Cyclic GMP and cGMP-binding Phosphodiesterase Are Required for Interleukin-1-induced Nitric Oxide Synthesis in Human Articular Chondrocytes*

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This study addressed the role of guanylyl cyclase (GC) and phosphodiesterase (PDE) in interleukin (IL)-1 activation of human articular chondrocytes. The GC inhibitors LY83583 and methylene blue dose-dependently inhibited IL-1-induced nitric oxide (NO) production, inducible NO synthase (iNOS) protein, and mRNA expression. These effects of GC inhibition were consistent with the rapid induction of cGMP by IL-1, which reached maximal levels after 5 min. The effects of GC inhibitors were selective as they did not reduce IL-1-induced cyclooxygenase II protein and mRNA. An inhibitor specific for soluble GC did not affect IL-1-induced NO production, and activators of soluble GC did not induce NO. However, the expression of iNOS mRNA was induced by atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), activators of particulate GC, indicating that particulate rather than soluble guanylyl cyclases were involved in iNOS induction. The expression of iNOS mRNA and the production of NO were induced by a slowly hydrolyzable analog of cGMP, 8-bromo-cGMP, but not by nonhydrolyzable analog, dibutylry cGMP, suggesting that PDE rather than cGMP-dependent protein kinase mediates the cGMP effects. Chondrocytes contained extensive cGMP PDE activity. This had PDE5 biochemical features and an inhibitor profile consistent with PDE5. Furthermore, the nonisoform-specific PDE inhibitor IBMX and PDE5-specific inhibitors suppressed IL-1-induced NO release and iNOS mRNA expression. PDE5 mRNA was constitutively expressed in chondrocytes. In addition to increasing PDE5 activities, IL-1 treatment reduced the sensitivity of PDE5 to several pharmacological inhibitors by up to 50-fold. In summary, inhibitors of either GC or PDE5 prevented IL-1 induction of iNOS; IL-1 increased the rates of both cGMP generation and hydrolysis; and exogenous PDE hydrolyzable cGMP analog induced iNOS and NO. These results suggest that increased cGMP metabolic flux is sufficient to induce iNOS, and GC and PDE5 activities are required for IL-1 induction of iNOS expression via increases in coupled cGMP synthesis and hydrolysis.

** IL-11 promotes cartilage catabolism through the induction of protases and the inhibition of extracellular matrix synthesis. IL-1 inhibits chondrocyte proliferation and suppresses collagen type II and aggrecan production (1). Some of these effects are mediated by the IL-1 induction of nitric oxide (2, 3). IL-1-induced signal transduction occurs via two IL-1 receptor subunits, the IL-1 receptor I (IL-1RI) and the IL-1 receptor accessory protein (IL-1RAcP) (4, 5). Diverse intracellular signaling pathways are activated by IL-1. Although there are tissue-specific differences in the IL-1-activated signaling pathways, even in the same cell type there are multiple IL-1-induced second messengers that differentially regulate subsets of IL-1-responsive genes. In smooth muscle cells, IL-1 can activate guanylyl cyclase in nitric oxide (NO)-dependent and NO-independent pathways (6, 7). IL-1 activates phospholipase A2 (8) and stimulates expression of the inducible cyclooxygenase (COX-2) (9), thus increasing levels of prostaglandin E2. The role of protein kinase C (PKC) in IL-1 activation of chondrocytes is unclear (10–13). IL-1 shares with tumor necrosis factor (TNF) the ability to induce the channel membrane by activating sphingomyelinase (14, 15). IL-1 activates mitogen-activated protein kinase (MAPK) and MAPK kinase (MKK) (16). Evidence for tyrosine kinase activation by IL-1 has been provided in several cell systems, including T lymphocytes (17) and chondrocytes (18). NO is a regulator of homeostatic cell function (19, 20) but can also cause cell damage and contribute to the pathogenesis of inflammatory diseases such as arthritis. Complementary DNA encoding inducible NOS from macrophages (21), hepatocytes (22), and chondrocytes (23, 24) have been cloned. The cytokines IL-1, TNF, and IFN-γ and bacterial LPS can induce NO synthesis. Studies on intracellular signals that regulate iNOS expression have shown cell-type specific differences. Increases in intracellular cAMP levels enhanced IFN-γ-induced NOS expression in vascular smooth muscle cells (25) but partially reduced LPS- and IFN-γ-induced NO production in murine macrophages (26). PKC appears to be involved in iNOS expression in macrophages and hepatocytes (27, 28). Tyrosine kinase inhibitors blocked LPS-induced NO release in murine macrophages (29). Articular chondrocytes activated by single stimuli such as IL-1 or LPS produce high levels of NO (30–32). Previous studies indicated that activation of protein tyrosine kinases but not of protein kinase A nor protein kinase C is

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¶ The abbreviations used are: IL-1, interleukin 1; 8-Br-cGMP, 8-bromo-cGMP; ANP, atrial natriuretic peptide; cGK, cGMP-dependent protein kinase; CNP, C-type natriuretic peptide; COX, cyclooxygenase; Bt2cGMP, dibutylry cGMP; GC, guanylyl cyclase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, S-nitrosoglutathione; IBMX, isobutylmethylxanthine; MB, methylene blue; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; RPA, ribonucleas probe assay; SNAP, S-nitroso-N-acetyl-penicillamine; SNP, sodium nitroprusside; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; MXZ, 4-[(3,4-methylenedioxybenzyl)amino]-6-methoxyquinazoline; IFN-γ, interferon γ; LPS, lipopolysaccharide.
required for iNOS mRNA expression in chondrocytes (18). Also, increased levels of intracellular calcium were not associated with the induction of NO synthesis. However, when IL-1-activated chondrocytes were treated with drugs that increase intracellular calcium, NO synthesis was completely suppressed via a reduction in iNOS mRNA half-life (33). In chondrocytes, IL-1 increases levels of cGMP, and this is at least in part NO-mediated (32). The role of increased cGMP levels in IL-1-induced gene expression and iNOS induction, as well as the downstream target(s) of cGMP, is unknown. The present study demonstrates that the activities of guanylyl cyclase and cyclic GMP phosphodiesterase are required for the induction of iNOS by IL-1. COX-2, also an IL-1-induced gene in chondrocytes, is not cGMP dependent. Pharmacological, biochemical and molecular characterization suggests that the chondrocyte cGMP phosphodiesterase (PDE) involved in iNOS induction is PDE5 and undergoes posttranslational modification in response to IL-1.

EXPERIMENTAL PROCEDURES

Chondrocyte Isolation and Culture—Cartilage was obtained at autopsy from donors with no history of joint disease. Chondrocytes were isolated by collagenase digestion of cartilage from the femoral condyles and tibial plateaus of the knee joints and cultured as described previously (34). All experiments were performed with primary or passage 1 and tibial plateaus of the knee joints and cultured as described previously (36). The sources of the PDE inhibitors were: trequinsin (HL-725), Hoechst-Roussel Pharmaceuticals, Inc. (Paris, France); rolipram, Schering AG (Berlin, Germany); indolilide, Eli Lilly Co. (Indianapolis, IN); zaprinast, May and Baker, Ltd.; CGS-9343B, Ciba-Geigy Corporation (Summit, NJ); LY38558, dipryridamole, MY5448 and MXZ from Calbiochem (La Jolla, CA). All inhibitors were dissolved in 100% Me2SO. The vehicle at the concentration used did not influence chondrocyte viability and PDE activity.

RESULTS

Induction of NO Release, iNOS Protein, and mRNA Expression Depend on Guanylyl Cyclase Activation—LY38558, an inhibitor of guanylyl cyclases (GC) was used to study the role of cGMP in IL-1-induced NO release and iNOS mRNA expression in cultures of primary human articular chondrocytes. LY38558 dose-dependently inhibited IL-1-induced NO release (Fig. 1A). Complete inhibition of NO release by LY38558 was observed at nanomolar concentrations. An IC_{50} of 250 nM was determined. The next set of experiments addressed whether the reduction in IL-1-induced NO release by LY38558 was because of a reduction in iNOS protein. Analysis by Western blot showed that LY38558 or methylene blue (MB), another GC inhibitor, reduced IL-1-induced iNOS protein almost to background levels in cultures of primary human articular chondrocytes. LY38558 at 10 μM reduced IL-1-induced NO release in chondrocytes although LY38558 completely blocked NO release in the same experiment (Fig. 2A). These results suggest that soluble GC appear not to be required for iNOS induction. Specific
activators of soluble or particulate GC were studied in the next set of experiments. Among the seven membrane GC (GC-A to -G) (43, 44), ligands have been characterized for three. They are ANP and brain natriuretic peptide (BNP) for GC-A; CNP for GC-B; and heat-stable enterotoxin (STa), guanylin, and uroguan for GC-C (43). GC-A and GC-B are expressed in rat chondrocytes, and ANP and CNP induced cGMP synthesis in these cells (45). Both, ANP and CNP peptides induced iNOS mRNA expression as detected by RPA although they were much less potent than IL-1 (Fig. 2B). Among three forms of soluble GC heterodimers, two, α1β1 and α2β1, are activated by NO (43); the activator for the third form is unknown. However, two NO generators, SNAP and GSNO, which generate NO in solution, failed to induce iNOS expression (Fig. 2B). Sodium nitroprusside (SNP), another NO donor, was also not able to induce iNOS (not shown). These results are consistent with our earlier observations using a soluble GC-specific inhibitor and suggest that particulate rather than soluble GC is involved in iNOS induction.

Cyclic GMP-dependent Protein Kinase and Ca²⁺ Channels Are Not Mediating cGMP Effects in IL-1 Activation of Chondrocytes—Cellular responses are regulated by cGMP through several different downstream mechanisms, including calcium influx by activation of calcium channels, protein phosphorylation by cGMP-dependent protein kinase (cGK) and hydrolysis of cGMP and/or cAMP by activation or inhibition of PDE (46). The next set of experiments was designed to examine the downstream targets of cGMP. The possible role of cGK and Ca²⁺ channels in NO release and iNOS mRNA expression was studied by using the cGMP analog Bt₂cGMP, which can mimic native cGMP to activate cGK and Ca²⁺ channels but is not hydrolyzed by PDE. This analog did not induce NO release (Fig. 2B) nor iNOS mRNA expression (not shown). Additionally, this analog did not reverse the effects of the GC inhibitor LY83583. Two cGK inhibitors, KT5823 and Rp-8-cPT-cGMP, did not reduce IL-1-induced NO release, iNOS protein, and mRNA expression (not shown). The calcium channel blockers nifedipine and diltiazem also had no effect on IL-1-induced NO release. Chondrocyte lysates were tested directly for cGK activity using the relatively selective substrate BPDEtide. The activity of cGK was not detectable in nonstimulated or IL-1-stimulated chondrocytes. These results suggest that cGK or Ca²⁺ channels do not function as downstream targets of cGMP in the IL-1 induction of iNOS.

Phosphodiesterases Are Involved in IL-1 Induction of NO—Because cGK or Ca²⁺ channels appeared not to be involved in the iNOS induction, the effect of another cGMP target protein, PDE, was examined by using a slowly hydrolyzable analog of cGMP (47, 48), 8-bromo-cGMP (8-b-cGMP). 8-b-cGMP dose-dependently induced NO release (Fig. 3A) although at much lower levels as compared with IL-1 (Fig. 3C). The time course of NO induction by 8-b-cGMP (Fig. 3B) was similar to that of IL-1 (18). 8-b-cGMP had additive effects with IL-1 in the induction of NO (Fig. 3C). In the same experiments, NO production was induced by 8-b-cGMP but not by Bt₂cGMP nor 8-Br-cAMP (Fig. 3D) at any of the doses tested. To further characterize the role of 8-Br-cGMP in NO induction, iNOS protein and mRNA were determined. 8-Br-cGMP did induce iNOS mRNA expression (Fig. 2B) and protein synthesis (not shown). These results further argue against a role of cGK and Ca²⁺ channels and suggest a role of cGMP PDE in IL-1-induced NO production. To further determine whether PDE is required for iNOS induction via mediating cGMP hydrolysis, the xanthine analog IBMX, a commonly used inhibitor of all PDEs, was not able to reverse the effects of the GC inhibitor LY83583. These results are consistent with our earlier observations using a soluble GC-specific inhibitor and suggest that particulate rather than soluble GC is involved in iNOS induction.
isoforms, was examined. IBMX alone did not induce NO release although it can increase intracellular cGMP concentrations by blocking cGMP hydrolysis. In contrast, it caused a dose-dependent reduction in NO release (Fig. 4A), iNOS mRNA expression (Fig. 4B), as well as iNOS protein levels (not shown) in IL-1-stimulated chondrocytes. IBMX did not reduce IL-1-induced COX-2 mRNA levels even at 1 mM dose, indicating that the inhibition of NO release and iNOS expression was not the consequence of nonspecific effects on the cells. These results suggested that PDE is involved in the cGMP-mediated iNOS induction. Increases in cGMP concentrations followed by hydrolysis by PDE rather than changes in steady state cGMP levels are sufficient to induce iNOS expression and are required for IL-1 induction of iNOS in chondrocytes.

Chondrocytes Contain Large Amounts of cGMP PDE Activity Which Increases after IL-1 Stimulation—Chondrocyte PDE was further characterized. Whole cell lysates, and pellets and supernatants obtained after ultracentrifugation of the whole cell lysates were tested for the major isoforms of PDE activity. Whole cell lysates contained large amounts of cGMP PDE that were approximately 100-fold higher than those of cAMP PDE (Table I). Most of the cGMP PDE activity was in the supernatant (30,388 pmol/min/mg in supernatant versus 7,060 pmol/min/mg in pellet), indicating that this PDE was in the cytosolic fraction (Table I). To determine whether GC and PDE are activated by IL-1 and whether these events are prior to iNOS mRNA induction, cGMP and cGMP PDE activity were analyzed. IL-1 induced a rapid and large increase in cGMP levels in chondrocytes. Total cellular cGMP was maximal within 5 min of IL-1 treatment and decreased to base line after 2 h (Fig. 5). The increased cGMP levels declined after 5 min of IL-1 stimulation followed by an increase in cGMP PDE activity (Fig. 5). Cyclic GMP PDE activity in the supernatant from the ultracentrifugation of the cell lysates was determined. IL-1 caused a time-dependent increase in cGMP PDE activity that was already evident after 5 min and approximately 10-fold higher than background after 60 min (Fig. 5). These results showed that chondrocytes contain large amounts of cGMP-specific PDE activity and that IL-1 increased both cGMP synthesis and hydrolysis by activation of guanylyl cyclase and cGMP PDE, respectively, and these events preceded iNOS induction.

Analysis of cGMP PDE Isoforms Required for iNOS Induction—The isoform of chondrocyte cGMP PDE was characterized by biochemical, pharmacological, and molecular criteria. Seven PDE isoforms have been identified based on cDNA sequences, modulator, and substrate specificity (49, 50). They are Ca2+/calmodulin-stimulated (PDE1), cGMP-stimulated (PDE2), cGMP-inhibited (PDE3), cAMP-specific (PDE4), cGMP-specific (PDE5), photoreceptor (PDE6), and high affinity cAMP-specific (PDE7). Neither calcium stimulation, nor stimulation or inhibition by cGMP of enzyme activity from the anion-exchange chromatography-isolated chondrocyte PDE was found, and the chondrocyte cGMP PDE did not hydrolyze cAMP (not shown). Thus, in chondrocytes the dominant isozyme is cGMP-specific PDE. For more detailed analysis of chondrocyte PDE isozymes, cytosolic cGMP PDE activity from unstimulated and IL-1-treated cells were isolated on anion-exchange chromatography and characterized using pharmacological inhibitors that are selective for the different PDE isoforms (Table II). Cyclic GMP PDE from unstimulated cells was most sensitive to the nonselective inhibitor IBMX (IC50 = 0.1 µM) and sensitive to the three PDE5-specific inhibitors dipyridamole, zaprinast, and E4021. Cyclic GMP PDE from IL-1-treated cells was most sensitive to the nonselective inhibitor IBMX (IC50 = 0.1 µM) and sensitive to the three PDE5-specific inhibitors dipyridamole, zaprinast, and E4021 with similar IC50 of the drugs except E4021. Chondrocyte cGMP PDE was not sensitive to the inhibitors of other PDE isoforms, CGS9943B (PDE1), indolidan (PDE3), rolipram (PDE4), and HL725, which is less selective. This rank order was similar in unstimulated and IL-1-treated
cells. However, there was a 30–50-fold increase in the IC₅₀ for nonselective and PDE5-specific inhibitors after IL-1 treatment (Table II). These results suggest that the chondrocyte cGMP PDE is PDE5. The role of PDE isoforms in chondrocyte activation by IL-1 was further determined in intact cells with isoform-specific inhibitors. CGS9343B, indolidan, and rolipram did not suppress IL-1-induced NO release (not shown). PDE5-specific inhibitors dipyridamole, MY5445, and MXZ reduced NO induction by IL-1 (Fig. 6A). The most commonly used PDE5 inhibitor dipyridamole (51), selectively reduced iNOS induction by IL-1 and LPS (Fig. 6B). In contrast, LPS and IL-1-induced COX-2 expression were extensively enhanced by this drug in the same cell cultures (Fig. 6C). These PDE5-specific inhibitors, like IBMX, also suppressed 8-Br-cGMP-induced NO production (Fig. 6D). PDE5 cDNAs from bovine and rat have been cloned (38, 52) but the human homologue has not been identified. Recently, a human PDE5 cDNA fragment corresponding to amino acids 146–401 of regulatory domains of bovine cGMP-binding, cGMP-specific PDE (PDE5) was cloned from human eye trabecular meshwork cells by using reverse transcription-PCR.² This 768-base pair human PDE5 cDNA fragment shares 94.4% sequence identity with bovine PDE5 cDNA. These PCR primers share 100% sequence identity with bovine PDE5 and were used to amplify chondrocyte cDNA. A single PCR product with predicted molecular size was amplified in nontreated human articular chondrocytes (Fig. 6D). A data base search showed that these primer sequences have no homology with other isoforms of PDE. These results demonstrate that chondrocyte cGMP PDE is biochemically, pharmacologically, and molecularly similar to cGMP-binding, cGMP-specific PDE (PDE5), and this isozyme mediates cGMP-induced iNOS expression.

**DISCUSSION**

This study addressed the role of cGMP in IL-1 activation of human articular chondrocytes. The observations demonstrate that IL-1 increased levels of cGMP and PDE activity and that cGMP and PDE are required for IL-1-induced iNOS expression. The cGMP effect on iNOS expression was related to increased cGMP metabolic flux. These results provide new insight into the role of cGMP in IL-1 activation of chondrocytes.

² (L. Zhou and W. J. Thompson, unpublished data.)
the regulation of iNOS expression, the role of cGMP and PDE in IL-1 signaling, and the activation of chondrocytes.

Cyclic GMP-induced iNOS Expression via Its Metabolic Flux—Chondrocytes produce high levels of NO in response to stimuli that cause cartilage degradation (30–32). Several studies suggest that there are cell-specific differences in the intracellular signals that regulate iNOS expression. Unlike macrophage and smooth muscle cells, in chondrocytes PMA and cAMP did not induce iNOS and PKC, and PKA had no detectable mediating effects on IL-1 or LPS induction of iNOS (18). IL-1- or LPS-induced iNOS mRNA in chondrocytes had a remarkably long duration of expression that persisted for several days following stimulation of the cells only at initiation of culture. As NO is a known activator of GC (39), the present study addressed the role of GCMP in the induction of iNOS and other genes in chondrocytes. An analog of cGMP, 8-Br-cGMP, induced iNOS mRNA and protein expression and NO production in chondrocytes. Guanylyl cyclase inhibitors blocked the IL-1-induced iNOS expression and NO release, did not modulate COX-2 expression, but increased c-fos mRNA. Cyclic GMP-mediated gene expression was only demonstrated in phaeochromocytoma PC12 cells in which c-fos and junB were induced by a cGMP analog, a soluble GC activator, and a PDE inhibitor (53). Several lines of evidence in this study indicate that increases in cGMP metabolic flux rather than changes in cGMP steady state levels are mediating iNOS expression. First, GC and PDE have opposite effects on steady state cGMP levels, but inhibitors of these two distinct enzymes equally blocked IL-1 induction of iNOS. Second, IL-1 increased the rates of both CGMP generation and cGMP hydrolysis by activation of GC and cGMP PDE, respectively. Third, increases in intracellular cGMP levels by suppression of cGMP hydrolysis with PDE inhibitors or by nonhydrolyzable cGMP analog 

was prevented by cGMP PDE inhibitors. Induction of gene expression by cyclic nucleotide via activation of protein kinase has been well characterized (54). However, there is no information to demonstrate that hydrolysis or metabolic flux of guanosine and/or adenosine nucleotides is involved in gene activation. Metabolic flux of cGMP has been demonstrated in phototransduction in which GC, PDE, G-proteins, and ion channels are involved (55–58). The GTP/cGMP metabolic cycle includes GC, PDE, and enzymes that regulate GMP, GDP, and GTP metabolism. Results from this study suggested that increases in coupled cGMP synthesis and cGMP hydrolysis, rather than the changes in cGMP steady state levels, are essential for the induction of iNOS. This could be explained by the following mechanisms. 1) Blocking the GTP/cGMP metabolic cycle changes the status of ion channels that affect membrane polarization (57, 58). 2) Changes in PDE-regulated GTPase activity or in GTP and GDP concentrations alter G-protein activity (57, 58). 3) Release of free energy or hydrolytic products affects cellular function (59, 60).

Particulate Rather Than Soluble Guanylyl Cyclase Mediates iNOS Expression—Several experimental observations suggest that particulate GC is involved in iNOS expression. The IL-1 induction of NO release, iNOS protein, and mRNA accumulation was prevented by the two GC inhibitors LY83583 and MB but not by the well characterized potent and specific soluble GC inhibitor ODQ (41, 42). Furthermore, several soluble GC activators, SNAP, SNAP, and GSNO, failed to induce iNOS. In contrast, particulate GC activators ANP and CNP that activate GC-A and GC-B, respectively, induced iNOS. The results obtained with GC inhibitors and activators were supported by the rapid increase in cGMP levels in chondrocytes. Cytosolic cGMP levels were up-regulated by ANP and CNP but not by any of the tested soluble GC activators, indicating that cGMP was predominantly produced by membrane GC in this cell type. Both GC-A and GC-B receptors are constitutively expressed in
rat chondrocytes, and the intracellular concentration of cGMP was increased in response to CNP and ANP (45), which is consistent with the observations in this study. Whether a soluble form of GC is expressed in chondrocytes is unknown. These results indicate that putative GC in neurons, including GC-D to -G, were identified recently (43, 44) and more recently, increasing numbers in the receptor GC family, in-membrane GC activity via its kinase domain will be further analyzed. Myelinase (14, 15), and G-protein (66). Whether these signals define a novel aspect of IL-1 signaling in which cGMP and PDE5 selectively modulate the expression of IL-1-responsive genes via cGMP metabolic flux.

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