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15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 Inhibits Bay 11-7085-induced Sustained Extracellular Signal-regulated Kinase Phosphorylation and Apoptosis in Human Articular Chondrocytes and Synovial Fibroblasts*

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We have previously shown that nuclear factor-κB inhibition by adenovirus expressing mutated IκB-α or by proteasome inhibitor increases human articular chondrocytes sensibility to apoptosis. Moreover, the nuclear factor-κB inhibitor BAY11-7085, a potent anti-inflammatory drug in rat adjuvant arthritis, is itself a proapoptotic agent for chondrocytes. In this work, we show that BAY 11-7085 but not the proteasome inhibitor MG-132 induced a rapid and sustained phosphorylation of extracellular signal-regulated kinases (ERK1/2) in human articular chondrocytes. The level of ERK1/2 phosphorylation correlated with BAY 11-7085 concentration and chondrocyte apoptosis. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) and its precursor prostaglandin (PG) D2 but not PGE2 and PGF2α rescued chondrocytes from BAY 11-7085-induced apoptosis. 15d-PGJ2 markedly inhibited BAY 11-7085-induced phosphorylation of ERK1/2. BAY 11-7085 also induced ERK1/2 phosphorylation and apoptosis in human synovial fibroblasts, and these reactions were down-regulated by 15d-PGJ2. Further analysis in synovial fibroblasts showed that only molecules that suppressed BAY 11-7085-induced phosphorylation of ERK1/2 (*i.e.* 15d-PGJ2, PGD2, and to a lesser extent, MEK1/2 inhibitor UO126, but not prostaglandins E2 and F2α or peroxisome proliferator-activated receptor-γ agonist ciglitazone) were able protect cells from apoptosis. These results suggested that the antiapoptotic effect of 15d-PGJ2 on chondrocytes and synovial fibroblasts might involve inhibition of ERK1/2 phosphorylation.

Joint diseases such as osteoarthritis (OA)[1] and rheumatoid arthritis lead to chondrocyte cell death and irreversible joint damage. Synovial tissue proliferation is one of the major detrimental events in rheumatoid disease. Cartilage is a highly specialized tissue that relies on only one cell type, the chondrocyte, and has very limited ability to regenerate (1). Chondrocyte survival is thus crucial for the preservation of cartilage and joint function (2).

Many biological functions of the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α are mediated by nuclear factor-κB (NF-κB) or mitogen-activated protein kinases, and deregulation of both pathways is involved in the pathogenesis of rheumatoid arthritis and OA (3). Because NF-κB controls the expression of pro-inflammatory molecules, such as cytokines, chemokines, nitric oxide, cyclo-oxygenase 2, metalloproteinases, and adhesion molecules (4), its inhibition by the super-repressor IκB, by NF-κB decoys (5), or by BAY11-7085 (6) is effective as treatment in animal model of experimentally induced arthritis. NF-κB activity, however, also has a protective role against apoptosis of primary (7) and dedifferentiated (8) chondrocytes. Although the proteasome and NF-κB inhibitor MG-132 merely sensitized chondrocytes to induced apoptosis, NF-κB inhibitor BAY 11-7085 was able to cause chondrocyte apoptosis directly (7). We thus searched for additional pathways triggered by BAY 11-7085 and for an anti-inflammatory molecule able to protect chondrocytes from BAY 11-7085-induced apoptosis.

In this study, we showed that, in human primary articular chondrocytes, BAY 11-7085 but not MG-132 induced a sustained ERK1/2 phosphorylation. Furthermore, we showed that BAY 11-7085-induced ERK1/2 phosphorylation as well as BAY 11-7085-induced chondrocyte apoptosis can be markedly inhibited by pretreatment with 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2). However, we also showed that 15d-PGJ2 has a pronounced antiapoptotic effect on synovial fibroblasts, suggesting that 15d-PGJ2 could also have pro-inflammatory effects on the cells from human joint.

EXPERIMENTAL PROCEDURES

Chondrocyte and Synovial Fibroblast Isolation—Human cartilage was obtained post-mortem from patients who had not been admitted to the hospital for joint diseases (used for experiments presented in Figs. 1–3), and from OA patients during joint replacement (used for experiments presented in Fig. 4). Cartilage was cut in 1–2-mm^3 explants and digested at 37 °C with gentle agitation with, in succession, 0.5 mg/ml hyaluronidase (Sigma-Aldrich) for 30 min, 1 mg/ml pronase (Merck KGaA) for 1 h, and 0.8 mg/ml collagenase IA (Sigma-Aldrich) for 16–20 h. After digestion, cells were filtered through 70 μM nylon membrane (Falcon; BD Biosciences), washed and seeded in Dulbecco's modified Eagle's medium (DMEM) lacking phenol red (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and supplemented with 10% fetal calf serum (FCS). Only primary chondrocytes were used (*i.e.* all experiments began on the first or second day after chondrocyte isolation and continued for a maximum of 48 h).

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[1] The abbreviations used are: OA, osteoarthritis; NF-κB, nuclear factor-κB; ERK1/2, extracellular signal-regulated kinases; 15d-PGJ2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PG, prostaglandin; MEK1/2, mitogen-activated protein kinase-kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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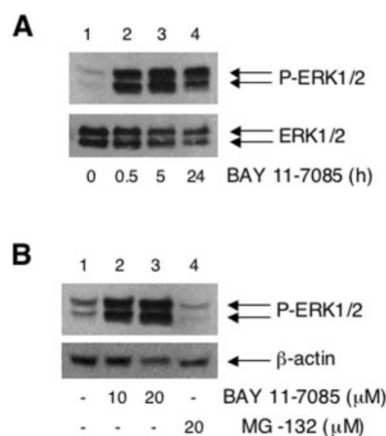

FIG. 1. **BAY 11-7085 induces ERK1/2 phosphorylation in human articular chondrocytes.** *A*, chondrocytes were treated with BAY 11-7085 (20 μM) for increasing times as indicated. *B*, chondrocytes were stimulated with BAY 11-7085 or MG-132 for 1 h at the indicated concentrations. The *arrows* indicate the expression of phospho-ERK1/2 (*P-ERK1/2*), ERK1/2, and β-actin as determined by Western blot analysis.

Synovial membranes were obtained from OA patients during joint replacement. For cell isolation, synovium was finely cut and digested 4 or 16 h with 0.5 or 1 mg/ml collagenase IA (Sigma-Aldrich), respectively. Synovial fibroblasts were filtered through 70 μM nylon membrane (Falcon), washed, and seeded in DMEM (Cambrex Bio Science Walkersville, Inc.) supplemented with 10% FCS. For experiments, synovial fibroblasts (passages 2–6) were seeded in DMEM supplemented with 1% FCS. Primary endothelial cells from human umbilical vein were purchased from Cambrex Bio Science Walkersville, Inc., and maintained in EGM2 medium (Cambrex Bio Science Walkersville, Inc.).

Cell Treatment—Isolated chondrocytes were cultured either in 96-well plates at the density of 5×10^4 cells/100 μl of DMEM supplemented with 10% FCS (cell surviving tests) or in 6-well plates at density of 10^6 cells/1.5 ml (protein extracts analysis). Synovial fibroblasts were cultured at the density of 10^4 cells/100 μl of DMEM supplemented with 1% FCS in 96-well plates or in 6-well plates at a density of 2.8×10^5 cells/1 ml (protein extracts analysis). Cells were pretreated with prostaglandin (PG) E2, PGF2α, PGD2, derivative 15d-PGJ2, peroxisome proliferator-activated receptor-γ agonist ciglitazone (BioMol, Plymouth Meeting, PA), or mitogen-activated protein kinase-kinase (MEK1/2)-specific inhibitor UO126 (Cell Signaling, Beverly, MA). Apoptosis was induced by NF-κB inhibitor BAY 11-7085 (Alexis Corporation, San Diego, CA) (6, 7), which was added to the cells for an additional 6–24 h. In some experiments, the proteasome and NF-κB inhibitor MG-132 (Alexis Corporation) was used.

Survival Assay—Cell survival was measured as mitochondrial NADH/NADPH-dependent dehydrogenase activity, resulting in the cellular conversion of methyltetrazolium salt MTS (Promega, Madison, WI) into a soluble formazan dye (9). An electron-coupling agent, phenazine methosulfate, was obtained from Sigma-Aldrich. Colorimetric measurement of formazan dye was performed at 490 nm.

Annexin V Labeling—Chondrocytes were seeded in 8-well chamber slides (Nalge Nunc International, Naperville, IL) and pretreated with 15d-PGJ2 for 24 h. BAY 11-7085 was then added for an additional 24 h. Cell supernatant was carefully discarded and chondrocytes labeled with annexin V-FITC using Annexin V fluos staining kit (Roche, Mannheim, Germany). Cell images were captured under fluorescence microscope.

Western Blotting—Cells were collected at 4 °C and lysed for 30 min on ice in 100 μl of buffer (25 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol) containing phosphatase inhibitors (1 mM Na_3VO_4, 25 mM β-glycerophosphate, and 1 mM NaF). Total proteins (20 μg) were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA). Phosphorylated ERK1/2, ERK1/2, and caspase-8 were detected with mouse monoclonal antibodies that recognize phosphorylated Tyr-204 (E4), rabbit polyclonal (K-23) antibodies (Santa Cruz Biotechnology), and rabbit polyclonal antibodies (BD Pharmingen), respectively, diluted 1:1000, in Tris-buffered saline-Tween 20 supplemented with 10% milk powder. β-Actin was detected with mouse monoclonal antibody (Sigma-Aldrich), diluted 1:1000 in Tris-buffered saline-Tween 20. Poly(ADP-ribose) polymerase was detected with mouse monoclonal antibody (BD Pharmingen) and diluted 1:1000 in Tris-buffered saline-Tween 20. In-

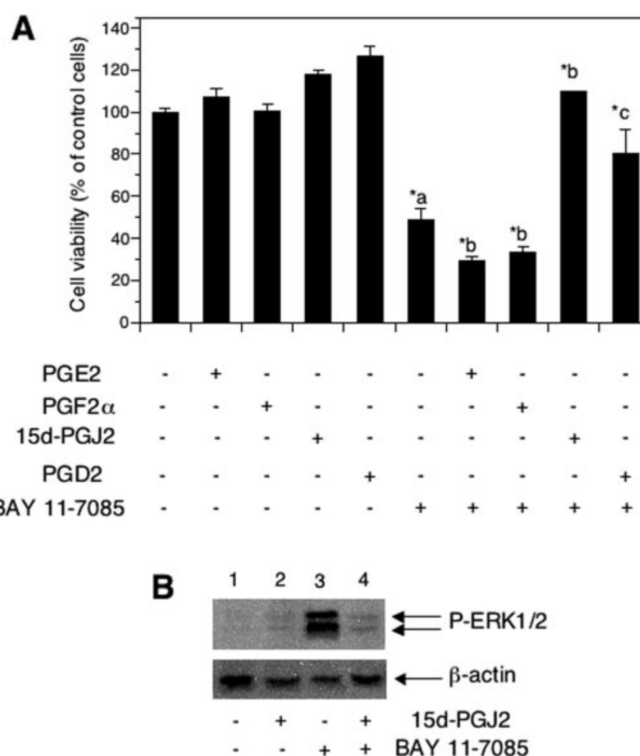

FIG. 2. **15d-PGJ2 inhibits BAY 11-7085-induced chondrocyte apoptosis and ERK1/2 phosphorylation.** *A*, Chondrocytes were pretreated with PGE2, PGF2, 15d-PGJ2, or PGD2 (20 μM) for 24 h. BAY 11-7085 (20 μM) was then added for an additional 24 h. Cell survival was estimated by an MTS test, and results were expressed as a percentage of surviving cells compared with control non-treated cells (100%). **a*, statistically different from the non-treated control ($p < 0.05$). **b*, statistically different from BAY 11-7085-treated cells ($p < 0.05$). **c*, statistically different from BAY 11-7085-treated cells in the presence of 15d-PGJ2 ($p < 0.05$). *B*, chondrocytes were pre-treated with 15d-PGJ2 (20 μM) for 24 h and BAY 11-7085 was then added for an additional 24 h. The *arrows* indicate the expression of phosphorylated ERK1/2 (*P-ERK1/2*) and β-actin as determined by Western blot analysis.

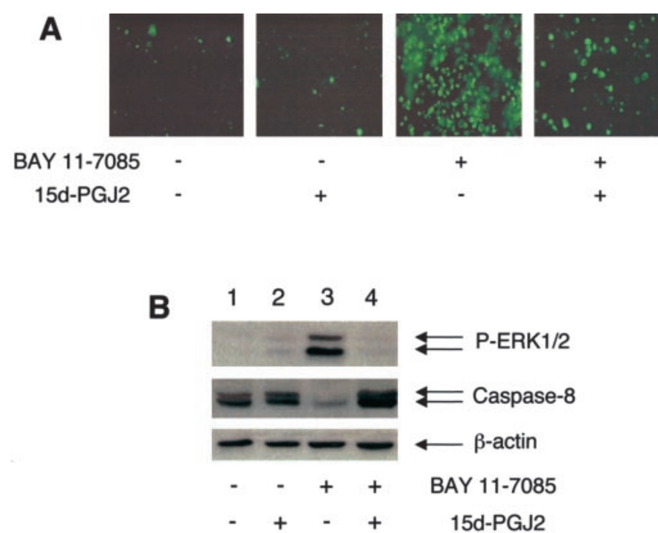

FIG. 3. **15d-PGJ2 inhibits BAY 11-7085-induced annexin V-fluorescein isothiocyanate binding and caspase-8 cleavage in human chondrocytes.** Chondrocytes were pretreated or not with 15d-PGJ2 (20 μM) and then exposed to BAY 11-7085 (20 μM) for an additional 24 h. *A*, annexin V-fluorescein isothiocyanate labeling was performed, and fluorescein isothiocyanate was detected by fluorescence microscope. *B*, Western blot of total chondrocyte extracts. The *arrows* indicate expression of phosphorylated ERK1/2 (*P-ERK1/2*), caspase-8 isoforms (10), and β-actin as determined by Western blot analysis.

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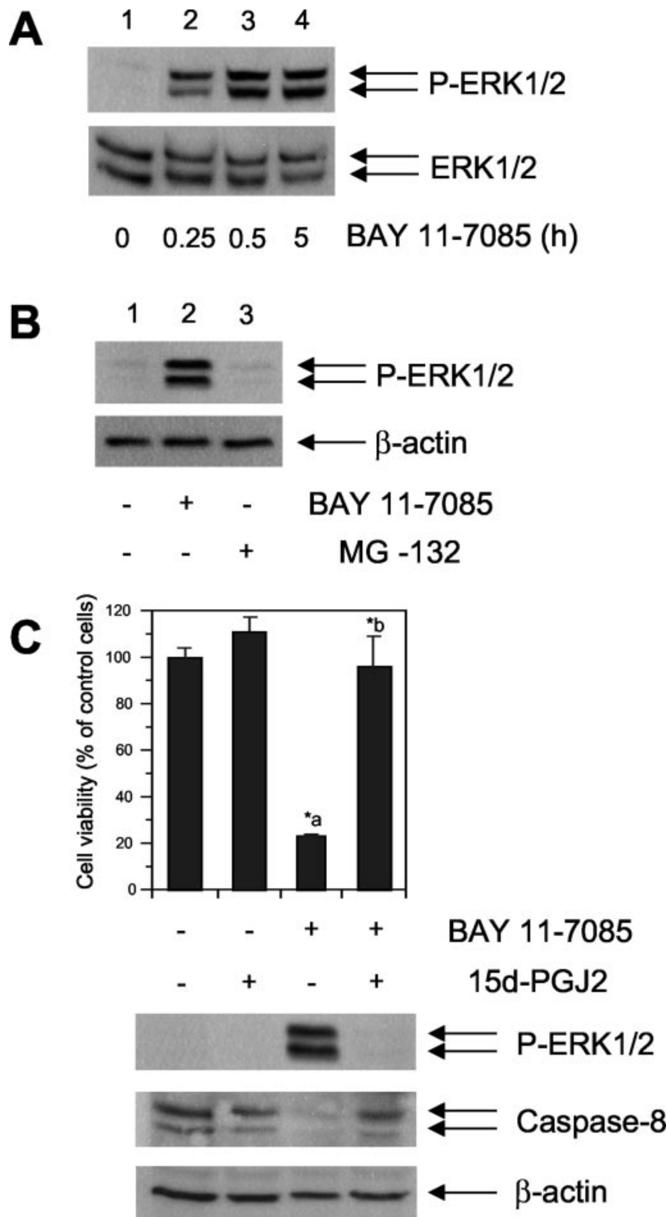

FIG. 4. **15d-PGJ2 inhibits BAY 117085-induced ERK1/2 phosphorylation and apoptosis in OA chondrocytes.** Chondrocytes were isolated from OA cartilage. *A*, OA chondrocytes were stimulated with BAY 11-7085 (20 μM) for the times indicated. The *arrows* indicate the expression of phospho-ERK1/2 (*P-ERK1/2*), and ERK1/2 as determined by Western blot analysis. *B*, OA chondrocytes were stimulated with BAY 11-7085 (20 μM) or MG-132 (20 μM) for 1 h. The *arrows* indicate the expression of phospho-ERK1/2, and β-actin, determined by Western blot analysis. *C*, OA chondrocytes were pretreated with 15d-PGJ2 (20 μM) for 24 h. BAY 11-7085 (20 μM) was then added for an additional 24 h. Cell survival (*top*) was estimated by an MTS test, and results were expressed as a percentage of surviving cells compared with control non-treated cells (100%). **a*, statistically different from the non-treated control ($p < 0.05$). **b*, statistically different from BAY 11-7085-treated cells ($p < 0.05$). The *arrows* indicate the expression of phospho-ERK1/2, caspase-8, and β-actin as determined by Western blot analysis.

cubation of membranes with primary antibodies was done at room temperature for 1–3 h. Western blots were revealed with 1:2000 diluted anti-mouse or anti-rabbit antibodies (DakoCytomation Denmark A/S, Glostrup, Denmark) and ECL chemiluminescent reagents (Amersham Biosciences).

Statistics—p values were obtained using the Mann-Whitney test and considered significant when lower than 0.05.

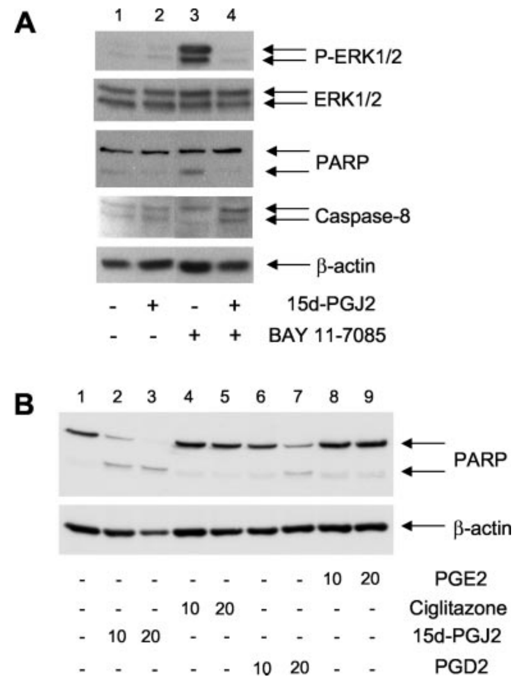

FIG. 5. **15d-PGJ2 inhibits BAY 117085-induced ERK1/2 phosphorylation and apoptosis in synovial fibroblasts.** Differential effect of 15d-PGJ2 on endothelial cells. *A*, synovial fibroblasts were pre-treated with 15d-PGJ2 (20 μM) for 24 h. BAY 11-7085 (20 μM) was then added for an additional 6 h. *B*, endothelial cells were treated with 15d-PGJ2, ciglitazone, PGD2, or PGE2 for 24 h at concentrations indicated. The *arrows* indicate the expression of phosphorylated ERK1/2 (*P-ERK1/2*), ERK1/2, poly(ADP-ribose) polymerase (*PARP*), caspase-8, and β-actin, as determined by Western blot analysis.

RESULTS

Bay 11-7085 but Not MG-132 Induces Sustained ERK1/2 Phosphorylation in Human Chondrocytes—We and others showed previously that inhibition of NF-κB by adenovirus-expressing mutated IκB-α or by proteasome inhibitor increases human chondrocyte sensitivity to apoptosis (7, 8). However, only BAY11-7085, not MG-132 or adenovirus-expressing mutated IκB-α, is itself a proapoptotic agent for chondrocytes. Thus, we searched for a signaling pathway distinct from NF-κB that was involved in BAY 11-7085-induced chondrocyte apoptosis. Results showed that in isolated human articular chondrocytes BAY 11-7085 (Fig. 1*A*), but not MG-132 (Fig. 1*B*), induced rapid and sustained ERK1/2 phosphorylation.

15d-PGJ2 Protects Chondrocytes from BAY 11-7085-induced Apoptosis and Suppresses BAY 11-7085-induced ERK1/2 Phosphorylation—We showed recently that tumor necrosis factor-α can protect chondrocytes from apoptosis in a cyclo-oxygenase-2-dependent fashion, suggesting that prostaglandins could exert an antiapoptotic action (7). Thus, we tested the effect of prostaglandins on chondrocyte survival in the presence of BAY 11-7085. Chondrocytes were pretreated with PGE2, PGF2α, PGD2, or 15d-PGJ2 for 24 h. Bay 11-7085 was then added for an additional 24 h. 15d-PGJ2 and PGD2 treatment significantly inhibited BAY 11-7085-induced apoptosis, whereas PGE2 and PGF2α did not have protective effect (Fig. 2*A*). It was interesting that pretreatment of chondrocytes with 15d-PGJ2 drastically inhibited Bay 11-7085-induced ERK1/2 phosphorylation (Fig. 2*B*), suggesting that suppression of ERK1/2 phosphorylation may rescue chondrocytes from Bay 11-7085-induced apoptosis. Moreover, 15d-PGJ2 markedly inhibited Bay 11-7085-induced annexin V-fluorescein isothiocyanate binding (Fig. 3*A*) and caspase-8 cleavage (Fig. 3*B*). Two isoforms of caspase-8 were detected in chondrocyte (Fig. 3*B*) and synovial fibroblast extracts (Fig. 5*A*) (10).

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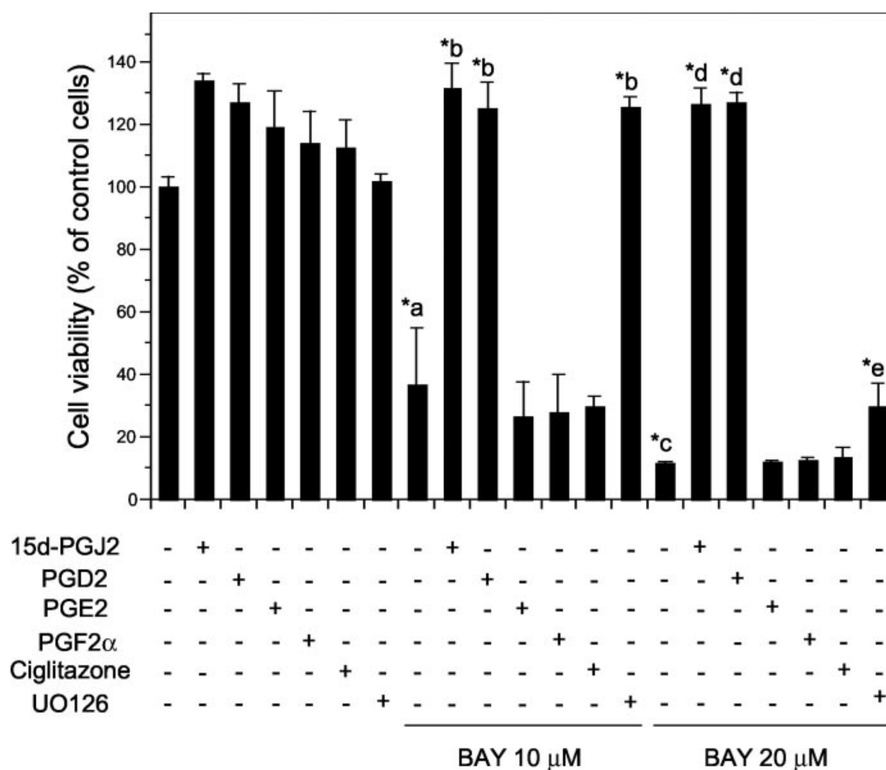

FIG. 6. **15d-PGJ2 inhibits BAY 11-7085-induced synovial fibroblast cell death.** Synovial fibroblasts were pre-treated with 15d-PGJ2, PGD2, PGE2, PGF2α, ciglitazone, or UO126 (20 μM) for 24 h. BAY 11-7085 (10 or 20 μM) was then added for additional 24 h. Cell survival was estimated by the MTS test, and results were expressed as a percentage of surviving cells compared with control non-treated cells (100%). **a* and **c*, statistically different from the non-treated control ($p < 0.05$). **b*, statistically different from BAY 11-7085 (10 μM) treated cells ($p < 0.05$). **d*, statistically different from BAY 11-7085 (20 μM) treated cells ($p < 0.05$). **e*, statistically different from BAY 11-7085 (20 μM) treated cells in the presence of 15d-PGJ2 ($p < 0.05$).

15d-PGJ2 Inhibits BAY 11-7085-induced ERK1/2 Phosphorylation and Apoptosis in Chondrocytes from Osteoarthritic Cartilage—To test further the positive effect of 15d-PGJ2 on chondrocyte survival, experiments were performed on chondrocytes isolated from cartilage of OA patients. As in chondrocytes isolated from non-osteoarthritic cartilage (Fig. 1), BAY 11-7085, but not MG-132, induced sustained ERK1/2 phosphorylation and apoptosis (Fig. 4, *A–C*). Furthermore, preincubation of osteoarthritic chondrocytes with 15d-PGJ2 inhibited both BAY 11-7085-induced apoptosis and ERK1/2 phosphorylation (Fig. 4*C*), and these effects were equivalent to the protective effects of 15-PGJ2 on non-osteoarthritic chondrocytes (Figs. 2 and 3*B*).

15d-PGJ2 Inhibits BAY 11-7085-induced ERK1/2 Phosphorylation and Apoptosis in Synovial Fibroblasts—We then tested the effect of prostaglandins on synovial fibroblasts and endothelial cells, two cell types whose proliferation have deleterious effects in joint rheumatoid disease. As in chondrocytes, Bay 11-7085-induced synovial fibroblast cell death was preceded with ERK1/2 phosphorylation that was down-regulated with 15d-PGJ2 (Fig. 5*A*). 15d-PGJ2 also down-regulated Bay 11-7085-induced poly(ADP-ribose) polymerase and caspase 8 cleavage, confirming its antiapoptotic effect. In contrast, 15d-PGJ2 caused apoptosis of endothelial cells (Fig. 5*B*), as previously reported (11). MTS tests showed that 15d-PGJ2, PGD2, and (only at lower BAY 11-7085 concentrations) UO126, but not PGE2 and PGF2α, markedly protected synovial fibroblasts from BAY 11-7085-induced cell death (Fig. 6). Ciglitazone showed no protective effect in chondrocytes (data not shown) or in synovial fibroblasts (Fig. 6), suggesting that 15d-PGJ2 effect may be peroxisome proliferator-activated receptor-γ-independent (11, 12). Moreover, Western blot showed that pretreatment with PGE2, PGF2α, or ciglitazone did not change BAY 11-7085-induced ERK1/2 phosphorylation, which was efficiently down-regulated with 15d-PGJ2 (Fig. 7*A*). Therefore, from the substances tested, only 15d-PGJ2 was able to prevent BAY 11-7085-induced caspase-8 cleavage (Fig. 7*A*). MEK1/2 inhibitor UO126 also down-regulated ERK1/2 phos-

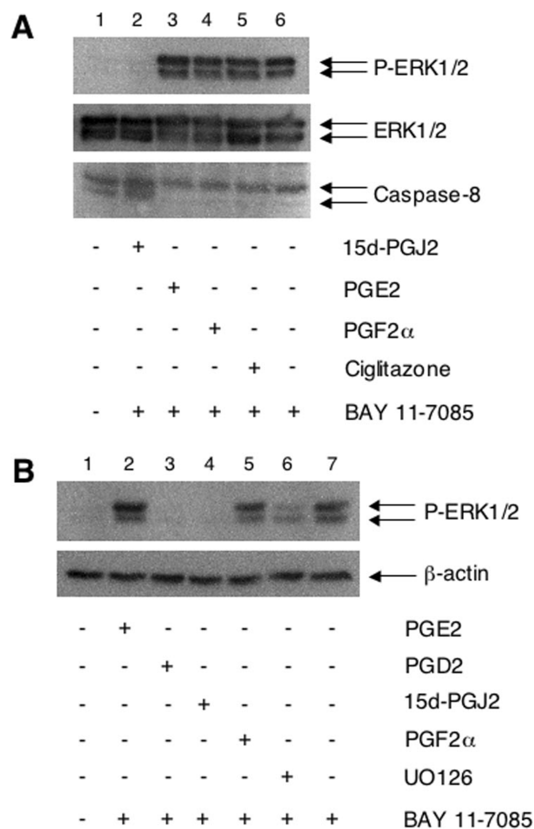

FIG. 7. **15d-PGJ2, PGD2, and MEK1/2 inhibitor, but not PGE2, PGF2α, or peroxisome proliferator-activated receptor-γ agonist, inhibit BAY 11-7085-induced ERK1/2 phosphorylation.** *A*, synovial fibroblasts were pre-treated with 15d-PGJ2, PGE2, PGF2α, or ciglitazone (20 μM) for 24 h. BAY 11-7085 was then added for an additional 24 h. *B*, synovial fibroblasts were pretreated with 15d-PGJ2, PGD2, PGF2α, PGE2, or UO126 (20 μM) for 24 h. BAY 11-7085 (20 μM) was then added for additional 8 h. The *arrows* indicate the expression of phosphorylated ERK1/2 (*P-ERK1/2*), ERK1/2, caspase-8, and β-actin, determined by Western blot analysis.

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phorylation; as in cell survival tests (Fig. 6), however, its effect was always less pronounced than that of15d-PGJ2 (Fig. 7*B*). These results suggested that sustained ERK1/2 phosphorylation might be necessary for BAY 11-7085-induced apoptosis.

DISCUSSION

We previously showed that the NF-κB inhibitor Bay 11-7085 induces chondrocyte apoptosis, whereas the proteasome inhibitor MG-132 and an adenovirus-expressing mutated IκB-α do not (7). It is noteworthy that BAY 11-7085 has been shown to be a potent anti-inflammatory drug in rat adjuvant arthritis (6). In this work, we showed that BAY 11-7085, but not MG-132, induced sustained ERK1/2 phosphorylation that preceded apoptosis of chondrocyte and synovial fibroblasts. Furthermore, we showed that the prostaglandin D2 metabolite 15d-PGJ2 down-regulated BAY 11-7085-induced ERK1/2 phosphorylation and rescued chondrocytes and synovial fibroblasts from apoptosis. These results suggest that BAY 11-7085-induced sustained ERK1/2 phosphorylation might be proapoptotic. Indeed, the ERK1/2 pathway, which regulates cellular growth and proliferation, has been shown to be pro- (12–17) or antiapoptotic (18–20), depending on experimental conditions and/or cell types. Five recent publications support our results (14, 21–24). ERK1/2 phosphorylation was detected during hydrogen peroxide (H_2O_2)-induced apoptosis in bovine articular chondrocytes (23), retinal pigment epithelial cells (14), and canine chondrocytes during canine experimentally induced osteoarthritis (21, 22). In addition, in retinal epithelial cells (14) and mouse hippocampal cells (24), oxidative stress-induced cell death was prevented with 15d-PGJ2 pre-treatment. However, the authors did not examine the effect of 15d-PGJ2 on ERK phosphorylation. We proposed here that the antiapoptotic effect of 15d-PGJ2 might be related to its inhibition of ERK1/2 activity. This inhibition seems to be, at least in part, distinct from MEK1/2, because the specific inhibitor UO126 showed significantly less antiapoptotic effect than 15d-PGJ2. Although MEK1/2 kinases phosphorylate ERK1/2 (25), uncoupled activation of two kinases was also documented in specific physiological conditions (26–28) and during mitosis (29).

15d-PGJ2 was also shown to have proapoptotic effect in several cell types: colon cancer cells (30), lung cancer cell (31), endothelial cells (11), and granulocytes (32). We also confirm here that 15d-PGJ2 induces apoptosis in endothelial cells, and our preliminary results showed that 15d-PGJ2 induces apoptosis in HCT-116 cancer cell line (data not shown).

It was shown previously that 15d-PGJ2 has anti-inflammatory effects on both human chondrocytes (33, 34) and rheumatoid synovial fibroblasts (35), mainly because of its inhibition of NF-κB (33, 34). The results presented in this work suggest that although 15d-PGJ2 may have a cartilage-protective effect, the antiapoptotic effect of 15d-PGJ2 on synovial fibroblasts, despite suppression of endothelial cell growth (11), may favor inflammation *in vivo*. However, although our results showed that 15d-PGJ2 has a strong antiapoptotic effect on synovial fibroblasts from osteoarthritis patients, others reported that 15d-PGJ2 can induce apoptosis in synovial fibroblasts from patients with rheumatoid arthritis (36) and can suppress adjuvant-induced arthritis in rats. The effect of 15d-PGJ2 on synovial fibroblasts may therefore be disease-dependent, and we are currently testing this hypothesis.

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