Published in final edited form as:

Endothelium. 2008 ; 15(1): 33–42. doi:10.1080/10623320802092260.

Differential Effects of Shear Stress and Cyclic Strain on Sp1 Phosphorylation by Protein Kinase C ζ Modulates Membrane Type 1–Matrix Metalloproteinase in Endothelial Cells

Ji Il Kim,

Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA; and Department of Surgery, Catholic University School of Medicine, Seoul, Korea

Alfredo C. Cordova,

Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA

Yo Hirayama,

Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA

Joseph A. Madri, and

Pathology, Yale University School of Medicine, New Haven, Connecticut, USA

Bauer E. Sumpio

Department of Vascular Surgery, Yale University School of Medicine, New Haven, Connecticut, USA; and Veteran Administration Connecticut Health System, West Haven, Connecticut, USA

Abstract

Membrane type 1–matrix metalloproteinase (MT1-MMP) plays a key role in extracellular matrix remodeling, endothelial cell (EC) migration, and angiogenesis. Whereas cyclic strain (CS) increases MT1-MMP expression, shear stress (SS) decreases MT1-MMP expression. The aim of this study was to determine if changes in levels of Sp1 phosphorylation induced by protein kinase C ζ (PKC ζ) in ECs exposed to SS but not CS are important for MT1-MMP expression. The results showed that SS increased Sp1 phosphorylation, which could be inhibited by pretreatment with PKC ζ inhibitors. In the presence of PKC ζ inhibitors, the SS-mediated decrease in MT1-MMP protein expression was also abolished. These data demonstrate that increased affinity of Sp1 for MT1-MMP's promoter site occurs as a consequence of PKC ζ -induced phosphorylation of Sp1 in response to SS, increasing Sp1 binding affinity for the promoter site, preventing Egr-1 binding, and consequently decreasing MT1-MMP expression.

Keywords

Hemodynamic Forces; MMP; PKC; sp1 phosphorylation

Matrix metalloproteinases (MMPs) constitute a family of structurally related zinc endopeptidases capable of proteolysis of numerous components of the extracellular matrix as well as a variety of cell surface proteins and cyto- and chemokines (Haas et al. 1998; Hiraoka

et al. 1998; Woessner 1994). MMP production and activation are strongly associated with endothelial cell (EC) migration, reduction of intercellular adhesion, and degradation of subendothelial matrix, which are all important components of the process of angiogenesis (Birkedal-Hansen H. 1995; Mignatti and Rifkin 1996). The activation of MMPs has been shown to be correlated with tumor- and inflammation-mediated angiogenesis (Haas et al. 1999, 2000; Rafty et al. 2001). Haas et al. (2000), using an EC culture model utilizing an extracellular matrix substrate, have shown that in particular membrane type 1–matrix metalloproteinase (MT1-MMP) and MMP-2 play important roles in inducing angiogenesis. MT1-MMP has been shown to be a key regulator of angiogenesis (Inoue et al. 1977; Kishimoto et al. 1980). It is one of the members of the family of matrix-bound MMPs having a C-terminal transmembrane domain and is identified as a physiologic activator of pro-MMP-2 on the cell surface as well as a functional enzyme that degrades a number of extracellular matrix components and bioactive molecules (McQuibban et al. 2000, 2001, 2002; Sato et al. 1994, 1997; Strongin et al. 1995; Tam et al. 2004; Van Den Steen et al. 2003; Zhang et al. 2003).

It is known that hemodynamic forces such as cyclic strain (CS) and fluid shear stress (SS) can modulate the expression of various genes in ECs (Davies et al. 1997; Yamaguchi et al. 2002). Both CS and SS are hypothesized to trigger angiogenesis in skeletal and coronary muscle in vivo (Hudlicka 1998; Yamaguchi et al. 2002). The up-regulation of MT1-MMP mRNA and protein occurs in microvascular ECs cultured in a three-dimensional collagen type I matrix as a consequence of increased production and promoter binding of the transcription factor Egr-1 (Haas et al. 1998; Haas 1999; Yun et al. 2002). Previous studies carried out by our laboratory (Yamaguchi et al. 2002; Yun et al. 2002) have shown that the mechanical force generated by CS increases the expression of MT1-MMP by displacing Sp1, with increased Egr-1 expression and binding to the promoter site (Yamaguchi et al. 2002; Yun et al. 2002). Although Egr-1 mRNA transcription and protein levels were induced in response to SS, SS was associated with decreased MT1-MMP mRNA transcription and protein levels in a time-dependent fashion. Egr-1 protein was increased after SS and CS, but Sp1 was serine phosphorylated only after SS. SS increased Sp1 DNA binding, which was inhibitable with calf intestinal phosphatases. Thus, SS appears to inhibit MT1-MMP expression despite Egr-1 up-regulation by inducing the serine phosphorylation of Sp1, which in turn increases its binding affinity for its site on the MT1-MMP promoter, reducing the ability of Egr-1 to displace it (Yun et al. 2002).

Sp1 is the founding member of a family of zinc finger transcription factors, which includes at least four Sp transcription factors (Black et al. 1999; Cook et al. 1999; Philipsen and Suske 1999). These transcription factors play important roles in a wide variety of physiological processes, including cell cycle regulation, hormonal activation, apoptosis, angiogenesis, etc. Sp proteins share several well-conserved functional domains, including the N-terminal transcription activation domains and the C-terminal zinc finger DNA-binding domain (Courey and Tjian 1988; Courey et al. 1989; Pascal and Tjian 1991). Among the Sp transcription factors, Sp1 is widely expressed in tissues and binds GC box motifs in promoters. Since the seminal study by (Jackson et al. 1993), several kinases involved in Sp1 phosphorylation have been identified. In renal cell carcinoma and human fibrosarcoma cells (Pal et al. 1998) and rat smooth muscle cells (SMCs) (Rafty et al. 2001), it has been proposed that protein kinase C ζ (PKC ζ) binds and phosphorylates the zinc finger region of Sp1, resulting in activation of Sp1-mediated transcription.

PKC is a member of the AGC (protein kinase A, G, and C) superfamily and has been implicated in playing significant roles in a wide range of physiological and pathological processes, including mitogenesis and proliferation of cells, protein synthesis, transcriptional regulation, gene expression, cell survival, apoptosis, remodeling of the actin cytoskeleton, and modulation of ion channels (Cenni et al. 2002; Chou et al. 1998; Liu and Heckman 1998; Nishizuka 1995; Toker 1998). It is composed of 12 identified members in mammalian cells divided into

three subfamilies: the conventional, the novel, and the atypical PKC (Toker 1998). PKC ζ , an atypical member of the PKC family of enzymes, is involved in a variety of processes, including mitogenesis, protein synthesis, cell survival, transcriptional regulation, and other biological functions (Black et al. 1999; Haas et al. 1998, 1999; Yun et al. 2002). Most PKC inhibitors do not decrease PKC ζ activity (Nakanishi et al. 1993; Pal et al. 2001), except Gö6983 at a concentration 60 nM, chelerythrine chloride at a concentration of 660 nM, and myristoylated PKC ζ pseudosubstrate at a concentration of 10 μ M when added to cells. These concentrations inhibit the conventional, novel PKCs, and PKC ζ (Cuschieri et al. 2004; Eichholtz et al. 1993; Sun et al. 2005). Like many other protein kinases, PKC ζ requires phosphorylation within its activation loops to express full catalytic potential (Chou et al. 1998; Keranen et al. 1995; Pal et al. 2001).

Thus, we hypothesize that PKC ζ may be involved in the signaling pathway that mediates MT1-MMP expression in cells exposed to CS and SS, being responsible for the increased affinity of Sp1 to the MT1-MMP promoter site as a consequence of phosphorylation of Sp1, displacing Egr-1 from the binding sites and thus inhibiting MT1-MMP transcription. This study demonstrates that PKC ζ is critical to regulation of MT1-MMP expression via differential Sp1 phosphorylation in ECs exposed to SS and CS.

METHODS AND MATERIALS

Cell Culture

Rat microvascular ECs were harvested from the epididymal fat pads of Sprague-Dawley rats and cultured as described previously (Madri and Williams 1983). Twelve rats were used per preparation, and experiments were performed on two separate isolations of cells. Cells were briefly grown on gelatin-coated tissue culture plates (1.5% gelatin in phosphate-buffered saline) and maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 25% sterile-filtered conditioned bovine aortic EC medium and 10% fetal bovine serum. For all experiments, ECs were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotic-antimycotic (penicillin 100 units/mL, streptomycin 100 μ g/mL, and amphotericin B 250 ng/mL [Invitrogen]) in a humidified atmosphere of 5% CO₂ on type I collagen coated 35-mm 6-well culture dishes at 37°C. Serum-containing medium was replaced by serum-free medium 24 h prior to each experiment. Cells were synchronized by serum-starving for 24 h.

Application of Inhibitors of PKC ζ

To determine the effects of PKC ζ on MT1-MMP protein expression and phosphorylation of Sp1, we used calphostin C (Calbiochem, San Diego, CA), Gö6983 (Calbiochem, San Diego, CA), chelerythrine chloride (Calbiochem, San Diego, CA), and myristoylated PKC ζ pseudosubstrate (Biomol, Plymouth Meeting, PA) as inhibitor of PKC. Cells were pretreated with 10 nM of Gö6983 or 50 nM of calphostin C or 10 μ M of myristoylated PKC ζ pseudosubstrate for inhibiting conventional and novel PKC isoforms 1 h before applying SS and CS. We used 60 nM of Gö6983 or 660 nM of chelerythrine chloride as inhibitors for all PKCs. These concentrations inhibit the conventional and novel PKCs, and PKC ζ (Cuschieri et al. 2004; Eichholtz et al. 1993; Sun et al. 2005).

Application of Cyclic Strain

ECs were seeded on flexible silicon membranes coated with type I collagen 6-well culture dishes (Flex-Cell International, McKeesport, PA) and synchronized as described above. ECs were exposed to cyclic strain using a Flexercell Strain Unit (Flexcell FX-4000 system; Flex-Cell International) that consists of a vacuum manifold with recessed ports controlled by a

computer program. The CS regime was carried out utilizing 150 mm Hg deformation at 60 cycles/min, resulting in 24% maximum strain (Yamaguchi et al. 2002).

Application of Shear Stress

ECs were seeded on type I collagen-coated 35-mm 6-well culture dishes. SS was applied to confluent cultures with an orbital shaker (Lab-Line, Melrose Park, IL). Applying a shaking frequency of 270 rpm results in a shear stress of 14 dynes/cm², which is normal level in arteries. Although this technique does not result in uniform application of laminar SS across the entire monolayer, the majority of cells are exposed to near-maximal SS (Dardik et al. 2005; Yun et al. 2002).

Western Blot Analysis

Whole-cell extracts prepared from ECs were lysed in 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM Na₃VO₄, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentration was measured using the modified Bradford technique and a DU-64 Spectrophotometer. The extracts were heat-denatured in Laemmli sample buffer, sized fractionated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane (Amersham Biosciences). After blocking for 1 h with Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% albumin bovine serum, the blots were exposed to polyclonal antibodies to phosphorylated (pPKCζ) or total (T) nPKCζ (Santa Cruz Biotechnology, Santa Cruz, CA), or MT1-MMP (Chemicon International, Temecula, CA). After rinsing three times for 15 min in TTBS, blots were incubated with a 1:1000 dilution of anti-rabbit immunoglobulin G's (IgG's) and anti-mouse IgGs for at least 1 h. After three additional 15-min rinses in TTBS, the resulting protein-antibody complexes were detected by enhanced chemiluminescence (ECL) (Amersham Biosciences).

Immunoprecipitation

ECs were washed three times with cold phosphate-buffered saline (PBS) and scraped into 500 µL of lysis buffer (50 mM Tris-HCl, pH 8.0, at 4°C, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 µg of leupeptin per milliliter). The cells were incubated at 4°C for 30 min with rotation. After centrifugation for 15 min, the supernatant was transferred to a fresh microcentrifuge tube, and the extract was precleared with 20 µL of protein G PLUS-agarose (Santa Cruz Biotechnology) for 1 h with rotation. The beads were pelleted, the supernatant was transferred to new tubes, and 20 µL of protein G PLUS-agarose beads conjugated to Sp1-specific antibody (Santa Cruz Biotechnology) was added. Immunoprecipitation was performed overnight at 4°C with rotation, after which the immunoprecipitated proteins were washed four times with lysis buffer. After the last wash, the beads were resuspended in 20 µL of 2× Laemmli sample buffer and boiled for 5 min. The samples were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane and probed with anti-phosphoserine-specific antibody (Zymed Laboratories, San Francisco, CA). Serine-phosphorylated Sp1 was detected using an enhanced chemiluminescent system as described above.

Protein Kinase Cζ Activity Assay

Whole-cell extracts prepared from ECs were lysed in 20 mM Tris/HCl (pH = 7.5), 150 mM NaCl, 0.25 M sucrose, 1.2 mM EGTA, 1 mM Na₃P₂O₇, 1 mM NaF, 1% Triton X-100, 0.5% Nonidet, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 20 µg/ml aprotinin, and 1 mM Na₃VO₄. Following incubation for 16 h with anti-pPKCζ antibody, precipitates were washed four times with 50 mM Tris/HCl (pH = 7.5), 5 mM MgCl₂, 100 µM Na₃P₂O₇, 1 mM NaF, 100 µM Na₃VO₄, and 100 µM phenylmethylsulfonyl fluoride. In the presence of PtdSer

(40 $\mu\text{g/mL}$), PKC ζ activation was assessed by measuring the incorporation of $\gamma^{32}\text{P}$ into glutathione *S*-transferase (GST). Aliquots containing the precipitate, PtdSer, the PKC substrate, and $\gamma^{32}\text{P}$ were incubated for 8 min at 30°C. The reaction was stopped using 5% acetic acid. Precipitates were collected on phosphocellulose filter paper. The filters were washed with 5% acetic acid and quantitated for $\gamma^{32}\text{P}$ using liquid scintillation spectroscopy.

Statistical Analysis

Data are presented as mean $\pm$ standard error (*SE*). The Student's *t* test and analysis of variance (ANOVA) with post hoc test were used for analyzing densitometric data for each experiment compared with the respective static control (time=0 min). Significance was set at $p < .05$.

RESULTS

Shear Stress But Not Cyclic Strain Increases PKC ζ Phosphorylation

ECs exposed to SS for up to 8 h exhibited increased levels of expression of phosphorylated PKC ζ protein compared to the static controls, as assessed by Western blot analysis. This effect has a peak after one hour of stimulation of 1.73 ± 0.3 (mean $\pm$ *SE*)-fold, which decreased to 1.4 ± 0.1 -fold and 1.2 ± 0.1 -fold at 4 and 8 h, respectively ($n = 5$; $p < .05$) (Figure 1A). In contrast, when the ECs were exposed to CS, there was no significant variation in the levels of expression of phosphorylated PKC ζ . These data suggest that SS, but not CS, influences PKC ζ phosphorylation.

Shear Stress But Not Cyclic Strain Increases PKC ζ Activity

ECs were exposed to SS for up to 8 h and the activity levels of PKC ζ were assessed. Lysates of cells exposed to SS or CS were immunoprecipitated with a specific antibody for PKC ζ , followed by the labeling of the samples with the isotope $\gamma^{32}\text{P}$. Following SS, there was a peak of 2.92 ± 0.39 -fold after 1 h of stimulation, which decreased with time to 1.71 ± 0.53 -fold and 1.43 ± 0.25 -fold at 4 and 8 h, respectively ($n = 3$; $p < .05$) (Figure 1B). In contrast, ECs exposed to CS exhibited no significant differences in the activity levels of PKC ζ compared to the static control group (Figure 1B).

Shear Stress in the Presence of PKC ζ Inhibitors Does Not Influence Sp1 Phosphorylation

Regulation of Sp1-dependent transcription could be affected by changes in Sp1 abundance, DNA binding activity, and/or transactivation activity (Black et al. 1999). Phosphorylation has also been implicated in changes in Sp1 binding and transcriptional activity (Friedl and Wolf 2003; Ihn et al. 2001). Although tyrosine residues are present, the vast majority of Sp1 phosphorylation in rat ECs is on serine residues (Black et al. 1999; Fojas de Borja et al. 2001; Ihn et al. 2001). Our previous data showed that Sp1 phosphorylation could be induced in ECs exposed to SS in a time-dependent manner (Yun et al. 2002).

We investigated the effects of SS with or without PKC ζ inhibitors on Sp1 phosphorylation. Lysates were prepared from ECs exposed to SS, or SS in the presence of 50 nM of calphostin C, 10 nM of Gö6983, 60 nM of Gö6983, or 660 nM of chelerythrine chloride, and immunoprecipitated with an Sp1 antibody prior to immunoblot with an anti-phosphoserine antibody. Total Sp1 levels remained relatively unchanged in all five groups, but SS stimulation of ECs led to an increased Sp1 phosphorylation (1.31 ± 0.14 -, 1.84 ± 0.14 -, and 2.14 ± 0.29 -fold increases at 1, 4, and 8 h, respectively; $n = 4$; $p < .05$) (Figure 2A). ECs pretreated with inhibitors at concentrations that do not affect PKC ζ (10 nM of Gö6983 or 50 nM of calphostin C) also exhibited increased levels of expression of phosphorylated Sp1 upon exposure to SS compared to the static control values (Figure 3A). In contrast, there was no change in the phosphorylation state of Sp1 in ECs exposed to SS with the addition of PKC ζ inhibitors at

concentrations known to inhibit PKC ζ (60 nM of Gö6983 or 660 nM of chelerythrine chloride) (Figure 2A). After 8 h of SS, the level of expression of phosphorylated Sp1 without PKC ζ inhibitors was significantly increased when compared with the level of expression of phosphorylated Sp1 in cultures containing PKC ζ inhibitors ($p < .05$). These data support our hypothesis for a critical role of PKC ζ in Sp1 phosphorylation in ECs exposed to SS.

Cyclic Strain with or without PKC ζ Inhibitors Does Not Influence Sp1 Phosphorylation

We also investigated the effects of CS on ECs with or without PKC ζ inhibitors on Sp1 phosphorylation. Lysates were prepared from ECs exposed to either CS or CS in the presence of 50 nM of calphostin C, 10 nM of Gö6983, 60 nM of Gö6983, or 660 nM of chelerythrine chloride, and immunoprecipitated with an anti-phosphoserine antibody. Total Sp1 levels remained relatively unchanged in all five groups, and the phosphorylation levels of Sp1 was not affected by CS regardless of the presence or absence of PKC ζ inhibitors (Figures 2B and 3B).

Down-Regulation of MT1-MMP Protein in ECs Exposed to Shear Stress Is Abolished in the Presence of PKC ζ Inhibitors

We determined whether ECs exposed to SS in the presence or absence of PKC ζ inhibitors induced change in MT1-MMP protein expression. Figures 4A and 5A confirmed our previous observation that SS decreased MT1-MMP protein expression in a time-dependent manner (Yun et al. 2002). In contrast, in the presence of PKC ζ inhibitors (60 nM of Gö6983, 660 nM of chelerythrine chloride, or 10 μ M of myristoylated PKC ζ pseudosubstrate), MT1-MMP protein expression was not diminished by SS (Figure 4A). These findings suggest that PKC ζ plays a crucial role in modulating MT1-MMP protein expression under SS conditions.

PKC ζ Inhibitors Do Not Affect MT1-MMP Protein Expression in ECs Exposed to Cyclic Strain

ECs exposed to CS increased MT1-MMP protein expression, as assessed by Western blot, in a time-dependent manner up through 8 h of exposure to CS. This rise in MT1-MMP protein reached maximum levels of 1.95 ± 0.5 -fold more than static controls at 8 h exposure to CS (mean $\pm$ SE; $n = 4$; $p < .05$) (Figure 4B). Similar expression was observed when cells were treated with PKC ζ inhibitors (60 nM of Gö6983, 660 nM of chelerythrine chloride, or 10 μ M of myristoylated PKC ζ pseudosubstrate) (Figure 5B; $n = 3$).

DISCUSSION

MMPs are capable of proteolysis of multiple extracellular matrix components, a wide variety of cell surface proteins and cyto- and chemokines (McQuibban et al. 2000, 2002; Tam et al. 2004; Van Den Steen et al. 2003). Their production and activation has been strongly associated with migration and invasion during development, following physiological and pathophysiological stimuli, and during angiogenesis (Birkedal-Hansen 1995; Mignatti and Rifkin 1996; Woessner 1994). Whereas most MMPs are secreted, MT1-MMP, also known as MMP-14, has a single transmembrane domain and is an integral membrane protein possessing an extracellular catalytic domain that has been suggested to be a key enzyme in tumor metastasis and angiogenesis (Friedl and Wolf 2003; Overall and Lopez-Otin). The process of angiogenesis, although critical for different physiological events such as wound healing and tissue remodeling, is also thought to be part of other pathological settings, such as chronic inflammatory diseases and tumorigenesis (Carmeliet and Jain 2000; Hanahan and Folkman 1996). In order for angiogenesis to occur, a complex series of events must take place, requiring ECs to coordinate a wide range of functions, including reduction of intercellular adhesion, degradation of subendothelial matrix, proliferation, migration, and formation of new capillary tubes (Risau 1997).

MT1-MMP in particular has been identified as a functional enzyme that degrades multiple extracellular matrix components and also functions as a physiological tether and activator of pro-MMP-2 (Sato et al. 1994; Strongin et al. 1995). To better understand of the MT1-MMP induction signaling pathway, multiples studies have been performed by our laboratory. Microvascular ECs were harvested, cultured (Madri and Williams 1983), and exposed to mechanical stimulation simulating the hemodynamic forces of SS and CS. The SS model is analogous to the effect of the laminar flow of blood on the vessel, whereas CS mimics the stretching that arterial walls undergo, caused by the force of the blood after each cardiac contraction. These hemodynamic forces, which have been reported to modulate the expression of various genes in ECs (Davies et al. 1997) and which elicit a variety of phenotypic responses, were hypothesized to be responsible, in part, for the differential induction of MT1-MMP.

Previously, our laboratory showed that the mRNA levels and protein expression of MT1-MMP is up-regulated with CS (Yamaguchi et al. 2002). On the other hand, SS down-regulated MT1-MMP mRNA and protein expression in a time-dependent fashion (Yun et al. 2002). It has been reported that the increased production and binding to the MT1-MMP promoter site by Egr-1 enhances MT1-MMP transcription (Haas et al. 1999; Yamaguchi et al. 2002; Yun et al. 2002). It is known that Egr-1 shares overlapping binding sites with Sp1. Additionally, we have shown that CS and SS dramatically induce the expression of Egr-1 mRNA and protein levels (Yamaguchi et al. 2002; Yun et al. 2002). Other studies (Akai et al. 1994; Khachigian et al. 1996; Schwachtgen et al. 1998) have also reported increased Egr-1 levels in ECs during SS, cyclic mechanical stretching, and upon wounding. However, we are not aware of other promoters that are sensitive to phosphorylated Sp1 in response to stress. SS does not affect Sp1 protein expression levels but increases the affinity of Sp1 for the MT1-MMP promoter, as assessed by mutant oligonucleotides containing GG to TA mutations within the Egr-1 binding site. These oligonucleotides blocked Egr-1 binding while not interfering with Sp1 binding (Yun et al. 2002). In contrast, it was shown that in ECs exposed to CS and then to mutant oligonucleotides and anti-Sp1 polyclonal antibodies, the Sp1 affinity to MT1-MMP was decreased (Yamaguchi et al. 2002). These findings suggest that despite the increased Egr-1 expression and the constant levels of Sp1 in ECs exposed to SS and CS, only SS is capable of enhancing Sp1 binding affinity to the MT1-MMP promoter site and thus inhibits the expression of MT1-MMP.

Sp1 is a ubiquitous transcription factor that recognizes GC-rich sequences present in many promoters including the MT1-MMP promoter (Azizkhan et al. 1993; Black et al. 1999). It is capable of inducible regulation by several diverse mechanisms. A recent study (Fojas de Borja et al. 2001) has demonstrated that serum increases Sp1 serine phosphorylation. There are many kinases that phosphorylate Sp1, including DNA-dependent protein kinase, protein kinase A, PKC ζ , casein kinase II, extracellular signal-regulated kinase (ERK), cyclin-dependent kinase, and others identified by only molecular weight and kinase activity (Black et al. 1999; Fojas de Borja et al. 2001; Jackson et al. 1993; Mignatti and Rifkin 1996; Pan and Hung 2002; Rafty and Khachigian 2001; Rohlf et al. 1997).

An association between PKC ζ and the activation or serine phosphorylation of Sp1 has been reported. Pal et al. used renal cell carcinoma and human fibrosarcoma cells to show the transcriptional regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), by the PKC ζ -mediated phosphorylation of Sp1, demonstrating that Sp1 interacts directly and specifically with PKC ζ in this process (Pal et al. 1998). Furthermore, PKC ζ was found to bind and phosphorylate the zinc finger region of Sp1 (Pal et al. 1998). In addition, Rafty and Khachigian showed the expression of platelet-derived growth factor (PDGF) in rat aortic smooth muscle cells is also mediated through PKC ζ -mediated Sp1 phosphorylation (Rafty and Khachigian 2001). In light of this, we hypothesized that PKC ζ may be responsible for the increased binding of Sp1 to the MT1-MMP promoter site in shear-stressed ECs and

thus the induction of MT1-MMP protein. Thus, we expected to find an increase in PKC ζ phosphorylation in SS, but not in CS, as Sp1 has only been shown to be activated by SS (Yun et al. 2002).

Using the same cell cultures and similar protocols and experimental design comparable to our previous studies (Yamaguchi et al. 2002; Yun et al. 2002), ECs showed an increase in the phosphorylated PKC ζ protein levels. In contrast, there was no significant change in the expression of phosphorylated PKC ζ and levels of activation of PKC ζ in ECs exposed to CS (Figure 1B). The discrepant time course of PKC ζ phosphorylation and PKC ζ activity is consistent with the findings of other laboratories that PKC ζ activity can be regulated by other factors (Gschwendt et al. 1996; Stempka et al. 1997). Likewise, the temporal pattern of PKC ζ phosphorylation of Sp1 is not clearly understood but may be a result of interplay with phosphatases that regulate this phosphorylation (Graesser et al. 1998; Standaert et al. 1997).

To investigate the role of PKC ζ on Sp1 phosphorylation, we used calphostin C, Gö6983 and chelerythrine chloride. Calphostin C inhibits only the conventional and novel PKAs (Sun et al. 2005). Gö6983 is specific to PKC and affects conventional, novel, and atypical PKC isoforms in a concentration dependent fashion (Gschwendt et al. 1996; Stempka et al. 1997). 2-[1-(3-Dimethylaminopropyl)-5-methoxindol-3-yl]-3-(1H-indol-3-yl) maleimide, termed Gö6983, has been shown to selectively inhibit several different PKC isozymes (IC₅₀ = 7 nM for PKC α and PKC β ; 6 nM for PKC γ ; 10 nM for PKC δ ; 60 nM for PKC ζ) (Gschwendt et al. 1996). Chelerythrine chloride is an inhibitor that inhibits all the PKC isotypes with IC₅₀ of 660 nM (Sun et al. 2005). Thus, conventional and novel PKC isoforms are inhibited at a concentration of 10 nM of Gö6983 or 50 nM of calphostin C. All isoforms, including PKC ζ , are affected at a concentration of 660 nM of chelerythrine chloride or 60 nM of Gö6983.

In order to demonstrate that inhibition of PKC ζ influences Sp1 phosphorylation on phosphoserine, we performed immunoprecipitation with an anti-serine antibody. In the presence of inhibitors of classic or novel PKC (10 nM of Gö6983 or calphostin C), low levels of phosphorylation were detected on CS, but serine phosphorylation was greatly increased in time dependent manner when cells were exposed to SS (Figure 3). However, in the presence of inhibitors of all isoforms of PKC (61 nM of Gö6983 or 660 nM of chelerythrine chloride), when SS or CS was applied, the levels of phosphorylation of Sp1 were unchanged from static conditions (Figure 2).

To assess the role of inhibition of PKC ζ on MT1-MMP protein expression, we performed Western blot analyses for MT1-MMP. In the presence or absence of inhibitors of all isoforms of PKC (60 nM of Gö6983 or 660 nM of chelerythrine chloride) and specific for PKC ζ (10 μ M of myristoylated PKC ζ pseudosubstrate), the MT1-MMP protein expression was increased in a time-dependent manner through 8 h of exposure to CS (Figures 4B, 5B). In contrast, SS decreased MT1-MMP protein expression in a time-dependent fashion. However, in the presence of PKC ζ inhibitors (60 nM of Gö6983, 660 nM of chelerythrine chloride, or 10 μ M of myristoylated PKC ζ pseudosubstrate), MT1-MMP expression was increased compare with static levels after SS (Figures 4A, 5A). Therefore, these data suggest that PKC ζ might be required for SS-induced Sp1 phosphorylation and MT1-MMP protein expression.

Thus, we hypothesize a role for PKC ζ -mediated Sp1 phosphorylation in the signaling pathway regulating MT1-MMP induction by SS. Our data are consistent with the requirement of PKC ζ phosphorylation and activation for serine phosphorylation of Sp1, increasing its affinity for and inhibiting Egr-1 binding of the MT1-MMP promoter site, ultimately leading to decreased MT-MMP induction (Figure 6). PKC ζ has been demonstrated to be involved in mitogenic signal transduction and most likely exerts its effects on gene transcription, at least in part, by activating the mitogen-activated protein kinase (MAPK) pathway. It has also been

implicated in insulin-stimulated signaling and may regulate glucose uptake through the glucose transporter GLUT4 (Graesser et al. 2000;Toker 1998). There is also evidence that PKC ζ participates in apoptosis and cell survival mechanisms. It has been shown that it specifically interacts with the product of par-4, a gene which is induced during apoptosis (Toker 1998). PKC ζ stimulates general protein synthesis, mediating the effect of insulin, insulin receptor substrate-signaling protein 1 (IRS-1), and phosphatidylinositol 3-kinase (PI3-K) (Graesser et al. 2000). PKCs are also involved in remodeling the cytoskeleton partly by phosphorylating specific PKC substrates such as MARCKS protein and pleckstrin. Thus, PKCs influence many aspects of cell function (Toker 1998) and a better understanding of PKC ζ function will perhaps lead to the development of drugs for the treatment of diseases in different pathological settings.

Acknowledgements

This work was supported in part by grants to B.E.S. from the National Institutes of Health (NIH; R01-HL47345) and Veterans Administration Merit Review Board, and NIH grants R37-HL28373 and R01-HL51018 to J.A.M.

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FIG. 1.

Western blot for PKC ζ phosphorylation and PKC ζ activity in rat microvascular endothelial cells (ECs) exposed to shear stress (SS) or cyclic strain (CS). (A) PKC ζ phosphorylation levels were assessed in ECs exposed for 0, 1, 4, and 8 h of SS or CS using total PKC ζ to normalize protein loading. After ECs were exposed to SS for up to 8 h, PKC ζ phosphorylation was noted to peak after 1 h of stimulation of 1.73 ± 0.3 (mean $\pm$ SE)-fold, which decreased with time of shear stress. In contrast, when the ECs were exposed to CS for the same time course (1, 4, and 8 h), there was no significant variation in the levels of expression of phosphorylated PKC ζ compared with static conditions. (B) The activity levels of PKC ζ as assessed by an activity assay where lysates of cells exposed to SS were immunoprecipitated with a specific antibody for PKC ζ , followed by the labeling of the samples with the isotope $\gamma^{32}\text{P}$. In SS, there was a

peak in activity after 1 h of stimulation of 2.92 ± 0.39 -fold ($n=3$; $p<.05$). In contrast, the activity levels of PKC ζ were not affected by CS in ECs. Results of *A* and *B* are mean $\pm$ SE from five different experiments. * $p<.05$ versus static cells.

FIG. 2.

Sp1 phosphorylation in ECs exposed to SS or CS in the presence or absence of PKC ζ inhibitors. Sp1 phosphorylation was assessed in ECs following SS or SS in the presence of PKC ζ inhibitors (Gö6983 at a concentration 60 nM or chelerythrine chloride at a concentration of 660 nM), CS, or CS in the presence of PKC ζ inhibitors (Gö6983 at a concentration 60 nM or chelerythrine chloride at a concentration of 660 nM) utilizing the same time course (0, 1, 4, and 8 h). Serine phosphorylation was determined by Sp1 immunoprecipitation followed by anti-phosphoserine-specific antibody immunoblot. (A) SS stimulation of ECs led to increased Sp1 phosphorylation (1.3-, 1.8-, and 2.1-fold; $n = 4$; $p < .05$). But in the presence of PKC ζ inhibitors, Sp1 phosphorylation was not affected by SS. (B) Phosphorylation levels of Sp1 were not affected by CS regardless of presence of PKC ζ inhibitors. Results are presented as mean $\pm$ SE of four independent experiments. * $p < .05$ versus static condition of SS; § $p < .05$ versus 8 h of SS.

FIG. 3.

Sp1 phosphorylation in ECs exposed to SS or CS in the presence of nonspecific PKC inhibitors. Sp1 phosphorylation was assessed in ECs following SS in the presence of either 10 nM of Gö6983 or 50 nM of calphostine C, or following CS in the presence of either 10 nM of Gö6983 or 50 nM of calphostine C utilizing the same time course (0, 1, 4, and 8 h). These concentrations of inhibitors inhibit only conventional and novel PKC isoforms. (A) In the presence of inhibitors, SS increases the phosphorylation of Sp1. (B) When the ECs are exposed to CS, the phosphorylation of Sp1 is not changed in the presence or absence of inhibitors.

FIG. 4.

Western blot for MT1-MMP protein expression in ECs exposed to SS or CS. MT1-MMP protein expression was assessed in ECs following SS, SS with PKC ζ inhibitors (Gö6983 at a concentration 60 nM or chelerythrine chloride at a concentration of 660 nM), CS, or CS with PKC ζ inhibitors (Gö6983 at a concentration 60 nM or chelerythrine chloride at a concentration of 660 nM) utilizing the same time course (0, 1, 4, and 8 h). We used actin to normalize for protein loading. (A) SS decreased MT1-MMP protein expression ($19\% \pm 2\%$, $45\% \pm 6\%$, and $48\% \pm 5\%$ reduction at 1, 4, and 8 h, respectively; $n=4$; $p<.05$). In the presence of PKC ζ inhibitors, MT1-MMP protein expression is not affected by SS. Results are presented as mean $\pm$ SE of four independent experiments. $*p<.05$ versus SS static level; $p<.05$ versus SS 4 h; $p<.05$ versus SS 8 h. (B) CS increased MT1-MMP protein expression in a time-dependent manner through 8 h of exposure to CS. MT1-MMP protein reached maximum levels of 1.95 ± 0.5 -fold more than static controls at 8 h exposure to CS (mean $\pm$ SE; $n=4$; $p<.05$). Similar expression was observed when cells were treated with PKC ζ inhibitors. Results are presented as mean $\pm$ SE of four independent experiments. $*p<.05$ versus CS static level; $p<.05$ versus CS with chelerythrine chloride at a concentration of 660 nM static levels.

FIG. 5.

Expression of MT1-MMP induced by SS and CS with or without myristoylated PKC ζ pseudo-substrate (PKC ζ -PS). MT1-MMP protein expression was assessed in ECs following SS, SS with 10 μ M of myristoylated PKC ζ , CS, or CS with 10 μ M of myristoylated PKC ζ utilizing the same time point (0, 1, 4, and 8 h). (A) SS decreased MT1-MMP protein expression (28% + 9%, 55% + 12%, 53% + 8% reductions at 1, 4, and 8 h, respectively; $n=3$; $p<.05$). In the presence of PKC ζ inhibitors, MT1-MMP protein expression is not affected by SS. Results are presented as mean + SE of three independent experiments. # $p<.05$ compared between SS and SS with 10 μ M of myristoylated PKC ζ pseudosubstrate (PKC ζ -PS); * $p<.05$ versus SS static level. (B) CS increased MT1-MMP protein expression in a time-dependent manner. Similar expression was observed when ECs were treated with 10 μ M of myristoylated PKC ζ pseudosubstrate (PKC ζ -PS). Results are presented as mean+SE of three independent experiments.

FIG. 6.

Working model of the effects of shear stress and cyclic strain endothelial cell MT1-MMP expression. Differential PKC ζ expression elicited by cyclic strain (A) and shear stress (B) results in differential serine phosphorylation of Sp1, which in turn affects its displacement by Egr-1 on the MT1-MMP promoter, ultimately resulting in differential MT1-MMP expression. Specifically, shear stress induces increased expression and activity of PKC ζ , which serine phosphorylates Sp1 (Sp1*), altering its affinity for the Egr-1/Sp1 consensus site on the MT1-MMP promoter, preventing Sp1 displacement and subsequent Egr-1 binding and induction of MT1-MMP mRNA and protein.