Osteopontin Inhibits Interleukin-1β-stimulated Increases in Matrix Metalloproteinase Activity in Adult Rat Cardiac Fibroblasts

ROLE OF PROTEIN KINASE C-ζ

Zhonglin Xie‡, Mahipal Singh‡, Deborah A. Siwik‡, William L. Joyner‡, and Krishna Singh‡

From the 3Department of Physiology, James H. Quillen College of Medicine, James H. Quillen Veterans Affairs Medical Center, East Tennessee State University, Johnson City, Tennessee 37614 and 5Boston University School of Medicine, Boston, Massachusetts 02118

We have shown that osteopontin (OPN), an extracellular matrix protein, plays an important role in post myocardial infarction (MI) remodeling by promoting collagen synthesis and accumulation. Interleukin-1β (IL-1β), increased in the heart following MI, increases matrix metalloproteinase (MMP) activity in cardiac fibroblasts in vitro. Here, we show that OPN alone has no effect on MMP activity or expression. However, it reduces IL-1β-stimulated increases in MMP activity and expression in adult rat cardiac fibroblasts. Pretreatment with bovine serum albumin had no effect on MMP activity or protein content, whereas GRGDs (glycine-arginine-glycine-aspartic acid-serine)-pentapeptide (which interrupts binding of RGD-containing proteins to cell surface integrins) and monoclonal antibody m7E3 (a rat β3 integrins antagonist) inhibited the effects of OPN. Inhibition of PKC using chelerythrine inhibited the activities of both MMP-2 and MMP-9. Stimulation of cells using IL-1β increased phosphorylation and translocation of PKC to membrane fractions, which was inhibited by OPN. OPN inhibited IL-1β-stimulated increases in translocation of PKC-ζ from cytosolic to membrane fractions. Furthermore, the levels of phospho-PKC-ζ were lower in the cytosolic fractions of OPN knock-out mice hearts as compared with wild type 6 days post-MI. Inhibition of PKC-ζ using PKC-ζ pseudosubstrate inhibited IL-1β-stimulated increases in MMP-2 and MMP-9 activities. These observations suggest that OPN, acting via β3 integrins, inhibits IL-1β-stimulated increases in MMP-2 and MMP-9 activity, at least in part, via the involvement of PKC-ζ. Thus, OPN may play a key role in collagen deposition during myocardial remodeling following MI by modulating cytoskeleton-stimulated MMP activity.

The dynamic synthesis and breakdown of extracellular matrix (ECM) proteins may play an important role in myocardial remodeling (1, 2). Osteopontin (OPN) is a glycosylated and phosphorylated ECM protein (3, 4). Although first isolated from mineralized matrix, OPN has since been shown to be synthesized by cardiac fibroblasts, endothelial cells, and myocytes (3, 5–7). Using spontaneously hypertensive and aortic-banded rats, we have shown increased expression of OPN coincident with heart failure (8). OPN interacts with specific integrins, namely α5β1, αvβ3, αvβ5, and also with CD44 receptors and affects many cellular processes including cell attachment, spreading, and migration (3, 4, 9). Recently, we have documented an increased expression of OPN in remote and infract regions of left ventricle following myocardial infarction (MI). Furthermore, using OPN knock-out mice and MI as a model of myocardial remodeling, we demonstrated that a lack of OPN results in greater left ventricle dilation and reduced collagen synthesis and accumulation 1 month post-MI, suggesting a role for OPN in ECM reorganization in the heart during myocardial remodeling (10). A critical role for OPN in the generation of interstitial fibrosis was observed in the skin incision model of wound healing and in the kidney following obstructive uropathy (11, 12). OPN has been shown to modulate the activity of matrix metalloproteinases (MMPs) in various cell types (13–15).

MMPs are a large family of zinc-containing endopeptidases involved in degradation of ECM components (1, 16–19). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are well known for their ability to degrade gelatin and type IV collagen. Because of their ability to initiate and continue the degradation of fibrillar collagens, the gelatinases are considered to play a major role in the remodeling of collagenous ECM (19). Since MMPs are capable of degrading ECM, the activity of these enzymes is under strict regulation. Within the heart, MMPs are synthesized by fibroblasts, inflammatory cells, and cardiac myocytes (19, 20). Normal heart expresses low basal levels of MMPs. Increased expression and activity of MMP-1, MMP-2, MMP-3, and MMP-9 have been demonstrated in human, rat, and porcine hearts during the remodeling process following MI (21–23). Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α are increased in the heart following MI (24, 25). Recently, using in vitro cultures of cardiac fibroblasts, Siwik et al. (26) demonstrated that IL-1β and tumor necrosis factor-α increase MMP-2 and MMP-9 expression and activities. However, signaling pathways involved in the regulation of MMP expression and activity have not yet been clarified. In cells of non-cardiac origin, activation of protein kinase C (PKC) is suggested to play a critical role in the

1 This work was supported in part by NHLBI, National Institutes of Health Grants HL-071519 and HL-057947 and a merit review grant from the Department of Veterans Affairs (to K. S.) and a fellowship from the American Heart Association, Southeast Affiliate (to Z. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Physiology, James H. Quillen College of Medicine, East Tennessee State University, P. O. Box 70576, Johnson City, TN 37614. Tel.: 423-439-2049; Fax: 423-439-2052; E-mail: singhk@mail.etsu.edu.

§ The abbreviations used are: ECM, extracellular matrix; OPN, osteopontin; MI, myocardial infarction; MMP, matrix metalloproteinase; KO, knock-out; WT, wild type; ANOVA, analysis of variance.
regulation of expression and activity of MMPs (27). Among the PKC isoforms, the activation of PKC-ζ (atypical isoform) is shown to be essential for MMP-1, MMP-3, and MMP-9 secretion in rabbit smooth muscle cells (28).

Since both OPN and IL-1β are increased during pathophysiological conditions of the heart, we hypothesized that OPN increases collagen accumulation in the heart following MI by inhibiting IL-1β-stimulated MMP activity. The data presented here suggest that OPN inhibits IL-1β-stimulated increases in MMP-2 and MMP-9 activity via the involvement of PKC-ζ.

EXPERIMENTAL PROCEDURES

Rat Cardiac Fibroblasts Culture and Treatments—Adult rat cardiac fibroblasts were isolated from the supernatant of adult rat cardiac myocytes as described previously (26). 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 of the experiments. The cells were treated with IL-1 (4 ng/ml) for 48 h to measure MMP activity or for 15–30 min to measure activation and translocation of PKC and PKC-ζ. OPN (50–200 nm, purchased from R&D systems), bovine serum albumin (BSA, 100 nm), GRGDS-pentapeptide (100 nm), or monoclonal antibody m7E3 Fab′ (2 μM, m7E3, 40–160 μg/ml) was added 30 min prior to the addition of IL-1β. The β1 integrin antagonist m7E3, kindly provided from Dr. Jonlow (Centocor, Inc.) was shown to inhibit MMP activity and smooth muscle cell migration (29). For experiments testing the role of PKC, cells were pretreated with chelerythrine (1–5 μM) or PKC-ζ pseudosubstrate (50 μM) for 30 min followed by treatment with IL-1β for 48 h.

In-gel Zymography—MMP activity in the conditioned media of fibroblasts was measured as described previously (26). The media were collected, centrifuged for 5 min at 500 × g to remove cells and debris, and lyophilized to dryness. The pellet was resuspended in water, and protein content was determined using Bradford assay (Bio-Rad). Samples (500 ng of protein) were loaded under non-reducing conditions onto 4% stacking and 10% separating SDS-polyacrylamide gel polymerized (500 ng of protein) were loaded under non-reducing conditions onto 1% Triton X-100 for 30 min with gentle shaking followed by a 30-min wash in distilled water. The gels were then incubated overnight at 37 °C in substrate buffer (50 mM Tris-HCl, pH 8.5, 5 mM CaCl₂, and 0.02% NaN₃), stained with Coomassie Blue R-250, and then destained using 7% acetic acid and 40% methanol solution. Clear bands were resolved by 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. The membrane was blocked using 10% nonfat milk in TBST containing (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). The samples were vortexed and centrifuged at 12,000 rpm for 10 min. Equal amounts of proteins from the supernatant were analyzed by Western blot using anti-phosho-PKC-ζ and anti-PKC-ζ antibodies. The loading of RNA was evaluated using Northern analysis of RNA using MMP-2 cDNA as a probe as described previously (5, 6, 31). Protein expression of MMP-2 and MMP-9 was measured in the conditioned media using monoclonal MM-2 or MMP-9 antibodies.

RNA Isolation and Northern Analysis—Total RNA from cells was extracted using the method of Chomczynski and Sacchi (32). RNA was size-fractionated on 1.0% formaldehyde-agarose gel and transferred to GeneScreen plus membrane. The blots were hybridized overnight using MMP-2 cDNA as probe as described previously (8). The loading of RNA in each lane was observed from ethidium bromide-stained gels.

Myocardial Infarction and Preparation of Tissue Extracts—MI was performed on age-matched OPN knock-out (KO) and wild-type (WT) mice as described previously (10). The sham animals underwent the same procedure without ligation of the coronary artery. All of the experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The mice were sacrificed 6 days post-MI. To prepare cytosolic fractions, tissue from non-infarcted left ventricle region was homogenized in lysis buffer (50 mM Tris-HCl, pH 7.6, 0.2 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35, and 0.02% NaN₃) using glass homogenizer as described previously (33). The samples were centrifuged at 4 °C for 5 min at 2,500 rpm. The supernatant was centrifuged again at 100,000 × g for 60 min. The supernatant was collected supernatant was considered a cytosolic fraction. The pellet was then resuspended in water, and lyophilized to dryness. The pellet was resuspended in water, and protein content was determined using Bradford assay (Bio-Rad). Samples (500 ng of protein) were loaded under non-reducing conditions onto 1% Triton X-100 for 30 min with gentle shaking followed by a 30-min wash in distilled water. The gels were then incubated overnight at 37 °C in substrate buffer (50 mM Tris-HCl, pH 8.5, 5 mM CaCl₂, and 0.02% NaN₃), stained with Coomassie Blue R-250, and then destained using 7% acetic acid and 40% methanol solution. Clear bands were resolved by 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. The membrane was blocked using 10% nonfat milk in TBST containing (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). The samples were vortexed and centrifuged at 12,000 rpm for 10 min. Equal amounts of proteins from the supernatant were analyzed by Western blot using anti-phosho-PKC-ζ and anti-PKC-ζ antibodies.

Statistical Analyses—All of the data are expressed as the mean ± S.E. Statistical analysis was performed using the Student's t test or a one-way analysis of variance (ANOVA) and a post hoc Tukey's test. Probability (p) values of <0.05 were considered to be significant.

RESULTS

OPN Inhibits IL-1β-stimulated Increases in MMP Activity—In-gel zymography of conditioned media from cardiac fibroblasts showed that IL-1β significantly increases MMP activity, confirming our previous finding (26). Treatment of cells with OPN in the presence of IL-1β dose dependently inhibited IL-1β-stimulated increases in MMP activity (Fig. 1A). The inhibition in MMP activity was detectable at 50 nM concentration of OPN; however, it was significant at 100 nM. Therefore, a 100 nM concentration was used for further experiments. Treatment of cells with OPN inhibited IL-1β-stimulated (folds change versus control) MMP-2 (bands corresponding to 72/66 kDa; IL-1β, 1.8 ± 0.1; IL-1β + OPN, 1.48 ± 0.1; p < 0.001, n = 12) and MMP-9 (bands corresponding to 95/88 kDa; IL-1β, 3.7 ± 0.4; IL-1β + OPN, 1.4 ± 0.4; p < 0.001, n = 12) activities. OPN alone had no effect on MMP activity. To confirm that the above observations are specific for OPN, cardiac fibroblasts were pretreated with BSA (100 nm) followed by treatment with IL-1β for 48 h. An analysis of conditioned media showed that pretreatment of cells with BSA did not significantly affect MMP-2 (IL-1β, 1.8 ± 0.1; IL-1β + BSA, 1.9 ± 0.2) and MMP-9 (IL-1β, 3.7 ± 0.4; IL-1β + BSA, 3.5 ± 0.2; see Fig. 1) activities.

Western blot analyses of the conditioned media demonstrated that IL-1β increases MMP-2 (Fig. 2A) and MMP-9 (Fig. 2B) protein contents by 7.9 ± 0.01- and 10.2 ± 1.7-folds, respectively. Pretreatment with OPN reduced MMP-2 and MMP-9 protein levels 0.8 ± 0.01 and 1.0 ± 0.1-folds (p < 0.05 versus IL-1β), respectively. Pretreatment with BSA had no significant effect on IL-1β-mediated increases in MMP2 and MMP-9 protein contents.

Northern analysis of RNA using MMP-2 cDNA as probe demonstrated increased expression of MMP-2 following 48 h of IL-1β stimulation. This increase in MMP-2 mRNA was inhibited by pretreatment with OPN (Fig. 2C).

OPN contains an arginine-glycine-aspartate (RGD) cell binding domain (3, 4, 9) and interacts with integrin and CD44 receptors. Therefore, to study the receptor-mediated effects of OPN, cells were pretreated with GRGDS-pentapeptide (100 nm). An analysis of conditioned media showed that pretreatment with GRGDS-pentapeptide significantly inhibits OPN-mediated decreases in MMP-2 (IL-1β, 2.0 ± 0.1; IL-1β + OPN, 1.3 ± 0.1; RGD + IL-1β + OPN, 1.7 ± 0.1-folds) and MMP-9 (IL-1β, 3.5 ± 0.3; IL-1β + OPN, 1.7 ± 0.1; RGD + IL-1β + OPN,
cells were pretreated with 40–160 μg/ml m7E3 in the presence of OPN for 30 min followed by treatment with IL-1β for 48 h. m7E3 dose dependently inhibited OPN-mediated decreases in MMP-2 and MMP-9 activity (data not shown). m7E3 at 160 μg/ml concentration almost completely inhibited the effects of OPN (Fig. 4, lane 5). To demonstrate that the effects of m7E3 are specific, cells were pretreated with BSA (160 μg/ml) in the presence of OPN (Fig. 4, lane 4).

Activation of PKC and Its Role in the Regulation of MMP Activity—To elucidate the role of PKC in IL-1β-stimulated increases in MMP activity, activation of PKC was measured in total cell lysates and membrane fractions using anti-phospho-PKC (pan) antibodies. Western analyses showed that IL-1β stimulation increases phosphorylation of PKC in total cell lysate (1.5 ± 0.1-fold versus CTL, p < 0.001, n = 3; Fig. 5, A and C) and membrane fractions (1.8 ± 0.05-fold versus CTL, p < 0.05, n = 3; Fig. 5, B and D). Pretreatment with OPN inhibited the IL-1β-stimulated increases in PKC activity in the lysate as well as in membrane fractions.

To assess whether activation of PKC plays a role in the regulation of IL-1β-mediated increases in MMP activity, cells were pretreated with chelerythrine (1 and 5 μM, an inhibitor of PKC). An analysis of conditioned media using in-gel zymography demonstrated that chelerythrine inhibits MMP activity (MMP-2 and MMP-9) in a dose-dependent manner where 1 μM partially inhibits MMP activity and a 5 μM concentration almost completely inhibits IL-1β-stimulated increases in MMP activity (Fig. 6).

Role of PKC-ζ in the Regulation of MMP Activity—PKC-ζ, an atypical isoform, has been suggested to play a critical role in the regulation of MMP activity in glioma cells and smooth muscle cells (28, 34). In smooth muscle cells, PKC-ζ is shown to be essential for secretion of cytokine-induced MMP-1, MMP-3, and MMP-9 (28). To elucidate the role of PKC-ζ, we measured phosphorylated PKC-ζ in the cytosolic and membrane fractions using anti-phospho-PKC-ζ antibodies. Western analyses

FIG. 2. OPN inhibits IL-1β-stimulated MMP protein and mRNA expression. Confluent cultures of cardiac fibroblasts were pretreated with OPN (100 nM) or BSA (100 nM) for 30 min followed by treatment with IL-1β (4 ng/ml) for 48 h. MMP-2 (A) and MMP-9 (B) protein levels in the conditioned media were analyzed by Western blotting using monoclonal anti-MMP-2 and anti-MMP-9 antibodies. The experiment was repeated three times with similar results. C, total cellular RNAs were analyzed by Northern analysis using MMP-2 cDNA probe. Pretreatment of cells with OPN prevents IL-1β-stimulated increases in MMP-2 mRNA (upper panel). Ethidium bromide-stained 18 S rRNA (lower panel) is used to show the differences in loading. CTL, control.

FIG. 3. OPN inhibits IL-1β-stimulated MMP activity. Confluent cultures of cardiac fibroblasts were pretreated with OPN (50–200 nM) (A), OPN (100 nM) or BSA (100 nM) (B) for 30 min followed by treatment with IL-1β (4 ng/ml) for 48 h. MMP activity in conditioned media was measured using in-gel zymography. C and D represent MMP-2 and MMP-9 activities, respectively, expressed as fold increase versus control (CTL). *, p < 0.001 versus CTL; #, p < 0.01 versus IL-1β; n = 12.
FIG. 3. GRGDS-pentapeptide inhibits the effects of OPN on IL-1β-stimulated increases in MMP activity. Confluent cultures of cardiac fibroblasts were pretreated with GRGDS-pentapeptide (RGD, 100 nM) for 30 min followed by treatment with OPN protein (100 nM) for 30 min. The cells then were stimulated with IL-1β (4 ng/ml) for 48 h. MMP activity in conditioned media was measured using in-gel zymography. Panel A represents zymography demonstrating the changes in MMP activity. Panels B and C represent MMP-2 and MMP-9 activities, respectively, expressed as fold increase versus control (CTL). *, p < 0.001 versus CTL; #, p < 0.01 versus IL-1β; @, p < 0.05 versus IL-1β+OPN (n = 4).

FIG. 4. β3 integrin antagonist m7E3 inhibits the effects of OPN on IL-1β-stimulated increases in MMP activity. Confluent cultures of cardiac fibroblasts were pretreated with m7E3 (160 μg/ml) or BSA (160 μg/ml) for 30 min followed by treatment with OPN protein (100 nM) for 30 min. The cells then were stimulated with IL-1β (4 ng/ml) for 48 h. MMP activity in conditioned media was measured using in-gel zymography.

showed that under basal (control) conditions, phosphorylated PKC-ζ is mainly present in the cytosolic fraction, not in the membrane fraction. IL-1β stimulation increases phosphorylated PKC-ζ in the membrane fractions while reducing the amount of phosphorylated PKC-ζ in the cytosolic fractions (Fig 7A). OPN inhibited IL-1β-stimulated translocation of PKC-ζ from cytosolic fractions to membrane fractions. OPN alone had no effect on PKC-ζ translocation.

An analysis of cytosolic fractions of hearts 6 days post-MI demonstrated higher levels of phosphorylated PKC-ζ in WT MI hearts as compared with WT sham and OPN KO MI hearts (Fig. 7B, fold change, WT sham 1.0 ± 0.16, n = 4; WT MI 1.8 ± 0.04*, n = 5; KO sham 0.9 ± 0.3, n = 3; KO MI 1.2 ± 0.09*, n = 3; *, p < 0.01 versus WT sham; *, p < 0.01 versus WT MI). Total heart lysates exhibited no apparent differences in the levels of phosphorylated PKC-ζ among these groups (data not shown).

To assess whether activation of PKC-ζ plays a role in the regulation of IL-1β-mediated increases in MMP activity, cells were pretreated with myristoylated PKC-ζ pseudosubstrate peptide. This peptide has been shown to inhibit PKC-ζ activity by interacting with the substrate binding pocket in the catalytic domain (35, 36). An analysis of conditioned media using in-gel zymography demonstrated that PKC-ζ pseudosubstrate inhibits MMP activity (Fig. 8).

DISCUSSION

Previously, we have shown that increased expression of OPN in the heart plays an important role in post-MI remodeling by promoting collagen synthesis and accumulation (10). The major new findings of this study are that OPN inhibits IL-1β-stimulated increases in MMP activity (MMP-2 and MMP-9) and expression in adult rat cardiac fibroblasts. OPN inhibits IL-1β-stimulated increases in PKC activity, specifically PKC-ζ activity, and inhibition of PKC-ζ activity inhibits IL-1β-stimulated increases in MMP activity.

The finding that OPN inhibits IL-1β-stimulated increases in MMP activity in adult rat cardiac fibroblasts is consistent with the evidence that OPN plays a role in the regulation of ECM remodeling (13–15). However, the inhibitory effects of OPN on MMP activity were observed only in the presence of IL-1β. Purified recombinant OPN protein (purchased from R & D systems) alone did not affect MMP-2 activity. Occasionally, we observed increased MMP-9 activity; however, the data were not found statistically significant. In human melanoma cells, recombinant OPN alone also failed to increase MMP-2 activity (37). In human aortic smooth muscle cells, purified OPN alone failed to affect MMP-2 or MMP-9 activity. However, OPN in the presence of platelet-derived growth factor increased MMP-9 but not MMP-2 activity (13). In contrast, purified human milk OPN protein alone increased MMP-2 production in murine melanoma cells (15). The differential effects of OPN on MMP activity in different cell types may reflect differences in the kinetics of coupling to different OPN receptors in different cell types. It is also important to emphasize that effects of OPN on MMP-2 in murine melanoma cells were observed at 5–10 μM, whereas in cardiac fibroblasts and aortic smooth muscle cells, the effects were observed at a 50–100 nM concentration.

The effects of OPN on MMP-2 and MMP-9 activity seem to be
PKC activation was studied by "dures. described under membrane fractions were prepared as de-
tion of MMPs activity (29). In fact, are representative blots obtained from 
total cell lysates and membrane fractions, respectively. Panels A and B are representative 
Western blot analyses using anti-phos-
pho-PKC (pan) antibodies. 
crease phosphorylation expressed as fold in-
treatment with IL-1
m7E3 inhibited MMP-2 and MMP-9 activity in carotid extracts 
specific since the same concentration of BSA (100 nM) had no effect on IL-1-β-stimulated MMP activity. Furthermore, the inhibitory effects of OPN were inhibited by GRGDS-pentapeptide and m7E3. GRGDS-pentapeptide alone had no effect on MMP-2 or MMP-9 activities. Consistent with our findings, Philip et al. (15) observed that RGD-containing non-cyclic and cyclic (GRGDSP and GPenGRDSPCA)-peptides had no effect on MMP-2 production and activation in murine melanoma cells. OPN contains an RGD cell binding sequence and has been shown to interact with αvβ3, αvβ5, αvβ1, and CD44 family of integrins (3, 7, 9). Adult cardiac fibroblasts express αv, β3, β5, and β3 integrins in vitro (38). Integrins and CD44 receptor signaling are known to modulate MMP expression and activity in various cell types (13, 39, 40). Using m7E3, an antagonist of rat β3 integrins, we identify β3 integrins as a potential candidate involved in OPN signaling in cardiac fibroblasts. The importance of β3 integrins, specifically αvβ3, in the regulation of MMPs has been suggested in various cell types. In smooth muscle cells, OPN binding to the αvβ3 receptors is shown to stimulate MMP-9 and MMP-1 synthesis (13). Treatment with m7E3 inhibited MMP-2 and MMP-9 activity in carotid extracts obtained from Sprague-Dawley rats 4 days after balloon catheter injury, suggesting a role for αvβ3 integrins in the regulation of MMPs activity (29). In fact, αvβ3 integrins play an important role in angiogenesis by binding and activating MMP-2 in endothelial cells (41).

MMP activity is regulated by both transcriptional and post-translational mechanisms (18, 19). Posttranslational regulation of MMPs occurs through the activation of latent proenzymes (ProMMPs) by plasmin, trypsin, chymase, elastase, and kallikrein. The membrane-type 1 MMP enzyme is also involved in the activation of MMPs. Conversely, MMP activity can be inhibited by interaction with naturally occurring inhibitors called tissue inhibitors of MMPs. In a previous study, Siwik et al. (26) provided evidence that IL-1β increases MMP activity because of increased mRNA and protein expression in cardiac fibroblasts. In this study, we provide evidence that OPN inhibits IL-1β-stimulated activity of MMPs, at least in part, by inhibiting their expression. IL-1-β-stimulated increases in both MMP-2 and MMP-9 protein levels were inhibited by OPN. Furthermore, IL-1β-stimulated increases in MMP-2 mRNA levels were also inhibited by pretreatment with OPN.

Expression of MMPs is normally low in adult heart tissue. The MMP expression is up-regulated during certain physiological and pathological remodeling processes of the heart (17, 19, 42, 43). Once activated, MMPs are collectively capable of degrading the complete ECM. Therefore, it is important that the activity of these enzymes is kept under tight control. In non-cardiac fibroblasts and tumor cell lines, activation of PKC in response to proinflammatory cytokines has been suggested to be necessary for the expression and activation of MMPs (27, 28, 44, 45). In this study, using PKC inhibitor chelerythrine, we show that activation of PKC plays a pivotal role in the regulation of IL-1-β-stimulated MMP activity in cardiac fibroblasts. Chelerythrine represents a unique class of PKC inhibitors that non-competitively inhibit the ATP binding site (46). The inhibition of IL-1-β-stimulated PKC activation by OPN provides indirect evidence that OPN may act through PKC to inhibit IL-1-β-stimulated increase in MMP activity. PKC has been suggested to play an important role in OPN-induced migration of human mammary epithelial cells (47).

In this study, we identify PKC-ζ as a key regulator of IL-1-β-stimulated MMP activity in adult rat cardiac fibroblasts. This is evident from the data that stimulation of cells with IL-1β increases translocation of phosphorylated PKC-ζ from cytosolic fractions to membrane fractions. IL-1-β-stimulated translocation of PKC-ζ is inhibited by OPN pretreatment. Furthermore, the levels of phosphorylated PKC-ζ were lower in the cytosolic fractions of OPN KO hearts as compared with WT 6 days post-MI, suggesting increased translocation of PKC-ζ to membrane fractions in the absence of OPN in the heart post-MI. In
Osteopontin Regulates MMP Activity in Cardiac Fibroblasts

A

B

WT KO

FIG. 6. Inhibition of PKC-ζ inhibits IL-1β-stimulated increases in MMP activity. Confluent cultures of cardiac fibroblasts were pretreated with PKC-ζ pseudosubstrate (PS-zeta; 50 μM) for 30 min followed by treatment with IL-1β for 48 h. MMP activity in the conditioned media was measured using in-gel zymography. The experiment was repeated three times with similar results.

adult rat cardiac fibroblasts, possibly through the inhibition of PKC-ζ pathway. Activation of PKC-ζ plays a critical role in the regulation of both MMP-2 and MMP-9 activity. Our findings of inhibition of IL-1β-stimulated MMP activity by OPN are consistent with the evidence that OPN plays an important role in collagen accumulation and suggest that one mechanism, by which OPN may promote collagen accumulation in the heart following MI, is by inhibiting cytokine-stimulated increases in MMP activity, possibly through the involvement of PKC-ζ.

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Zhonglin Xie, Mahipal Singh, Deborah A. Siwik, William L. Joyner and Krishna Singh

J. Biol. Chem. 2003, 278:48546-48552.
doi: 10.1074/jbc.M302727200 originally published online September 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302727200

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