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Differential Regulation of Matrix Metalloproteinase-2 and -9 Expression and Activity in Adult Rat Cardiac Fibroblasts in Response to Interleukin-1 β *

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Matrix metalloproteinases (MMPs), a family of endopeptidases, are implicated in cardiac remodeling. Interleukin-1 β (IL-1 β), which is increased in the heart following myocardial infarction, increases expression and activity of MMP-2 (gelatinase A) and -9 (gelatinase B) in cardiac fibroblasts. Previously, we have shown that IL-1 β activates ERK1/2, JNKs, and protein kinase C (PKC). However, signaling pathways involved in the regulation of MMP-2 and -9 expression and activity are not yet well understood. Using adult rat cardiac fibroblasts, we show that inhibition of ERK1/2 and JNKs inhibits IL-1 β -stimulated increases in MMP-9, not MMP-2, expression and activity. Chelerythrine, an inhibitor of PKC, inhibited activation of ERK1/2 and JNKs and expression and activity of both MMPs. Selective inhibition of PKC- α / β 1 using Gö6976 inhibited JNKs activation and the expression and activity of MMP-9, not MMP-2. Inhibition of PKC- θ and PKC- ζ using pseudosubstrates inhibited IL-1 β -stimulated activation of ERK1/2 and JNKs and the expression and activity of MMP-2 and -9. Inhibition of PKC- ϵ had no effect. IL-1 β activated NF- κ B pathway as measured by increased phosphorylation of IKK α / β and I κ B- α . Inhibition of ERK1/2, JNKs, and PKC- α / β 1 had no effect on NF- κ B activation, whereas inhibition of PKC- θ and PKC- ζ inhibited IL-1 β -stimulated activation of NF- κ B. SN50, NF- κ B inhibitor peptide, inhibited IL-1 β -stimulated increases in MMP-2 and -9 expression and activity. These observations suggest that 1) activation of ERK1/2 and JNKs plays a critical role in the regulation of MMP-9, not MMP-2, expression and activity; 2) PKC- α / β 1 act upstream of JNKs, not ERK1/2; 3) PKC- ζ and - θ , not PKC- ϵ , act upstream of JNKs, ERK1/2, and NF- κ B; and 4) activation of NF- κ B stimulates expression and activity of MMP-2 and -9.

Matrix metalloproteinases (MMPs),¹ a family of endopeptidases, have the ability to degrade extracellular matrix pro-

teins, and therefore, play a fundamental role in tissue remodeling (1–6). MMPs are implicated in the remodeling processes of the heart during chronic heart failure and following myocardial infarction (6–10). MMP-2 (gelatinase A) and MMP-9 (gelatinase B), also called type IV collagenases, contain fibronectin-like domains for collagen binding (11) and are known to cleave matrix substrates including gelatin and collagen Type I, IV, V, VII, and X (5). Cardiac fibroblasts in culture constitutively express MMP-2, not MMP-9. Inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α , increase expression of MMP-2 and -9 (12, 13). Although both MMP-2 and -9 are activated in the heart during myocardial remodeling, the temporal changes in the expression of these enzymes are different (14). MMP-9 activity increases 1 day after MI, whereas MMP-2 activity starts to increase within 4 days after MI. Furthermore, MMP-9 abundance increases in both ischemic and non-ischemic dilated cardiomyopathy, whereas the abundance of MMP-2 increases only in non-ischemic dilated cardiomyopathy (9). These observations suggest differential regulation of MMP-2 and -9 in the heart.

Inflammatory cytokines such as IL-1 β and tumor necrosis factor- α are increased during chronic heart failure (15) and following myocardial infarction (16, 17). We have shown that IL-1 β activates ERK1/2 and JNKs, not p38 kinase in adult rat cardiac fibroblasts (18). We have also provided evidence that IL-1 β activates PKC, specifically PKC- ζ , in cardiac fibroblasts and that activation of PKC plays a critical role in the regulation of MMP-2 and -9 (12). In addition to the activation of MAPKs and PKC, IL-1 β has been shown to activate NF- κ B in several different cell types (19–21). The present study was undertaken to investigate the signaling pathways, including MAPKs (ERK1/2 and JNKs), PKC (α , β 1, ζ , θ , and ϵ), and NF- κ B, involved in the expression and activity of MMP-2 and -9 in response to IL-1 β in adult rat cardiac fibroblasts. The data presented here suggest that different isoforms of PKC differentially activate ERK1/2, JNKs, and NF- κ B and that activation of ERK1/2, JNKs, and NF- κ B differentially modulates the expression and activity of MMP-2 and -9.

MATERIALS AND METHODS

Antibodies and Reagents—Rabbit polyclonal anti-phospho-ERK1/2, anti-phospho-c-Jun, anti-phospho-IKK α /IKK β , and mouse monoclonal anti-phospho-I κ B- α antibodies were purchased from New England Biolabs (Beverly, MA). Monoclonal anti-phospho-JNK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas monoclonal anti-MMP-2 and MMP-9 antibodies were purchased from Oncogene Research (Boston, MA). The myristoylated PKC- ζ pseudosubstrate (PKC- ζ -PS) was purchased from BIOSOURCE International (Camarillo, CA). Chelerythrine, Gö6976, PKC- ϵ translocation inhibitor peptide (PKC- ϵ -TIP), myristoylated PKC- θ pseudosubstrate inhibitor (PKC- θ -PS), PD98059, SP600125, and SN50 (peptide inhibitor of NF- κ B translocation) were purchased from Calbiochem.

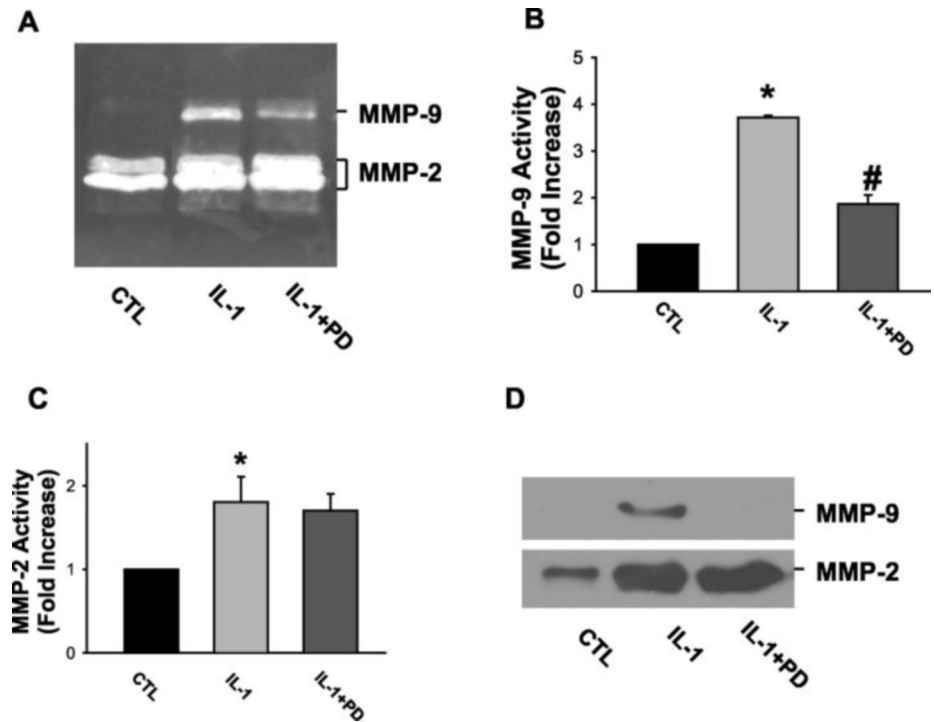
Rat Cardiac Fibroblast Culture and Treatment—Adult rat cardiac

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¹ The abbreviations used are: MMP, matrix metalloproteinase; PKC, protein kinase C; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; IKK, I κ B kinase; IL-1 β , interleukin-1 β ; PS, pseudosubstrate; TIP, translocation inhibitor peptide; MI, myocardial infarction; MT1, membrane-type 1; PD, PD98059; SP, SP600125.

FIG. 1. Inhibition of ERK1/2 inhibits MMP-9 activity. Confluent cultures of cardiac fibroblasts were pretreated with PD (10 μ M) for 30 min followed by stimulation with IL-1 β (4 ng/ml) for 48 h. MMP activity and protein levels in conditioned media were measured using in-gel zymography and Western blot analysis, respectively. *A* is a representative zymogram demonstrating the changes in MMP activities. *CTL*, control. *B* and *C* represent MMP-9 and MMP-2 activities, respectively, expressed as -fold increase versus control ($n = 4$); *, $p < 0.001$ versus control; #, $p < 0.01$ versus IL-1 β . *D*, Western blot analysis of conditioned media using monoclonal anti-MMP-2 and anti-MMP-9 antibodies. The experiments were repeated three times with similar results.



fibroblasts were isolated from the supernatant of adult rat cardiac myocytes as described (13). The cells were grown to confluence and serum-starved for 48 h before use. Cells from the first and second passages were used for all the experiments. The cells were treated with IL-1 β (4 ng/ml) for 48 h to measure MMP activity or for 15 min to measure activation of MAPKs, IKK α/β , and I κ B- α . To study the role of MAPKs, PKC isoforms, or NF- κ B, cells were pretreated with PD98059 (10 μ M), SP600125 (10 μ M), chelerythrine (5 μ M), Gö6976 (5 or 10 nM), PKC- ϵ -TIP (5 μ M), PKC- θ -PS inhibitor peptide (50 μ g/ml), PKC- ζ -PS (25 μ M), or SN50 (100 μ g) for 30 min followed by treatment with IL-1 β .

Western Blot Analyses—Activation of MAPKs (ERK1/2 and JNKs), IKK α/β , and I κ B- α was studied using Western blot analyses as described (18). Cells lysates were prepared using lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 0.5% Nonidet P-40), and protein contents were measured using the Bradford assay (Bio-Rad). Equal amounts of protein (30 μ g) were subjected to SDS-PAGE, and proteins were transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were then probed with phospho-specific ERK1/2, JNKs, c-Jun, IKK α/β , or I κ B- α antibodies. The membranes were then incubated with appropriate secondary antibody and analyzed as described (18). The protein loading of each sample was verified by staining the membrane with 0.1% Ponceau S solution. Protein levels for MMP-2 and -9 were measured in the conditioned media using monoclonal anti-MMP-2 and anti-MMP-9 antibodies.

In-gel Zymography—MMP activity in conditioned media was measured as described (13). Briefly, the media were collected, centrifuged for 5 min at 500 \times g to remove cells and debris, and lyophilized to dryness. The pellet was resuspended in water, and protein content was determined by Bradford assay (Bio-Rad). Samples (500 ng of protein) were loaded under non-reducing conditions onto 4% stacking, 10% separating SDS-polyacrylamide gel polymerized with 1 mg/ml gelatin (type A from porcine skin, Sigma) as a substrate and electrophoresed at 15–20 mA. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min with gentle shaking followed by a 30-min wash in distilled water. The gels were incubated overnight at 37 $^{\circ}$ C in substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl $_2$, and 0.02% NaN $_3$), stained in Coomassie Blue (R-250), and then destained using 7% acetic acid and 40% methanol solution. Clear and digested regions representing MMP activity were quantified using a Kodak gel documentation system (Eastman Kodak Co.), and molecular weights were estimated using prestained molecular weight markers.

Statistical Analyses—The data are expressed as mean \pm S.E. Statistical analysis was performed using the Student's *t* test or a one-way

analysis of variance followed by a post hoc Tukey's test. Probability (p) values of <0.05 were considered to be significant.

RESULTS

Inhibition of ERK1/2 and JNKs Inhibits MMP-9, Not MMP-2, Expression and Activity—Members of the MAPK superfamily (ERK1/2, JNKs, and p38 kinase) are known to regulate MMP activity in various cell types (22, 23). Recently, we have shown that IL-1 β activates ERK1/2 and JNKs, but not p38, in cardiac fibroblasts (18). To determine whether activation of ERK1/2 and JNKs plays a role in the regulation of MMP expression and activity, cells were pretreated with PD98059 (PD, a selective inhibitor of ERK1/2 pathway, 10 μ M) or SP600125 (SP, a selective inhibitor of JNKs, 10 μ M) for 30 min followed by treatment with IL-1 β for 48 h. Analysis of conditioned media demonstrated that PD98059 significantly inhibits MMP-9 activity (-fold increase versus control; IL-1 β , 3.7 \pm 0.04; IL-1 β + PD, 1.9 \pm 0.2, $p < 0.001$; $n = 4$; Fig. 1, *A* and *B*) but has no significant effect on MMP-2 activity (-fold increase versus control; IL-1 β , 1.8 \pm 0.3, IL-1 β + PD, 1.7 \pm 0.2, $p =$ not significant; $n = 4$; Fig. 1, *A* and *C*). Western blot analysis of conditioned media demonstrated that IL-1 β increases MMP-2 and -9 protein levels. Pretreatment with PD98059 inhibited MMP-9 protein but had no significant effect on MMP-2 levels (Fig. 1*D*). Inhibition of JNKs using SP600125 also significantly inhibited MMP-9 activity (-fold increase versus control; IL-1 β , 4.0 \pm 0.1; IL-1 β + SP, 1.6 \pm 0.06, $p < 0.001$; $n = 4$; Fig. 2, *A* and *B*) but had no significant effect on MMP-2 activity (-fold increase versus control; IL-1 β , 1.8 \pm 0.3; IL-1 β + SP, 1.6 \pm 0.1; $p =$ not significant; $n = 4$; Fig. 2, *A* and *C*). Western blot analysis of conditioned media demonstrated that pretreatment with SP600125 inhibits MMP-9, not MMP-2, protein levels (Fig. 2*D*).

Previously, we have shown that PD98059 and SP600125 specifically inhibit IL-1 β -stimulated increases in ERK1/2 and JNKs activity, respectively. SP600125 from 2–20 μ M concentration had no effect on IL-1 β -stimulated activation of ERK1/2 (18). To explore the possibility of whether a combination of PD98059 and SP600125 could be effective in the inhibition of MMP-2 activity and protein expression, cells were pretreated

FIG. 2. Inhibition of JNKs inhibits MMP-9 activity. Confluent cultures of cardiac fibroblasts were pretreated with SP (10 μ M) for 30 min followed by stimulation with IL-1 β (4 ng/ml) for 48 h. MMP activity and protein levels in conditioned media were measured using in-gel zymography and Western blot analysis, respectively. *A* is a representative zymogram demonstrating the changes in MMP activities. *CTL*, control. *B* and *C* represent MMP-9 and MMP-2 activities, respectively, expressed as -fold increase versus control ($n = 4$); *, $p < 0.001$ versus control; #, $p < 0.01$ versus IL-1 β . *D*, Western blot analyses of conditioned media using monoclonal anti-MMP-2 and anti-MMP-9 antibodies. The experiments were repeated three times with similar results.

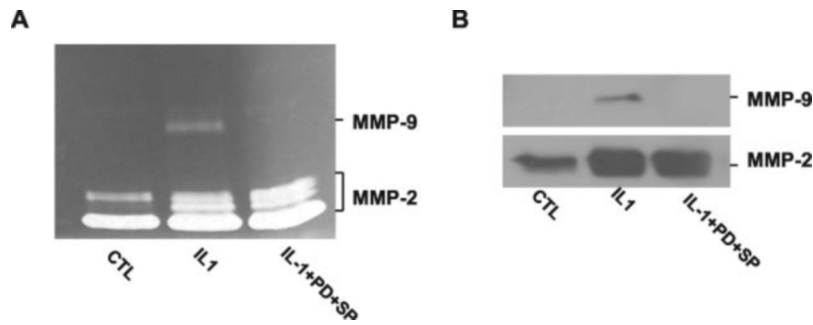
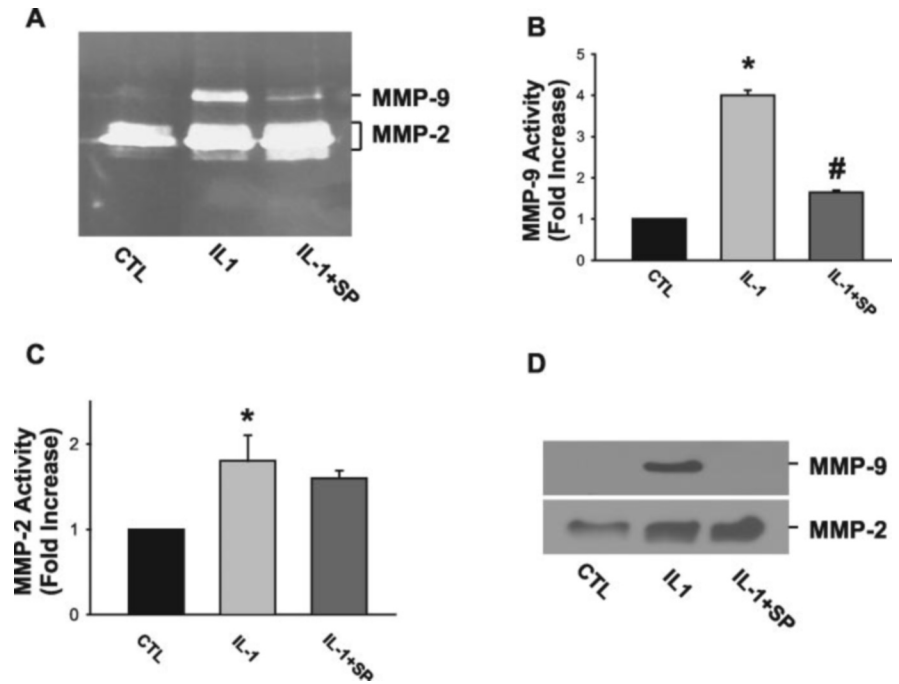


FIG. 3. Inhibition of ERK1/2 and JNKs inhibits MMP-9, not MMP-2, activity and protein levels. Confluent cultures of cardiac fibroblasts were pretreated with a combination of PD (10 μ M) and SP (10 μ M) for 30 min followed by treatment with IL-1 β for 48 h. MMP activities (*A*) and protein levels (*B*) in conditioned media were measured using in-gel zymography and Western blot analysis, respectively. The experiments were repeated three times with similar results. *CTL*, control.

with PD98059 + SP600125 (PD + SP) followed by treatment with IL-1 β for 48 h. In-gel zymography and Western blot analyses indicated that inhibition of both ERK1/2 and JNKs together significantly inhibits IL-1 β -stimulated MMP-9 activity and protein levels but has no significant effect on MMP-2 (Fig. 3, *A* and *B*).

Inhibition of PKC Inhibits IL-1 β -stimulated Activation of ERK1/2 and JNKs—Recently, we have shown that IL-1 β -stimulated activation of PKC plays an important role in the expression and activity of MMP-2 and -9 (12). Activated PKC may act upstream in the activation of ERK1/2 and JNKs (24). To explore this possibility, cells were pretreated with chelerythrine (5 μ M) for 30 min followed by stimulation with IL-1 β for 15 min. Analysis of cell lysates using phospho-specific antibodies indicated that inhibition of PKC almost completely inhibits IL-1 β -stimulated activation of ERK1/2 (Fig. 4*A*) and JNKs (Fig. 4*B*, as reflected by the phosphorylation of c-Jun).

Chelerythrine is a specific (but not isoform-selective) PKC inhibitor and represents a unique class of PKC inhibitors that competitively interfere with the phosphate acceptor site and non-competitively inhibits the ATP-binding site (25). To identify PKC isoforms involved in the activation of ERK1/2 and JNKs, we used isoform-specific inhibitors of PKC. Gö6976, a selective inhibitor for PKC- α and - β 1 ($IC_{50} = 2.3$ and 6.2 nM, respectively) at 5 and 10 nM, inhibited IL-1 β -stimulated in-

creases in c-Jun phosphorylation but had no significant effect on ERK1/2 phosphorylation (Fig. 4, *C* and *D*). PKC- ϵ translocation inhibitor peptide (PKC- ϵ -TIP) had no significant effect on IL-1 β -stimulated activation of ERK1/2 and JNKs (Fig. 4, *C* and *D*). PKC pseudosubstrates (PS) are specific inhibitory peptides and are known to suppress PKC activity by interacting with the substrate-binding pocket in the catalytic domain (26). Pretreatment with PKC- ζ -PS completely inhibited IL-1 β -stimulated activation of ERK1/2 and JNKs (as reflected by c-Jun phosphorylation), whereas PKC- θ -PS exhibited a partial effect (Fig. 4, *C* and *D*).

PKC Isoforms Differentially Modulate Expression and Activity of MMP-2 and -9—To determine the role of PKC isoforms on MMP-2 and -9 expression and activity, cells were pretreated with Gö6976 (5 and 10 nM), PKC- ϵ -TIP, PKC- ζ -PS, and PKC- θ -PS for 30 min followed by treatment with IL-1 β for 48 h. In-gel zymographic analysis of conditioned media demonstrated that Gö6976 almost completely inhibits IL-1 β -stimulated increases in MMP-9 (-fold increase versus control; IL-1 β , 4.0 ± 0.6 ; IL-1 β + Gö6976, 1.0 ± 0.4 ; $p < 0.01$; $n = 4$; Fig. 5*A*) activity but had no significant effect on MMP-2 activity (-fold increase versus control; IL-1 β , 2.0 ± 0.1 ; IL-1 β + Gö6976, 1.6 ± 0.2 ; $p =$ not significant; $n = 4$; Fig. 5*A*). PKC- ϵ -TIP had no significant effect on IL-1 β -stimulated increases in MMP-2 or -9 activity. Both PKC- ζ -PS and PKC- θ -PS almost completely in-

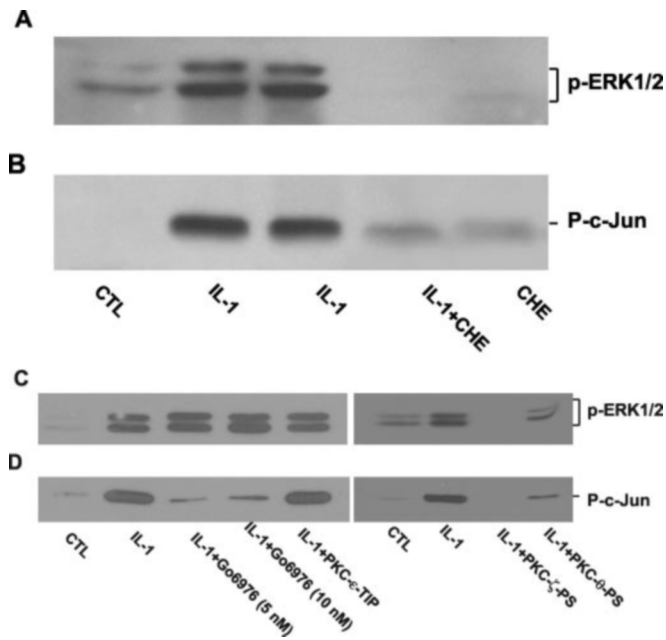


FIG. 4. Effects of inhibition of PKC isoforms on IL-1 β -stimulated activation of ERK1/2 and JNKs. Confluent cultures of cardiac fibroblasts were pretreated with chelerythrine (*CHE*, 5 μ M), Gö6976 (5 nM, 10 nM), PKC- ϵ -TIP (5 μ M), PKC- θ -PS (50 μ g/ml), or PKC- ζ -PS (25 μ M) for 30 min followed by treatment with IL-1 β for 15 min. Total cell lysates were analyzed for activation of ERK1/2 and JNKs using phospho-specific ERK1/2 (*p-ERK1/2*) (A and C) or phospho-specific c-Jun (*p-c-Jun*) (B and D) antibodies, respectively. The experiments were repeated three times with similar results. *CTL*, control.

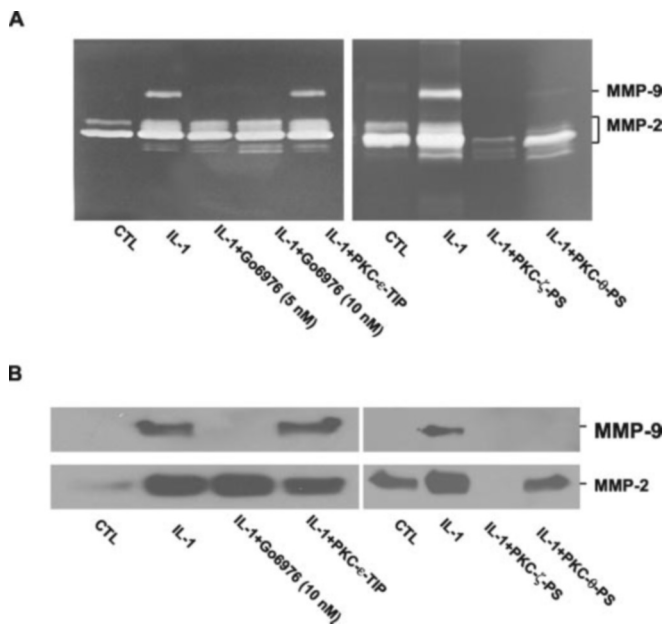


FIG. 5. Effects of PKC isoforms on IL-1 β -stimulated increases in MMP activity and protein levels. Confluent cultures of cardiac fibroblasts were pretreated with Gö6976 (5 or 10 nM), PKC- ϵ -TIP (5 μ M), PKC- θ -PS (50 μ g/ml), or PKC- ζ -PS (25 μ M) for 30 min followed by treatment with IL-1 β for 48 h. MMP activity (A) and protein levels (B) in the conditioned media were measured using in-gel zymography and Western blot analysis, respectively. The experiments were repeated three times with similar results. *CTL*, control.

hibited IL-1 β -stimulated activation of MMP-9 (-fold increase *versus* control; IL-1 β , 4.0 \pm 0.6; IL-1 β + PKC- ζ -PS, 0.2 \pm 0.1; IL-1 β + PKC- θ -PS, 0.8 \pm 0.5; p < 0.05; n = 4; Fig. 5A) and -2 (-fold increase *versus* control; IL-1 β , 2.0 \pm 0.1; IL-1 β + PKC- ζ -PS, 0.2 \pm 0.07; IL-1 β + PKC- θ -PS, 0.9 \pm 0.07; p < 0.05; n = 4; Fig. 5A). Western blot analysis of conditioned media demon-

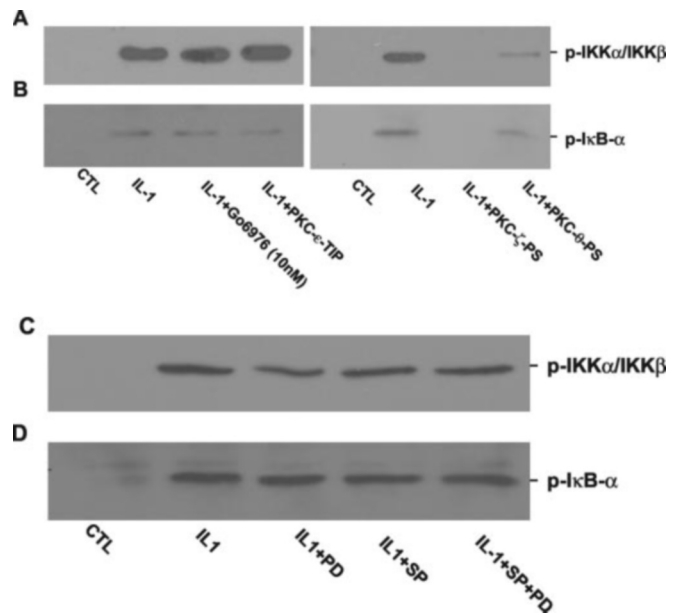


FIG. 6. Effects of PKC isoforms and MAPKs on activation of IKK α/β and I κ B- α . Confluent cultures of cardiac fibroblasts were pretreated with Gö6976 (10 nM), PKC- ϵ -TIP (5 μ M), PKC- θ -PS (50 μ g/ml), PKC- ζ -PS (25 μ M), PD (10 μ M), or SP (10 μ M) for 30 min followed by treatment with IL-1 β for 15 min. Total cell lysates were analyzed for activation of IKK α/β and I κ B- α using phospho-specific IKK α/β (*p-IKK α/β*) (A and C) or I κ B- α (*p-I κ B- α*) (B and D) antibodies, respectively. The experiments were repeated three times with similar results. *CTL*, control.

strated that Gö6976 also reduces MMP-9, not MMP-2, protein levels (Fig. 5B). Pretreatment with PKC- ζ -PS and PKC- θ -PS almost completely inhibited IL-1 β -stimulated increases in MMP-9 and -2 protein levels, although PKC- ζ -PS seemed more effective than PKC- θ -PS since it also inhibits the basal protein levels of MMP-2.

PKC- ζ and - θ Act Upstream of NF- κ B, and Inhibition of NF- κ B Inhibits IL-1 β -stimulated Expression and Activity of MMP-2 and -9—Inflammatory cytokines are known to activate NF- κ B in different cell types (19–21). To determine whether IL-1 β activates NF- κ B and the role of PKC in the activation of NF- κ B, we studied phosphorylation of IKK α/β and I κ B- α . Western blot analysis demonstrated that IL-1 β increases phosphorylation of both IKK α/β and I κ B- α within 15 min of treatment, suggesting activation of NF- κ B (Fig. 6, A–D). Pretreatment with Gö6976 and PKC- ϵ -TIP failed to inhibit IL-1 β -stimulated increases in IKK α/β and I κ B- α phosphorylation. PKC- ζ -PS completely inhibited IL-1 β -stimulated increases in IKK α/β and I κ B- α phosphorylation, whereas PKC- θ -PS only exhibited a partial effect (Fig. 6, A and B). To test whether IL-1 β -induced activation of NF- κ B is mediated through MAPKs, cells were pretreated with PD98059 and SP600125. Pretreatment of cells with PD98059 and SP600125 alone or in combination had no effect on IL-1 β -stimulated increases in IKK α/β and I κ B- α phosphorylation (Fig. 6, C and D).

To determine the role of NF- κ B in the regulation of IL-1 β -stimulated increases in MMP-2 and -9 activity and protein levels, cells were pretreated with SN50, an inhibitor of NF- κ B. In-gel zymographic analysis of conditioned media indicated that SN50 almost completely inhibits IL-1 β -stimulated increases in MMP-9 (-fold increase *versus* control; IL-1 β , 4.0 \pm 0.3; IL-1 β + SN50, 0.5 \pm 0.3; p < 0.001; n = 4; Fig. 7, A and B) and MMP-2 activity (-fold increase *versus* control; IL-1 β , 1.8 \pm 0.2; IL-1 β + SN50, 0.8 \pm 0.02; p < 0.05; n = 4; Fig. 7, A and C). Western analysis demonstrated similar decreases in MMP-9 and -2 protein levels (Fig. 7D).

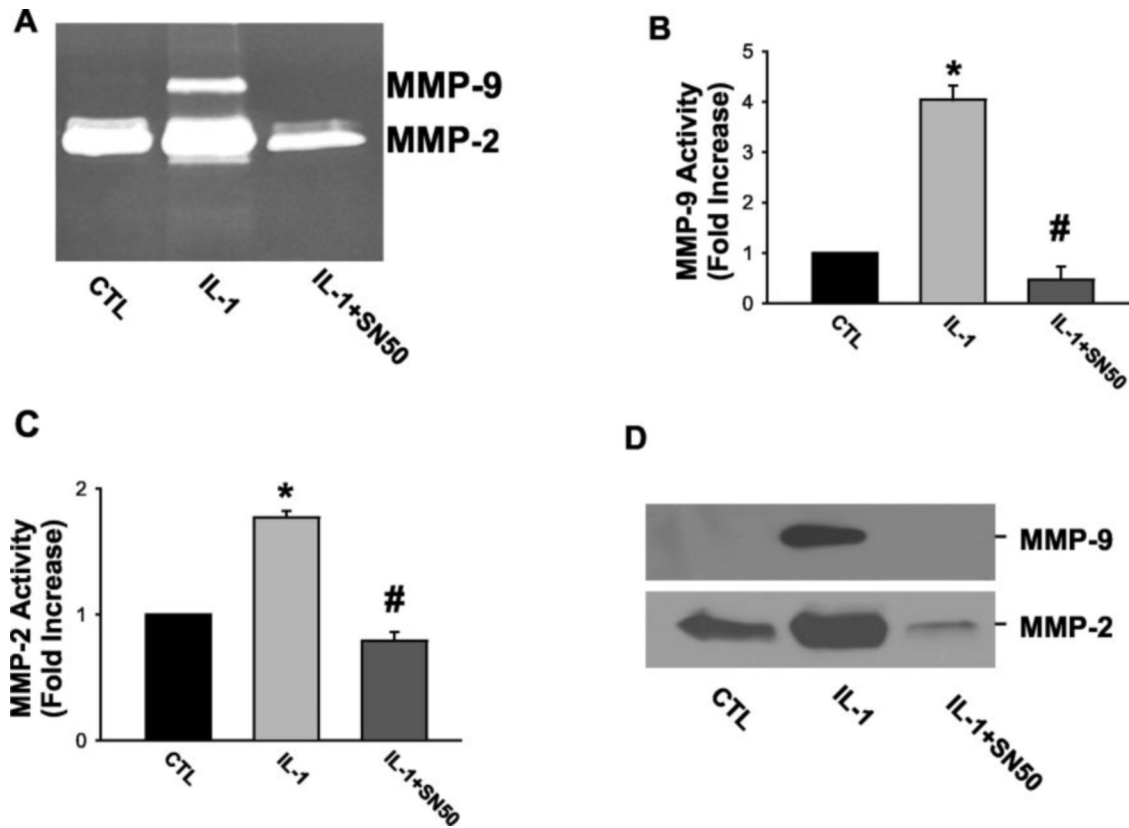


FIG. 7. Inhibition of NF- κ B inhibits IL-1 β -stimulated increases in MMP-2 and -9 activity and protein levels. Confluent cultures of cardiac fibroblasts were pretreated with SN50 (100 μ g/ml) for 30 min followed by treatment with IL-1 β for 48 h. MMP activities and protein levels in conditioned media were measured using in-gel zymography and Western blot analysis, respectively. A is a representative zymogram demonstrating the changes in MMP activities. CTL, control. B and C represent MMP-9 and MMP-2 activities, respectively, expressed as -fold increase versus control ($n = 4$); *, $p < 0.001$ versus control; #, $p < 0.01$ versus IL-1 β . D, Western blot analysis of conditioned media using monoclonal anti-MMP-2 and anti-MMP-9 antibodies. The experiments were repeated three times with similar results.

DISCUSSION

IL-1 β activates ERK1/2 and JNKs, not p38 kinase, in adult rat cardiac fibroblasts (18). Recently, we found that IL-1 β activates PKC and that activation of PKC plays a critical role in the regulation of MMP-2 and -9 expression and activity (12). The major new findings of this study are that 1) activation of ERK1/2 and JNKs plays a critical role in the regulation of MMP-9, not MMP-2, expression and activity; 2) PKC- α/β 1 act upstream of JNKs, not ERK1/2; 3) PKC- ζ and - θ , not PKC- ϵ , act upstream of ERK1/2, JNKs, and NF- κ B; and 4) activation of NF- κ B stimulates expression and activity of MMP-2 and -9.

MMPs are proposed to play a critical role in myocardial remodeling process following MI (10). IL-1 β , increased in the heart following MI, increases expression and activity of MMP-2 and -9 in adult rat cardiac fibroblasts (12). Here, we show that inhibition of ERK1/2 and JNKs alone or in combination significantly, but partially, inhibits IL-1 β -stimulated increases in MMP-9, not MMP-2, protein levels and activity. These data suggest the involvement of MAPK-independent pathway(s) in the regulation of both MMP-2 and -9 expression and activity. These findings are consistent with the observations that inducible MMPs (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13) contain one or more AP-1-binding site(s) in their promoter, whereas the promoter region of constitutive MMPs (MMP-2 and MMP-11) does not contain an AP-1-binding site (27, 28). The transcription factor AP-1 is formed by dimerization of different members of Fos and Jun protein family members. The expression of Fos and Jun is mainly regulated via the activation of ERK1/2 and JNKs, respectively. It is interesting to note that activation of ERK1/2 pathway is shown

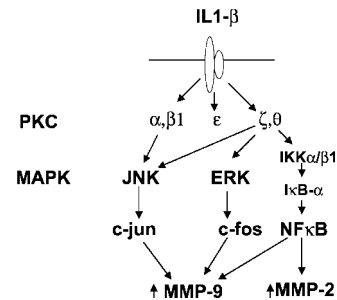


FIG. 8. Schematic representation of signaling pathways involved in the regulation of IL- β -stimulated increases in MMP-2 and -9. IL-1 β -stimulated increase in MMP-2 protein levels and activity is regulated via PKC- θ , PKC- ζ , and NF- κ B pathways, whereas the regulation of MMP-9 involves PKC- α/β 1, PKC- ϵ , PKC- ζ , ERK1/2, JNKs, and NF- κ B signaling pathways.

to play a critical role in the regulation of MMP-2 activity in transformed cells (29).

The possibility of involvement of MAPK-independent pathway(s) and that transcription factors, other than AP-1, are involved in the regulation of both MMP-2 and -9 is supported by our recently published data that inhibition of PKC using chelerythrine completely inhibits IL-1 β -stimulated increases in MMP-2 and -9 expression and activities (12). Human MMP-9 promoter has a binding site for transcription factor polyoma enhancer activator-3 (PEA-3), which is regulated by PKC (28, 30). The human MMP-2 promoter contains AP-2 and SP-1 transcription factor-binding sites. In hepatoma cells, activation of PKC and protein kinase A activated AP-2 and MMP-2 gene

expression (28, 31). PKC activation is suggested to be necessary for the induction of *MMP* gene expression in response to proinflammatory cytokines, such as IL-1 β and tumor necrosis factor- α in non-cardiac fibroblasts and tumor cell lines (32–35). PKC comprises a large family of serine-threonine kinases and plays an important role in the regulation of cardiovascular functions. The documented 12 members of the PKC family are classified according to their structure and substrate requirements (24, 36, 37). In this study, using PKC isoform-selective inhibitors, we show that PKC- α/β play a critical role in the regulation of MMP-9 expression and activity, whereas activation of PKC- ζ and PKC- θ plays a critical role in the regulation of expression and activity of MMP-2 and -9. The data presented here suggest that different isoforms of PKC differentially regulate the expression and activity of MMPs in cardiac fibroblasts in response to IL-1 β .

Various isoforms of PKC are known to modulate various signaling pathways (24). In cardiac myocytes, endothelin-1 stimulated activation of ERK1/2 is selectively mediated via PKC- ϵ , whereas PKC- δ preferentially activated JNKs and p38 kinase (38, 39). The data presented here suggest that PKC- α and - β act upstream of JNKs, whereas PKC- ζ and - θ act upstream of both ERK1/2 and JNKs. Activation of neither ERK1/2 nor JNKs requires PKC- ϵ . The differential effects of PKC isoforms on the regulation of MAPK activity in different cell types may reflect differences in activation of different isoforms of PKC and their downstream coupling. Of note, IL-1 β has been shown to activate ERK1/2, JNKs, and p38 kinase via PKC-independent pathways in intestinal myofibroblasts (40).

Another interesting finding of this study is that PKC- ζ and - θ , not PKC- α , - β , or - ϵ , act upstream in the activation of NF- κ B signaling pathway and that inhibition of NF- κ B inhibits IL-1 β -stimulated increases in MMP (MMP-2 and -9) expression and activity. Here, activation of NF- κ B was studied using increased phosphorylation of IKK α/β and I κ B- α kinase as an index. Increased phosphorylation and activation of IKK α/β increases phosphorylation of I κ B- α . Upon phosphorylation, the I κ B- α protein is rapidly degraded by the proteasome, thereby allowing NF- κ B to enter into the nucleus (41). Inhibition of ERK1/2 and JNKs failed to inhibit IL-1 β -stimulated increases in phosphorylation of IKK α/β and I κ B- α , suggesting activation of NF- κ B independent of MAPK pathways. The data presented here suggest both PKC- ζ and - θ are involved in the activation of NF- κ B. However, the effect of PKC- ζ seemed more prominent than PKC- θ . These data are consistent with the findings of Anrather *et al.* (42) in which PKC- ζ has been shown to be essential for the transcriptional activity of NF- κ B in endothelial cells.

NF- κ B has been suggested to play a role in the regulation of MMP expression (43, 44). The proximal stimulatory region of the MMP-9 promoter has a functional NF- κ B site (28). NF- κ B activity is shown to be required for the inducibility of MMP-9 production in smooth muscle cells in response to cytokines (45, 46). Inhibition of NF- κ B activation using SN50 inhibits MMP-9 expression and activity induced by IL-1 β in adult cardiac fibroblasts. However, our data that NF- κ B activity plays an essential role in the regulation of IL-1 β -stimulated increases in MMP-2 in cardiac fibroblasts are interesting because no consensus NF- κ B-binding site has been identified in MMP-2 promoter (28). Activation of latent MMP-2 is regulated by membrane-type 1 MMP (MT1-MMP) in conjunction with tissue inhibitors of MMPs (47). MT1-MMP has an NF- κ B-binding site in its promoter (44). Therefore, it is possible that IL-1 β -stimulated activation of NF- κ B increases MMP-2 activity indirectly via the increased expression of MT1-MMP. It is also possible that NF- κ B directly modulates MMP-2 expression and activity

via its interaction with other transcription factors that regulate MMP-2 expression. The later possibility is supported by our data that inhibition of NF- κ B also inhibits protein levels as well as activity of MMP-2.

The data presented here indicate differential regulation of MMP-2 and -9 expression and activity in adult rat cardiac fibroblasts. PKC- α/β activates JNKs, leading to increased protein levels and activity of MMP-9, whereas PKC- θ and PKC- ζ activate JNKs, ERK1/2, and NF- κ B, resulting in increased protein levels and activity of both MMPs (Fig. 8). Thus, IL-1 β -stimulated increases in MMP-9 expression and activity involve PKC- α/β , PKC- θ , PKC- ζ , ERK1/2, JNKs, and NF- κ B signaling molecules, whereas IL-1 β -stimulated increases in MMP-2 expression and activity are regulated via PKC- θ , PKC- ζ , and NF- κ B pathways. Although these observations are made *in vitro*, activation of PKC, NF- κ B, and AP-1 has been observed in the non-infarcted myocardium of rats after MI (48, 49). NF- κ B and AP-1 are also activated in congestive heart failure due to ischemic and dilated cardiomyopathy (49). We have observed increased phosphorylation of PKC- ζ in mice after MI (12), whereas others have shown increased expression of PKC- α and PKC- δ , not PKC- ϵ , in rat after MI (48). Thus, our findings raise the possibility that activation of these signaling pathways could contribute to pathologic remodeling of the heart by differentially regulating the expression and activity of MMP-2 and -9. A better understanding of the regulation of individual MMP is essential for the development of pharmacological strategies for the prevention of excessive matrix degradation and remodeling during chronic heart failure and after MI.

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