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A new player in cartilage homeostasis: adiponectin induces nitric oxide synthase type II and pro-inflammatory cytokines in chondrocytes[1]

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Summary

Objective: Recent studies revealed a close connection between adipose tissue, adipokines and articular degenerative inflammatory diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA). The goal of this work was to investigate the activity of adiponectin in human and murine chondrocytes and to study its functional role in the modulation of nitric oxide synthase type II (NOS2). For completeness, interleukin (IL)-6, IL-1β, matrix metalloproteinase (MMP)-2, MMP-3, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1, prostaglandin E2 (PGE2), leukotriene B4 (LTB4), tumor necrosis factor alpha (TNF)-α and monocyte chemoattractant protein-1 (MCP-1) accumulation have been evaluated in adiponectin-stimulated chondrocytes cell culture supernatants.

Methods: Murine ATDC5 cell line, C28/I2, C20A4, TC28a2 human immortalized chondrocytes, and human cultured chondrocytes were used. Nitrite accumulation was determined by Griess reaction. Adiponectin receptors (AdipoRs) expression was evaluated by immunofluorescence microscopy and confirmed by reverse transcriptase-polymerase chain reaction. NOS2 expression was evaluated by Western blot analysis whereas cytokines, prostanoids and metalloproteinases production was evaluated by specific enzyme-linked immunosorbent assays.

Results: Human and murine chondrocytes express functional AdipoRs. Adiponectin induces NOS2. This effect is inhibited by aminoguanidine, dexamethasone and by a selective inhibitor of phosphatidylinositol 3-kinase. In addition, adiponectin is able to increase IL-6, MMP-3, MMP-9 and MCP-1 by murine cultured chondrocytes whereas it was unable to modulate TNF-α, IL-1β, MMP-2, TIMP-1, PGE2 and LTB4 release.

Conclusions: These results bind more closely the interactions between fat-derived adipokines and articular inflammatory diseases, and suggest that adiponectin is a novel key element in the maintenance of cartilage homeostasis which might be considered as a potential therapeutical target in joint degenerative diseases.
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Key words: Adipokines, Adiponectin, Nitric oxide synthase, PI3-Kinase, Chondrocytes.

Introduction

The concept of white adipose tissue (WAT) as a passive tissue in charge of maintaining only whole body energy reserve has been radically modified after the discovery of leptin and it's wide ranging physiological activity in 1994[1]. So, WAT is emerging as a dynamic sharer in the regulation of physiopathological processes including immunity and inflammation and it is no longer viewed as a simply storage depot for body energy[2].

WAT has been described to produce and secrete a plethora of peptides that participates in diverse metabolic processes *via* endocrine, paracrine, autocrine and juxtacrine models of action. In addition, some of these factors have been acknowledged as pro-inflammatory mediators with cytokine-like activity, now called adipokines[3]. The grade of present knowledge is that adipose tissue is a critical

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component in modulating the crosstalk among several tissues and organs including adrenal, central and peripheral nervous system, and immune system.

Thus, WAT is now considered as a source of pro-inflammatory peptides that significantly contributes to the so-called "low grade inflammatory state" noted particularly in subjects with obesity and metabolic syndrome[4]. This cluster of metabolic abnormalities is characterized by insulin resistance, dyslipidemia, alteration of coagulation, and it is associated with increased risk of cancer, type II diabetes, cardiovascular complications and autoimmune inflammatory diseases. It is dutiful to underline that WAT also produces, likely as an adaptive response, anti-inflammatory factors such as interleukin (IL)-1 receptor antagonist (IL1-RA) and IL-10, circulating levels of both are also elevated in obese individuals[5].

The basis for obesity as pro-inflammatory state is that several markers of inflammation, including pro-inflammatory cytokines and acute phase proteins, are elevated in obese subjects[6]. Apparently simple, the physiology of adipocytes has been ignored along decades, but several evidences revealed to be extremely complex and finely tuned. Following the seminal discovery of leptin, many research efforts have been devoted to the identification of other proteic factors specifically secreted by adipocytes, and, in the last 10 years more than 50 factors have been characterized and constituted the so-called "adipokinome".

Therefore, it is of particular interest that recent studies have revealed multiple links among adipose tissue, adipokines, and inflammatory joint diseases. For instance, pro-inflammatory cytokines have been described to be produced by the infrapatellar fat pad of osteoarthritic subjects[7]. Moreover, studies aimed to determine adipokine levels in inflammatory articular diseases have demonstrated high levels of adiponectin and resistin in the synovial fluid of patients with osteoarthritis (OA) and rheumatoid arthritis (RA)[8].

Adiponectin, also called GBP-28, apM1, Acrp 30 or AdipoQ, is a 244 aa adipose tissue specific protein that has structural homology to collagen VIII and X and complement factor C1q[9,10]. Thus, it belongs to the C1q-tumor necrosis factor (TNF) super-family, whose members are thought to be derived from a common ancestral molecule and to share common "pro-inflammatory" properties[11]. Adiponectin circulates in the blood in large amounts and constitutes about 0.01% of total plasma proteins. Adiponectin is present in serum as oligomeric isoforms constituted prevalently by trimers, hexamers but also by high molecular weight isoforms (12−18 mer)[12].

Adiponectin has been investigated primarily in the context of energetic substrate metabolism (fatty acids and glucose)[13] and for its role on the cardiovascular system, wherein it has been suggested to act as anti-atherosclerotic factor and to exert, in general, anti-inflammatory properties at the endothelial level[14,15]. At cartilage level, Chen *et al.* suggested that adiponectin is involved in the modulation of cartilage destruction in chondrocytes by up-regulating tissue inhibitor of metalloproteinase (TIMP)-2 and down-regulating IL-1β-induced matrix metalloproteinase (MMP)-13[16].

In contrast, our findings of elevated adiponectin levels in RA patients[17], confirmed also by other authors[8], which demonstrated elevated adiponectin levels in synovial fluid of RA patients suggested a pro-inflammatory role for this adipokine. In addition, recent reports, demonstrated that adiponectin is expressed in synovial fibroblasts[18], and that adiponectin enhances IL-6 production *via* a complex pathway involving specific adiponectin receptors (AdipoRs) and intracellular intermediates[18,19].

Given the fact that other members of the C1q-TNF super-family are involved in articular degenerative diseases pathophysiology, and considering that other adipokines, such as leptin, have been described to act as pro-inflammatory factor at joint level[20−22], it was conceivable to hypothesize that other adipokines, and particularly adiponectin, might play a distinct pathogenic role in inflammatory joint diseases by acting at the unique cellular component of articular cartilage: the chondrocyte.

Therefore, in this study we investigated the functional role of adiponectin as modulator of inflammatory response by using as molecular target the nitric oxide synthase type II (NOS2). In addition, other relevant factors involved in the inflammatory response in chondrocytes upon adiponectin stimulation have been evaluated.

Methods

REAGENTS

All culture reagents were purchased from SIGMA (St Louis, MO, USA), except Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium and Trypsin−ethylenediaminetetraacetic acid (EDTA) that was from Lonza (Lonza Group Ltd, Basel, Switzerland). Reverse transcriptase-polymerase chain reaction (RT-PCR) products were supplied by Invitrogen (Life Technologies, Carlsbad, CA, USA) and Stratagene (Cedar Creeck, TX, USA) and full-length adiponectin by Biocat (BioVision Research Products, Mountain View, CA, USA).

CELL CULTURE AND TREATMENTS

The clonal chondrogenic cell line ATDC5 was chosen for these studies because it has been shown to be a useful *in vitro* model for examining the multistep differentiation of chondrocytes. Undifferentiated ATDC5 cells proliferate rapidly until they reach confluence, at which point they undergo growth arrest. When treated with insulin, transferrin and sodium selenite, confluent ATDC5 cells re-enter a proliferative phase and form cartilaginous matrix nodules (mature chondrocytes). As differentiation progresses, these cells undergo a late differentiation phase, becoming hypertrophic, calcifying chondrocytes that synthesize type X collagen and osteopontin − a marker of terminal chondrocyte differentiation[23]. ATDC5 cells were a kind gift from Dr Agamemnon E Grigoriadis (Department of Craniofacial Development, King's College, London Guy's Hospital, London, UK). Unless otherwise specified, cells were cultured in DMEM/Ham's F12 medium supplemented with 5% fetal bovine serum (FBS), 10 μg/ml human transferrin, 3×10^{-8} mol/l sodium selenite and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). ATDC5 cells were used in both undifferentiated and differentiated stage as they have shown a similar behavior in all differentiation stages, at least in term of nitric oxide (NO) type II activation[20]. The immortalized human juvenile costal chondrocytes cell lines TC28a2, C20/A4, and C28/I2 were cultured in DMEM/F12 as described previously[24] supplemented with 10% FBS, L-glutamine, and antibiotics. Human normal articular cartilage samples (a kind gift of Mary B. Goldring, Hospital for Special Surgery, NYC, USA) were obtained from knee joints of patients undergoing leg amputations from above the knee because of peripheral vascular disease. (Permission from the local ethical committee was granted.) None of the patients had a clinical history of arthritis or any other pathology affecting the cartilage, and the specimens appeared normal on morphological examination (no change in color and no fibrillation). Articular osteoarthritic cartilage samples were obtained from femoral condyles of patients with clinical history of OA undergoing leg amputations as above described. Samples were taken from the main defective area of maximal load. Patients with RA (or other autoimmune disease), infection-induced OA and posttraumatic OA have not been considered for cartilage sample harvesting. For chondrocyte isolation, aseptically dissected cartilage was subjected to sequential digestion with pronase (catalog number 165921; Roche Molecular Biochemicals, Indianapolis, IN, USA) and collagenase P (catalog number 1213873; Roche Molecular Biochemicals) at a final concentration of 1 mg/ml in DMEM/F12 plus 10% FBS and sterilized by filtration, in accordance with the manufacturer's instructions. Cartilage specimens were finely diced in phosphate-buffered saline (PBS), and after removing PBS diced tissue was incubated for 30 min with pronase in a shaking water bath at 37°C. Pronase was subsequently removed from the digestion flask and the cartilage pieces were washed with PBS. After removal of PBS, digestion was continued with addition of collagenase P; this was done over 6−8 h in a shaking water bath at 37°C. The resulting cell suspension was filtered through a 40-μm nylon cell strainer (BD Biosciences Europe, Erembodegem, Belgium) in order to remove debris. Cells were centrifuged and washed twice with PBS, counted and plated in

24-well tissue culture plates for chondrocyte culture. Cells were sub-cultured from a chondrocyte primary culture to obtain a sufficient number of cells and used between the first and second passages to warrant chondrocyte phenotype.

For NO accumulation studies, enzyme-linked immunosorbent assay (ELISA), and Western Blot (WB) analysis, cells were seeded in P24 multiwell plates up to complete adhesion (85–90% confluence) and starved overnight in serum free conditions (for WB analysis cells were seeded in P6 multiwell plates as above described). Cells were treated with adiponectin (0.1 and 1 μg/ml) in the presence of polymixin B (10 μg/ml) in order to neutralize the eventual presence of endotoxin (which is <0.1 ng/μg of recombinant adiponectin, as stated by provider's analysis certificate). Different specific pharmacological inhibitors were added 1 h before stimulation: aminoguanidine (1 mM) for NOS2 activity blockade, LY294002 (10 μM) for phosphatidylinositol 3-kinase (PI3-K) inhibition and dexamethasone (10 μM) to inhibit NOS2 *de novo* synthesis. All treatments were performed at least in three independent experiments.

IMMUNOFLUORESCENCE MICROSCOPY

Cell slide preparation

Cells were seeded on glass microslides and unless otherwise specified, all cell lines were fixed in 90% ethanol for 10 min and mounted on Dako adhesion microslides (DakoCytomation, Carpinteria, CA, USA).

Primary antibodies and procedure

Rabbit anti-adiponectin receptor 1 (AdipoR1) and anti-adiponectin receptor 2 (AdipoR2) (Phoenix Pharmaceuticals, Inc, Belmont, CA, USA) diluted 1:100, for 1 h at room temperature; mouse anti-collagen II (Chemicon, Temecula, CA, USA) diluted 1:20, overnight at 4°C. Collagen II epitope retrieval was done by microwave oven in 0.05 M Tris EDTA buffer pH 9 (20 min at 700 W) and proteinase K (40 ml in 2 ml 0.05 M Tris HCl pH 7.5, Dako) 5 min at 37°C. In the single immunofluorescence, primary antibodies were incubated as above described. Detection system used in this case was an anti-rabbit antibody (Ab) conjugated with Alexa 488 (1:200, for 1 h at room temperature; Molecular Probes, Carlsbad, CA, USA) for AdipoR1 and AdipoR2; anti-mouse Ab conjugated with Cyanine 3 (Sigma, St Louis, MO, USA) 1:100 for 1 h for collagen II. For double immunofluorescence the protocol was as follows: (1) epitope retrieval: 20 min microwave oven in Tris EDTA buffer, pH 9 and proteinase K (5 min at 37°C); (2) first primary Ab mouse anti-collagen II 1:20 overnight at RT; (3) anti-mouse Cyanine 3 (1:100, 1 h); (4) second primary Ab: AdipoR1 or AdipoR2; (5) and anti-rabbit conjugated with Alexa 488 (1:200, 1 h). Between steps, slides were washed twice for 5 min with tris buffered saline (TBS) (Tris 20 mM, NaCl 137 mM) and after step 5 in distilled water. At the end, slides were mounted with Immuno-Fluore mounting medium (ICN, Aurora, OH, USA). The sections were observed and photographed by using a Provis AX70 microscope (Olympus Corp., Tokyo, Japan).

RNA ISOLATION AND RT-PCR

RNA was isolated from cell culture by TRIzol LS, according to the manufacturer's instructions (Gibco BRL Life Technologies, Grand Island, NY, USA) as previously described[21]. Briefly, total RNA (2–5 μg) was used to perform RT-PCR. Complementary DNA (cDNA) was synthesized using 200 units of Moloney murine leukemia virus (MMLV) RT (Gibco BRL) and 6 μl of deoxynucleotide triphosphate (dNTP) mix (10 mM of each dNTP), 6 μl of first-strand buffer (250 mM Tris HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; Gibco BRL), 1.5 μl of 50 mM $MgCl_2$, 0.17 μl of random hexamer solution (3 μg/μl; Gibco BRL), and 0.25 μl of RNAseOut (recombinant ribonuclease inhibitor, 40 units/μl; Gibco BRL), in a total volume of 30 μl. Reaction mixtures were incubated at 37°C for 50 min and at 42°C for 15 min. The RT reaction was terminated by heating at 95°C for 5 min, and the mixture was subsequently quick-chilled on ice. Three microliters of the RT reaction mixture was used for PCR amplification. The amplification conditions were as follows: 5 μl of PCR buffer (200 mM Tris HCl, pH 8.4, and 500 mM KCl; Gibco BRL), 1.5 μl of 50 mM $MgCl_2$, 4 μl of dNTP mix, 150 ng of upstream primer, 150 ng of downstream primer and 1.25 units of Taq DNA polymerase (Gibco BRL). Amplification conditions were as shown in Table I.

Negative controls consisted of omitting the RT reaction mixture for each sample and amplifying samples of RT reaction mixture without MMLV. To exclude a competitive amplification of genomic DNA, RT-PCR was performed on mouse genomic DNA. The identity of the amplimer was confirmed by performing RT-PCR together with positive controls. PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, examined with ultraviolet light, and visualized with a Typhoon 9410 documentation system (Amersham Pharmacia Biotech, Little Chalfont, UK).

WB

Human cultured chondrocytes and ATDC5 murine chondrogenic cells were seeded as above described. Cells were stimulated with adiponectin at different doses (0.1 and 1 μg/ml) and different times (5, 10, 30, 120 min for 5′AMP activated protein kinase (AMPK) activation studies, and 48 h for NOS type II and PI3-K studies). In some experiments, lipopolysaccharide (LPS) (500 ng/ml), was used as positive control. After pertinent stimulations, cells were rapidly washed with ice cold PBS and scraped in Radio Immuno Precipitation Assay (RIPA) lysis buffer. Lysed cells were centrifuged at 13,000*g* for 15 min. Lysates from control or stimulated cells were collected and separated by SDS-PAGE on a 10% polyacrylamide gel. Proteins were subsequently transferred to a polyvinylidene difluoride transfer membrane (Hybond TM-P; Amersham International, Little Chalfont, UK).

Blots were incubated with the pertinent primary Ab: (1) anti-NOS2 diluted 1:1000 (Upstate, Lake Placid, NY, USA), (2) AMPK diluted 1:1000, (3) Phospho-AMPK diluted 1:1000 (two and three from Cell Signalling Technology, Inc, Dambers, MA, USA), (4) anti-β-actin diluted 1:2500 (Sigma, St Louis, MO, USA) and (5) anti-PI3-K diluted 1:1000 (Upstate, Lake Placid, NY, USA). Immunoblots were visualized using ECLPlus detection Kit (GE Healthcare, Little Chalfont, UK) using anti-rabbit or anti-mouse horseradish peroxidase labeled secondary Ab. Images were acquired with an EC3 Imaging System (UVP, Upland, CA, USA) and densitometred using the ImageMaster TotalLab tool (GE Healthcare, Little Chalfont, UK).

NITRITE ASSAY

Nitrite accumulation was measured in the culture medium by Griess reaction as previously described[20–22].

ELISAS

MMP-2 (DMP200), MMP-3 (MMP300), MMP-9 (MMP900), and TIMP-1 (MTM100) ELISA kits were purchased from R&D Systems Europe, Abingdon, UK. IL-1β (EMIL1B), IL-6 (EM2IL6), monocyte chemoattractant protein (MCP)-1 (EMMCP1), TNF-α (EMTNFA), prostaglandin E2 (PGE2) (EHPGE2) and leukotriene B4 (LTB4) (EHLTB4) were purchased from Pierce Biotechnology, Rockford, USA. Assays were performed according to manufacturer's protocol. Range, sensitivity and inter-assay precisions were as described by manufacturer's technical sheets.

STATISTICAL ANALYSIS

All results are expressed as mean ± S.E.M. of at least three independent experiments, each with at least three independent observations. Statistical

Table I
Primer sequences and amplification conditions for PCR reaction

Amplification conditions	Mouse AdipoR1	Mouse AdipoR2	Human AdipoR1	Human AdipoR2	Mouse GAPDH	Human GAPDH
Annealing temperature (°C)	60	60	62	62	Same conditions of target	Same conditions of target
Product size	132	72	71	76	376	376
Number of cycles	40	40	35	35	Same conditions of target	Same conditions of target
Accession number	BC014875	XM_132831	NM_015999	NM_024551	M32599	NM_002046
Forward	470–491	564–586	1100–1121	1119–1140	531–554	593–616
Accession number	BC014875	XM_132831	NM_015999	NM_024551	M32599	NM_002046
Reverse	592–602	614–635	1151–1170	1177–1194	884–907	946–969

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

analysis was performed by analysis of variance (ANOVA) followed by the Student–Newman–Keuls test and Bonferroni or Dunnet multiple comparison test. A *P* value less than 0.05 was considered as significant.

Results

ADIPONECTIN RECEPTORS EXPRESSION

AdipoR1 and AdipoR2 immunofluorescence

As shown in Fig. 1 (left panel) the cytoplasm of murine ATDC5 cultured cell line and human C28/I2 immortalized chondrocytes showed intense immunoreactivity with anti-AdipoR1 (a and e) and anti-AdipoR2 antibodies (b and f). ATDC5 differentiated chondrocytes showed strong immunoreactivity for AdipoR1 and AdipoR2 (c and d). As shown in Fig. 1 (right panel), immunoreactivity for AdipoR1 was found in both human normal (a) and osteoarthritic (g) chondrocytes. A similar pattern was found for AdipoR2 (d and j). All analyzed cells were immunoreactive for collagen type II (b, e, h and k). The possibility that the human cultured cells were chondrocytes, rather than other cell types, was ruled out in co-localization studies showing positivity for AdipoR1 and AdipoR2, by their immunoreactivity with Ab against collagen type II (c, f, i and l).

AdipoR1 and AdipoR2 mRNA expression

Data above exposed were further confirmed by studies aimed to analyze AdipoR1 and AdipoR2 mRNA expression by RT-PCR. As shown in Fig. 2, murine AdipoR1 and AdipoR1 mRNA was found in ATDC5 (AT) cells (Fig. 2, panel A). Human cultured normal chondrocytes (hC), as well as human immortalized chondrocyte cell lines TC28a2 (TC), C28/I2 (C28) and C20A4 (C20) also expressed AdipoR1 and AdipoR2 mRNA (Fig. 2, panel B). Skeletal muscle (SM) and liver (Liv) were used as positive control for AdipoR1 and AdipoR2 expressions, respectively. To note, functionality of AdipoRs was tested in separate experiments, performed on ATDC5 cells, that showed rapid phosphorylation of the downstream AMPK in adiponectin-stimulated cells, as evaluated by WB analysis (Fig. 2, panel C1) and quantified by densitometric analysis (Fig. 2, panel C2).

Adiponectin effect on NOS2 expression and activity

As shown in Fig. 3 (panel A), adiponectin stimulation of ATDC5 murine cells with different doses (0.1 and 1 μg/ml) and LPS (500 ng/ml) as positive control, provoked a significant increase in nitrite accumulation in the culture supernatant. This effect was dependent on the dose and was evaluated at 24 and 48 h. This result was confirmed also in terms of NOS2 protein expression.

To confirm whether NO formation was produced *via* NOS2, ATDC5 cells and human chondrocytes were incubated for 48 h with adiponectin in the presence of the NOS2 inhibitor aminoguanidine (1 mM), added 1 h before adiponectin administration. Aminoguanidine strongly decreased nitrite

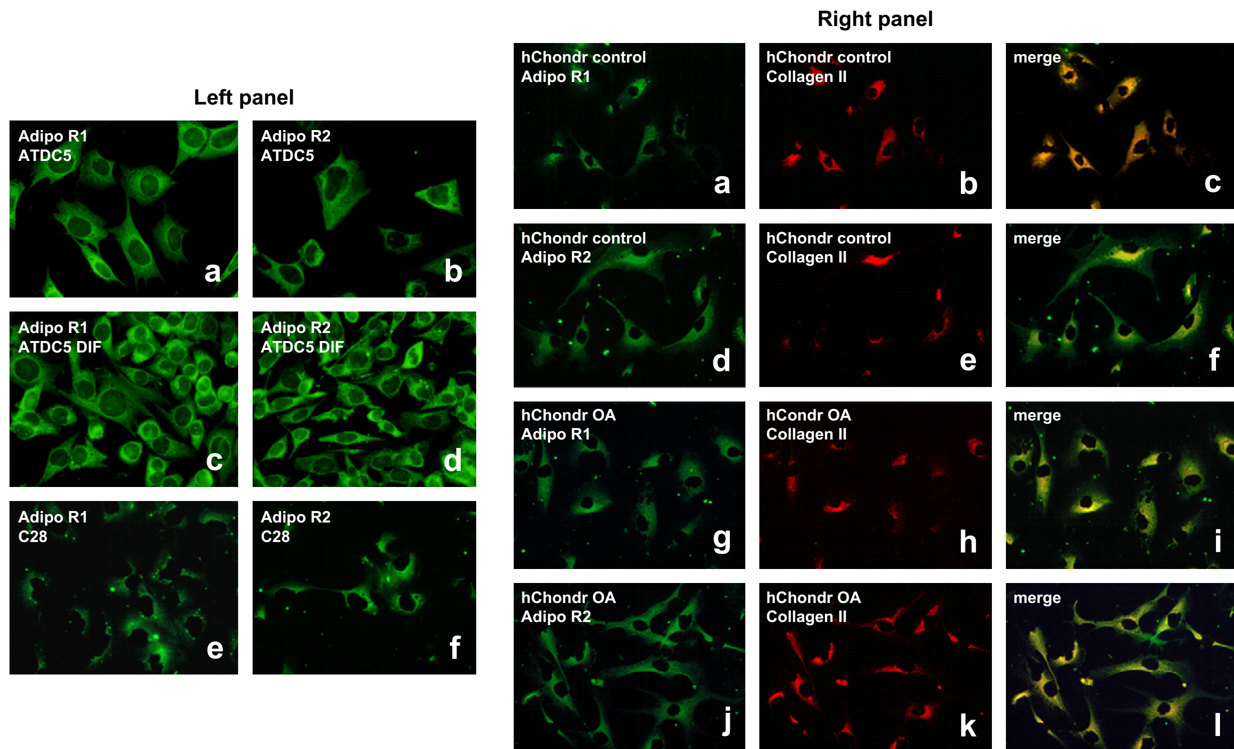

Fig. 1. Adiponectin receptors expression in chondrocytes. Left panel: AdipoR1 and AdipoR2 are expressed in undifferentiated ATDC5 murine cell line (a and b, respectively), in differentiated ATDC5 murine cell line (c and d, respectively), and in C28/I2 human immortalized chondrocytes (e and f, respectively). Right panel: AdipoR1 is expressed in human normal cultured chondrocytes (a) and in human cultured chondrocytes from osteoarthritic patients (g). AdipoR2 is expressed in human normal cultured chondrocytes (d) and in human cultured chondrocytes from osteoarthritic patients (j). All cultured chondrocytes showed positive immunofluorescence for collagen type II as chondrocyte specific marker (b, e, h and k). Co-localization of AdipoRs and collagen type II is shown in c, f, i and l.

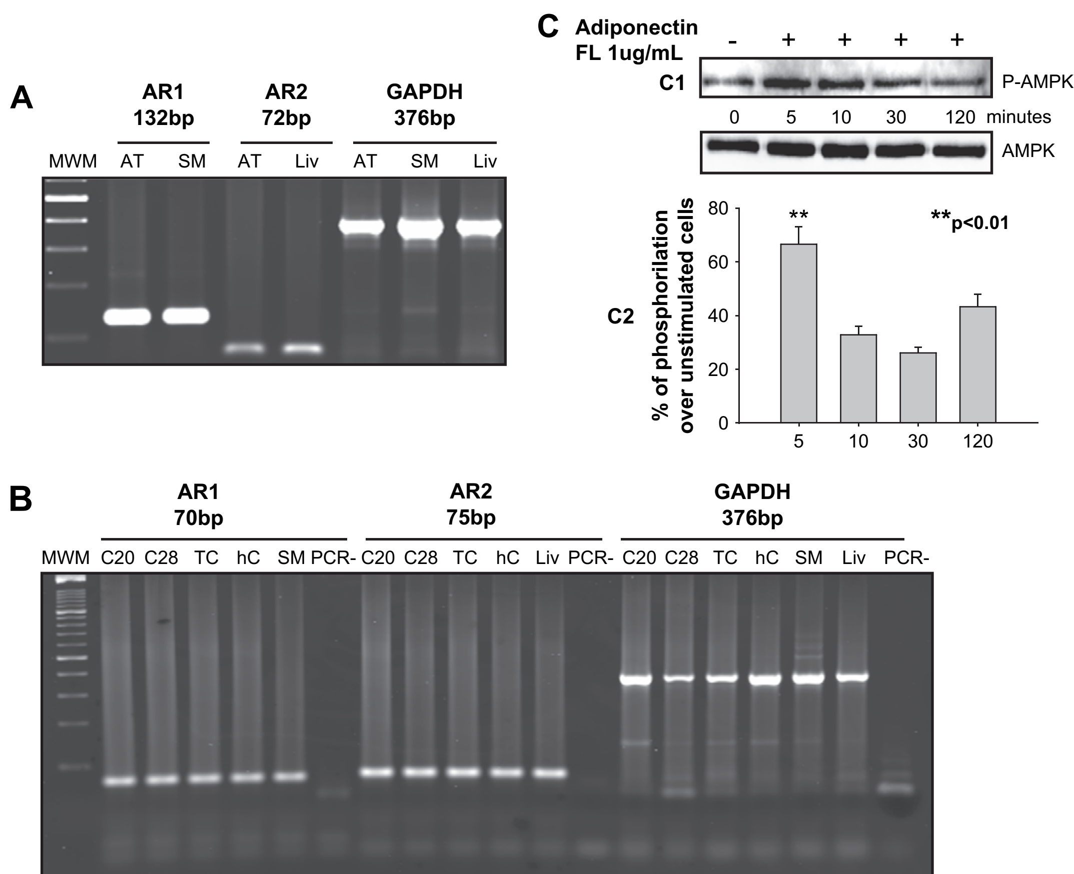

Fig. 2. AdipoRs expression and functionality in chondrocytes. Panel A: AdipoR1 and AdipoR2 mRNA expression in ATDC5 (AT) cells determined by RT-PCR. SM and Liv were used as positive controls. Panel B: AdipoR1 and AdipoR2 mRNA expression in C20A4 (C20), C28/I2 (C28), TC28a2 (TC) and human cultured chondrocytes (hC), determined by RT-PCR. SM and Liv were used as positive controls. Panel C: WB for phosphorylated AMPK. Phosphorylated AMPK was identified by phospho-AMPKα Ab Thr172 (Cell Signalling catalog number 2531, 62 kDa) in lysates coming from ATDC5 cells stimulated with adiponectin (1 μg/ml). Phosphorylation levels were evaluated, in time-dependent experiments, and compared to total AMPK (AMPKα Ab, Cell Signalling catalog number 2532, 62 kDa). Blot is representative of at least four independent experiments (C1). Densitometric analysis of WB experiments is reported in panel C2. Data are represented as percent ± S.E.M. over basal AMPK phosphorylation in unstimulated cells.

accumulation in the culture supernatant of ATDC5 cells (Fig. 3, panel B) and fully inhibited NO accumulation in human cultured chondrocytes (Fig. 3, panel C). To note, pre-treatment with dexamethasone (10 μM), a classic *de novo* NOS2 inhibitor, also blocked nitrite accumulation in adiponectin–ATDC5 stimulated cells (Fig. 3, panel B).

PI3-K inhibition blocks adiponectin-induced NO production and NOS2 protein expression.

We also investigated the role played by PI3-K in nitrite accumulation and NOS2 expression evoked by adiponectin in murine and human chondrocytes by using LY294002 (10 μM). The dose of LY294002 was selected according to previous published literature[20–22]. As shown in Fig. 3 (panel B1), PI3-K is phosphorylated after chondrocyte treatment with recombinant adiponectin. Pre-treatment with LY294002 specifically blocked adiponectin-induced nitrite accumulation in, respectively, murine and human cultured chondrocytes (Fig. 3, panel B and C). This result was confirmed also in terms of NOS2 expression since cell pre-treatment with LY294002 significantly decreases NOS2 protein expression (Fig. 3, panel B2 and C1) and completely inhibited PI3-K phosphorylation (Fig. 3, panel B1).

Adiponectin effect on cytokines, metalloproteinases and prostanoids

As shown in Fig. 4, adiponectin was able to significantly increase IL-6, MCP-1, MMP-3 and MMP-9 in the supernatant of adiponectin-stimulated ATDC5 cells for 48 h (Fig. 4, panels A–F, respectively), whereas was unable to modulate IL-1β, MMP-2 (Fig. 4, panels C and D), TIMP-1, LTB4, PGE2 and TNF-α [Fig. 5(A–D), respectively]. Inhibition of PI3-K pathway by LY294002 strongly blunted adiponectin induction of IL-6 and MMP-9 (Fig. 4, panels A and F). In addition, LY294002 decreased, *per se*, MMP-2 basal spontaneous accumulation (Fig. 4, panel D). Intriguingly, this PI3-K pharmacological blocker was able to partially inhibit adiponectin-induced MCP-1 when a minimal dose of adiponectin was used whereas was unable to counteract higher adiponectin doses (Fig. 4, panel B). A similar pattern was observed for MMP-3 (Fig. 4, panel E), where surprisingly, co-stimulation with LY294002 and adiponectin

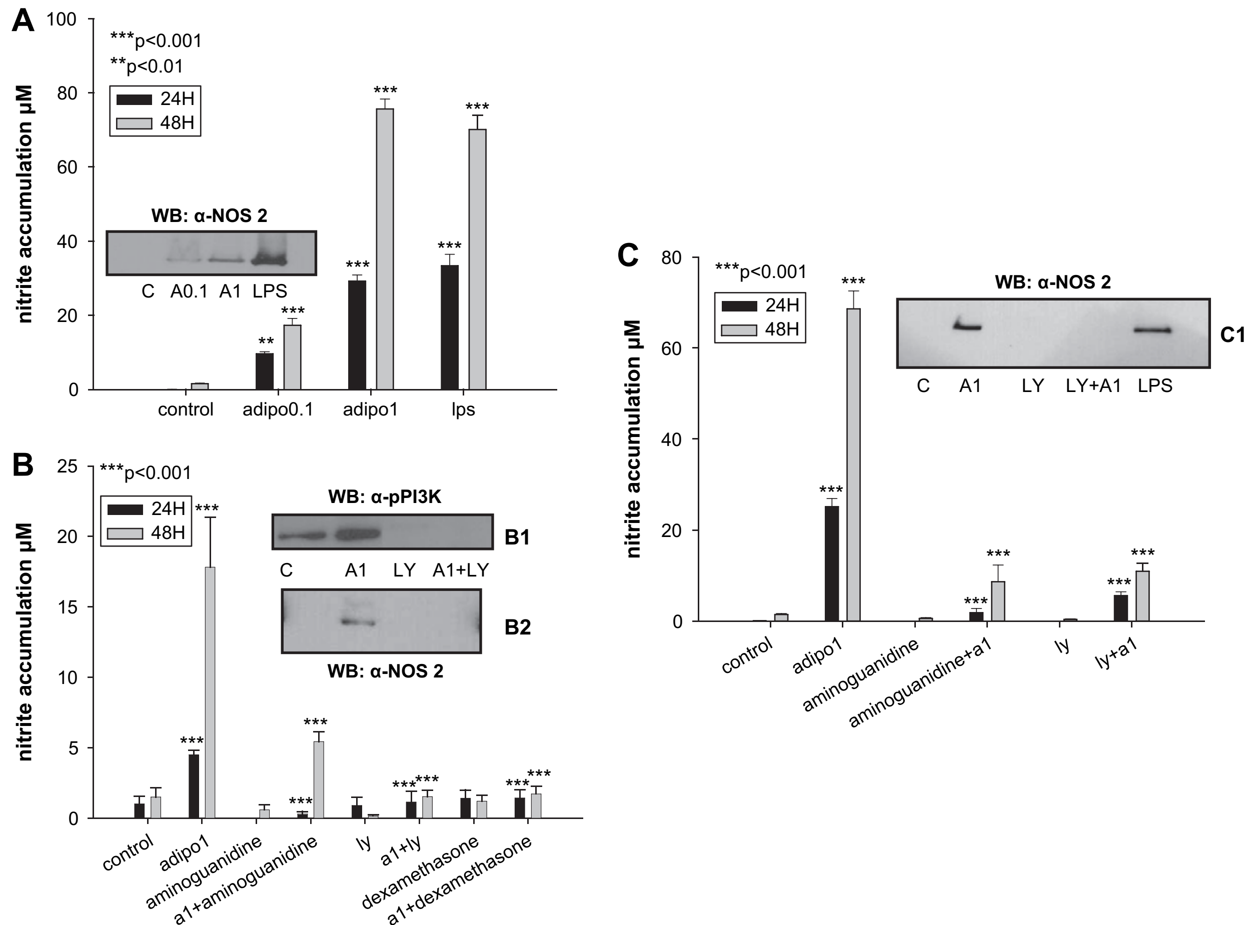

Fig. 3. NOS2 induction by adiponectin. Panel A: Adiponectin (0.1 and 1 μg/ml) induces nitrite accumulation in ATDC5 cells in a dose-dependent manner. LPS (500 ng/ml) was used as a positive control. Nitrite has been evaluated at 24 (black bars) and 48 h (gray bars). This result was confirmed by WB. Adiponectin-induced NOS2 synthesis in a dose-dependent manner and LPS was used as a positive control at 48 h (panel inside). Panel B: Adiponectin-induced (1 μg/ml) nitrite accumulation in ATDC5 cells was modulated by different specific pharmacological inhibitors [aminoguanidine (1 mM), LY294002 (10 μM) and dexamethasone (10 μM)]. All inhibitors used 1 h before adiponectin treatment, reduced significantly nitrite accumulation whereas NOS2 synthesis is decreased by LY294002 (B2). PI3-K is phosphorylated upon adiponectin treatment. Adiponectin-induced phosphorylation of PI3-K is prevented by LY294002 (B1). Panel C: Nitrite accumulation in human cultured chondrocytes induced by adiponectin (1 μg/ml) in presence or not of different specific pharmacological inhibitors [aminoguanidine (1 mM), LY294002 (10 μM)] at 24 (black bars) and 48 h (gray bars). NOS2 protein expression is selectively inhibited by LY294002 (C1).

seemingly synergized to increase MMP-3 production by cultured chondrocytes.

Discussion

Conversely to the wide range of data in the field of endocrinology and cardiovascular physiopathology, very little is known about the role of adipose tissue and adipokines in articular degenerative inflammatory and/or autoimmune diseases such as RA and OA. Less is known about the expression and the physiopathological role of adiponectin in chondrocytes, the unique cartilage cell component. With regards to joints, only recent findings demonstrated that articular adipose tissue and synovial fibroblasts from patients with joint inflammatory diseases are a local source of adiponectin[18]. This is also reflected by the presence of adiponectin in synovial fluids from RA and OA patients[8]. In addition other authors recently reported that adiponectin levels in synovial fluids from RA patients are significantly higher than in OA patients[16,25]. Therefore, the aim of this study was to investigate the functional role of adiponectin in chondrocytes.

Of interest, our data show that murine ATDC5 cells and both human cultured and immortalized chondrocytes express the two isoforms of adiponectin receptors, which are necessary to transfer the adiponectin-dependent signals. Therefore, we investigated the effect of adiponectin in cultured chondrocytes. Up to now, most of the endocrinological and cardiovascular studies, aimed to delineate the functional role of adiponectin, reported a protective rather than a pro-inflammatory or detrimental role of this molecule. One previous report postulated potential roles of adiponectin in cartilage, suggesting that joint tissues might be targets for this adipokine, where it may exert several modulatory effects including up-regulation of TIMP-2 and down-regulation of IL-1β-induced MMP-13[16]. So, these authors suggested that adiponectin might have a potential protective role in osteoarthritic joints[16].

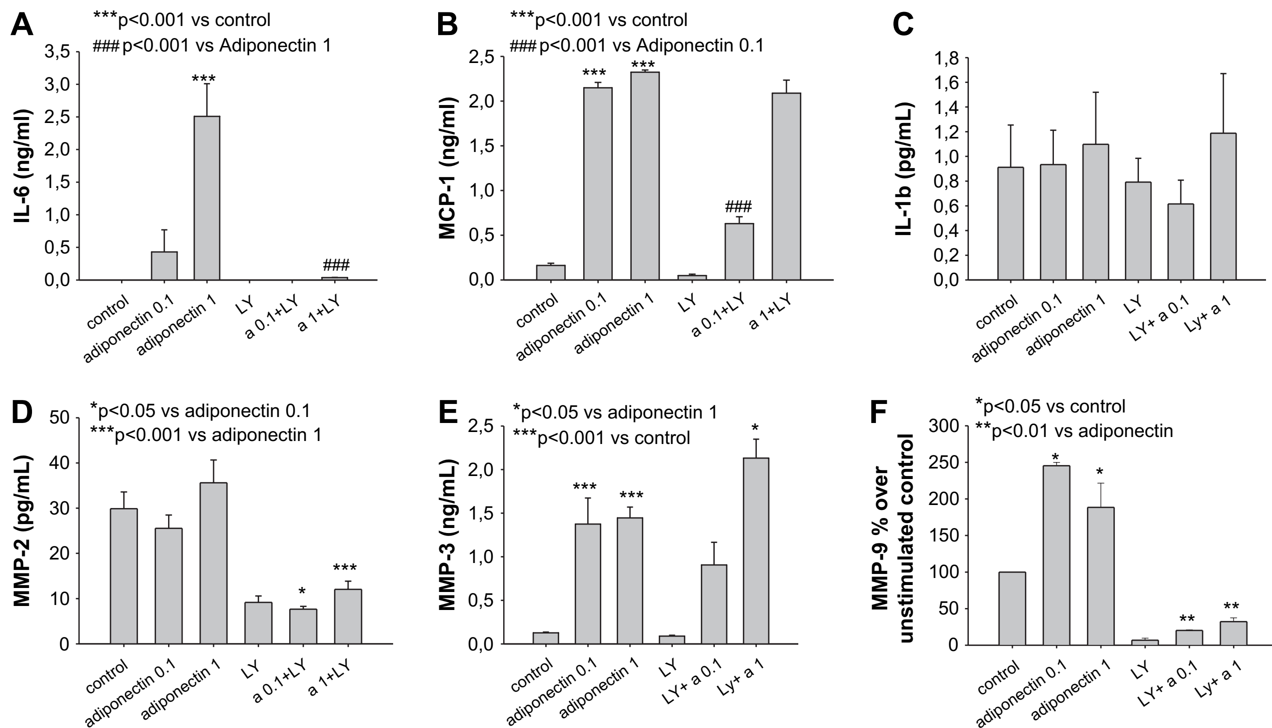

Fig. 4. Adiponectin induces pro-inflammatory cytokines. Adiponectin (0.1 and 1 μg/ml) induces a significant increase in IL-6, MCP-1, MMP-3, and MMP-9 production by murine ATDC5 cells in a dose-dependent manner (panels A–F). This effect is strongly decreased by the PI3-K inhibitor LY294002 (10 μM) for IL-6, and MMP-9 (panels A and F). LY294002 partially attenuated adiponectin-induced MCP-1 (B). Adiponectin is unable to modulate IL-1β, MMP-2 levels (panels C and D).

By contrast, our study demonstrates, for the first time, that adiponectin might also exert significant pro-inflammatory effects in chondrocytes by inducing the expression of NOS2 and also by strongly stimulating IL-6, MMP-3, MMP-9 and MCP-1 release.

NO, produced in great amounts by the NOS2 inducible synthase, controls a variety of cartilage functions, including loss of chondrocyte phenotype, chondrocyte apoptosis, and extracellular matrix degradation[26]. *In vitro*, human articular cartilage is able to produce large amounts of NO[27], which can be enhanced by pro-inflammatory cytokines. In addition, NO production can be significantly increased by the presence of leptin, as shown in our previous works[20–22]. The ability of chondrocytes to respond to adiponectin, by increasing specifically the expression and activity of NOS2, appears to be highly selective. Indeed, its induction is blocked by aminoguanidine, a well known NOS2 activity inhibitor, and also by dexamethasone (an inhibitor of NOS2 *de novo* synthesis). Our data regarding adiponectin-induced factors production by chondrocytes are partially in agreement with data published by Chen *et al.*, at least for those regarding MCP-1 and TIMP-1[16]. In contrast to data reported by these authors, adiponectin, in our hands, was able to strongly induce IL-6 and MMP-3. This discrepancy might be due to the different adiponectin doses used in our experimental set (0.1 and 1 μg/ml, which are in the physiological range of adiponectin levels), in comparison to the dose used by Chen *et al.* (30 μg/ml). Another possibility might be represented by the different technologies used in the measurement of these parameters.

The ability to respond to adiponectin is not restricted to chondrocytes. Indeed, other cell types such as adipocytes, synovial fibroblasts, and normal fibroblasts exhibit the potential to secrete IL-6 upon stimulation with adiponectin *in vitro*[18,19]. To note, all the above mentioned cells are of mesenchymal origin. Thus, it is conceivable that the ability to mount a pro-inflammatory response when stimulated by adiponectin could be a common characteristic of mesenchymal-derived cells.

To address the question which molecular mechanism might mediate the induction of NOS2 and cytokines production in response to adiponectin, we examined a key pathway of intracellular signaling. Actually, previous studies addressing intracellular effects of adiponectin in energy substrate metabolism, as well as in cardiovascular diseases, revealed that one of the intermediates upon adiponectin binding to its respective receptors is the PI3-K[28,29]. To note, PI3-K has been previously described as a relevant intracellular signaling molecule involved also in the NOS2 induction in chondrocytes elicited by several pro-inflammatory cytokines including leptin[5]. Consistent with this hypothesis, we demonstrated that PI3-K pharmacological blockade by LY294002 resulted in a marked reduction of adiponectin-induced NOS2 expression, as well as in a decrease of adiponectin-induced IL-6 and MMP-9 production. Intriguingly, LY294002 was able to partially attenuate adiponectin-induced MCP-1 and MMP-3, suggesting that other alternate and/or convergent signaling pathways, rather than PI3-K, are at play. Finally, LY294002 was able to strongly decrease basal MMP-2 production in chondrocytes, *per se*. Very recently, it has been reported that LY294002 significantly reduces adiponectin-induced nuclear factor kappa B (NFκB) activity in monocytic cells U937 where adiponectin provoked, among other actions, the induction of MCP-1[30].

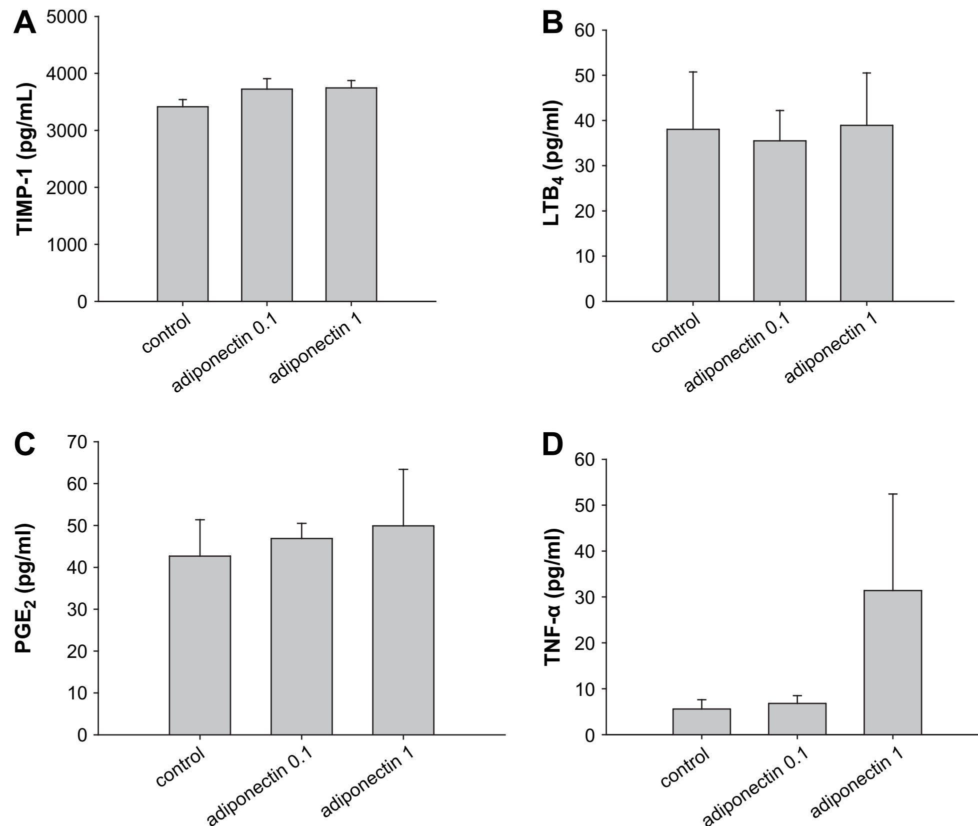

Fig. 5. Adiponectin does not modulate prostanoids, TIMP-1 or TNF-α. Adiponectin (0.1 and 1 μg/ml) does not induce any significant modulation in TIMP-1 (A), LTB4 (B), PGE_2 (C) and TNF-α (D).

Finally, recent data by Tang *et al.*, indicated that adiponectin-induced IL-6 production in synoviocytes occurred *via* p38 and NFκB activation[19]. To note, the activation of this nuclear transcription factor is a key element for the triggering of NOS2 in several cell lineages[31].

In conclusion, these data show for the first time that adiponectin is a frank modulator of the inflammatory response in chondrocytes. Upon these cells, adiponectin is able to induce key mediators of cartilage degeneration such as NO, IL-6, MCP-1 and metalloproteinases such as MMP-3 and MMP-9, whose induction appears to be modulated by at least one limited regulating mechanism represented by PI3-K.

Conflict of interest

The authors have no conflict of interest.

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