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).
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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

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 TCCAT-GACGTTCCTGATGCT [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 Biosiences 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′-TCCCAGGAAGATGA-AGGGTTTAT-3′, AdipoR2 reverse primer, 5′-TTCCATTCGTTC-CATAGCATGA-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) anti-

body, 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
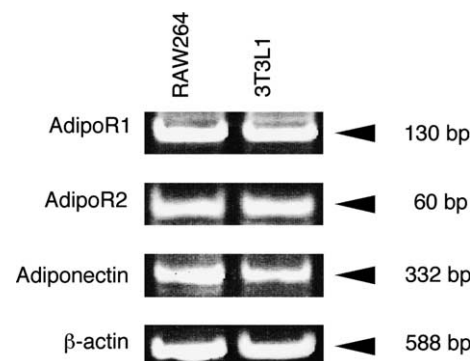

Fig. 1. RT-PCR analysis of mouse adiponectin receptors (AdipoR1 and AdipoR2), adiponectin, and β-actin in RAW264 and 3T3-L1 cells.
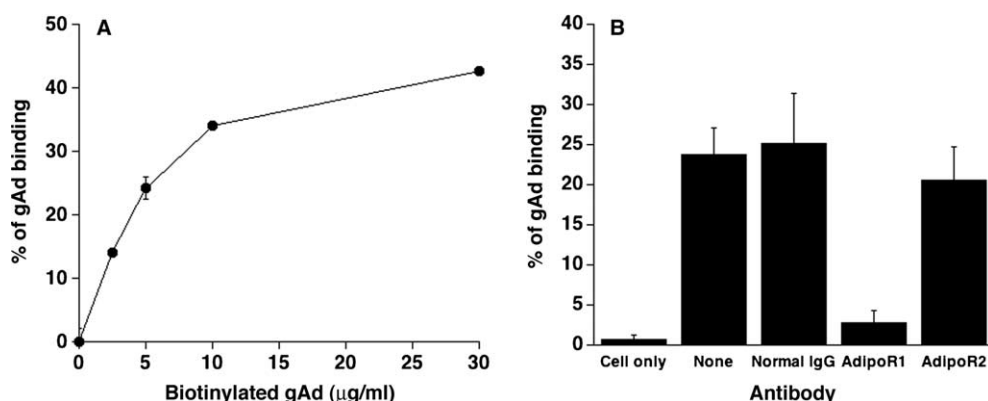
Fig. 2. Binding of gAd to RAW264 cells. (A) RAW264 cells (10^6) were incubated for 30 min at 37 °C with 2.5–30 µg/ml biotinylated gAd. The cells were then washed with Hanks' balanced salt solution, stained with streptavidin-phycoerythrin for 15 min at room temperature, and analyzed by flow cytometry. Data are shown as the means ± S.D. (B) Effect of anti-adiponectin receptor antibodies on binding of gAd to RAW264 cells. The cells (10^6) were pretreated with 10 µg/ml anti-mouse AdipoR1 antibody, anti-mouse AdipoR2 antibody or non-immune rabbit IgG for 1 h at 37 °C. The cells were then incubated for 30 min at 37 °C with 10 µg/ml biotinylated gAd. The results are expressed as the means ± S.D. Representative results from 3 experiments are shown.

and that intracellular signaling by them is induced via TLRs. Although several recent studies [5,6,23] have suggested that adiponectin acts as a potent anti-inflammatory cytokine, it had still not been known whether the cytokine is able to regulate negatively TLR-mediated intracellular signaling pathways. Therefore, it was of interest to us to explore this possibility. Firstly, we measured LPS-induced NF-κB activity in RAW264 cells by conducting a luciferase assay. Although the data are not shown, LPS induced the highest NF-κB activity in RAW264 cells at 6 h after initiation of LPS treatment at 0.3 µg/ml in the presence of 10% FBS. Under these culture conditions, we examined whether gAd could inhibit this LPS-induced NF-κB activity. Consequently, we observed that gAd markedly inhibited LPS-induced NF-κB activity when the cells were pretreated for 6 h with the cytokine fragment (Fig. 3). In addition, we wanted to know whether gAd could also inhibit NF-κB activity in RAW264 cells when such activity was induced via other TLRs. Therefore, using LTA and CpG, which are ligands of TLR2 and 9, respectively, we examined whether gAd would inhibit NF-κB activation induced by them. As expected, gAd pretreatment significantly inhibited LTA- and CpG-induced NF-κB activation in a dose-dependent manner (Fig. 3).

3.4. Effect of cycloheximide on the gAd inhibition of LPS-induced NF-κB activation

Also we considered it is very important to address whether the gAd-mediated inhibition of TLR intracellular signaling occurred directly or indirectly via some endogenous product(s). Therefore, we examined the effect of cycloheximide on the inhibition by gAd. The cells were pretreated or not with the protein synthesis inhibitor at 20 ng/ml for 1 h prior to gAd treatment and subsequently incubated in the presence or absence of LPS (0.3 µg/ml) for 6 h. Then, we measured LPS-induced NF-κB activation under these conditions. Consequently, we observed that the cycloheximide pretreatment significantly blocked the inhibitory action of gAd (Fig. 4). These observations suggest that some endogenous product may be involved in the gAd inhibitory action toward LPS-induced NF-κB activity.

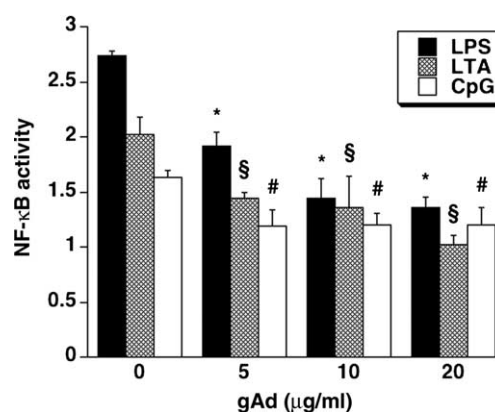Fig. 3. Attenuation of NF-κB-induced reporter gene activity by gAd in RAW264 cells. The cells were pretreated with gAd (5–20 µg/ml) for 6 h before the addition of LPS (0.3 µg/ml, closed bars), LTA (5 µg/ml, meshed bars), or CpG-DNA (25 µg/ml, open bars) for 6 h. Then, the cells were harvested and luciferase activities were analyzed by using a Dual-Luciferase Reporter Assay System. Activity was expressed relative to the untreated control. Values represent means ± S.D. for triplicate cultures. The experiments were performed three times, and similar results were obtained in each experiment. *, $P < 0.05$ versus basal LPS-induced NF-κB activity; §, $P < 0.05$ versus basal LTA-induced NF-κB activity; #, $P < 0.05$ versus basal CpG-induced NF-κB activity.

3.5. gAd inhibition of LPS-induced IκB phosphorylation

To determine if this unknown endogenous product might act to block the phosphorylation of IκB, thereby preventing NF-κB transport to the nucleus, we explored the effect of cycloheximide on inhibitory action of gAd for IκB phosphorylation in LPS-treated RAW264 cells. Fig. 5 shows that although the peak of LPS-stimulated IκB-α phosphorylation was observed at 10 min after LPS addition, gAd pretreatment significantly inhibited the LPS-stimulated IκB-α phosphorylation. Importantly, as shown in Fig. 6, the inhibitor of protein synthesis clearly blocked the inhibitory effect of gAd on phosphorylation of IκB-α in LPS-treated cells. The gAd treatment alone had no effect on the IκB phosphorylation (data not shown). These observations strongly support our suggestion that some gAd-
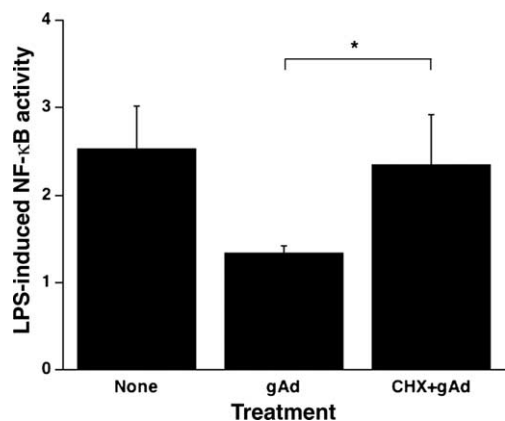
Fig. 4. Pretreatment with cycloheximide blocks the inhibitory effects of gAd on LPS-induced NF-κB activation. The cells were pretreated or not with cycloheximide (CHX; 20 ng/ml) for 1 h prior to gAd treatment. They were then treated with gAd (20 µg/ml) for 6 h and washed. After the culture medium had been replaced with fresh medium, the cells were stimulated with LPS (0.3 µg/ml) for an additional 6 h. Data are shown as the means ± S.E. of the means for three experiments performed with triplicate cultures. *, $P < 0.05$ comparing the gAd-treated group with the cycloheximide and gAd-pretreated group.
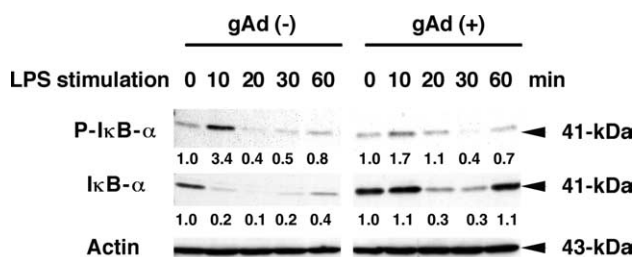

Fig. 5. Effects of gAd on LPS-induced signaling in RAW264 cells. Time courses of phosphorylation of IκB (top) and IκB (middle) induced by LPS (0.3 µg/ml) without gAd or with 20 µg/ml of gAd. Actin levels (bottom) are also shown. Whole-cell lysates were immunoblotted with anti-phospho-IκB-α antibody, anti-IκB-α antibody or anti-actin antibody. Relative band intensities were determined by using NIH Image version 1.62 software. The values below the bands are the mean fold increases in the expression levels of RAW264 cells incubated with LPS compared with that of unstimulated RAW264 cells.
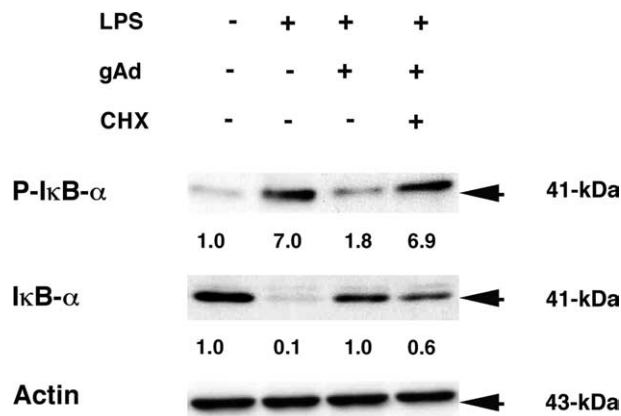

Fig. 6. Pretreatment with cycloheximide blocks the inhibitory effects of gAd on LPS-induced IκB phosphorylation. The cells were pretreated or not with cycloheximide (CHX; 20 ng/ml) for 1 h prior to gAd (20 µg/ml) for 6 h, and then stimulated with LPS (0.3 µg/ml) for 10 min. Then the cells were lysed with detergent (1% Nonidet P-40 in 0.15 M NaCl). The proteins from each cell lysate were electrophoresed on a 12.5% SDS gel. After the proteins on the gels had been blotted onto nitrocellulose membranes, fluorographs were developed after incubation (overnight at 4 °C) with either anti-phospho-IκB-α antibody, anti-IκB-α antibody or anti-actin antibody. Relative band intensities were determined by using NIH Image version 1.62 software. The values below the bands are the mean fold increases in the expression levels of RAW264 cells incubated with LPS, gAd and CHX compared with that of unstimulated RAW264 cells.
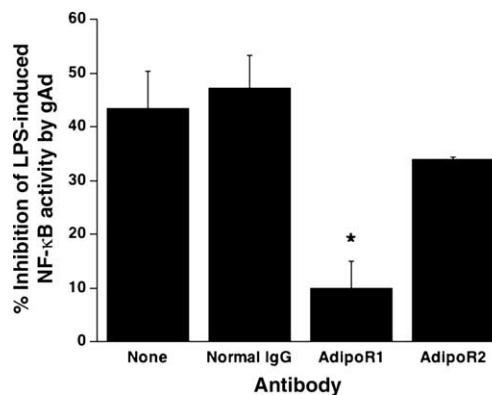

Fig. 7. Anti-AdipoR1 antibody blocks gAd-mediated inhibition of LPS-induced NF-κB activation. The cells were pretreated with 10 µg/ml of normal rabbit IgG, 10 µg/ml of anti-AdipoR1 antibody, or 10 µg/ml of anti-AdipoR2 antibody for 1 h at 37 °C, and were then incubated with 20 µg/ml gAd for 6 h. After having been washed with phosphate-buffered saline and replaced with fresh medium, the cells were stimulated with 0.3 µg/ml LPS for 6 h at 37 °C. NF-κB activity was measured by using a Dual-Luciferase Reporter Assay System. Activity was expressed relative to the untreated control. Data are shown as the means ± S.E. of the means for three experiments performed in triplicate. *, $P < 0.05$ versus the other groups.

elicited endogenous product(s) acts to block the phosphorylation of IκB, thereby inhibiting NF-κB activation in LPS-treated cells.

3.6. gAd inhibits LPS-induced NF-κB activation and IκB phosphorylation via AdipoR1

We have already shown that gAd binding to RAW264 cells is dramatically inhibited by pretreating the cells with anti-AdipoR1 antibody, but not with anti-AdipoR2 antibody (Fig. 2B). Therefore, we explored which receptor is involved in the gAd-mediated inhibition of LPS-induced NF-κB activation and IκB phosphorylation. As shown in Figs. 7 and 8, although anti-AdipoR1 antibody significantly blocked the inhibitory effects of gAd, such significant inhibition was not observed with anti-AdipoR2 antibody. These results suggest that gAd inhibition of TLR-mediated NF-κB signaling is predominantly mediated via AdipoR1.

4. Discussion

In the present study, we demonstrated that gAd clearly inhibited LTA, LPS, and CpG DNA-induced NF-κB activation in RAW264 cells. Interestingly, this observation suggests that gAd acts as a negative regulator of TLR-mediated inflammatory responses.

Recently, two adiponectin receptors (AdipoR1 and AdipoR2) were cloned [17]. AdipoR1 is abundantly expressed in
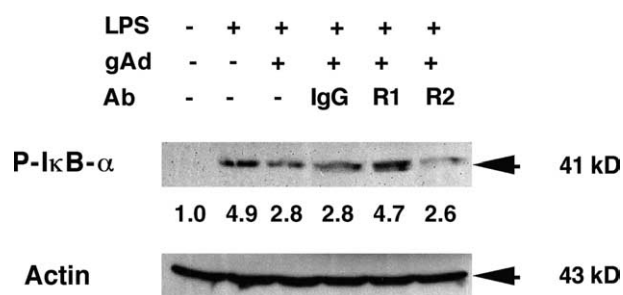
Fig. 8. Anti-AdipoR1 antibody blocks gAd-mediated inhibition of LPS-induced IκB phosphorylation. The cells were pretreated with 10 μg/ml of normal rabbit IgG, 10 μg/ml of anti-AdipoR1 antibody, or 10 μg/ml of anti-AdipoR2 antibody for 1 h prior to gAd (20 μg/ml) for 6 h, and then stimulated with LPS (0.3 μg/ml) for 10 min. Whole-cell lysates were immunoblotted with anti-phospho-IκB-α antibody or anti-actin antibody. Relative band intensities were determined by using NIH Image version 1.62 software. The values below the bands are the mean fold increases in the expression levels of RAW264 cells incubated with LPS, gAd and each antibody compared with that of unstimulated RAW264 cells.

skeletal muscle and appears as a high-affinity receptor for gAd [24,25] and as a low-affinity receptor for full-length adiponectin, whereas AdipoR2 is predominantly found in liver and serves as an intermediate-affinity receptor for both forms of adiponectin [17]. Although we observed that our anti-AdipoR1 antibody completely inhibited the binding of gAd to RAW264 cells and blocked gAd-mediated inhibition of TLR-mediated IκB phosphorylation and NF-κB activation, the AdipoR2 antibody had no such effects. Therefore, these observations suggest that AdipoR1 is the predominant receptor for the binding of gAd to RAW264 cells (Fig. 2B) and may play an important role in adiponectin signaling of macrophages (Figs. 7 and 8).

Several investigations [26,27] have demonstrated that TLRs initiate their signaling cascade via the sequential recruitment of MyD88, IRAK, and TRAF6, which in turn activate downstream mediators such as mitogen-activated protein kinase and NF-κB, though Akira et al. [28] also showed the existence of a MyD88-independent signaling pathway of TLR. Since TLR-mediated signaling pathways are strongly involved in both the initiation and progression of inflammatory responses induced by microbial infections, it is very important to define the negative regulatory molecule(s) of TLR-mediated signaling cascades. Regarding this point, several studies [29–33] have actually identified negative molecules such as IRAKM, SOCS1, Tollip, SIGIRR, A20 and so on. Therefore, our interest was to explore gAd as another inhibitor of this TLR-mediated signaling.

We herein showed that gAd acted as a potent inhibitor of TLR-induced signaling in RAW264 cells (Fig. 3). Since we found that gAd-mediated inhibition of TLR-induced NF-κB activity and IκB phosphorylation in RAW264 cells was prevented by pretreatment with cycloheximide (Figs. 4 and 6), some endogenous protein(s) may be involved in this inhibition by gAd. Therefore, it is of interest to explore in a future study what proteins are endogenously induced in gAd-treated cells and how they function to inhibit the TLR signaling pathway.

NF-κB-inducing kinase (NIK) phosphorylates the IκB kinase complex, leading to IκB phosphorylation and subsequent NF-κB activation [34]. gAd specifically suppressed TLR family-induced NF-κB activation of the IκB-α–NF-κB pathway, suggesting that the target molecule of gAd inhibition of the TLR family-induced NF-κB signaling pathway may be one of the signaling molecules that act between NIK and IκB-α.

Furthermore, a recent study has suggested that obesity is one of the risk factors for developing postoperative mediastinitis (POM) due to Staphylococcus aureus [10]. The association between obesity and risk for POM has been demonstrated [11–13]. Since adiponectin levels in plasma are decreased in obesity, it is interesting to note the possibility that therapeutic applications of adiponectin could function as a negative regulator against microbial infections via TLRs in obese patients.

In conclusion, the data presented herein suggest that gAd acts as a potent negative regulator of macrophage-like cell responses to TLR ligands via a newly synthesized endogenous product(s). Our present results also suggest a novel function of adiponectin as an anti-inflammatory molecule acting in bacterial and virus infections.

References

[1] Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T. and Matsuzawa, Y. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem. Biophys. Res. Commun. 257, 79–83.

[2] Halleux, C.M., Takahashi, M., Delporte, M.L., Detry, R., Funahashi, T., Matsuzawa, Y. and Brichard, S.M. (2001) Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. Biochem. Biophys. Res. Commun. 288, 1102–1107.

[3] Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N., Maeda, K., Nishida, M., Kihara, S., Sakai, N., Nakajima, T., Hasegawa, K., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Hanafusa, T. and Matsuzawa, Y. (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler. Thromb. Vasc. Biol. 20, 1595–1599.

[4] Fruebis, J., Tsao, T.S., Javorschi, S., Ebbets-Reed, D., Erickson, M.R., Yen, F.T., Bihain, B.E. and Lodish, H.F. (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc. Natl. Acad. Sci. USA 98, 2005–2010.

[5] Yokota, T., Oritani, K., Takahashi, I., Ishikawa, J., Matsuyama, A., Ouchi, N., Kihara, S., Funahashi, T., Tenner, A.J., Tomiyama, Y. and Matsuzawa, Y. (2000) Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood 96, 1723–1732.

[6] Ouchi, N., Kihara, S., Arita, Y., Okamoto, Y., Maeda, K., Kuriyama, H., Hotta, K., Nishida, M., Takahashi, M., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Funahashi, T. and Matsuzawa, Y. (2000) Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-κB signaling through a cAMP-dependent pathway. Circulation 102, 1296–1301.

[7] Aderem, A. and Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. Nature 406, 782–787.

[8] Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. and Akira, S. (1999) Differential roles of

TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11, 443–451.

[9] Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745.

[10] Dodds Ashley, E.S., Carroll, D.N., Engemann, J.J., Harris, A.D., Fowler Jr., V.G., Sexton, D.J. and Kaye, K.S. (2004) Risk factors for postoperative mediastinitis due to methicillin-resistant *Staphylococcus aureus*. Clin. Infect. Dis. 38, 1555–1560.

[11] Milano, C.A., Kesler, K., Archibald, N., Sexton, D.J. and Jones, R.H. (1995) Mediastinitis after coronary artery bypass graft surgery. Risk factors and long-term survival. Circulation 92, 2245–2251.

[12] Ridderstolpe, L., Gill, H., Granfeldt, H., Ahlfeldt, H. and Rutberg, H. (2001) Superficial and deep sternal wound complications: incidence, risk factors and mortality. Eur. J. Cardiothorac. Surg. 20, 1168–1175.

[13] Olsen, M.A., Lock-Buckley, P., Hopkins, D., Polish, L.B., Sundt, T.M. and Fraser, V.J. (2002) The risk factors for deep and superficial chest surgical-site infections after coronary artery bypass graft surgery are different. J. Thorac. Cardiovasc. Surg. 124, 136–145.

[14] Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H. and Lipford, G.B. (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. USA 98, 9237–9242.

[15] Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T. and Matsuzawa, Y. (2002) Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat. Med. 8, 731–737.

[16] Yamaguchi, N., Tsuda, H., Yamashita, Y. and Koga, T. (1998) Binding of the capsule-like serotype-specific polysaccharide antigen and the lipopolysaccharide from *Actinobacillus actinomycetemcomitans* to human complement-derived opsonins. Oral Microbiol. Immunol. 13, 348–354.

[17] Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R. and Kadowaki, T. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762–769.

[18] Hazel, M., Cooksey, R.C., Jones, D., Parker, G., Neidigh, J.L., Witherbee, B., Gulve, E.A. and McClain, D.A. (2004) Activation of the hexosamine signaling pathway in adipose tissue results in decreased serum adiponectin and skeletal muscle insulin resistance. Endocrinology 145, 2118–2128.

[19] Yamaguchi, N., Kubo, C., Masuhiro, Y., Lally, E.T., Koga, T. and Hanazawa, S. (2004) Tumor necrosis factor alpha enhances *Actinobacillus actinomycetemcomitans* leukotoxin-induced HL-60 cell apoptosis by stimulating lymphocyte function-associated antigen 1 expression. Infect. Immun. 72, 269–276.

[20] Yamaguchi, N., Kieba, I.R., Korostoff, J., Howard, P.S., Shenker, B.J. and Lally, E.T. (2001) Maintenance of oxidative phosphorylation protects cells from *Actinobacillus actinomycetemcomitans* leukotoxin-induced apoptosis. Cell. Microbiol. 3, 811–823.

[21] Sato, S., Takeuchi, O., Fujita, T., Tomizawa, H., Takeda, K. and Akira, S. (2002) A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways. Int. Immunol. 14, 783–791.

[22] Schnare, M., Holt, A.C., Takeda, K., Akira, S. and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Curr. Biol. 10, 1139–1142.

[23] Wulster-Radcliffe, M.C., Ajuwon, K.M., Wang, J., Christian, J.A. and Spurlock, M.E. (2004) Adiponectin differentially regulates cytokines in porcine macrophages. Biochem. Biophys. Res. Commun. 316, 924–929.

[24] Motoshima, H., Wu, X., Mahadev, K. and Goldstein, B.J. (2004) Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL. Biochem. Biophys. Res. Commun. 315, 264–271.

[25] Wu, X., Motoshima, H., Mahadev, K., Stalker, T.J., Scalia, R. and Goldstein, B.J. (2003) Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. Diabetes 52, 1355–1363.

[26] Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S. and Janeway Jr., C.A. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell 2, 253–258.

[27] Muzio, M., Polentarutti, N., Bosisio, D., Manoj Kumar, P.P. and Mantovani, A. (2000) Toll-like receptor family and signalling pathway. Biochem. Soc. Trans. 28, 563–566.

[28] Akira, S., Hoshino, K. and Kaisho, T. (2000) The role of Toll-like receptors and MyD88 in innate immune responses. J. Endotoxin. Res. 6, 383–387.

[29] Li, X. and Qin, J. (2005) Modulation of Toll-interleukin 1 receptor mediated signaling. J. Mol. Med. 83, 258–266.

[30] Gingras, S., Parganas, E., de Pauw, A., Ihle, J.N. and Murray, P.J. (2004) Re-examination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling. J. Biol. Chem. 279, 54702–54707.

[31] O'Neill, L. (2000) The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. Biochem. Soc. Trans. 28, 557–563.

[32] Wald, D., Qin, J., Zhao, Z., Qian, Y., Naramura, M., Tian, L., Towne, J., Sims, J.E., Stark, G.R. and Li, X. (2003) SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat. Immunol. 4, 920–927.

[33] Gon, Y., Asai, Y., Hashimoto, S., Mizumura, K., Jibiki, I., Machino, T., Ra, C. and Horie, T. (2004) A20 inhibits toll-like receptor 2- and 4-mediated interleukin-8 synthesis in airway epithelial cells. Am. J. Respir. Cell. Mol. Biol. 31, 330–336.

[34] Malinin, N.L., Boldin, M.P., Kovalenko, A.V. and Wallach, D. (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 385, 540–544.