Role of Ca\(^{2+}\)-Independent Phospholipase A\(_2\) in the Regulation of Inducible Nitric Oxide Synthase in Cardiac Myocytes

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Abstract—We have previously shown that the regulation by interleukin-1β (IL-1β) of inducible nitric oxide synthase (iNOS) involves phospholipase A\(_2\) (PLA\(_2\)) metabolites in neonatal ventricular myocytes. Based on studies in which ONO-RS-082 is used to inhibit secretory PLA\(_2\) and methyl arachidonyl fluorophosphonate is used to inhibit cytosolic PLA\(_2\), our data suggest that a secretory PLA\(_2\) metabolite was involved in the regulation by IL-1β of iNOS. In addition, a third PLA\(_2\) isoform, which is Ca\(^{2+}\) independent (iPLA\(_2\)), has also been detected in cardiac myocytes and shown to be regulated by cytokines. We tested whether iPLA\(_2\) metabolites are involved in the regulation by IL-1β of iNOS with the use of bromoenol lactone (BEL), a specific and irreversible inhibitor of iPLA\(_2\). For this, we measured IL-1β–stimulated nitrite (NO\(_x\)) production with use of the Griess reagent, prostaglandin E\(_2\) (PGE\(_2\)) production with use of an enzyme immunoassay, and arachidonic acid release in the presence and absence of BEL. We also detected iNOS and iPLA\(_2\) proteins by Western blotting. Treatment with IL-1β (5 ng/mL) for 24 hours stimulated NO\(_x\) production by 8-fold and iNOS protein levels by at least 10-fold. In addition, arachidonic acid release was increased by 1.6-fold and PGE\(_2\) production was increased by 300-fold. When neonatal ventricular myocytes were treated with 10 μmol/L BEL, both IL-1β–stimulated PGE\(_2\) production and arachidonic acid release were inhibited. BEL inhibited IL-1β–stimulated NO\(_x\) production and iNOS protein by 88% and 93%, respectively. Lysoosphatidic acid, but not arachidonic acid or lysophosphatidylcholine, stimulated iNOS expression. Our results indicate that an iPLA\(_2\) metabolite, perhaps lysophosphatidic acid, may be involved in the IL-1β–signaling pathway, regulating the synthesis of iNOS. (Hypertension. 2000;35[part 2]:249-254.)

Key Words: nitric oxide ■ arachidonic acids ■ interleukins ■ myocytes

Much recent research has focused on regulation of the cytokine-inducible nitric oxide (NO) synthase isoform (iNOS). The iNOS isoform produces large quantities of NO over prolonged periods of time. In the cardiovascular system, NO produced by iNOS is a major pathophysiological mediator of septic shock: it has been shown to mediate the negative inotropic effects of cytokines, it plays a role in myocardial cell death after ischemia, and it may be a factor in the development of heart failure.1–8

Proinflammatory cytokines are released locally in a variety of conditions associated with myocardial inflammation, including cardiac allograft rejection, myocardial infarction, myocarditis, and idiopathic cardiomyopathy.9–11 In addition, elevated levels of circulating cytokines have been described in advanced heart failure.12,13 These proinflammatory cytokines are potent inducers of NOS in cardiac myocytes.14–16 Interleukin-1β (IL-1β) is released primarily by monocytes and macrophages, as well as by nonimmune cells such as fibroblasts and endothelial cells, during injury, infection, invasion, and inflammation17,18 and is a major regulator of iNOS in neonatal15 and adult16 cardiac myocytes.

In addition to iNOS, IL-1β induces the synthesis or stimulates the activity of a number of mediators of the inflammatory response, including cyclooxygenase-2 (COX-2)19,20 and phospholipase A\(_2\) (PLA\(_2\)) isozymes.21,22 PLA\(_2\) acts on membrane phospholipids to release arachidonic acid (AA), which is involved in eicosanoid production by cyclooxygenases, lipoxygenases, and P450 monooxygenases. Other PLA\(_2\) metabolites include lysophospholipid products, which may be associated with cytokine-induced inflammation and cell injury.18

Several different PLA\(_2\) isozymes have been identified in the heart, including low-molecular-weight secretory PLA\(_2\) (sPLA\(_2\)), high-molecular-weight cytosolic PLA\(_2\) (cPLA\(_2\)), and Ca\(^{2+}\)-independent intracellular PLA\(_2\) (iPLA\(_2\)).20–23 Most PLA\(_2\) activity in the mammalian myocardium involves iPLA\(_2\), and the 80- and 40-kDa iPLA\(_2\) isoforms are the most well characterized.21,24,25 iPLA\(_2\) has been shown to play an important role
in (1) signal transduction in response to several agonists, (2) plasma membrane remodeling, and (3) cell injury during myocardial ischemia.\textsuperscript{21,23,25} In addition, studies suggest that IL-1\(\beta\) can increase iPLA\(_2\) activity in adult ventricular myocytes.\textsuperscript{22}

Prostanoids\textsuperscript{36} and AA metabolites\textsuperscript{19} have been shown to modulate IL-1\(\beta\) induction of iNOS. Previously, we showed that an inhibitor of sPLA\(_2\), but not of cPLA\(_2\), prevented IL-1\(\beta\)-stimulated NO production and iNOS synthesis.\textsuperscript{19} Because of the abundance of iPLA\(_2\) in the heart, we extended these studies to determine whether iPLA\(_2\) metabolites are involved in the stimulation by IL-1\(\beta\) of iNOS and NO production. In addition, using the selective iPLA\(_2\) inhibitor bromoenol lactone (BEL), we determined whether iPLA\(_2\) contributes to AA release and PGE\(_2\) production in neonatal ventricular myocytes (NVMs) treated with IL-1\(\beta\).

## Methods

### Chemicals

BEL and rabbit iPLA\(_2\) polyclonal antiserum were purchased from Cayman Chemical Co. The iNOS antibody was obtained from Santa Cruz. IL-1\(\beta\) was obtained from Promega. Protein molecular weight markers, horseradish peroxidase–linked anti-mouse and anti-rabbit secondary antibodies, and enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham. Laboratory supplies and chemicals were obtained from Sigma and Fisher.

### Cell Culture

Primary cultures of NVMs were derived from digestion of 1- or 2-day-old neonatal Sprague-Dawley rat hearts as described previously.\textsuperscript{27} Female Sprague-Dawley rats with pups were obtained from Charles River. The protocol was approved by the Henry Ford Hospital Committee for Care and Use of Experimental Animals. Cells were plated at a high density in DMEM (GIBCO BRL) plus 10\% FBS (Hyclone) onto 6-well (1\(\times\)10\(^6\) cells/well) or 12-well (0.5\(\times\)10\(^6\) cells/well) dishes for 40 hours, after which serum-free medium supplemented with glutamine, insulin, selenium, and transferrin was added for 24 hours before the test compounds were added. Cells were pretreated for 30 minutes with the iPLA\(_2\) inhibitor BEL (10 \(\mu\)mol/L) before the addition of IL-1\(\beta\) for 24 hours.

### NO Production

Nitrite (NO\(_x\)), an index of NO production, was measured in medium samples with use of the Griess reaction. A 1-mL aliquot of medium from each well was dried down and resuspended in 0.15 mL UltraPure water (Cayman). Duplicate 50-\(\mu\)L aliquots were assayed for NO\(_x\). Values from triplicate wells (in mmol/mL) were averaged for each experiment. Data are expressed as mean±SEM.

### Enzyme Immunoassay for Measurement of PGE\(_2\)

Aliquots of dried medium previously assayed for NO\(_x\) were diluted 1:20 to 1:2,000 and assayed for prostaglandin E\(_2\) (PGE\(_2\)) with the use of an enzyme immunoassay kit from Cayman according to the manufacturer’s protocol. Data from triplicate wells were averaged and expressed in pg/mL.

### Isolation of Protein and Western Blot Analysis

Protein was isolated from ventricular myocytes through the use of buffers and protease inhibitors as described previously.\textsuperscript{19,28} Lysate protein (50 \(\mu\)g/lane) was separated through electrophoresis onto an 8\% SDS–polyacrylamide gel and transferred to an Immobilon-P PVDF membrane (Millipore). For detection of the 130-kDa iNOS protein, we used 0.0001 mg/mL polyclonal iNOS antibody (SC 650; Santa Cruz). A polyclonal antibody (used at 1:1,000 dilution) generated against the amino-terminal domain of iPLA\(_2\) detected an 80- to 85-kDa protein. The appropriate secondary antibody linked to horseradish peroxidase was used for chemiluminescence detection of the iNOS and iPLA\(_2\) proteins. The signal was detected through exposure to Fuji RX film and analyzed with scanning densitometry. In most cases, the densitometry value for IL-1\(\beta\)-treated cells was assigned a value of 1, and values for all treatments were normalized to 1 (fold change versus IL-1\(\beta\)).

## Measurement of AA Release

IL-1\(\beta\)-stimulated AA release from radiolabeled membrane phospholipids was determined through the measurement of \(\text{[^{3}H]}\)AA in the NVM medium as described previously.\textsuperscript{22} Briefly, NVMs (0.5\(\times\)10\(^6\) cells in 1 mL culture medium) were incubated at 37\(^\circ\)C with 3 \(\mu\)Ci \(\text{[^{3}H]}\)AA for 24 hours to label phospholipids in the membranes. This resulted in \(\approx 50\%\) incorporation of radiolabeled AA into membrane phospholipids (47±9%; \(n=7\)). Labeling medium was removed, and the cells were washed 3 times with serum-free DMEM containing 1\% BSA (fatty acid free). After incubation at 37\(^\circ\)C for 15 minutes, myocytes were treated with IL-1\(\beta\) alone or with IL-1\(\beta\) and BEL for 24 hours. At the end of the treatment period, the medium was removed from the myocyte cultures and centrifuged to remove contaminating cells, and triplicate 100-\(\mu\)L aliquots were counted in 1 mL of scintillation fluid. NVMs were scraped from the wells into 1 mL of serum-free DMEM containing 1\% BSA and pelleted, and the pellets were dissolved in 200 \(\mu\)L of 10\% SDS. A 100-\(\mu\)L aliquot of the pellet was counted in 1 mL of scintillation fluid with the use of a Packard 3320/3330 TRI-CARB \(\beta\)-counter. The amount of radioactivity in the medium (M) plus that in the cell pellet (C) represents the total amount of \(\text{[^{3}H]}\)AA in each sample. AA release was calculated as the amount of radioactivity in the medium divided by total radioactivity: \(\text{[M/(C+M)]}\times100\%\). For each experiment, cpm in the control sample was set at 100\%, and samples containing IL-1\(\beta\) and IL-1\(\beta\) plus BEL were normalized to control.

### Statistical Analysis

Values are mean±SEM, with N values representing the number of experiments. Statistical significance was evaluated with Student’s \(t\) test or ANOVA with the appropriate correction for multiple comparisons (Newman-Keuls method). \(P<0.05\) was considered significant (compared with control unless otherwise specified).

## Results

### Effect of the iPLA\(_2\) Inhibitor on PGE\(_2\) Production and AA Release in IL-1\(\beta\)-Treated NVMs

We have previously shown that inhibition of sPLA\(_2\) with ONO-RS-082 prevents IL-1\(\beta\)-stimulated PGE\(_2\) production but inhibition of cPLA\(_2\) with methyl arachidonyl fluorophosphonate (MAFP) does not.\textsuperscript{19} We questioned whether the BEL-sensitive iPLA\(_2\) isoform is also involved in the hydrolysis of membrane phospholipids and the release of AA to form PGE\(_2\). For these experiments, we used BEL at a concentration of 10 \(\mu\)mol/L, which is known to significantly inhibit iPLA\(_2\).\textsuperscript{29} Figure 1A shows that the exposure of NVMs to 5 ng/mL (0.3\(\times\)10\(^{-7}\) mol/L) IL-1\(\beta\) for 24 hours increased PGE\(_2\) production by 300-fold (IL-1\(\beta\) 275±43 ng/mL, \(n=5\), control 0.9±0.05 ng/mL). Pretreatment with 10 \(\mu\)mol/L BEL reduced IL-1\(\beta\)-stimulated PGE\(_2\) production by \(>99\%\) (BEL/IL-1\(\beta\) 4.2±1.3 ng/mL). When BEL was added for just 1 to 2 hours instead of being present for the entire IL-1\(\beta\) treatment period, it still inhibited PGE\(_2\) production, which is consistent with its role as an irreversible inhibitor (data not shown).

To test whether the inhibition of PGE\(_2\) production by BEL was due to a decrease in AA release, we next examined the effect of the iPLA\(_2\) inhibitor on IL-1\(\beta\) stimulation of AA release. Figure 1B shows that exposure of cardiac myocytes to IL-1\(\beta\) for 24 hours resulted in a 1.6-fold increase in AA
Effect of the iPLA2 Inhibitor on IL-1β Stimulation of NO Production and iNOS Synthesis

Because the inhibition of iPLA2 by BEL blocked AA release and PGE2 production and because our previous results suggested that AA metabolites are involved in the regulation of iNOS IL-1β,19 we next tested the effect of iPLA2 inhibition on IL-1β regulation of iNOS synthesis and NOx production. When ventricular myocytes were pretreated with BEL and then treated with IL-1β, IL-1β stimulation of nitrite production was inhibited by 88% (IL-1β 25.2 ± 0.9 nmol/10^6 cells, BEL/IL-1β 3.1 ± 0.3 nmol/10^6 cells; Figure 2). To test whether the decrease in NO production was the result of a decrease in iNOS synthesis, protein samples from cell lysates were analyzed by Western blotting. BEL decreased IL-1β-stimulated iNOS by 93% (BEL/IL-1β 0.07 ± 0.02-fold, IL-1β 1-fold; Figure 3). To test whether BEL was nonspecifically inhibiting general protein synthesis, we reprobed several blots with an antibody to β-actin but found no effect. Thus, the induction of iNOS by IL-1β seems to require metabolites generated by a BEL-sensitive PLA2 isoform.

**Figure 1.** Effect of BEL on IL-1β-stimulated PGE2 production and AA release. A, PGE2 production. The y-axis represents ng/mL PGE2 produced by 1 × 10^6 cells, and the x-axis represents treatment. Each bar represents the mean ± SEM of 4 to 12 separate experiments. **P < 0.01, IL-1β vs BEL/IL-1β. B, AA release. The y-axis represents [3H]AA released into the medium by 0.5 × 10^6 cells and is expressed as a -fold increase vs control (CONT). Control value for each experiment has been set at 1, and all treatments are normalized to it. Average percentage of AA released during the control period was 29 ± 3% (n = 6). Each bar represents the mean ± SEM of 5 or 6 separate experiments. **P < 0.01, IL-1β vs BEL/IL-1β. CONT indicates control; IL-1β, 5 ng/mL IL-1β for 24 hours; BEL, 10 μmol/L BEL.

**Figure 2.** Effect of BEL on IL-1β-stimulated NO production. Results are expressed as NO production (nmol/mL per 10^6 cells). Each bar represents the mean ± SEM of 4 to 10 separate experiments. ***P < 0.001, IL-1β vs BEL/IL-1β. CONT indicates control; IL-1β, 5 ng/mL IL-1β for 24 hours; BEL, 10 μmol/L BEL.

**Figure 3.** Effect of BEL on regulation of iNOS by IL-1β. A, Denitometry data for 6 separate Western blots (each bar is mean ± SEM). The y-axis represents iNOS protein level expressed as -fold change vs IL-1β (arbitrarily set at 1), and the x-axis represents treatment. B, Representative Western blot. iNOS is indicated as a 130-kDa protein. CONT indicates control; IL-1β, 5 ng/mL IL-1β for 24 hours; BEL, 10 μmol/L BEL. ***P < 0.001, IL-1β vs BEL/IL-1β.
Regulation of iNOS by PLA₂ Metabolites

Because the inhibition of iPLA₂ prevented the stimulation of iNOS by IL-1β, we hypothesized that either AA itself or other PLA₂ metabolites, such as lysophosphatidylcholine (LPC) or lysophosphatic acid (LPA), might be involved in the regulation of iNOS by IL-1β. AA alone (25 μmol/L) was unable to stimulate iNOS expression (Figure 4); therefore, we tested the involvement of LPC and LPA (10 μmol/L). LPA alone stimulated iNOS protein by at least 8.4-fold, whereas LPC had no effect (Figure 4). LPA was also able to stimulate iNOS expression in the presence of IL-1β and BEL (data not shown), suggesting that BEL was not nonspecifically inhibiting critical signaling molecules, like activation of protein kinase C (PKC). Thus, the induction of iNOS by IL-1β seems to involve metabolites generated by iPLA₂, such as LPA.

Effect of IL-1β on iPLA₂ Synthesis

Because IL-1β is known to stimulate the activity of a Ca²⁺-independent iPLA₂ isoform, we used Western blotting to test whether synthesis of the 80- to 85-kDa iPLA₂ isoform is regulated by IL-1β. In contrast to our expectations, IL-1β decreased iPLA₂ protein (IL-1β 0.46 ± 0.06-fold, control 1-fold; Figure 5).

Discussion

The principal new finding of the present study is that the induction of iNOS by the inflammatory cytokine IL-1β is mediated by PLA₂ metabolites that are generated by a BEL-sensitive iPLA₂ isoform in cardiac myocytes. These data extend our previous finding that an AA metabolite, perhaps generated via a lipoxygenase, but not a cyclooxygenase, pathway, is involved in the regulation of iNOS. Because IL-1β induces a number of inflammatory mediators in cardiac myocytes besides NO, including COX-2 products, AA, and PLA₂ metabolites, and because all of them can act as signaling molecules, it is not surprising that a number of these products enhance or mediate IL-1β stimulation of iNOS.

IL-1β elicits its multiple biological actions via several signal transduction pathways, and its receptor may be coupled to phospholipases, including PLA₂. PLA₂ catalyzes the hydrolysis of fatty acids esterified at the sn-2 position of phospholipids, generating AA. Other products of this reaction include LPA, LPC, and platelet-activating factor. Different isoforms of PLA₂ have been identified in a variety of cells. In NVMs, we identified cPLA₂ through Western blotting and sPLA₂ through RT-PCR (E.I. and M.C.L., unpublished observations, 1999). In the present study, we examined the role of a BEL-sensitive Ca²⁺-independent isoform in the regulation of iNOS and detected an 80- to 85-kDa iPLA₂ isoform by Western blotting. We also determined that a BEL-sensitive isoform is important in AA release and PGE₂ formation. The iPLA₂ isoform has been implicated in AA release by several types of cells; however, Murakami et al. produced stable transfectants of sPLA₂-/-, cPLA₂-/-, and iPLA₂ overexpressing cells and found that despite the ability of iPLA₂ to release AA, it was unable to supply AA for PGE₂ generation by COX-2. Our data on BEL suggest that AA released by iPLA₂ is metabolized into PGE₂ by COX-2. However, an alternative explanation is that an iPLA₂ metab-
olite is involved in IL-1β-stimulated COX-2 synthesis and that a decrease in COX-2 will result in inhibition of PGE₂ formation. In support of this, preliminary data from our laboratory indicate that BEL totally inhibits IL-1β-stimulated COX-2 (E.I. and M.C.L., unpublished data, 1999). Thus, in NVMs, IL-1β stimulates the release of AA through a BEL-sensitive PLA₂ isofrom, but whether AA generated via this mechanism can directly couple to PGE₂ by generating DAG and inhibiting protein kinase C activity is unclear.

At least 2 different iPLA₂ isoforms exist in cardiac myocytes. Although there is some confusion in the literature regarding the subcellular location of these isoforms, studies indicate that both 80- and 40-kDa iPLA₂ isoforms can be either cytosolic or membrane-associated. McHowat and Liu reported that IL-1β increases the activity of a membrane-associated Cal²⁺-independent iPLA₂ in adult rat ventricular myocytes when plasmencolylcholine is used as a substrate and that this BEL-sensitive enzyme releases AA. In a subsequent study, these authors showed that the membrane-associated iPLA₂ isoform was 82 kDa. Using crude homogenates of NVMs and the same polyclonal antibody, we also detected an 80- to 85-kDa iPLA₂ isoform. We found that IL-1β decreased the level of this iPLA₂ isoform, which might argue against its involvement in either AA release or PGE₂ production and implicate another isoform in these processes. In support of this, it has been reported that the 80- to 85-kDa isoform is inhibited by both BEL and MA6P and that the 40-kDa form is inhibited by BEL. We have previously shown that MA6P, which inhibits cPLA₂, and some iPLA₂ isoforms, has very little effect on IL-1β-stimulated PGE₂ production. Because our present study shows that BEL inhibits AA release and PGE₂ production, it is possible that the BEL-sensitive 40-kDa isoform described by Hazen et al., or some entirely new isoform, is involved in our system; additional studies will be required to identify the BEL-sensitive isoform found to be active in the present study.

By acting through multiple signaling pathways, IL-1β regulates iNOS expression. We have previously shown that tyrosine kinases and AA metabolites, presumably derived from a lipoxigenase pathway, are involved in this regulation. An iPLA₂ isoform has been shown to generate the second-messenger lipids LPA and LPC. Here, for the first time, we have implicated the PLA₂ metabolite LPA in iNOS regulation. Xu et al. have shown that phosphatidic acid, but not LPA, induces protein synthesis in adult myocytes and that this process involves the activation of phospholipase C, tyrosine kinase, and PKC. Whether phosphatidic acid also regulates iNOS in NVMs is presently unknown; nevertheless, activation of kinases such as tyrosine kinase and PKC by LPA may mediate the regulation of iNOS by IL-1β.

With regard to the possible involvement of PLA₂ metabolites in the regulation of iNOS by IL-1β, we have data that indicate both sPLA₂ and iPLA₂ play roles. There are several possibilities to explain these results. It is possible that BEL and ONO-5024, at the concentrations used in these cell culture experiments, inhibit PLA₂ activity nonspecifically, but this seems unlikely based on a number of publications. Balsinde and Dennis tested the effect of BEL on PLA₂ isoforms in P388D, macrophages and found that it had no effect on either cPLA₂ or sPLA₂ activity in cellular homogenates. BEL can reportedly also inhibit the enzyme that converts phosphatidic acid to diacylglycerol (DAG), potentially resulting in decreased DAG levels and inhibition of DAG-sensitive enzymes, such as the activation of PKC. To address this issue, we added LPA to IL-1β- and BEL-treated myocytes and found that LPA could overcome the BEL-induced decrease in iNOS. This indicates that BEL is not causing a generalized inhibition of critical signaling molecules. In addition, it is possible that iPLA₂ and sPLA₂ are both required for the regulation of iNOS by IL-1β, iPLA₂ and sPLA₂ may act in parallel signaling pathways, both of which are necessary for the regulation of iNOS by IL-1β. Alternatively, it is possible that there is cross-talk between sPLA₂ and iPLA₂ and that iPLA₂ is activated first, resulting in the activation or induction of synthesis of sPLA₂. If induction or activation of iPLA₂ is needed for the action of sPLA₂, this would explain why IL-1β fails to induce iNOS in the presence of BEL. Although we are unaware of any studies indicating cross-talk between sPLA₂ and iPLA₂, there are numerous studies demonstrating that cPLA₂ and sPLA₂ interact in cell signaling.

In conclusion, our data suggest that a BEL-sensitive PLA₂ isoform releases AA from membrane phospholipids in NVMs and that one of its metabolites, perhaps LPA, mediates the regulation of iNOS and NO production by IL-1β. We must determine the specific BEL-sensitive PLA₂ isoform involved in this process, as well as the signaling mechanisms employed. Because iPLA₂ is involved in the release of AA and the subsequent formation of many important secondary messengers and because it plays a role in phospholipid remodeling, an understanding of its role in the regulation of iNOS by IL-1β may provide some insight into the roles of both proteins in cardiac pathophysiology.

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References


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