5 μg/ml or 10 μg/ml adiponectin pre-treated foam cells were separately normalized to no adiponectin pretreatment controls in each group. Experimental data were presented as mean ± range from three separate experiments (n=3). *, $p < 0.05$

Figure 5. Lipid accumulation in APPL1 knockdown THP-1 cells

APPL1 lentiviral-shRNAs were introduced into THP-1 cells by viral infection. **(A)** APPL1 protein expression in THP-1 monocytes from pools of stable knockdown cell lines generated with different lentiviral-shRNA constructs or controls. **(B)** APPL1 and APPL2 protein expressions in control or APPL1 knockdown THP-1 monocytes. **(C)** PMA induced THP-1 macrophages were pre-treated with 5 μ g/ml adiponectin followed by exposing to 100 μ g/ml ox-LDL. Cholesterol accumulations in control (gray bar) or APPL1 knockdown (slashed bar) foam cells pre-treated with or without adiponectin. Experimental data were presented as mean $\pm$ range from three separate experiments (n=3). *, $p < 0.05$

Figure 6. Regulation of gene expression by adiponectin during macrophage foam cell transformation in APPL1 knockdown cells

Protein expression levels were examined by western blot analyses, and compared between control (Control) or APPL1 knockdown (APPL1) foam cells pre-treated with 5 μ g/ml adiponectin followed with 100 μ g/ml oxLDL stimulation. (A) Western blot pictures. (B, C, D) Density analysis of (A) using NIH Image J software for relative values. Gene expression levels in control (Control) or APPL1 knockdown (APPL1) THP-1 foam cells were examined by quantitative PCR following with different concentrations of adiponectin pre-treatment (0, 5, 10 μ g/ml). (E) SR-AI (F) IL-1Ra (G) TNF (H) MCP-1 mRNA relative expression levels in 0, 5 or 10 μ g/ml adiponectin pre-treated foam cells transfected with control shRNA (square) or APPL1 shRNA (triangle). mRNA relative expression levels in 5 μ g/ml or 10 μ g/ml adiponectin pre-treated foam cells were separately normalized to no adiponectin pretreatment controls in control or APPL1 shRNA transfected cells. Experimental data were presented as mean $\pm$ range from three separate experiments (n=3). *, $p < 0.05$; **, $p < 0.01$.

Table 1

Quantitative PCR primer sets sequences

Gene names	Primer Sequences
AdipoR1	F: 5' TTCTTCCTCATGGCTGTGATCT 3' R: 5' AAGAAGCGCTCAGGAATTCG 3'
AdipoR2	F: 5' ATAGGGCAGATAGGCTGGTTGA 3' R: 5' GGATCCGGGCAGCATACA 3'
SR-AI	F: 5' CCTCGTGTTTGCAGTTCTCA 3' R: 5' CCATGTTGCTCATGTGTTCC 3'
IL-1Ra	F: 5' AATCCATGGAGGGAAGATGTGCCT 3' R: 5' TGTCTGCTTTCTGTTCTCGCTCA 3'
TNF α	F: 5' GCAGGACGTGGACCATTACT 3' R: 5' TTCTGGGGTTTGGAGATTG 3'
MCP-1	F: 5' TCGCTCAGCCAGATGCAATCAATG 3' R: 5' AGTTTGGGTTTGCTTGCCAGGTG 3'
GAPDH	F: 5' GAAGGTGAAGGTCGGAGTC 3' R: 5' GAAGATGGTGATGGGATTTC 3'