# Synergy between adiponectin and interleukin-1β on the expression of interleukin-6, interleukin-8, and cyclooxygenase-2 in fibroblast-like synoviocytes

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## Abstract

To determine whether adiponectin may have synergistic effects in combination with the proinflammatory cytokine interleukin (IL)-1β regarding the production of proinflammatory mediators during arthritic joint inflammation, synovial cells from rheumatoid arthritis (RA) patients were treated with adiponectin, IL-1β, and their combination for 24 h. Culture supernatant was collected and analyzed by enzyme-linked immunosorbent assay for levels of IL-6, IL-8, prostaglandin $E_2$ ($PGE_2$), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs). Adiponectin-mediated intracellular signaling pathways were investigated to elucidate the molecular mechanisms underlying their synergy. The association of proinflammatory mediators with adiponectin was investigated in the synovial fluid of arthritis patients. Adiponectin functioned synergistically with IL-1β to activate IL-6, IL-8, and $PGE_2$ expression in RA fibroblast-like synoviocytes; Levels of VEGF, MMP-1, and MMP-13 were not synergistically stimulated. Adiponectin and IL-1β each increased the expression of both adiponectin receptor 1 and IL-1 receptor 1. However, adiponectin and IL-1β did not synergistically support the degradation of IκB-α or the nuclear translocation of NF-κB. Synergistically increased gene expression was significantly inhibited by MG132, an NF-κB inhibitor. Supporting the *in vitro* results, IL-6 and IL-8 levels were positively associated with adiponectin in synovial joint fluid from patients with RA, but not osteoarthritis (OA). In conclusion, adiponectin and IL-1β may synergistically stimulate the production of proinflammatory mediators through unknown signaling pathways during arthritic joint inflammation. Adiponectin may be more important to the pathogenesis of RA than previously thought.

Keywords: adiponectin; interleukin-1β; obesity; osteoarthritis; rheumatoid arthritis; synovial fluid; synovial membrane

## Introduction

Adipose tissue, which once was simply recognized as a lipid storage and release depot, is now considered an endocrine tissue (Ronti *et al*., 2006; Halberg *et al*., 2008). This tissue secretes various substances (known as adipokines), including tumor necrosis factor-α (TNF-α), interleukin (IL)-6, leptin, adiponectin, resistin, visfatin, omenetin, and many others (Matsuzawa *et al*., 1999; Henry and Clarke, 2008). Much attention has been paid to the role of adiponectin in the past 10 yr because of its relationship with insulin sensitivity, glucose, and lipid metabolism.

In addition, adiponectin, which circulates at relatively high levels in the bloodstream, exhibits potent anti-inflammatory (Wulster-Radcliffe *et al.*, 2004), atheroprotective (Matsuda *et al.*, 2002), and antidiabetic effects (Maeda *et al.*, 2002). Adiponectin has also demonstrated beneficial effects against the development of obesity-related vascular diseases (Giannessi *et al.*, 2007), and stimulates angiogenesis (Ribatti *et al.*, 2007).

Recently, research interests have been devoted to elucidating the role of adiponectin in the pathophysiology of arthritis. Adiponectin may be involved in the pathogenesis of rheumatoid arthritis (RA) by stimulating the expression of IL-6, IL-8, matrix metalloproteinase (MMP)-1, and MMP-13 in RA fibroblast-like synoviocytes (FLSs) (Ehling *et al.*, 2006; Choi *et al.*, 2009; Kitahara *et al.*, 2009). However, adiponectin has been suggested to have anti-inflammatory effects in the context of arthritis. It has been shown to significantly inhibit IL-1β-stimulated synovial cell proliferation in collagen-induced arthritic mice (Lee *et al.*, 2008), and high-grade inflammation in RA patients has been negatively correlated with circulating adiponectin concentrations (Gonzalez-Gay *et al.*, 2008). Furthermore, adiponectin has been negatively associated with leukocyte count in RA synovial fluid (Senolt *et al.*, 2006). The controversy regarding adiponectin's biological role may be explained by adiponectin isoform-specific responses. Although there are three types of adiponectin (low molecular weight [LMW] trimeric form, middle molecular weight [MMW] exameric form, and high molecular weight [HMW] multimer), it circulates mainly as a LMW hexamer, which displays anti-inflammatory properties, and an HMW multimer, which may be responsible for pro-inflammatory effects (Neumeier *et al.*, 2006).

IL-1β is known to strongly stimulate various proinflammatory mediators and MMPs, which bring about joint destruction, in various types of cells (Dayer *et al.*, 1986; Kay and Calabrese, 2004). In particular, it greatly stimulates the production of cytokines that perpetuate inflammation and proteases that contribute to cartilage destruction in FLSs. Therefore, IL-1β is regarded as a key effector in rheumatoid arthritis (Bartok and Firestein, 2010). Meanwhile, both IL-1β and adiponectin levels have been shown to be elevated in the synovial fluid and sera of patients with RA (Symons *et al.*, 1989; Schaffler *et al.*, 2003; Otero *et al.*, 2006).

In this study, to better understand the role of adiponectin in arthritis, we questioned whether adiponectin and IL-1β act synergistically in the production of proinflammatory mediators (IL-6, IL-8, and prostaglandin E$_2$ [PGE$_2$]), VEGF, and MMPs in RA FLSs.

## Results

### Synergistic effect of adiponectin on the production of IL-6, IL-8, and PGE$_2$ in IL-1β-stimulated RA FLSs

To evaluate the synergistic effects of adiponectin and IL-1β on inflammation and joint destruction in arthritic joints, cultured synovial cells were treated with IL-1β (10 or 100 pg/ml) and adiponectin (10 μg/ml) for 24 h. Supernatants were harvested and analyzed for levels of MMP-1, MMP-13, VEGF, IL-6, IL-8, and COX-2. As shown in Figure 1, adiponectin (10 μg/ml) stimulated the production of VEGF and MMP-1 similarly to IL-1β (100 pg/ml), while MMP-13 was stimulated more by adiponectin than by IL-1β. When synovial cells were stimulated with adiponectin and IL-1β at the same time, the protein expression of VEGF, MMP-1, and MMP-13 was not synergistically stimulated by the two cytokines in RA FLSs. In contrast, the levels of the proinflammatory mediators IL-6, IL-8, and PGE$_2$ were further increased by
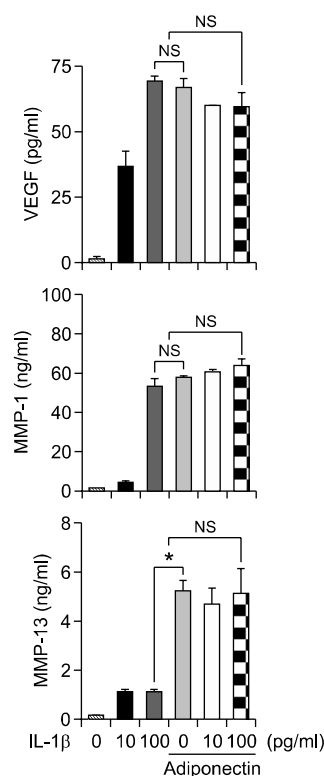


**Figure 1.** No synergistic effect of adiponectin plus IL-1β on the production of VEGF, MMP-1, or MMP-13 in RA FLSs. Synovial cells (2.5 × 10$^5$ cells/60 mm dish/2 ml serum-free media) were stimulated with IL-1β for 24 h at 10 or 100 pg/ml in the absence or presence of 10 μg/ml adiponectin, as described in the Materials and Methods section. The levels of VEGF, MMP-1, and MMP-13 were measured by ELISA from culture supernatants. Three independent experiments were performed in quadruplicate with FLSs from each patient. The data shown are representative of three independent experiments, and similar results were obtained from all three. Values are expressed as mean ± S.E.M.
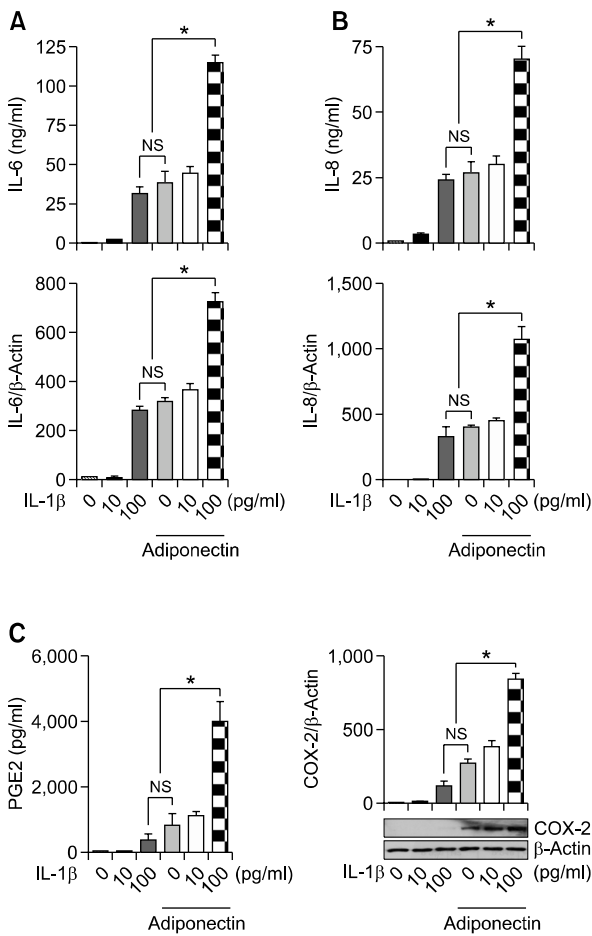
**Figure 2.** The synergistic effect of adiponectin plus IL-1β on the production of IL-6, IL-8, and PGE$_2$ in RA FLSs. Culture supernatants were evaluated for (A) IL-6, (B) IL-8, and (C) PGE$_2$ by ELISA, as in Figure 1. The cells were used for mRNA extraction and Western blot to detect COX-2 expression. The mRNA levels were measured by real-time RT-PCR. Three independent experiments were performed in quadruplicate with FLSs from each patient. The data shown are representative of three independent experiments, and similar results were obtained from all three. Values are expressed as mean ± S.E.M. *$P < 0.05$ versus treatment with IL-1β (100 pg/ml) or adiponectin (10 μg/ml) alone.



**Figure 3.** Molecular mechanisms underlying the synergistic effects of adiponectin and IL-1β. (A) The expression of IL-1 receptor 1 (IL-1R1) and adiponectin receptor 1 (AdipoR1) were determined by semi-quantitative PCR. (B) Time course activation of various signaling pathways in adiponectin-stimulated RA FLSs. FLSs cultured ($2.5 \times 10^5$ cells) in 60 mm dishes were serum-starved overnight and stimulated with either adiponectin or IL-1β for a given time. The cells were prepared for Western blot analysis. (C) IκB-α levels. (D) Nuclear levels of NF-κB in adiponectin and/or IL-1β-stimulated RA FLSs. IκB-α level was analyzed by Western blot after 60 min of stimulation. The nuclear level was evaluated after 90 min of stimulation, as described in the Materials and Methods section. For the Western analysis, three independent experiments were performed in one plate with FLSs from each patient. For the analysis of NF-κB level, three independent experiments were performed in quadruplicate with FLSs from each patient. The data shown are representative of three independent experiments, and similar results were obtained from all three. Values are expressed as mean ± S.E.M. *$P < 0.05$ versus treatment with IL-1β (100 pg/ml) or adiponectin (10 μg/ml) alone.

concomitant stimulation with adiponectin and IL-1β compared with stimulation by either cytokine alone (Figure 2). In particular, PGE$_2$ level was further increased approximately twofold by concomitant treatment with the two cytokines compared with accumulated treatment with individual cytokines. Consistent with the PGE$_2$ level, the levels of mRNA and protein (COX-2), which were evaluated by real-time PCR and Western blot, respectively, were increased in a pattern identical to that of PGE$_2$. More importantly, while the level of COX-2 measured in cells stimulated with 100 pg/ml of IL-1β was almost undetectable, the COX-2 level measured in cells stimulated with 10 μg/ml of adiponectin 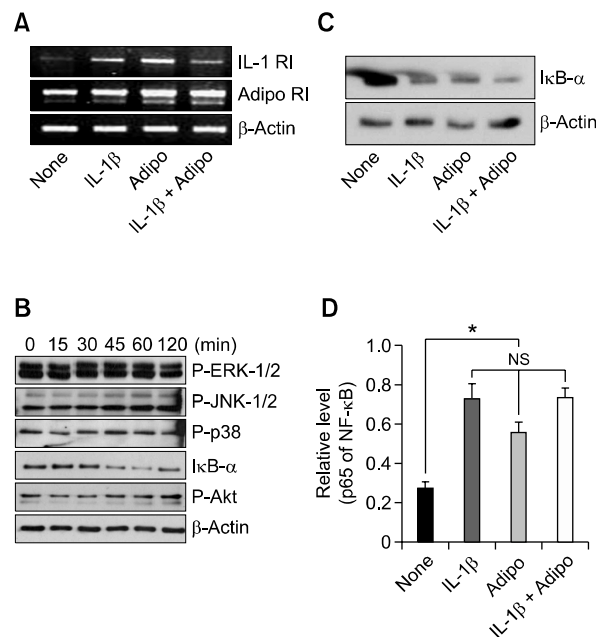was detected weakly by Western blot. Combined treatment increased the COX-2 level approximately threefold compared with treatment with adiponectin alone.

## Molecular mechanisms underlying the synergistic effects of adiponectin and IL-1β

To study the molecular mechanisms by which adiponectin and IL-1β synergistically enhance the production of proinflammatory mediators, first we checked whether adiponectin and IL-1β increased the expression of IL-1 receptor (IL-1R1) and adiponectin receptor (AdipoR1), respectively. As shown in Figure 3A, adiponectin and IL-1β each increased the expression of both AdipoR1 and IL-1R1. This finding suggests that increased expression of their receptors may be a possible mechanism underlying the synergy between adiponectin and IL-1β regarding the expression of IL-6, IL-8 and
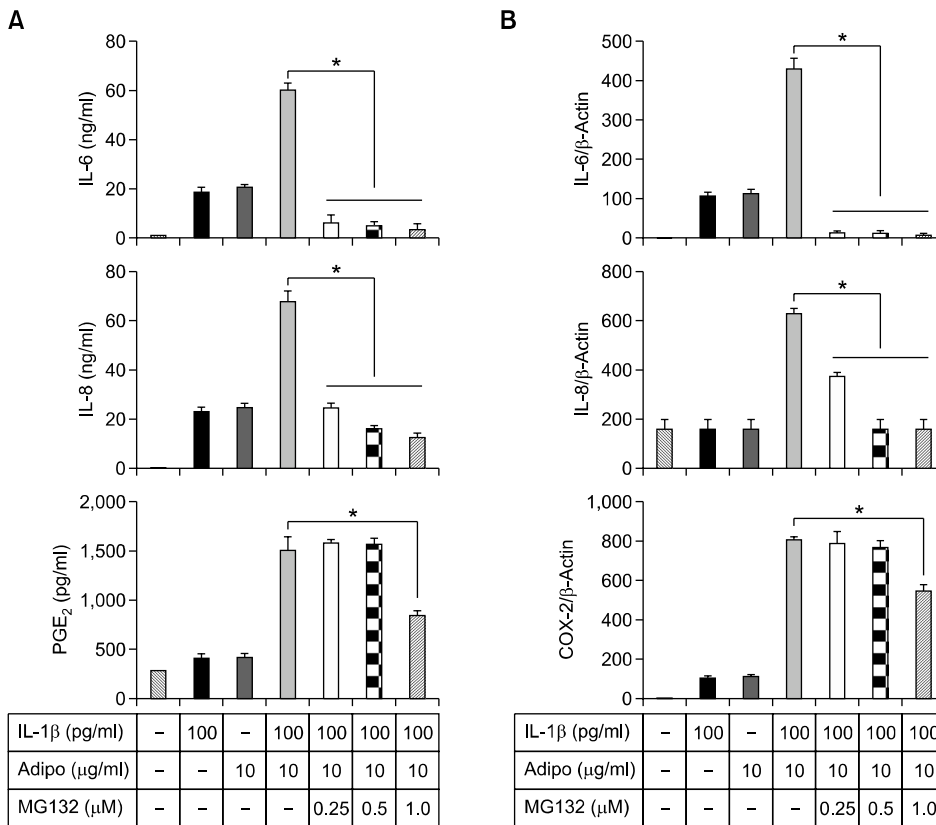
**Figure 4.** The effects of the NF-κB inhibitor MG132 on the production of proinflammatory mediators such as IL-6, IL-8, and PGE$_2$. Synovial cells (2.5 × 10$^5$ cells/60 mm dish/2 ml serum-free media) were stimulated with IL-1β (100 pg/ml) and/or adiponectin for 24 h in the presence of MG132 at concentrations of 0.25-1.0 μM. The supernatants and cells were used for ELISA (A) and real-time PCR (B), respectively. Three independent experiments were performed in quadruplicate with FLSs from each patient. The data shown are representative of three independent experiments, and similar results were obtained from all three. Values are expressed as mean ± S.E.M.

COX-2.

Next, we investigated adiponectin-mediated signaling pathways in RA FLSs. As shown in Figure 3B, adiponectin treatment (10 μg/ml) degraded IκB-α maximally at 60 min, while ERK1/2, P-38, and JNK-1/2 were not significantly phosphorylated in this system. Combined treatment with adiponectin and IL-1β could not significantly lead to the synergistic degradation of IκB-α (Figure 3C). In keeping with these results, the combined treatments did not act synergistically to increase the level of nuclear NF-κB (Figure 3D). Next, we determined whether the increase of IL-6, IL-8, and PGE$_2$ levels by adiponectin plus IL-1β could be blocked by the NF-κB inhibitor MG132 (Figure 4). This inhibitor effectively inhibited the increase of IL-6, IL-8, and PGE$_2$ levels produced by the combined stimulation of adiponectin and IL-1β at both protein and mRNA levels.

**Expression levels and association of IL-6, IL-8, and PGE$_2$ with adiponectin in the synovial fluid of arthritic patients**

To evaluate whether the synergistic effect of adiponectin and IL-1β on the production of proinflammatory mediators is demonstrable *in vivo*,

we investigated the expression levels and association of IL-6, IL-8, and PGE$_2$ with adiponectin in the synovial fluid of patients with arthritis. As shown in Figure 5, the levels (mean ± S.D.) of adiponectin (1,358 ± 655.4 vs. 606.1 ± 231.1 ng/ml), IL-6 (17,868 ± 20,484 vs. 1,054 ± 1,435 pg/ml), IL-8 (7,238 ± 7,164 vs. 79.58 ± 129.6 pg/ml), and PGE$_2$ (1,061 ± 642.8 vs. 650.6 ± 327.6 pg/ml) were significantly higher in RA patients than in OA patients. Next, we studied the association of IL-6, IL-8, and PGE$_2$ levels with adiponectin level (Figure 6). In OA patients, the levels of none of these proinflammatory mediators were significantly associated with adiponectin level. However, in RA patients, IL-6 and IL-8 levels correlated positively with adiponectin level, while PGE$_2$ level was not significantly correlated with adiponectin level.

## Discussion

Adiponectin has been known to play various roles in the pathogenesis of various diseases. In our previous report, we suggested that adiponectin is a stimulant of MMP-1 and MMP-13 expression as strong as IL-1β, even though adiponectin is also an inducer of proinflammatory mediators (Choi *et al.*,
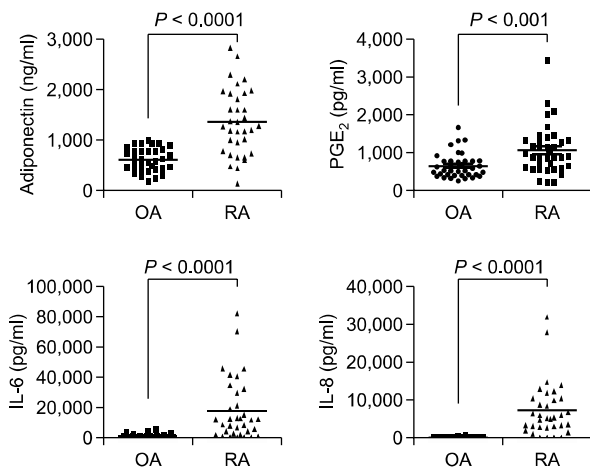
**Figure 5.** Expression levels of IL-6, IL-8, PGE₂, and adiponectin in synovial fluid from patients with RA or OA. Collected joint fluid from 34 patients with RA or OA was dispensed into 1-ml aliquots and treated with hyaluronidase (50 μg/ml) for 1 h at room temperature. The joint fluid was diluted with diluent buffer to achieve the proper detection range for ELISA. The levels of proteins of interest in joint fluid were measured using a commercially available ELISA kit, according to the manufacturer's instructions.



**Figure 6.** Association of adiponectin with IL-6, IL-8, or PGE₂ in synovial fluid from patients with RA or OA. To determine the degree of linearity between 2 variables, data were compared using Spearman's correlation test (2-tailed). Differences were considered significant at $P < 0.05$.

2009). Regarding adiponectin's synergy with IL-1β, Kusunoki *et al.* reported that adiponectin stimulates PGE₂ production in RA synovial fibroblasts (Kusunoki *et al.*, 2010). Consistent with that report, our data also found that adiponectin stimulated the PGE₂ production and COX-2 expression, and greatly increased the COX-2 expression in the presence of IL-1β. The production of IL-6 and IL-8 by adiponectin in RA FLSs has already been reported (Ehling *et al.*, 2006; Kitahara *et al.*, 2009); however, the synergy of adiponectin and IL-1β in the production of proinflammatory mediator is, to our knowledge, reported here for the first time. Contradictory to adiponectin's synergy with IL-1β, adiponectin treatment mitigates the severity of arthritis in mice with collagen-induced arthritis; additionally, *in vitro* experiments with RA FLSs indicated that adiponectin significantly inhibits IL-1β-induced RA FLS proliferation, while increasing IL-6 expression in IL-1β-stimulated RA FLSs (Lee *et al.*, 2008). All of these findings suggest that adiponectin's synergy with IL-1β may not necessarily lead to more severe inflammation in arthritic joints, but may sometimes be overwhelmed by other factors at the local inflammatory site. In addition, the above controversial reports of adiponectin may be due to a potential dual effect of adiponectin, having an anti-inflammatory and pro-inflammatory action according to its exposure time and concentration (Brochu-Gaudreau *et al.*, 2010).

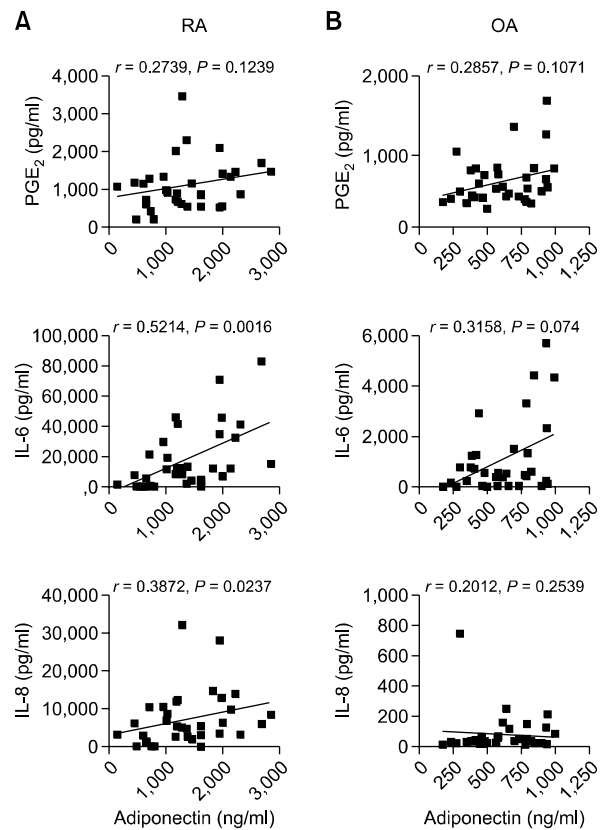Adiponectin activates intracellular signaling pathways through the adiponectin receptors AdipoR1 and AdipoR2. COX-2 expression is significantly inhibited when these receptors are expressed in RA FLSs and reduced by RNA interference (RNAi) technology, suggesting that adiponectin activates intracellular signaling pathways through adiponectin receptors (Kusunoki *et al.*, 2010). However, adiponectin-mediated intracellular signaling pathways are not much studied by Western blot in RA FLSs. Therefore, various inhibitors of MAPK signaling pathways have been tested to indirectly evaluate adiponectin-induced intracellular signaling pathways. In particular, p38 MAPK inhibitor SB203580 treatment resulted in a significant reduction of adiponectin-dependent IL-6 synthesis in RA FLSs (Ehling *et al.*, 2006). Furthermore, adiponectin-induced activation of p38 and NF-κB pathways has been demonstrated by Western blot in human chondrosarcoma cells (Chiu *et al.*, 2009). Recently, adiponectin-induced MCP-1 secretion was downregulated by p38 MAPK and protein kinase C inhibitors (Frommer *et al.*, 2010). All of these findings emphasized that adiponectin stimulates proinflammatory mediators through the p38 signaling pathway. However, in the present

study, Western blot data revealed that NF-κB signaling pathways are likely to be more important in adiponectin-induced gene expression of RA FLSs than other pathways. In contrast, 10 ng/ml IL-1β strongly stimulated MAPK and IκB pathways in our previous report (Bang *et al*., 2009). In this study, the NF-κB inhibitor MG132 greatly inhibited IL-6, IL-8 and PGE$_2$ production in adiponectin- and IL-1β-stimulated RA FLSs. However, the specific synergistic mechanisms are not explained here. Unknown signaling pathways other than IκB or MAPK pathways seem to be involved in the synergistic action in this system. The MMP-1, MMP-13, and VEGF genes, which are not synergistically stimulated, may be expressed by a set of transcription factors through the common signaling pathways activated by adiponectin or IL-1β. Expression of IL-6, IL-8, and COX-2 may be synergistically increased through transcription factors of different signaling pathways activated by adiponectin and IL-1β. In addition, 10 ng/ml IL-1β is not a physiological concentration; rather, 10-100 pg/ml IL-1β is a more physiological concentration in the synovial fluid of RA patients (Kahle *et al*., 1992). Therefore, the combined stimulation of adiponectin (10 μg/ml) and IL-1β (100 pg/ml) is feasible in physiological conditions. Their synergistic effect on the production of proinflammatory mediators may shed some light on the question of how to properly control their level during the pathogenesis of inflammatory arthritis.

To provide greater insight into their synergistic effects, we investigated the levels of proinflammatory mediators in synovial fluids and their association with adiponectin. The level of IL-1β in synovial fluid was below the detection limit for ELISA; therefore, we could not measure the level of IL-1β. However, the positive association of IL-6 and IL-8 with adiponectin in RA synovial fluids can, at least in part, tell us that adiponectin plays an important role in the production of these proinflammatory mediators during joint inflammation. In support of this hypothesis, IL-6 levels are strongly associated with IL-8 levels in RA synovial fluid (Spearman r = 0.6999, *P* < 0.0001), but not in OA synovial fluid, suggesting that the expression levels of adiponectin, IL-6, and IL-8 are associated with each other in inflammatory arthritic joints. Meanwhile, various cell types in arthritic joints contribute to the production of proinflammatory mediators such as IL-6, IL-8, and PGE$_2$. Therefore, the synergistic effect of adiponectin and IL-1β on the production of such proinflammatory mediators should be tested in other cell types, such as endothelial cells and immune cells. Because other various factors such as hypoxia are involved in the production of proinflammatory mediators, we are unable to

declare their synergistic effect on the basis of this correlation study. In conclusion, we demonstrate for the first time that adiponectin and IL-1β may act synergistically on the production of proinflammatory mediators such as IL-6, IL-8, and PGE$_2$ in inflammatory arthritic joints. Considering that there are still some patients who are refractory to the biologics, such as blockers of TNF-α, IL-1β, and IL-6, new biologics are necessary to improve arthritis treatment. Therefore, adiponectin can be considered an additional potential therapeutic target to control inflammation in arthritic joints. A blocker against adiponectin may be useful to supplement the effective biologics that are currently available.

## Methods

### Reagents

Recombinant adiponectin and IL-1β expressed in *E. coli* was purchased from ProSpec (Rehovot, Israel). MG132 was obtained from Sigma-Aldrich Korea (Young-In, Korea).

### Synovial cell culture and collection of synovial fluids from patients

All *in vitro* experiments were carried out with fibroblast-like synoviocytes derived from patients with rheumatoid arthritis. After obtaining informed consent, synovial tissues were collected from RA patients. They met the 1987 American College of Rheumatology criteria for the diagnosis of RA and had been treated with nonbiological disease-modifying antirheumatic drugs (DMARDs) and were underwent therapeutic joint surgery. FLSs were isolated and grown in Dulbecco's Modified Essential Medium (DMEM, low glucose) (Gibco-BRL, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1× Antibiotic-Antimycotic (Gibco-BRL) as described previously (Kim *et al*., 2007). After the cells had grown to confluence, they were split at a 1:4 ratio. FLS passages 3-6 from three patients were used for all experiments. The synovial joint fluids were collected from 34 RA and OA patients. This study was approved by the Institutional Review Board of Kyung Hee University Hosipital at KANGDONG. The joint fluid was collected at Kyung Hee Hospital, Seoul, Korea.

### ELISA

Synovial cells (2.5 × 10$^5$ cells/60 mm dish/2 ml serum-free media) were treated with recombinant adiponectin (10 μg/ml), IL-1β (10 or 100 pg/ml) or their combination (ProSpec, Rehovot, Israel). Conditioned media was collected 24 h later as described previously (Choi *et al*., 2009). Three independent experiments were performed in quadruplicate. Each experiment was performed using synovial cells from different patients. For the assessment of MMP-1, MMP-13, VEGF, IL-6, IL-8, PGE$_2$, adiponectin levels in joint fluid, the collected joint fluid from 34 patients

with RA or OA were dispensed into 1 ml aliquots and treated with hyaluronidase at 50 μg/ml for 1 h at room temperature. The joint fluid was diluted with diluent's buffer for the proper detection range with ELISA. The levels of proteins of interest in joint fluid were measured using a commercial ELISA kit from R&D Systems. For the measurement of transcription factor, NF-κB, in the nucleus, FLSs were seeded ($5 \times 10^6$ cells) into 100 mm dishes and grown to 80% confluence. The cells were serum-starved overnight and stimulated by adiponectin, IL-1β or their combination for 90 min at the concentration as described above. Subsequently, the cells were washed twice in PBS and treated with lysis buffer and the extraction of transcription factors from the nucleus was performed according to the manufacturer's protocol (Active Motif, Seoul, Korea).

## RT-PCR

FLSs ($2.5 \times 10^5$ cells) were cultured overnight in 60 mm dishes containing 2 ml of media. Cells were incubated with serum-free media for 2 h and new serum-free media was replaced just prior to the addition of adiponectin or IL-1β and cultured for 24 h. Supernatants were collected for ELISA and the cells were used for RT-PCR. Trizol was used to extract total RNA from the cells. Complementary DNA was synthesized from 1 μg of total RNA in a 20 μl reverse transcription reaction mixture. For semi-quantitative PCR, aliquots of cDNA were amplified in a 25 μl PCR mixture according to the protocol provided by the manufacturer (TaKaRa Bio, Kyoto, Japan). The PCR conditions for the IL-1 receptor 1 (IL-1R1) and adiponectin receptor (AdipoR1) were as follows: 35 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s. PCR products were subjected to electrophoresis on 1.0% agarose gels containing ethidium bromide, and the bands were visualized under UV light.

For real-time quantitative PCR, the reaction was carried out using the LightCycler PCR system (Roche Diagnostics, Meylan, France) using the DNA binding SYBR Green I dye and primers to detect the PCR products as described previously (Choi *et al.*, 2009, 2010). The results are calculated as ratios of gene transcripts to β-actin transcripts, with the quantity of transcripts in each sample expressed as a copy number. The ratio of IL-6, IL-8 and COX-2 /β-actin mRNA was assigned a value of 100%, with the corresponding results calculated as relative percentages. The primer were synthesized by Bioneer Co. Ltd. (Seoul, Republic of Korea), and their sequences are listed in Supplemental Data Table S1.

## Western blot analysis

FLSs cultured ($2.5 \times 10^5$ cells) in 60 mm dishes were serum-starved overnight and stimulated by adiponectin or IL-1β for a given time. As described previously (Choi *et al.*, 2009), the cells are prepared for Western blot analysis and the samples were separated using 12% SDS-PAGE, and were then transferred to Hybond-ECL membranes (Amersham, Arlington Heights, IL). The membranes were first blocked with 6% nonfat milk dissolved in TBST buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20). The blots were then probed with various rabbit polyclonal antibodies for IκB-α, p-ERK1/2, p-P38, p-JNK-1/2, COX-2 and β-actin (Cell Signaling Technology, Beverly, MA) diluted 1:1000 in TBS at 4°C for overnight, and incubated with 1:1000 dilutions of goat anti-rabbit IgG secondary antibody coupled with horseradish peroxidase. The blots were developed using the ECL method (Amersham). For re-probing, the blots were incubated in the stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) at 50°C for 30 min with occasional agitation.

## Statistical analysis

The *in vitro* experimental data are expressed as the mean ± standard error of the mean (SEM) of quadruplicate samples. Differences between groups were compared with the Mann-Whitney test. The level of IL-6, IL-8, PGE$_2$, and adiponectin in joint fluid of RA and OA patients was compared between groups with the Mann-Whitney test. To determine the degree of linearity between 2 variables, data were compared using Spearman's correlation test (2-tailed). Prism software 4 (Graphpad Software, San Diego, CA) was used for statistical analysis and graphing. Differences were considered significant at $P < 0.05$.

## Supplemental data

Supplemental data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-7-04.pdf.

## Competing interests

The authors declare that they have no competing interests.

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