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Cyclic Strain Stimulates Monocyte Chemotactic Protein-1 mRNA Expression in Smooth Muscle Cells

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Abstract Hemodynamic forces are important determinants for the formation of atherosclerotic plaques. The recruitment of circulating monocytes into the arterial wall is an important step during atherogenesis. Monocyte chemotactic protein-1 (MCP-1) has been shown to be a key factor for monocyte transmigration. This study examined the effects of cyclic strain on MCP-1 mRNA expression levels of cultured rat aortic smooth muscle cells. The MCP-1 mRNA levels of aortic smooth muscle cells first increased as the duration of cyclic strain increased, reaching the maximum at 6–12 h, maintained at high levels throughout the 48-h strain period. To explore signaling pathways mediating cyclic strain-stimulated MCP-1 mRNA expression, we examined the involvement of tyrosine kinase and protein kinase C (PKC). Tyrosine kinase inhibitors, genistein and tyrphostin 51, at 50 μM blocked cyclic strain-stimulated MCP-1 mRNA expression. Preincubation with a PKC activator, phorbol 12-myristate 13-acetate (PMA), 2 μM, for 24 h to downregulate PKC did not decrease cyclic strain-induced MCP-1 mRNA expression. A 6-h incubation with 0.1 μM PMA to activate PKC, which stimulated MCP-1 expression when applied alone, abolished the stimulatory effects of cyclic strain. A specific PKC inhibitor, calphostin C (0.1 μM), diminished cyclic strain-stimulated MCP-1 mRNA expression. Angiotensin II at 10 or 1,000 nM induced a moderate upregulation of MCP-1 mRNA, and no synergistic effects were observed between angiotensin II and cyclic strain. These results indicate that cyclic strain stimulates MCP-1 mRNA expression in smooth muscle cells through signaling pathway(s) mediated by tyrosine kinase activation. J. Cell. Biochem. 76:303–310, 1999.

Key words: monocyte chemotactic protein-1; cyclic strain; vascular smooth muscle cell; tyrosine kinase; protein kinase C

Atherosclerotic plaques exhibit a focal distribution in the arterial tree, most often occurring in areas surrounding arterial bifurcations. The distribution of hemodynamic forces has been implicated in the higher occurrence rate of early atherosclerotic lesions on the outer segments of bifurcations, characterized by lower and more variable shear stress than on the inner segments that are exposed to shear stress of higher magnitude with less temporal fluctuation [Zarins et al., 1983; Asakura and Karino, 1990]. The role of hemodynamic forces in atherogenesis has been supported by studies performed in cultured vascular cells. In endothelial cells, shear stress has been demonstrated to induce the production and transient expression of genes encoding vasoactive peptides, growth factors, chemotactic factors, adhesion molecules, and protooncogenes [Frangos et al., 1985; Diamond et al., 1989; Yoshizumi et al., 1989; Hsieh et al., 1992, 1993; Kuchan and Frangos, 1994; Shyy et al., 1993, 1994]. In addition, cyclic mechanical strain has also been reported to stimulate a prolonged expression of a variety of vasoactive factors [Carosi et al., 1992; Rosales and Sumpio, 1992; Wang et al., 1993, 1995; Howard et al., 1997]. By contrast, cyclic strain results in more variable effects in cultured smooth muscle cells depending on cell source, culture conditions, and the magnitude and frequency of the cyclic strain [Sumpio and Banes, 1988; Kulik and Alvarado, 1993; Sudhir et al., 1993; Wilson et al., 1993; Yang et al., 1993; Lyall et al., 1994].

The recruitment of circulating monocytes into subendothelial space of arteries is thought to be an important early event in atherogenesis. Monocyte chemotactic protein-1 (MCP-1), a glycoprotein with an apparent molecular mass of 9,000–15,000, is a major chemotactic factor for

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monocyte migration [for review, see Leonard and Yoshimura, 1990]. Several lines of evidence indicate that MCP-1 might play an important role in the advancement of atherosclerotic lesions. MCP-1 expression was found to be stimulated in atherosclerotic plaques of human and hypercholesterolemic animals [Yla-Herttuala et al., 1991; Nelken et al., 1991; Yu et al., 1992; Takeya et al., 1993; Chen et al., 1999]. Minimally modified low-density lipoprotein (LDL) can activate MCP-1 expression both in cultured vascular cells and in mice models in vivo [Cushing et al., 1990; Liao et al., 1991]. Furthermore, transmigration of monocytes into the subendothelial space induced by modified LDL was inhibited by an antibody to MCP-1 [Navab et al., 1991]. The increased adhesive force between monocytes and cultured endothelial cells stimulated by MCP-1 might contribute to its effects on monocyte transmigration [Shyy et al., 1993].

In accordance with a role for mechanical strain in promoting atherogenesis, the expression of MCP-1 mRNA was found to be stimulated transiently by laminar flow in cultured endothelial cells [Shyy et al., 1994]. In addition, mechanical strain was shown to stimulate a persistent MCP-1 expression and secretion by endothelial cells [Wang et al., 1995; Wung et al., 1996]. Previous experiments performed in our laboratories showed that MCP-1 expression was stimulated to a great extent in arterial smooth muscle cells along with fatty streak development of hypercholesterolemic rabbits [Chen et al., 1999]. Because cyclic transmural strain represents the major physical force for arterial smooth muscle cells in the physiological environment, it is conceivable that changes in mechanical strain may activate arterial smooth muscle cells. Whether cyclic strain affects MCP-1 expression in smooth muscle is unknown. Therefore, the present study examined effects of cyclic mechanical strain on cultured aortic smooth muscle cells. Our results showed that cyclic strain induced prolonged expression of MCP-1 mRNA through signaling pathways mediated by the activation of tyrosine kinase(s).

MATERIALS AND METHODS

Smooth Muscle Cell Culture

Rat aortic smooth muscle cells (RASMC) were obtained by an enzyme digestion method [Gunther et al., 1982]. Briefly, thoracic aorta segments were incubated in Hank's balanced salt solution (HBSS) containing 200 U/ml collagenase (type II, Sigma Chemical Co., St. Louis, MO), 1 mg/ml bovine serum albumin (BSA) and antibiotics at 37°C for 30 min to loosen media-adventitia binding. After the removal of tunica adventitia and endothelium, media was cut into segments approximating 1×3 mm^2 and incubated in HBSS containing 240 U/ml collagenase, 50 U/ml elastase (type III, Sigma), 1 mg/ml BSA, and 1 mg/ml soybean trypsin inhibitor at 37°C for 75 min. Arterial segments were pipetted 60 times to facilitate the release of isolated smooth muscle cells from arterial wall and filtered through a 105-μm polypropylene membrane. Undigested arterial segments were further incubated for 30 min at 37°C in fresh digestion solution with reduced collagenase concentration (120 U/ml). Cells were collected from the filtrate by centrifugation (180*g*, 6 min), counted, and seeded at 1×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) for 3 h. Cells were maintained in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml) in a 5% CO_2 incubator.

Application of Cyclic Strain to Cultured RASMC

Cells were grown to confluence in six-well silicone elastomer-bottomed culture plates coated with type I collagen (Flexcell Corp., McKeesport, PA). Confluent cells were placed in the serum-free medium, which is a mixed medium (1:1) of DMEM and Ham's F12 medium containing insulin (0.5 μM), transferrin (5 μg/ml), and ascorbic acid (0.2 mM) [Libby and O'Brien, 1983], for at least 24 h before being subjected to mechanical deformation with the Flexercell strain unit (Flexcell Corp.). The strain unit, which is a modification of the unit originally described by Banes et al. [1985], consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. Vacuum (-2 $-$ 17 kPa) was applied repetitively to culture plates via the baseplate at a frequency of 60 cycles/min. The negative pressure produced by vacuum caused a downward deformation of the membrane, exerting maximal stretching effect at the outer annulus with a geomatric progression of strain toward zero at the center of the membrane. A strain of 15 kPa applied in this study was calculated to produce a maximum of 21% elongation at the outer annulus [Gilbert et al., 1989].

RNA Isolation and Northern Blot Analysis

RASMC were lysed in a 4 mol/L guanidinium thiocyanate solution containing 15 mmol/L sodium acetate and 10 mmol/L dithiothreitol. Total RNA was isolated from the cell lysate by repeated phenol-chloroform extraction, followed by ethanol precipitation [Chomczynski and Sacchi, 1987]. RNA samples (15 μg per lane) were electrophoresed on 1% agarose gels in a buffer containing 20 mmol/L MOPS, pH 7.4, 1 mmol/L EDTA, and 0.22 mol/L formaldehyde. After electrophoresis, RNA was transferred onto a nylon membrane (Nytran 0.45 μm, Schleicher & Schuell, Dassel, Germany), and fixed by ultraviolet (UV) irradiation. DNA probes were labeled with deoxycytidine 5′-[α-^{32}P]triphosphate (10^8 cpm/μg, Rediprime DNA labeling system, Amersham, Arlington Heights, IL). Membranes were prehybridized for 1 h at 55°C in prehybridization solution (ExpressHyb solution, Clontech, Palo Alto, CA). Hybridization was performed in the ExpressHyb solution containing 4×10^6 cpm/mL ^{32}P-labeled MCP-1 probes at 60°C for 2 h. Blots were washed four times (10 min each) at room temperature in 2× SSC (standard saline citrate, 1× SSC contains 0.15 mol/L NaCl and 15 mmol/L sodium citrate, pH 7.0) and 0.05% sodium dodecyl sulfate (SDS), followed by two washes in 0.1× SSC and 0.1% SDS at 50°C for 20 min per wash. Blots were covered in Saran Wrap and exposed to X-ray film (X-OMAT, Kodak, Rochester, NY) at −70°C for autoradiography. The intensity of each hybridized band on XAR films was determined by optical densitometry (PDI Instruments, Huntington Station, NY). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels served as the internal standard to normalize the MCP-1 signals. The cDNA probes used in these experiments were human MCP-1 (0.738 kb) and rat GAPDH, 1.3 kb, respectively.

Anti-phosphotyrosine Immunoblotting

Tyrosine phosphorylation levels of cyclic strain-stimulated RASMCs were assessed according to a published method [Schwartz et al., 1993]. Upon the termination of cyclic strain, medium was removed immediately. Cells were washed and extracted with 2% SDS, 100 mM Tris, pH 7.0, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 mM sodium fluoride, and 10 μg/ml aprotinin and leupeptin. Cell extract was stored at −70°C until ready to use. To perform immunoblotting, cell extract was heated to 95°C for 5 min, electrophoresed on 10% polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were probed by sequential incubation with monoclonal mouse anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY), horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody, and detected by ECL chemiluminescence detection kit (Amersham).

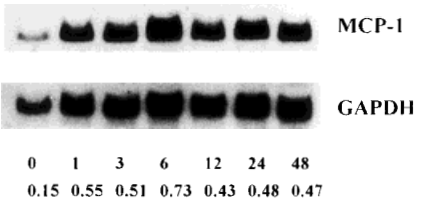


Fig. 1. Time course of cyclic strain-stimulated MCP-1 mRNA expression levels in RASMCs. Smooth muscle cells were placed in the serum-free medium for 24 h before being subjected to cyclic strain for 1, 3, 6, 12, 24, or 48 h. MCP-1 mRNA levels were normalized with GAPDH mRNA levels. The figure represents one of the four independent experiments.

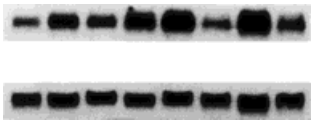
RESULTS

Effects of Cyclic Strain Duration on MCP-1 mRNA Expression Levels

The expression of MCP-1 mRNA levels increased as the duration of cyclic strain increased, reaching the maximum at 6–12 h (Fig. 1). During the 48-h period cyclic strain was conducted, MCP-1 mRNA expression levels remained markedly elevated over control in all the experiments performed.

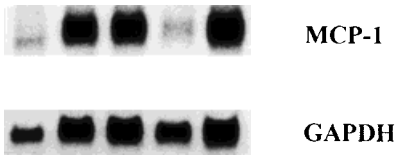
Role of PKC Activation in Cyclic Strain-Stimulated MCP-1 Expression

The expression of MCP-1 homologue *JE* gene in smooth muscle cells has been shown to be stimulated by a PKC activator, phorbol 12-myristate 13-acetate (PMA) [Taubman et al., 1992]. Thus, we examined the possible involvement of protein kinase C in cyclic strain-stimulated MCP-1 expression. When RASMC was pretreated with 2 μM PMA for 24 h in order to downregulate PKC activity, cyclic strain-induced stimulation on MCP-1 mRNA levels was not inhibited (Fig. 2). Interestingly, incubation with 0.1 μM PMA during the last 6 h of cyclic strain diminished the stimulatory effects of cyclic strain though such treatment alone stimulated MCP-1 expression. The PKC inhibitor, calphostin C, at 0.1 μM was found to partially inhibit cyclic strain-stimulated MCP-1



PMA (0.1μM,6h)	-	+	-	-	-	+	-	-
PMA (2μM, 48h)	-	-	+	-	-	-	+	-
Calphostin (0.1μM)	-	-	-	+	-	-	-	+
Flex (24h)	-	-	-	-	+	+	+	+
MCP-1/GAPDH	0.57	1.46	0.80	1.42	2.03	0.67	2.00	0.95

Fig. 2. Effects of protein kinase C activation/inhibition on cyclic strain-stimulated MCP-1 mRNA levels. Smooth muscle cells were either pretreated with 2 μM PMA for 24 h before 24-h cyclic strain in the presence of PMA or treated with 0.1 μM PMA for 6 h during the 24-h cyclic strain stimulation period. Calphostin C (0.1 μM) was pretreated for 1 h under the light before cyclic strain. Similar results were obtained with six independent experiments.



Flex (24h)	-	+	+	+	+
Genistein (μM)	-	-	5	50	-
Cyto D (μM)	-	-	-	-	1
MCP-1/GAPDH	0.38	1.05	1.07	0.27	1.53

Fig. 3. Effects of tyrosine kinase inhibitors on cyclic strain-stimulated MCP-1 mRNA levels, representing one of three independent experiments. Smooth muscle cells were stimulated with cyclic strain for 24 h in the presence or absence of tyrosine kinase inhibitor genistein (5 or 50 μM) or actin polymerization inhibitor cytochalasin D (1 μM).

mRNA expression (Fig. 2). When the concentration of calphostin C was increased beyond 0.1 μM, cells detached readily from the substratum and rendered further assessment difficult.

Effects of Tyrosine Kinase Inhibitors on Cyclic Strain-Stimulated MCP-1 mRNA Expression

The involvement of tyrosine kinase activation in cyclic strain-stimulated MCP-1 mRNA expression was examined by the addition of genistein and tyrphostin. Pretreating RASMC with genistein (50 μM) for 30 min inhibited 24-h cyclic strain-stimulated MCP-1 mRNA expression while lower concentration (5 μM) was not effective (Fig. 3). The addition of another tyrosine kinase inhibitor, tyrphostin 51 (50 μM), resulted in a similar inhibition on cyclic strain-stimulated effects (data not shown). In contrast to tyrosine kinase inhibitors, cytochalasin D at 1 μM, which blocked actin filament polymerization, did not affect strain-induced MCP-1 mRNA expression (Fig. 3).

Tyrosine Phosphorylated Proteins During Cyclic Strain

Because tyrosine kinase inhibitors significantly inhibited cyclic strain-stimulated MCP-1 mRNA expression, we examined proteins undergoing tyrosine phosphorylation during cyclic strain stimulation. The rate of tyrosine phosphorylation was much faster than that of MCP-1 mRNA expression, peaking within minutes of cyclic strain, and decreasing afterward (data not shown). Tyrosine phosphorylation as detected by a monoclonal antibody against phosphotyrosine occurred in several proteins, approximating 40, 80, 95, 110, 140, and 180 kDa (Fig. 4).

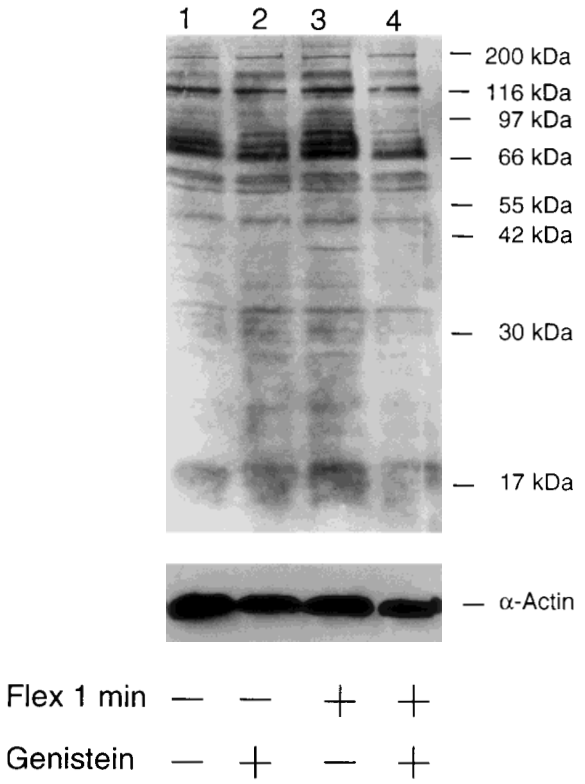
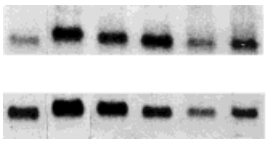


Fig. 4. Tyrosine phosphorylation of RASMC stimulated by cyclic strain. Smooth muscle cells were stimulated with cyclic strain for 1 min in the presence or absence of tyrosine kinase inhibitor genistein (25 μg/ml). Smooth muscle-specific α-actin was later determined as the internal control for each sample. Similar results were obtained with six independent experiments.

Interaction Between Cyclic Strain and Angiotensin II on the Expression of MCP-1 mRNA

Angiotensin II, a potent vasoconstrictor as well as a stimulator for smooth muscle growth, has been reported to have synergistic effects



Flex (24h)	-	-	-	+	+	+
Ang II (nM)	-	10	10^3	-	10	10^3
MCP-1/GAPDH	0.16	0.45	0.40	0.95	0.52	0.80

Fig. 5. Effects of angiotensin II and cyclic strain on MCP-1 mRNA levels, representing one of the two independent experiments. Smooth muscle cells were treated with 10 or 1,000 nM angiotensin II in the presence or absence of 24-h cyclic strain.

with cyclic strain on smooth muscle mitogenesis [Sudhir et al., 1993]. We examined the possible interaction or overlapped signaling pathways between angiotensin II and cyclic strain on MCP-1 expression. As shown in Figure 5, incubation of RASMC with 10 or 1,000 nM angiotensin II for 24 h stimulated MCP-1 mRNA expression, although to a smaller extent compared with that induced by cyclic strain. When angiotensin II treatment was performed simultaneously with cyclic strain for 24 h, no additive effects on MCP-1 mRNA expression were detected, suggesting that similar mechanisms are probably involved in MCP-1 upregulation by both stimuli.

DISCUSSION

Atherosclerotic lesions exhibit a focal pattern of distribution in the arterial tree, preferentially occurring around bends and arterial bifurcations where the hemodynamic forces are disturbed. The results from this study showed that cyclic strain induced a sustained MCP-1 mRNA expression by arterial smooth muscle cells which account for the majority of the vascular wall. These findings are in concert with recent reports showing that in endothelial cells mechanical strain stimulated both MCP-1 mRNA expression and protein secretion which persisted through the period of strain [Wang et al., 1995; Wung et al., 1996]. It is also noteworthy that fluid shear forces induced a transient response of MCP-1 messenger RNA expression in cultured endothelial cells [Shyy et al., 1994]. Taken together, these results demonstrate that disturbed flow and transmural stress can both stimulate the expression of MCP-1 by vascular cells, providing a molecular basis for disturbed flow-induced formation of atherosclerotic plaques. Our previous results showing a pronounced upregulation of MCP-1 expression by arterial smooth muscle cells along with fatty streak development in hypercholesterolemic rabbits are also consistent with a role for mechanical stress-induced MCP-1 expression of smooth muscle in atherogenesis [Chen et al., 1999].

The inhibition of cyclic strain-stimulated MCP-1 mRNA expression by two types of tyrosine kinase inhibitor clearly indicates that tyrosine phosphorylation is involved in the activation pathways stimulated by cyclic strain. Several lines of evidence have indicated that in smooth muscle cells actin filament reorganization and cell growth stimulated by mechanical strain may be mediated by tyrosine phosphorylation. Cyclic strain was shown to stimulate cell proliferation via the induction of the autocrine secretion of PDGF in a neonatal cell line [Wilson et al., 1993] and to induce DNA synthesis by increasing FGF-2 release from cultured human smooth muscle cells [Cheng et al., 1996, 1997]. PDGF is known to activate mitogenesis through tyrosine autophosphorylation of its receptors [Ullrich and Schlessinger, 1990]. In concert with this notion, tyrosine kinase inhibitor herbimycin A was shown to diminish smooth muscle cell growth stimulated by cyclic deformation [Davis et al., 1994]. In airway smooth muscle, cyclic mechanical strain was found to stimulate tyrosine phosphorylation of two focal adhesion proteins, the focal adhesion kinase (FAK) and paxillin, which was associated with the reorganization of actin cytoskeleton. Furthermore, tyrosine kinase inhibitors were effective in inhibiting mechanical strain-induced cytoskeletal reorientation and elongation [Smith et al., 1997, 1998] (Y.J. Yu, Y.L. Chen and M.J. Jiang, unpublished results). Whereas both actin reorganization and MCP-1 mRNA expression are induced by mechanical strain and involve tyrosine kinase activation, our results indicated that intact actin filaments are not required for the stimulatory effects of mechanical strain on MCP-1 mRNA expression (Fig. 3). This finding does not, however, exclude the possibility that similar initial signaling pathways are shared by these two events. Whether the inhibitory effects of tyrosine kinase inhibitors on cyclic strain-induced MCP-1 mRNA expression involve inactivation of any particular tyrosine kinase(s) merits further investigation.

Reactive oxygen species have been demonstrated to function as second messengers dur-

ing cell activation by various stimuli, including angiotensin II and mechanical strain [Griendling et al., 1994; Howard et al., 1997; Wung et al., 1997]. Mechanical strain-induced stimulation on MCP-1 mRNA expression in endothelial cells was shown to be mediated via the interaction of tissue plasminogen activator-responsive element (TRE) with activator protein 1 through reactive oxygen species [Wung et al., 1997]. Similarly, MCP-1 gene expression induced by angiotensin II in smooth muscle cells was recently shown to involve the formation of H_2O_2 and requires the activation of tyrosine kinase and mitogen-activated protein kinase [Chen et al., 1998]. Whether TRE element is involved in angiotensin II-stimulated MCP-1 gene transcription remains unclear. Our results that no synergistic effects on MCP-1 expression were observed between cyclic strain and angiotensin II in RASMC (Fig. 5) are consistent with the notion that similar signaling pathways might mediate MCP-1 mRNA upregulation by these two stimuli.

PKC activation has been demonstrated during mechanical stimulation of cardiac myocytes [Sadoshima and Izumo, 1993] and endothelial cells [Hsieh et al., 1992; Rosales and Sumpio, 1992; Wang et al., 1995]. In the present study, a protein kinase C activator, PMA, was found to stimulate MCP-1 mRNA expression, in agreement with the results of a previous study (Fig. 2) [Taubman et al., 1992]. The inhibitory effects of calphostin C on cyclic strain-stimulated MCP-1 mRNA expression suggest that PKC activation may mediate, at least in part, the stimulatory effect of cyclic strain on MCP-1 mRNA expression. Surprisingly, the preincubation of RASMC with 2 μM PMA for 24 or 48 h had no inhibitory effect on cyclic strain-induced stimulation of MCP-1 mRNA expression. Instead, the incubation of RASMC with 0.1 μM PMA during the last 6-h period of cyclic strain abolished cyclic strain-stimulated MCP-1 mRNA expression (Fig. 2). Calphostin C was shown to inhibit PKC by competing at the phorbol ester-binding sites of the regulatory subunit [Bruns et al., 1991]. The apparent discrepancy between the results obtained with calphostin C and those by prolonged PMA treatment suggests that 24- or 48-h incubation with PMA may not be sufficient to completely downregulate PKC activities under our experimental conditions. By contrast, the surprising results that 6-h PMA treatment abolished cyclic strain-stimulated MCP-1 expression indicate that PKC is likely to be involved in multiple pathways associated with mechanical strain, exerting a more complex role. Whether these apparently conflicting results reflect the involvement of different PKC isozymes remains to be examined.

Monocyte adherence to the endothelium and subsequent transmigration into subendothelial space is an important event during the initial development of atherosclerotic plaques. Our results clearly demonstrate that cyclic strain stimulates RASMC to express MCP-1 mRNA via activation pathways that are mediated by tyrosine kinases and involve PKC as well. The stimulation of MCP-1 expression in RASMC by mechanical strain may provide a chemotactic factor gradient to attract circulating monocytes to enter subendothelial space, promoting the development of atherosclerotic plaques in regions with disturbed flow.

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