Regulation of Smooth Muscle Myosin Heavy Chain Gene Expression in Cultured Vascular Smooth Muscle Cells by Growth Factors and Contractile Agonists

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Abstract

Stimulation of vascular smooth muscle cells (VSMC) by the growth factors TGFβ and IGFII consistently resulted in upregulation of smooth muscle myosin heavy chain (SMHC) mRNA levels by 50-70% greater than control. PDGF BB had no effect whereas most other growth factors and serum downregulated SMHC mRNA levels to as low as 5-10% of control. Surprisingly, the expression of the SM1 and SM2 SMHC isoforms was differentially regulated so that mRNA levels for each isoform showed a unique pattern of expression following growth factor stimulation. Changes in SMHC expression were not simply related to mitogenicity of factors tested since the strong mitogen PDGF BB had no effect whereas the non-mitogenic PDGF AA decreased SMHC levels to less than 50% of control. To determine whether the growth factors tested were having a transcriptional effect on the SMHC gene promoter, CAT activity driven by the SMHC promoter was measured in transiently transfected primary VSMC following growth factor treatment. The results showed that TGFβ consistently raised CAT activity ~70% greater than control whereas the other factors tested decreased CAT activity to 30-70% of control. These studies therefore suggest that SMHC expression is differentially regulated by growth factors in vitro with controls being exerted on the SMHC promoter and splicing of the SM1 and SM2 isoforms.

Key words: myosin, promoter, gene, vascular, growth.

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Smooth muscle cells demonstrate a plasticity in their phenotype which can be modulated during growth *in vitro* and *in vivo*. Growth of smooth muscle cells via hyperplasia and/or cell hypertrophy is regulated during development [21, 23] and equally dramatically in response to work overload [8, 22] and during disease [17]. It is also well known that the phenotype of primary smooth muscle cells grown in culture can oscillate between a "contractile" and "synthetic" state depending on cell density and culture conditions [6]. Furthermore hypertrophy of cultured smooth muscle cells can be induced by the contractile agonists angiotensin II [5, 9] and arginine-vasopressin [10, 24].

The proliferation and hypertrophy of vascular smooth muscle cells (VSMC) are two major biological responses which result in remodelling of smooth muscle tissue in cardiovascular disease but the underlying mechanisms are not well understood [19]. The transition of quiescent VSMC from a contractile to a synthetic state has been shown to coincide with new rounds of cell division [19, 21] but it is not clear whether inhibition of myogenesis is a prerequisite for VSMC to re-enter the cell cycle. Unlike the biological role of the MyoD family of genes and other muscle-specific transcription factors which regulate growth and differentiation of skeletal muscle [26], little is known about the factors regulating the development of smooth muscle.

Smooth muscle myosin heavy chain (SMHC) gene expression has been shown to be a very sensitive and key marker of the differentiated state of VSMC [11, 20]. To investigate the regulation of myogenesis in VSMC, we previously isolated the SMHC gene [3, 16] and a number of splicing variants [1, 4, 15, 27]. Expression of SMHC was found to be tissue-restricted [4, 16], developmentally regulated [13] and modulated by culture conditions [2, 11, 20]. Furthermore it was shown that SMHC expression was

altered following damage to the blood vessel wall [12]. Despite these changes, little is known about the molecular mechanisms regulating SMHC expression in VSMC. Here, we show that SMHC expression in primary VSMC cultures from rabbit aorta is regulated in different ways by specific growth factors and contractile agonists. Control appears to be exerted both at the level of transcription and splicing of the SM1 and SM2 SMHC isoforms.

Materials and Methods

Reagents

Collagenase (type IV), elastase (type IV), transferrin, insulin, sodium selenite, ascorbic acid, interleukin-1β (IL-1β), transforming growth factor α (TGF-α), and phorbol myristate acetate (PE) were obtained from Sigma. M199 medium, DMEM/HamsF12 medium, fetal calf serum (FCS), glutamine, penicillin, streptomycin, fungizone and transforming growth factor β (TGF-β1) were obtained from GibcoBRL. ^{3}H-Thymidine (70-90 Ci/mmol) was obtained from NEN. DNaseI and the reporter plasmids pCAT Basic, pCAT Control and pSVβgal were obtained from Promega. Platelet derived growth factor AA (PDGF-AA), PDGF-AB, PDGF-BB, basic fibroblast growth factor (bFGF), angiotensin II (AII), arginine vasopressin (AVP), insulin growth factor I (IGF-I), IGF-II and epidermal growth factor (EGF) were obtained from Bachem.

VSMC culture

Primary VSMC from adult NZW rabbit (2 kg) aorta were isolated by mechanical stripping followed by enzyme dispersion with collagenase and elastase. Transverse strips (~1 mm) of medial smooth muscle including endothelium were incubated in 0.05% trypsin for 10 minutes at 37°C in a 5% CO_2 incubator. Endothelial cells were removed by incubation of strips with collagenase (2 mg/ml) in M199 medium for 60 minutes at 37°C. VSMC were dispersed by incubation with elastase (0.25 mg/ml) for 60 minutes at 37°C, followed by addition of collagenase (2 mg/ml) and DNaseI (2.5 mg/ml) for a further 1-2 hours. VSMC were resuspended in M199 medium containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone. VSMC routinely showed > 95% viability and were routinely negative for von Willebrand antigen. Cells were seeded at 1 x 10^4/cm^2 and medium changed every two days. Cells were split 1:3 and used at passage 2. VSMC were made quiescent by transfer to serum-free medium (SFM) containing: DMEM/Hams F12 (1:1), 5 µg/ml transferrin, 5 µg/ml insulin, 5 ng/ml sodium selenite, 0.2 mM ascorbic acid, 2 mM glutamine, 0.25 µg/ml fungizone, 100 U/ml penicillin and 100 µg/ml streptomycin. VSMC were maintained in SFM for 48 hours prior to stimulation with growth factors.

Growth factors and contractile agonists were added at the concentrations given in figure legends. VSMC were incubated with factors for 48 hours after which cells were harvested for mRNA analysis. In parallel experiments, VSMC were pulsed with ^{3}H-Thymidine for estimation of DNA synthesis rates. RNase protection analysis was used to determine SM1 and SM2 isoform mRNA levels [4] and quantitation was performed by densitometric scanning.

DNA transfections

VSMC cultures were transiently transfected with DNA using the modified calcium phosphate-BES method (Kallmeier et al, in press). Chloramphenicol acetyl transferase (CAT) and galactosidase (β-gal) enzyme activities were measured according to the Promega protocols. Confluent VSMC at passage 1 were split 1:3 and replated on 6 cm dishes 24 hours prior to transfection. Optimal reporter activity occurred when VSMC were exposed to DNA for 18 hours, transferred to 10% serum-containing medium and harvested 48 hours post-transfection. In the present experiments with growth factors, transfected cells were transferred to SFM not serum containing medium for 48 hours and were then stimulated with growth factors for a further 48 hours.

Plasmids

A series of 5' end deletion mutants of the rabbit SMHC gene promoter [3] were previously constructed in the reporter vector pCAT Basic (Kallmeier et al, in press). The longest promoter construct (designated pRSMHC-2,305) containing 2.3 kb of upstream sequence was used in these studies. The construct pRSMHC-2,305 was cotransfected with the reporter vector pSVβgal to correct for transfection efficiency. VSMC were cotransfected with 10 µg CAT plasmid DNA and 5 µg β-gal reporter. Experiments with growth factors were repeated as many as six times using 2-3 dishes per transfection. Results were expressed relative to VSMC which were maintained in SFM and did not receive any treatment.

Results

Differential expression of SM1 and SM2 isoforms in VSMC in response to growth factor stimulation

Although it is known that the contractile phenotype of VSMC can be manipulated by serum and cell contact *in vitro*, it is unclear if expression of contractile protein genes is regulated by unique or similar mechanisms. The results in Fig 1 show for the first time a unique pattern of SM1 and SM2 SMHC isoform expression in response to growth factor stimulation. Sensitive RNase protection assays were used to follow the simultaneous pattern of expression of SM1 and SM2 mRNAs in order to determine the extent to which growth factor stimulation affected the contractile phenotype.

In these experiments, rabbit VSMC which were maintained in SFM expressed a ratio of SM1:SM2 mRNAs (90:10) which was similar to the ratio in cultured rat VSMC [2]. When VSMC were stimulated with the specific growth factors shown in Fig 1, the SM1 and SM2 isoforms were affected in different ways suggesting that SMHC expression was a sensitive marker of growth factor stimulation. Compared to cells maintained in SFM, very low

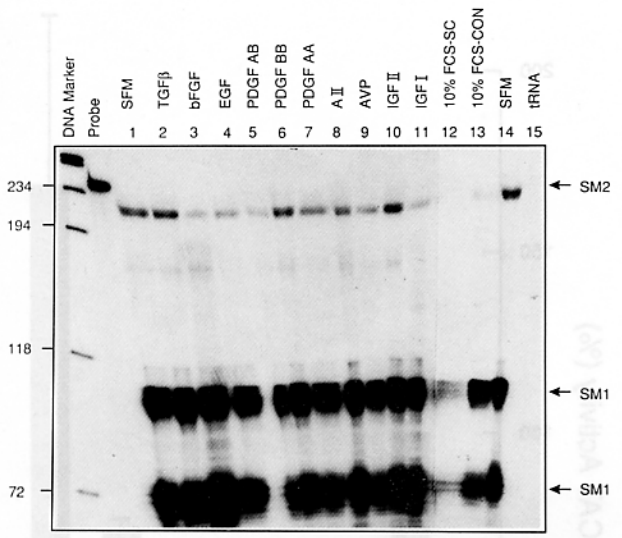

Figure 1a. Effect of growth factors, serum and contractile agonists on SM1 and SM2 SMHC mRNA levels in cultured VSMC. Cells were stimulated with TGFβ (5 ng/ml), EGF (100 ng/ml), bFGF, PDGF-AB, PDGF-BB, PDGF-AA, IGF-I and IGF-II (all 10 ng/ml), A-II and AVP (both 1 μM). SC = subconfluent, CON = confluent. RNase protection gel shown is representative of n = 3 experiments.

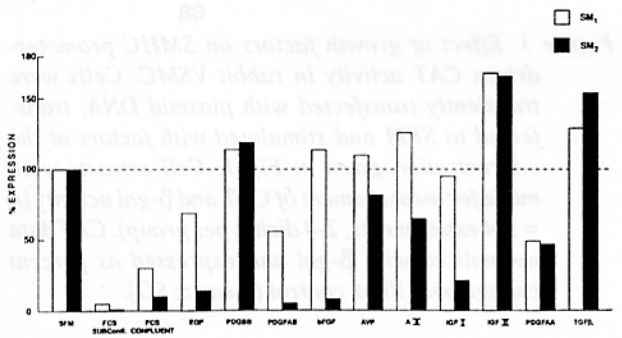

Figure 1b. Densitometric scanning data of SM1 and SM2 mRNA levels from representative RNase protection gel shown in Figure 1a. Data is expressed relative to mRNA levels obtained from VSMC maintained in SFM as control.

levels (< 10%) of SMHC expression were detectable in subconfluent VSMC grown in 10% FCS. SMHC expression was only partially restored to about 25% of control when cells were grown to confluency, suggesting that factors in serum had a dominant effect on SMHC expression.

When VSMC were stimulated with specific growth factors, PDGF-AA had the greatest effect on SM1 expression by decreasing it to about 50% of control; PDGF-AA also decreased SM2 mRNA level by the same amount. PDGF-AB and EGF treatment also led to lower SM1 mRNA levels (~60-70% of control) but these factors appeared to have a more profound effect on SM2 mRNA. The results in Fig 1b show that SM2 mRNA levels decreased to 5-10% of control thus indicating that the SM2 isoform was more sensitive to treatment with PDGF-AB and EGF. This idea was reinforced by the observation that SM2 mRNA levels fell to 10-20% of control following stimulation of VSMC by bFGF and IGFI even though these two factors had no significant effect on the SM1 isoform. The contractile agonists AVP and AII also reduced SM2 mRNA levles to 70-80% of control without any significant effect on SM1, thus pointing to the SM2 isoform as a primary target for the effect of these factors.

Whereas PDGF-AA and AB both significantly reduced SM1 and SM2 mRNA levels, PDGF-BB had no effect (Fig 1b) indicating that mitogenic signalling via this pathway was not important for SMHC expression in rabbit VSMC. This observation contrasts with the reported negative effect of PDGF-BB on smooth α-actin [7] expression in rat VSMC.

Furthermore although it has been reported that AVP and AII increase total protein content in rat VSMC and increase expression of specific proteins such as smooth α-actin [24], the results in Fig 1b show that these agonists had little effect (10-20%) on SMHC expression in rabbit VSMC.

The only cases where the levels of SM1 and SM2 mRNAs were consistently increased above control, were following stimulation with IGFII and TGF-β (Fig 1b). The greatest effect was seen with IGFII which increased SM1 and SM2 mRNA levels to 60-70% above control (n = 3); TGF-β increased the levels to 30-50% above control (n = 3).

Mitogenicity of growth factors does not correlate with SMHC expression in rabbit VSMC

It is known that serum induced growth of primary VSMC correlates with loss of the contractile phenotype [6] but the effect of specific mitogens on the expression of SMHC has not been studied. The results in Fig 2 show the effect of specific mitogens and agonists on ^{3}H-thymidine uptake into rabbit VSMC. Compared to serum, the factors EGF and PDGF-BB were the most mitogenic for rabbit VSMC. However, despite the similar degree of mitogenicity of EGF and PDGF-BB, their effects on SMHC expression were surprisingly different. EGF significantly downregulated SM1 and SM2 mRNA levels whereas PDGF-BB had no effect.

Moreover, the results in Fig 2 show that although PDGF-AB induced a relatively low level of ^{3}H-thymidine incorporation into rabbit VSMC and PDGF-AA had little or no effect, they both exert a major effect on SMHC expression by reducing mRNA levels to 50% or less of control. These results therefore suggest that mitogenicity does not directly correlate with the SMHC phenotype in VSMC. This is strengthened by the observation in Fig 2 showing that whereas IGFII, PDGF-AA and TGF-β are all effectively non-mitogenic for rabbit VSMC, PDGF-AA has the opposite effect to IGFII and TGF-β on SMHC expression.

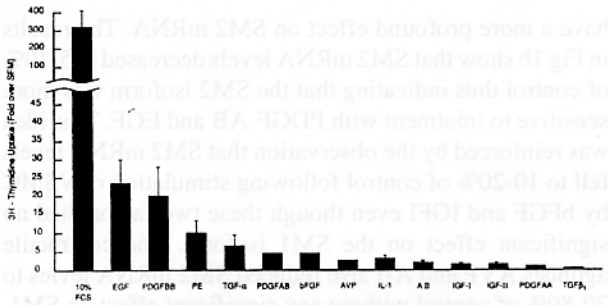

Figure 2. Estimation of DNA synthesis rates using ^{3}H-thymidine (n = 3-4 experiments, 2-3 dishes per group) in quiescent VSMC stimulated with various growth factors and other agents. Concentrations of factors as in Figure 1a. (PE = phorbol ester, 10 nM; TGFα = 10 ng/ml; IL-1 = interleukin 1, 5 U/ml). Data expressed as fold uptake (mean ± SD) over SFM control. (SD bars in some cases too small for presentation).

Growth factors exert an effect on the SMHC gene promoter in VSMC

Since it has been known that adult quiescent VSMC can depart from a contractile phenotype and undergo new rounds of cell division, efforts have been made to understand the regulation of myogenesis in VSMC. In the present experiments we attempted to address this question by investigating factors regulating activity of the SMHC gene promoter in rabbit VSMC.

Rabbit VSMC were transiently transfected with the plasmid pRSMHC-2,305 and cells were transferred to SFM for 48 hours before stimulation with factors for 48 hours. The results in Fig 3 show that compared to control cells, the growth factors tested had either a positive or negative effect on CAT activity driven by the SMHC promoter. Stimulation of VSMC with TGF-β resulted in an increase in CAT activity to 170 ± 47% ($p < 0.05$) of control. A similar range of CAT activity (165 - 185% greater than control) was observed with shorter SMHC promoter constructs down to pRSMHC-1,332 (data not shown). SMHC promoter constructs shorter than pRSMHC-1,332 did not respond to TGF-β (data not shown), thus suggesting that DNA elements distal to the minimal SMHC promoter (Kallmeier et al, in press) responded to TGF-β.

As a negative control in these experiments, we tested the activity of pRSMHC-2,305 in VSMC following stimulation with 10% FCS. The data in Fig 1 pointed to inhibition of SMHC expression in cultured VSMC grown in 10% FCS and this was partially supported by the result in Fig 3. Stimulation of VSMC with 10% FCS led to a decrease in CAT activity to 45 ± 17% of control ($p < 0.05$), thus raising the possibility that the SMHC promoter was adversely sensitive to factors in serum.

The data in Fig 2 indicated that EGF and PDGF-BB were strongly mitogenic for rabbit VSMC although they had differential effects on SMHC expression (Fig 1). Although EGF decreased and PDGF-BB had no effect on SMHC

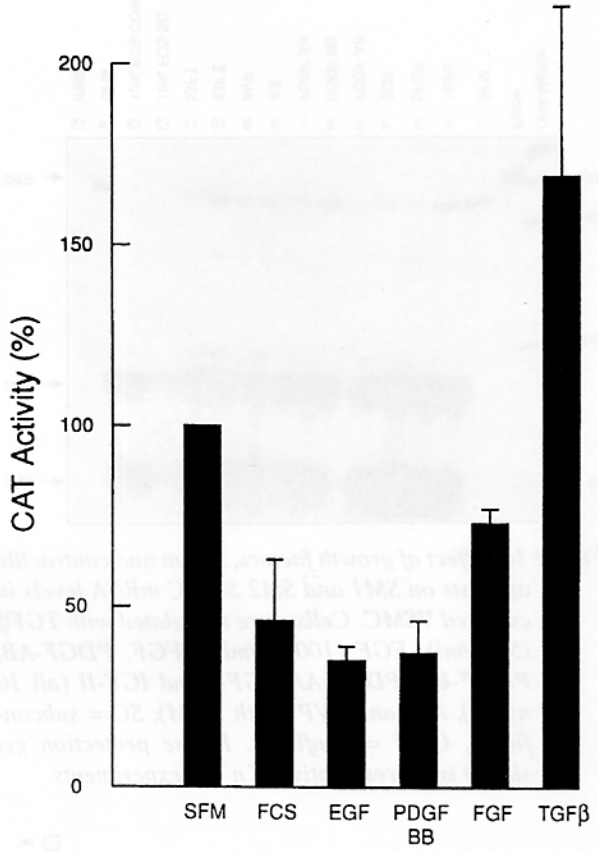

Figure 3. Effect of growth factors on SMHC promoter-driven CAT activity in rabbit VSMC. Cells were transiently transfected with plasmid DNA, transferred to SFM and stimulated with factors at the concentration given in Fig 1. Cell extracts were made for measurement of CAT and β-gal activity (n = 3-4 experiments, 2-4 dishes per group). CAT data normalized with β-gal and expressed as percent change over SFM control (mean ± SD).

expression, both mitogens were able to decrease CAT activity driven by the SMHC promoter to 34 ± 5% ($p < 0.05$) and 37 ± 10% ($p < 0.05$) of control respectively (Fig 3). These observations therefore indicate that SMHC expression may be controlled at different levels by specific growth factors.

Discussion

The present results show that expression of SM1 and SM2 SMHC isoforms in rabbit VSMC is differentially regulated by specific growth factors, is not directly correlated with mitogenicity and occurs via transcriptional and post-transcriptional processing mechanisms.

It is now known that at least three isoforms of SMHC are produced in smooth muscle tissues [1, 4, 27] by alternative splicing mechanisms thus resulting in the production of unique peptides with presently undetermined function. The combinatorial forms of the SMHC isoforms may play functional roles in thick filament assembly and regulation

of ATPase activity. In the present study, the ratio of SM1:SM2 isoforms showed a variable pattern of expression in response to VSMC stimulation by specific growth factors (Fig 1) but it is unknown if these changes in isoform expression result in altered VSMC function. The level of SM2 mRNA was apparently most sensitive to stimulation by the mitogens EGF, PDGF-AB and bFGF although PDGF-BB had no effect thus suggesting that mitogenicity and SM2 expression were not directly correlated. The observation (Fig 1) that the decrease in SM2 mRNA levels was relatively greater than SM1 following stimulation by EGF, PDGF-AB and bFGF suggested that SM2 expression may be controlled primarily by post-transcriptional processing events. The reason for this selective downregulation of SM2 expression in VSMC is unknown.

Although it has been suggested that the contractile phenotype of VSMC is compromised when cells undergo mitosis [6, 21, 23], the present results indicate that this effect may be mitogen-specific as indicated by the contrasting effects of PDGF-BB and PDGF-AA (Figs 1 and 2) on SMHC expression. Furthermore, the effects may also be gene-specific since the effect of PDGF-BB on smooth α-actin expression [7] is apparently opposite to SMHC. However, despite the observation that most growth factors tested had a negative effect on SMHC expression, we found that IGFII and TGF-β could augment SMHC expression in cultured rabbit VSMC (Fig 1). TGF-β has previously been shown to promote differentiation and hypertrophy of VSMC [18] and the similar effect observed for IGFII (Fig 1) suggests that both factors play a role in VSMC differentiation. These data together therefore point to the complex control of the contractile phenotype in VSMC.

To determine whether the SMHC promoter was transcriptionally active during stimulation of VSMC by growth factors and agonists, SMHC-driven CAT activity was measured in transiently transfected cells. VSMC were maintained in SFM for 48 hours before stimulation, thus ensuring that most cells were synchronized with respect to the cell cycle. Although the finding that CAT activity fell to < 50% of control following 10% FCS treatment is new, it is not entirely surprising and supports the view that SMHC expression is tightly coupled to factors in serum (Fig 1). The data in Fig 3 indicate that the growth factors EGF and PDGF-BB were as equally potent as serum in decreasing CAT activity but whereas EGF also decreased SMHC expression (Fig 1), PDGF-BB had no effect. One possible explanation for this effect is that the stability of SM1 and SM2 mRNAs is increased following PDGF-BB stimulation.

Whereas serum and the mitogens EGF, PDGF-BB and bFGF all decreased CAT activity from the SMHC promoter (Fig 3), TGF-β had the opposite effect by increasing CAT activity to ~170% of control. The magnitude of this change was similar to the increase in SMHC expression shown in Fig 1 thus providing indirect evidence for upregulation of SMHC expression by a transcriptional mechanism following stimulation of VSMC with TGF-β. The contrasting effects of different growth factors on promoter activity were recently demonstrated for the smooth α-actin promoter [25]. In this work CAT activity driven by the actin promoter was increased following stimulation of VSMC by angiotensin II but this upregulation was blocked by PDGF. This negative effect of PDGF on the smooth α-actin promoter therefore resembles the effect of PDGF-BB on SMHC promoter activity shown in Fig 3 of the present study. These data together indicate that PDGF negatively regulates transcription of contractile protein genes in VSMC.

In the present study, the level of CAT activity was similar for three other shorter SMHC promoter constructs which included sequences from -2,305 to -1,332 bp. Constructs made with even shorter 5' end deletion mutants down to the level of the minimal promoter did not respond to TGF-β treatment (data not shown), although we did not test the effect of more prolonged (72-96 hour) treatment of VSMC with TGF-β. These findings therefore indicate that TGF-β may exert an effect on the SMHC promoter via a distal DNA regulatory element. These and previous data [18] therefore support a role for TGF-β in regulating VSMC differentiation. The recent findings showing that SMHC exclusively marks the smooth muscle lineage during mouse embryogenesis [14] indicates that the SMHC promoter may provide an important tool for studying growth of normal and diseased smooth muscle tissues.

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