cAMP Response Element-binding Protein Content Is a Molecular Determinant of Smooth Muscle Cell Proliferation and Migration*

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We hypothesized that cAMP response element-binding protein (CREB) could function as a molecular determinant of smooth muscle cell fate. In arterial sections from the systemic and pulmonary circulation, CREB content was high in proliferation-resistant medial subpopulations of smooth muscle cells and low in proliferation-prone regions. In vessels from neonatal calves exposed to chronic hypoxia, CREB content was depleted and smooth muscle cell (SMC) proliferation was accelerated. Induction of quiescence by serum deprivation in culture led to increased CREB content. Highly proliferative SMC in culture were observed to have low CREB content. Exposure to proliferative stimuli such as hypoxia or platelet-derived growth factor decreased SMC CREB content. Assessment of CREB gene transcription by nuclear run-on analysis and transcription from a CREB promoter-luciferase construct indicate that CREB levels in SMC are in part controlled at the level of transcription. Overexpression of wild type or constitutively active CREB in primary cultures of SMC arrested cell cycle progression. Additionally, expression of constitutively active CREB decreased both proliferation and chemokinesis. Consistent with these functional properties, active CREB decreased the expression of multiple cell cycle regulatory genes, as well as genes encoding growth factors, growth factor receptors, and cytokines. Our data suggest a unique mode of cellular phenotype determination at the level of the nuclear content of CREB.

The vessel wall, once seen as simply a mechanical conduit for blood flow, is a complex organ whose dysfunction is responsible for the leading cause of death in the United States, cardiovascular disease. The lumen of blood vessels is lined with endothelial cells, which communicate with the underlying medial cell layer. For many years, the arterial media was considered to be made-up of a homogeneous population of smooth muscle cells (SMC) arising from a common lineage. Thus the diverse activities of contraction, proliferation, migration, and extracellular matrix production were thought to reflect SMC response to either normal or pathological stimuli. Vascular remodeling is the compensatory response of the vasculature to stress or injury. Under pathological circumstances (hypoxia, mechanical injury, hyperlipidemia, and oxidative stress), SMC in the intimal and medial compartments of the arterial wall become proliferative, migratory, and produce excess matrix proteins. This switch in SMC phenotype is termed phenotypic modulation and is considered to play a key pathogenic role in atherosclerosis and pulmonary hypertension. While the stimuli for SMC activation are well known, the molecular events, which permit activation of SMC, remain poorly understood.

Cyclic nucleotides (cAMP and cGMP) promote SMC quiescence in vitro and in vivo. β-Adrenergic stimulation of cAMP signaling is important for SMC quiescence and contractile function under normal conditions. It is believed that cAMP acts as a gate to prevent SMC mitogenic response to growth factors in the intact vessel wall (1–3). Elevation of cyclic AMP and cyclic GMP leads to inhibition of MAP kinase, p70 S6 kinase, and cdk4, which effectively blocks entry into G1 from the G0 phase of the cell cycle (4–10). One important nuclear target of cyclic nucleotide signaling is the transcription factor CREB. cAMP response element-binding protein. CREB is a widely expressed DNA-binding protein and it is a downstream target of cAMP (and in some reports cGMP). Classically, the CREB functional state is regulated by phosphorylation on serine 133, which permits binding to the co-activator protein CREB-binding protein (CBP) and leads to gene regulation (11). Numerous CREB kinases and phosphatases have been identified which determine the CREB phosphorylation state (12). Our laboratory has demonstrated that CREB is a key regulator of adipocytic differentiation and neuronal survival. Thus CREB may be a target of cyclic nucleotide signaling that is important for determination of SMC phenotype.

We hypothesized that CREB could function as a molecular determinant of smooth muscle cell fate. In this article we dem-

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¶ The abbreviations used are: SMC, smooth muscle cells; CREB, cAMP response element-binding protein; SM-1, smooth muscle myosin; SM-2, smooth muscle actin; 1 The abbreviations used are: SMC, smooth muscle cells; CREB, cAMP response element-binding protein; SM-1, smooth muscle myosin; CBP, CREB-binding protein, PDGF, platelet-derived growth factor; MPA, main pulmonary arteries; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; MEM, minimal essential medium; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; CMV, cytomegalovirus; m.o.i. multiplicity of infection; PKA, protein kinase A; FACS, fluorescence-activated cell sorter; β-gal, β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
onstrate a strong link between CREB content and proliferative capacity in vitro and in vivo. Overexpression of wild-type or constitutively active CREB blocked mitogen- and serum-stimulated proliferation and chemokinesis. Active CREB also decreased the expression of a number of cell cycle and mitogenic genes, providing a potential mechanism for the impact of CREB on SMC behavior.

**MATERIALS AND METHODS**

**Bovine Animal Models**—Pulmonary hypertension was induced in newborn male Holstein calves by placing animals at 1 day of age in a hyperbaric chamber with barometric pressure of 445 mm Hg (which is equivalent to an elevation of 4300 m). This exposure has previously been reported to produce severe neonatal pulmonary hypertension with characteristic hemodynamic and structural changes (13–17). Age-matched controls were kept indoors at ambient altitude (1500 m, 640 mm Hg). Animals were sacrificed at 14 days of age (n = 3 of each age, hypoxia and control) and tissue samples were isolated immediately. All animal studies were performed after review and approval of the University of Colorado and Colorado State University Animal Care and Use Committees.

Freshly isolated arterial rings (~1.0 cm long) from the main and the right or left main pulmonary arteries (MPA) and portions of lung tissue were taken from 14-day-old calves with hypoxia-induced pulmonary hypertension or from age-matched control animals (n = 3 in each group). In addition, specimens of aortic arch were obtained from each animal at the same level and distance from the heart as for MPA. For immunofluorescence analysis, arterial rings from the neonatal calves were cut in half and both sections of the artery were then processed. Arterial segments were embedded in O.C.T. Compound (Miles Inc., Kankakee, IL), frozen slowly in cold hexane to prevent tissue fracturing, and stored at −70°C until use. Fixed tissues were dehydrated, embedded in paraffin, and sectioned at 4 μm thickness. Frozen tissue samples were cut at 5 μm, air-dried, fixed in absolute acetone for 5 min at room temperature, and processed for immunofluorescence staining as further described.

**Immunohistochemistry in Bovine Aorta and Pulmonary Arteries**—For indirect single-label horseradish peroxidase staining, acetone-fixed lung tissue sections were incubated in blocking solution (phosphate-buffered saline (PBS) containing 5% calf serum) for 5–10 min and then incubated with polyclonal antibodies to CREB for 1 h at room temperature. Immune complexes were visualized using VECTASTAIN ABC Peroxidase kit and DAB reagents according to the manufacturer’s instructions. Controls were performed in which primary antibodies were replaced by nonimmune calf serum. Photomicrographs were made with a Nikon Eclipse E600 microscope equipped with a 35-μm camera on Kodak Royal Gold 200 film. For double-label immunofluorescence staining, the antibodies were applied to MPA sections in the following order: staining with Ki-67 antibody was accomplished first using a Texas Red-conjugated anti-mouse secondary antibody, then staining with polyclonal CREB antibodies was performed using fluorescein isothiocyanate-conjugated anti-rabbit IgG as secondary antibodies. Digital images were made on a Nikon AXIophot microscope equipped with a chilled CCD video camera. Digital images were captured to a Macintosh G3 computer, and contrast enhanced in Adobe Photoshop to cover the full 8-bit range of the image. The monochrome images were pseudocolored by loading into RGB color channels.

**Immunofluorescence Staining of Aortic Sections** was performed using monoclonal SMMS-1 antibodies recognizing both SM-1 and SM-2 smooth muscle myosin heavy chain isoforms, but not nonmuscle isoforms, as previously reported (18). Multiple phenotypically distinct SMC populations exist in the adult and developing bovine pulmonary arterial media in vivo (18, 19). Affinity purified rabbit polyclonal anti-CREB antibodies were employed at 1:100 dilution (New England Biolabs, Beverly, MA). Horse anti-mouse IgG conjugated to Alexa-488 green fluorescence dye (Molecular Probes, Eugene, OR), goat anti-rabbit IgG conjugated to biotin (Dako Corp., Carpinteria, CA), and streptavidin conjugate with Alexa-594 red fluorescence dye were applied at dilutions recommended by the suppliers. Double-label immunofluorescence of SM-myosin heavy chains and CREB was performed as follows. Acetone-fixed tissue sections were preincubated in full calf serum as a blocking solution for 20 min at room temperature, and then incubated with a mixture of monoclonal anti-SM-myosin SMMS-1 and polyclonal anti-CREB antibodies for 1 h at room temperature. After 3 washes in PBS, cells were incubated with a mixture of biotinylated anti-rabbit IgG and Alexa-488-conjugated anti-mouse IgG for 1 h at room temperature. The staining was accomplished by incubation with Streptavidin-Alexa-594. Finally, fluorescently labeled tissue sections were embedded with VectaShield mounting medium with DAPI (blue fluorescence dye) (Vector Laboratories, Inc., Burlingame, CA). Controls were performed in which primary and secondary antibodies were omitted and mechanically stripped of adventitia. To ensure complete removal of the adventitia, a thin portion of the outermost media was also discarded. Endothelium was removed by gentle scraping of the luminal surface of the vessel with a scalpel blade. The arterial media was then separated into three previously described layers: 1) a very thin subendothelial layer; 2) an intermediate-thickness middle-media layer; and 3) a thick outer layer. We found that these three medial layers could be mechanically separated from one another because of distinct mechanical properties of each layer, apparently due to specific patterns of cell arrangement and elastic lamellar distribution. After separation of the media into three layers, cells were grown from the subendothelial and middle media layer by explant (17). Tissue explants were maintained in complete Dulbecco’s modified Eagle’s medium supplemented with 200 units/ml penicillin, 0.2 mg/ml streptomycin, 10% fetal calf serum.

Since our goal was to obtain pure subpopulations of smooth cells we selectively isolated individual cell colonies with a distinct, although uniform, morphological appearance from primary culture using cloning cylinders. We then examined expression of smooth muscle-specific markers in each isolated cell subpopulation. Only cell subpopulations with uniform morphological appearance and uniform patterns of expression of smooth muscle markers were selected for the proposed experiments. To isolate individual cell colonies growing from tissue explants in primary culture, cloning cylinders (5-10 mm in diameter, greased on the bottom) were placed over each cell colony of interest. Cells within the ring were trypsinized and transferred to a 24-well multwell plate for expansion. All studies will be carried out using cells at passages 1–8. Cell cultures will be tested for mycoplasma contamination using a Gen-Probe Mycoplasma T. C. Rapid Detection System (Gen-Probe Inc.).

For experiments, SMCs were seeded at ~500,000 cells/10-cm plate. Control cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, and incubated under 20% O2 and 5% CO2 (this gas mixture was used for 8.5 hours and trace atmospheric gasses). Cells subjected to hypoxia were grown in the same medium, but placed in sealed, pleixglass chambers flushed with a mixture of gasses containing 3% O2 and 5% CO2. For low serum conditions, cells were transferred to Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum.

Rat aortic SMCs were isolated from aortic tissue harvested from eight adult Sprague-Dawley rats. Aortic tissue was minced and cells released by collagenase (7000 units/ml in MEM Eagle) and perfused to create a suspension of cells. Cells subjected to hypoxia were grown in the same medium, but placed in sealed, pleixglass chambers flushed with a mixture of gasses containing 3% O2 and 5% CO2. After 8 hours, cells were harvested and grown in cell culture media containing 10% fetal calf serum. DNA content was determined by FACS analysis and expressed as a percentage of the DNA content in untreated control cells. DNA content was determined at each time point in triplicate, and the results represent the average of triplicate determinations.

**Western Blot Analysis of Extracts Prepared from Cultured SMC**—Following experimental treatment, SMC were harvested by scraping in Laemmli sample buffer, and extracts are sonicated and boiled. Protein concentrations of samples were assessed by Bradford protein assay, and 40 μg of cellular protein fractionated on 12% SDS-polyacrylamide gels. Proteins are electrophoretically transferred to nylon membranes, and the equivalent of protein loading was assessed by staining of membrane-bound proteins by Ponceau stain, and subsequently probed with protein-specific primary antisera. Immunologically identified proteins are recognized using alkaline phosphatase-conjugated, species-specific IgG and CDP-Star Enhanced Chemiluminescence (New England Biolabs, Beverly, MA). Autoradiographic results are quantified densitometrically using a Fluor-S MultiImager and Quantity One Software (Bio-Rad).

**Nuclear Run-on Analysis**—Bovine pulmonary and aortic SMCs were propagated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. For assays, cells were grown in normal medium or in medium containing only 0.1% fetal calf serum for 48 h prior to harvesting. Approximately 8 × 106 cells were used for each data point. Cell
harvest, nuclei recovered, and nuclear run-ons were performed as previously described (20). The full-length open reading frame for CREB-327, bound to nitrocellulose, was used to recover 32P-labeled CREB RNA from the reactions.

**Transfections for CREB Promoter-Reporter Construct**—Bovine pulmonary artery SMCs were transiently transfected using Superfect Transfection reagent (Qiagen, Germany) according to the manufacturer’s specifications. SMCs were transfected with a plasmid containing the full-length (1264 to +51) CREB gene promoter linked to firefly luciferase gene (the generous gift of William W. Walker, University of Pittsburgh, Pittsburgh, PA) or the control plasmid pCIS-CK (Stratagene; identical to the CREB promoter construct but lacking CREB promoter elements). Transfections were determined by co-transfecting the cells along with a plasmid containing the enhancease herpes simplex virus-thymidine kinase gene promoter linked to Renilla luciferase, whose activity is measured subsequent to the activity of the firefly enzyme using the Dual Luciferase Assay System by Promega (Madison, WI) on a Turner Designs TD 2020 luminoimeter.

**Transfection of All Other Plasmids**—Early passage cultures (P1-P5) of SMCs were transfected in 6-well culture dishes at a density of 1.4 x 10^5 cells/cm^2 and maintained in medium containing 10% serum for 18 h. Transfection is performed using LipofectAMINE Plus Transfection Reagent (Life Technologies, Inc., Grand Island, NY) as described by the manufacturer. In addition to specific chimeric promoter-luciferase plasmid constructs, SMCs are co-transfected with a constitutively expressed β-galactosidase reporter plasmid (pCMVL-β-gal). The LipofectAMINE Plus transfection plus serum containing medium is left on cells for 3 h. The cells are allowed to recover in fresh medium with 10% serum overnight. SMC cells are subsequently serum-starved in 1 x MEM (minimal essential medium containing 1 x nonessential amino acids and 0.4 mM glutamine) for 24 h. Agonist treatment is performed in 1 x MEM for durations of 4–24 h, and cells are subsequently extracted in 1 x Reporter Lysis Buffer (Promega Corp., Madison, WI) for analysis of reporter gene expression. Luciferase reporter activity is corrected for differences in transfection efficiency, cell number, and extract recovery, using β-galactosidase activity determined in the same cellular extract.

**Stable Transfection of SMC with Muristerone-inducible CREB Expression Plasmids**—Subendothelial bovine aortic SMCs were stably transfected with muristerone-inducible WT CREB and grown to confluency. This construct has been described in previously published work (21, 22). SMCs were subsequently exposed to serum in 6-well culture dishes at a density of 0.1 or 10% with and without exposure to muristerone to induce CREB expression. Cells were fixed in ice-cold ethanol and stained with propidium iodide. Cell cycle was assessed by fluorescence flow cytometry. Results are expressed as cells in stages S + G2,M relative to cells in stages G0,G1.

**Extraction and Purification of SMC RNA and Northern Blot Analysis**—were grown to confluence and serum deprived 5 or 7 days in MEM containing 0.1% FBS. RNA was isolated using TRIzol reagent (Life Technologies, Inc.). 10 μg of total RNA was separated on agarose gels and transferred on Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary transfer in 20 x SSC. The blot was hybridized with full size CREB probe labeled with Prime-It RT Labeling Kit (Stratagene, La Jolla, CA) at 42 °C overnight. Hybridization solution contained 1 x hybridization solution (Sigma) and 50% formamide. After hybridization, the blot was washed twice with low stringency wash solution (2 x SSC, 0.1% SDS) and twice with high stringency solution (0.2 x SSC, 0.1% SDS) at 42 °C (15 min each). Autoradiography was performed with a 20 h film exposure.

**Construction and Preparation of Recombinant, Replication-deficient Adenoviruses Expressing CRE-binding Proteins**—To generate recombinant adenoviruses, wild type or mutated CREB DNA was microinjected into p95ACCMVpLpASR. The adenovirus construct downstream of a CMV promoter (p95ACCMVpLpASR). The complete VP16-CREB plasmid coding sequence was confirmed by nucleotide sequence of the expression of VP16-CREB protein was confirmed by Western blot analysis using the anti-CREB antibody (Sigma). Function was confirmed by infection of HEK 293 cells co-transfected with pCMVβ-luc reporter plasmid (Stratagene). A similar strategy was used to generate wild type CREB and dominant-negative KCREB chimeric plasmid constructs. For construction of the adenovirus transfection chimeric plasmid for production of the constitutively active CREB-DI自从luc (DCREB) adenovirus, the DCREB DNA was excised from the plasmid pCMVLpASR, which was subsequently subcloned as a HIIIbXb fragment into the adenovirus transfer vector (p95ACCMVpLpASR).

Chimeric CREB-adenovirus transfer plasmids were co-transfected with right-arm viral DNA into HEK 293 cells using calcium phosphate precipitation. Full cytopathic effect was observed 7–14 days after transfection. In this stage cells were disrupted to collect virus by cycles of freeze-thawing. HEK 293 cells were infected with serially virus dilutions followed by an overlay with 1.5% agarose. Single plaques were observed 4–10 days after infection. Plaques of each recombinant virus were collected (6–12 for each construct), amplified, and the expression of recombinant proteins confirmed by Western blot analysis. A second construct was performed and recombinant virus by cycles of freeze-thawing and were infected with serially virus dilution by centrifugation in CaCl2 gradient.

All constructs were created using the same adenoviral DNA backbone. Each virus also has a FLAG tag for assessment of expression in infected cells. Experiments were performed to determine the number of cells that were actively expressing recombinant proteins following adenoviral infection. SMCs were plated 7 x 10^5 cells per well on sterile coverslips in 24-well plates. The next day cells were infected with recombinant adenovirus (1 x 10^6 m.o.i. equivalent to 1 x 10^6 m.o.i. per 400,000 cells), expressing different CREB mutants or β-galactosidase as a control. Forty-eight hours after infection, cells were fixed in 4% paraformaldehyde for 30 min at room temperature and incubated with antibodies that recognize FLAG epitope (Sigma, 1:400 dilution). Cells were washed and subsequently incubated with biotinylated anti-mouse IgG, 1:100 dilution (Jackson ImmunoResearch Laboratories) for FLAG epitope detection. Results indicate that 42–70% of cells in these cultures expressed visually detectable FLAG peptide in cells infected with the same m.o.i. of different CREB-FLAG adenovirus constructs (data not shown). When cells were infected with an adenoviral construct expressing green fluorescent protein (GFP), more than 90% of cells were infected. The observation that infection and expression of recombinant proteins at a rate less than 100% of cells indicates that the impact of these proteins on SMC behavior may actually be understated by these experiments.

**CREB Promoter Reporter Transactivation**—SMCs were transiently transfected with a CREBlue reporter construct (Stratagene) and a control plasmid containing a constitutively expressed β-galactosidase gene (under control of the CMV promoter) using LipofectAMINE Plus Reagents (Life Technologies, Inc.). After 12 h recovery in 10% serum, cells were placed in serum-reduced medium (MEM + 0.1% fetal bovine serum) and were infected with 300 μl of crude adVP16CREB. After 24 h of virus exposure, medium was changed to remove virus and incubation continued for 24 h. Cell extracts were analyzed for luciferase and β-galactosidase activities.

**Estimation of Rates of DNA Synthesis Using Incorporation of [3H]Thymidine into Acid-insoluble DNA**—SMCs were cultured as described above, incubated in serum-reduced medium for 24 h, then infected as indicated with recombinant adenoviruses encoding either VP16CREB or β-galactosidase. During the final 24 h of incubation, half of the treatment groups were exposed to growth factor-BB (PDGF) at a final concentration of 0.1 nm. During the final 3 h of the 24 h PDGF treatment, 2 μCi/ml [3H]thymidine were added to the cultures. Cells were assayed for thymidine incorporation by standard methods. Results are expressed as dpm/μg of protein.

**SMC Chemokinesis**—Chemokinetic behavior of SMC cells, defined as non-directed migration of SMC in response to a chemical stimulus, was assessed using a 24-well micro-Boroden chamber apparatus (Transwell Apparatus, Costar, Corning, NY). Chemotactic compounds, such as PDGF, are diluted in serum-free medium and added to the lower chamber of the apparatus. PolvinyiJJpyrrolidine-free filter with 8-mm pores were coated with a Type I collagen. Tryptinized cells were resuspended in serum-free medium containing either chemotactic compounds or vehicle, and plated at a density of 30,000 cells per well in a volume of 100 μl. After 15 min to allow for cell attachment, PDGF was added to the bottom chamber of half the wells at a final concentration of 0.1 nm. Cells were allowed to migrate for 3 h, and were then fixed and stained using Dif Quik solutions (Dade Behringer, Newark, DE). Once dry, the cells on the peting surface were swabbed away and the remaining migrating cells on the membrane underside were counted. Data is expressed as a fold-stimulation of chemotaxis by PDGF with data sets collected as cells migrating per microscopic field.
Alto, CA). These probes were used for hybridization with separate Rat cDNA Array membranes using protocols and reagents provided by the manufacturer (CLONTECH). Arrays were subjected to autoradiography at $80^\circ$C using Kodak Lightning Plus Screens. Scanned arrays were analyzed using Atlas Image Software, comparing relative intensities of specific cDNA "spots" which were corrected for differences in the relative intensities of housekeeping genes between membranes prior to analysis. Three separate array analyses were performed with probes generated from RNA resulting from 2 different experiments. Results for cDNAs depicted below are the mean of these three separate determinations. Data is presented as mRNA content in adVP16CREB-treated cells relative to mRNA content in adBetaGal-treated cells.

RESULTS

CREB Distribution in SMC of the Walls of the Pulmonary Artery and Aorta Correlates Inversely with Proliferative Capacity—Under basal conditions, little to no SMC proliferation is occurring in the vessel, yet there are cell medial populations with strikingly different proliferative potential. Frid et al. (19, 23, 24) have defined at least four subpopulations of cells in the inner, middle, and outer compartments of the arterial media. Cells in or derived from these different regions demonstrate differences in morphological appearance, expression of muscle specific proteins, and growth capabilities in vitro and in vivo. Cells in the intimal layer which are just beneath the endothelium express little of the smooth muscle cell markers such as $\alpha$ smooth muscle actin and smooth muscle myosin (Fig. 1A) and have been reported by our group and others to have extremely high growth potential (19). These are the cells believed important for neointimal proliferation when the endothelium is in-
jured. Cells in the middle media are resistant to mitogenic stimulation in culture compared with the subendothelial population of cells. In light of the role of cyclic nucleotides as critical modulators of SMC phenotype and CREB as a modulator of overall cellular phenotype in multiple tissue types, we examined CREB distribution in arterial tissue. Immunohistochemical staining of bovine aorta revealed homogeneous cellularity (DAPI) and the expected high content of smooth muscle myosins (SM-1) in the middle medial cell layer with very little subendothelial staining for this SMC differentiation marker (Fig. 1A). CREB content was high in the SM-1 staining middle medial layer (known to have low proliferative capacity) and it was negligible in the proliferation prone subendothelial cells. We expected that the level of CREB phosphorylation would be different in the various SMC compartments, as phosphorylation is the classic mode of regulation of CREB transcriptional activity. Instead we observed dramatically higher levels of CREB protein in the well differentiated, proliferation resistant L2 SMC regions and significantly less CREB protein in proliferation prone regions (Fig. 1A). Of note, the amount of CREB phosphorylation correlates directly with CREB protein content.

Under normal circumstances very little proliferation is occurring in the vasculature. It is possible to stimulate SMC proliferation in vivo by exposure to hypoxia. Under hypoxic conditions in neonatal cows, brisk proliferation is observed in the pulmonary vasculature. We therefore examined vascular CREB content in this model system. Bovine pulmonary arteries were examined from control animals and animals dwelling in a hypoxic chamber for 14 days (see “Materials and Methods” for details). Under control conditions, pulmonary vessels had strong staining for CREB and low levels of staining for the proliferation marker Ki-67 (Fig. 1C). Exposure to a chronic hypoxic environment led to increased cellularity (DAPI) and increased proliferation (Ki-67). This increased cellularity was associated with decreased CREB staining (Fig. 1, B and C). Decreased CREB and phosphorylated CREB content in the pulmonary vascular stroma from animals exposed to hypoxia were confirmed by Western analysis (Fig. 1D). Thus an inverse correlation of CREB content with both proliferative capacity and proliferation was observed in vivo. These histological sections demonstrate an inverse correlation of CREB content with proliferative capacity in both the pulmonary and arterial circulation.

**CREB Content and Proliferation**—Primary smooth muscle cells in culture have been used to examine proliferation and migration (two markers of activated SMC function believed important in atherosclerosis and pulmonary hypertension). It is well established that treatment of primary SMC with serum, mitogen, or toxins will increase proliferation and that serum deprivation (0.1% fetal calf serum) will lead to quiescence. We examined CREB protein as well as serine 133 phosphorylated CREB in SMC exposed to hypoxia (mitogenic stimulus) and serum deprivation (Fig. 2). Serum deprivation increased CREB and phosphorylated CREB protein levels (Fig. 2A). In contrast, we observed a decrease in CREB and phosphorylated CREB by Western analysis in propagated hypoxic conditions. To confirm the serum deprivation and mitogen stimulation were affecting proliferation as expected in our system, cells were stained with propidium iodide and cell cycle progression was assessed by FACSCAN. As expected, serum deprivation decreased G2/M phase from 25.6 to 6.6%. Frid et al. (19, 23, 24) have previously described a population of medial “SMC” with high proliferative capacity that do not respond to serum deprivation by decreasing proliferation. These cells (Fig. 2A, lanes 3 and 4) have no detectable CREB and fail to increase CREB content or significantly decrease proliferation with serum deprivation. These data indicate that CREB content and activity correlate inversely with proliferation in vitro.

**CREB Content Is Transcriptionally Regulated**—The experiments described thus far demonstrate that CREB content correlates inversely with proliferative capacity and suggest that CREB content is regulated. To determine whether this regulation is a transcriptional event, we assessed the content of CREB mRNA as well as the activity of the CREB gene promoter. In SMC isolated from either the systemic or pulmonary circulation nuclear run-on and CREB promoter-reporter activity were increased by serum deprivation (Fig. 3A). In parallel experiments, low serum conditions had no effect on luciferase production from the control pGL2-Basic plasmid (similar to pCREB-luc but lacking CREB promoter elements) indicating that the effects of low serum are mediated via the CREB gene promoter (Fig. 3A). These results were confirmed by Northern analysis and quantitative RT-PCR (Fig. 3, B and C). These data indicate that the increase in CREB protein content observed with serum deprivation is regulated at least in part at the transcriptional level.

**CREB Activity Modulates Proliferation**—The observation that CREB content was high in SMC with a proliferation resistant phenotype suggested that CREB could play a role in determining SMC phenotype, or that it could be a previously undescribed marker of SMC differentiation. To test this hypothesis that CREB activity directly regulates SMC activation (proliferation and chemokinesis), we modulated cellular content of CREB. To assess the effect of wild type CREB and VP16-CREB on SMC proliferation, the two proteins were introduced into SMCs by two methods. The first method employed the edysone-inducible expression system, in which SMCs were stably transfected with plasmids from which CREB or VP16-CREB...
expression was induced with the insect hormone homolog, muristerone. As a control, cells inducibly expressing a LacZ protein were also generated. To validate this system, we first treated stable transfectants for CREB and VP16-CREB with increasing concentrations of muristerone and measured the expression of these proteins by Western blot. As shown in Fig. 4A, no expression of VP16-CREB was observed in untreated cells, but increasing amounts of VP16-CREB were readily apparent in cells treated with increasing levels of muristerone. Similar data were recorded in CREB-expressing cells with the exception that basal levels of endogenous CREB were present in untreated cells (data not shown). To ensure that the expressed proteins were functional, the cells were transfected with a plasmid containing the CRE-containing somatostatin gene promoter linked to luciferase. Transcription (luciferase production) from this promoter was stimulated by muristerone at concentrations from 0.1 to 1.0 μM (Fig. 4A, lower panel). Higher levels of muristerone (10 μM) stimulated transcription from the somatostatin gene promoter, but the response was weaker than at 1.0 μM, perhaps due to transcriptional “squelching” from excess VP16-CREB protein. Finally, when cells carrying the inducible LacZ expression system were treated with muristerone, well over 95% of the cells stained blue with X-gal reagent, whereas untreated cells exhibited no LacZ expression (Fig. 4B). Together, these data show that the ec dysone-inducible system is capable of generating stably transfected cells in which active proteins can be expressed in virtually all the cells in the selected population.

In the initial studies using the strategies described above, we examined the effect of CREB overexpression upon serum-induced proliferation. First we made stable clones of primary SMC with inducible expression vectors encoding either wild type CREB or constitutively activity CREB (VP-16-CREB). Stably transfected cells were stimulated with either 10 or 0.1% FBS and examined via FACS analysis for the proportion of cells progressing through cell cycle. Treatment of these cells with muristerone, which induced expression of either CREB or active CREB, significantly decreased the proportion of cells progressing through cell cycle (Fig. 4C). This effect was also noted in cells treated with 0.1% FBS and proliferating at a lower level. No effect of muristerone was noted in vector-only transfected cells (data not shown). A parallel set of studies was undertaken using adenoviral constructs for constitutively active CREB. Cells were infected with either control virus encoding (β-galactosidase) or virus expressing constitutively active CREB (VP-16-CREB) and examined for proliferation. Viral infection with VP16-CREB decreased the proportion of cells in G2/M phase in the presence of both high (10%) and low (0.1%) serum. The proportion of cells in G2/M in high serum was reduced from 34 to 18% by expression of VP16-CREB when compared with cells infected with control virus. In low serum medium, the proportion of cells in G2/S was reduced from 14% in cells expressing β-galactosidase to 7.4% with expression of VP16-CREB. PDGF is a potent stimulus for SMC proliferation contained in serum. In additional studies, PDGF-mediated proliferation was examined using thymidine incorporation. Infection with active CREB significantly decreased PDGF-mediated thymidine incorporation (Fig. 4D). Thus, CREB activation is capable of decreasing both serum and mitogen-induced DNA synthesis and cell cycle progression. These data support the hypothesis that CREB is a determinant of SMC proliferation.

**PDGF-stimulated Chemokinesis with CREB Isomers**—A second important parameter of SMC behavior under pathological conditions is the stimulation of SMC migratory behavior. We have examined chemokinesis, defined as nondirected migration of SMC in response to a chemical stimulus, as an indicator of migratory behavior in SMC. PDGF is also one of the key factors in serum known to be a potent stimulus of proliferation. Adenoviral-expressed VP16-CREB had no significant impact upon basal SMC chemokinesis, but significantly reduced PDGF stimulated chemokinesis (Fig. 5). Theoretically the VP16-CREB fusion protein will activate all CREB-binding protein (CBP). Thus, the observed responses to VP16-CREB overexpression can represent either responses regulated by CREB, or actions mediated by other CRE-binding proteins. We therefore tested additional forms of constitutively active and dominant negative CREB on mitogen-stimulated chemokinesis. The recently reported constitutively active CREB mutant, CREB-DIEDML (DCREB), was cloned into adenovirus (11). DCREB binds to CBP independent of CREB serine 133 phosphorylation and activates CRE-mediated transcription. Two dominant negative recombinant adenoviruses were also generated: a phosphorylation deficient serine 133 to alanine CREB mutant, MC Revenge, and a defective DNA binding mutant, KCREB. Adenovirus mediated expression of DCREB and VP16-CREB (constitutively active) blunted PDGF stimulated SMC chemokinesis, whereas expression of MCREB and KCREB (dominant-nega-
tive) enhanced mitogen-stimulated SMC chemokinesis (Fig. 5). These more specific tools indicate that CREB is the CRE-binding protein responsible for the observed changes in SMC chemokinesis.

Impact of CREB on Gene Expression—The above studies demonstrate a clear correlation between increased CREB content and proliferative and migratory capacity. The mechanism of CREBs impact on SMC phenotype is likely to be at the level of gene expression, as the primary function of CREB is transcriptional regulation. A final series of experiments were undertaken to investigate the impact of VP16-CREB overexpression on a set of genes known to be important for cell cycle, growth, and chemokinesis. Initial screening of alterations in SMC gene expression was performed using Atlas cDNA Array analysis. Analysis to determine the impact of VP16-CREB expression on mRNA content in SMC (relative to /H9252-gal controls) indicated that numerous mRNA levels were altered by 25% or more. Twenty-eight mRNAs demonstrated changes in all

Fig. 4. CREB regulates SMC proliferation. Validation of the ecdysone-inducible expression system. Stably transfected bovine pulmonary SMCs containing either an ecdysone-inducible LacZ or VP16-CREB expression systems were prepared as described under “Experimental Procedures.” A, inducible expression of VP16-CREB protein was verified by preparing nuclear extracts from SMCs treated with the indicated levels of muristerone for 24 h. Twenty-five μg of protein from each extract was resolved on 10% polyacrylamide-SDS gels, and transferred to nitrocellulose. After the blots were blocked they were incubated with antibody to VP16. The upper panel shows a representative Western blot of induced VP16-CREB expression. In the lower panel, SMCs, stably transfected with the inducible VP16-CREB expression system, were transfected with a plasmid containing the CRE-containing somatostatin gene promoter linked to a luciferase reporter gene using Superfect reagent. As indicated, VP16-CREB expression was induced with the concentrations of muristerone indicated in A for 24 h. Luciferase expression was measured in lysates as an index of transcriptional activity, and levels are shown relative to luciferase activity in cells not treated with muristerone. Levels were corrected for transfection efficiency by co-transfecting cells with a plasmid containing the Rous sarcoma virus (RSV) long terminal repeat linked to a β-galactosidase reporter. The data shown are averaged from three separate assays. B, SMCs, stably transfected with the inducible LacZ expression system were treated overnight with 1.0 μm muristerone, rinsed with PBS, and fixed with 3.5% formaldehyde in PBS for 10 min. After an additional three rinses in PBS the monolayers were incubated in PBS containing 5 mM potassium ferrocyanide, 2 mM MgCl₂, and 1 mg/ml X-gal. The figure shows representative photomicrographs of untreated (No Add’n) and muristerone-treated cells. C, stably transfected adult, bovine pulmonary artery SMCs inducibly expressing wild type CREB (CREB), VP16-CREB, or the LacZ gene product (Ctrl) were prepared as described under “Experimental Procedures.” Cells were transferred to medium containing either 10 or 0.1% FBS as indicated for 24 h and then expression of each protein was initiated by adding muristerone to the media at a final concentration of 1 μM, and incubation was continued an additional 24 h. Cells were lifted from the plates with trypsin, fixed, stained with propidium iodide, and subjected to flow cytometric analysis as described elsewhere. D, uninfected (No Virus) adult, bovine aorta SMCs or cells infected with adenoviruses (1 × 10⁹ m.o.i. per 400,000 cells) expressing β-galactosidase or VP16-CREB were grown for 24 h in serum reduced medium. SMC were subsequently treated with PDGF for 24 h, with [³H]thymidine added to the medium for the final 3 h of the PDGF treatment. Cells were washed with cold PBS and fixed in ice-cold 5% trichloroacetic acid to extract unincorporated radionucleotide. Acid-insoluble material from SMC was dissolved in 250 μl of 1 N sodium hydroxide, neutralized with 1 N HCl, and assessed for [³H]thymidine incorporation by liquid scintillation spectroscopy. Incorporation of label was normalized with cell protein content. Results are presented as PDGF-stimulated incorporation relative to unstimulated controls (each expressed as [³H]thymidine incorporation per mg of protein); n = 4 each experimental group.
concentration of 0.1 nM. Cells were allowed to migrate for 3 h, and were
placed on Boyden chamber membranes (Transwells, CoStar). After 15 min to allow for cell attachment,
proliferation, consistent with the role of cyclic nucleotides.

Support a model wherein the role of CREB is to restrain SMC
attachment; this effect of CREB is not surprising as CREB is a
target of cAMP/PKA signaling pathways. It is well
understood that cyclic nucleotides such as cAMP and phosphodiesterase-inhibitory drugs (aminophylline
and amrinone) in rats markedly inhibited neointimal formation following balloon injury. The growth inhibitory effect
was completely reversed by inhibition of PKA. Trapidil is a
pharmacological agent that has been shown in clinical trials to
reduce post-angioplasty restenosis (7).

Fig. 5. CREB regulates migratory capacity. Cells were cultured and infected with virus as described above (1 × 10^6 m.o.i. per 400,000 cells), with the exception that all recombinant viruses other than VP16CREB were purified from high titer virus stocks. Cells were kept in virus-containing serum-reduced medium for 24 h. Virus containing serum-reduced medium was replaced with fresh serum-reduced medium and incubation continued for an additional 24 h. Cells were trypsinized and resuspended in serum-free medium at a concentration of 30,000 cells per 100 µL which was plated on Boyden chamber membranes (Transwells, CoStar). After 15 min to allow for cell attachment, PDGF was added to the bottom chamber of half the wells at a final concentration of 0.1 nM. Cells were allowed to migrate for 8 h, and were
then fixed and stained using Diff Quik solutions (Dade Behring). Once
dry, the cells on the plating surface were swabbed away and the re-
maining migrating cells on the membrane underside were counted.

The data is expressed as fold stimulation of chemokinesis by PDGF with data sets collected as cells migrating per microscopic field. Each exper-
iment was repeated 3 times, with n = 3 for each experimental variable per repetition. # indicates significant decrease relative to β-ga-
lactosidase control; * indicates significant increase relative to control (two-tailed Student’s t test, p < 0.05).

Array analysis is a screening tool and only those results with relevance to growth, cell cycle, and matrix modification are reported in Fig. 6. From the overall analysis we report three sets of gene products: 1) growth factor, cytokine, and growth factor receptor genes were down-regulated by VP16-
CREB; 2) cell cycle proteins were down-regulated by VP16-
CREB; and 3) matrix remodeling proteins were up-regulated by VP16-CREB (Fig. 6). Decreased expression of endothe-lin-1 receptor and platelet-derived growth factor receptor-α (PDGFRα) were detected. These results have been confirmed by Northern and Western analysis. This mRNA profile is consistent with the changes noted in proliferation and chemokinesis. Inhibition of transcription by an active CREB construct is not unexpected. It has been previously reported that CREB can decrease expres-
sion of cyclin D1 (25) (verified on our screen). Our screen
reveals down-regulation of a number of cyclins and CDK4
consistent with the slowing of cell cycle observed in cells over-
expressing active CREB. Increased expression of myosin heavy chain would be expected as cells become quiescent. These data support a model wherein the role of CREB is to restrain SMC proliferation, consistent with the role of cyclic nucleotides.

Discussion

Understanding the mechanism of SMC proliferation and mi-
gation is of great importance, because of the high incidence of
SMC proliferation disorders such as atherosclerosis, restenosis,
and pulmonary hypertension. Despite this, little is known of
the molecular events releasing quiescent SMC to migrate and
proliferate. In this article we demonstrate that CREB content
not only correlates with proliferative capacity in vivo and in vitro, but also that genetic manipulation of active CREB content can restrain SMC proliferation and chemokinesis. This paper shows that the differential expression of CREB in vascular medial subpopulations may underlie the differential sen-
sitivity of SMC to growth promoting stimuli. Finally, using
cDNA array analysis, we demonstrate that overexpression of active CREB results in decreased expression of a number of cell cycle and growth related gene products, providing a potential mechanism for the anti-proliferative effects of CREB. These observations indicate that CREB is a regulator of SMC activity and provides a potential therapeutic target for treatment of SMC proliferative disorders.

Perhaps this effect of CREB is not surprising as CREB is a
nuclear target of cAMP/PKA signaling pathways. It is well
known that cyclic nucleotides such as cAMP/Ca2+ inhibit vas-
cular smooth muscle cell proliferation under basal conditions.
In fact, Indolfi et al. (26) showed that the local administration of cAMP and phosphodiesterase-inhibitory drugs (aminophylline and amrinone) in rats markedly inhibited neointimal formation following balloon injury. The growth inhibitory effect was completely reversed by inhibition of PKA. Trapidil is a
pharmacological agent that has been shown in clinical trials to
reduce post-angioplasty restenosis (7). In vitro, this agent in-
increases cyclic nucleotides and stimulates protein kinase A (PKA) activity. It is postulated that the increase in PKA disrupts PDGF-mediated ERK activation and consequent mitogenesis. SMC cGMP levels are suppressed post-angioplasty. Restoration of cGMP by adenoviral transfer of eNOS dramatically reduced post-injury luminal narrowing (5, 7). Thus, the observation that CREB is the nuclear target mediating these effects is consistent with published observations regarding cAMP and PKA in SMC.

Serine 133 phosphorylation of CREB was initially attributed to activation of protein kinase A (27). Since that initial observation, the ability of numerous signaling cascades to enhance CREB phosphorylation has been reported (27–29). Numerous tyrosine kinase growth factors described by our group and others enhance CREB phosphorylation on serine 133. These growth factors include a set of factors believed important for SMC proliferation (e.g. PDGF, insulin-like growth factor-1, insulin, and epidermal growth factor). Additionally non-tyrosine kinase mitogens such as angiotensin II increase CREB phosphorylation. Neurotoxic states such as acute ethanol exposure, formalin-induced nerve injury, hyperglycemia, and hypoxia induce CREB phosphorylation acutely in neuronal tissues (30, 31). Chronic ethanol exposure decreases this acute CREB response (31). Vascular toxins such as hyperglycemia and reactive oxidant stress also initially phosphorylate CREB (30). These observations add complexity to the data set presented in this article. We observe that constitutively active CREB blocks PDGF-stimulated chemokinesis and proliferation (Figs. 4 and 5). However, Abbott et al. (32) demonstrate PDGF-BB induced CRE-dependent transcription in SMC. PDGF-BB was also observed to lead to serine 133 phosphorylation of CREB in Schwann cells (33). We observe an initial serine 133 CREB phosphorylation in response to PDGF. Yet 72-h treatment with PDGF leads to depletion of CREB content similar to that observed with hypoxia (data not shown). While there is currently no simple explanation for the difference in response to mitogen versus cAMP activation of CREB, this result is not unique to the vasculature. In the central nervous system, nerve growth factor and insulin-like growth factor-1 enhance CREB phosphorylation, a central target for both neuronal differentiation and survival (28). Neuronal toxins such as glutamate, seizure activity, and hyperosmolar stress also activate CREB (30, 31). These agents decrease expression of the CRE-dependent gene bcl-2 and decrease neuronal CREB responsiveness the neurotrophins. CRE-regulated gene transcription is a complex process involving CREB binding to the CRE, CREB phosphorylation, and interaction with the coactivator protein CBP/p300. Additional cell or signal-specific opportunities for regulation exist at the level of CRE–binding protein competition for occupancy of the CRE (AP-1, CREM (CREM regulatory element modulator), and ATFs (activating transcription factors)) and competition for CBP with other nuclear proteins such as NF-κB and steroid hormone receptors. It may also be that additional more potent mitogenic pathways are activated by PDGF. The current observations support the hypothesis that CREB activity (which is basally high in SMC and correlates with cAMP content) promotes SMC quiescence. Characterizing the differences between mitogen and cAMP activation of CREB in SMC is an ongoing area of investigation in the laboratory.

The studies presented in this article were conducted using medial arterial cells from a number of sources including bovine aorta, pulmonary artery, neonatal pulmonary artery, and rat aorta. Cells isolated from numerous vascular beds and different developmental stages had similar proliferative and migratory responses to modulation of CREB activity. This consistent response to CREB activation suggests that CREB could play a role for restraining SMC proliferation in numerous beds. It would be of interest to look at developmental regulation of vascular CREB content.

The observation that CREB can mediate SMC quiescence (considered by some to be a form of SMC differentiation) is consistent with CREB function in numerous tissues including adipocytes, neurons, and cardiac myocytes. Our group demonstrated that CREB is necessary and sufficient for adipocyte differentiation. Depletion of CREB in cardiac myocytes is observed with β-adrenergic overstimulation. This is associated with induction of a number of early developmental genes and the development of congestive heart failure. Targeted overexpression of dominant negative CREB recapitulates this effect (34). In neurons in culture CREB is necessary but not sufficient of differentiation (35, 36). Mice with a truncated form of CBP have defects in hematopoiesis and vasculoagenesis (37). Thus, a role for CREB in differentiation in numerous tissues is well established.

In a recent review by Mann and Dzau (38) it was reported that transcription factors and synthetic tools that modulate gene regulatory function hold great promise as future therapeutic interventions. The studies presented in this article are the first report of a transcription factor with histologically distinct patterns of expression in intact healthy vessels (Fig. 1) whose content confers a significant impact upon SMC cell function. In summary, CREB is an important determinant of SMC proliferation and migration. CREB content is up- or down-regulated in SMC in culture by serum deprivation and down-regulated by stress (hypoxia and PDGF). This regulation also occurs in SMC in the vessel. As such CREB can be viewed as a molecular “off switch” for SMC activation and a potential target for interventions for the treatment of SMC proliferative disorders.

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cAMP Response Element-binding Protein Content Is a Molecular Determinant of Smooth Muscle Cell Proliferation and Migration

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