Antisense \( \text{fosB} \) RNA Inhibits Thrombin-Induced Hypertrophy in Cultured Pulmonary Arterial Smooth Muscle Cells

Chuanyi Lu, MD; Frank J. Giordano, MD; Xuping Bao, MD; Kemberlyn C. Morris, BS; Abraham Rothman, MD

**Background**—We have previously reported that \( \text{fosB} \) mRNA is induced by hypertrophic stimuli (thrombin, angiotensin II) but not proliferative stimuli (platelet-derived growth factor, basic fibroblast growth factor) in pulmonary arterial smooth muscle cells (PASMCs) (*J Biol Chem*. 1994;9:6399–6404). Our aim in the present study was to investigate the potential role of FosB in PASMC hypertrophy.

**Methods and Results**—Adenoviruses carrying sense or antisense \( \text{fosB} \) RNA expression cassettes were used to infect cultured PASMCs with the aim of increasing or inhibiting \( \text{fosB} \) expression, respectively. We examined whether \( \text{fosB} \) expression modification affected the growth of quiescent PASMCs, thrombin-induced hypertrophy, or platelet-derived growth factor–induced proliferation. PASMC growth was assessed by daily cell number count, determination of \([\text{H}]\)leucine incorporation, and quantification of total cellular protein. Neither an increase nor a decrease in FosB protein expression caused a significant change in the growth of quiescent PASMCs over a period of 96 hours, indicating that FosB alone is not sufficient to induce hypertrophy. Modification of FosB levels did not affect platelet-derived growth factor–induced PASMC proliferation. An increase in FosB expression did not augment thrombin-induced hypertrophy; however, inhibition of FosB expression resulted in a diminution of thrombin-induced hypertrophy by 58±6% (\( P<0.005 \)).

**Conclusions**—These results suggest that FosB is necessary but not sufficient for thrombin-induced hypertrophy in cultured PASMCs. (*Circulation*. 1998;98:596-603.)

**Key Words:** muscle, smooth ■ hypertrophy ■ growth substances ■ RNA

Vascular smooth muscle cells normally exist in a growth-arrested state, functioning to maintain vascular tone. Abnormal VSMC growth has been described in several disease processes, including systemic hypertension,\(^{1,2}\) atherosclerosis,\(^3,4\) restenosis after balloon angioplasty,\(^5\) and pulmonary hypertension.\(^6\) Interest has therefore focused on the regulators and intracellular signaling pathways involved in VSMC growth in normal and pathological states.

A variety of growth factors, cytokines, and vasoregulatory molecules have been linked to hyperplasia of VSMCs during the development of vascular diseases.\(^6,7\) Other factors, including angiotensin II,\(^8\) thrombin,\(^9,10\) TGF-\(\beta\)1,\(^11\) thromboxane \(\text{A}_2\),\(^12\) bFGF,\(^12\) PDGF-\(\text{AA}\),\(^13,14\) serotonin,\(^15\) vasoconstrictor prostanoids,\(^16\) and arginine vasopressin,\(^17\) have been shown to induce primarily hypertrophy in VSMCs in vitro. The angiotensin II–induced hypertrophic response in VSMCs has been shown to be associated with increased expression of PDGF-\(\text{AA}\),\(^13,18\) smooth muscle isoenzyme,\(^17\) and autocrine TGF-\(\beta\)1 production.\(^19\) Nevertheless, the precise signal transduction pathways and intracellular determinants involved in VSMC proliferation and hypertrophy remain unknown.

Induction of cell growth in VSMCs and fibroblasts triggers a rapid, sequential, and transient expression of IEGs.\(^9,20,21\) Several IEG families encode putative transcription factors involved in complex signaling cascades that control cell growth and differentiation.\(^22\) As a member of the IEGs, the \( \text{fosB} \) gene encodes a nuclear protein that shares 70% sequence homology with \( \text{c-fos} \), a more widely studied transcription factor of the Fos family. The \( \text{fosB} \) gene expression is rapidly and transiently induced after growth stimuli in fibroblasts and VSMCs.\(^9,23\) FosB protein appears to be a strong transcriptional activator in fibroblasts.\(^24-26\) We have previously demonstrated that the mRNAs of several IEGs, including \( \text{c-fos} \), \( \text{c-jun} \), \( \text{junB} \), \( \text{junD} \), \( \text{fra-1} \), and \( \text{egr-1} \), are induced in PASMCs by both hypertrophic (thrombin and angiotensin II) and proliferative (PDGF) stimuli; however, \( \text{fosB} \) mRNA is induced exclusively by the hypertrophic stimuli.\(^9\) This finding suggests that FosB may be an intermediary or a marker of the hypertrophic response.

To further define the function of FosB in PASMC hypertrophy, adenovirus vectors were used to deliver sense and
antisense fosB RNA expression cassettes to PASMCs with the goal of modifying FosB levels. We examined the effects of alteration in fosB gene expression on cellular growth, as well as thrombin-induced (a G protein–coupled receptor agonist) and PDGF-induced (a tyrosine kinase–coupled receptor agonist) growth responses. Our results suggest that FosB is a necessary but not sufficient intermediary of thrombin-induced hypertrophy in PASMCs. To the best of our knowledge, this report is the first to describe the direct involvement of a specific IEG product in VSMC hypertrophy.

Methods

Generation of Recombinant Adenoviral Vectors
The E1 region–deleted recombinant adenoviral vectors carrying either sense or antisense fosB cDNA, called Ad.S.fosB or Ad.A.fosB, were constructed as previously described.13 The shuttle vector ACCMVPLPA (8.8 kb) (provided by Dr Robert Gerard, University of Texas Southwestern Medical Center, Dallas) was prepared by insertion of the constitutive cytomegalovirus (CMV) early–gene promoter (0.76 kb), the pUC19 polylinker, and the SV40 polyadenylation signal (0.47 kb) into the pAC vector. A 1.8-kilobase-pair fragment of full-length fosB cDNA23 (provided by Dr Rodrigo Bravo, Bristol-Myers Squibb, Trenton, NJ) was then subcloned in sense or antisense orientation into the ACCMVPLPA shuttle vector to yield the sense expression construct SR.fosB and the antisense expression construct SR.A.fosB. SR.S.fosB and SR.A.fosB were then independently cotransfected with pJM17 (provided by Dr Frank L. Graham, McMaster University, Ontario, Canada) into 293 cells by calcium phosphate/DNA coprecipitation. For viral plaque assays, the cotransfected 293 cells were overlaid with 0.65% agarose (prepared with 1× DMEM) every 3 to 4 days. The pJM17 contains the full-length Ad5 DNA (36 kb) and pBHR, a 4.3-kb insert placed in the E1 region, thus exceeding by nearly 2 kb the maximum packaging limit of DNA into the Ad capsid. Homologous recombination between the expression construct (SR.S.fosB or SR.A.fosB) and pJM17 in 293 cells replaced the E1 region and the pBHR insert with the expression cassette from the expression constructs. The growth of these E1-deleted Ads is limited to 293 cells,17 a human embryonic kidney cell line that has been transformed by Ad5 genome and expresses the E1 region. Individual viral plaques were isolated and amplified in 293 cells. The polymerase chain reaction assay was used for identification and differentiation of the recombinant Ad vectors.

Successful recombinant Ad vectors then underwent 2 rounds of plaque purification. Purified viral plaques were propagated in 293 cells, and viral stocks were prepared from lysates of infected 293 cells. The titers of the adenoviral stocks used in this study were 2×10^9 to 3×10^10 pfu/mL. By using the same procedure, we also generated another 2 control Ad vectors, Ad.A.junD and Ad.dE1 (empty vector). The Ad.A.junD carries the antisense junD cDNA expression cassette. The Ad.dE1 does not carry a foreign gene insert.

Cell Culture and Adenovirus Infection
Cloned PASMCs, PAC1 cells,23 were grown in medium 199 (M199) (Gibco BRL) supplemented with 10% (vol/vol) FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 0.07 mmol/mL streptomycin in a 37°C, 5% CO₂ incubator. The medium was changed every 3 to 4 days. Cells were harvested with trypsin and passaged at a split ratio of 3 to 4 when they reached 80% to 90% confluence. In preparation for experiments, the cells were plated into 6-well culture dishes (Costar Corp) at 3×10⁴ cells/well and grown in 2 mL M199 containing 10% FBS until ~40% to 50% confluence. Cells were then switched to the same medium containing 0.5% FBS and incubated for another 48 hours to make the cells quiescent.9 The medium was changed again just before each experiment. Cells were divided into 12 groups: (1) control, (2) thrombin (1 U/mL) (Sigma), (3) PDGF (2.5 ng/mL) (Sigma), (4) Ad.S.fosB (3 pfu/cell), (5) Ad.A.fosB (3 pfu/cell), (6) Ad.dE1 (3 pfu/cell), (7) Ad.S.fosB (3 pfu/cell)+thrombin (1 U/mL), (8) Ad.A.fosB (3 pfu/cell)+thrombin (1 U/mL), (9) Ad.dE1 (3 pfu/cell)+thrombin (1 U/mL), (10) Ad.S.fosB (3 pfu/cell)+PDGF (2.5 ng/mL), (11) Ad.A.fosB (3 pfu/cell)+PDGF (2.5 ng/mL), and (12) Ad.dE1 (3 pfu/cell)+PDGF (2.5 ng/mL). In groups 7 through 12, adenoviral infection was performed 24 hours before PDGF or thrombin stimulation. After addition of the adenovirus to the culture medium, the cells were incubated with gentle swirling every 20 to 30 minutes for the first 90 minutes. After 24
hours, thrombin (1.0 U/mL) or PDGF (2.5 ng/mL) was added to the culture medium.

RNA Isolation and Northern Blotting
Total cellular RNA was extracted from PASMCs by the acid guanidium–phenol-chloroform standard method. RNA was denatured with 3% formaldehyde and fractionated in 1% agarose gels before being transferred to nylon membranes (Micron Separation Inc.). Nylon filters with uniformly transferred RNA were then hybridized with the [32P]dCTP-labeled probes in a hybridization solution containing 50% formamide, 6×SSC (1×SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate), 0.5% SDS, and 0.1 mg/mL calf thymus DNA at 42°C. After 20 to 24 hours, the membranes were washed once with 1×SSC/0.1% SDS for 20 minutes at room temperature and once with 0.5×SSC/0.1% SDS for 20 minutes at 45°C before being exposed to Kodak X-OMat AR films.

The following cDNA probes were labeled by nick translation using [32P]dCTP (ICN Radiochemicals) to a specific activity of 1.0×106 cpm/µg DNA: fosB,23 TGF-β1,11 PDGF-AA,22 bFGF,31 IGF-114 (ATCC), and a vascular smooth muscle–specific probe derived from the 3’ untranslated region of the rat smooth muscle α-actin mRNA (pRVαA-3’UT-DP).35

Protein Preparation and Western Blotting
The cells were washed twice with PBS, lysed at 4°C with lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 1% (vol/vol) Nonidet P-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L NaF, 1 µg/mL aproatin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin A) (for FoxB) or SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, 50 mmol/L DTT, and 0.1% wt/vol bromphenol blue] (for MAPKs). Cellular lysate was scraped, transferred into a microtube, and sonicated for 10 seconds, followed by centrifugation (30 minutes, 4°C, 14 000 rpm). Protein concentration in the supernatant was determined by the Bio-Rad colorimetric protein assay method (Bio-Rad Laboratories). Twenty micrograms of total cellular protein was size-fractionated in a 10% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. The membrane was stained with Ponceau S (Sigma) to confirm equal amounts of protein in each lane and homogenous transfer. After the membrane was washed with TBS (20 mmol/L Tris-HCl, 0.5 mmol/L NaCl, pH 7.4) to remove the stain, the filters were blocked with 5% nonfat dry milk in TBS overnight at 4°C, incubated with diluted specific rabbit anti-rat FosB antibody (Santa Cruz Biotechnology, Inc.) or rabbit anti-human phospho-specific MAPK (ERK1 and ERK2) antibody (New England Biolabs, Inc) for 1 hour, and incubated with horseradish peroxidase–conjugated goat anti-rabbit antibody for 1 hour. The filters were then washed twice with TBBS (0.05% Tween-20 in TBS, pH 7.4) for 5 minutes each and once with TBS. Protein-antibody conjugates were detected by chemiluminescence (super signal CL-HRP, Pierce Chemical Corp).

Cellular Growth Assessment
Cellular growth was assessed by daily cell counts, new protein synthesis, and total protein quantification for 4 days. Cells counts were performed by hemocytometry immediately after the cells were harvested with trypsin. For new protein synthesis assessment, the cells were switched to L-leucine–deprived culture medium (Gibco BRL) and treated with [3H]leucine (10 µCi/mL) (DuPont Corp) simultaneously with growth factor stimulation as described above. At the time of harvest, the cells were washed twice with cold PBS and once with 10% (wt/vol) cold TCA and incubated with 10% TCA at 4°C for 30 minutes. Cells were then scraped off the plates and centrifuged. The pellet was washed once with 10% TCA and once with 95% ethanol, dried, and dissolved with 0.1 mol/L sodium hydroxide at room temperature for 2 hours. Radioactivity was determined with a liquid scintillation counter. For total protein quantification, PASMCs were washed twice with PBS, lysed with 0.2 mol/L NaOH, and harvested into microtubes. Protein was quantified by the Bio-Rad colorimetric protein assay method (Bio-Rad Laboratories).

Statistical Analysis
The results are presented as mean±SEM. Unpaired t test, single-factor ANOVA, and the Newman-Keuls post hoc test were used for statistical analyses. A value of P<0.05 was considered to be significant.

Results
Modification of fosB mRNA and Protein Levels
Adenovirus vectors were used to deliver sense and antisense fosB mRNA expression units with the goal of increasing and inhibiting FosB expression, respectively. In preliminary experiments, dose-dependent cytopathic effects occurred in PASMCs infected with adenovirus at 10 pfu/cell or higher multiplicity of infection, and FosB could be detected in nearly 95% of PASMCs infected with Ad.S.fosB at 3 pfu/cell.27 Therefore, a ratio of 3 pfu/cell was chosen in this study. The fosB mRNA and protein expression were examined 25 hours after adenoviral infection by Northern and Western blotting, respectively. As shown in Figures 1 and 2, Ad.S.fosB-infected cells expressed abundant fosB mRNA and protein. Only small amounts of fosB mRNA (the double-stranded fosB cDNA was labeled and used as the hybridization probe) and no FosB protein were detected in the Ad.A.fosB-infected PASMCs. Neither fosB mRNA nor FosB expression could be detected in the Ad.dE1 (empty Ad vector)–infected PASMCs. In the Ad.S.fosB-infected PASMCs, 2 bands of FosB of 37 and 48 kDa were detected, corresponding to 2 isoforms of FosB produced by alternative splicing of fosB transcripts.24,36,37

One hour after growth stimulation, thrombin but not PDGF induced a marked increase in fosB mRNA and protein in serum-deprived, quiescent PASMCs (Figures 1 and 2). The FosB isoforms induced by thrombin and Ad.S.fosB had similar molecular sizes.

To determine whether infection with Ad.S.fosB or Ad.A.fosB modified thrombin-induced fosB gene expres-
sion, quiescent PASMCs were incubated for 24 hours with Ad.S.fosB, Ad.A.fosB, or Ad.dE1 at 3 pfu/cell and then treated with thrombin (1 U/mL). FosB expression was examined 1 hour after thrombin stimulation. As shown in Figures 1 and 2, no significant difference in fosB mRNA and protein levels was observed among Ad.S.fosB-infected (lane 4), thrombin-stimulated (lane 2), Ad.S.fosB-infected + thrombin-stimulated (lane 7), and Ad.dE1-infected + thrombin-stimulated (lane 8) PASMCs. However, preinfection with Ad.A.fosB significantly inhibited thrombin-induced fosB mRNA and protein expression (lane 8). Therefore, adenovirus-mediated expression of antisense fosB RNA effectively blocked the stimulatory effect of thrombin on fosB mRNA or protein levels compared with the levels in cells infected with these Ad vectors alone (lanes 4 through 6).

**Effect of FosB Expression Modification on PASMC Growth**

PDGF caused a 90% increase in cell number over a period of 96 hours but no significant change in [3H]leucine incorporation or protein content per cell ([protein]/cell) compared with unstimulated serum-deprived PASMCs. In contrast, thrombin did not cause an increase in cell number but caused a 100% increase in [3H]leucine incorporation and a 42% increase in [protein]/cell over 96 hours (Figures 3 and 4). These results corroborated our previous observation that PDGF induced proliferation and thrombin caused hypertrophy in the PASMCs.

We then examined whether adenovirus-mediated modification of FosB expression alone would result in a change in cell number and cell growth. Serum-deprived PASMCs were infected with Ad.S.fosB, Ad.A.fosB, or Ad.dE1 at 3 pfu/cell, and cellular growth was assessed daily for 4 days. Compared with the growth of control PASMCs, infection with Ad.S.fosB, Ad.A.fosB, or Ad.dE1 alone did not cause a significant increase in cell count, new protein synthesis, or [protein]/cell (data not shown). The results indicated that an increase in the level of FosB alone was not sufficient to induce hypertrophy in PASMCs.

To investigate whether adenovirus-mediated modification of fosB expression affected PDGF- or thrombin-induced growth, serum-deprived PASMCs were incubated for 24 hours with Ad.S.fosB, Ad.A.fosB, or Ad.dE1 (each at 3 pfu/cell) and then treated with PDGF or thrombin. Over the next 4 days, neither Ad.S.fosB, Ad.A.fosB, nor Ad.dE1 altered PDGF-induced PASMC proliferation (Figure 3). Similarly, preinfection with Ad.S.fosB or Ad.dE1 did not affect thrombin-induced hypertrophic growth. When PASMCs were preinfected with Ad.A.fosB, however, the thrombin-induced increases in new protein synthesis and [protein]/cell were inhibited by 68% (n=9, P<0.025) and 58% (n=9, P<0.005), respectively (Figure 4).

To ensure that the inhibitory effect of Ad.A.fosB was not due to a nonspecific effect of a sense-antisense RNA-RNA duplex, we constructed another control Ad vector, Ad.A.junD. JunD mRNA is expressed at very low levels in serum-deprived PASMCs and is upregulated by 20% FBS plus cycloheximide (10 μg/mL). Infection with Ad.A.junD effectively blocked the upregulation of junD mRNA by 20% FBS plus cycloheximide (data not shown). We infected PASMCs with Ad.A.junD for 24 hours and then stimulated the infected cells with thrombin. As shown in Figure 4, preinfection with Ad.A.junD did not affect the thrombin-induced hypertrophic growth response.

To examine whether adenoviral infection caused a contractile protein phenotype change in PASMCs, we examined the expression of vascular smooth muscle–specific α-actin mRNA in the cells. No significant difference in α-actin mRNA levels was observed in any of the experimental groups (Figure 1C), suggesting that adenoviral infection did not cause a major phenotypic change in the PASMCs.

**Other Potential Intermediaries of the Thrombin-Induced fosB Expression and Hypertrophy in PASMCs**

Because FosB protein appeared to be important for thrombin-induced hypertrophy in PASMCs, we sought to further delineate potential signaling pathways and mediators involved in the thrombin-induced fosB gene expression and...
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Several factors have been identified as substrates for MAPKs. We examined the expression of TGF-β, a cytokine whose upregulation is closely associated with increased gene expression of TGF-β1, PDGF-AA, bFGF, and IGF-1 between thrombin- and PDGF-stimulated PASMCs. MAPKs, also known as ERKs, appear to act as a point of convergence or integration for various growth stimuli. Transcriptional factors encoded by c-fos, c-jun, and c-myc have been identified as substrates for MAPKs. We examined the activation of MAPKs by PDGF and thrombin using Western blotting and a phospho-specific MAPK antibody, which exclusively detects the phosphorylated MAPKs. As shown in Figure 5, activation of MAPKs was examined with a phospho-specific MAPK antibody, which detects ERK1 and ERK2 only when its tyrosine kinase domain is catalytically phosphorylated. Lanes: 1, control (serum deprivation only); 2, sham (culture dishes were moved out of incubator, swirled, and placed back into incubator); 3, thrombin; 4, PDGF; 5, phorbol ester; and 6, FBS.

Therefore, we sought to examine whether thrombin induced the expression of these factors and whether FosB is involved. Both thrombin and PDGF augmented TGF-β1 mRNA levels as early as 8 hours after stimulation of quiescent PASMCs (Figure 6). In contrast, PDGF-AA, bFGF, and IGF-1 mRNAs were undetectable in both PDGF- and thrombin-stimulated PASMCs (data not shown). Thus, TGF-β1, PDGF-AA, bFGF, and IGF-1 do not appear to mediate the thrombin-induced hypertrophy in these cells.

Discussion

Whereas acute systemic hypertension models (such as aortic coarctation) are associated with VSMC proliferation, chronic hypertension models, including the spontaneously hypertensive rat (SHR, a genetic model of hypertension) and the 2-kidney, 1-clip Goldblatt hypertensive rat, exhibit aortic smooth muscle cell hypertrophy and polyploidy. In an animal model of pulmonary hypertension, the predominant pathological feature appears to be hypertrophic growth of precursors of smooth muscle cells—intermediate cells and pericytes—in the distal pulmonary arterial circulation. Similarly, hypertrophy of smooth muscle cells characterizes pulmonary arterial wall thickening after administration of monocrotaline pyrrole to rats. VSMC hypertrophy has also been demonstrated to be 1 of the key vascular pathological features in human systemic hypertension and pulmonary hypertension. All of these findings suggest that VSMC hypertrophy is important in the pathogenesis of hypertensive vascular diseases.

Several factors have been demonstrated to induce smooth muscle cell hypertrophy in vitro, but the intracellular determinants of this growth process remain unknown. Al-

![Figure 4](image)

**Figure 4.** Effects of adenoviral infection on thrombin-induced growth response in PASMCs. Serum-deprived cells were infected with Ad.S.fosB, Ad.A.fosB, Ad.A.junD, or Ad.dE1 at 3 pfu/cell, followed 24 hours later by thrombin stimulation. Cellular growth was assessed 24, 48, 72, and 96 hours after thrombin stimulation. Top, Daily cell number; middle, new protein synthesis ([³H]leucine incorporation); bottom, cell protein content. Shown are growth curves of Ad.A.fosB- and Ad.A.junD-infected cells. Growth curves of Ad.S.fosB and Ad.dE1-infected cells (not shown) were similar to that of Ad.A.junD-infected cells. Results are expressed as mean±SEM. ○, Control (n=9); ●, thrombin (n=9); □, Ad.A.fosB+thrombin (n=9); △, Ad.A.junD+thrombin (n=6); ▲, PDGF (n=9).

![Figure 5](image)

**Figure 5.** Western blotting assay of phosphorylation of 42- and 44-kDa MAPKs (ERK1 and ERK2) in PASMCs. Cells were serum-deprived for 48 hours and then treated with thrombin (1 U/mL), PDGF (2.5 ng/mL), phorbol 12-myristate 13-acetate, (phorbol ester, 1 μmol/L), or FBS (10% in volume). Total cellular proteins were harvested 12 minutes after treatments. Level of MAPK activation was examined with a phospho-specific MAPK antibody, which detects ERK1 and ERK2 only when its tyrosine kinase domain is catalytically phosphorylated. Lanes: 1, control (serum deprivation only); 2, sham (culture dishes were moved out of incubator, swirled, and placed back into incubator); 3, thrombin; 4, PDGF; 5, phorbol ester; and 6, FBS.

![Figure 6](image)

**Figure 6.** Northern blotting assay of TGF-β1 mRNA levels in PASMCs. Serum-deprived, quiescent PASMCs were stimulated with thrombin, PDGF, phorbol 12-myristate 13-acetate, (phorbol ester, 1 μmol/L) or FBS (10% in volume). Total cellular RNA was isolated 48 hours later. Lanes: 1, control (culture dishes stayed in incubator); 2, sham (culture dishes were moved out of incubator, swirled, and placed back into incubator); 3, thrombin; 4, PDGF; 5, FBS; 6, PDGF; and 6, thrombin.
though an increase in atrial natriuretic factor expression has been regarded as a marker of cardiac myocyte hypertrophy, there is no recognized marker for VSMC hypertrophy. On the basis of our previous finding that fosB mRNA is induced exclusively by hypertrophic but not proliferative stimuli in PASMCs, we hypothesized that FosB could be a potential mediator or marker of VSMC hypertrophy.

Thrombin has been shown to have prominent effects on vascular cellular function. In the absence of endothelium, thrombin is a potent vasoconstrictor for VSMCs. It is also a well-established promoter of VSMC proliferation in vitro, with a similar role suggested but not yet proven in vivo. However, thrombin has also been shown to induce hypertrophic growth in certain types of VSMCs. In our PASMCs, we showed previously and again in this study that thrombin causes hypertrophy.

In contrast to previous VSMC hypertrophy studies, which focused on the involvement of extracellular mediators of hypertrophy, the goal of the present study was to identify a potential intracellular mediator or marker of VSMC hypertrophy. FosB is 1 member of the family of Fos proteins, which include c-Fos, FosB, Fra-1, and Fra-2. The Fos protein occurs in heterodimers with the Jun proteins (c-Jun, JunD, JunB) and functions as an AP-1-responsive element located in the 5′ upstream region of other genes. Varying amounts of FosB probably alter the proportion of different Jun/Fos heterodimers and provide the cells with a fine-tuning mechanism for controlling the expression of other genes. Recent reports on fosB knockout mice demonstrated that FosB plays an important and specific role in transcriptional regulation. FosB expression has been linked to VSMC growth because rapid increases in fosB mRNA and protein occur in VSMCs after growth stimulation and experimental balloon angioplasty. In cultured PASMCs, the expression of fosB mRNA is generally undetectable in quiescent cells, increases rapidly with hypertrophic growth stimuli (thrombin and angiotensin II), and returns to basal levels within 2 to 4 hours.

To determine whether FosB is involved in PASMC hypertrophy, we successfully modified the intracellular FosB protein levels by adenovirus-mediated delivery of sense and antisense fosB RNA expression units, respectively. Our major finding was that FosB is necessary but not sufficient for the thrombin-induced PASMC hypertrophy. Increase or inhibition of FosB expression alone did not cause a change in the growth pattern of serum-deprived unstimulated cells; however, inhibition of FosB expression significantly diminished the thrombin-induced hypertrophy, by 58±6%. Supportive data for the specificity of antisense fosB RNA on PASMC hypertrophy include the following: (1) fosB gene expression was abolished in Ad.A.fosB-infected cells; (2) among Ad.S.fosB, Ad.A.fosB, and Ad.dE1, only infection with Ad.A.fosB caused inhibition of thrombin-induced hypertrophy; (3) Ad.A.junD did not inhibit hypertrophy; and (4) neither Ad.S.fosB, Ad.A.fosB, Ad.A.junD, nor Ad.dE1 caused a change in PDGF-induced PASMC proliferation.

The intracellular signaling pathways through which diverse extracellular signals regulate cellular growth are complex and are known to include activation of phospholipase C, phosphoinositide metabolism, protein kinase C, MAPK and other protein kinases, increases in intracellular calcium, and sequential expression of IEGs. We have previously identified several intracellular signaling differences in PASMCs stimulated by thrombin and PDGF, including the following: (1) PDGF exclusively stimulates an increase in phosphatidylinositol 3,4,5-triphosphate (PI-3,4,5-P3), whereas thrombin causes a predominant increase in inositol 1,4-biphosphate (1,4-IP2); and (2) the induction of IEG expression appears to require thapsigargin-sensitive intracellular Ca2+ stores in response to thrombin but not to PDGF. To further investigate how FosB is involved in the thrombin-induced hypertrophic growth, we studied MAPKs. MAPKs are activated by tyrosine kinase–coupled receptor agonists (eg, PDGF), G protein–coupled receptor agonists (eg, angiotensin II), and protein kinase C activators (eg, phorbol esters). Both p44 and p42 MAPKs (ERK1 and ERK2) function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation. Activation of MAPKs occurs through phosphorylation of threonine and tyrosine by a single upstream MAPK kinase (MEK). We found that PDGF, but not thrombin, rapidly activated MAPKs in PASMCs. This finding indicates that activation of intracellular pathways involving MAPKs may not be required for the thrombin-induced fosB gene expression and hypertrophic growth in PASMCs and provides another difference in signaling cascades activated by thrombin and PDGF. Our results raise the possibility that thrombin induces hypertrophy by activating specific pathways (eg, FosB), which are not activated by PDGF. Conversely, thrombin does not appear to activate signaling pathways (eg, MAPK) that may be required for progression of the cell cycle and proliferation.

To further explore the role of FosB induction in thrombin-induced PASMC hypertrophy, we compared thrombin- and PDGF-induced expression of other genes in PASMCs, with the goal of finding other mediators of hypertrophy and their relationship with FosB. TGF-β1, an AP-1 responsive gene, has been shown to be a major determinant of whether aortic smooth muscle cells grow by hypertrophy or hyperplasia. TGF-β1, PDGF-AA, IGF-1, and bFGF have also been shown to be closely associated with SMC hypertrophy. In the PASMCs, however, both thrombin and PDGF caused an increase in the expression of TGF-β1 but not PDGF-AA, bFGF, or IGF-1. Therefore, TGF-β1, PDGF-AA, bFGF, and IGF-1 are unlikely to be exclusive mediators of the thrombin-induced PASMC hypertrophy. In addition, in separate experiments, we found that thrombin did not cause a selective increase in the expression of several major cellular structural and functional proteins, including collagen I, collagen III, fibronectin (data not shown) and α-actin, suggesting that the thrombin-induced PASMC hypertrophy is probably due to a generalized increase in cellular proteins rather than a selective increase in specific proteins.

The incomplete inhibition of hypertrophy by our antisense fosB RNA raises several possibilities: (1) in addition to the pathway involving FosB protein, there may be other parallel pathways that contribute to the hypertrophic response; (2) preinfection with Ad.A.fosB may not have blocked fosB gene expression completely, even though FosB protein was...
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unnecessary by the Western blotting assay; or (3) other nuclear transcriptional factors may compensate for the function of FosB protein. The lack of a hypertrophic growth response in PASMCs infected with Ad.S fosB alone suggests that other factors, such as Jun proteins, may be necessary simultaneously to cause hypertrophy. Further studies are in progress to determine whether a simultaneous increase in FosB and Jun proteins leads to PASMC hypertrophy.

There are limitations to this study. One is the uncertainty of whether thrombin-induced hypertrophy in PASMCs is comparable to smooth muscle cell hypertrophy in pulmonary hypertension in vivo. Also, while thrombin induces hypertrophy in some VSMC types, it causes proliferation in other cell types. The cells used in the present study were cloned PASMCs that were originally derived from rat pulmonary arteries and were shown to maintain many differentiated properties through multiple subcultures. VSMCs derived from different tissues or from different regions of the same organ have been shown to be phenotypically diverse and respond differently to growth stimuli. These observations may explain in part the heterogeneous biological responses of different VSMCs.

In summary, our studies are beginning to define distinct pathways for proliferation and hypertrophy in a cloned smooth muscle cell line. PDGF induces predominantly phosphatidylinositol trisphosphate (PIP3), activates ERK1 and ERK2, and does not induce fosB gene expression. Thrombin induces predominantly inositol bisphosphate (IP2), does not activate ERK1 and ERK2, and causes a significant increase in fosB mRNA and protein. Further studies are necessary to elucidate the specific role of FosB in PASMC hypertrophy in vitro and during vascular remodeling in pulmonary hypertension in vivo.

References


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