Osteoarthritis is a debilitating and painful disease that is anticipated to affect 59 million Americans by 2020.\(^1\) In the past, osteoarthritis was often thought to be a noninflammatory form of arthritis completely separate from rheumatoid arthritis, but results of recent studies indicate that they share more similarities than previously thought. Populations of clonally expanded T-cells are found in the synovium of osteoarthritis patients,\(^2\) and factors that promote the proliferation and differentiation of adaptive immunity, including IL-2 and interferon-\(\gamma\), were found in the synovium of 50% of osteoarthritis patients examined. Tumor necrosis factor-\(\alpha\) and IL-1\(\beta\) appear to be the primary inflammatory mediators of the initiation and perpetuation of degenerative pathways in osteoarthritis.\(^3\) These 2 pleiotropic cytokines directly or indirectly promote a multitude of inflammatory responses involved in osteoarthritis, including iNOS, COX-2, and PGE\(_{2}\). It has been reported that PPAR\(\gamma\) agonists do attenuate, at least in part, some of the inflammatory pathways associated with osteoarthritis. Fahmi et al\(^4\) reported that PGJ2 decreases IL-1\(\beta\)-induced production of NO and MMP-13 in human articular cartilage. In the same study,\(^4\) PGJ2 inhibited IL-1, IL-6, tumor necrosis factor-\(\alpha\), IL-8, monocyte chemotactic protein-1, NO, and MMP-9 production by synovial macrophages and IL-2 from T-cells in a dose-dependent manner. Similar effects associated with PGJ2 have been reported by several other researchers investigating osteoarthritis and rheumatoid arthritis.\(^5\)–\(^7\)

**Evaluation of anti-inflammatory and chondroprotective effects of peroxisome proliferator-activated receptor gamma agonists in cartilage and synovial explants from dogs**

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**Objective**—To evaluate the effects of peroxisome proliferator–activated receptor gamma (PPAR\(\gamma\)) agonists on tissue metabolism in cartilage and synovial explants from dogs.

**Sample Population**—Cartilage-synovial membrane explants from 12 dogs.

**Procedures**—Explants were cultured for 21 days without (negative control) or with interleukin (IL)-1\(\beta\) (positive control) or with IL-1\(\beta\) and 2 concentrations of 2 PPAR\(\gamma\) agonists (15-deoxy-\(\Delta\)12,14-prostaglandin J2 [PGJ2] and pioglitazone). Media were collected on days 3, 7, 9, 12, 15, 18, and 21 and assessed for glycosaminoglycan (GAG), nitric oxide (NO), and prostaglandin E\(_2\) (PGE\(_2\)) concentrations. Tissue GAG and hydroxyproline concentrations were determined in cartilage explants collected on day 21.

**Results**—The GAG concentrations of cartilage explants cultured in IL-1\(\beta\) (100 ng/mL) with 2 concentrations of PGJ2 were significantly higher than those in all other groups, whereas media GAG concentrations were significantly lower in the high-concentration PGJ2-treated groups, compared with all other groups. The PGE\(_2\) concentrations were significantly lower in the PGJ2 treatment groups, compared with the positive control and the pioglitazone treatment groups on days 3 to 21. The NO concentrations were significantly lower in PGJ2 treatment groups, compared with the other groups on days 3 and 12 to 21.

**Conclusions and Clinical Relevance**—PGJ2, an endogenous PPAR\(\gamma\) agonist, may have anti-inflammatory and chondroprotective effects in an osteoartiotic joint environment.

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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
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<tr>
<td>DMMB</td>
<td>1,9-dimethylmethylene blue</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PGE(_2)</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGJ2</td>
<td>15-deoxy-(\Delta)12,14-prostaglandin J2</td>
</tr>
<tr>
<td>PPAR(\gamma)</td>
<td>Peroxisome proliferator–activated receptor gamma</td>
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DMMB assay—The GAG concentrations in cartilage explants after 21 days of culture were determined via DMBB assay. Briefly, a 5-µL aliquot of the tissue digest solution was assayed for total GAG concentration (dry weight) by addition of 245 µL of DMBB solution and spectrophotometric determination of absorbance at 325 nm. The GAG loss from the explant was determined by calculating GAG concentration in the liquid media as a percentage of total GAG in the explant for each sample.

Hydroxyproline analysis—For collagen matrix evaluation, total collagen concentrations of the cartilage explants were estimated by measurement of hydroxyproline by use of a described colorimetric procedure. Briefly, a 50-µL aliquot of the tissue digest solution was mixed with 50 µL of 4N NaOH, and the mixture was autoclaved for 20 minutes at 121°C to hydrolyze the sample. The sample was then mixed with chloromine T reagent and incubated at 25°C for 25 minutes. The sample was mixed with Ehrlich aldehyde reagent, and the chlorophore was developed at 65°C for 20 minutes. After development, the absorbance was read at 550 nm and the samples were compared with a hydroxyproline standard to determine the hydroxyproline concentration of the sample. Results were standardized to tissue dry weight and are reported as micrograms of hydroxyproline per milligram of tissue (dry weight).

NO assay—The NO concentration of the media was obtained by determining NO$^-$ concentration according to manufacturer's instructions.

PGE$\_2$ determination via ELISA—The PGE$\_2$ concentration of the media was determined via ELISA according to manufacturer’s instructions. Because the concentration of PGE$\_2$ in the samples was beyond the range of the assay, the media samples were diluted in plain Dulbecco modified eagle medium to obtain concentrations within the range of the assay. Samples were initially tested at a dilution of 1:250. If needed, further dilutions were made to obtain appropriate concentrations. The maximum dilution conducted in this study was 1:1,300.

Liquid chromatography–mass spectrometry—Samples of media were submitted to the University of Missouri mass spectrometry core facility for analysis for metabolites. The liquid chromatography–mass spectrometry analyses were performed by use of electrospray ionization on a triple-quadrupole mass spectrometer with an integrated liquid chromatography system consisting of a quaternary liquid chromatography pump and vacuum degasser, an autosampler, and a diode-array detector. A column with dimensions of 4.6 × 150 mm and 5-µm packing was used for the separation.
The mobile phase was composed of high-performance liquid chromatography-grade solvents and consisted of 0.1% formic acid in water (A) and methanol (B) flowing at 1 mL/min; the gradient was as follows: 0 minutes, 100% A; 16 minutes, 100% B; 20 minutes, 100% B; 21 minutes, 100% A; and 25 minutes, 100% A. The photodiode array detector was set to acquire spectra over the 190- to 420-nm range. On the mass spectrometer, the electrospray needle voltage was 4.5 kV and the heated inlet capillary was set at 250°C. Nitrogen sheath and auxiliary gases were provided by use of liquid nitrogen. All other voltages and settings were optimized to maximize ion transmission and minimize unwanted fragmentation as determined during the regular tuning and calibration of the instrument.

Statistical analysis—All statistical analyses were performed by use of a computer software program. A 1-way ANOVA was performed to determine significant differences among treatment groups with respect to each assay at each collection time. When significant differences among groups were detected, an all-pairwise multiple comparison (Tukey test) was performed. If the data failed a normality test, a Mann-Whitney rank sum test was performed to determine significance among groups. Significance was set at \( P < 0.05 \).

Results

To determine the in vitro effects of PPARγ agonists on cartilage health, tissue GAG and hydroxyproline concentrations were examined in cartilage explants. The GAG concentration (dry weight) of the explants cultured in IL-1β (100 ng/mL) plus PGJ2 (30μM and 15μM) was significantly higher than that of all other groups. There were no significant differences in tissue hydroxyproline concentrations in PPARγ-treated groups, compared with the negative controls (media alone) or the positive controls (media plus IL-1β alone; Figure 1).

To further delineate the nature of higher tissue GAG concentration in PGJ2 groups, media GAG concentrations were analyzed. The GAG concentration in the 30μM PGJ2 group was significantly lower than the concentration in all other groups on days 3 to 18, except for the 15μM PGJ2 group on day 18. The 15μM PGJ2 group had significantly lower GAG concentration than the positive control group on days 15 and 18 (Figure 2). The ratios of the total tissue GAG concentration (explants) on day 21 to the sum of the tissue GAG concentration on day 21 and the total media GAG concentration over all time points for each respective group revealed that the groups treated with either concentration of PGJ2 had significantly greater retention of GAG in the tissue, compared with all other groups (Figure 3). The retention of GAG for the 30μM PGJ2 group was significantly greater than that for the 15μM PGJ2 group (\( P < 0.001 \)).

The PGE\(_2\) concentrations were significantly (\( P \leq 0.001 \)) lower in the negative control, compared with the positive control and treatment groups, at all time points except for the 30μM PGJ2 group on day 18 (Figure 4). The 30μM PGJ2 treatment group had significantly lower PGE\(_2\) than the 15μM PGJ2 group on days 7, 9, and 15 and the 4.2μM and 2.5μM pioglitazone groups on days 3 to 9, 15, and 21. The PGE\(_2\) concentrations of the 4.2μM and 2.5μM pioglitazone groups were not significantly different from the positive control group at any time point.

To determine whether pioglitazone was being adequately metabolized in the coculture system, samples of media from pioglitazone treatment groups were analyzed for metabolites. No metabolites were detected.

Nitric oxide and PGE\(_2\) were examined as mediators of inflammation. The NO concentration of the negative control was significantly lower than that of the positive control group at all time points (Figure 5). The NO concentration of the 30μM PGJ2 treatment group was significantly lower than that of the negative control group on day 3 and days 12 to 21 and the positive control group at all time points (days 3 to 21 (\( P < 0.001 \))).
The NO concentration in the 15μM PGJ2 treatment group was significantly lower than that in the positive control group at all time points except day 7 and greater than the negative controls at days 3, 7, and 9. Nitric oxide concentration in the 4.2μM pioglitazone treatment group was significantly lower than that in the positive control and the 2.5μM pioglitazone groups at days 7 to 21.

**Discussion**

For quantitation of the GAG concentration of the media and cartilage explants, the DMMB assay was used. In a previous study, the GAG concentration of the positive controls decreased significantly, compared with the negative control. In the present study, an overall decrease occurred but it was not significant, possibly because the positive control values were more variable. Nevertheless, the GAG concentration was significantly greater in both PGJ2 treatment groups, compared with all other groups, in explants harvested on day 21.

To gain a better understanding of the nature of the GAG retention in the explants, ratios of the total tissue GAG concentration (explants) on day 21 to the sum of the tissue GAG concentration on day 21 and the total media GAG concentration over all time points for each respective group were examined. This revealed that both PGJ2 groups had significantly greater retention of GAG, compared with all other groups (Figure 3). This indicated that PGJ2, but not pioglitazone, contributed to the maintenance of GAG within the cartilage explant. Also, there was a significantly decreased media GAG concentration in PGJ2 groups, which suggested that the maintenance of cartilage GAG concentration in these groups might be attributed to inhibition of proteoglycan degradation, although definitive statements regarding GAG synthesis or degradation cannot be made.

Nitric oxide and PGE2 are potent inflammatory mediators in osteoarthritis. Excess production of NO has been linked with inhibition of collagen synthesis, proteoglycan synthesis, and IL-1 receptor antagonist production as well as interference with integrin signaling, induction of chondrocyte apoptosis, stimulation of metalloprotease production and activation, and inactivation of tissue inhibitors of MMPs.13 The PPARγ agonists inhibit NO, and the mechanism for inhibition of NO has been reported to be associated with effects on mitogen-activated protein kinase kinase kinase-1 induction of iNOS promoter activity.14 Fahmi et al14 reported that PGJ2 inhibited delta MEKK-1–induced activator protein-1 and nuclear factor-kappa B-luciferase reporter plasmid activation through a PPARγ-dependent pathway. Colville-Nash et al14 reported that PGJ2 may also affect the stress protein, heme oxygenase-1, and in doing so inhibit iNOS.

In the present study, the NO concentration measured via the Griess reaction in the positive control group was greatly increased, compared with that in the negative control group, consistent with previous findings. The 4.2μM pioglitazone treatment group had significantly...
reduced NO concentration, compared with the positive group and 2.5\(\mu\)M pioglitazone group, whereas the NO concentration in the 2.5\(\mu\)M pioglitazone group was not significantly different from the positive control group, suggesting a possible dose-related effect of pioglitazone. The PGJ2 treatment groups also had a similar dose relationship. The lower NO concentration in the high-concentration PGJ2 group was of particular note because it was less than that of the negative control group even though it was treated with IL-1\(\beta\).

Although it is unclear whether the alteration of NO production in the PGJ2 groups caused the changes in GAG concentration, NO production was greatly reduced by administration of PPAR\(\gamma\) agonists. Not only did the higher concentration of the PGJ2 treatment (30\(\mu\)M) appear to be more efficient at reducing NO production than did the lower concentration treatment (1.5\(\mu\)M), but it also induced particularly striking results by reducing the NO concentration to less than that of the negative controls. This also supported previous findings that iNOS induction is suppressed in a dose-dependent manner by PGJ2. Interestingly, the 4.2\(\mu\)M pioglitazone treatment also appeared to consistently reduce NO production, compared with the positive controls, but the 2.5\(\mu\)M pioglitazone treatment did not.

The significant reduction in PGE\(_2\), in association with PPAR\(\gamma\) agonists could also prove beneficial in osteoarthritis by reducing inflammation and related degradation. Several mechanisms have been proposed for the action of PPAR\(\gamma\) agonists as anti-inflammatory drugs. In 2002, Fahmi et al. examined human osteoarthritis chondrocytes and noted that PGJ2 did not inhibit PGE\(_2\) through a COX-2 pathway. Recently, it was reported that PGJ2 can act directly on mesenchymal PGE synthase type 2 to prevent PGE\(_2\) production in colorectal cells.

In the present study, the PGE\(_2\) concentration of the positive control group was greatly increased, compared with the negative control group, consistent with previous findings. The 30\(\mu\)M PGJ2 treatment group had significantly lower PGE\(_2\) concentration, compared with the IL-1\(\beta\) positive control group at most time points and all other PPAR\(\gamma\) treatments at multiple time points. The 4.2\(\mu\)M and 2.5\(\mu\)M pioglitazone groups did not have significantly reduced PGE\(_2\) concentration compared with the positive control group, at any time point. The data confirmed previous findings in regard to PGJ2, but it was still unclear why pioglitazone had no observed suppressive effect.

The major limitation of this study was its in vitro nature, and although the system has been determined to closely mimic in vivo conditions, it would be presumptuous to assume that the interactions in a culture system completely explain the complex conditions in vivo. Notable difference in the experimental system used in this study, compared with the previous study, is that it incorporated not only cartilage but also synovium in an attempt to resemble the entire joint environment as closely as possible.

We hypothesized that PGJ2 and pioglitazone would be both anti-inflammatory and anticytotoxic in the coculture system. The PGJ2 reduced NO and PGE\(_2\) production while preserving tissue GAG concentration, compared with the positive control. Pioglitazone reduced NO production, compared with the positive control, but failed to preserve GAG concentration or reduce PGE\(_2\) production. Therefore, despite apparent beneficial effects of PGJ2 on joint health, pioglitazone did not markedly reduce inflammatory responses and matrix alterations in cartilage exposed to IL-1\(\beta\). Pioglitazone did not cause detrimental effects in the cartilage, compared with the negative control, but it appears that pioglitazone does not have substantial chondroprotective effects in this in vitro system despite recent notable reports in animal models. This could be attributable to the fact that metabolism to more active forms may be required because pioglitazone is a prodrug and must be bioconverted to active forms. This bioconversion is not facilitated in a culture system where proper metabolic pathways for bioconversion, usually found in the liver and kidneys, may not be sufficient or even present. To definitively answer the question of whether pioglitazone was metabolized in this culture model, we conducted liquid chromatography–mass spectrometry analysis of multiple samples of media in both pioglitazone concentration groups and found only trace amounts of metabolized pioglitazone. This finding confirmed that the metabolites were not produced in substantial amounts, and we believe this is the most likely reason that results for the pioglitazone groups did not more closely resemble those for the PGJ2 groups.

Taken together, these data suggest that targeting PPAR\(\gamma\) would be a potential treatment strategy for osteoarthritis. The endogenous PPAR\(\gamma\) agonist, PGJ2, appears to induce anti-inflammatory and chondroprotective effects in a simulated osteoarthritis joint environment; however, we were not able to conclude that the synthetic PPAR\(\gamma\) agonist, pioglitazone, has analogous effects. Further investigation is needed to explain this finding and elucidate the actions of PPAR\(\gamma\) agonists in osteoarthritis.

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\footnotesize
a. Becton-Dickinson, Franklin Lakes, NJ.
\item b. BD Biosciences, San Jose, Calif.
\item c. Sigma-Aldrich, St Louis, Mo.
\item d. Promega Corp, Madison, Wis.
\item e. Cayman Chemical, Ann Arbor, Mich.
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\item g. GL Sciences Inc, Tokyo, Japan.
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