Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters

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Submitted 22 August 2006; accepted in final form 18 September 2006

Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters. Am J Physiol Cell Physiol 292: C886–C895, 2007. First published September 20, 2006; doi:10.1152/ajpcell.00449.2006.—A hallmark of smooth muscle cell (SMC) phenotypic switching is suppression of SMC marker gene expression. Although myocardin has been shown to be a key regulator of this process, the role of its related factors, MKL1 and MKL2, in SMC phenotypic switching remains unknown. The present studies were aimed at determining if: 1) MKL factors contribute to the expression of SMC marker genes in cultured SMCs; and 2) platelet-derived growth factor-BB (PDGF-BB)-induced repression of SMC marker genes is mediated by suppression of MKL factors. Results of gain- and loss-of-function experiments showed that MKL factors regulated the expression of single and multiple CArG [CC(AT-rich)6GG]-containing SMC marker genes, such as smooth muscle (SM) α-actin and telokin, but not CArG-independent SMC marker genes such as smoothelin-B. Treatment with PDGF-BB reduced the expression of CArG-containing SMC marker genes, as well as myocardin expression in cultured SMCs, while it had no effect on expression of MKL1 and MKL2. However, of interest, PDGF-BB induced the dissociation of MKL factors from the CArG-containing region of SMC marker genes, as determined by chromatin immunoprecipitation assays. This dissociation was caused by the competition between MKL factors and phosphorylated Elk-1 at early time points, but subsequently by the reduction in acetylated histone H4 levels at these promoter regions mediated by histone deacetylases, HDAC2, HDAC4, and HDAC5. Results provide novel evidence that PDGF-BB-induced repression of SMC marker genes is mediated through combinatorial mechanisms, including downregulation of myocardin expression and inhibition of the association of myocardin/MKL factors with CArG-containing SMC marker gene promoters.

Yoshida T, Gan Q, Shang Y, Owens GK. Platelet-derived growth factor-BB represses smooth muscle cell marker genes via changes in binding of MKL factors and histone deacetylases to their promoters. Am J Physiol Cell Physiol 292: C886–C895, 2007. First published September 20, 2006; doi:10.1152/ajpcell.00449.2006.—A hallmark of smooth muscle cell (SMC) phenotypic switching is suppression of SMC marker gene expression. Although myocardin has been shown to be a key regulator of this process, the role of its related factors, MKL1 and MKL2, in SMC phenotypic switching remains unknown. The present studies were aimed at determining if: 1) MKL factors contribute to the expression of SMC marker genes in cultured SMCs; and 2) platelet-derived growth factor-BB (PDGF-BB)-induced repression of SMC marker genes is mediated by suppression of MKL factors. Results of gain- and loss-of-function experiments showed that MKL factors regulated the expression of single and multiple CArG [CC(AT-rich)6GG]-containing SMC marker genes, such as smooth muscle (SM) α-actin and telokin, but not CArG-independent SMC marker genes such as smoothelin-B. Treatment with PDGF-BB reduced the expression of CArG-containing SMC marker genes, as well as myocardin expression in cultured SMCs, while it had no effect on expression of MKL1 and MKL2. However, of interest, PDGF-BB induced the dissociation of MKL factors from the CArG-containing region of SMC marker genes, as determined by chromatin immunoprecipitation assays. This dissociation was caused by the competition between MKL factors and phosphorylated Elk-1 at early time points, but subsequently by the reduction in acetylated histone H4 levels at these promoter regions mediated by histone deacetylases, HDAC2, HDAC4, and HDAC5. Results provide novel evidence that PDGF-BB-induced repression of SMC marker genes is mediated through combinatorial mechanisms, including downregulation of myocardin expression and inhibition of the association of myocardin/MKL factors with CArG-containing SMC marker gene promoters.

Differentially smooth muscle cells (SMCs) in adult vessels exhibit a unique repertoire of gene expression, such as smooth muscle (SM) α-actin, SM-myosin heavy chain (SM-MHC), and SM22α, while expression of these SMC marker genes is decreased in response to vascular injury (28). This plasticity of SMC phenotype is referred to as SMC phenotypic switching and plays a critical role in the development of atherosclerosis and restenosis after percutaneous coronary interventions (28). Platelet-derived growth factor-BB (PDGF-BB) is a key mediator of SMC phenotypic switching. It has been shown to potently suppress expression of SMC marker genes, as well as to increase the rate of proliferation and migration in cultured SMCs (4, 8, 20). Expression of PDGF-BB and PDGF-β receptor has been shown to be increased in the vascular lesions following mechanical injury and during atherosclerosis in vivo (8, 20). Moreover, inhibition of PDGF-BB or PDGF-β receptor signaling has been shown to attenuate neointimal thickening in several models of vascular injury and atherosclerosis (8, 20). Given such a critical involvement of PDGF-BB in vascular lesion formation, elucidation of mechanisms whereby PDGF-BB represses SMC marker gene expression is likely to provide key insights towards understanding of the development of vascular disease.

Most SMC marker genes, including SM α-actin, SM-MHC, and SM22α, contain multiple CArG [CC(AT-rich)6GG] elements in the promoter-enhancer regions, and expression of these genes is controlled by the ubiquitously expressed transactivation factor, serum response factor (SRF), and its coactivator, myocardin (28). Results of previous studies by our laboratory and others showed that myocardin was exclusively expressed in SMCs and cardiomyocytes, and markedly induced the transcription of multiple CArG-containing SMC marker genes in the presence of SRF (3, 6, 24, 29), but failed to induce CArG-independent SMC marker genes such as smoothelin-B, acid carboxypeptidase-like protein (ACLP), and focal adhesion kinase-related nonkinase (FRNK) (30). Suppression of myocardin by either dominant-negative forms or short interfering RNA (siRNA) in cultured SMCs was associated with marked decreases in transcription of CArG-containing SMC marker genes (6, 29). Moreover, Li et al. (11) demonstrated that myocardin-deficient mice died by embryonic day 10.5 and showed no evidence of vascular SMC differentiation. However, myocardin does not appear to be necessarily required for the formation of SMCs, since recent studies using chimeric mice from wild-type blastocysts injected with myocardin-null embryonic stem (ES) cells revealed that myocardin-null ES cells still gave rise to SMCs in vivo (18). Of particular interest, PDGF-BB-induced suppression of SMC marker genes has been shown to be mediated, at least in part, through inhibition of formation of the CArG-SRF-myocardin complex (13, 26). Wang et al. (26) showed that PDGF-BB induced phosphorylation of a ternary complex factor, Elk-1, and that phosphorylated Elk-1 inhibited the binding of myocardin to SRF, thereby reducing the transcription of CArG-dependent SMC marker genes in cultured A7r5 SMCs. We also showed that PDGF-BB...
repressed SMC marker gene expression through downregulating myocardin expression and interfering with SRF binding to CArG elements in intact chromatin of cultured SMCs (13). As such, the preceding results provide compelling evidence that regulation of formation of the CArG-SRF-myocardin complex plays a key role in the control of SMC marker gene expression.

MKL1 (also referred to as MAL, BSAC, MRTF-A) (2, 16, 21, 25) and MKL2 (also referred to as MRTF-B) (22, 25) have been identified as members of the family of myocardin-related transcription factors. Although both MKL factors have been shown to interact with SRF, MKL factors are widely expressed in many cell types in contrast to the SMC-cardiomyocyte-restricted expression pattern of myocardin (2, 7, 22, 25). It has been shown by cotransfection/reporter assays that MKL1 and MKL2 induced the transcription of multiple CArG-containing SMC marker genes, including SM α-actin and SM22α (2, 7, 22, 25). Du et al. (7) showed that a dominant-negative form of MKL1 decreased MKL1-induced transcription of the SM22α gene in ES cells, although this truncated protein also decreased myocardin-induced transcription of this gene such that effects may not have been MKL1-dependent. Selvaraj and Prywes (22) showed that a truncated form of MKL2, which behaves as a dominant-negative protein for both MKL1 and MKL2, suppressed skeletal muscle differentiation in C2C12 skeletal myoblasts. Moreover, results of studies by Li et al. (10) and Oh et al. (17) showed that MKL2 knockout mice exhibited cardiovascular defects, including the abnormal patterning of branchial arch arteries and a reduction in SM α-actin expression in neural crest-derived SMCs. In addition, recent studies showed that MKL1 knockout mice exhibited a failure to nurse their offspring due to an impaired expression of CArG-dependent SMC marker genes in mammary myoepithelial cells (12, 23). The preceding results suggest that MKL factors also play a key role in transcription of multiple CArG-containing SMC marker genes, and indicate that they can play both redundant but also unique roles depending on the SMC subtype. However, as yet, no studies have been reported investigating the role or mechanisms by which MKL factors contribute to downregulation of SMC marker genes during SMC phenotypic switching.

Therefore, in the present studies, we investigated the involvement of MKL factors in PDGF-BB-induced suppression of SMC marker genes. We first determined the contribution of MKL factors to the expression of CArG-dependent and CArG-independent SMC marker genes, using a combination of direct siRNA-induced gene suppression and overexpression studies, and then tested if PDGF-BB-induced repression of CArG-dependent SMC marker genes is mediated by the inhibition of MKL function in cultured SMCs.

MATERIALS AND METHODS

Plasmid constructs and siRNA duplexes. Rat SM α-actin-luciferase (−2.6/+2.8 kb) construct and its triple CArG mutant were described previously (29). Mouse telokin-luciferase (−198/+180 bp) construct and its CArG mutant were provided by Dr. B. Paul Herring (Indiana University, Indianapolis, IN) (31). Expression plasmids for FLAG-tagged myocardin, FLAG-tagged MKL1, and FLAG-tagged MKL2 were provided by Dr. Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, TX) (24) and Dr. Ron Prywes (Columbia University, New York, NY) (2, 22). Expression plasmids for siRNA specific for myocardin (pMighty-αMyo) and its control (pMighty-αScr) were described previously (29). siRNA expression plasmids specific for MKL1 and MKL2 were constructed by inserting specific oligonucleotides (MKL1: CCAACGAGCTGAGTCTGA; MKL2: GACATCCCAAGAATTCAAA) into pMighty-Empty (29). Expression plasmids for histone deacetylase 1 (HDAC1) to HDAC7 were provided by Dr. Stuart L. Schreiber (Harvard University, Cambridge, MA), Dr. Edward Seto (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), and Dr. Hung-Ying Kao (Case Western Reserve University, Cleveland, OH). siRNA targets were used for enhanced green fluorescent protein (EGFP) (9, 29), myocardin (29), MKL1 (CCAAACGAGUCAGACGUGAA), MKL2 (GACATT-CCAAATATCAGAA), HDAC2 (GAUAAUCAGAGAGCUUA), HDAC4 (GAACAUAAUAAGACGAGC), and HDAC5 (GAGAGAACUGUGAUAGU) were purchased from MWG-BIOTECH (High Point, NC).

Cell culture, transient transfection, and PDGF-BB stimulation. Rat aortic SMCs, A404 cells, 10T1/2 cells, and NIH/3T3 cells were cultured as described previously (29, 30). Transfection of DNA plasmids was performed by using FuGene6 (Roche Diagnostics, Indianapolis, IN). To examine the effects of PDGF-BB in rat aortic SMCs (4), cells were seeded at 10,000 cells/cm² in DMEM-Ham’s F12 medium with 10% fetal bovine serum (HyClone, Logan, UT). After a 24-h incubation, the medium was changed to serum-free DMEM-Ham’s F12 medium supplemented with 5 μg/ml transferrin, 6.25 ng/ml sodium selenite, and 0.2 mmol/lL-ascorbic acid, and cells were transfected with DNA plasmids or siRNA duplexes if required. Twenty-four hours later, SMCs were treated with 30 ng/ml PDGF-BB (Upstate, Lake Placid, NY) or vehicle for indicated times. Transfection of siRNA duplexes was performed by using Oligofectamine (Invitrogen, Carlsbad, CA).

Reverse transcription (RT)-PCR and luciferase assays. Total RNA was prepared from cultured cells after the induction of differentiation or after the transfection and was used for semiquantitative or real-time RT-PCR analyses. Primer and probe sequences for SM α-actin, SM-MHC, SM22α, smoothelin-B, ACLP, myocardin, GAPDH, and 18S rRNA were described previously (30). Primer and probe sequences for MKL1 and MKL2 were as follows: MKL1-sense, 5’-TCCAGGCCAAGCAGTGAA-3’; MKL1-antisense, 5’-GTCCTACCTGGCCCAATGAT-3’; MKL1-probe, 5’-AGATTGCCAAGGGCTTCGGCCCA-3’. MKL2-sense, 5’-TGGCTCCAGCCTGAGCTGCAC-3’. MKL2-antisense, 5’-GGCGGCCTCCCGATTTGCTG-3’. MKL2-probe, 5’-AGAAACACTGTTGACAGGGCGAT-3’. Luciferase assays were performed as previously described (29).

Western blotting, immunofluorescence, and coimmunoprecipitation assays. Western blotting, immunofluorescence, and coimmunoprecipitation assays were performed as previously described (9, 29, 30). Antibodies used were as follows: SM α-actin (1A4; Sigma Chemical, St. Louis, MO), Telokin (provided by Dr. B. Paul Herring), FRNK (Santa Cruz Biotechnology, Santa Cruz, CA), MKL1 (Santa Cruz Biotechnology), SRF (Santa Cruz Biotechnology), GAPDH (Chemicon International, Temecula, CA), phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA), phospho-Erk-1 (Cell Signaling Technology), Elk-1 (Santa Cruz Biotechnology), HDAC2 (Zymed Laboratories, South San Francisco, CA), HDAC4 (Cell Signaling Technology), HDAC5 (Cell Signaling Technology), and FLAG (M2; Sigma Chemical).

Chromatin immunoprecipitation (ChIP) assays. SMCs were fixed with 1% formaldehyde for 10 min at 37°C to cross-link protein-DNA and protein-protein interactions within intact chromatin. Cells were harvested and the cross-linked chromatin was sonicated to shear chromatin fragments to 200–600 bps as described previously (9, 14). Chromatin-protein complexes were immunoprecipitated with antibodies against MKL1, SRF, Elk-1, acetylated histone H4 (Upstate), HDAC2, HDAC4, or HDAC5, and salmon sperm DNA/protein A agarose was added. As a negative control, the antibody was excluded from the immunoprecipitation reaction. Samples were washed, reverse cross-linked, and purified. Recovered DNA was quantified by
fluorescence with picogreen reagent (Molecular Probes, Eugene, OR). Real-time PCR was performed to amplify the CArG-containing region of the SM α-actin and SM-MHC promoters. Primer sequences were previously described (14). Data in ChIP assays are representative of three independent experiments.

RESULTS

MKL1 and MKL2 induced expression of single and multiple CArG-containing SMC marker genes, but not CArG-independent SMC marker genes. The initial aim of the present studies was to determine the expression and localization of MKL1 and MKL2 in our cultured SMCs derived from rat thoracic aorta. As shown in Fig. 1A, transcripts of MKL1 and MKL2 were widely expressed in a variety of cells examined, including rat aortic SMCs, A404 cells, 10T1/2 cells, and NIH/3T3 cells, whereas myocardin transcript was expressed in a cell-specific manner. Intracellular localization of MKL factors was also examined in cultured SMCs by transfecting FLAG-tagged expression plasmids for myocardin, MKL1, and MKL2. In contrast to myocardin, which was expressed exclusively in the nucleus, both MKL factors were localized both within the nucleus and the cytoplasm (Fig. 1, B–D). However, MKL1 was mostly localized in the nucleus, whereas MKL2 was predominantly localized in the cytoplasm. The distribution of MKL1 in cultured SMCs was further confirmed by detecting endogenous MKL1 using anti-MKL1 antibody (Fig. 1E). These results clearly indicate that both MKL factors were expressed in cultured SMCs, although intracellular localization of MKL factors was different from that of myocardin.

Although results of previous studies suggest that both MKL factors were capable of inducing multiple CArG-containing SMC marker genes (2, 7, 22, 25), it remains unknown whether MKL factors have effects on single CArG-containing SMC marker genes and CArG-independent SMC marker genes. To address this question, expression plasmids for MKL1 and MKL2 were transiently transfected into NIH/3T3 cells and the induction of various SMC marker genes examined by semi-quantitative RT-PCR or Western blotting (Fig. 2, A and B). Overexpression of MKL1 and MKL2 induced mRNA expression of multiple CArG-containing SMC marker genes including SM α-actin, SM-MHC, and SM22α. Neither MKL1 or MKL2 had any effect on expression of CArG-independent SMC marker genes, including smoothelin-B, ACPL, and FRNK. Of interest, MKL1 and MKL2, induced expression of telokin, a single CArG-containing SMC marker gene, although the effect of MKL2 was modest compared with myocardin and MKL1. Effects of myocardin, MKL1, and MKL2 on the transcription of the SM α-actin and telokin genes were also examined by luciferase assays (Fig. 2, C and D). The SM α-actin promoter activity was significantly induced by myocardin (864-fold) and MKL1 (3,046-fold). The transcriptional activity of the telokin gene was also induced by myocardin (332-fold) and MKL1 (404-fold). MKL2 also induced the transcriptional activity of these genes (SM α-actin by 424-fold and telokin by 40-fold), although effects were weaker than myocardin and MKL1. The effects of MKL factors were CArG-dependent, since mutation of CArG elements in both SM α-actin and telokin promoters abolished the responsiveness to MKL1 and MKL2. Taken together, results suggest that both MKL1 and MKL2, as well as myocardin, were capable of inducing expression of single and multiple CArG-containing SMC marker genes, but not inducing expression of CArG-independent SMC marker genes.

siRNA-induced suppression of MKL factors reduced CArG-dependent SMC marker gene expression in cultured SMCs. To test whether endogenous MKL1 and MKL2 contribute to the expression of SMC marker genes in SMCs, effects of siRNAs specific for MKL1 and MKL2 (Fig. 3A) on the transcription of SMC marker genes were examined in cultured SMCs. Suppression of MKL1 and MKL2 reduced the transcriptional activity of the SM α-actin gene in SMCs by 75% and 42%, respectively, whereas suppression of myocardin reduced expression by 54% (Fig. 3B). Likewise, suppression of MKL1 and MKL2 reduced the telokin promoter activity by 64% and 20%, respectively, whereas suppression of myocardin reduced the transcription by 20%.

Myocardin-, MKL1-, and MKL2-specific siRNA duplexes were transfected into cultured SMCs, and expression of endogenous SMC marker genes measured by real-time RT-PCR (Fig. 3C). Expression of myocardin, MKL1, and MKL2, respectively, was successfully reduced by the appropriate siRNA duplex. Suppression of myocardin, MKL1, and MKL2 was associated with decreases in expression of endogenous SM

Fig. 1. Expression of myocardin, MKL1, and MKL2 in cultured cells. A: myocardin, MKL1, and MKL2 mRNA expression in a variety of cells, including rat aortic smooth muscle cells (SMCs) (RASMC), undifferentiated and differentiated A404 cells, 10T1/2 cells, 10T1/2 cells treated with 1 ng/ml transforming growth factor-β1, and NIH/3T3 cells was determined by semi-quantitative RT-PCR. B–D: cultured SMCs were transfected with expression plasmids for FLAG-tagged myocardin, MKL1, and MKL2, and intracellular localization of these factors was examined by anti-FLAG antibody. E: endogenous MKL1 expression in cultured SMCs was examined by anti-MKL1 antibody.
α-actin by 50%, 52%, and 55% and SM-MHC by 67%, 62%, and 68%, respectively. However, suppression of myocardin or MKL factors did not decrease smoothelin-B expression. In addition, there was no change in expression of the rest of myocardin/MKL factors to compensate for the lack of each factor. These results clearly indicate that each of the MKL factors contributes to the expression of CArG-dependent SMC marker genes in cultured SMCs.

**PDGF-BB elicits the dissociation of MKL factors from CArG-containing SMC marker gene promoters in cultured SMCs.** PDGF-BB has been shown to repress expression of CArG-dependent SMC marker genes, including SM α-actin and SM-MHC (4). We tested the hypothesis that suppression of SMC marker genes by PDGF-BB was in part mediated through the inhibition of MKL factors. First, the effect of PDGF-BB on expression of MKL factors was tested in cultured SMCs. Although PDGF-BB reduced mRNA expression of myocardin and CArG-dependent SMC marker genes, it did not decrease mRNA expression of MKL1 and MKL2 (Fig. 4A). Treatment of SMCs with PDGF-BB also had no effect on MKL1 protein expression (Fig. 4B). Intracellular localization of MKL factors in SMCs was also unaffected by PDGF-BB (data not shown).

To determine if PDGF-BB altered the association of MKL factors with the CArG-containing region of SMC marker genes, ChIP assays were performed in PDGF-BB-treated cultured SMCs. These studies utilized an anti-MKL1 antibody, since, at present, no anti-MKL2 antibody is available that works in ChIP assays. Of major interest, the association of MKL1 with the CArG-containing region of SM α-actin (Fig. 4C) and SM-MHC (data not shown), was markedly decreased by PDGF-BB treatment at both 0.5 and 24 h. In contrast, the binding affinity of SRF to these CArG-containing regions was unchanged at 0.5 h, but significantly decreased at 24 h of PDGF-BB treatment. We also tested if PDGF-BB decreased the association of MKL2 with the CArG-containing region in FLAG-MKL2 expressing SMCs. ChIP assays using anti-FLAG antibody revealed that PDGF-BB decreased the association of FLAG-MKL2 with the CArG-containing region of the SM α-actin promoter in a similar manner to endogenous MKL1 (data not shown). Results thus indicate that repression of SMC marker genes by PDGF-BB is in part mediated through the inhibition of accessibility of MKL factors to the CArG-containing SMC marker gene promoters. That is, although expression of MKL factors was not reduced by PDGF-BB, as is the case with myocardin, their binding to CArG-containing SMC marker gene promoters within intact chromatin was reduced.
PDGF-BB induced displacement of MKL factors from SRF by phosphorylated Elk-1 at early time points. Wang et al. (26) previously showed that PDGF-BB induced phosphorylation of Elk-1, and that phosphorylated Elk-1 displaced myocardin from SRF in cultured A7r5 SMCs. To determine if this mechanism also contributes to PDGF-BB-induced decreases in association of MKL factors with CArG-containing SMC promoters, we first determined the time course of Elk-1 phosphorylation. 

Fig. 3. siRNA-induced suppression of myocardin, MKL1, and MKL2 decreased expression of CArG-dependent SMC marker genes. A: efficiency and specificity of short interfering RNAs (siRNAs) were tested. Rat aortic SMCs were cotransfected with 250 ng of pMighty-αMyo, pMighty-αMKL1, pMighty-αMKL2, or pMighty-αScr and 250 ng of expression plasmids for FLAG-tagged myocardin, MKL1, or MKL2. Expression of FLAG-tagged proteins was examined by Western blotting with anti-FLAG antibody. B: rat aortic SMCs were cotransfected with 100 ng of pMighty-αMyo, pMighty-αMKL1, pMighty-αMKL2, or pMighty-αScr and 300 ng of SM α-actin-luciferase or telokin-luciferase construct. Luciferase activities were measured and normalized to protein contents. An arbitrary value of 100 was assigned to the activity of the cells transfected with pMighty-αScr and the reporter plasmid. C: cultured SMCs were transfected with siRNA duplexes specific for myocardin, MKL1, and MKL2 and incubated for 48 h. Endogenous expression of SMC marker genes (SM α-actin, SM-MHC, smoothelin-B), myocardin, MKL1, and MKL2 was determined by real-time RT-PCR. An arbitrary value of 100 was assigned to the expression of cells transfected with siRNA specific to enhanced green fluorescent protein (EGFP). Values represent the means ± SE.

Fig. 4. PDGF-BB did not alter expression of MKL factors, but it decreased the association of MKL1 with the CArG-containing region of the SM α-actin promoter within intact chromatin, as determined by chromatin immunoprecipitation (ChIP) assays. Cultured SMCs were treated with PDGF-BB (B) or vehicle (V) for indicated times. A: mRNA expression of SMC marker genes, myocardin, MKL1, and MKL2 was determined by real-time RT-PCR. The ratio of PDGF-BB-treated SMCs to vehicle-treated SMCs are shown. B: expression of MKL1, serum response factor (SRF), and GAPDH was examined by Western blotting. C: association of MKL1 and SRF with the CArG-containing region of the SM α-actin promoter was determined by ChIP assays. Values represent the means ± SE.
lation after PDGF-BB treatment by Western blotting. As shown in Fig. 5A, phosphorylation of Elk-1 was transiently induced by PDGF-BB treatment being present at 0.5 h, but not later time points. In contrast, phosphorylation of ERK1/2 was present at 0.5 h and sustained through 24 h. Based on coimmunoprecipitation assays, there was evidence of binding between Elk-1 and SRF at 0.5 h after PDGF-BB treatment (Fig. 5B), consistent with the time course of Elk-1 phosphorylation. In contrast to the very transient interaction between Elk-1 and SRF, PDGF-BB treatment virtually abolished binding between MKL1 and SRF at both 0.5 and 24 h. Moreover, ChIP assays revealed that PDGF-BB elicited the association of Elk-1 with the CArG-containing region of SMC marker gene promoters only at 0.5 h, but not at 24 h following PDGF-BB treatment (Fig. 5C). These results suggest that PDGF-BB causes the dissociation of MKL factors from SRF by recruiting phosphorylated Elk-1 to SRF at early time points, but that alternative mechanisms must be required for mediating sustained dissociation of MKL factors from CArG-containing regions of SMC marker gene promoters.

PDGF-BB-induced repression of SMC marker genes was mediated through the recruitment of HDAC2, HDAC4, and HDAC5 to the CArG-containing SMC marker gene promoters. Results of previous studies in our laboratory showed that treatment of SMCs with PDGF-BB for 24 h induced hypoacetylation of histone H4 at the CArG-containing region of SMC marker genes (14). Hypoacetylation of histone H4 has been demonstrated to restrict the accessibility of transcription factors to cognate DNA elements by forming the condensed chromatin structure, thereby suppressing gene transcription (15). Although hypoacetylation is normally mediated by HDACs, it is unknown which HDACs mediate PDGF-BB-induced suppression of SMC marker gene expression. Thus, the following experiments were performed to examine the possible involvement of HDACs in this process. First, the effect of PDGF-BB on acetylation levels of histone H4 was examined by ChIP assays. PDGF-BB caused a rapid and sustained reduction in acetyl histone H4 levels at the CArG-containing SMC marker gene promoters (Fig. 6A). To determine which HDACs were capable of mediating this modification, expression plasmids for HDAC1 to HDAC7 were cotransfected into SMCs along with the SM α-actin-luciferase construct. Results showed that HDAC2, HDAC4, and HDAC5 markedly reduced transcription of SMC marker genes (Fig. 6B). However, the expression levels of these HDACs were not altered by the treatment with PDGF-BB (Fig. 6C). Therefore, the association of these HDACs with the CArG-containing region of SMC marker genes was examined by ChIP assays (Fig. 6D). Treatment of SMCs with PDGF-BB was associated with recruitment of HDAC2, HDAC4, and HDAC5 to the CArG-containing SMC marker gene promoters within 0.5 h of PDGF-BB treatment, and the association of HDAC2 and HDAC5 was sustained through 24 h. Effects of knockdown of these HDACs on PDGF-BB-mediated suppression of SMC marker genes were then tested. Results showed that PDGF-BB-induced suppression of SM α-actin gene expression was markedly attenuated in SMCs transfected with siRNA duplexes specific for HDAC2 and HDAC5, while the effect of HDAC4 siRNA was somewhat less (i.e., a 89% reduction in control αEGFP-treated SMCs compared with 50%, 82%, and 60%...
reductions, respectively, in SMCs treated with αHDAC2, αHDAC4, and αHDAC5 siRNAs) (Fig. 7). Taken together, results provide evidence that PDGF-BB recruits HDAC2, HDAC4, and HDAC5 to the CArG-containing regions of SMC marker genes, and that HDAC-induced hypoacetylation of histone H4 inhibits the accessibility of MKL factors to the CArG-containing regions within intact chromatin, and thereby reduces transcription of SMC marker genes.

**DISCUSSION**

Although MKL2 has been shown to be required for SM α-actin expression in neural crest-derived SMCs during the early embryonic development (10, 17), its role in adult SMCs or local mesoderm-derived SMCs is unknown. Likewise, although Du et al. (7) showed that a truncated mutant of MKL1 repressed MKL1-induced activation of the SM22α gene in a dominant-negative manner, the effect of MKL1 on SMC marker gene expression is still unclear because the dominant-negative MKL1 used in these studies also inhibited effects of myocardin. Results of the present studies clearly demonstrate the contribution of both MKL factors, as well as myocardin, to SMC marker gene expression in cultured SMCs, based on experiments involving siRNA-induced direct targeting of individual myocardin and MKL factors. Knockdown of myocardin, MKL1, and MKL2 reduced the expression of CArG-dependent SMC marker genes. These observations are novel and significant in that they indicate that each of the myocardin/MKL family members contributes to basal expression of SMC marker genes but cannot completely compensate for each other. Results are consistent with a large body of evidence that expression of SMC marker genes is controlled by the coordinated interaction of multiple transcription factors and cofactors rather than a single factor (28). Nevertheless, results are surprising given clear evidence that all three factors are coexpressed in cultured SMCs and that each is a very potent SRF coactivator. Indeed, the results of the present studies are the first to provide direct evidence that there are interdependencies between myocardin/MKL factors, although this was suggested by recent studies by Li et al. (10) and Oh et al. (17) showing that MKL2 is required for differentiation of neural crest-derived SMCs within branchial arch arteries, which also express myocardin and MKL1. That is, myocardin and MKL1 were apparently unable to compensate for loss of MKL2. In contrast, recent joint studies between Olson’s laboratory and ours (18) showed that myocardin-null ES cells were capable of differentiating into vascular SMCs in the context of chimeric knockout mice, indicating that cell-autonomous myocardin is not an absolute requirement for SMC lineage. The most likely explanation for this observation is that MKL factors compensated for loss of myocardin within chimeric knockout mice, although there is no proof that this is the case. Results in the present and previous studies suggest that distinct intracellular localization of myocardin and MKL factors may contribute to the differential functional roles of these factors (7, 16).
addition, there is evidence that myocardin/MKL factors can form homodimers or heterodimers with each other (7, 22), although it is unclear whether homodimeric or heterodimeric myocardin-MKL complexes are predominant and whether this varies in distinct SMC subtypes or varies in response to different environmental cues. Taken together, results indicate that extensive further studies are needed to precisely define the similarities and differences in functional roles of individual members of this very intriguing gene family.

Previous studies showed that PDGF-BB-induced suppression of SMC marker genes was mediated in part through displacement of myocardin from SRF triggered by phosphorylated Elk-1, downregulation of myocardin expression, and reduced SRF binding to CArG elements of SMC marker gene promoters (13, 26). The present studies extend these results by testing if multiple repressor mechanisms contribute to PDGF-BB-induced suppression of SMC marker genes and if these vary at multiple time points after PDGF-BB exposure. Our observations can be summarized as follows (Fig. 8). First, we found that the mechanism whereby phosphorylated Elk-1 displaced myocardin from SRF was applicable to MKL factors, but this mechanism was only present at very early time points. Second, we showed that, although expression of myocardin was decreased, expression of MKL factors was unaltered by PDGF-BB treatment. Third, we showed that the association of MKL factors with CArG-containing SMC marker gene promoters within intact chromatin was reduced by PDGF-BB treatment not only at early time points, but also at later time points. Fourth, we provided evidence that HDAC-induced hypoacetylation of histone H4 contributed to PDGF-BB-induced repression of SMC marker genes. The results that myocardin expression, but not expression of MKL factors, was downregulated by PDGF-BB are quite interesting and may provide a mechanism for target gene selectivity. That is, previous studies have shown that the immediate early genes such as c-fos and c-jun are target genes of MKL factors, but are not regulated by myocardin (2). As such, the differential effects of PDGF-BB on myocardin vs. MKL factors may be necessary, since MKL factors might be required for the induction of these immediate early genes in response to PDGF-BB.

Results of the present studies also showed that HDAC2, HDAC4, and HDAC5 were involved in the repression of SMC marker genes by PDGF-BB. Indeed, ChIP assays revealed the recruitment of these HDACs to the CArG-containing SMC marker gene promoters by treatment with PDGF-BB in cultured SMCs. Moreover, we showed that siRNA-induced

Fig. 7. siRNA-induced suppression of HDACs attenuated the repression of SMC marker genes by PDGF-BB. A: efficiency and specificity of siRNAs were tested. Rat aortic SMCs were transfected with siRNA duplex specific for HDAC2, HDAC4, and HDAC5, and endogenous expression of HDACs was examined by Western blotting. B: rat aortic SMCs were transfected with siRNA duplex specific for EGFP, HDAC2, HDAC4 or HDAC5, and treated with PDGF-BB or vehicle for 24 h. Expression of SM α-actin was determined by real-time RT-PCR. An arbitrary value of 100 was assigned to the cells transfected with EGFP siRNA and treated with vehicle. Values represent the means ± SE.

Fig. 8. Simplified model illustrating mechanisms whereby PDGF-BB represses CArG-dependent SMC marker genes. A: MKL1 and MKL2, as well as myocardin, contribute to the transcription of CArG-dependent SMC marker genes in cultured SMCs. B: PDGF-BB induces phosphorylation of Elk-1, and phosphorylated Elk-1 displaces myocardin/MKL factors from SRF at early time points. PDGF-BB also recruits HDAC2, HDAC4, and HDAC5 to the CArG-containing region of the SMC promoters, thereby reducing histone H4 acetylation levels. C: at late time points after PDGF-BB treatment, the association of HDACs with the CArG-containing SMC promoters and the resultant hypoacetylation of histone H4 are sustained. As a result, SRF and myocardin/MKL factors are dissociated from the CArG-containing SMC promoters, and transcription of SMC marker genes is continuously repressed.
knockdown of these HDACs attenuated PDGF-BB-mediated repression of SM α-actin gene expression. Although the present studies were the first to identify which HDACs were employed to repress SMC marker genes by PDGF-BB, results of several previous studies support our results. First, Qiu and Li (19) showed that treatment of 10T1/2 embryonic fibroblasts with trichostatin A, an HDAC inhibitor, increased the activity of the SM22α gene, whereas it did not increase the activity of CArg-mutation construct of the SM22α gene. Their results are consistent with ours in that HDACs modulated the activity of SMC marker genes via the CArg-SRF complex. Second, it has been shown that HDAC4 interacted with SRF and this interaction was enhanced by angiotensin II or elevation of intracellular calcium concentrations in cardiomyocytes (5). Although studies showed that the augmented interaction by the activation of calcium signaling was accompanied by the translocation of HDAC4 from the nucleus to the cytoplasm, intracellular redistribution of HDAC4 was not seen in PDGF-BB-treated SMCs. Third, Zhou et al. (32) showed evidence that HDAC4 was the downstream target of the ERK1/2 pathway, which is consistent with our results showing that PDGF-BB-induced activation of ERK1/2 was associated with the recruitment of HDAC4 to the CArg-containing SMC marker gene promoters. Fourth, HDAC4 and HDAC5 have been shown to interact with myocardin (1). However, the importance of these interactions in PDGF-BB-mediated repression of SMC marker genes remains unclear, because we showed the decrease in myocardin expression after the treatment with PDGF-BB. Finally, HDAC2 has been shown to directly interact with Elk-1 (27). As such, multiple studies support our results showing the contribution of HDACs to SMC phenotypic switching, including PDGF-BB-induced suppression of SMC marker genes. Currently, multiple HDAC inhibitors are in clinical trials for treatment of cancer (15). Vascular lesion-specific delivery of HDAC inhibitors might be a new therapeutic strategy for vascular diseases such as atherosclerosis.

In summary, results of the present studies provide novel evidence showing that both MKL1 and MKL2 contribute to the expression of single and multiple CArg-containing SMC marker genes in cultured SMCs using a combination of gain- and loss-of-function experiments. Moreover, we showed that PDGF-BB-induced suppression of CArg-dependent SMC marker genes is in part mediated by the inhibition of MKL factors, which is caused by the competition between MKL factors and phosphorylated Elk-1 for SRF binding at early time points, followed by sustained HDAC-mediated hypoacetylation of histone H4, which restricts the accessibility of MKL factors (and myocardin) to the CArg-containing SMC marker gene promoters within intact chromatin. Further studies using combinatorial and conditional gene knockout approaches are needed to define the precise roles of MKL factors and myocardin in vivo during SMC phenotypic switching in response to vascular injury and disease, as well as to clearly elucidate the differential functions of these factors in different SMC subtypes.

ACKNOWLEDGMENTS

We thank Rupande Tripathi for technical assistance.

GRANTS

This study was supported by National Heart, Lung, and Blood Institute Grants R01-HL-19242, R01-HL-38854, and R37-HL-57353 to G. K. Owens and by American Heart Association National Scientist Development Grant 063523N to T. Yoshida.

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