Phospholipase A2 Metabolites Regulate Inducible Nitric Oxide Synthase in Myocytes

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Abstract—The proinflammatory cytokine interleukin-1β (IL) stimulates inducible nitric oxide synthase (iNOS) mRNA, protein, and nitric oxide (NO) production in neonatal ventricular myocytes (NVM). In other types of cells, IL also activates phospholipase A2 (PLA2), which liberates arachidonic acid for the pathways involved in eicosanoid production, and induces the cyclooxygenase-2 (COX-2) isoform, which increases prostanoid production. Since NO has been shown to directly stimulate COX activity and the resulting prostanoids to modulate IL induction of iNOS, we questioned whether PLA2 and/or COX products are involved in IL regulation of iNOS and NO production in NVM. We first found that IL induced COX-2 mRNA and protein, resulting in ≈200-fold and 15-fold increases in PGE2 and 6-keto-PGF1α (the stable metabolite of PG12), respectively. IL-stimulated prostanoid production was inhibited by the COX-2-specific inhibitor NS-398, as well as the nonspecific COX inhibitor indomethacin (INDO). We next studied the involvement of the PLA2 inhibitor ONO-RS-082 (ONO) and the COX inhibitor INDO in IL regulation of iNOS. Pretreatment with ONO blocked IL-stimulated NO production and iNOS protein, suggesting that PLA2 products are involved in regulation of iNOS synthesis. Unlike ONO, the COX inhibitor INDO had little effect on IL-stimulated NO. In addition to the COX pathway, arachidonic acid (AA) is also metabolized by the lipooxygenase (LO) pathway. The LO inhibitor nordihydroguaiaretic acid (NDGA) decreased IL-stimulated NO and iNOS synthesis. These data suggest that (1) IL upregulates COX-2 expression and prostanoid production in NVM, and (2) AA metabolites other than COX products, possibly products of the LO pathway, are involved in IL regulation of iNOS.

Key Words: cardiac myocytes • cyclooxygenase • arachidonic acid • lipooxygenase

We and others have previously shown that the cytokine interleukin-1β (IL) and endotoxin (bacterial lipopolysaccharide) stimulate inducible nitric oxide synthase (iNOS) mRNA, protein, and nitric oxide (NO) production in cardiac myocytes.1-4 iNOS is also upregulated in the heart following infarction, during the development of heart failure, and in inflammatory diseases such as allograft rejection, contributing to cardiac dysfunction.5-8

In addition to iNOS, IL stimulates the expression of a number of other gene products including the inducible cyclooxygenase (COX) isoform, COX-2 (also known as prostaglandin synthase-2 or prostaglandin-endoperoxide synthase-2), and the type II secretory form of phospholipase A2 (sPLA2) (reviewed in 9,10). Stimulation of sPLA2 activity and/or expression can result in enhanced generation of arachidonic acid (AA), which is an important substrate for COX isofoms, lipooxygenases (LO), which are involved in the production of hydroxyeicosatetraenoic acids (HETES) and leukotrienes, and p450 monooxygenases, which are involved in the formation of dihydroxy acids and epoxidecontaining metabolites. Induction of COX-2 results in overproduction of prostanoids, including prostaglandins (PGE2, PG12, and PGE3) and thromboxane. The eicosanoids produced by these three pathways are involved in the regulation of vascular tone and smooth muscle cell growth, platelet aggregation, and inflammation.11,12 While COX-2 and PLA2 have been implicated in a number of inflammatory diseases, such as osteo- and rheumatoid arthritis,9 a pathophysiological role for COX-2 induction in the heart has not been described to our knowledge. In contrast, some studies have shown that inhibition of PLA2 protects the ischemic or ischemic/reperfused heart from injury.13,14 Thus, the role of cytokine-inducible COX-2 and PLA2 in cardiac dysfunction could prove to be of interest.

Both iNOS and COX-2 are stimulated following cytokine treatment of many different types of cells, and, in many cases, NO has been implicated in the activation of COX and PG2 synthesis.15-19 In contrast, studies with COX inhibitors indicate that prostanoids either have no effect on cytokine induction of iNOS,16,20,21 stimulate it,15 or inhibit it19 Since IL induces iNOS and since studies have implicated iNOS in the pathophysiology of several cardiac diseases, we questioned (1) whether IL also induces COX-2 mRNA and (2) whether COX products or other PLA2 metabolites are involved in the regulation of iNOS. Our findings indicate that COX-2 is greatly induced in NVM and that PLA2 metabolites, possibly LO products, are involved in IL regulation of iNOS.
Cells were plated at a density of $1 \times 10^5$ cells/cm$^2$. 1 X $10^4$ cells/well of a h-well were trypsinized and digested with trypsin and DNAse to prepare cultures of ventricular myocytes as described previously. 

**Results**

Eagle medium (DMEM, Gibco) plus 10% fetal bovine serum (HyClone) was used to culture the primary cultures of ventricular myocytes as described previously. 

**2-day-old neonatal Sprague-Dawley rats** (Charles River, Kalamazoo, MI) were used to isolate RNA and Northern Blotting. 

**Isolation of RNA and Northern Blot**

Total RNA was isolated from NVM using buffers and protease inhibitors as described previously. 

**NO Production**

Nitrite (NO$^−$) production, an index of NO production, was measured in media samples by the Greiss reaction.$^{25}$ Values (nmol/mL) from trichloroacetic acid (TCA) pellets were averaged for each experiment. In most of the experiments, controls (untreated cells) were assigned a value of 1, and values for all treatments were normalized to 1 (fold increase versus control). 

**Isolation of Protein and Western Blot**

Protein was isolated from NVM using buffers and protease inhibitors as described previously. 1 Lysate protein (50 μg per lane) was separated by electrophoresis on an 8% SDS-polyacrylamide gel and then transferred to an Immobilon-P PVDF membrane (Millipore). Detection of the 130-kilodalton (kD) iNOS protein was identical to our previous report$^3$ except that we used 0.0001 mg/mL polyclonal iNOS antibody (SC #650, Santa Cruz) and developed the blot with a chemiluminescent kit (ECL, Amersham). To detect the 72-kD COX-2 protein, we used either 0.0001 mg/mL of an anti-goat COX-2 polyclonal antibody (Santa Cruz) or 5 μL/mL of an anti-rabbit COX-2 polyclonal antibody (Cayman). 

**Enzyme Immunoassay for Measurement of PGE$_2$ and the Stable PGI$_2$ Metabolite 6-keto PGE$_{1a}$**

Approximately 1 X $10^5$ cells were cultured in each well of a 6-well plate at 1 mL of serum-free DMEM. Cells were pretreated with PLA$_2$ and COX inhibitors for 1 hour prior to treatment with IL for 24 hours. A 1-mL aliquot of medium from each well was dried down and resuspended in 0.15 mL buffer. Aliquots were diluted anywhere from 1:20 to 1:500 and assayed for PGE$_2$ and 6-keto PGE$_{1a}$ (the stable metabolite of PGI$_2$) using ELISA (enzymee immunoassay) kits from Cayman according to the manufacturer’s protocols. According to Cayman, intra- and interassay variability are all ±10% Data from duplicate wells were averaged and expressed as ng/mL. Differences in mean values among treatment groups were analyzed by one-way ANOVA, pairwise multiple comparisons were made by the Student-Newman-Keuls method. $P<0.05$ was considered significant. 

**Chemicals**

Indomethacin and arachidonic acid were obtained from Sigma, ONO-107, and 2-(3-hexanoyloxy)amino-4-chlorobenzoic acid and basacalcin from Biocline, and NS-398, NDGA (nordihydroguaretic acid) and MAPF (methyl arachidonoyl fluorophosphonate) from Cayman. Interleukin-1β was obtained from Promega. Routine laboratory supplies and chemicals were obtained from Fisher Scientific.

**Results**

**IL Stimulates COX-2 and Prostanoid Production**

We have previously shown that IL stimulates NO production in NVM.$^4$ IL stimulated PGE$_2$ production from control values of 1.02 ± 0.1 ng/mL (P < 0.0001) but synthesis of PGE$_2$ is higher in IL-stimulated cells.$^6$ Thus, we tested for COX-2 upregulation in NVM. Treatment of NVM with 5 ng/mL IL for 24 hours induced COX-2 mRNA (Fig 1A) and protein (Fig 1B). IL stimulated PGE$_2$ production from a control value of 1.02 ± 0.1 ng/mL to 210 ± 28 ng/mL in 24 hours, a 200-fold increase (Fig 1C). In a separate study, we determined the time course of PGE$_2$ production and found that PGE$_2$ did not increase in the medium until between 6 and 24 hours of IL treatment (24-hour control = 0.72 ± 0.07 ng/mL, 6-hour IL = 1.0 ± 0.4 ng/mL, 24-hour IL = 1.32 ± 0.26 ng/mL, n = 3). We also measured the PGL$_2$ metabolite 6-keto PGE$_{1a}$. Its production in the same media samples also increased, but less dramatically than PGE$_2$ (control = 2.3 ± 0.4 ng/mL, 24-hour IL = 33 ± 5.6 ng/mL, a 15-fold increase, P < 0.0001). These data indicate that basal production of PGI$_2$ is greater than PGE$_2$ (2.3 ± 0.4 versus 1.02 ± 0.1 ng/mL, P < 0.0001), but synthesis of PGE$_2$ is higher in IL-stimulated cells.

Since IL induces type II sPLA$_2$ mRNA in many types of cells and enhanced synthesis of this enzyme can release arachidonic acid (AA), which is a substrate for cyclooxygenase, we postulated that inhibition of sPLA$_2$ activity would prevent IL-stimulated PGE$_2$...
PLA₂ and Regulation of iNOS

Involvement of PLA₂ Metabolites and/or Cyclooxygenase Products in IL Regulation of iNOS

To find out whether PLA₂ metabolites and/or COX products are involved in IL regulation of iNOS, NVM were pretreated with either the sPLA₂ inhibitor ONO (5 and 10 μmol/L), the cPLA₂ inhibitor MAFP (25 μmol/L), or the COX inhibitor indomethacin (10 and 25 μmol/L) and then stimulated with IL for 24 hours. We found that 10 μmol/L ONO blocked IL-stimulated NO production (Fig 3A), and this effect was at the level of iNOS synthesis as IL-stimulated NO production (Fig 3B) was completely inhibited. To exclude nonspecific effects of ONO, four blots were reprobed with reagents to detect COX-2 protein. The data were analyzed by scanning densitometry and indicated that there was no effect of ONO on IL stimulation of COX-2 (IL-stimulated COX-2 protein; 10 μmol/L ONO+IL=1.2±0.3; n=4).

The cPLA₂ inhibitor MAFP was less effective in suppressing NO production and iNOS synthesis. IL-stimulated NO production was reduced by 40% (Fig 3C) when NVM were pretreated with 25 μmol/L MAFP, and there was also a small effect on iNOS protein (Fig 3D). Data from 3 Western blots...
Figure 3. Effect of PLA₂ inhibitors on NO and iNOS synthesis.

A. Effect of the sPLA₂ inhibitor ONO on NO production. The y axis is nitrite (NOx) production, expressed as fold increase vs control (which is arbitrarily set to 1), and the x axis is treatment. n=5 to 10. *P<.05 vs IL and ***P<.01 vs IL. CONT, control; IL=interleukin-1β; 5=5 μmol/L ONO; 10=10 μmol/L ONO. B. Western blot showing the effect of ONO on iNOS protein. In 4 of 5 separate experiments, 10 μmol/L ONO totally inhibited IL-stimulated iNOS protein. C. Effect of the cPLA₂ inhibitor MAFP on IL-stimulated NO production. The y axis is nitrite (NOx) production expressed as nmol/mL, and the x axis is treatment. n=9 wells from 3 separate NVM preparations. *P<.05 vs IL. D. Western blot showing the effect of MAFP on iNOS protein. Laser scanning densitometry indicates that MAFP reduced IL-stimulated iNOS protein by 20%. Western blot data for MAFP are representative of 3 separate experiments and confirm the NO data.

Figure 4. Effect of the COX inhibitor INDO on IL-stimulated NO.

A. Effect of INDO on NO production. The y axis is nitrite (NOx) production expressed as fold increase vs control (which is arbitrarily set to 1), and the x axis is treatment. n=3. CONT, control; IL=interleukin-1β; 25=25 μmol/L INDO. B. Northern blot showing the effect of INDO on iNOS mRNA. C. Western blot showing the effect of INDO on iNOS protein. The Northern and Western blot data are representative of at least 3 separate experiments and confirm the NO data.

Discussion

Two important findings of our studies are that IL induces COX-2 and PGE₂ production in cardiac myocytes, and that a PLA₂ metabolite, possibly a product of the LO but not the COX pathway, is involved in IL regulation of iNOS. Inhibition of sPLA₂ and LO affects IL stimulation of iNOS at the level of gene expression.

Cytokines, growth factors, phorbol ester, and bacterial endotoxin (LPS) induce COX-2 in many types of cells. To our knowledge, our studies represent the first demonstration of IL induction of COX-2 in cardiac myocytes. IL has been shown to regulate COX-2 at the transcriptional level in the pancreas, and at the post-transcriptional level to increase mRNA stability in chondrocytes and rat mesangial cells. The AUUUA motif in the 3' untranslated region of the COX-2 mRNA seems to contribute to its instability, and it is a target for binding of stabilizing factors whose activity is regulated by IL. IL regulation of COX-2 in myocytes is likely to involve both mechanisms, although we did not address this question directly. We did observe variable but small amounts of COX-2 mRNA and protein in untreated myocytes, which suggests that it is continuously present at low levels, and these levels were increased at least 10-fold by IL. The presence of COX-2 in untreated myocytes was not the result of LPS
contamination of our cell culture system, since we did not observe iNOS mRNA or protein in these control samples.

Induction of COX-2 resulted in 200-fold and 15-fold increases in PGE2 and PGI2, respectively, such that PGE2 became the dominant IL-stimulated prostanoid, as described for other types of cells as well.29 Studies with the specific inhibitor NS-398, as well as the large increases in COX-2 mRNA and protein, would seem to indicate that COX-2 was responsible for much of the enhanced prostanoid production. However, we cannot exclude an effect of NS-398 on PGE2 production by COX-1, since Panara et al have shown that 10 μmol/L NS-398 inhibits thromboxane B2 synthesis (primarily a COX-1 product) by 50% in human whole blood.30 We also found that PGI2 was the major prostanoit secreted by control NVM, which is consistent with the recent study by Oudot et al.31 They found that PGI2 was the major prostanoit secreted into the medium, followed by PGE2, PGE3, and thromboxane B2, and that the profile of prostanoit production changed during hypoxia (increase in PGE2) and reoxygenation (increase in PGI2), reflecting in vivo studies of ischemic/reperfused hearts.32

Our data indicate that upregulation and/or activation of type II sPLA2 are necessary for enhanced eicosanoid production in IL-stimulated NVM. The substrate for eicosanoids is AA liberated by hydrolysis of the sn-2 fatty acyl bond of membrane phospholipids (primarily phosphatidylcholine, -serine, -inositol and -ethanolamine) by PLA2. This process also results in the liberation of lysophospholipids, which themselves are effector molecules. There are several groups of PLA2s, which differ in localization, regulation, and function. Two groups seem to be able to release AA from phospholipids, II A sPLA2 and IV cPLA2.33 cPLA2 is generally considered to be more selective for releasing AA from phospholipids and thus contributing to eicosanoid synthesis in many types of cells.34 However, in our studies, the sPLA2 inhibitor ONO totally eliminated IL induction of PGE2 in NVM, but the cPLA2/sPLA2 inhibitor MAFP did not, suggesting that sPLA2 is the major AA-liberating enzyme in IL-treated cardiac myocytes. IL has been shown to induce type II sPLA2 in mesangial cells, chondrocytes, hepatoma, and macrophages,35-39 which occurs at the level of gene expression and requires several hours of stimulation. In contrast, IL and other growth factors rapidly activate cPLA2.40 Thus, although we did not measure sPLA2 mRNA or AA release, our data, based on our inhibitor and time course studies and indicating that PGE2 does not appear in the cell culture medium of cells until 6 to 24 hours after IL treatment, point to IL induction of sPLA2.

Previous studies have shown that prostanoitds can modulate cytokine regulation of iNOS in different types of cells.15,16,19,21 Our studies are the first to show that PLA2 metabolites, but not cyclooxygenase products, may be involved in IL regulation of iNOS. These results suggest that IL regulation of iNOS in cardiac myocytes is mediated or modulated by (1) AA itself, (2) lysophospholipid products or derivatives (lyso phosphatidylcholine, LPC; platelet-activating factor, PAF; and lysolecithin, LPA), or (3) an eicosanoid product(s) of the LO and/or p450 monooxygenase pathways. Although AA has been shown to modulate signaling pathways and activate transcription factors,41,42 it was not able to stimulate NO production or induce iNOS in cardiac myocytes. While we cannot absolutely exclude lysophospholipid products as a factor in IL regulation of iNOS, we have indirect evidence that they are not. PAF, LPA and LPC actions are mediated by phospholipase C and protein kinase C (PKC).43-45 Our previous work indicates that PKC inhibition by staurosporine does not block IL stimulation of NO in cardiac myocytes,3 and thus we do not believe these agents are major mediators of IL regulation of iNOS. In contrast, our data would suggest that an eicosanoid product is most likely responsible. Preliminary studies with the p450 monooxygenase inhibitors 17-ODYA and ketroxozole seem to eliminate this pathway as a mediator/modulator of IL regulation of iNOS, but additional experiments are required to confirm this (M.C.L. and J.R.S., unpublished observations). In contrast, the LO inhibitor NDGA reduced IL-stimulated iNOS mRNA, protein and NO production, demonstrating the importance of this pathway in the regulation of iNOS gene expression.

Studies have suggested that NDGA has a number of non-specific effects, including inhibition of mitochondrial respiration and antioxidant properties.46 If NDGA is acting as an antioxidant, its mechanism of action is completely different from that of the antioxidant N-acetylcysteine, which we have found has no effect on IL stimulation of iNOS synthesis in cardiac myocytes.47 Moreover, we have tested the effect of a second LO inhibitor, baicalein, and found that it also inhibits iNOS mRNA.

Our studies have not identified the major LO product (5-, 12-, 15-HETE or leukotrienes generated by 5-, 12-, and 15-lipoxygenase) and the mechanism by which this product mediates/modulates IL regulation of iNOS. LO products have been implicated in stimulation of mitogen-activated protein...
kinase (MAPK) and c-Jun kinase (JNK) signaling cascades,\(^1\)\(^-\)\(^4\) induction of c-fos and c-jun mRNA\(^5\)\(^-\)\(^8\) and activation of transcription factors such as AP-1\(^9\)\(^-\)\(^14\) and NFκB.\(^15\)\(^-\)\(^20\) We have shown previously that a tyrosine kinase signaling cascade is required for IL induction of iNOS,\(^1\) and that IL activates NFκB in cardiac myocytes,\(^2\) but only NFκB has been implicated in the transcriptional regulation of iNOS.\(^2\) Of interest, a tyrosine kinase signaling pathway activates both cPLA\(_2\)\(^5\)\(^-\)\(^6\) and 5-LO,\(^4\)\(^-\)\(^12\) causing both to translocate to the nuclear membrane,\(^4\) where they may play a novel role in transcriptional regulation of genes involved in the inflammatory response. Future studies will determine whether IL stimulation of a LO product results in activation of the MAPK pathway and directly regulates NFκB and/or other nuclear effectors.

In conclusion, the proinflammatory cytokine IL induces a wide array of inflammatory mediators in cardiac myocytes, including iNOS, AA and other sPLA\(_2\) metabolites, COX-2 protein and prostanoids, and LO products. One of the novel results reported here is that COX-2 is a major protein stimulated by IL in cardiac myocytes, resulting in a 200-fold increase in PGE\(_2\). Moreover, it would appear that IL also induces LO products that are able to mediate/modulate IL regulation of iNOS, a process heretofore not reported for iNOS. The excessive production of NO and eicosanoids by IL-stimulated myocytes, coupled with crosstalk between the NO-forming and eicosanoid-forming pathways, may contribute to myocyte dysfunction and tissue injury in cardiac pathologies involving the inflammatory response, such as infarction and heart failure.

Acknowledgments

This work was supported by NIH grants HL 03188 and 28982 to MCL. The authors thank Kim Sadaqo for her excellent technical assistance.

References

Regulation of iNOS


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_Hypertension_. 1998;31:218-224
doi: 10.1161/01.HYP.31.1.218

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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