Increased Intracellular Ca\textsuperscript{2+} Selectively Suppresses IL-1-induced NO Production by Reducing iNOS mRNA Stability

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Abstract. This study addresses the role of intracellular calcium (Ca\textsuperscript{2+}) in the expression of iNOS, an IL-1 inducible gene in human articular chondrocytes.

The calcium ionophore A23187 and ionomycin did not induce NO release or iNOS expression but inhibited dose dependently IL-1-induced NO release with IC\textsubscript{50} of 200 nM and 100 nM, respectively. Increased intracellular Ca\textsuperscript{2+} induced by thapsigargin or cyclopiazonic acid, inhibitors of the endoplasmic reticulum Ca\textsuperscript{2+} ATPase, had similar inhibitory effects with IC\textsubscript{50} of 1 nM and 3 \mu M, respectively. LPS and TNF\alpha induced NO production were also suppressed by these Ca\textsuperscript{2+} elevating drugs.

Levels of IL-1-induced iNOS protein were reduced by A23187, thapsigargin, and cyclopiazonic acid. These drugs as well as Bay K 8644 and KCl inhibited IL-1-induced iNOS mRNA expression. To analyze the role of Ca\textsuperscript{2+} in the expression of other IL-1 responsive genes in chondrocytes, these Ca\textsuperscript{2+} modulating drugs were tested for effects on COXII. In contrast to the inhibitory effects on iNOS mRNA, these drugs induced COXII mRNA expression and in combination with IL-1, enhanced COXII mRNA levels. Ca\textsuperscript{2+} mediated increases in COXII mRNA expression were associated with an increase in COXII protein.

The kinetics of Ca\textsuperscript{2+} effects on IL-1-induced iNOS mRNA levels suggested a posttranscriptional mechanism. Analysis of iNOS mRNA half life showed that it was 6–7 h in IL-1-stimulated cells and decreased by A23187 to 2–3 h.

In conclusion, these results show that Ca\textsuperscript{2+} inhibits IL-1-induced NO release, iNOS protein, and mRNA expression in human articular chondrocytes by reducing iNOS mRNA stability. Under identical conditions increased Ca\textsuperscript{2+} enhances IL-1-induced COXII gene and protein expression.

Nitric oxide (NO)\textsuperscript{1} is involved as a signaling and effector molecule in diverse biological systems (24, 33). It is an important regulator in homeostatic cell functions; increased levels of NO production may cause cell damage and contribute to the pathogenesis arthritis, diabetes and other conditions (39, 41). NO is enzymatically formed from L-arginine by NO synthases (NOS) which have binding sites for NADPH, tetrahydrobipterin, and molecular oxygen. Constitutive NOS are primarily regulated by calcium/calmodulin (27). Phosphorylation of cNOS by protein kinase C (PKC), cAMP dependent protein kinase (PKA) and calcium/calmodulin dependent protein kinase (CaM-K) has been shown to modulate enzymatic activity (2, 4, 26, 32). In contrast, inducible NOS (iNOS) activity appears slowly after exposure of cells to cytokines and bacterial products, is sustained, depends on RNA and protein synthesis and the enzyme functions independently of calcium and calmodulin (27).

cDNAs encoding constitutive enzymes (1b, 3, 20, 23, 35) and inducible NOS from macrophages (44), hepatocytes (12), and chondrocytes (8, 25) have been cloned. In addition to these latter three cell types, iNOS expression has been observed in vascular smooth muscle cells, keratinocytes, and renal mesangial cells (6, 16, 29). Inducible NO synthesis has been characterized with respect to the identification of extracellular stimuli and a series of proinflammatory cytokines, including IL-1, TNF, interferon-\gamma (IFN\gamma), and bacterial lipopolysaccharide (LPS) can induce NO synthesis. Intracellular signals that regulate iNOS expression have been characterized in a small number of cell types. Increases in intracellular cAMP levels enhanced IFN\gamma-induced NOS expression in vascular smooth muscle cells (21) but partially reduced LPS and IFN\gamma-induced NO production in murine macrophages (5). PKC appears to be involved with iNOS expression in macrophages and hepa-

\textsuperscript{1}Abbreviations used in this paper: COXII, cyclooxygenase II; CPA, cyclopiazonic acid; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; PKA, cAMP dependent protein kinase; PKC, protein kinase C; RPA, RNAse protection assay; TG, Thapsigargin.

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tytocytes (17, 18, 36). Tyrosine kinase inhibitors blocked LPS or IL-1-induced NO release in murine macrophages and pancreatic islet cells (9, 10).

Articular chondrocytes activated by single stimuli such as IL-1 or LPS produce high levels of NO (28, 30, 38). In a previous study we reported that activation of protein tyrosine kinases is required for iNOS mRNA expression in these cells (14). Activation of protein kinase A and protein kinase C did not result in iNOS expression and was not required for the effects of IL-1 or LPS. As Ca$^{2+}$ is an important signal in the regulation of constitutive NOS and there are Ca$^{2+}$-sensitive genes in chondrocytes, we addressed in this study the role of Ca$^{2+}$ in the regulation of IL-1-induced iNOS expression in chondrocytes. We show that Ca$^{2+}$ differentially regulates gene expression in chondrocytes. Under identical activation conditions, increased intracellular levels of Ca$^{2+}$ inhibit iNOS expression but increased the expression of other IL-1 inducible genes such as cyclooxygenase II. The Ca$^{2+}$ effects on iNOS are related to a reduction in steady state mRNA levels by lowering iNOS mRNA stability.

Materials and Methods

Chondrocyte Isolation and Culture
Cartilage was obtained at autopsy from donors without known history of joint disease. For all experiments reported here, cartilage from the femoral condyles and tibial plateaus of the knee joints was used. Chondrocytes were isolated by collagenase digestion of cartilage and cultured as previously described (22). All experiments were performed with primary or passage 1 cells. For studies on the production of NO or iNOS mRNA expression, chondrocytes were plated in 96- or 24-well plates at 40,000 or 250,000 cells per well, respectively, and cultured in serum-free DMEM supplemented with L-glutamine and antibiotics.

Quantification of Nitrates
The concentration of nitrates, the stable end products of cellular NO breakdown, in conditioned media from chondrocytes was determined by the Griess reaction using NaNO$\text{}_2$ as standard. All results are expressed as mMoles nitrates per 100,000 cells.

Western Blotting
Chondrocytes were plated in 6-well plates and stimulated for 24 h. Cells were washed once with cold PBS. Cell pellets were lysed by boiling for 5 min in 20 μl lysis buffer (1% SDS, 10 mM Tris, pH 7.4), and microfuged for 5 min. The supernatants were separated on 10% SDS-PAGE and the proteins transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., NC™, Keene, NH). The blots were blocked with 5% BSA overnight at 4°C, and incubated with primary antibody to iNOS or COX-2. For iNOS a murine monoclonal antibody raised against a COOH-terminal fragment of iNOS (Transduction Laboratories, Lexington, KY) was used. COX-2 was detected with a rabbit anti-prostaglandin H synthase II (COX-2) polyclonal antibody (Cayman Chemical Co., Ann Arbor, MI) at 1:1000 dilution for 1 h at room temperature. After five washes with TBS/Tween (125 mM NaCl, 25 mM Tris, pH 8.0, 0.1% Tween 20), the blots were incubated with horseradish peroxidase-conjugated goat anti–rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBS with 1% BSA for 1 h at room temperature. After 30 min washing with TBS/Tween, the blots were incubated with ECL substrate solution (Amersham Corp., Arlington Heights, IL) for 1 min according to the manufacturer's instructions. After 30–60 min, the blots were exposed to X-ray film for 10–30 s. To document the amounts of protein loaded in the different lanes, the blots were probed with antibody to MAP kinase (Zymed Laboratories, South San Francisco, CA). Although IL-1 induces phosphorylation of MAP kinase in chondrocytes, it does not alter the levels of MAP kinase proteins (Geng, Y., and M. Lozzi, unpublished).
were dissolved in RNA loading buffer, denatured at 90°C and electrophoresed on 6% polyacrylamide gels. After transfer to chromatography paper, the gels were exposed to Kodak film overnight.

**Analysis of mRNA Stability**

Chondrocytes were stimulated with IL-1 for 12 h and then treated with A23187 for 2 h. The cells were then treated with actinomycin D (5 µg/ml) and harvested from replicate flasks 0-12 h later for RNA isolation. Levels of iNOS and GAPDH mRNA were analyzed by RPA.

**Reagents**

Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN). Thapsigargin, cyclopiazonic acid, and Bay K 8644 were purchased from Sigma Chem. Co., calcium ionophore A23187 and ionomycin from Calbiochem (La Jolla, CA). Thapsigargin, cyclopiazonic acid, A23187, and ionomycin were dissolved in dimethyl sulphoxide (DMSO) at 5 mg/ml.

**Results**

**Increased Intracellular Ca²⁺ Is Associated with Inhibition of IL-1-induced NO Release**

The calcium ionophore A23187 when added in the absence of other stimuli to primary cultures of human articular chondrocytes did not induce NO release or iNOS mRNA expression (14). In combination with IL-1, A23187 inhibited dose dependently the release of NO (Fig. 1 A) with an IC50 of 200 nM. Ionomycin had similar inhibitory effects with IC50 of 100 nM (not shown). These results suggested that increased intracellular Ca²⁺ inhibits inducible NO synthesis. To confirm this, other mechanisms to increase intracellular Ca²⁺ were tested. Thapsigargin (TG) and cyclopiazonic acid (CPA) increase intracellular Ca²⁺ by specifically inhibiting the endoplasmic reticulum Ca²⁺ ATPase (34, 42). These reagents also failed to induce NO release (not shown) but inhibited the IL-1 effect with IC50 of 1 nM and 3 µM, respectively (Fig. 1, Band C). LPS and TNFα are potent NO inducers in chondrocytes and their effects were also inhibited by the Ca²⁺ elevating drugs (not shown).

**Inhibition of IL-1-induced iNOS Protein Levels by Calcium Ionophore and ATPase Inhibitors**

The Ca²⁺-mediated inhibition of NO synthesis could be mediated by different mechanisms such as inhibition of co-factor synthesis, posttranslational modifications of the iNOS enzyme or effects on iNOS protein synthesis or mRNA accumulation. The next set of experiments examined iNOS protein levels by Western blotting. Unstimulated chondrocytes did not contain detectable levels of iNOS protein. In response to IL-1, there was a strong increase in the intensity of the iNOS band at 145 kD. When IL-1 was combined with A23187 or TG, only a weak iNOS protein signal was detectable (Fig. 2). These findings show that in the presence of drugs which increase intracellular Ca²⁺, IL-1-induced iNOS protein levels are reduced and this may account for the observed inhibition of NO release.

**Increased Intracellular Ca²⁺ Is Associated with Selective Inhibition of iNOS and Enhanced COXII Protein Expression**

Since prolonged increases in intracellular Ca²⁺ levels can induce apoptosis by the activation of endonucleases, chondrocyte viability was assessed by trypan blue dye exclusion and cells were examined for apoptotic changes. This showed

![Figure 2](image-url)

**Figure 2.** Effect of increased intracellular Ca²⁺ on iNOS protein synthesis. Chondrocytes were stimulated with IL-1β (1 ng/ml) alone or in the presence of A23187 (500 nM) or TG (100 nM). Cell lysates were prepared after 24 h and analyzed by Western blotting. Blotting of the identical filter for MAP kinase shows that the different lanes contain similar amounts of protein.

**Figure 3.** Ca²⁺ differentially regulates IL-1-induced iNOS and COXII protein synthesis. Chondrocytes were stimulated with IL-1β (1 ng/ml) alone or in the presence of the indicated concentrations of CPA. Cell lysates were prepared after 24 h and analyzed by Western blotting with antibody to COXII. The same blot was subsequently probed with antibody to iNOS. The weak bands at 71 kD in the iNOS figure are traces of the COXII signal. The different lanes contained identical amounts of protein as demonstrated by Western blotting for MAP kinase (not shown).
that neither A23187 nor the ATPase inhibitors in combination with IL-1 were causing cell death at the doses used in these experiments. To address selectivity of the Ca\(^{2+}\) effects, the synthesis of cyclooxygenase 2 (COXII), a Ca\(^{2+}\) inducible protein, was examined. COXII and iNOS proteins were analyzed in the identical cell lysates. Neither COXII nor iNOS were detectable in unstimulated chondrocytes. IL-1 induced the synthesis of both proteins. However, when the Ca\(^{2+}\) ATPase inhibitor CPA was combined with IL-1, there was a dose-dependent increase in COXII protein synthesis and a simultaneous decrease in iNOS protein synthesis (Fig. 3). These results suggested specificity and selectivity of Ca\(^{2+}\) effects, since under identical conditions the synthesis of two IL-1 inducible proteins was differentially regulated.

**Ca\(^{2+}\) Effects on IL-1-induced iNOS and COXII mRNA Accumulation**

iNOS protein synthesis can be modulated under conditions where steady state mRNA levels are unchanged (43). A RNAse protection assay was used to quantify iNOS mRNA and determine effects of increased intracellular Ca\(^{2+}\) on steady state mRNA levels of IL-1-induced genes. To determine selectivity of the Ca\(^{2+}\) modulating drug effects, iNOS mRNA was quantified in the same hybridization solutions with COXII and GAPDH. iNOS and COXII mRNA were not expressed in unstimulated cells. After 12 h both mRNAs were readily detectable (Fig. 4). A23187 and the two Ca\(^{2+}\) ATPase inhibitors almost completely inhibited IL-1-induced iNOS mRNA accumulation. Bay K 8644, a Ca\(^{2+}\) channel agonist, was less potent but still resulted in a 40% reduction and KCl caused 90% inhibition. In contrast to this inhibition of iNOS, all of the Ca\(^{2+}\) regulators increased COXII mRNA levels.

These findings indicate Ca\(^{2+}\) suppression of NO release and iNOS protein levels are associated with inhibition of iNOS mRNA accumulation. Under identical conditions increased levels of intracellular Ca\(^{2+}\) differentially regulate IL-1-induced COXII gene expression in chondrocytes while not affecting the constitutively expressed GAPDH mRNA.

**Increased Intracellular Ca\(^{2+}\) Reduces iNOS mRNA Stability**

To further characterize the Ca\(^{2+}\) effects on IL-1-induced iNOS gene expression, incubation times of IL-1 and A23187 as well as the sequence of their addition were modified and iNOS mRNA levels were examined at different time points. When RNA was isolated 6 h after IL-1 stimulation, addition of A23187 2 h before or 3 h subsequent to IL-1 did not reduce iNOS mRNA levels. Only when the cells were preincubated with A23187 for 4 h, there was a moderate reduction in IL-1-induced iNOS mRNA levels (Fig. 5 A). 

IL-1-induced iNOS mRNA in chondrocytes shows pro-

![Figure 4. Ca\(^{2+}\) effects on IL-1-induced iNOS and COXII mRNA expression. Chondrocytes were stimulated with IL-1B (1 ng/ml) in the presence of A23187 (1 μM), CPA (25 μM), TG (1 μM), Bay K 8644 (25 μM), and KCl (50 mM). Cells were harvested after 12 h for RNA extraction. iNOS, COXII, and GAPDH mRNA levels were determined by RNAse protection assay in the same hybridization reactions. The autoradiograph shows the expected 236 bp and 194-bp protected bands for iNOS and COXII.](https://jcb.rupress.org/content/jcb/129/6/1654.full)

![Figure 5. Kinetics of A23187 effects on IL-1-induced iNOS mRNA expression. Chondrocytes were stimulated with IL-1B (1 ng/ml) and A23187 (1 μM) was added 1–4 h before IL-1 (+1,+2,+4), at the same time as IL-1 (0) or 1–12 h later than IL-1 (−1 to −12). Cells were harvested 6 h after IL-1 addition (A) or 24 h after IL-1 (B). iNOS and GAPDH mRNA levels were analyzed by RNAse protection assay.](https://jcb.rupress.org/content/jcb/129/6/1654.full)
Ca\textsuperscript{2+} and iNOS Expression

Figure 6. Increased intracellular Ca\textsuperscript{2+} and IL-1 induced iNOS mRNA stability. Chondrocytes were stimulated with IL-1β (1 ng/ml) for 12 h, followed by the addition of A23187 (500 nM). After 2 h actinomycin D (5 μg/ml) was added and cells from replicate flasks were harvested 0–12 h later. RNA expression was analyzed by RPA. The autoradiographs were subjected to densitometry and the iNOS values were normalized on the basis of the values for GAPDH. A value of 5 was arbitrarily assigned to iNOS mRNA levels at time 0. A is representative of three separate experiments. B shows mean values from the three experiments.

Discussion

This study addresses the role of intracellular Ca\textsuperscript{2+} in the regulation of inducible NO synthesis in human articular chondrocytes. Intracellular levels of Ca\textsuperscript{2+} increased by Ca\textsuperscript{2+} ionophore or inhibitors of Ca\textsuperscript{2+} ATPase inhibit NO release, iNOS protein synthesis, and iNOS steady state mRNA levels. This is associated with a Ca\textsuperscript{2+}-mediated reduction of iNOS mRNA stability. We also show that under identical conditions where increased intracellular Ca\textsuperscript{2+} has these inhibitory effects on iNOS, it enhances the IL-1-induced expression of COXII. NOS isoforms are distinct with respect to their sensitivity to Ca\textsuperscript{2+} levels. The activities of the constitutive enzymes purified from brain and endothelial cells are regulated by the intracellular free Ca\textsuperscript{2+} concentration and the Ca\textsuperscript{2+}-binding protein calmodulin. Macrophage iNOS is Ca\textsuperscript{2+} independent. In contrast to most CaM-dependent enzymes, macrophage iNOS binds CaM tightly without a requirement for elevated Ca\textsuperscript{2+} (27). Endotoxin-induced NOS from liver differs from the inducible enzyme found in macrophages and is unusual in that it is stimulated by calmodulin with little dependence on the Ca\textsuperscript{2+} concentration (12). iNOS activity in extracts from IL-1-stimulated human articular chondrocytes is not dependent on calcium (28).

Transcriptional regulation of iNOS shows cell type and stimulus-dependent differences. In chondrocytes iNOS mRNA expression is induced by proinflammatory cytokines such as IL-1, TNF, and by bacterial lipopolysaccharide and remains detectable at high levels for prolonged time periods (28, 30, 38). We showed that the second messenger agonists PMA, cAMP, and A23187 did not induce NO release or iNOS mRNA expression. By using specific kinase inhibitors we found that PKA and PKC were not required for the IL-1 induction of iNOS mRNA (14). However, the induction of iNOS mRNA and NO release by IL-1 and LPS were dependent on activation of tyrosine kinases which are also important for iNOS expression in macrophages and renal mesangial cells (9, 10).

With this background the present study addressed the role of Ca\textsuperscript{2+} in the regulation of NO production in human articular chondrocytes. We found that thapsigargin and cyclopiazonic acid which increase intracellular Ca\textsuperscript{2+} by inhibiting Ca\textsuperscript{2+} ATPase did not induce NO synthesis or iNOS mRNA expression and thus had similar effects as A23187 (14). To address a possible involvement of Ca\textsuperscript{2+} in the IL-1 induction of NO synthesis, Ca\textsuperscript{2+} chelators were tested and found not to affect IL-1-induced NO release or iNOS mRNA levels (Geng, Y., and M. Lotz, unpublished). Collectively, these results suggest that in chondrocytes, Ca\textsuperscript{2+} does not induce NO synthesis and is not required for the IL-1 effects.

We then examined whether Ca\textsuperscript{2+} has negative regulatory effects on NO synthesis. These studies showed that all drugs which increase Ca\textsuperscript{2+} inhibited IL-1–induced NO synthesis and reduced iNOS protein and steady state mRNA levels. Although an effect of Ca\textsuperscript{2+} on iNOS enzymatic activity cannot be excluded, it appears that it acts primarily by altering iNOS mRNA levels. Studies on the kinetics of
the Ca\(^{2+}\) effects suggested posttranscriptional effects and a significant reduction of iNOS mRNA half life by A23187 was demonstrated. The effects of Ca\(^{2+}\) on iNOS mRNA were slow in onset, possibly suggesting a protein synthesis-dependent mechanism. These results support the hypothesis that Ca\(^{2+}\) regulates a protein which controls stability of iNOS mRNA. The 3\(^{\prime}\) untranslated region of murine and human iNOS contains a conserved region which is almost identical to the UUAUUUAU octanucleotide sequence in the TNF mRNA (11). This region confers mRNA instability and represents a binding motif for labile, cyclhexamide-sensitive proteins (7, 31, 37). Consistent with this, the half life of iNOS mRNA is prolonged in the presence of CHX and in some cell types such as chondrocytes and smooth muscle cells CHX alone in the absence of other iNOS inducers causes the accumulation of iNOS mRNA (11). Whether the cyclhexamide and Ca\(^{2+}\) act via the same protein will require analysis of modified 3\(^{\prime}\) ends of the iNOS mRNA. However, the present findings provide further support for the importance of mRNA stability in the regulation of iNOS. These results also represent to our knowledge the first example where Ca\(^{2+}\), which is well known to stabilize mRNAs, causes more rapid mRNA degradation.

The inhibitory effects of Ca\(^{2+}\) modulating drugs on IL-1-induced gene expression in chondrocytes were not global but selective. In contrast to the inhibition of iNOS, under identical conditions (identical cells, culture conditions, incubation times) Ca\(^{2+}\) induced the expression of COXII and significantly enhanced the IL-1 effect. These results are consistent with the regulation of COXII as an immediate early gene which is known to be induced by elevated levels of intracellular Ca\(^{2+}\) (13). It is furthermore of interest that COXII can be induced by PKC activation via PMA and that this pathway does not lead to iNOS expression. These findings suggest that within the set of IL-1 inducible genes in a given cell type, the same second messenger can be a stimulus for one and an inhibitor for another. Thus, although Ca\(^{2+}\) may not be part of IL-1-initiated second messengers, the present results demonstrate that the Ca\(^{2+}\) levels can profoundly alter the cellular response to this cytokine. Information on Ca\(^{2+}\) in IL-1-activated chondrocytes is limited but available evidence suggests that although IL-1 strongly altered the pattern of phosphorylated proteins it did not induce protein kinases activated by Ca\(^{2+}\)/calmodulin (19). The present findings of increased levels of intracellular Ca\(^{2+}\) being associated with inhibition of iNOS are also consistent with the role of Ca\(^{2+}\) and NO in the regulation of chondrocyte mitogenesis. Growth factors such as platelet-derived growth factor increase inositol phosphate hydrolysis and cytoplasmic Ca\(^{2+}\) in chondrocytes (40). In contrast, IL-1 inhibits chondrocyte proliferation induced by serum or growth factors (15). These inhibitory effects of IL-1 are mediated by NO as NO derived from exogenous sources inhibits cell proliferation and the antiproliferative effects of IL-1 are neutralized by the NOS inhibitor NMA (Blanco, F., and Lotz, M. unpublished). Thus, in the regulation of cell proliferation increased levels of Ca\(^{2+}\) are associated with the suppression of NO synthesis.

In conclusion, the present results suggest that intracellular levels of Ca\(^{2+}\) differentially modulate chondrocyte gene expression as demonstrated for iNOS and COXII, two IL-1 inducible genes. The effects on iNOS are related to a decrease in mRNA stability and are consistent with the role of NO and Ca\(^{2+}\) in the regulation of chondrocyte function.

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