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TLR2 Signaling in Chondrocytes Drives Calcium Pyrophosphate Dihydrate and Monosodium Urate Crystal-Induced Nitric Oxide Generation

Ru Liu-Bryan,*† Kenneth Pritzker,‡ Gary S. Firestein,† and Robert Terkeltaub2*†

Microcrystals of calcium pyrophosphate dihydrate (CPPD) and monosodium urate (MSU) deposited in synovium and articular cartilage initiate joint inflammation and cartilage degradation in large part by binding and directly activating resident cells. TLRs trigger innate host defense responses to infectious pathogens, and the expression of certain TLRs by synovial fibroblasts has revealed the potential for innate immune responses to be triggered by mesenchymally derived resident cells in the joint. In this study we tested the hypothesis that chondrocytes also express TLRs and that one or more TLRs centrally mediate chondrocyte responsiveness to CPPD and MSU crystals in vitro. We detected TLR2 expression in normal articular chondrocytes and up-regulation of TLR2 in osteoarthritic cartilage chondrocytes in situ. We demonstrated that transient transfection of TLR2 signaling-negative regulator Toll-interacting protein or treatment with TLR2-blocking Ab suppressed CPPD and MSU crystal-induced chondrocyte release of NO, an inflammatory mediator that promotes cartilage degeneration. Conversely, gain-of-function of TLR2 in normal chondrocytes via transfection was associated with increased CPPD and MSU crystal-induced NO release. Canonical TLR signaling by parallel pathways involving MyD88, IL-1R-associated kinase 1, TNF receptor-associated factor 6, and IκB kinase and Rac1, PI3K, and Akt critically mediated NO release in chondrocytes stimulated by both CPPD and MSU crystals. We conclude that CPPD and MSU crystals critically use TLR2-mediated signaling in chondrocytes to trigger NO generation. Our results indicate the potential for innate immunity at the level of the articular chondrocyte to directly contribute to inflammatory and degenerative tissue reactions associated with both gout and pseudogout.


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2 Address correspondence and reprint requests to Dr. Robert Terkeltaub, Veterans Affairs Medical Center, 3350 La Jolla Village Drive, San Diego, CA 92161. E-mail address: rtterkeltaub@ucsd.edu

3 Abbreviations used in this paper: MSU, monosodium urate; CPPD, calcium pyrophosphate dehydrate; IKK, IκB kinase; IRAK, IL-1R-associated kinase; MMP, matrix metalloproteinases; OA, osteoarthritis; PBD, protein binding domain; PDTC, pyridoline dihydrocarbamate; Pyk2, proline-rich tyrosine kinase; Toll, Toll-interacting protein; TRAF6, TNF receptor-associated factor 6; HEMA, 2-hydroxyethylmethacrylate.
P3K, and the downstream protein kinase Akt also transduces TLR2 signaling to NF-κB activation (15). Interestingly, both P3K and Akt are involved in MSU and CPPD crystal-induced stimulation of neutrophils (16, 17).

Although many TLR ligands are microbial products (11), nonbacterial ligands for certain TLRs have been identified, such as heat shock protein 70, fatty acids, etc. (18, 19). In addition, MSU crystals appear to directly engage certain integrins and the FcR CD16 in cells of hemopoietic origin (20, 21). Recently, the expression of certain TLRs was discovered in normal and rheumatoid arthritis synovial fibroblasts (22, 23), indicative of potential innate immune responses driven by mesenchymally derived cells in arthritis. Hence, we hypothesized that normal articular chondrocytes also normally express TLRs and that MSU and CPPD crystals use TLRs to stimulate chondrocytes. Below, we demonstrate constitutive expression by articular chondrocytes of TLR2, and we observe that TLR2 signaling centrally mediates CPPD and MSU crystal-induced NO release in chondrocytes. Our findings identify an innate immune response pathway of the articular cartilage chondrocyte that may contribute to inflammatory and degenerative tissue reactions in the joint in both gout and pseudogout.

Materials and Methods
Reagents
All chemical reagents were obtained from Sigma-Aldrich, unless otherwise indicated. Monoclonal CPPD crystals were prepared as previously described (24) and treated for 2 h at 200°C after crystalization, followed by suspension at 25 mg/ml in sterile, endotoxin-free 10 mM sodium PBS, pH 7.4. Triclinic MSU crystals were prepared under pyrogen-free conditions using uric acid treated for 2 h at 200°C before crystalization (25) and were suspended at 25 mg/ml in PBS. The suspended MSU and CPPD crystals were verified to be free of detectable LPS contamination (<0.025 endotoxin unit/ml) by the Limulus amebocyte lysate assay (BioWhittaker).

The pharmacological inhibitor to PI3K LY294002, and pyrrolidine dithiocarbamate (PDTC) were purchased from Calbiochem. Phosphospecific Akt (Ser473), p56 NF-κB (Ser536), and IκBα (Ser32) polyclonal Abs and total Akt and p65 NF-κB polyclonal Abs were purchased from Cell Signaling Technology. mAb to TLR2 was purchased from Upstate Biotechnology. Polyconal Abs to IKK1, IKK2, Rac1, and MyD88; mAb to TLR2; and HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG were obtained from Santa Cruz Biotechnology. Functional blocking mAb to TLR2, and the isotype control mouse IgG2a were obtained from eBioscience.

Wild-type and mutant P3K p85α cDNAs in SRα vector were obtained from Dr. M. Kasuga (Kobe University School of Medicine, Kobe, Japan). Wild-type and mutant Akt cDNAs in pET 17b vector were obtained from Dr. R. Roth (Stanford University School of Medicine, Stanford, CA). Human TLR1, TLR2, TLR6, and TLR9 cDNAs in pFlag.CMV vector, Myctagged wild-type and dominant negative mutants of MyD88, and IRAK1 and TRAF6 in pRK5 vector were obtained from Dr. T.-H. Chuang (The Scripps Research Institute, La Jolla, CA). The pUNO-Toll-interacting protein (pUNO-Tollip) construct was purchased from eBioscience.

Cell culture and transfection and assay of NO production
Normal human knee articular chondrocytes and adult bovine knee articular chondrocytes (Animal Technologies) were isolated as described previously (6). Primary chondrocytes were maintained in DMEM high glucose medium with 10% FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37°C for 5 days, then transferred to nonadherent culture conditions in poly-2-hydroxyethylmethacrylate (HEMA)-coated plates as previously described (6), where first passage chondrocytes in DMEM supplemented with 1% FCS, streptomycin, and penicillin, as described above, were stimulated with MSU and CPPD crystals. For transfection of bovine chondrocytes, aliquots of 4 x 10⁵ primary cells were plated in 60-mm dishes and allowed to adhere for 18 h, after which cells were transfected using FuGene 6 and hyaluronidase, as previously described (26). Transfection efficiency, evaluated in control samples via β-galactosidase transfection and staining (26), was >25%. Twenty-four hours after transfection, medium was replaced with fresh complete DMEM high glucose medium containing 10% FCS, and the cells were allowed to recover for another 24 h. NO production was measured as the concentration of nitrates in conditioned medium by the Griess reaction (27) using NaNO2 as standard.

Where a higher transfection efficiency was needed for plasmids, chondrocytes were transfected using the Nucleofection system (Amaxa), following the manufacturer’s protocol optimized for chondrocytes. The Amaxa Nucleofection system yielded a transfection efficiency of >70%. Adenoviral gene transfer for expression of wild-type and dominant negative IKK1 and IKK2 and control adenoviral GFP (provided by Dr. B. Benett, Celgene, Signal Research Division) was performed in chondrocytes, with transfection efficiency >80%

We cultured the human embryonic kidney cell line HEK293 (American Type Culture Collection) in DMEM with 10% FCS. For transfection, aliquots of 3 x 10⁵ HEK293 cells were plated in each well of six-well plates overnight. Cells were then cotransfected with plasmids including the NF-κB binding site-containing reporter construct ELAM-1-luciferase, pRLTK (used as an internal control), and TLR2 using the transfection reagent SuperFect (Invitrogen Life Technologies) according to the manufacturer’s instructions, which yielded a transfection efficiency of >80%.

Immunohistochemistry
Frozen sections from human knee cartilage sections (5 μm) were obtained from normal joints at autopsy or from osteoarthritic joints at the time of total knee arthroplasty, prepared as previously described (28). Sections were fixed with ice-cold acetone, washed with PBS, and treated with 0.1% H2O2 in PBS for 10 min at room temperature. After serial washes in PBS, the sections were blocked with PBS containing 10% goat serum and incubated with TLR2 Ab at 4°C overnight. Washed sections were then incubated for 1 h at room temperature with biotinylated goat anti-mouse IgG, followed by a 1-h incubation with peroxidase-conjugated avidin. Peroxidase activity was detected using the Sigma Fast 3,3-diaminobenzidine staining kit, according to the manufacturer’s instructions.

Assays for TLR family member mRNA expression
RT-PCR analyses for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, CD14, and the housekeeping gene L30 were performed on RNA isolated from chondrocytes using TRizol (Invitrogen Life Technologies). Primers used for RT-PCR are shown in Table I. Aliquots of 600 ng of total RNA were reverse transcribed as previously described (6). PCRs were performed for 30 cycles; 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C 5 min.

Preparation of cell lysates, immunoprecipitation, SDS-PAGE/Western blot analyses, and in vitro kinase assays
Preparation of cell lysates and SDS-PAGE/Western blot analyses were performed as previously described in detail, using HRP-conjugated secondary Ab (Santa Cruz Biotechnology) and detection by the ECL system (Pierce) (6). For immunoprecipitation assays, 250 μg of protein of cell lysates was used as previously described (6). For IKK1 or IKK2 in vitro kinase assays, the cell lysates (250 μg) were first precipitated with IKK1

Table I. Primers for RT-PCR analyses

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<tr>
<th>TLR</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>PCR Products (bp)</th>
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<td>ATCGGAAATCACCAGGCTTATTGAGCCAG</td>
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</table>
or IIK2 Ab. IKK1 or IKK2 activity was then determined using GST-IκBα as a substrate as described previously (29). For PI3K assay, aliquots of 250 μg of cell lysate protein were precipitated with p85α Ab using protein A/G agarose (Santa Cruz Biotechnology). PI3K activity was examined using the method described previously (30).

Rac1 activation assay

Cell lysates (250 μg) were subjected to affinity precipitation with GST-protein binding domain (GST-PBD; 10 μM) in binding buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 30 mM MgCl2, 40 mM NaCl, and 1% Nonidet P-40, proteins bound to GST-PBD beads were separated by SDS-PAGE and immunoblotted for bound Rac1.

Statistical analysis

Numerical data were uniformly expressed as the mean ± SD. Statistical analyses were performed using two-tailed Student’s t test.

Results

Constitutive expression of TLR2 in human articular chondrocytes and knee cartilage in situ

We consistently detected TLR2 mRNA expression in different donors of normal human primary articular chondrocytes by RT-PCR (Fig. 1A). However, TLR1, TLR3, TLR4, TLR6, and TLR9 mRNA expression were consistently below limits of detection in human primary chondrocytes by RT-PCR analysis under these conditions (Fig. 1A). Immunohistochemical analysis demonstrated constitutive TLR2 expression in normal human knee articular cartilages (Fig. 1B).

TLR2 mediates induction of NO production by MSU and CPPD crystals in chondrocytes

We confirmed that pyrogen-free MSU crystals induced NO release by normal bovine knee articular chondrocytes (6) and observed a similar response to pyrogen-free CPPD crystals (Fig. 2A). Next, we transfected bovine articular chondrocytes with Tollip, an adapter protein known to inhibit both TLR2- and TLR4-mediated signaling (31, 32). Chondrocytes transfected with Tollip and then stimulated with either MSU or CPPD crystals for 24 h demonstrated marked inhibition of NO production in response to both MSU and CPPD crystals (Fig. 2A). TLR2 functional blocking Ab also suppressed MSU and CPPD crystal-induced NO production (Fig. 2B). Next, using a gain-of-function approach, we observed that overexpression of TLR2 (but not TLR1, TLR3, or TLR9) via transient transfection in bovine articular chondrocytes was associated with a significant increase in NO production in response to both MSU and CPPD crystals (Fig. 2C).

Chondrocyte MyD88, IRAK-1, and TRAF6 mediate NF-κB activation and induction of NO by MSU and CPPD crystals

Cheminoluminescent assays of bovine chondrocytes stimulated with MSU crystals demonstrated rapidly up-regulated NF-κB p65 subunit activity, which reached a peak by 60 min (Fig. 3A). Furthermore, pretreatment of bovine chondrocytes with the NF-κB inhibitor PDTC markedly inhibited both MSU and CPPD crystal-induced NO production (Fig. 3B). MyD88, IRAK, and TRAF6 have been observed to mediate TLR2 signaling that promotes NF-κB activation (11). We observed that dominant negative mutants of MyD88, IRAK1, and TRAF6 inhibited NF-κB transcriptional activation and NO generation induced by both crystal types (Fig. 3, C and D).

IKK2 mediates induction of NO production by MSU and CPPD crystals in chondrocytes

IKKs function downstream of TRAF6 in mediating TLR2 signaling to activate NF-κB-dependent genes (11). Via in vitro kinase assays, we demonstrated that both IKK1 and IKK2 were activated by MSU crystals (Fig. 4A), with similar results seen for IKK activation by CPPD crystals (not shown). To assess whether IKK1 or IKK2 mediates NF-κB action by MSU crystals, bovine chondrocytes were infected with recombinant adenovirus of kinase-dead mutants of IKK1 and IKK2 before stimulation with the
NF-κB crystals (0.5 mg/ml) at the times indicated, and cell lysates were prepared as described in Materials and Methods. Nonradioactive chemiluminescent NF-κB p65 transcription factor assays determined the activity of p65 subunit of NF-κB, as described in Materials and Methods. Bovine primary chondrocytes cultured under nonadherent conditions on poly-HEMA-coated plates in medium containing 1% FCS were treated with MSU crystals (0.5 mg/ml) for 24 h with and without the pretreatment with the NF-κB inhibitor PDTC (100 μM). NO release was determined in the conditioned medium as described above. C, HEK293 cells were cotransfected with TLR2, pELAM-Luc, pRLTK (internal control), and dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals using the same conditions as described in Fig. 3B. NO release was determined in the conditioned medium as described above. All data are representative of three independent experiments with three different donors. *, p < 0.05.

FIGURE 3. MyD88, IRAK1, and TRAF6 signaling mediates NF-κB activation and NO production by MSU and CPPD crystals in chondrocytes. A, Bovine knee articular chondrocytes cultured under nonadherent conditions on poly-HEMA-coated plates in medium containing 1% FCS were treated with MSU crystals (0.5 mg/ml) at the times indicated, and cell lysates were prepared as described in Materials and Methods. Nonradioactive chemiluminescent NF-κB p65 transcription factor assays determined the activity of p65 subunit of NF-κB, as described in Materials and Methods. B, Bovine primary chondrocytes cultured under nonadherent conditions on poly-HEMA-coated plates in medium containing 1% FCS were treated with MSU crystals (0.5 mg/ml) for 24 h with and without the pretreatment with the NF-κB inhibitor PDTC (100 μM). NO release was determined in the conditioned medium as described above. C, HEK293 cells were cotransfected with TLR2, pELAM-Luc, pRLTK (internal control), and dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. The cells were then stimulated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals using the same conditions as described in Fig. 3B. NO release was determined in the conditioned medium as described above. All data are representative of three independent experiments with three different donors. *, p < 0.05.

Rapid signaling complex assembly of TLR2, MyD88, Rac1, and PI3K in chondrocytes stimulated with MSU crystals

Upon stimulation with TLR2 ligands, MyD88 is recruited to the cytosolic domain of TLR2 (11), and PI3K subunit p85α, Rac1, and the cytosolic domain of TLR2 form a stimulus-dependent signaling complex (15). Immunoprecipitation studies (Fig. 5) demonstrated rapid signaling complex assembly that involved TLR2, MyD88, IRAK1, and TRAF6 in chondrocytes stimulated with MSU crystals (Fig. 5). Transient association of p85α with MyD88 was detected and reached a maximum at 15–30 min; a similar pattern of association of p85α with TLR2 was also observed, whereas the association of p85α and Rac1 was rapid and transient within 5 min (Fig. 5).

PI3K and Akt mediate induction of NO production by MSU and CPPD crystals in chondrocytes

MSU and CPPD crystals both triggered rapid PI3K activation in chondrocytes (Fig. 6A). Inhibition of PI3K using the selective pharmacological inhibitor LY294002 or by transient transfection of chondrocytes with dominant negative mutant of PI3K subunit p85α (Δp85α) prevented NO production in response to MSU and CPPD crystals (Fig. 6B). MSU and CPPD crystals also induced rapid phosphorylation of Akt in chondrocytes, which was demonstrated to be PI3K-dependent via inhibition using LY294002 (Fig. 7A). Inhibition of Akt activity by transfection with a kinase-dead mutant of Akt (Akt-KM) also attenuated the induction of NO production by MSU and CPPD crystals in chondrocytes (Fig. 7B).

Rac1 critically mediates induction of NO production by MSU and CPPD crystals in chondrocytes

Rac1 was activated in chondrocytes stimulated with MSU or CPPD crystals (Fig. 8A). Rac1 not only can regulate the activation of PI3K (35), but also can be activated by PI3K (36). We observed that activation of Rac1 induced by MSU crystals was not inhibited by LY29004 (Fig. 8B), consistent with PI3K being a downstream target of Rac1 in MSU crystal-stimulated chondrocytes. Transfection of the constitutively activated Rac1V12 mutant slightly enhanced both MSU and CPPD crystal-induced NO production, whereas transfection with the dominant negative Rac1N17 significantly attenuated induction of NO production expression by both crystals (Fig. 8C). Furthermore, Rac1N17 and PI3K Δp85α, inhibited MSU and CPPD crystal-induced phosphorylation of NF-κB p65 (Fig. 8D), consistent with a joint role of Rac1 and PI3K in crystal-induced NF-κB activation.

Collectively, the results obtained in this study were consistent with a model of crystal-induced chondrocyte activation via TLR2-mediated parallel signaling chains in which NF-κB activation was a critical downstream event in NO production (Fig. 9).

Discussion

In this study we demonstrated that human knee articular cartilage chondrocytes constitutively express TLR2. Moreover, we observed that TLR2 signaling is centrally used to stimulate chondrocytes by microcrystals of CPPD and MSU. This conclusion were
cytokine phagocytes were lessened by blocking Abs specific for the leukocyte integrin CD11b/CD18 (21) and FcR CD16 (21), which mediates activation of chondrocytes, but we have not excluded a role for rapid crystal-induced release of one or more endogenous TLR2 ligands, such as heat shock protein 70 or specific saturated fatty acids (18, 19).

IKK2, but not IKK1, has been shown to be critical for cytokine-induced NF-κB activation and expression of a variety of NF-κB-regulated genes in fibroblast-like synovial lining cells (41). In this study, crystal-induced TLR2-mediated signaling cascades culminating in NF-κB activation included activation of both IKK1 and IKK2 in chondrocytes, but only IKK2 activation was required to mediate crystal-induced NO production. It should be noted that the IL-1R and TLRs share a common signaling pathway, leading to NF-κB activation involving MyD88, IRAK, TRAF6, and IKKs (11). Furthermore, incubation of chondrocytes with MSU crystals induces IL-1β expression (6). Significantly, the triggering of MSU crystal-induced NO production in chondrocytes is not IL-1 dependent in chondrocytes, as we demonstrated previously using soluble IL-1R antagonist (6). However, it remains possible that autocrine signaling by cytokines, including effects of IL-1 through MyD88, could amplify NO production in chondrocytes after the early responses to MSU and CPPD crystals.

We demonstrated transient complex formation among TLR2, MyD88, Rac1, and PI3K in chondrocytes. Bovine chondrocytes cultured under nonadherent conditions with the extracellular domain of TLR2 mediates activation of chondrocytes, but we have not excluded a role for rapid crystal-induced release of one or more endogenous TLR2 ligands, such as heat shock protein 70 or specific saturated fatty acids (18, 19).

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We demonstrated transient complex formation among TLR2, MyD88, Rac1, and PI3K in chondrocytes. Bovine chondrocytes cultured under nonadherent conditions with the extracellular domain of TLR2 mediates activation of chondrocytes, but we have not excluded a role for rapid crystal-induced release of one or more endogenous TLR2 ligands, such as heat shock protein 70 or specific saturated fatty acids (18, 19).

IKK2, but not IKK1, has been shown to be critical for cytokine-induced NF-κB activation and expression of a variety of NF-κB-regulated genes in fibroblast-like synovial lining cells (41). In this study, crystal-induced TLR2-mediated signaling cascades culminating in NF-κB activation included activation of both IKK1 and IKK2 in chondrocytes, but only IKK2 activation was required to mediate crystal-induced NO production. It should be noted that the IL-1R and TLRs share a common signaling pathway, leading to NF-κB activation involving MyD88, IRAK, TRAF6, and IKKs (11). Furthermore, incubation of chondrocytes with MSU crystals induces IL-1β expression (6). Significantly, the triggering of MSU crystal-induced NO production in chondrocytes is not IL-1 dependent in chondrocytes, as we demonstrated previously using soluble IL-1R antagonist (6). However, it remains possible that autocrine signaling by cytokines, including effects of IL-1 through MyD88, could amplify NO production in chondrocytes after the early responses to MSU and CPPD crystals.
FIGURE 6. PI3K mediates the induction of NO in response to MSU and CPPD crystals in chondrocytes. A, Bovine primary chondrocytes cultured under nonadherent conditions in medium containing 1% FCS, as described above, were treated with MSU crystals (0.5 mg/ml) for the times indicated. The PI3K assay was conducted on cell lysates, as described in Materials and Methods. B, Bovine chondrocytes were stimulated with MSU crystals (0.5 mg/ml) for 24 h with or without pretreatment with the PI3K inhibitor LY294002 (25 μM) for 1 h. Where indicated, the cells were transiently transfected (using FuGene 6 and hyaluronidase) with wild-type and mutant PI3K subunit p85α (specifically p85α-WT and Δp85α, which lacks a region that is necessary for its association with PI3K activity) or vector control (Vec). Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. The data are representative of three independent experiments with three different donors.

FIGURE 7. Akt mediates induction of NO in response to MSU and CPPD crystals in chondrocytes. A, Bovine chondrocytes cultured under nonadherent conditions in medium containing 1% FCS were stimulated with MSU crystals (0.5 mg/ml) for the times indicated with or without pretreatment with the PI3K inhibitor LY294002 (25 μM) for 1 h. Cell lysates were analyzed by SDS-PAGE/Western blotting using Abs to phospho-specific Akt (Ser473) and total Akt. B, Bovine chondrocytes were transiently transfected with wild-type and kinase-dead mutant-type Akt (Akt-WT and Akt-KM) or vector control (Vec) using FuGene 6 and hyaluronidase, as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. The data are representative of three independent experiments with three different donors.

FIGURE 8. Rac1 acts upstream of PI3K to mediate induction of NO in response to MSU and CPPD crystals in chondrocytes. A and B, Bovine chondrocytes cultured under nonadherent conditions in medium containing 1% FCS were stimulated with MSU or CPPD crystals (0.5 mg/ml) for the times indicated. Where indicated, pretreatment with LY294002 (25 μM) was performed. Rac1 activation (pull-down) assay was performed on cell lysates (250 μg) prepared from these cells using GST-PBD, as described in Materials and Methods. SDS-PAGE/Western blotting analyses were then performed on the GST-PBD pull-down samples as well as the control cell lysates (aliquots of 30 μg of protein) from each sample using Rac1 Ab. C, Bovine chondrocytes were transiently transfected with constitutively activated or dominant negative Rac1 (Rac1V12 and Rac1N17, respectively) or vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of Rac1 (Rac1N17) and p85α (Δp85α) or vector control (Vec) using the Amaxa Nucleofection system, as described in Materials and Methods. Two days after transfection, the cells were transferred to nonadherent culture conditions in medium containing 1% FCS, then stimulated with MSU or CPPD crystals (0.5 mg/ml) for 1 h. Phosphorylation of NF-κB was determined by SDS-PAGE/Western blot analysis from cell lysates using Abs to phosphor-specific p65 (Ser536) and total p65. Data are representative of two independent experiments with two different donors. *, p < 0.05.
to induce NO production in chondrocytes. In previous studies of chondrocytes, we observed that MSU crystals induce tyrosine phosphorylation of paxillin (6), an adaptor protein for focal adhesion kinases, including Pyk2 (42). MSU crystal-induced activation of Pyk2 is pivotal for downstream p38 MAPK activation involved in the induction of NO production and MMP-3 expression in chondrocytes (6). Significantly, MyD88-dependent phosphorylation of paxillin by TLR2 ligands has been observed in macrophages (43), and we have observed that the dominant negative mutant of MyD88 inhibits phosphorylation of paxillin induced by both MSU and CPPD crystals in chondrocytes (R. Liu-Bryan, unpublished observations). Because paxillin can associate with Pyk2, it will be of interest to determine whether Pyk2 activation by the crystals is also mediated through TLR2 signaling.

We speculate that the strikingly similar modes by which pyrogen-free MSU and CPPD crystals were shown in this study to induce NF-κB activation and NO production in chondrocytes may be mirrored in the remarkable phenotypic similarity of synovitis in acute gout and pseudogout. Both of these forms of joint inflammation are mediated by transcriptionally NF-κB-regulated cytokines, such as TNF-α and IL-8 (25, 44–47). However, this study was based on observations with cultured cells. We stimulated chondrocytes with crystals under nonadherent culture conditions, rather than less physiologic monolayer culture conditions in this study. Nevertheless, crystal-induced cell-signaling events observed in this study in cultured chondrocytes could differ from those in chondrocytes in their cartilage matrix in situ. Another limitation of this study was the primary reliance on transfection of cultured cells. Due to the limitations imposed by the low yields of primary articular chondrocytes (e.g., <1000 cells/knee) (8) from mouse joints, studies of crystal-induced activation of TLR2+/− chondrocytes have not yet been performed.

Innate immunity, modulated directly by MSU crystal-induced activation of the membrane attack complex of complement, was recently identified as a major mediator of neutrophil ingress in experimental acute gouty knee synovitis in the rabbit model (48). Taken together with findings in this study, we conclude that innate immunity, including TLR2-mediated activation of chondrocytes within articular cartilage triggered by free MSU and CPPD crystals, has the potential to contribute to degradation of cartilage matrix and to joint inflammation in gout and pseudogout.


