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Suppression of Fibroblast Metalloproteinases by Ajulemic Acid, a Nonpsychoactive Cannabinoid Acid

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Abstract Production of matrix metalloproteinases (MMP) in joint tissue of patients with inflammatory arthritis facilitates cartilage degradation and bone erosion, and leads to joint deformities and crippling. Thus, MMPs are important targets for agents designed to treat inflammatory arthritis. Oral administration of ajulemic acid (AjA), a synthetic, nonpsychoactive cannabinoid acid, prevents joint tissue injury in rats with adjuvant arthritis. AjA binds to and activates PPARγ directly. Therefore, we investigated the influence of AjA on MMP production in human fibroblast-like synovial cells (FLS), and examined the role of PPARγ in the mechanism of action of AjA. FLS, treated or not with a PPARγ antagonist, were treated with AjA then stimulated with TNFα or IL-1α. Release of MMPs-1, 3, and 9 was measured by ELISA. The influence of AjA on MMP-3 release from stimulated PPARγ positive ($PPAR^{+/-}$) and PPARγ null ($PPAR^{-/-}$) mouse embryonic fibroblasts (MEF) was also examined. Addition of AjA to FLS suppressed production of MMPs whether or not PPARγ activation was blocked. Secretion of MMP-3 was also suppressed by AjA in both TNFα- and IL-1α-stimulated $PPAR\gamma^{+/-}$ and $PPAR\gamma^{-/-}$ MEF. Suppression of MMP secretion from FLS by AjA appears to be PPARγ independent. Prevention by AjA of joint tissue injury and crippling in the rat adjuvant arthritis model may be explained in large part by inhibition of MMPs. These results suggest that AjA may be useful for treatment of patients with rheumatoid arthritis and osteoarthritis. J. Cell. Biochem. 100: 184–190, 2007.

Key words: inflammation; metalloproteinases; cannabinoids

Fibroblast-like synovial cells (FLS), the stromal cells of the joint capsule, are important to joint structure and lubrication, and participate in immune/inflammatory responses. In patients with chronic inflammatory arthritis, FLS express a hyperplastic, inflammatory, cartilage- and bone-destructive phenotype which includes secretion of cytokines and matrix metalloproteinases (MMP) [Aupperle et al., 1998]. It is the production of MMP in joint tissue of patients with inflammatory arthritis that facilitates cartilage degradation and bone erosion, and leads to joint deformities and crippling [Brinckerhoff and Matrisian, 2002]. Among the several MMPs, stromelysin-1 (MMP-3) has the broadest substrate specificity, and it can also activate other MMPs. MMP-3 normally is not expressed to an appreciable extent, but it is induced by cytokines, including IL-1 and TNFα [Nagase and Woessner, 1999]. Expression of MMP-9 (92 kD gelatinase) in joint cartilage from patients with osteoarthritis suggests that MMP-9 is involved in progressive articular cartilage degradation in these patients [Mohtai et al., 1993], and elevated serum levels of MMP-9 are associated with a high risk for coronary artery events [Ferroni et al., 2003]. MMP-1 is detected in synovium shortly after onset of symptoms in patients with inflammatory arthritis [Cunnane et al., 1999], and it is abundant at the bone-implant interface of failed prosthetic joints [Pap et al., 1999].

The Cannabis plant has been a source of medicinal preparations since the earliest

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written records on pharmacobotany [Abel, 1980]. A major obstacle to broad acceptance of cannabinoids as therapeutic agents is their potent psychoactive effects. A class of cannabinoids, the carboxyl tetrahydrocannabinols, which are metabolites of tetrahydrocannabinol (THC), shows promise as therapeutic agents that are free of cannabimimetic central nervous system activity [Burstein, 1999]. These compounds, called cannabinoid acids, include all the carboxylic acid metabolites of the cannabinoids and their synthetic analogs. One analog, 1′1′-dimethylheptyl-THC-11-oic acid, termed ajulemic acid (AjA), is a potent antiinflammatory and analgesic agent in several animal models [Burstein, 1999; Dajani et al., 1999; Zurier et al., 2003]. In addition, AjA is not psychoactive in mice. In fact, AjA suppresses THC-induced catalepsy in mice [Burstein, 1999]. Moreover, administration of 80 mg/day AjA for 1 week to patients with neuropathic pain relieved symptoms and did not induce behavioral changes [Karst et al., 2003].

Oral administration of AjA to rats with adjuvant arthritis prevents joint tissue injury in this animal model [Zurier et al., 1998]. AjA binds to and activates the nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) in vitro [Liu et al., 2003]. Therefore, we investigated the influence of AjA on release of MMPs from stimulated human FLS and mouse embryonic fibroblasts (MEF), and we examined the role of PPARγ in the mechanism of action of AjA.

MATERIALS AND METHODS

Reagents

AjA was obtained from Organix (Woburn, MA). Its purity was monitored on high-pressure liquid chromatography by comparison with material synthesized previously [Burstein et al., 1992]. The sample was 97% chemically pure, and was >99% chirally pure in the R, R enantiomer. AjA was dissolved in DMSO, then diluted with minimal essential medium (MEM) 2% fetal bovine serum (FBS) to achieve appropriate concentrations. The concentration of DMSO was kept constant at 0.3%. Recombinant human TNFα and IL-1α (R&D Systems, Minneapolis, MN) was prepared in MEM/2% FBS (Gibco). Vehicle control was MEM/2% FBS with 0.3% DMSO. GW 9662 and troglitazone were from Biomol Laboratories, Inc.

Cell Culture

Human FLS were isolated from synovial fluid as we have described [Stebulis et al., 2005]. Synovial fluid was aspirated from joints of patients with rheumatoid arthritis (RA), psoriatic arthritis, or osteoarthritis (OA). Fluid was collected in heparinized syringes, then centrifuged at 1,200 rpm for 15 min. The resulting cell pellet was resuspended in 7 ml of growth medium (MEM with 15% heat-inactivated FBS, 1% nonessential amino acids, 1% penicillin/streptomycin solution) and plated in 25-ml tissue culture flasks. Cultures were incubated at 37°C with 5% CO_2 for 24–48 h, after which medium was aspirated and cultures were washed with phosphate buffered saline (PBS) to remove nonadherent cells. Growth medium was replaced every 3 to 4 days. After 10 to 14 days adherent cells were removed from flasks by trypsinization, washed, and transferred to 6 well tissue culture plates in fresh growth medium. FLS were passaged (split 1:3) when they reached confluence, generally at 10 to 14 days. Passages 2 through 6 were used for experiments. Experiments were done on FLS rested overnight in low serum medium (MEM/2% FBS). Medium was replaced before beginning the experiments.

$PPAR^{+/-}$ (wild-type) and $PPAR^{-/-}$ (null) mouse embryonic fibroblasts (MEF, provided by Dr. Bruce Spiegelman, Dana Farber Cancer Institute, Boston, MA) were maintained in 10% FBS. Cells were grown to confluence in 6-well culture plates and cells were rested in low-serum medium (MEM with 0.5% FBS) for 18–24 h before an experiment. Cells (FLS or MEF) were exposed to AjA for 60 min, then stimulated 18–24 h with 10 ng/ml rhIL-1α or rhTNFα.

MMP Assays

Supernatants were collected and analyzed for MMPs by ELISA (R&D). Standards and diluted samples were incubated for 2 h at room temperature and then washed. Samples were then incubated with conjugate for 2 h at room temperature, then washed. Substrate solution was then added to the samples for 30 min. Samples were then read on a microplate reader at 450 nm with background correction at 540 nm. Conditions were run in triplicate, and experiments were repeated at least three times.

Viability of Cells

The integrity of the FLS at the end of experiments was assessed by exclusion of trypan blue. Cells were diluted in 0.5% trypan blue. Nonviable cells lost their ability to exclude trypan blue, and stained blue. In no instance did the proportion of nonviable cells exceed 5%.

Statistical Analysis

Data were analyzed and compared by paired Student's test or by Student's *t*-test difference of means of independent groups.

RESULTS

Release of MMPs From FLS

Release of MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (92 kD gelatinase) from FLS stimulated with TNFα in vitro was suppressed by AjA in a dose-dependent manner (Fig. 1). Results in Figure 1 are from one representative experiment. In a series of three experiments MMP-3 secretion was reduced from 37.5 ± 2.5 ng/ml to 4.5 ± 2.5 ng/ml (mean $\pm$ SD) by 10 μm AjA ($P = 0.002$ vs. untreated control). Although MMP-1 and MMP-9 release were also reduced significantly by AjA, total amounts of both metalloproteinases released were more variable than for MMP-3, and were not as sensitive to reduction by AjA (Table I).

We then tested the hypothesis of PPARγ dependence in human FLS exposed to the irreversible PPARγ antagonist GW9662. AjA suppressed MMP-3 release from IL-1α- and TNFα-stimulated cells whether or not PPARγ activity was blocked by GW9662 (Fig. 2).

In a separate experiment, the known PPARγ activator troglitazone [Zingarelli and Cook, 2005] did not suppress MMP-3 release (Table II), and suppression of MMP-3 release by AjA was not blocked by GW9662. The results suggest that the action of AjA on MMP-3 secretion from human FLS is PPARγ independent.

Release of MMP-3 From Mouse Embryonic Fibroblasts (MEF)

To further investigate PPARγ independence, we studied MEF. We observed (Fig. 3) that AjA reduced secretion of MMP-3 from TNFα- and IL-1α-stimulated MEF in which PPARγ was intact ($PPAR^{+/-}$) and from the same cells which do not have active PPARγ ($PPAR^{-/-}$). The results suggest that the influence of AjA on MMP-3 release from the MEF was also PPARγ independent. We did not anticipate that MMP production in these cells would be so dependent on PPARγ (Fig. 3).

DISCUSSION

Cartilage degradation and bone erosion are major features in joints of patients with rheumatoid arthritis (RA). Although many mediators of inflammation contribute to joint tissue injury in RA patients, the final insult to the integrity of the joint is due to the actions of MMPs secreted from synovial cells and chondrocytes [Yamanishi and Firestein, 2001]. These zinc-dependent endopeptidases have the capacity to destroy the extracellular matrix of cartilage and bone [Murphy et al., 2002] and are therefore also integral to the joint destruction seen in patients with osteoarthritis. Although several cell types migrate to the synovial lining in RA patients, studies in animals and humans suggest that joint damage can proceed with participation of synovial cells alone [Muller-Ladner et al., 1995].

We have shown that oral administration of AjA at a dose of 0.1 mg/kg three times weekly reduces significantly the severity of adjuvant induced polyarthritis in rats, although periarticular inflammation did occur in treated rats [Zurier et al., 1998]. Histomorphological evaluation of the joints indicates that synovial inflammation also occurred in AjA-treated animals, but that it did not progress to cartilage degradation, bone erosion, and distortion of joint architecture, as was observed in rats given placebo. These findings suggested that the final common pathway of joint tissue injury—activation of MMPs—might be blocked.

Results of experiments presented here indicate that the antiinflammatory, nonpsychoactive, synthetic cannabinoid acid AjA does in fact suppress secretion of several MMPs from activated human FLS. Experiments done with mouse embryonic fibroblasts lacking PPARγ activity, and with FLS in which PPARγ activity is blocked, indicate that suppression of MMPs by AjA does not depend on PPARγ activation. In addition, the known selective PPARγ agonist, troglitazone, did not suppress MMP release.

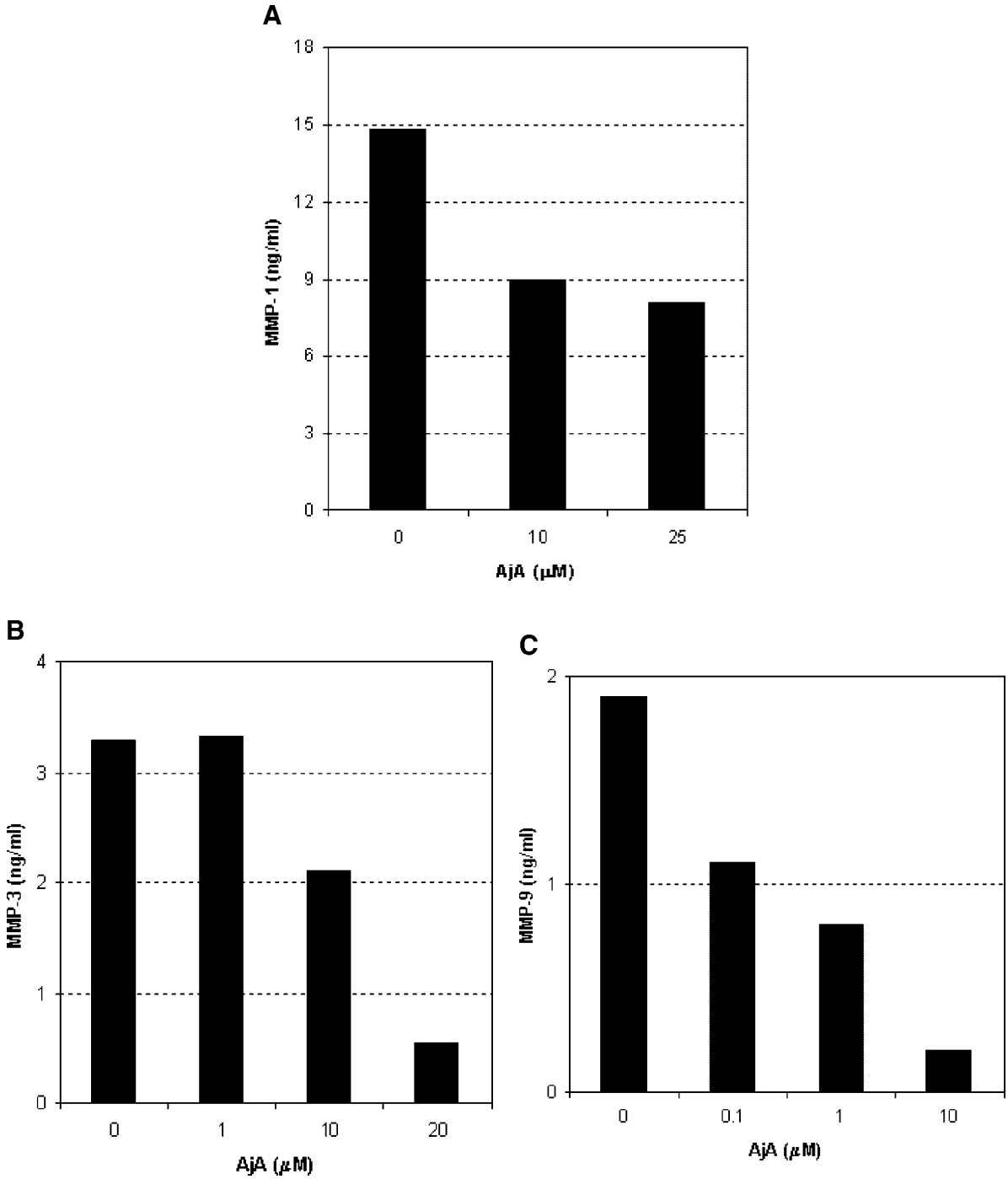

Fig. 1. Influence of AjA on TNFα-stimulated secretion of MMPs from human FLS. Cells were treated with AjA (0.1–30 μM) for 1 h, then stimulated with 10 ng/ml TNFα for 18 h. MMP secreted into the supernatant was measured by ELISA. Individual samples were assayed in duplicate, and each experiment was run in triplicate. Data are from one representative experiment. **A**: MMP-1 (collagenase); (**B**) MMP-3 (stromelysin-1); (**C**) MMP-9 (gelatinase).

Other PPARγ ligands such as prostaglandin J2 (PGJ_2) can also affect cell function in a PPARγ-independent manner [Scher and Pillinger, 2005].

We have shown [Liu et al., 2003] that AjA activates PPARγ in HEK293 cells, and we have observed the same effect of AjA in human FLS (unpublished). PPARs were first cloned as

TABLE I. Reduction by AjA of MMP Release From Human FLS Stimulated With TNFα*

Enzyme	Percent reduction		*P* versus untreated control cells
	Mean	SD	
MMP-1	53.2	36.3	0.035
MMP-3	88.0	2.5	0.002
MMP-9	52.8	24.3	0.004

*Cells stimulated for 18 h with 10 ng/ml TNFα after 1-h exposure to 10 μM AjA.
Supernatant MMP measured by ELISA. n = 4 for MMPs 1 and 9; n = 3 for MMP-3.

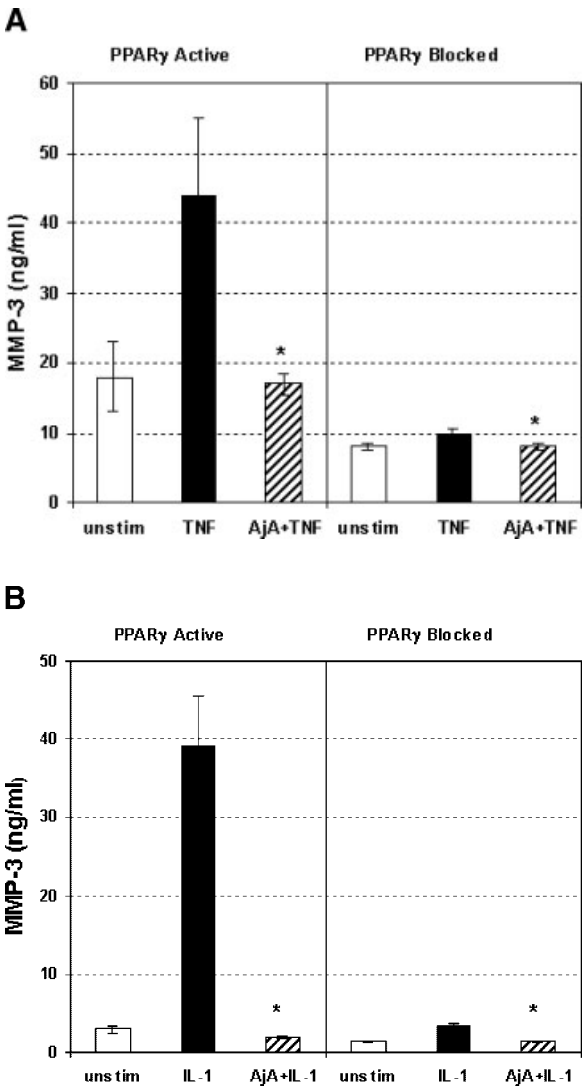

Fig. 2. Influence of PPARγ on MMP-3 release from AjA treated, TNFα- and IL-1α-stimulated human FLS. PPARγ blocked by incubation of cells with 10 μM of the PPARγ antagonist GW9662 60 min before treatment with 10 μM AjA. Data expressed as mean ± SD of three experiments. *$P < 0.01$ versus untreated control. **A**: Cells stimulated with 10 ng/ml TNFα. **B**: Cells stimulated with 10 ng/ml IL-1α.

TABLE II. Influence of PPARγ on MMP-3 Release From Human FLS Stimulated With TNFα*

Condition	MMP-3 (ng/mL)
Untreated control	1,998
Troglitazone (10 μM)	2,047
AjA (10 μM)	720
AjA + GW9662 (10 μM)	651

*Cells treated for 1 h with each agent before stimulation with 10 ng/ml TNFα for 18 h.
Supernatant MMP-3 measured by ELISA. Mean baseline MMP-3 = 493 ng/ml.

nuclear receptors that mediate the effects on gene transcription of synthetic compounds called peroxisome proliferators. Interest in PPARs increased dramatically when they were shown to be activated by medically relevant compounds including nonsteroidal antiinflammatory drugs [Lehmann et al., 1997]. Upregulation of PPARγ reduces expression of several mediators of inflammation [Jiang et al., 1998] including MMP-1 in FLS stimulated with IL-1β [Fahmi et al., 2002a]. However, responses of cells to PPAR ligands can be due to activation of PPAR or can be PPAR independent [Fahmi et al., 2002b] actions which appear to be cell and stimulus, and perhaps ligand specific. It is not unlikely that PPARγ activation by AjA contributes to the therapeutic effect of the cannabinoid. However, it appears from the studies presented here that the anti-MMP action of AjA exhibited in vitro is not due to PPARγ activation.

Of course, it is difficult to correlate results of experiments done in vitro with studies done in vivo with animal models. However, the evidence from animal studies [Zurier et al., 1998] and experiments done in vitro which indicate that AjA suppresses monocyte IL-1β production and enhances T cell apoptosis [Bidinger et al., 2003; Zurier et al., 2003], and results presented here suggest that AjA may have value for the treatment of RA and osteoarthritis. Successful therapy of joint destructive arthritis will require modification of several aspects of host defense responses with agents that can be given safely for long periods of time. Cannabinoid acids meet those criteria.

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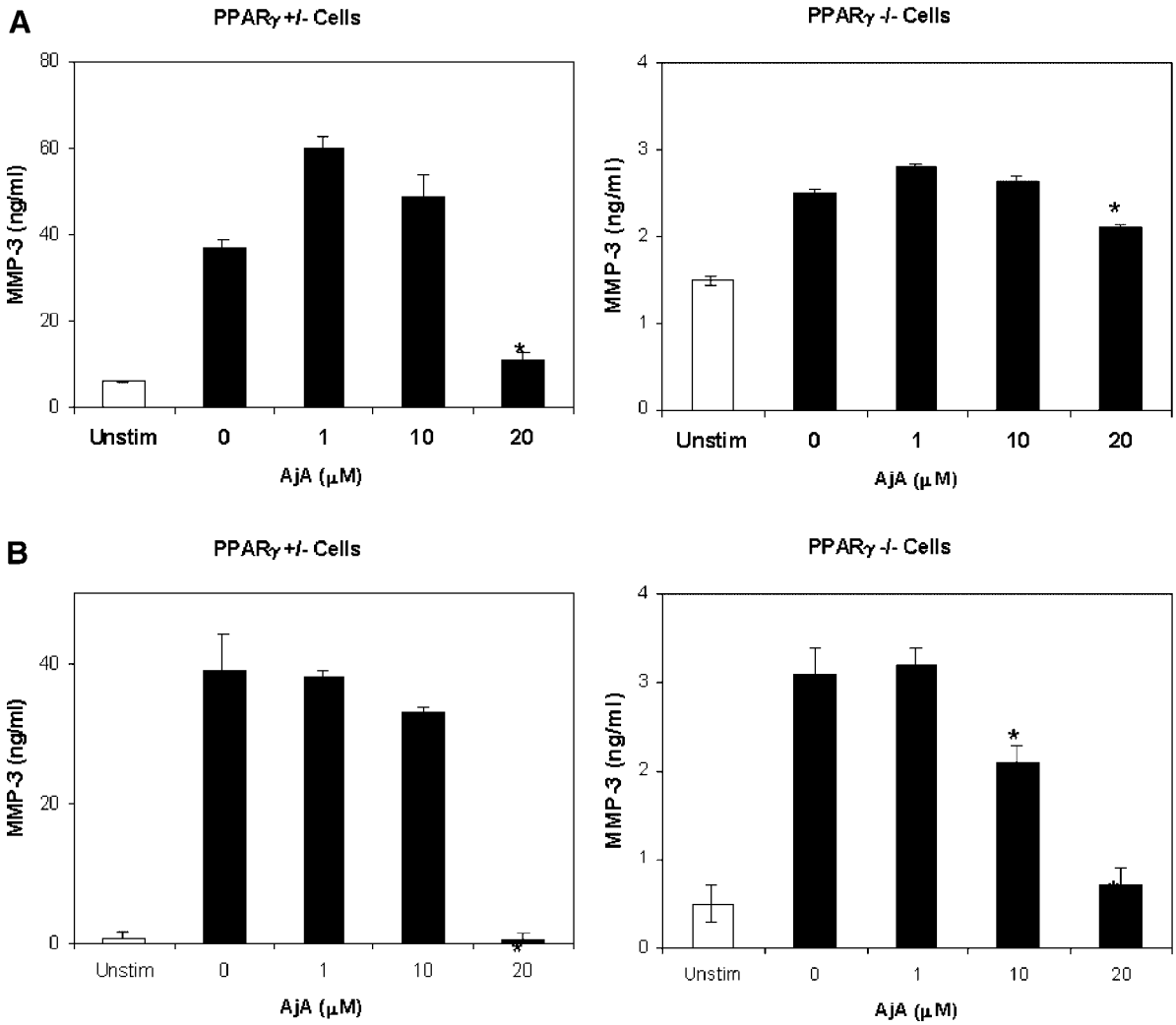

Fig. 3. Influence of PPARγ on MMP-3 release from AjA treated, TNFα- and IL-1α-stimulated wild-type ($PPAR\gamma^{+/-}$) and PPARγ null ($PPAR\gamma^{-/-}$) mouse embryonic fibroblasts (MEF). Cells were treated with AjA for 1 h, then stimulated 18 h with 10 ng/ml TNFα or IL-1α. Data expressed as mean ± SD of three experiments. *$P < 0.005$ versus untreated control. **A**: $PPAR\gamma^{+/-}$ and $PPAR^{-/-}$ cells stimulated with TNFα. **B**: $PPAR\gamma^{+/-}$ and $PPAR^{-/-}$ cells stimulated with IL-1α.

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