Adiponectin inhibits Toll-like receptor family-induced signaling

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Abstract **Recent studies have shown that adiponectin, an adipocyte-derived cytokine, acts as a potent inhibitor of inflammatory responses. It has been also demonstrated that bacterial and viral signalings in host cells are triggered via Toll-like receptor (TLR) molecules. Therefore, in the present study, we investigated whether globular adiponectin (gAd) would be able to inhibit TLR-mediated nuclear factor-κB (NF-κB) signaling in mouse macrophages (RAW264). gAd predominantly bound to the AdipoR1 receptor and suppressed TLR-mediated NF-κB signaling. gAd-mediated inhibition of TLR-mediated IκB phosphorylation and NF-κB activation was eliminated by the pretreatment of cycloheximide. Also their inhibitions of gAd were blocked by preincubation of the cells with an antibody against AdipoR1, but not with an antibody against AdipoR2. Taken together, these findings indicate that adiponectin negatively regulates macrophage-like cell response to TLR ligands via an unknown endogenous product(s).**

Keywords: Adiponectin; Toll-like receptor; Nuclear factor-κB; Inhibitor κB phosphorylation; Murine macrophage-like cell; Adiponectin receptor

1. Introduction

Adiponectin, a 30-kDa adipocyte complement-related protein (ACRP30), is an adipocyte-derived hormone. Although adiponectin is abundantly present in plasma of healthy humans (1.9–17 μg/ml) [1], interestingly, it has been shown that the levels of adiponectin mRNA and its protein in plasma are decreased in obesity and type 2 diabetes [2,3]. Adiponectin consists of two structurally distinct domains, an amino-terminal collagen-like domain and a carboxyl-terminal globular domain. Fruebis et al. [4] detected both the globular domain of adiponectin (gAd) and the full-length form of adiponectin in mammalian plasma. They also demonstrated that gAd exhibits much more potent biological activities than the full-length adiponectin.

Recent studies have shown that adiponectin is involved in the modulation of inflammatory responses by inhibiting the proliferation of myelomonocytic cells [5] and that the hormone also suppresses tumor necrosis factor alpha (TNF-α)-mediated inflammatory responses in human aortic endothelial cells [6]. Adiponectin is also able to inhibit the phagocytic activity of and lipopolysaccharide (LPS)-stimulated TNF-α production in macrophages [5]. Together, these observations suggest that adiponectin acts as a potent anti-inflammatory cytokine. Importantly, many studies [7–9] have demonstrated that intracellular signaling induced by microorganism cell components is triggered by their binding to members of the Toll-like receptor (TLR) family. However, since it has not been demonstrated whether adiponectin acts as a negative regulator of TLR-mediated signaling, it is very important to demonstrate this point; because such a demonstration would suggest a novel action of adiponectin in the host defense mechanism. Furthermore, although several researchers have suggested that obesity is a potential risk factor of wound infection, the precious mechanisms by which obese patients are predisposed to wound infection are not known [10–13]. It is possible that adiponectin will function as a regulatory factor of bacterial infections via TLRs in obese subjects.

We investigated herein, using murine macrophage-like cells (RAW264), whether gAd negatively regulates the intracellular signaling induced by three different Toll-like receptors (TLR2, TLR4 and TLR9). Our data suggest a novel function of adiponectin as a potent negative regulator of the TLR-signaling pathway.

2. Materials and methods

2.1. Cells and reagents

Murine macrophage-like cell line RAW264 (RCB0535; RIKEN Cell Bank, Ibaragi, Japan) was maintained in RPMI 1640 medium (Sigma–Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Trace Ltd., Melbourne, Australia), 2 mM L-glutamine, and 50 μg/ml gentamicin. The murine preadipocyte cell line 3T3-L1 (JCRB9014) was purchased from Health Science Research Resources Bank (Osaka, Japan) and its cells were grown in

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Abbreviations: ACRP30, a 30-kDa adipocyte complement-related protein; CpG, deoxycytidylate-phosphate-deoxyguanylate; FBS, fetal bovine serum; gAd, globular adiponectin; GST, glutathione *S*-transferase; IκB, inhibitor κB; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; ODN, oligodeoxynucleotide; RT-PCR, reverse transcriptase-PCR; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha

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Dulbecco's Modified Eagle's Medium (Sigma–Aldrich Corp.) containing 10% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin. Cultures were maintained at 37 °C under 5% CO_2.

Lipopolysccharide from *Escherichia coli* O111:B4 (LPS), lipoteichoic acid from *Bacillus subtilis* (LTA), and cycloheximide were purchased from Sigma–Aldrich Corp. An oligodeoxynucleotide (ODN) containing the proper deoxycytidylate-phosphate-deoxyguanylate (CpG)-DNA motif was commercially synthesized by Operon Biotechnologies, Inc. (Tokyo, Japan). The sequence of the ODN was TCCATGACGTTCCTGATGCT [14]. Rabbit anti-mouse AdipoR1 antibody and rabbit anti-mouse AdipoR2 antibody were obtained from Alpha Diagnostic International (San Antonio, TX, USA).

2.2. Purification of recombinant protein

Glutathione *S*-transferase (GST) fusion vector [pGEX-6P-1 (Amersham Biosiences Corps., Piscataway, NJ, USA)] containing the globular domain of mouse ACRP30 (gACRP30) was provided by Dr. I. Shimomura (Osaka University, Osaka, Japan). Recombinant gAd was prepared as described previously [15]. Briefly, GST-gACRP30 protein was produced in *E. coli* strain BL21 and purified with glutathione Sepharose 4B (Amersham Biosiences Corps.). GST was cleaved from GST-gACRP30 protein by PreScission Protease (Amersham Biosiences Corps.). The isolated protein was applied to an Affi-Prep polymyxin column (Bio-Rad Laboratories, Heracules, CA, USA) to remove endotoxin contaminants as described previously [16].

2.3. Reverse transcriptase (RT)-PCR

Total RNA (5 μg) from RAW264 cells or 3T3-L1 cells was isolated by using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). The RNA samples were reverse-transcribed to cDNA by use of Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences Corps.), and the cDNAs for AdipoR1/R2 and adiponectin were amplified by PCR. PCR primers were partly designed from recently published sequences [17,18] and were as follow: AdipoR1 forward primer, 5′-ACGTTGGAGAGTCATCCCGTAT-3′, AdipoR1 reverse primer, 5′-CTCTGTGTGGATGCGGAAGAT-3′ (product size: 130 bp); AdipoR2 forward primer, 5′-TCCCAGGAAGATGAAGGGTTTAT-3′, AdipoR2 reverse primer, 5′-TTCCATTCGTTCCATAGCATGA-3′ (product size: 60 bp); adiponectin forward primer, 5′-GCCCAGTCATGCCGAAGA-3′, adiponectin reverse primer, 5′-TCTCCAGCCCCACACTGAAC-3′ (product size: 332 bp); β-actin forward primer, 5′-ATGGATGACGATATCGCT-3′, β-actin reverse primer, 5′-ATGAGGTAGTCTGTCAGGT-3′ (product size: 588 bp). The resulting amplification products were electrophoresed on a 12.5% acrylamide gel and stained with ethidium bromide. The bands were visualized by illumination with UV light.

2.4. Adiponectin binding assay

gAd biotinylation with NHS-LC-biotin (Pierce Chemical, Rockford, IL, USA) and flow cytometric analysis of gAd binding to RAW264 cells were performed as described by Yamaguchi et al. [19].

2.5. NF-κB luciferase assay

RAW264 cells (2×10^6) were incubated with a mixture of pTKκB2Luc (reporter gene, 8 ng), pRL-TK (internal control, 2 ng), and PolyFect transfection reagent (80 μl, QIAGEN K.K., Tokyo, Japan) for 24 h in 10-cm plastic plates with RPMI 1640 medium containing 10% FBS. The cells were harvested, cultured in a 24-well plastic plate, and preincubated with the desired amounts of gAd, and then were treated or not for an additional 6 h with a stimulant. Thereafter, the treated cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). The Dual-Luciferase Reporter Assay System (Promega) was used to quantify the expression of the firefly luciferase and *Renilla* luciferase. The firefly luciferase was normalized to the *Renilla* and presented as values relative to the control.

2.6. Western blotting

Whole-cell lysates (20 μg of protein) were resolved on 12.5% SDS–PAGE gels, and then electrophoretically transferred to nitrocellulose membranes. Immunoblotting was performed as described previously [20]. The membranes were first exposed to primary antibodies, and then to secondary antibodies conjugated with horseradish peroxidase. The primary antibodies used were anti-phospho-IκB-α (Ser32) antibody, anti-IκB-α antibody (Cell Signaling Technology, Inc.; Beverly, MA, USA), and anti-actin antibody (MP Biomedicals, Inc.; Aurora, OH, USA).

2.7. Statistical analysis

Comparisons between groups were performed by using Tukey's multiple range tests. Statistical difference was indicated by $P < 0.05$. Student's *t*-test was used to determine the statistical significance of differences between results obtained for the gAd-pretreated groups versus those for the cycloheximide and gAd-pretreated group.

3. Results

3.1. Adiponectin receptor mRNA levels in RAW264 and 3T3L1 cells

Since it is of importance to know whether RAW264 and 3T3L1 cells used in this study constitutively express adiponectin and its two different receptors (AdipoR1 and AdipoR2), firstly using RT-PCR we examined the expression of these genes at the mRNA level. As shown in Fig. 1, adiponectin, AdipoR1, and AdipoR2 genes were expressed in both types of cells.

3.2. Binding of gAd to RAW264 cells via AdipoR1

Our next interest was to address whether adiponectin could directly bind to adiponectin receptors on the cells. Therefore, we analyzed, by use of flow cytometry, the binding of gAd to RAW264 cells. The association of biotinylated gAd to RAW264 cells occurred in a saturable manner over a physiological concentration range (2.5–30 μg/ml) of gAd (Fig. 2A). Importantly, we observed that the saturable binding of gAd to RAW264 cells was dramatically inhibited by pretreatment of the cells with anti-AdipoR1 antibody. However, such inhibitory action was not observed when anti-AdipoR2 antibody was used (Fig. 2B). On the other hand, although data not shown, the binding of gAd to the cells was also significantly inhibited by pretreating the cytokine with anti-gAd antibody. Together, these observations suggest that gAd predominantly bound to AdipoR1 on RAW264 cells, not to AdipoR2.

3.3. gAd inhibition of TLR ligands-induced NF-κB activation

Many recent studies [8,21,22] have demonstrated that cell components of microorganisms including bacteria, viruses, fungi, and so on are recognized by TLR family receptors

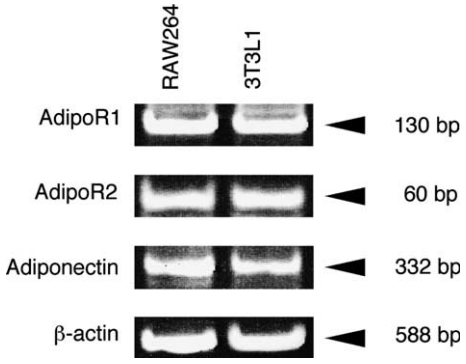

Fig. 1. RT-PCR analysis of mouse adiponectin receptors (AdipoR1 and AdipoR2), adiponectin, and β-actin in RAW264 and 3T3-L1 cells.