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Basic Fibroblast Growth Factor Decreases Elastin Gene Transcription In Aortic Smooth Muscle Cells

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Abstract The extracellular matrix (ECM) protein elastin plays an essential role in the cardiovascular system by imparting elasticity to blood vessel wall. In this study, we examined the effect of basic fibroblast growth factor (bFGF) on the expression of elastin in aortic smooth muscle cells (SMC) to gain insight into events associated with cardiovascular diseases. The results show that bFGF treatment of SMC causes a significant decrease in elastin mRNA and secreted tropoelastin levels. Nuclear run-on analyses demonstrate that the downregulation is due to a decrease in the level of elastin gene transcription. Transient transfections of SMC with wild-type and mutated elastin gene promoter/chloramphenicol acetyl transferase (CAT) constructs show that a previously identified activator protein-1-cAMP response element (AP1/CRE) (−564 to −558-bp) within the elastin promoter mediates the bFGF-dependent downregulation of elastin gene transcription in SMC. Addition of bFGF to SMC activates the extracellular signal-regulated kinases 1/2 (ERK1/2) resulting in their translocation into the nucleus and subsequent induction of Fra-1. The addition of PD-98059, an inhibitor of ERK1/2 kinase, abrogates the bFGF-dependent decrease of elastin mRNA in SMC. The described inhibitory effect of bFGF on elastin gene expression in SMC may significantly contribute to the inefficient repair of elastin in early stages of vascular wall injury. J. Cell. Biochem. 85: 592–600, 2002.

Key words: tropoelastin; transcription; extracellular matrix; bFGF; SMC; atherosclerosis

Abbreviations used: AP1/CRE, activator protein-1-cAMP response element; bFGF, basic fibroblast growth factor; BAPN, β-aminopropionitrile; bp, base pair; BSA, bovine serum albumin; CAT, chloramphenicol acetyl transferase; CLSM, confocal laser scanning microscopy; Cy3, indocarbocyanine; DMEM, Dulbecco's Modified Eagles Media; DFP, diisopropyl fluorophosphate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; HEPES, N-2[hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; kDa, kilodaltons; MAPK, mitogen activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; NP-40, Nonidet P-40; PBS, phosphate buffered saline; pBS, pBluescript; PMSF, phenylmethylsulphonylfluoride; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SSC, sodium chloride sodium citrate; SMC, smooth muscle cells; TBS, tris-buffered saline; TBST, tris-buffered saline with 0.1% triton.

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The aortic wall is adapted to receive and transmit the pulsatile output of the left ventricle by virtue of possessing a relatively large amount of elastin in the extracellular matrix (ECM). In the aortic wall, elastin is found in fenestrated concentric cylinders with the most prominent of these rings being the internal elastic lamella that marks the boundary between the medial and intimal layer of smooth muscle cells (SMC), major producers of elastin [Kadar, 1974].

Thickening of the vessel wall during restenosis associates with increased synthesis of ECM molecules by SMC [Boyd et al., 1988; Nikkari et al., 1994; Thyberg et al., 1995]. Other pathologies, like atherosclerosis and aneurysm, are characterized by the decrease of ECM content in the vascular wall, particularly due to the fragmentation and loss of elastin by increased elastolytic activity [Kramsch et al., 1971; Partridge and Keeley, 1974; Campa et al., 1987; Ross, 1993; Ghorpade and Baxter, 1996]. However, whether the decrease of elastin might be also associated with the inability of SMC to resynthesize elastin is currently unclear.

Within the environment of the developing arterial wall lesion, SMC are exposed to a number of cytokines and growth factors released by infiltrated inflammatory cells and injured vascular SMC. Basic fibroblast growth factor (bFGF) is one of such molecules implicated in vascular wall remodeling [Lindner and Reidy, 1993]. This growth factor promotes proliferation and migration of vascular SMC [Winkles et al., 1987; Lindner and Reidy, 1991; Casscells et al., 1992; Edelman et al., 1992], decreases expression of type I, III and V/XI collagen genes [Majors and Ehrhart, 1993; Kennedy et al., 1995; Kypreos and Sonenshein, 1998], and activates collagenase expression by SMC [Pickering et al., 1997].

bFGF is released in elastase-treated pulmonary fibroblast cultures and signals to repress elastin gene expression in these cells [Brettell and McGowan, 1994; Rich et al., 1996]. In this report, we examine the effect of bFGF on elastin gene expression in cultured aortic SMC. We find that addition of bFGF decreases elastin mRNA and protein, and this downregulation is controlled at the level of elastin gene transcription via signaling through the extracellular signal regulated kinase 1/2 (ERK1/2) pathway.

MATERIALS AND METHODS

Reagents

Human recombinant bFGF (18 kDa) was obtained from Scios-Nova (Mountain View, CA). Rat tropoelastin DNA was generated by the digestion of a chimeric pBluescript (pBS) vector containing the rat tropoelastin cDNA with EcoR1 [Rich and Foster, 1989]. Fra-1 and c-Jun rabbit polyclonal antibodies and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal elastin antibody, ST1 clone, was previously described by Foster et al. (1976). PD-98059 was purchased from New England Biolabs (Beverly, MA). Indocarbocyanine (Cy3) goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (West Grove, PA). The complementary single stranded oligonucleotide to the elastin promoter sequence from the −573 to −546-bp (5′-GGCAGAACCTGTCTCTAGCCAGACCTG-3′) was synthesized by the DNA Protein Core Facility at Boston University Medical Center (Boston, MA). Duplex oligomer was prepared as previously described [Jensen et al., 1995].

Isolation of Aortic SMC and Treatment Conditions

Aortic SMC were isolated from 2–3 days old Sprague–Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) as previously described by Oakes et al. (1982). Cells were seeded at 2×10^4 cells/cm^2 in Dulbecco's Modified Eagles Media (DMEM) and 10% fetal bovine serum (FBS), maintained in this medium for 2 weeks and changed twice a week. After 2 weeks, cells were starved in serum-free media for 72 h. For transfection experiments, cells were seeded at 3×10^4 cells/cm^2 and grown overnight in DMEM/FBS, then the medium was changed and the calcium phosphate/DNA precipitate was added. Once the transfection was stopped, cells were starved in serum-free media for 24 h. Starved cells were ready for bFGF treatment and/or other treatments as indicated. For bFGF treatment 10 or 50 ng/ml or an equal amount of saline were used for the indicated periods of time. The inhibitor PD-98059 (50 μM) or an equal amount of the solvent dimethyl sulfoxide (DMSO) was added to the cells 30 min before the treatment with bFGF. The elastin crosslink inhibitor β-aminopropionitrile (BAPN) (25μg/ml) was added at the same time as bFGF.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted following the guanidinium thiocyanate-phenol-chloroform single step method [Chomczynski, 1993] using TRIzol Reagent (Gibco Lifesciences, Gaithersburg, MD). Equal amounts of RNA were fractionated in a 1% agarose, 1.8% formaldehyde gel, and capillary transferred to a nylon membrane (MSI, Westboro, MA). The membrane was incubated first in pre-hybridization solution (50% formamide, 5 × sodium chloride sodium citrate (SSC), 0.5% SDS, 5 × Denhardt's, 10 μg/ml of heat denatured salmon sperm DNA) for 2 h at 42°C, and then in hybridization solution (50% formamide, 5% SSC, 0.5% SDS, 10% Dextran sulfate) containing the heat denatured [(α-^{32}P] labeled probe. Incubation proceeded overnight at 42°C. The membrane was washed in 1 × SSC, 0.1% SDS at 55°C, and exposed to an X-ray film (X-OMAT AR, Kodak). The autoradiographic signal was quantitated using a laser densitometer (Molecular Dynamics, Sunnyvale, CA) and results were normalized to the level of 18S rRNA.

Transcription Run-On Analysis

Intact nuclei were isolated according to the method of Dean et al. (1986). The transcription assay was performed in the presence of [α-^{32}P] UTP for 20 min at 30°C essentially as described by Levine et al. (1986). Ten micrograms of pBS DNA and chimeric pBS DNA containing cDNAs for elastin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were applied in duplicate onto nitrocellulose (Schleicher and Schuell, Keene, NH) using a slot-blot apparatus. Prehybridized filters were hybridized with the resultant transcription run-on solutions at 65°C for 72 h, then membranes were washed and exposed to an X-ray film for visualization and quantitation of the signal on a laser densitometer (Molecular Dynamics).

Transient Transfection and Chloramphenicol Acetyl Transferase (CAT) Assay

Cells were transfected with 30 μg of pCAT900, pCAT900M, or pCATBasic by the calcium phosphate co-precipitation method as previously reported [Rich et al., 1996]. Transfection efficiencies were assessed by co-transfection with 5 μg of pCMV-β-galactosidase and measuring the resultant β-galactosidase activity from the cell lysates according to the method of Herbomel et al. (1984). Cell lysates containing equal amounts of β-galactosidase activity (100–300 μg of protein) were assayed for CAT reporter activity using 5 μl of [^{14}C]chloramphenicol substrate, and the acetylated and nonacetylated forms of the [^{14}C]chloramphenicol were separated by thin layer chromatography [Donoghue et al., 1988]. The resultant plate was air-dried and exposed to an X-ray film for visualization and quantitation of the signal on a laser densitometer (Molecular Dynamics).

Preparation of Nuclear and Total Cell Extracts

Nuclear extracts were prepared as previously described [Rich et al., 1996]. Aliquots were stored at −80°C in extraction buffer consisting of 20 mM HEPES, pH 7.9, 0.35 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 25% glycerol, 0.5 mM dithiothreitol (DTT), 1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin, 0.2 mM sodium vanadate, 100μM sodium fluoride, 1 μM diisopropylfluorophosphate. Total cell lysates were prepared from cells washed twice with ice-cold phosphate buffered saline (PBS) and then extracted for 10 min at 4°C with ice-cold cell lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EGTA (pH 9.0), 0.5% NP-40, 0.4 mM phenylmethylsulphonylfluoride (PMSF), and 0.2 mM sodium vanadate]. The cells were scraped and pelleted at 18,000*g* at 4°C for 10 min, and the supernatant was stored at −80°C. Total protein for each sample was determined using the BCA protein assay (Pierce Chemical, Rockford, IL).

In-Gel Kinase Assay

An in-gel myelin basic protein kinase assay was performed as described by Carreras et al. (2001) with minor modifications. Briefly, treated cells were lysed in ice-cold buffer containing 10 mM Tris-Cl (pH7.5), 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.4 mM PMSF, and 0.2 mM sodium vanadate. Forty micrograms of heat denatured lysates were electrophoresed in a 12% SDS–PAGE that had been polymerized with 0.4 mg/ml of myelin basic protein. After electrophoresis, the separating gel was washed with 20% isopropanol in 100 mM Tris pH 8.0, followed by wash in 100 mM Tris pH 8.0 containing 5 mM 2-mercaptoethanol. Then, the gel was denatured in 6 M guanidinium HCl followed by a renaturation in 0.04% Tween 40. The gel was incubated at room temperature in kinase buffer containing 20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol for 30 min followed by another incubation in kinase buffer containing 50 μM ATP and 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) (NEN, Boston MA) for 60 min at room temperature. The gel was washed with 1% sodium pyrophosphate in 5% trichloroacetic acid, stained with Coomassie Blue R-250, and dried. Autoradiography was performed for 6–24 h at −80°C with an intensifying screen.

Western Blot Analysis

Fifty micrograms of protein were fractionated on a 12% SDS–polyacrylamide gel and electrotransferred to nitrocellulose as previously described [Laemmli, 1970; Towbin et al., 1982]. Membranes were stained with Ponceau S solution (Sigma, St. Louis, MO) to assess equal protein loading and transfer and to locate protein standards. Membranes were treated as we have described previously [Jensen et al., 1995]. Primary antibodies recognizing elastin, Fra-1, and c-Jun were used at 1:1,000 dilution. Secondary antibodies were conjugated to

horseradish peroxidase diluted at 1:5,000 for elastin and 1:2,000 for Fra-1 and c-Jun. Immunodetected proteins were visualized by the chemiluminescence method according to manufacturer's instructions (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Immunocytochemistry and Confocal Analysis

Aortic SMC were cultured on glass cover slips in 12-well plates and maintained for 3 or 5 days. After the 72 h starvation period, cells were treated with bFGF (10 ng/ml) for 15 min and then processed for immunocytochemical analysis. Cells were washed once with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at 4°C. Fixed cells were washed twice with tris-buffered saline with 0.1% triton (TBST) and incubated in blocking buffer (PBS, 5% normal goat serum) for 45 min at room temperature. Incubation with anti-phospho-ERK1/2 (New England Biolab, Beverly, MA) diluted to 1:250 in tris-buffered saline (TBS), 3% bovine serum albumin (BSA) proceeded overnight at 4°C. Cells were washed in TBST (TBS, 0.1% Triton), and incubated with Cy3-conjugated anti-rabbit IgG diluted to 1:800 in TBST, 3% BSA for 1 h at room temperature. Immunostained cells were examined using a Zeiss LSM-510 confocal laser scanning microscope (CLSM). Control cells, not incubated with primary antibody, were used to establish CLSM voltage settings such that no fluorescence was visible within the control cultures and these same settings were used to analyze the primary antibody-containing cultures.

RESULTS

bFGF Decreases Elastin Protein and mRNA Levels in SMC

We first examined whether treatment of SMC with bFGF affects the level of elastin protein expression. SMC were treated with bFGF (50 ng/ml) in the presence or absence of β-aminoproprionitrile (BAPN), a specific inhibitor of lysyl oxidase, in order to suppress elastin crosslinking. Culture media were obtained and analyzed by Western blotting using antibodies to tropoelastin, the soluble form of elastin (Fig. 1A). Treatment of SMC with bFGF decreased the steady state level of tropoelastin in both BAPN treated and untreated cultures. The higher basal level of tropoelastin in BAPN-treated cultures is consistent with the ability of BAPN to suppress elastin crosslinking.

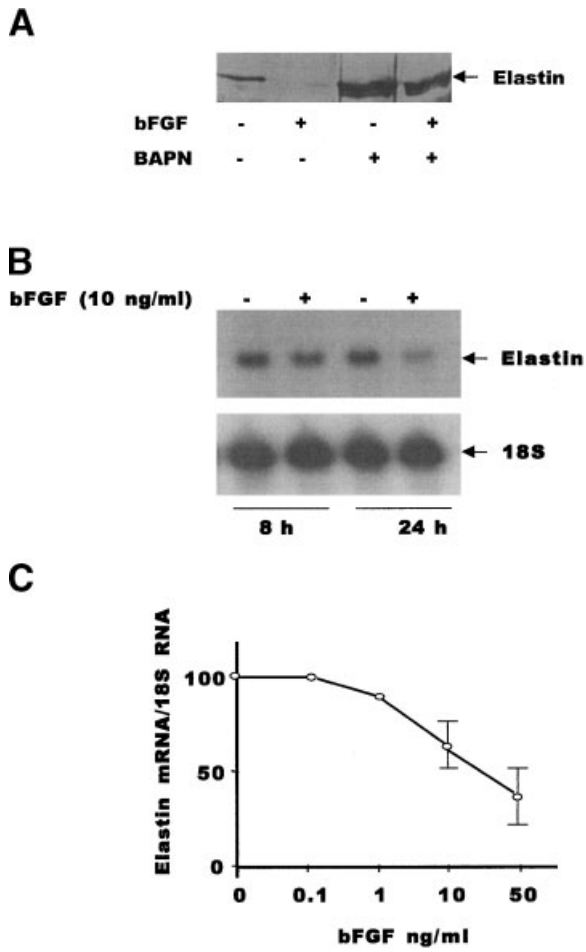

Fig. 1. Effect of bFGF on the steady-state levels of elastin mRNA in aortic SMC. **A**: Serum starved SMC were treated with BAPN (25 μg/ml) and/or bFGF (50 ng/ml) for 24 h. Two hundred microliters of media were loaded on a 9% SDS–polyacrylamide gel and analyzed by Western blot with an elastin antibody. **B**: Serum starved SMC were treated with 10 ng/ml bFGF (+) or with saline (–) for 8 and 24 h. Total RNA was isolated and analyzed by Northern blot using ^{32}P-labeled rat elastin cDNA and 18S rRNA probes. **C**: Serum starved SMC were treated with different concentrations of bFGF (0–50 ng/ml) for 24 h. Total RNA was obtained and analyzed by Northern blot for elastin mRNA and 18 S rRNA. Data is expressed as a percent of elastin mRNA normalized to the 18S rRNA. Error bars represent SD of results from three independent experiments.

In order to gain insight into the mechanism responsible for the bFGF-dependent decrease in tropoelastin, elastin mRNA was measured in response to bFGF addition. Figure 1B provides a Northern blot of elastin mRNA at various specified times after bFGF addition to SMC. The results show that the levels of elastin mRNA decrease by 50% after 24 h. The bFGF-dependent decrease of elastin mRNA is dose

dependent (Fig. 1C) and is detectable at a concentration as low as 1 ng/ml. Treatment of cells with increasing concentrations of bFGF resulted in further decrease in the mRNA level, reaching 40% of the original level with 50 ng/ml of bFGF. Together these results show that bFGF decreases elastin mRNA and tropoelastin synthesis in aortic SMC.

bFGF Downregulates Elastin Gene Transcription

To determine whether bFGF affects the stability of the elastin mRNA, the decay rate of elastin mRNA in control and bFGF-treated cells was studied after addition of RNA polymerase II inhibitor 5,6-dichlorobenzimidazole riboside (DRB). Linear regression analysis of elastin mRNA dynamics in transcriptionally arrested SMC did not show any significant change in the half life of elastin mRNA in control ($t_{1/2} = 17$ h) versus bFGF-treated cells ($t_{1/2} = 16$ h), suggesting that bFGF is not altering transcript stability (data not shown).

To determine if bFGF may act at the level of elastin gene transcription, nuclear run-on assays were performed on nuclei isolated from SMC cultures treated with or without bFGF. Figure 2A provides a nuclear run-on blot obtained from a typical analysis performed and Figure 2B provides a quantitative analysis of data obtained from three separate sets of SMC. We found that bFGF downregulates elastin transcription levels by approximately 50%. The transcription level of GAPDH does not change significantly with bFGF addition suggesting that the bFGF-dependent decrease of elastin transcription is specific. Overall these results show that the inhibitory effect of bFGF on the elastin gene transcription accounts in great part, if not completely, for the observed decrease in the elastin mRNA in SMC.

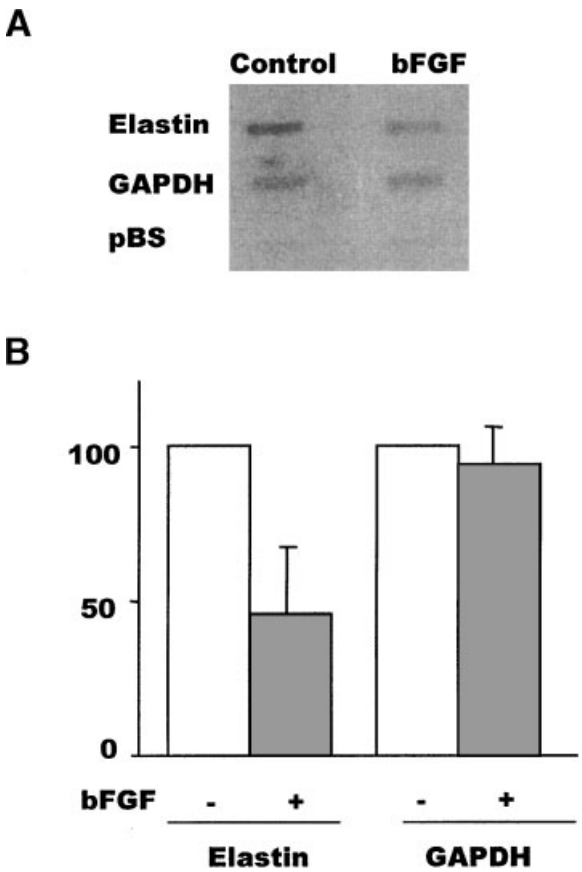

Fig. 2. Effect of bFGF on the transcriptional activity of the elastin gene in aortic SMC. Serum starved SMC were treated with saline (control) or bFGF (50 ng/ml) for 24 h, nuclei were isolated, and nuclear run-on assays performed in the presence of [α-^{32}P]UTP for 20 min. The nascent ^{32}P-labeled transcripts were hybridized to slots of membrane-bound elastin and GAPDH cDNAs. The pBS DNA was also included to control for nonspecific hybridization. **A**: Representative run-on slot blot autoradiography. **B**: Quantitation of elastin and GAPDH transcription levels of bFGF-treated and control aortic SMC. Data were obtained from laser densitometric analysis of hybridization signal of membranes from three independent experiments. The transcriptional level for each gene in the control cultures is given as 100% and the levels obtained after addition of bFGF to the cells are plotted as a percent of that control.

Activator Protein-1-cAMP Response Element (AP1/CRE) (−564 to −558) in the Elastin Promoter Mediates the bFGF-Dependent Decrease in Elastin Gene Transcription

Previous studies on the effect of bFGF on cultured pulmonary fibroblasts showed that bFGF represses elastin gene transcription through a hybrid AP1/CRE element in the distal region of the elastin promoter [Rich et al., 1996; Rich et al., 1999]. In order to determine if the same cis-acting element is functional in aortic SMC, transient transfections followed by CAT assays were performed using a wild type elastin promoter-CAT contruct (900wt) and a construct mutated at the AP1/CRE element (900M) [Rich et al., 1999]. The results of three separate transfection experiments are given in Figure 3. The data show that addition of bFGF decreases wild type elastin promoter activity by 50%. Mutation of the AP1/CRE element resulted in a more than two times higher basal activity of the promoter, and complete loss of its sensitivity to bFGF. These results confirm the transcriptional downregulation by bFGF and further implicate the

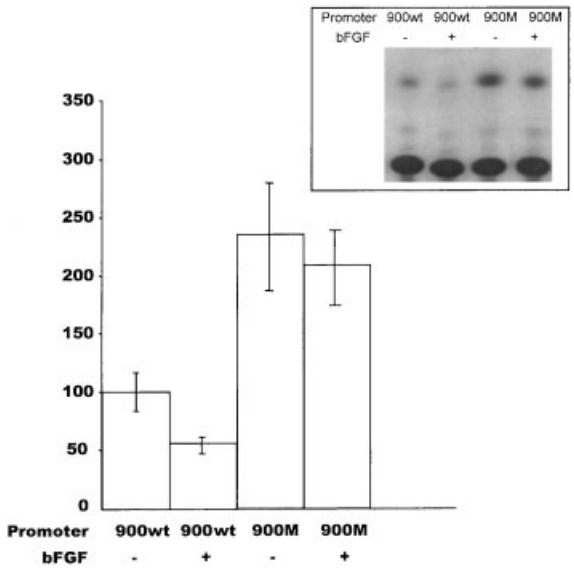

Fig. 3. Effect of bFGF on the elastin promoter activity in aortic SMC. Rat aortic SMC were transiently co-transfected with 30 μg of wild type (900 wt) or mutated (900 M) elastin/CAT promoter constructs together with 5 μg of pCMV-β-gal. Transfected cells were treated with 50 ng/ml of bFGF (+) or with saline (−) for 24 h. Equal amounts of β-galactosidase activity were subjected to a thin layer chromatography in order to separate ^{14}C-acetylated chloramphenicol derivatives. The air-dried plate was exposed to a X-ray film (inset). Quantification was performed by laser densitometric analysis of autoradiographic film. Error bars represent SD of results from three separate transfection experiments using two different preparations of plasmid DNA.

AP1/CRE cis-element (−564 −558) in the distal promoter of the elastin gene.

Fra-1 Is Induced by bFGF in Aortic SMC

Previous work in pulmonary fibroblasts showed that the downregulation of elastin gene transcription by bFGF was due to the upregulation of Fra-1 expression leading to the formation of the Fra-1/c-Jun heterodimer that bound to a hybrid AP1/CRE element in the elastin promoter [Rich et al., 1999]. The levels of Fra-1 and c-Jun as a function of time after bFGF addition to aortic SMC were examined. Representative Western blots are shown in Figure 4. Fra-1 was barely detectable in control cells but was increased 2 h after the addition of bFGF, reached its maximum level at 4 h, and remained elevated 8 h after the treatment started. On the other hand, high levels of c-Jun expression remained unaffected by the addition of bFGF. These results suggest that the bFGF-dependent decrease in elastin gene transcription is mediated by a Fra-1/c-Jun heterodimer similar to

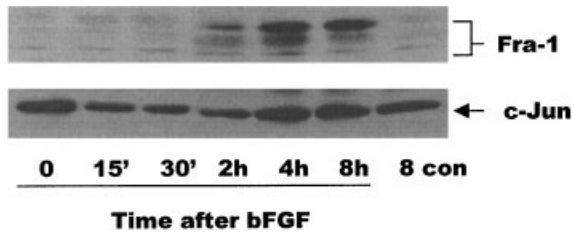

Fig. 4. Effect of bFGF on Fra-1 and c-Jun protein levels in aortic SMC. Rat aortic SMC were treated with 50 ng/ml of bFGF for various times as indicated. Total cell protein were extracted and 50 μg per sample were subjected to Western blot analysis using antibodies to Fra-1 and c-Jun. A control sample in which the cells were treated with saline for 8 h (8 con) was also included.

the mechanism reported in pulmonary fibroblasts [Rich et al., 1999].

Activation of ERK1/2 Mediates the Downregulation of Elastin mRNA by bFGF in Aortic SMC

The canonical signal transduction pathway initiated by the majority of growth factors signaling through their specific tyrosine kinase receptors is the ERK1/2 cascade. Thus, the ability of bFGF to activate ERK1/2 kinases in aortic SMC was studied by an in-gel kinase assay (Fig. 5A). Addition of bFGF activates ERK1/2 kinases within 5 min with the highest activity reached at 30 min, followed by a lower activity level that persists for the 6 h period examined. Immunocytochemistry was performed with phospho-ERK1/2 specific antibodies. Figure 5B depicts the confocal microscopic images showing that 15 min after administration of bFGF activated ERK1/2 are present in the nucleus where they are poised to act on potential nuclear substrates.

PD-98059, a specific inhibitor of ERK1/2 kinase, was used to determine whether the bFGF-dependent activation of ERK1/2 may signal to downregulate elastin gene transcription. Preincubation of SMC in the presence of PD-98059 inhibited the bFGF-dependent activation of ERK1/2 (Fig. 6A). Additional experiments were performed to examine the effect of PD-98059 on bFGF-dependent elastin mRNA downregulation. As shown in Figure 6B, the bFGF-dependent downregulation of elastin mRNA is abrogated by the ERK1/2 kinase inhibitor.

DISCUSSION

The SMC is a unique muscle cell because of its ability to change phenotypes [Thyberg, 1998].

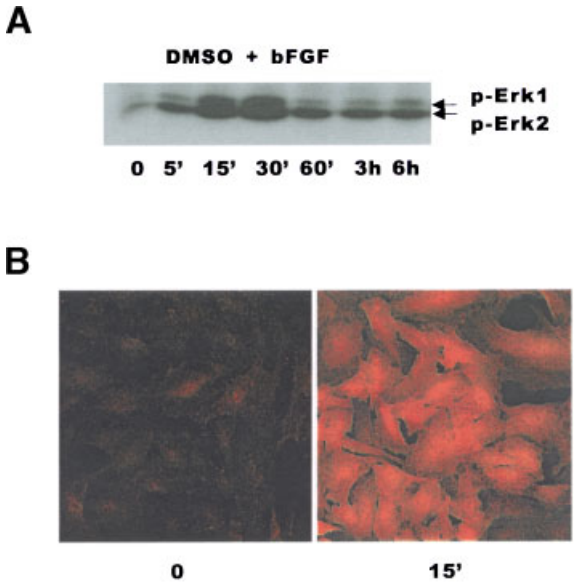

Fig. 5. Kinase activation of ERK-1 and ERK-2 by bFGF in SMC. **A**: Serum starved SMC were treated with bFGF (10 ng/ml) for the indicated periods of time after a 30 min incubation with DMSO (solvent for PD-98059). Total cell proteins were extracted. Forty micrograms of protein per sample were analyzed by in-gel kinase assay using myelin basic protein and [γ-^{32}P] ATP as detailed under Materials and Methods. Kinase activity was visualized by exposing the dried gel to an X-ray film. **B**: SMC grown on glass cover slips were serum starved and treated with or without bFGF (10 ng/ml) for 15 min. Cells were fixed and immunostained with antibodies to phospho-ERK1/2 and a Cy3-conjugated secondary antibody. Fluorescent images were obtained by confocal laser scanning microscopy (magnification = 25 ×). Cells stained with the secondary Cy3-conjugated antibody only were used to establish the settings in which all the samples were analyzed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

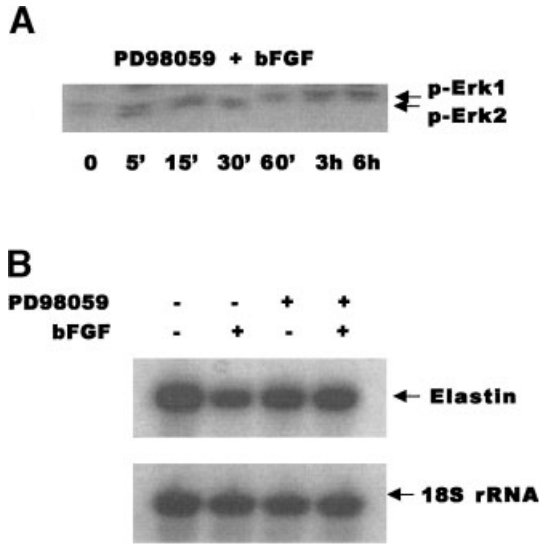

Fig. 6. Effect of PD98059 on the bFGF-dependent downregulation of elastin mRNA in SMC. **A**: Serum starved SMC were treated with bFGF (10 ng/ml) for the indicated periods of time after a 30 min incubation with PD-98059. Forty micrograms of protein per sample were analyzed by in-gel kinase assay as done with samples from Figure 5A. **B**: Serum starved SMC were treated with PD98059 (+) to a final concentration of 50 ng/ml or with an equal volume of solvent DMSO (−) for 30 min followed by treatment with bFGF (10 ng/ml) for 24 h. Total RNA was extracted and the steady-state levels of elastin mRNA and 18S rRNA were assessed by Northern blot analysis.

These various phenotypes are tightly controlled in development and important in response to injury. Atherosclerosis is characterized by the fragmentation of elastic fibers; however, animal models of restenosis show an increased elastin synthesis by neointimal SMC. The discrepancy in the state of elastin in these two pathologies may relate to the differential availability or effect of growth factors that alter the SMC phenotype. Disparate responses of medial SMC versus neo-intimal SMC to growth factors has been reported [Reidy et al., 1992; Koyama et al., 1998].

The ability of bFGF to downregulate elastogenesis in aortic SMC may contribute to the efficacy of elastin repair in early stages of vascular disease. In the vascular extracellular space, bFGF binds to heparan sulfate proteoglycans where it is poised to respond to vascular injury in a more localized and regulated manner [Benezra et al., 1993]. During injury to the vessel wall, recruited inflammatory cells and SMC themselves release elastases. Interestingly, the action of these elastases not only results in the proteolysis of elastin fibers but also in the release of bFGF in a biologically active form [Rich et al., 1996; Thompson and Ravinowitch, 1996]. Breakdown of the matrix, and specifically the internal elastin lamella, appears to be necessary for the initial migration of SMC from the medial to the intimal layer [Davies, 1996; Carragher et al., 1999]. Released bFGF appears to lead an orchestrated response to allow the SMC migration to the intima.

Results from this study show that the ERK pathway signals the downregulation of elastin gene expression triggered by bFGF. The ERK pathway is commonly associated with the interaction of growth factors with their receptors and appears to be critical for cell division. The kinase activity of ERK1/2 is rapidly increased in balloon-injured artery and is associated with activation of the transcription factor AP1 complex and cell replication of medial SMC [Kim et al., 1998; Koyama et al., 1998]. bFGF appears to be involved in these injury related events [Koyama et al., 1998]. Several in situ studies have been

shown an inverse correlation between SMC proliferation and elastin expression [Wachi et al., 1995; Belknap et al., 1996; James et al., 1998; Belknap et al., 1999]. Therefore, the bFGF-dependent activation of ERK1/2 can control both elastin expression and proliferation in aortic SMC thereby permitting a tighter regulation of these two events.

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