Abstract—The genes encoding inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2, also known as prostaglandin-endoperoxide synthase-2) are induced in many types of cells in response to proinflammatory cytokines. We have previously shown that interleukin-1β (IL) stimulates iNOS and COX-2 mRNA in cardiac myocytes. Because IL has been shown to activate mitogen-activated protein kinase (MAPK) signaling pathways in many different cells, we tested whether the p42/44 and p38 MAPK pathways were involved in IL stimulation of iNOS and COX-2, using a specific inhibitor of p42/44 activation, PD98059 (PD), and the p38 inhibitor SB203580 (SB). Nitrites were measured using the Griess reagent, prostaglandin PGE2 by an enzyme immunoassay, iNOS and COX-2 protein by Western blot analysis, and iNOS mRNA by Northern blot analysis. Tested separately, the p38 kinase and MAPK inhibitors partially reduced IL stimulation of nitrite, iNOS protein, and iNOS mRNA; used together, they completely abolished the effect of IL. SB and PD inhibited IL-stimulated COX-2 protein by 60% and 80%, respectively, and IL-stimulated COX-2 protein was totally prevented by the combination of inhibitors. PGE2 production was inhibited more than 99% by either drug alone, suggesting a posttranslational effect on enzyme activity. To test whether this posttranslational effect involved the cytosolic phospholipase A2 (cPLA2) isoform, Western blots were probed for cPLA2 protein. Results indicated that IL stimulated cPLA2 activity and synthesis, which was inhibited by SB but not PD. These data indicate that (1) IL induction of iNOS synthesis depends on both the p42/44 and p38 signaling pathways, acting primarily at the level of transcriptional regulation; and (2) IL regulation of COX-2 synthesis involves the p42/44 and p38 signaling pathways, with an additional level of regulation occurring posttranslationally, perhaps at the level of activation of the cPLA2 isoform, which may be involved in intracellular signaling, as well as regulation of arachidonic acid release for COX-2 activity. (Hypertension. 1999;33[part II]:276-282.)

Key Words: prostaglandins ■ phospholipases ■ nitric oxide ■ cell signaling

Inducible nitric oxide synthase (iNOS) has been implicated in the pathogenesis of myocardial infarction, heart failure, and inflammatory cardiac diseases.1–3 Recent study also demonstrates the induction of cyclooxygenase-2 (COX-2) in myocardium of failing human hearts.4 The synthesis of interleukin-1β (IL) and other cytokines is also induced in the ischemic myocardium.5,6 In neonatal rat cardiac myocytes in vitro, IL is the primary cytokine regulating the induction of iNOS and the production of nitric oxide (NO).7 In addition to iNOS, IL also regulates synthesis of COX-2 and generation of prostanoids (PGE2 and PGI2) in myocytes, a process that depends on the release of arachidonic acid from membrane phospholipids by the secretory phospholipase A2 (sPLA2) isoform.8 Interactions between sPLA2 and cytosolic PLA2 (cPLA2) have been reported, with cPLA2 involved in cell signaling, and both cPLA2 and sPLA2 required for release of arachidonic acid needed for prostanooid production by COX-2.9,10 COX-2 and PLA2 have been implicated in inflammatory diseases such as arthritis,11 and induction of PLA2 in the heart during ischemia or ischemia-reperfusion seems deleterious.12,13 Thus it is also possible that both COX-2 and PLA2 contribute to cardiac dysfunction in diseases involving an inflammatory response.

IL signaling involves numerous intracellular mediators: depending on the type of cell, these may include the sphingomyelin-ceramide pathway, serine-threonine kinases, tyrosine kinases, and phosphatidylinositol 3-kinase.14–16 Although IL preferentially activates the c-Jun kinase (JNK) and p38 kinase pathways,17 it has also been reported to activate the p42/44 mitogen-activated protein kinase (MAPK) pathway.18 The p42/44, JNK, and p38 MAPK signaling pathways are distinct serine-threonine kinase cascades each consisting of 3 enzymes, MAPK kinase (MAPKK), MAPK kinase (MAPKKK, MEK, MKK), and MAPK. Upstream activators of the MAPK pathways include small GTPases of the Ras family, and downstream effectors include transcription factors and other kinases.17,19

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p38 kinase is involved in regulation of cytokine synthesis by monocytes,\textsuperscript{20} as well as COX-2 synthesis by monocytes,\textsuperscript{21} mesangial cells,\textsuperscript{22} fibroblasts,\textsuperscript{23} and vascular endothelial cells.\textsuperscript{24} IL has also been shown to stimulate the synthesis of sPLA\textsubscript{2} and cPLA\textsubscript{2}.\textsuperscript{25,26} Moreover, cPLA\textsubscript{2} is regulated posttranslationally by p42/44 MAPK phosphorylation.\textsuperscript{25} Based on these studies and our own showing that the general tyrosine kinase inhibitor genistein inhibits IL regulation of iNOS in cardiac myocytes,\textsuperscript{7} we hypothesized that IL regulation of iNOS and COX-2 would involve MAPK pathways in myocytes and that activation of cPLA\textsubscript{2} might be an important intermediate signal. To test this hypothesis, we used a relatively specific pharmacological inhibitor of p38 kinase, SB203580 (SB), and compared it with an inhibitor of p42/44 MAPK activation, PD98059 (PD), in terms of (1) IL regulation of iNOS synthesis and NO production, and (2) COX-2 synthesis and PGE\textsubscript{2} production by cardiac myocytes. Additionally, we examined whether either the p38 or p42/44 kinase inhibitor prevented activation or synthesis of cPLA\textsubscript{2}.

### Methods

#### Cell Culture

Primary cultures of neonatal ventricular myocytes were derived from digestion of 1- to 2-day-old neonatal Sprague-Dawley rat hearts as described previously.\textsuperscript{27} The protocol was approved by the Henry Ford Hospital Committee for Care and Use of Experimental Animals. Cells were plated in DMEM (Gibco BRL) plus 10% fetal bovine serum (HyClone) in a 6-well dish (1 x 10\textsuperscript{5} cells/well) or a 10-cm dish (5.8 x 10\textsuperscript{5} cells) for 40 hours. Serum-free medium supplemented with glucose, insulin, selenium, and transferrin was then added for 24 hours before adding test compounds. Cells were pretreated with inhibitors for 1 hour before addition of IL for 24 hours. Several concentrations of PD and SB were tested for efficacy and toxicity. Results are shown for the maximum concentration that had no toxic effect (25 \textmu mol/L PD and 10 \textmu mol/L SB).

#### Determination of Nitrites and PGE\textsubscript{2}

Nitrite production, an index of NO synthesis, was measured in the medium by the Griess reaction as described previously.\textsuperscript{8} The same samples were diluted and assayed for PGE\textsubscript{2} using an enzyme immunoassay (EIA) from Cayman Chemical as described previously.\textsuperscript{8} Untreated control samples were arbitrarily assigned a value of 1, and values for all treatments were normalized to 1 (fold increase versus control). Data from multiple experiments were expressed as mean±SE, and differences in mean values were analyzed by one-way analysis of variance with pairwise multiple comparisons made by the Student-Newman-Keuls method. A P value <0.05 was considered significant (compared with control unless otherwise specified).

#### Isolation of Protein and Western Blot Analysis

Protein was isolated from ventricular myocytes using buffers and protease inhibitors as described previously\textsuperscript{7} and subjected to electrophoresis, immunoblotting, and chemiluminescent detection of iNOS and COX-2.\textsuperscript{27,28} Polyclonal antibodies against both proteins were obtained from Santa Cruz. A monoclonal antibody (used at 1:1000 dilution) generated against the amino terminal domain of human cPLA\textsubscript{2} was obtained from Santa Cruz and detected a 97-kDa protein. The appropriate secondary antibody linked to horseradish peroxidase was used for chemiluminescent detection of the iNOS, COX-2, and PLA\textsubscript{2} proteins. Signals on x-ray films were quantified by laser densitometry.

#### Isolation of RNA and Northern Blot Analysis

Isolation of total RNA and Northern blotting of iNOS have been described previously.\textsuperscript{7,28} mRNA levels were quantified by laser densitometry and corrected to GADPH mRNA.

#### Chemicals and Supplies

PD98059 and SB203580 were obtained from Calbiochem. Dr John C. Lee of SmithKline Beecham Pharmaceuticals also kindly provided SB 203580. IL was obtained from Promega. Routine chemicals and laboratory supplies were obtained from Sigma and Fisher.

### Results

#### MAPKs in the Regulation of iNOS

The p42/44 MAPK signaling pathway is activated by growth factors and other stimuli, whereas the p38 kinase pathway is preferentially activated by cytokines and stress. PD inhibits MEK, the upstream activator of p42/44, whereas SB inhibits p38 kinase activity. When ventricular myocytes were pretreated with 25 \textmu mol/L PD for 1 hour and then treated with IL (5 ng/mL) for 24 hours, iNOS protein and mRNA were reduced by 60%, and NO production by 50% (Figure 1). Similarly, when myocytes were pretreated with 10 \textmu mol/L SB, IL-stimulated iNOS protein was reduced by 75%, iNOS mRNA by 60% and NO production by 65% (Figure 2). Cotreatment with SB and PD completely inhibited both IL-stimulated iNOS and NO (Figure 3). These data indicate that IL stimulation of iNOS and NO involves both the p38 and p42/44 MAPK signaling pathways.

#### MAPKs in the Regulation of COX-2

Because IL also stimulates COX-2 synthesis and PGE\textsubscript{2} and PGI\textsubscript{2} production in cardiac myocytes,\textsuperscript{8} we examined whether the regulatory mechanisms are similar to IL stimulation of iNOS. In contrast to regulation of iNOS, PD reduced IL-stimulated COX-2 protein levels by 80%, but totally inhibited production of PGE\textsubscript{2} (Figure 4). Similarly, the p38 kinase inhibitor SB reduced IL-stimulated COX-2 protein by only 60%, but PGE\textsubscript{2} production by 100% (Figure 5A and B). As with IL regulation of iNOS, combined treatment with both SB and PD completely blocked IL stimulation of COX-2 protein and thus PGE\textsubscript{2} production (Figure 5C and D).

Because inhibition of either p38 or p42/44 MAPK only partially reduced COX-2 synthesis while totally preventing IL-stimulated PGE\textsubscript{2}, this suggested that both kinases could be acting at a posttranslational step, perhaps at the level of COX-2 activity or availability of its substrate. We have previously shown that inhibition of PLA\textsubscript{2} activity prevents IL-stimulated PGE\textsubscript{2} production.\textsuperscript{8} Cytokines and growth factors can also induce the synthesis of sPLA\textsubscript{2} and sPLA\textsubscript{2} and activation of cPLA\textsubscript{2} is critical for cell signaling, regulation of sPLA\textsubscript{2}, release of arachidonic acid, and generation of PGE\textsubscript{2} by COX-2.\textsuperscript{9,10,24,25} Therefore, we examined whether cPLA\textsubscript{2} or the level of COX-2 enhanced in IL-treated myocytes and whether p38 and p42/44 kinases mediate this effect. Activation of cPLA\textsubscript{2} by its phosphorylation causes a shift to a higher molecular weight during electrophoresis.\textsuperscript{29–31} IL treatment resulted in such a shift, as indicated by Western blotting (Figure 6A). This shift was inhibited by SB but not PD (Figure 6A, compare lanes 2, 3, and 4). In addition, SB but not PD inhibited IL stimulation of cPLA\textsubscript{2} protein synthesis (Figure 6B). Thus p38 kinase is also involved in IL regulation of cPLA\textsubscript{2}.
Discussion

We believe our data on cardiac myocytes demonstrate that both p38 and p42/44 kinase pathways are necessary for IL regulation of iNOS and COX-2 synthesis, as well as PGE\(_2\) and NO production. The p38 pathway also regulates cPLA\(_2\) activation and synthesis. Both p38 and p42/44 pathways are involved in posttranslational regulation of COX-2, perhaps at the level of regulation of arachidonic acid release. Because of its effect on COX-2 synthesis, cPLA\(_2\) activation and synthesis, and PGE\(_2\) production, p38 MAPK is a critical regulator of cytokine-induced prostaglandin synthesis. These data provide evidence for crosstalk be-
The fact that IL-induced iNOS and COX-2 synthesis, as well as production of NO and PGE₂, were all blocked by cotreatment with SB and PD indicates that both p38 and p42/44 kinase pathways are necessary for their expression. Combined p38 and p42/44 inhibition of iNOS has also been demonstrated in primary glial cultures and isolated rat islets of Langerhans; however, neither pathway seems important for cytokine regulation of iNOS in an epithelial-like colon carcinoma cell line, whereas in rat mesangial cells p38 kinase negatively modulates IL-stimulated iNOS. As with iNOS expression, inhibition of p38 kinase alone does not completely prevent cytokine induction of COX-2. Thus multiple cytokine-regulated and cell-specific pathways are critical for the transcriptional regulation of both iNOS and COX-2.

Regulation of iNOS and COX-2 gene expression by multiple signaling pathways would be expected on the basis of the complexity of their promoters. To date several different transcription factors have been implicated in the regulation of iNOS by inflammatory mediators, including STAT1α, NFkB, IRF-1, C/EBP, and CREB, whereas CREB, NF-IL6, and NFkB are important for regulation of COX-2. Transcription factors targeted by MAPK signaling pathways include Elk1, which is activated by p42/44 and binds to the serum-
response element; c-Jun and ATF-2, which are activated by JNK and bind to AP-1 or CRE sites; and ATF-2 and -6 and MEF-2C, which are activated by p38.17,19,46 Other than activation of Jun and ATF family members, which can bind to CRE sites, it is not clear how p42/44 and p38 kinases are involved in regulation of the iNOS and COX-2 genes. Because regulation of gene expression involves complex combinatorial interactions among transcription factors, it is possible that p42/44 and p38 MAPKs activate factors that dimerize or otherwise interact with some of the known transcriptional regulators of iNOS and COX-2. The fact that IL signals through many different pathways also adds complexity to p38 and p42/44 MAPK-activated transcriptional mechanisms.

Our data indicate that both p38 and p42/44 MAPK pathways are necessary for COX-2 synthesis, but that either pathway alone regulates PGE2 production, suggesting that these kinases may regulate COX-2 activity. Generation of prostanoids by COX-2 is functionally linked to the regulation of arachidonic acid release by PLA2 isoforms, most notably sPLA2 and cPLA2. We have previously shown that sPLA2 is involved in the prolonged generation of PGE2 in IL-

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**Figure 5.** Effect of the p38 kinase inhibitor SB203580 alone and in combination with the MEK inhibitor PD on IL regulation of COX-2.

A. Effect of PD on COX-2 protein. Top, Data generated from densitometry of 7 separate Western blots (each bar is mean±SE). The y axis is percent IL stimulation (100% equals the COX-2 protein stimulated by IL) and the x axis is treatment. Bottom, Representative Western blot. B, PGE2 production. The y axis is PGE2 level expressed as fold increase vs control (arbitrarily set to 1). Each bar represents the mean±SE of 7 separate experiments. C, Effect of PD and SB on COX-2 protein. Top, Data generated from densitometry of 3 separate Western blots (each bar is mean±SE). The y axis is percent IL stimulation (100% equals the COX-2 protein stimulated by IL) and the x axis is treatment. Bottom, Representative Western blot. D, PGE2 production. The y axis is PGE2 expressed as percent IL stimulation. Each bar represents the mean±SE of 3 separate experiments. Abbreviations as in Figures 1 and 2.
would totally inhibit PGE2 production despite only partial cPLA2 activity by phosphorylation, and depending on the sPLA2 isoforms involved in arachidonic acid release or elevated calcium and then either regulating synthesis of those nuclear and endoplasmic reticulum membranes in response to a critical role in intracellular signaling, translocating to the cytosol (phosphorylated) form. Lane 1 indicates control; 2, IL; 3, IL + PD; 4, IL + SB; 5, PD; and 6, SB. The upper band has a molecular weight of 70–90 kDa. B, IL stimulation of cPLA2 protein. Each bar is the mean ± SE of 3 (PD and PD + IL) or 4 (CONT, IL, SB, and IL + SB) experiments. The y axis is fold increase in cPLA2 protein (percent of IL stimulated level) and the x axis is treatment. Abbreviations as in Figures 1 and 2. **P < 0.01 vs IL.

Figure 6. Effect of p38 and MEK inhibitors on cPLA2. A, Western blot showing IL stimulation of cPLA2 gel shift to a higher molecular weight form. &cPLA2 represents the activated (phosphorylated) form. Lane 1 indicates control; 2, IL; 3, IL + PD; 4, IL + SB; 5, PD; and 6, SB. The upper band has a molecular weight of 70–90 kDa. B, IL stimulation of cPLA2 protein. Each bar is the mean ± SE of 3 (PD and PD + IL) or 4 (CONT, IL, SB, and IL + SB) experiments. The y axis is fold increase in cPLA2 protein (percent of IL stimulated level) and the x axis is treatment. Abbreviations as in Figures 1 and 2. **P < 0.01 vs IL.

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Effect on cPLA2 activity, it prevents sPLA2 synthesis and hence blocks both arachidonic acid release and PGE2 production. Although some of these details of this model have yet to be tested, numerous studies suggest that there are complex, cell-specific functional interactions between sPLA2 and cPLA2 in the generation of arachidonic acid and production of PGE2 by COX-2. We are currently investigating the regulation of sPLA2 in cardiac myocytes to more fully understand this functional crosstalk.

Cardiac dysfunction and tissue injury in response to ischemia, infarction, and heart failure may involve the localized induction of iNOS, COX-2, and cytokines in the heart. We have previously shown that induction of iNOS reduces infarct size by 40% in the rat heart after coronary artery occlusion, suggesting that NO contributes to tissue injury. Whether COX-2 is also involved is presently unknown. Our studies indicate that both p38 and p42/44 MAPK are necessary for IL induction of iNOS and COX-2. p38 and p42/44 MAPK also seem to target COX-2 activity at a posttranslational level, with p38 involved in cPLA2 activation and synthesis. Thus IL activation of p38 and p42/44 MAPK results in multiple levels of regulation of the synthesis of the inflammatory mediators NO and PGE2 in myocytes. By understanding these mechanisms, we may be able to design therapeutic strategies to reduce cardiac dysfunction and tissue injury in ischemia, infarction, and heart failure.

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IL Regulation of iNOS and COX-2


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