Calcineurin Promotes the Expression of Monocyte Chemoattractant Protein-1 in Vascular Myocytes and Mediates Vascular Inflammation

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Abstract—Although the role of the calcineurin-dependent pathway in the development of cardiac hypertrophy has been intensively studied, little is known of its role in vascular inflammatory diseases such as atherosclerosis and restenosis after angioplasty. To help elucidate the role of calcineurin in vascular inflammation, we infected cultured vascular smooth muscle cells (VSMCs) with an adenovirus construct expressing a constitutively active mutant of calcineurin, and examined its effect on the expression of monocyte chemoattractant protein-1 (MCP-1). We also examined the role of calcineurin in vivo using a transluminal wire injury model of the rat femoral artery. Forced activation of calcineurin significantly increased the expression of MCP-1 both at the transcriptional and protein levels. Angiotensin II (Ang II) also significantly stimulated MCP-1 expression, and this increase was significantly inhibited by cyclosporin A (CyA). Constitutive activation of calcineurin stabilized MCP-1 mRNA without enhancing MCP-1 promoter activity. In accordance with the results, Ang II–induced increase of MCP-1 promoter activity was not suppressed by CyA. Ang II stabilized MCP-1 mRNA, and this effect of Ang II was diminished by CyA. CyA suppressed MCP-1 expression in the femoral artery after the transluminal mechanical injury. CyA also inhibited macrophage infiltration and neointimal formation in the wire-injured femoral arteries. These results suggested that calcineurin mediates vascular inflammation via stimulation of MCP-1 expression in VSMCs and macrophage infiltration. (Circ Res. 2004;94:693-700.)

Key Words: atherosclerosis □ angioplasty □ angiotensin □ signal transduction □ inflammation

It has become apparent that inflammation of the blood vessel wall plays a pivotal role in the initiation and maintenance of vascular diseases such as atherosclerosis and restenosis after angioplasty. Among various forms of inflammation, infiltration of monocytes into the vessel wall is an event that is observed in the early phase of atherosclerosis. This process appears to be mediated by chemokines such as monocyte chemoattractant protein-1 (MCP-1). MCP-1 is a peptide that induces migration of monocytes and is reportedly implicated in the formation of vascular diseases. MCP-1 is widely expressed in atherosclerotic lesions including vascular endothelial cells, smooth muscle cells, and macrophages. It was reported that the disruption of the MCP-1 receptor, in addition to apolipoprotein E, markedly attenuated atherosclerotic lesions by inhibiting macrophage infiltration in these double knockout mice. It has also been shown that neutralization of MCP-1 by an anti–MCP-1 antibody in rats and blockade of MCP-1 function using a dominant-negative mutant of MCP-1 in rabbit effectively prevent neointimal formation after balloon injury of the carotid artery. Although many studies have been performed so far to clarify the intracellular signaling pathways leading to the activation of the MCP-1 gene in macrophages and vascular endothelial cells, the pathways that function in vascular myocytes have not been intensively studied.

Accumulated evidence suggests that the renin-angiotensin system is implicated in the pathogenesis of atherosclerosis. It also seems to be involved in the pathogenesis of restenosis after angioplasty. It has been shown that angiotensin II (Ang II) promotes the expression of MCP-1 in vascular smooth muscle cells (VSMCs), suggesting Ang II plays a role in vascular inflammation.

Recently, the mechanism of the development of cardiac hypertrophy has started to be unveiled. A phosphatase called calcineurin is activated in a Ca2+/calmodulin-dependent manner, and activated calcineurin then dephosphorylates transcription factors called nuclear factors of activated T cells (NFAT), which in turn promotes nuclear translocation of NFAT. The NFAT transcription factors then cooperate with nuclear transcription factors such as GATA-4 and stimulate the transcriptional activation of various genes that are involved in the development of cardiac hypertrophy.
ever, little is known about the role of the calcineurin/NFAT-dependent pathway in vascular diseases such as atherosclerosis and restenosis after angioplasty.

In the present study, we examined whether activation of calcineurin would promote the expression of MCP-1, and if so, the mechanism by which calcineurin enhanced the expression of MCP-1. We also studied the role of the calcineurin-dependent pathway in vivo using a wire injury model of the rat femoral artery.

Materials and Methods

Reagents

Cyclosporin A (CyA) and valsartan (Vals) were kindly supplied by Novartis Pharma AG. Angiotensin II was purchased from Pepptide Institute. Actinomycin D was obtained from Wako Pure Chemical.

Cell Culture

Rat VSMCs were cultured from rat thoracic aortas following the explant method, as previously described.12

Plasmids

Details of the cloning of human calcineurin A 1–398 (CalA), a constitutively active mutant lacking the carboxyl-terminal calmodulin binding domain, and its subcloning into an expression plasmid (pcDNA3HA-CalΔC) were previously described.13 Construction of human MEK6AA, in which serine 207 and threonine 211 of human MEK6 (mitogen-activated protein kinase kinase 6) were replaced with alanine, was also previously described.13 The promoter region of human MCP-1 gene (2644 bp) was isolated by polymerase chain reaction (PCR). Three consecutive fragments (designated F1, F2, and F3 in the sense, 5'-AAACAAATCAACCTCAGGTCTCTGGGCTTCGTGGC-3'; F2 sense, 5'-CAAGAGGCGCCCTGCGAGTGTAGTTTGTTGTC-3'; F3 antisense, 5'-TCTAGAGATTGGAAGGCTGCTCTGACATACG-3'; F3 sense, 5'-GTACCAAAGACAAACAGTTCACTGGTAAATACTGTC-3'; F3 antisense, 5'-AGATCTTCCTCGTGTCCTCGTCGTCGTC-3'; F1 was digested with KpnI and XhoI, and F2 was digested with XhoI and Xbal. These fragments were ligated together in the pBluescript vector (Stratagene) at KpnI and XhoI sites, and the sequence was determined. Then, the large fragment was digested with KpnI and NheI, and F3 was digested with NheI and BglII. These fragments were ligated together in the pGL2 vector (Promega) at KpnI and BglII sites (pGL2–2644 human MCP-1 promoter). The entire coding region of rat MCP-1 cDNA and a partial coding region of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were isolated by reverse transcription PCR. One microgram of total RNA was reverse-transcribed and subjected to PCR. The PCR primers used were as follows: MCP-1 sense primer, 5'-GGATCCATGCAGGTCTCTGTCACGCT-3'; MCP-1 antisense primer, 5'-TCAGACGGCTAGTCTCTGGTACGCT-3'; pRL-TK sense primer, 5'-CTGAGCTAGTCTCTGGTACGCT-3'; pRL-TK antisense primer, 5'-CTGAGCTAGTCTCTGGTACGCT-3'; GAPDH sense primer, 5'-GGATCCATGCAGGTCTCTGTCACGCT-3'; and GAPDH antisense primer, 5'-CTGAGCTAGTCTCTGGTACGCT-3'. The PCR conditions were 1 minute at 95°C, 1 minute at 59°C, and 30 seconds at 72°C for 35 cycles. The PCR-amplified products were digested with BamHI and XhoI and subcloned into the pcDNA3 vector (Invitrogen). These plasmids were used as the positive control for real-time PCR analysis. The entire DNA sequence was determined by cycle sequence reaction using a CEQ8800 DNA sequencer (Beckman Coulter).

RNA Extraction and Real-Time PCR

Total RNA was extracted using TRIzol Reagent (Gibco-BRL), according to the instructions provided by the manufacturer. To extract total RNA from the rat femoral artery, the femoral artery was homogenized in the TRIzol Reagent. After phenol/chloroform extraction, a small amount of total RNA was coprecipitated with transfer RNA (SIGMA). Total RNA was subjected to reverse transcription using an Omniscript RT kit (QIAGEN). Expression of MCP-1 and GAPDH was examined by real time PCR using a SYBR Green dye. Primers used were as follows: Rat MCP-1 sense, 5'-CTCAGGCAATGCATGTTAATGCG-3'; Rat MCP-1 antisense, 5'-CTCAGGCAATGCATGTTAATGCG-3'; Rat GAPDH sense, 5'-GTATGACTCTTACCCAGGCAGATG-3'; Rat GAPDH antisense, 5'-TTCCCTTGATGACCAAGTCT-3'. Real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). To confirm that no significant amounts of primer dimers were formed, dissociation curves were analyzed. PCR-amplified products were also electrophoresed on 2% agarose gels to confirm single bands were amplified.

Transient Transfection

pRL-TK, which encodes the SeaPansy luciferase gene, was purchased from Toyo Ink and used as the internal control for the luciferase assays. Rat VSMCs were transiently transfected with 1.5 µg of pGL2–2644 human MCP-1 promoter and 0.25 µg of pRL-TK using lipofectAMINE (Life Technologies). In some experiments, cells were cotransfected with 1.5 µg of an expression plasmid encoding CalΔC (pcDNA3HA-CalΔC) or MEK6AA (pcDNA3HA-MEK6AA). The total amounts of plasmid DNA transfected in VSMCs were adjusted using the expression vector pcDNA3. Cells were serum-starved for 48 hours after the transfection and some cells were stimulated with 2.0 × 10−7 mol/L Ang II for 12 hours. Cells were harvested and a dual luciferase assay was conducted using a luminometer (Lumat LB 9507, Berthold). SeaPansy luciferase activity was used as the internal control to normalize the MCP-1 promoter activity.

Construction of a Replication-Defective Adenovirus

Construction of a replication-defective adenovirus that expresses a constitutively active mutant of human calcineurin A (AdCalΔC) was described previously.13 A replication-defective adenovirus that expresses MEK6AA (AdMEK6AA) was constructed according to the method previously described using an AdMax kit (Microbix Biosystems Inc). In brief, the protein-coding region of MEK6AA was ligated to the pDC516 vector and cotransfected into 293 cells along with pBHgfrΔE1.3FLP. Recombinant adenoviruses were generated through homologous recombination and propagated in 293 cells, and finally purified by CsCl gradient ultracentrifugation. A recombinant adenovirus expressing green fluorescent protein (AdGFP) was purchased from Quantum Biotechnologies.

ELISA

MCP-1 concentrations in culture medium were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Inc) according to the manufacturer’s instructions. Confluent rat VSMCs were infected with AdCalΔC or AdGFP in low-serum medium for 72 hours. Cells were then washed twice with phosphate-buffered saline and medium was replaced with phenol red-free medium to start incubation. In some experiments, cells were stimulated with Ang II for 12 hours. Medium was collected after the incubation period and centrifuged at 10,000 rpm for 1 minute. The supernatants were stored at −80°C until the assay.

Rat Femoral Artery Injury

Transluminal mechanical injury to the rat femoral artery was performed as previously described with slight modifications.14 Male Wistar rats (8 to 10 weeks old, 10 rats in each group) were obtained from Charles River (Wilmington, Mass) and used in accordance with the guidelines for animal care of the Tokyo University and the NIH. Rats were anesthetized with pentobarbital injected intraperitoneally and a groin incision was made under a surgical microscope. A guide wire (0.46 mm in diameter) was introduced through a small muscular branch of the femoral artery proximally to the aortic bifurcation and withdrawn. Either CyA (10 mg/kg body weight) dissolved in olive oil or vehicle was administered orally once daily for 14 days starting just after the injury.
Histochemical Analysis
The femoral arteries were fixed by perfusing 4% paraformaldehyde and processed for paraffin embedding. Cross sections (2 µm) were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin. For immunohistochemistry, sections were incubated with mouse anti-rat ED1 antibody (Serotec) diluted at 1:800. Sections were then incubated with biotinylated anti-mouse secondary antibody and finally horseradish peroxidase-labeled streptavidin according to the instructions provided by the manufacturer (DAKO, Copenhagen, Denmark). The sections were counterstained with hematoxylin.

Statistical Analysis
The values are the mean±SEM. Statistical analyses were performed using analysis of variance followed by the Student-Neumann-Keul’s test. Differences with a value of P<0.05 were considered statistically significant.

Results
Forced Activation of Calcineurin Upregulates MCP-1 mRNA Expression in VSMCs
We examined by real-time PCR analysis whether forced activation of calcineurin promoted the expression of MCP-1 mRNA in VSMCs using an adenovirus expressing a constitutively active mutant of human calcineurin A. AdCalAΔC infection at a multiplicity of infection (MOI) of 10 significantly upregulated MCP-1 mRNA expression as compared with that observed in AdGFP infection, whereas AdGFP infection did not significantly enhance MCP-1 mRNA expression as compared with that observed in noninfected control (Figure 1A). Preincubation with 10⁻⁶ mol/L CyA, a calcineurin inhibitor, for 24 hours significantly suppressed AdCalAΔC-induced upregulation of MCP-1 mRNA expression, whereas it did not affect MCP-1 mRNA expression in noninfected control cells or AdGFP-infected cells, suggesting that at this concentration CyA did not cause cytotoxic effects in VSMCs. We also confirmed by ethidium bromide staining that a single product was amplified by PCR (Figure 1B).

Angiotensin II–Induced MCP-1 Expression Is Inhibited by CyA in VSMCs
To examine whether endogenous vasoactive substances utilize the calcineurin-dependent pathway to induce MCP-1 expression, we stimulated cultured VSMCs with Ang II, because we have demonstrated that Ang II activates the calcineurin/NFAT-dependent pathway in VSMCs. To investigate whether MCP-1 expression induced by AdCalAΔC infection was regulated at the level of transcription, a reporter plasmid containing a 2644-bp MCP-1 promoter region upstream of the luciferase gene was transfected into cultured rat VSMCs along with an expression plasmid encoding a constitutively active mutant of human calcineurin A. Surprisingly, this cotransfection did not significantly increase the promoter activity of the MCP-1 gene (Figure 3). Ang II significantly increased this promoter activity and this increase was significantly inhibited by pretreatment with Vals or cotransfection with an expression plasmid encoding MEK6AA. In contrast, Ang II–induced increase of the promoter activity was not significantly suppressed by pretreatment with CyA. These results suggested that the calcineurin-dependent pathway enhanced MCP-1 expression, at least partly, at the posttranscriptional level.

Statistical Analysis
The values are the mean±SEM. Statistical analyses were performed using analysis of variance followed by the Student-Neumann-Keul’s test. Differences with a value of P<0.05 were considered statistically significant.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Figure 1. Forced activation of calcineurin increases the expression of MCP-1 mRNA in VSMCs. A, Cultured rat VSMCs were infected with AdGFP or AdCalAΔC at an MOI of 10 for 3 days in low-serum medium and total RNA was extracted. CyA (10⁻⁶ mol/L) was added during the last 24 hours. Amount of MCP-1 mRNA and GAPDH mRNA was determined by real-time PCR analysis. Histograms show the relative amount of MCP-1 mRNA. *P<0.01 vs AdGFP infection; #P<0.01 vs AdCalAΔC infection (n=3). B, Typical ethidium bromide staining of the PCR-amplified products.

Overexpression of the Constitutively Active Mutant of Calcineurin Has No Effect on MCP-1 Promoter Activity in VSMCs
To investigate whether MCP-1 expression induced by AdCalAΔC infection was regulated at the level of transcription, a reporter plasmid containing a 2644-bp MCP-1 promoter region upstream of the luciferase gene was transfected into cultured rat VSMCs along with an expression plasmid encoding a constitutively active mutant of human calcineurin A. Surprisingly, this cotransfection did not significantly increase the promoter activity of the MCP-1 gene (Figure 3). Ang II significantly increased this promoter activity and this increase was significantly inhibited by pretreatment with Vals or cotransfection with an expression plasmid encoding MEK6AA. In contrast, Ang II–induced increase of the promoter activity was not significantly suppressed by pretreatment with CyA. These results suggested that the calcineurin-dependent pathway enhanced MCP-1 expression, at least partly, at the posttranscriptional level.
MCP-1 mRNA Is Stabilized by Constitutive Activation of Calcineurin in VSMCs

We, therefore, hypothesized that forced activation of calcineurin inhibited the degradation of MCP-1 mRNA. To test this hypothesis, cultured rat VSMCs were infected with 10 MOI of AdGFP or AdMEK6AA and serum-starved for 3 days. VSMCs were then restimulated with Ang II for 8 hours in the presence and absence of pretreatment with CyA (10⁻⁶ mol/L) for 24 hours or Vals (100 nmol/L) for 15 minutes. Real-time PCR analysis was performed to detect the amount of MCP-1 and GAPDH mRNA. Histograms show the relative amount of MCP-1 mRNA. *P<0.01 vs AdGFP infection, #P<0.05 vs Ang II stimulation, and ##P<0.01 vs Ang II stimulation (n=3).

MCP-1 mRNA Is Stabilized by Constitutive Activation of Calcineurin in VSMCs

We, therefore, hypothesized that forced activation of calcineurin inhibited the degradation of MCP-1 mRNA. To test this hypothesis, cultured rat VSMCs were infected with 10 MOI of AdGFP or AdMEK6AA and serum-starved for 3 days. VSMCs were then restimulated with Ang II for 8 hours in the presence and absence of pretreatment with CyA (10⁻⁶ mol/L) for 24 hours or Vals (100 nmol/L) for 15 minutes. Real-time PCR analysis was performed to detect the amount of MCP-1 and GAPDH mRNA. Histograms show the relative amount of MCP-1 mRNA. *P<0.01 vs AdGFP infection, #P<0.05 vs Ang II stimulation, and ##P<0.01 vs Ang II stimulation (n=3).

Activation of Calcineurin Increases the Expression of MCP-1 Protein in VSMCs

To examine whether constitutive activation of calcineurin increased the expression of MCP-1 at the protein level as well as at the transcriptional level, cultured rat VSMCs were infected with AdCalAΔC or AdGFP for 3 days, and culture medium was replaced with fresh one to start incubation. MCP-1 content in the culture medium started to increase 8 hours after the beginning of the incubation, and continued to increase during up to 16 hours in AdCalAΔC-infected cells (Figure 5A). We, therefore, incubated cells for 12 hours and the content of MCP-1 in the culture medium was assayed. The MCP-1 content in the culture medium from AdCalAΔC-infected cells was significantly higher than that in the control cells (Figure 5B, left). When cultured rat VSMCs were pretreated with 10⁻⁶ mol/L CyA for 24 hours, this upregulation was completely abolished. We also examined the effects of Ang II on the expression of MCP-1 protein. Ang II significantly increased the MCP-1 content in the culture medium and this increase was completely suppressed by pretreatment with CyA for 24 hours (Figure 5B, right).
CyA Inhibits Mechanical Injury–Induced Neointimal Formation and Macrophage Infiltration in the Rat Femoral Artery

It has been reported that MCP-1 is required for macrophage infiltration and neointimal formation after balloon injury.4,5 To study the significance of the calcineurin-dependent upregulation of MCP-1 expression in vivo, we examined the effect of CyA on macrophage infiltration and neointimal formation in the rat femoral artery after transluminal wire-induced injury. CyA administration did not significantly alter blood pressure, which was measured by the tail cuff method 10 days after the injury (systolic blood pressure: vehicle, 169.8±1100.6 mm Hg versus CyA, 165.7±1103.8 mm Hg, no significant difference). We first checked whether CyA administration inhibited MCP-1 expression in this transluminal wire-induced injury model. MCP-1 mRNA expression in the femoral artery was significantly enhanced 3 days after the injury, and this increase was significantly suppressed by CyA administration, suggesting that CyA also inhibited MCP-1 mRNA expression in vivo (Figure 6). Neointimal formation [the ratio of the intimal to medial area (I/M ratio)] was significantly inhibited by CyA (Figures 7A and 7C). Infiltration of macrophages was observed mainly in the intima. The number of macrophages infiltrated in the intima was significantly attenuated by CyA (Figures 7B and 7D). Finally, to study whether CyA inhibited neointimal formation via its direct suppressive effect on the proliferation of VSMCs, we

Figure 4. Activation of calcineurin stabilizes MCP-1 mRNA. A and B, Cultured rat VSMCs were infected with AdGFP or AdCalAΔC at an MOI of 10 for 3 days in low-serum medium, and 5 μg/mL of actinomycin D was added. Some cells were pretreated with CyA (10⁻⁶ mol/L) for 24 hours. Total RNA was extracted at the indicated time points. Real-time PCR analysis was performed to examine the stability of MCP-1 mRNA. Relative amount of MCP-1 mRNA is indicated in the graph. ■, AdGFP infection; △, AdCalAΔC infection with CyA pretreatment; and ●, AdCalAΔC infection. Shown is a representative result of 2 independent experiments in which the same result was obtained. B, Typical ethidium bromide staining of the PCR-amplified products. C and D, Cultured rat VSMCs were serum-starved for 3 days and restimulated with Ang II for 12 hours in the presence and absence of CyA pretreatment. Actinomycin D was then added and total RNA was extracted at the indicated time points. Real-time PCR analysis was performed. Relative amount of MCP-1 mRNA is indicated in the graph. ■, nonstimulated control; ●, Ang II stimulation with CyA pretreatment; and ◼, Ang II stimulation. Shown is a representative result of 2 independent experiments in which the same result was obtained. D, Typical ethidium bromide staining of the PCR-amplified products.

CyA Inhibits Mechanical Injury–Induced Neointimal Formation and Macrophage Infiltration in the Rat Femoral Artery

It has been reported that MCP-1 is required for macrophage infiltration and neointimal formation after balloon injury.4,5 To study the significance of the calcineurin-dependent upregulation of MCP-1 expression in vivo, we examined the

Figure 5. Activation of calcineurin enhances MCP-1 protein expression in VSMCs. A, Cultured rat VSMCs were infected with AdGFP (open bars) or AdCalAΔC (filled bars) at an MOI of 10 for 3 days in low-serum medium and medium was replaced with a new one to start incubation. Medium was collected at the indicated time points and MCP-1 content in the medium was measured by ELISA. *P<0.05, #P<0.01, and †P<0.001 vs AdGFP infection at each time point (n=3). B, Experiments were performed in the same way as described in A. Medium was collected 12 hours after the medium change. CyA was added during the last 24 hours of serum starvation and the incubation periods. Some cells were stimulated with Ang II for 12 hours. *P<0.0001 vs AdGFP infection; #P<0.0001 vs AdCalAΔC infection; **P<0.01 vs control; and ##P<0.01 vs Ang II stimulation (n=4).
examined the effect of CyA on endothelin (ET)-1–induced 3H-thymidine uptake in cultured rat VSMCs. We used ET-1 in this case, because Ang II is not a potent mitogen for VSMCs. CyA did not significantly inhibit ET-1–induced increase of 3H-thymidine incorporation (online Figure in the online data supplement at http://circres.ahajournals.org).

Discussion

Although the role of the calcineurin/NFAT-dependent pathway in the development of cardiac hypertrophy has been intensively studied, much less is known about its role in blood vessels. However, it has been recently demonstrated that disruption of both NFATc4 and NFATc3 genes is fatal in mice because blood vessel formation is abnormal.18 It was also shown in the same study that a mutation of the calcineurin B gene that prevents its activation by Ca2+/H11001 signals resulted in abnormalities in vascular formation as observed in the NFATc3/c4 null mice. Furthermore, we have shown that Ang II stimulates the DNA binding activity of NFAT and NFAT-dependent transcriptional activation of genes in VSMCs.13 We have also demonstrated that Ang II–induced expression of nonmuscle-type myosin heavy chain B is mediated by the calcineurin-dependent pathway.13 Thus, it appears that the calcineurin/NFAT-dependent pathway has important roles in VSMCs, although its role remains to be fully elucidated.

In the present study, we showed that forced activation of calcineurin using a constitutively active mutant of calcineurin A stimulated the expression of MCP-1 both at the transcription and protein levels in VSMCs. We also showed that Ang II–induced increase of the MCP-1 expression was inhibited by CyA. These results suggested that the calcineurin-dependent pathway was implicated in MCP-1 expression and that endogenous agonists such as Ang II utilized the calcineurin-dependent pathway to induce MCP-1 expression. Our results also suggested a link between Ca2+/H11001 signals and vascular inflammation. It has been reported that mitogen-activated protein kinases such as extracellular signal-regulated kinase and p38, and Janus kinase are involved in the activation of the MCP-1 gene.15,17,19 To our knowledge, this is the
first report that shows that calcineurin is implicated in the activation of MCP-1 gene in VSMCs (Figure 8).

Surprisingly, forced expression of a constitutively active mutant of calcineurin A did not increase the MCP-1 promoter activity in VSMCs. Instead, forced activation of calcineurin stabilized the MCP-1 transcripts. Accumulated evidence has suggested that the expression of MCP-1 is largely regulated at the level of transcription and that a variety of cis-elements in the promoter region of the MCP-1 gene, such as nuclear factor-κB (NF-κB) sites, a binding site for signal transducers and activators of transcription (STAT), AP-1 sites, and SP-1 sites, are implicated in the transcriptional activation of the gene.20–22 Although the promoter region of the MCP-1 gene we used in this study was large enough to contain the whole NF-κB, STAT, AP-1 and SP-1 sites, we did not find any induction of the promoter activity by forced activation of calcineurin. Furthermore, Ang II-induced increase of the MCP-1 promoter activity was not inhibited by CyA, although MEK6AA inhibited this induction. Interestingly, it was reported that hyperoxia stimulated MCP-1 expression, at least partly, through the stabilization of the MCP-1 transcripts in monocytes.23 Thus, it appeared that expression of the MCP-1 gene was regulated, at least in part, at the posttranscriptional level, although we do not exclude the possibility that the calcineurin-dependent transcriptional activation of the MCP-1 gene was not detected, because some as-yet unidentified cis-elements responsible for the calcineurin-induced activation of the MCP-1 gene might have been missing in the promoter region we used in this study.

To study the significance of the calcineurin-dependent increase of MCP-1 expression in vivo, we examined the effects of CyA on the expression of MCP-1 and the neointimal formation of the rat femoral artery after transluminal mechanical injury. We found that CyA significantly inhibited MCP-1 expression in the rat femoral artery. We also found that CyA significantly suppressed both macrophage infiltration and neointimal formation. It has been shown that macrophages infiltrating the blood vessel wall play critical roles in neointimal formation. Inactivation of macrophages by an anti-CD4 antibody or bisphosphonate-containing liposomes, which kill macrophages after phagocytosis, resulted in significant suppression of neointimal formation after transluminal endothelial injury.24,25 Furthermore, inactivation of MCP-1 function by using a neutralizing antibody for MCP-1 or by expressing a dominant-negative mutant of MCP-1, has been demonstrated to result in decreases of macrophage infiltration and neointimal formation after balloon injury.4,5 Although it was possible that CyA inhibited neointimal formation by directly suppressing proliferation and/or hypertrophy of VSMCs, the effect of CyA on the proliferation of VSMCs remains controversial. CyA inhibited the proliferation of VSMCs in some studies, but not in others.26,27 We examined the effect of CyA on endothelin-1–induced proliferation of VSMCs as assessed by 3H-thymidine uptake. We did not find any suppressive effect of CyA on VSMC proliferation (online Figure 1). Some positive results might be due to the cytotoxic effect of CyA. In our previous report, CyA did not inhibit Ang II–induced vascular hypertrophy as assessed by 3H-leucine uptake.13 Thus, CyA did not seem to have a potent effect on the proliferation and/or hypertrophy of VSMCs. Although CyA inhibits the proliferation and activation of T cells, the role of T cells in neointimal formation remains controversial. Several studies showed that depletion of T cells using athymic rats or a cytolytic anti–T cell antibody did not affect the extent of neointimal formation after balloon injury as compared with that in control rats, rather it enhanced neointimal formation.28,29 Thus, it seems unlikely that CyA inhibited neointimal formation via its suppressive effect on T cell function. Taken together, it is probable that CyA inhibited macrophage infiltration and neointimal formation by suppressing MCP-1 production in the vessel wall. In accordance with our results, several reports showed inhibitory effects of CyA on neointimal formation in rats.27,30 However, some authors reported that CyA did not inhibit neointimal formation in rabbits.26,31 Although the reason for this discrepancy is not clear, it might result from a difference in the animal species. We and others, who showed positive effects of CyA on neointimal formation, used rats, whereas rabbits were used in most studies in which negative effects of CyA were reported. Another possibility is that not only the endothelium but also the media of the rat artery might be easily injured by transluminal mechanical injury compared with the rabbit artery, because the luminal diameter and wall thickness of rat arteries are smaller than those of rabbit arteries. Because the media consists of vascular myocytes and can be a source of MCP-1 production, MCP-1 production in the media might be induced more potently in rats than in rabbits, and the inhibitory effect of CyA might be stronger in rats. Thus, although future studies are required to clarify the reason for the inconsistency, CyA seems to inhibit macrophage infiltration and neointimal formation after transluminal mechanical injury, at least, in rats.

In summary, we have shown that the calcineurin-dependent pathway mediates MCP-1 expression in VSMCs and vascular inflammation. Calcineurin may be a novel target to modulate vascular inflammation in conditions such as atherosclerosis and restenosis after angioplasty.
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References


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MATERIALS AND METHODS

Reagents: Human endothelin (ET)-1 was obtained from Peptide Institute (Osaka, Japan). BQ123, a specific antagonist for ET\textsubscript{A} receptor, was purchased from SIGMA-ALDRICH Co. (St. Louis, MO).

Measurement of \(^3\)H-thymidine incorporation: Cultured rat vascular smooth muscle cells (VSMCs) plated on 24-well plates were serum starved for three days and stimulated with 10\(^{-7}\) mol/L ET-1 for 16 hrs. Some cells were preincubated with 10\(^{-6}\) mol/L cyclosporin A (CyA) for 12 hrs or 10\(^{-5}\) mol/L BQ123 for 1 hr. \(^3\)H-thymidine (2 \(\mu\)Ci/ml, Amersham) was added to each well 2 hrs before the end of the incubation period. Cells were washed twice with ice-cold phosphate-buffered saline and incubated with ice-cold 10\% trichloroacetic acid for 30 min. After washing them twice with distilled water, the cells were lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and subjected to liquid scintillation counting.

RESULTS

ET-1 significantly increased \(^3\)H-thymidine incorporation as compared with the nonstimulated control level (basal level), and this increase was significantly suppressed by pretreatment with BQ123, while CyA did not inhibit ET-1-induced increase of \(^3\)H-thymidine uptake. CyA did not suppress the basal level of \(^3\)H-thymidine uptake, either (Online Figure 1).

FIGURE LEGEND

Online Figure 1  CyA does not inhibit ET-1-induced DNA synthesis. Cultured rat VSMCs were serum starved for three days and stimulated with ET-1 in the presence and absence of pretreatment with CyA or BQ123. DNA synthesis was assessed by \(^3\)H-thymidine incorporation. *: P<0.001 vs. nonstimulated control and #: P<0.001 vs. ET-1 stimulation.