Published in final edited form as:

Biochem Pharmacol. 2007 December 3; 74(11): 1636–1641.

Requirement for Protein Kinase R in Interleukin-1 α -stimulated Effects in Cartilage

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Abstract

Interleukin-1 (IL-1) has pleiotropic effects in cartilage. The interferon-induced, double-stranded RNA-activated protein kinase PKR that phosphorylates eukaryotic initiation factor 2 (eIF2) α has been implicated in cytokine effects in chondrocytes. A compound was recently identified that potentially suppresses PKR autophosphorylation (IC₅₀ approximately 200 nM) and partially restores PKR-inhibited translation in a cell-free system with significant effect in the nanomolar range. The objectives of this study were to exploit this potent PKR inhibitor to assess whether PKR kinase activity is required for catabolic and proinflammatory effects of IL-1 α in cartilage and to determine whether IL-1 α causes an increase in eIF2 α phosphorylation that is antagonized by the PKR inhibitor. Cartilage explants were incubated with the PKR inhibitor and IL-1 α . Culture media were assessed for sulfated glycosaminoglycan as an indicator of proteoglycan degradation and for prostaglandin E₂. Cartilage extracts were analyzed by Western blot for cyclooxygenase-2 and phosphorylated signaling molecules. Nanomolar concentrations of the PKR inhibitor suppressed proteoglycan degradation and cyclooxygenase-2 accumulation in IL-1 α -activated cartilage. The PKR inhibitor stimulated or inhibited PGE₂ production with a biphasic dose response relationship. IL-1 α increased the phosphorylation of both PKR and eIF2 α , and nanomolar concentrations of PKR inhibitor suppressed the IL-1 α -induced changes in phosphorylation. The results strongly support PKR involvement in pathways activated by IL-1 α in chondrocytes.

Keywords

cartilage; interleukin-1; protein kinase R; degradation; cyclooxygenase-2; eukaryotic initiation factor 2

1. Introduction

Articular cartilage is the resilient connective tissue covering the ends of bones within joints, enabling load bearing and mobility [1]. Interleukin-1 (IL-1) has pleiotropic effects on cartilage, activating proinflammatory and catabolic pathways and inhibiting the synthesis of specialized extracellular matrix macromolecules that are essential for biomechanical function. IL-1 is among the most studied cytokine regulators of cartilage metabolism, and IL-1 and other cytokines, such as tumor necrosis factor α , have been implicated in pathological changes to

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the cartilage in degenerative joint diseases [2,3]. IL-1 activates mitogen- and stress-activated protein kinases and transcription factors, such as nuclear factor κ B (NF- κ B) and AP-1 [4,5], but the signal transduction pathways linking IL-1's interaction with cell surface receptors to the diverse metabolic effects in cartilage have not been thoroughly defined.

In response to diverse cellular stresses, phosphorylation of the key translational factor eukaryotic initiation factor-2 (eIF2) on serine 51 of the α subunit causes a global down regulation of protein synthesis in mammalian cells. Four protein kinases catalyze the specific phosphorylation of eIF2 α , including the endoplasmic reticulum stress-activated kinase PERK and the double stranded RNA-activated protein kinase R (PKR). Accumulating evidence supports diverse roles for PKR in regulating cellular functions, including anti-viral response, apoptosis, cell stress response, cell growth and differentiation, cytokine signaling, and inflammation [6–8]. PKR effects are mediated by eIF2 and other direct or indirect targets of PKR, including NF- κ B, activating transcription factor, and signal transducers and activators of transcription (STATs).

PKR is constitutively expressed at low levels in chondrocytes, as well as in a variety of other cell types. Binding of PKR to double stranded RNA or a protein activator results in its autophosphorylation, dimerization, and kinase activity. One of the known PKR-activating proteins is PACT [9], which is overexpressed and hyperphosphorylated in tissues of aging mice [10] and overexpressed in susceptible regions of cartilage in a spontaneous model of osteoarthritis [11]. Co-stimulation of bovine articular cartilage with TNF α and IL-1 α causes a prolonged increase in the phosphorylation of PKR [12]. By immunochemical techniques, specifically phosphorylated eIF2 α was detected in chondrocytes 3 hours after TNF α stimulation [12]. Treatment of immortalized chondrocytes of the C28/I2 cell line with IL-1 β resulted in the prolonged phosphorylation of eIF2 α , postulated to be part of the ER stress response catalyzed by the kinase PERK [13]. The PKR inhibitor 2-aminopurine blocked TNF α and C2-ceramide-stimulated cartilage degradation, chondrocyte apoptosis, and secretion and activation of matrix metalloproteases; however, only high concentrations that are known to inhibit other protein kinases were effective [14–16].

These results suggest that PKR may play a role in pathways associated with tissue destruction in arthritis; therefore, it is crucial to better understand the mechanisms and consequences of PKR activation in articular cartilage. Cytokines have been implicated in the pathogenesis of arthritis, with evidence from in vivo antagonist studies pointing to IL-1 as a major culprit in cartilage degeneration in rheumatoid arthritis and osteoarthritis [2,3]. The objectives of this study were to exploit a potent inhibitor of PKR to assess the necessity of this kinase for IL-1 α -stimulated catabolic and proinflammatory effects in cartilage and to determine whether IL-1 α causes an increase in eIF2 α phosphorylation that is antagonized by the PKR inhibitor.

2. Materials and methods

2.1. Materials

Human recombinant IL-1 α was a gift from the Biological Resources Branch of National Cancer Institute. The PKR inhibitor (catalogue # 527450) was from EMD Biosciences, La Jolla, CA. Monoclonal antibodies to human COX-1 and COX-2 were from Cayman Chemical, Ann Arbor, MI. Antibodies to β -actin were from Sigma, St. Louis, MO. Antibodies to phosphorylation-specific and total eIF2 α were from Cell Signaling Technology, Beverly, MA. Phospho-specific antibody to PKR phosphorylated on threonine 451 of the human sequence was purchased from Biosource International, Inc., Camarillo, CA. Dimethyl methylene blue was from Serva Biochemical, Heidelberg, Germany or Polysciences, Warrington, PA. Dulbecco's modified Eagle's medium, ITS supplement, and antibiotics were from Mediatech,

Herndon, GA. Complete Protease Inhibitor Cocktail Tablets were from Roche Molecular Biochemicals, Mannheim, Germany. Phosphatase Inhibitor Cocktail 2 was from Sigma.

2.2. Cartilage explant culture

Cartilage cores (4-mm diameter) were taken from radiocarpal joints of one- to two-week-old calves, and 1-mm-thick disks were cut from the articular surfaces. The disks of cartilage were washed and placed in serum-free Dulbecco's modified Eagle's medium containing 5 µg/ml human recombinant insulin, 5 µg/ml human transferrin, 5 ng/ml selenious acid, 100 U/ml penicillin and 100 µg/ml streptomycin (DMEM). Prior to initiating the experiments, disks were equilibrated to culture conditions (5% CO₂, 95% humidified air 37 °C) in DMEM for 5 days. Medium was changed at one- to three-day intervals and 24 hours before initiating experiments.

2.3. Experimental treatment of cartilage disks

DMEM for experiments was supplemented with 10 µg/ml bovine serum albumin (BSA) to prevent nonspecific absorption of low abundance proteins. Cartilage disks were distributed as individual replicates in 96-well plates. Disks were preincubated with PKR inhibitor for 2 hours and then incubated with or without human rIL-1α (10 ng/ml) for the indicated time. Disks and media were separated and stored at -70° C for subsequent analyses.

2.4. Proteoglycan release

Cartilage proteoglycan degradation was assessed by measuring sulfated glycosaminoglycan (GAG) released into culture media using dimethyl methylene blue with chondroitin sulfate as a standard [17]. Results are expressed as µg of GAG released per disk.

2.5. Prostaglandin E₂ Release

Aliquots of culture media from cartilage disks were diluted at least 1:5 and analyzed for prostaglandin E₂ (PGE₂) using Parameter Competitive Elisa Kits purchased from R&D Systems, Minneapolis, MN. In samples of media where PGE₂ was below the limit of detection even at 1:5 dilution, values are recorded as zero. PGE₂ release is reported as pg/disk.

2.6. Cytotoxicity assays

The CytoTox-One assay (Promega Corp. Madison, WI) was used to measure lactate dehydrogenase (LDH) released into culture medium from non-viable cells in the cartilage disks as previously described [18].

2.7. Preparation of cartilage disk extracts and Western blot analysis

Cellular proteins were extracted from disks using 50 µl per disk of detergent-containing buffer (20 mM Tris HCl pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT), supplemented with protease inhibitor and phosphatase inhibitor cocktails) by gentle agitation at 4°C for 4 hours. Proteins in aliquots of disk extract equivalent to one half of one disk were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes. Transferred proteins were stained with Ponceau S to monitor protein loading and transfer. Proteins of interest were detected using specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies with chemiluminescent detection. Blots were scanned and analyzed for net signal intensity using Kodak Image Station 440 software. Signals for phosphorylated eIF2α were normalized to signals obtained using antibody to β-actin or an antibody that recognizes eIF2α regardless of its phosphorylation status on parallel immunoblots. Membranes examined for specifically phosphorylated PKR were reprobbed using an antibody to β-actin, and signals for phosphorylated PKR were normalized to the β-actin signals.

2.8. Statistical analysis

Statistical analysis was carried out using GraphPad Prism software. One-way analysis of variance (ANOVA) with Tukey post-hoc comparison of groups was used to test for significant effects of the PKR inhibitor on IL-1 α -stimulated effects in cartilage. IC50 was estimated by linear regression of GAG release (8 individual replicates for each condition) versus log concentration with standard slope and without weighting.

3. Results

3.1. Inhibition of IL-1 α -stimulated effects in cartilage by a potent PKR inhibitor

PKR has been reported to play a role in cartilage degradation activated by TNF α or C2-ceramide [14]. However, PKR involvement in the catabolic effects of IL-1 α was not addressed, and 2-aminopurine, the PKR inhibitor that supported this contention, is known to inhibit other processes at the doses used. To further investigate the issue, a potent cell permeable inhibitor of PKR kinase activity was tested for its effect on the degradation of proteoglycan in IL-1 α -stimulated bovine articular cartilage. The measurement of GAG released into culture medium is a well-characterized assay for catabolism of proteoglycan in the cartilage extracellular matrix. IL-1 α -stimulated GAG release was inhibited in dose dependent manner by the PKR inhibitor ($P < 0.001$, estimated IC50 of 267 nM), with complete inhibition at 1 μ M ($P > 0.05$ relative to basal GAG release) (Fig. 1). IL-1-stimulated proteoglycan degradation is a metabolic process that requires live chondrocytes. There was no significant effect of the PKR inhibitor on the release of LDH at concentrations of PKR inhibitor up to 6 μ M, and therefore the inhibition of GAG release by the PKR inhibitor was not due to toxicity.

IL-1 stimulation of cartilage leads to the production of proinflammatory small molecule mediators such as PGE₂ and nitric oxide that are generated through the action of the inducible enzymes cyclooxygenase-2 (COX-2) and nitric oxide synthase II (NOS II). In order to investigate whether PKR is required for IL-1 activation of the eicosanoid pathway, the effect of the PKR inhibitor on COX-2 levels in IL-1 α -activated cartilage was examined by Western blot analysis of disk extracts. COX-2 was not detected in protein extracts prepared from disks of cartilage incubated in the absence of IL-1 α . IL-1 α stimulation for 24 hours resulted in the accumulation of COX-2 in the disks, and the PKR inhibitor reduced IL-1 α -induced COX-2 accumulation in a dose dependent manner with suppression evident at the lowest dose tested (100 nM) and almost complete inhibition at 500 nM (Fig. 2). PGE₂ is the principal COX-2-dependent prostanoid mediator in chondrocytes, and the PKR inhibitor at 5 μ M significantly suppressed IL-1 α -stimulated PGE₂ production. Surprisingly, concentrations of the PKR inhibitor in the nanomolar range increased IL-1 α -stimulated PGE₂ production with a significant ($P < 0.05$) effect at 500 nM (Fig.3).

3.2. Phosphorylation of eIF2 α in IL-1 α -activated cartilage is sensitive to the PKR inhibitor

Previous studies showed an increase in the specific phosphorylation of PKR in bovine articular cartilage detectable 6 hours after co-stimulation with TNF α and IL-1 α , and phosphorylated eIF2 α was evident by immunocytochemical staining of chondrocytes 3 hours after TNF α stimulation. In order to assess whether IL-1 α alone activates PKR and leads to the phosphorylation of eIF2 α , cartilage disks were preincubated for 2 hours with the PKR inhibitor and then stimulated with IL-1 α for various time intervals. DTT (3 mM) was used as a positive control for eIF2 α phosphorylation, because it reportedly activates PKR and PERK in other cell types [15,19]. Despite the presence of insulin to counter the stresses of serum-free culture [20,21] and consistent with previous reports, low level phosphorylation of PKR and eIF2 α was apparent under basal conditions. IL-1 α treatment increased PKR and eIF2 α phosphorylation 2-fold within 30 minutes (fig. 4A). Importantly, the PKR inhibitor at concentrations as low as 20 nM almost totally prevented the change in phosphorylation of PKR and eIF2 α in IL-1 α -

activated cartilage. DTT treatment of cartilage disks for 30 minutes modestly increased the phosphorylation of eIF2 α but not PKR. Furthermore, eIF2 α phosphorylation was not sensitive to the PKR inhibitor in this system. Hyperphosphorylated eIF2 α was still evident in cartilage that had been incubated with IL-1 α for 24 hours (fig. 4B).

4. Discussion

The diverse effects of IL-1 in cartilage are elicited through pathways that remain poorly defined but may involve PKR. The PKR inhibitor that was identified by Jammi and colleagues [22] inhibits IL-1 α -stimulated cartilage proteoglycan degradation and suppresses the production of COX-2 in IL-1 α -activated bovine articular cartilage. The potency of this inhibitor against these IL-1 α -stimulated effects in cartilage explant assays is at least three orders of magnitude greater than for 2-aminopurine and is comparable to inhibition of PKR autophosphorylation in the cell free system [22]. Low level basal phosphorylation of both PKR and eIF2 α was evident in unstimulated bovine articular cartilage explants, in agreement with previous reports [12]. IL-1 α treatment increased the phosphorylation of both PKR and eIF2 α in cartilage. PKR inhibitor concentrations as low as 20 nM blocked the changes in phosphorylation of PKR and eIF2 α , defining PKR as the IL-1 α -activated eIF2 α kinase.

These data support and extend the work of Gilbert and colleagues, providing strong evidence that the kinase activity of PKR is required for at least some of the effects of IL-1 α in cartilage. The confirmation is important because the inhibition by 2-aminopurine, which is known to affect other processes and kinases including MAPKs [15,16], provided the primary support for a PKR role in cytokine-activated cartilage catabolism. The discovery of a potent selective inhibitor for PKR in a library of ATP-binding site directed compounds has provided a powerful new tool for dissecting the role of PKR in IL-1-activated cartilage. Compound #16 in the library partially restored translation inhibited by a PKR kinase domain construct in a rabbit reticulocyte cell free translation system and inhibited poly(rI: rC)-activated PKR autophosphorylation (IC₅₀ approximately 200 nM) [22]. By comparison, the IC₅₀ for 2-aminopurine was 10 mM.

The PKR inhibitor has previously been shown to be effective in cultured cells at approximately the concentrations that blocked double stranded RNA-activated PKR autophosphorylation and rescued PKR-inhibited translation. It protected against tunicamycin-induced apoptosis, prevented PKR autophosphorylation, and reduced caspase-3 activation in cultured neuroblastoma cells [23,24]. In bronchial epithelial cells, either rhinovirus infection or stimulation with a double stranded RNA analogue activated cytokine production, and this potent PKR inhibitor significantly blocked these effects at 20 nM [25]. IL-1 has been implicated in a variety of pathological conditions, including cancer, inflammation, degenerative diseases, and cardiovascular diseases [26–30]. Therefore, it is important to determine whether PKR kinase activity is involved in IL-1-activated effects in other cell types.

Although the PKR inhibitor has been shown to be selective and potent, additional studies with purified molecules are necessary to assure its specificity. IL-1 α activates catabolic, anti-anabolic, and proinflammatory pathways in cartilage, and we have investigated whether PKR is required in a limited subset of these effects. PKR is likely to be involved in other IL-1 α -activated pathways that would therefore be susceptible to similar modulation by the PKR inhibitor. Certainly this should be addressed. For reasons that are unclear, the PKR inhibitor affected PGE₂ production with a biphasic dose response relationship; nanomolar PKR inhibitor concentrations that suppressed COX-2 accumulation paradoxically stimulated PGE₂ production, while low micromolar concentrations were inhibitory.

The complex regulation of the eicosanoid metabolic pathway and the possibility for diverting among alternative eicosanoids complicate identification of the mechanisms involved in the

approximately 2-fold stimulation of PGE₂ release by nanomolar concentrations of PKR inhibitor. Cyclooxygenases convert arachidonic acid liberated from cell membranes by phospholipase A2 to PGG₂ and PGH₂. Further conversion to various eicosanoid mediators requires the action of specific synthases; for PGE₂ formation, this step is catalyzed by a prostaglandin E synthase (PGES). Low dose PKR inhibitor may modulate the levels or activities of enzymes in this pathway, leading to increased PGE₂ despite the reduction in COX-2. We considered the possibility of an inhibitor-activated increase in the constitutive cyclooxygenase COX-1, but preliminary Western blot analysis did not support this mechanism (data not shown). The concept of cross talk between COX-2 and NOS II pathways remains controversial, but nitric oxide, reactive oxygen species, and derivatives have been shown to either activate or inhibit cyclooxygenase catalytic activity and PGE₂ formation [31–36]. This suggests a possible NOS II-mediated mechanism whereby nanomolar PKR inhibitor concentrations could increase enzymatic activity and PGE₂ formation. Additional studies are clearly warranted.

The early and prolonged increase in PKR inhibitor-sensitive hyperphosphorylation of eIF2 α reported here, together with the prolonged activation of PKR after co-stimulation with IL-1 α and TNF α [14], suggest that PKR activity could play roles in initial signal transduction as well as the delayed effects of IL-1 α . The relevant target(s) of PKR kinase activity in the suppression of IL-1 α -stimulated effects in cartilage may be eIF2 α or some other substrate, and the molecular pathways remain to be clarified.

The data presented here demonstrate that this potent PKR inhibitor counteracts IL-1 α -activated catabolic and proinflammatory effects in cartilage. IL-1 is probably the most important cytokine in the pathological destruction of cartilage in osteoarthritis, and perhaps in rheumatoid arthritis as well [3]. It increases the production and activity of degradative enzymes that damage the extracellular matrix; perhaps more importantly, it potently disrupts tissue repair by inhibiting the synthesis of the cartilage specific macromolecules, type II collagen and aggrecan, that are essential for biomechanical function. Therefore, it is critical to understand the molecular pathways that are activated by IL-1 in chondrocytes. If PKR is found to be an essential element for IL-1-stimulated effects in human articular cartilage, it should be viewed as a potential target for pharmaceutical invention in osteoarthritis. There are currently no therapeutic agents with proven disease modifying properties for this common age-related joint disease.

Acknowledgement

This work was supported in part by the National Institute of Aging (AG20987) and The Wechsler Fund.

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Fig. 1.

The PKR inhibitor suppresses IL-1 α -stimulated cartilage proteoglycan degradation. Cartilage disks ($n = 8$) were placed individually in 200 μ L DMEM supplemented with 10 μ g/ml BSA in the wells of a 96-well plate. Disks were preincubated for 2 hours with the PKR inhibitor (PKRi) at the indicated concentration. IL-1 α (10 μ g/ml) was added (black bars) or not (open bar), and incubation was continued for 24 hours. Proteoglycan degradation was assessed by assaying aliquots of culture media for GAG. Values are reported as mean $\pm$ S. E. M. Significance of difference of IL-1 α -stimulated GAG release in PKR inhibitor-treated disks relative to non-inhibited disks: ***, $p < 0.001$; ns, not significant.

Fig. 2.

The PKR inhibitor reduces the accumulation of COX-2 in IL-1 α -activated cartilage. Cartilage disks (n = 8) were preincubated for 2 hours with the indicated concentration of the PKR inhibitor in DMEM supplemented with BSA. IL-1 α was added to selected wells, and incubation was continued for 24 hours. Protein extracts were prepared from cartilage disks for analysis by Western blotting using antibody to COX-2 with anti- β -actin to monitor loading and transfer. COX-2 accumulation was dependent on IL-1 α and inhibited by the PKR inhibitor.

Fig. 3. The PKR inhibitor has biphasic dose-dependent effects on IL-1 α -stimulated PGE₂ production. Cartilage disks (n = 8) were preincubated for 2 hours with the PKR inhibitor at the indicated concentration. IL-1 α (10 ng/ml) was added (black bars) or not (open bar not visible), and incubation was continued for 24 hours. Harvested culture media were assayed for PGE₂ by ELISA. Significance of difference of IL-1 α -stimulated PGE₂ release in PKR inhibitor-treated disks relative to non-inhibited disks: *, $p < 0.05$; ns, not significant.

Fig. 4.

The PKR inhibitor prevents the IL-1 α -stimulated increase in phosphorylation of PKR and eIF2 α in cartilage. (A) Cartilage disks were preincubated for 2 hours with or without 20 η M PKR inhibitor. IL-1 α (10 η g/ml) or 3 mM DTT was added as indicated, and the incubation was continued for 30 minutes. Protein extracts of the disks were analyzed by Western blot for phosphorylated eIF2 α , phosphorylated PKR, and β -actin. Signals for phosphorylated proteins were normalized to signals obtained using antibody to β -actin. Fold-stimulation shown below the Western blot for each phosphorylated protein is calculated as the ratio of each normalized signal relative to the normalized signal for the extract from unstimulated cartilage disks shown in lane 1. (B) Disks were preincubated for 2 hours with the indicated concentrations of PKR inhibitor before the addition of IL-1 α to selected wells. The incubation was continued for 24 hours. Phosphorylated and total eIF2 α in disk extracts were analyzed by Western blot, and the signals for the phosphorylated form were normalized to signals for total eIF2 α . Fold-stimulation is determined as in the legend for Fig. 4B. IL-1 α caused a rapid and prolonged increase in eIF2 α phosphorylation that was inhibited in disks treated with the PKR inhibitor.