G-protein-coupled-receptor activation of the smooth muscle calponin gene

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A hallmark of cultured smooth muscle cells (SMCs) is the rapid down-regulation of several lineage-restricted genes that define their *in vivo* differentiated phenotype. Identifying factors that maintain an SMC differentiated phenotype has important implications in understanding the molecular underpinnings governing SMC differentiation and their subversion to an altered phenotype in various disease settings. Here, we show that several G-protein coupled receptors [α -thrombin, lysophosphatidic acid and angiotensin II (AII)] increase the expression of smooth muscle calponin (SM-Calp) in rat and human SMC. The increase in SM-Calp protein appears to be selective for G-protein-coupled receptors as epidermal growth factor was without effect. Studies using AII showed a 30-fold increase in SM-Calp protein, which was dose- and time-dependent and mediated by the angiotensin receptor-1 (AT₁ receptor). The increase in SM-Calp protein with

AII was attributable to transcriptional activation of SM-Calp based on increases in steady-state SM-Calp mRNA, increases in SM-Calp promoter activity and complete abrogation of protein induction with actinomycin D. To examine the potential role of extracellular signal-regulated kinase (Erk1/2), protein kinase B, p38 mitogen-activated protein kinase and protein kinase C in AII-induced SM-Calp, inhibitors to each of the signalling pathways were used. None of these signalling molecules appears to be crucial for AII-induced SM-Calp expression, although Erk1/2 may be partially involved. These results identify SM-Calp as a target of AII-mediated signalling, and suggest that the SMC response to AII may incorporate a novel activity of SM-Calp.

Key words: differentiation, promoter, signalling, smooth muscle cell, transcription.

INTRODUCTION

Smooth muscle cell (SMC) differentiation involves the orchestrated transcriptional control of several cell-restricted genes whose encoded proteins impart the unique physiological characteristics of this cell type. Most of these genes have been cloned and characterized both in terms of transcriptional regulation and their expression in various cellular, developmental, and pathological contexts [1]. Of particular note is the *in vitro* and *in vivo* down-regulation of such SMC-restricted genes as the smooth muscle isoforms of α -actin, myosin heavy chain and calponin [2–4]. Much effort has been directed towards understanding the nature of such changes in gene expression inasmuch as restoring their expression levels may not only yield a more reliable experimental model system to work from, but could provide a rational approach to treat SMC-associated diseases [5–7]. Towards this end, we and others have used the smooth muscle calponin (SM-Calp) gene as a model system to begin dissecting out the rules governing its expression *in vitro* and *in vivo* [4,8,9].

Several features of SM-Calp distinguish it from most other SMC-restricted markers. First, the proximal 5'-regulatory region of SM-Calp gene does not confer SMC-specific promoter activity *in vitro* and SMC regulatory elements commonly found in this region (e.g., CarG box) are absent [9,10]. Second, SM-Calp protein appears to serve multiple roles in the cell and is localized in different intracellular compartments, thus complicating a clear-cut assignment of function [11–14]. Finally, while the recent genetic inactivation of SM-Calp appears to support an earlier *in vitro* observation of SM-Calp function [15], an unexpected phenotype was discovered in healing bones [16]. These findings

point to layers of complexity surrounding SM-Calp's gene regulation and *in vivo* functions.

In a recent genomics study [17], we observed a dramatic increase in steady-state SM-Calp mRNA upon stimulation with the G-protein-coupled receptor (GPCR) α -thrombin. Here we extend these observations to another GPCR, angiotensin II (AII). While this manuscript was in preparation, another independent report showed a similar up-regulation of SM-Calp with AII [18]. Our results confirm the latter study, and extend it by showing that AII-induced increases in SM-Calp are transcriptionally mediated through *cis*-elements located in the first intron of SM-Calp. We further show that increases in SM-Calp occur, at least partially, through an extracellular signal-regulated kinase 1/2 (Erk1/2) pathway. These results demonstrate that SM-Calp is a target of AII-mediated signalling and suggest that elevations in SM-Calp may participate in the SMC response to this GPCR. Moreover, AII-treated SMC afford a unique opportunity to assess SM-Calp function in a cellular context where levels of the endogenous protein are ordinarily very low.

EXPERIMENTAL

Materials

AII was obtained from Peninsula Laboratories (Belmont, CA, U.S.A.). Monoclonal antibodies against human SM-Calp (clone hCP, C-2687) and smooth muscle α -actin (clone IA4, A-2547) were from Sigma. The hCP SM-Calp antibody has been shown to be specific for the smooth muscle isoform of calponin [9].

Abbreviations used: AII, angiotensin II; AT₁, angiotensin receptor-1; AT₂, angiotensin receptor-2; EGF, epidermal growth factor; Erk1/2, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphoinositide 3-kinase; PKB/Akt, protein kinase B; PKC, protein kinase C; SM-Calp, smooth muscle calponin; SMC, smooth muscle cell; RASMC-WKY, Primary-derived aortic SMCs from Wistar-Kyoto rats; –549, 5'-proximal promoter construct containing 549 nt upstream of initiation site; –549Int, –594 promoter construct plus the first intron of SM-Calp; –549Int, –549Int construct with a mutated CarG box.

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An antibody against c-Fos was from Santa Cruz Biotechnology (sc-052). Phospho-specific antibodies against Erk1/2, protein kinase B (PKB/Akt) and p38 mitogen-activated protein kinase (MAP kinase) were from New England Biolabs. Antibodies against Erk1/2 were a gift from Dr Michael J. Dunn (Medical College of Wisconsin, Milwaukee, WI, U.S.A.). Losartan (DUP753) was from Merck. PD123319 and PD098059 were from Research Biochemicals International (Natick, MA, U.S.A.) and Sigma. Actinomycin D, cycloheximide, LY294002, wortmannin and SB203580 were from Calbiochem.

SMC cultures

Primary-derived aortic SMCs from Wistar–Kyoto rats (RASMC-WKY) were obtained by enzymatic dissociation of the aortas as described previously [19]. These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 10% calf serum, 2 mM L-glutamine and antibiotics and used between passage numbers 5 and 15. Primary-derived aortic SMCs from Sprague–Dawley rats were obtained by a modified explant method as described previously [9], and used between passage number 10 and 20. These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine and antibiotics. Human coronary artery SMCs were obtained from a commercial vendor (Clonetics, San Diego, CA, U.S.A.) and used between passages 5 and 10. All SMC cultures were shown to express the SM-Calp and SM22 genes, two markers that characterize the SMC phenotype [20]. All the SMC types were grown to subconfluence and then growth-arrested in medium containing 0.2% calf serum for 36 h before addition of the following agonists: AII (0.1 nM–1 μ M), 2 units/ml of α -thrombin, 10 μ M LPA, 100 μ M carbachol and 100 ng/ml of EGF.

Western blotting

After stimulation of quiescent cells with the desired agonist, cells were lysed in buffer containing 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 μ M sodium orthovanadate and protease inhibitors (1 μ g/ml of leupeptin, 1 μ g/ml of aprotinin and 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20000 *g* for 10 min, subjected to 10% PAGE, transferred to nitrocellulose, analysed by Western blotting with the desired primary antibodies followed by horse radish peroxidase-conjugated secondary antibodies (Calbiochem), and developed by enhanced chemiluminescence reaction (Pierce).

RNase-protection assay

Following AII stimulation, duplicate cultures of RASMC-WKY were processed for total RNA isolation by the acid-guanidinium isothiocyanate procedure [21] and processed for the RNase protection assay [9]. The riboprobes used were a 200-bp 5'-fragment of the rat SM-Calp cDNA and a 465-bp fragment of the rat α -tubulin cDNA. The results shown are representative of two independent studies.

Transient transfection of SM-Calp luciferase reporter genes

To assess whether the SM-Calp locus was a target of AII signalling, we transfected RASMC-WKY with several SM-Calp promoter constructs we have studied previously [9,22]. Briefly, subconfluent cells grown in 24-well dishes were transfected with

LIPOFECTAMINE[™] Plus reagent (Gibco BRL) complexed with 1 μ g of an SM-Calp luciferase reporter [with either the 5'-proximal promoter construct containing 549 nt upstream of initiation site (–549), the –594 promoter construct plus the first intron of SM-Calp (–549Int) or the –549Int construct with a mutated CarG box (–549uInt)] and 50 ng of a thymidine kinase–Renilla reporter (to control for varying transfection efficiency, pipetting error, etc). Following a 4 h incubation, the transfection medium was adjusted to full volume with low FBS-containing medium and the incubation carried out for an additional 20 h. Cells were then washed and stimulated for 24 h with 200 nM AII. Cell lysates were then prepared with a commercial kit (Dual Luciferase Assay, Promega, Madison, WI, U.S.A.), and analysed for bioluminescence with an AutoLumat LB 953 luminometer (EG&G Berthold, Gaithersburg, MD, U.S.A.). All transfections were performed in quadruplicate and repeated in one additional experiment, which showed the same results reported here. Results were analysed with GraphPad Prism Software (Version 3.0, GraphPad Software, San Diego, CA, U.S.A.) using one-way ANOVA and Tukey's post-hoc test for intergroup comparisons. Results were considered statistically significant at $P < 0.05$.

RESULTS

GPCR activation of SM-Calp

In a previous study [17], we noted a dramatic increase in SM-Calp mRNA upon α -thrombin stimulation of SMC. To determine whether SM-Calp was induced by other GPCRs and if this response was conserved across different species of SMC, we measured SM-Calp protein expression following AII, lysophosphatidic acid (LPA) or α -thrombin stimulation in two rat aortic SMC types as well as human coronary artery SMC. Figure 1 shows variable elevations in SM-Calp protein across all three SMC types stimulated with various GPCRs agonists. In contrast, little change in expression of SM α -actin protein levels was observed in any of the SMC types studied (Figure 1B and results

Figure 1 Expression of SM-Calp in vascular smooth muscle cells in response to various agonists

Vascular SMCs derived from aorta of Wistar–Kyoto rats (RASMC-WKY, **A–C**), from aorta of Sprague–Dawley rats (RASMC-SD, **D**) and from human coronary artery (HCASMC, **E**), were serum-starved for 24 h followed by stimulation with 100 nM AII, 2 units/ml α -thrombin, 10 μ M LPA, 100 μ M carbachol and 100 ng/ml EGF for 24 h (**A, B, D and E**) or for 5 min (**C**). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp (**A, D and E**), SM α -actin (**B**), or with phospho-specific anti-ERK1/2 antibodies (**C**).

Figure 2 AII-induced up-regulation of SM-Calp in RASMC-WKY

Serum-starved RASMC-WKY were stimulated with various concentrations of AII for 24 h (A), or with 100 nM AII for various times (B), or were preincubated with or without 2 μ M DUP753 or 2 μ M PD123319 followed by stimulation with 100 nM AII for 6 h (C). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp. (D) Samples from the 6 h time point of 100 nM AII stimulation were serially diluted with the sample buffer and were analysed side-by-side, together with the control (zero time point). Abbreviation: C, control.

not shown). The muscarinic receptor agonist carbachol failed to increase SM-Calp protein in all three SMC types and to stimulate MAP kinase phosphorylation in RASMC-WKY (Figure 1C), suggesting the lack of muscarinic receptors in these cells. A receptor tyrosine kinase ligand, epidermal growth factor (EGF), had no effect on the expression of SM-Calp (Figure 1), although it potently stimulated Erk1/2 phosphorylation (Figure 1C). Taken together, the results of Figure 1 show a general trend of GPCR-mediated induction of SM-Calp protein in several distinct SMC lineages.

AII-induced expression of SM-Calp protein

Because SM-Calp was most strongly up-regulated in response to AII in RASMC-WKY (Figure 1A), we focused on this cellular model in order to investigate the mechanism of AII-induced expression of SM-Calp. Figure 2(A) shows a dose-response to AII, wherein SM-Calp expression was detectable at 10 nM AII and reached a maximum at 100 nM AII. Kinetically, AII elicited robust increases in SM-Calp protein at 3 h with levels reaching a maximum at 6 h (Figure 2B). Increases in steady-state SM-Calp protein persisted up to 24 h (Figure 2B) and 2 days (results not shown). The effect of AII on SM-Calp protein induction was entirely mediated by the AT₁ receptor, since losartan (DUP753) abolished expression whereas the AT₂ receptor antagonist PD123319 was without effect (Figure 2C). In order to estimate quantitatively the maximal effect of AII, we used a serial-dilution approach, wherein the cell lysates from the 6 h time point of AII treatment were serially diluted to approximate an SM-Calp signal similar to control intensity. Using this approach, we estimated that the maximal effect of AII on SM-Calp expression was approximately 30-fold over control levels (Figure 2D).

Figure 3 AII-induced upregulation of SM-Calp mRNA

Serum-starved RASMC-WKY were stimulated with 100 nM AII for the indicated times, then harvested for total RNA isolation and RNase protection assay using riboprobes specific to SM-Calp and the housekeeping gene, α -tubulin. Normalized densitometry revealed a peak increase in SM-Calp of 2.7-fold over baseline at 6 h after AII stimulation. The Figure shown is representative of two independent studies.

Figure 4 Effect of AII on SM-Calp promoter activity

Serum-starved RASMC-WKY were transfected as described in the Experimental section with the indicated plasmids, and then stimulated for 24 h with 100 nM AII. The results represent luciferase activity (relative light units) normalized to the Renilla reporter gene. *, significantly different from control; #, significantly different from -549Int plus AII ($P < 0.05$ in both cases). These results were reproduced in a second independent assay.

AII-induced expression of SM-Calp is transcriptionally mediated

To examine whether the induction of SM-Calp protein was regulated at the level of mRNA synthesis, we assessed the effect of AII on steady-state SM-Calp mRNA levels. The RNase protection assay demonstrated a > 2-fold increase in SM-Calp mRNA beginning 3 h after AII stimulation with mRNA levels remaining above baseline over a 24 h time period (Figure 3A). In contrast, AII had no significant effect on the expression of the housekeeping gene, α -tubulin (Figure 3B). We next investigated whether AII-induced expression of SM-Calp was regulated at the level of transcription using a transiently expressed luciferase reporter gene under control of the SM-Calp promoter. Two regions of the SM-Calp locus were examined: (i) a 5'-proximal promoter containing 549 nt upstream of the initiation site (-549), and (ii) the same -549 promoter construct plus the first intron of SM-Calp (-549Int). We recently identified a CArG box within the first intron of SM-Calp, which was responsible, in part, for serum-response-factor-mediated up-regulation of SM-Calp [22]. Therefore, we also used a construct with a mutated CArG box (-549uInt) in order to assess the potential role of the

Figure 5 Effect of actinomycin D and cycloheximide on AII-induced expression of SM-Calp

Serum-starved RASMC-WKY were pretreated for 30 min with 2 μ g/ml of actinomycin D (+ActD) or 5 μ g/ml of cycloheximide (+CHX) followed by stimulation with AII for 3 or 6 h as indicated. The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp.

Figure 6 AII-induced phosphorylation of Erk1/2, PKB/Akt and p38 MAP kinase

Serum-starved RASMC-WKY were stimulated with 100 nM AII for various times, lysed, followed by immunoblotting of cell lysates with phospho-specific Erk1/2 antibodies (A), normal Erk1/2 antibodies (B), phospho-specific PKB/Akt antibodies (C), and phospho-specific p38 MAP kinase antibodies (D).

consensus CArG box in AII-induced up-regulation of SM-Calp. As shown in Figure 4, AII caused no statistically significant change in -549 promoter activity. In contrast, we observed a statistically significant 2-fold increase in -549 Int activity, which was partially attenuated in the context of a mutated consensus CArG box (Figure 4). Taken together, these results indicate that in RASMC-WKY, addition of AII results in a modest up-regulation of both SM-Calp mRNA and promoter activity, the latter of which required elements (e.g. CArG box) within the first intron of SM-Calp.

Further evidence suggesting that the increase in SM-Calp protein expression was transcriptionally mediated is provided in Figure 5. The RNA polymerase II inhibitor actinomycin D completely blocked AII-induced expression of SM-Calp protein at 3 and 6 h. Cycloheximide treatment similarly inhibited elevations in SM-Calp protein levels. These results suggest that while the elevation in SM-Calp mRNA is rather modest (approx. 2-fold), the increase is apparently sufficient to account for the rise in SM-Calp protein.

Role of MAP kinase signalling in AII-induced SM-Calp expression

To further examine the mechanism of AII-induced expression of SM-Calp, we investigated the possible roles of Erk1/2, PKB/Akt, p38 MAP kinase, and protein kinase C (PKC) whose involvement in the regulation of gene expression is well established. In RASMC-WKY, AII stimulated a transient phosphorylation of

Figure 7 Effect of PD098059 on AII-induced SM-Calp expression and Erk1/2 phosphorylation

Serum-starved RASMC-WKY were preincubated with increasing concentrations of PD098059 for 1 h as indicated, followed by stimulation with 100 nM AII for 6 h (A and C) or for 5 min (B). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp (A), Erk1/2 (B) or c-Fos (C).

Figure 8 Effect of LY294002 and wortmannin on AII-induced SM-Calp expression and PKB/Akt phosphorylation

Serum-starved RASMC-WKY were preincubated with increasing concentrations of LY294002 or wortmannin (Wortm) for 1 h as indicated, followed by stimulation with 100 nM AII for 6 h (A) or for 5 min (B). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp (A) or with phospho-specific antibodies against PKB/Akt (B).

p42 and p44 isoforms of Erk (Figures 6A and 6B), PKB/Akt (Figure 6C) and p38 MAP kinase (Figure 6D), as determined by immunoblotting with corresponding phospho-specific antibodies (Figures 6A, 6C and 6D), as well as by gel retardation of phosphorylated Erk1/2 (Figure 6B). Erk1/2 is generally activated by MAP-kinase kinase ('MEK1'), whereas PKB/Akt is activated by a phosphoinositide-3-kinase-dependent mechanism. Therefore to assess the role of Erk1/2 and PKB/Akt in AII-induced SM-Calp expression, we employed an inhibitor of MAP-kinase kinase, PD098059, and inhibitors of phosphoinositide 3-kinase (PI 3-kinase) (LY294002 and wortmannin). The role of p38 MAP kinase was examined employing its inhibitor SB203580. Finally, the role of PKC was examined by 24 h preincubation of cells with PMA resulting in depletion of phorbol-sensitive PKC isoforms.

Preincubation of RASMC-WKY with increasing doses of PD098059 resulted in a modest, but consistent, attenuation of AII-induced SM-Calp expression, reaching approx. 2-fold inhibition at PD098059 concentrations of 30 μ M (Figure 7A). The dose-response of SM-Calp expression to PD098059 was similar to that of Erk1/2 phosphorylation (Figure 7B), suggesting that AII-induced up-regulation of SM-Calp could be partially

Figure 9 Effect of SB230580 on AII-induced SM-Calp expression and p38 MAP kinase phosphorylation

Serum-starved RASMC-WKY were preincubated with increasing concentrations of SB230580 for 1 h as indicated, followed by stimulation with 100 nM AII for 6 h (A) or for 5 min (B). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp (A) or with phospho-specific antibodies against p38 MAP kinase (B).

Figure 10 Effect of PMA on AII-induced SM-Calp expression and ERK phosphorylation

Serum-starved RASMC-WKY were preincubated with or without 1 μM PMA for 24 h as indicated, followed by stimulation with 100 nM AII or 100 nM PMA for 6 h (A) or for 5 min (B). The cells were lysed, and equal amounts of cell lysates were subjected to immunoblotting with antibodies against SM-Calp (A) or with phospho-specific ERK1/2 antibodies (B).

mediated by Erk1/2. However, PD098059 was much more potent in the inhibition of AII-induced c-Fos expression, having completely abrogated the effect of AII at concentrations of 10 μM (Figure 7C).

Preincubation of cells with the PI 3-kinase inhibitor LY294002 resulted in a dose-dependent inhibition of AII-induced SM-Calp expression, reaching nearly complete inhibition at concentrations of 50 μM (Figure 8A). However, there was no correlation between the ability of LY098059 to attenuate SM-Calp expression and PKB/Akt phosphorylation, since the latter was completely abolished by LY098059 at as low a concentration as 5 μM (Figure 8B). This suggests that LY098059 elicits a non-specific effect on SM-Calp expression which is not related to the activation of PKB/Akt. Moreover, another inhibitor of PI 3-kinase, wortmannin, had no effect on SM-Calp expression when used at concentrations of up to 1 μM (Figure 8A), whereas it completely abolished PKB/Akt phosphorylation at concentrations of 0.1 μM (Figure 8B). Taken together, these results suggest that PI 3-kinase and PKB/Akt are not involved in AII-induced up-regulation of SM-Calp. Similarly, the inhibitor of p38 MAP kinase, SB230580, significantly attenuated AII-induced SM-Calp expression only at very high concentrations (30 μM) (Figure 9A), whereas phosphorylation of p38 MAP kinase was abolished at as

low a concentration of SB230580 as 1 μM (Figure 9). Interestingly, 30 μM SB230580 also inhibited AII-induced phosphorylation of Erk1/2, which may explain its effect on SM-Calp expression at high concentrations (results not shown). Finally, an activator of PKC, PMA, had no effect on SM-Calp expression (Figure 10A). Moreover, depletion of phorbol-sensitive PKC isoforms by long-term treatment of SMC with PMA did not affect AII-induced SM-Calp expression (Figure 10A), but abrogated PMA-stimulated (PKC-mediated) Erk1/2 phosphorylation (Figure 10B). This suggests that phorbol-sensitive PKCs are also not involved in AII-induced SM-Calp expression. In addition, in RASMC, AII-induced Erk1/2 phosphorylation was not dependent on phorbol-sensitive PKCs, because pretreatment of cells with PMA was without effect (Figure 10B).

DISCUSSION

The results of this study show that the GPCR ligand, AII, potentially activates the expression of SM-Calp in cultured SMCs. In a recent report [18] that was published while this manuscript was in preparation, a similar activation of SM-Calp by AII was observed in aortic SMCs derived from Sprague-Dawley rats. Both the latter report and the results here demonstrate that AII-mediated activation of SM-Calp occurs through the AT₁ receptor. Here we have extended these general observations by providing several lines of evidence that support a role for the transcriptional activation of SM-Calp in facilitating AII-induced SM-Calp protein expression. First, transient transfection studies indicate a 2-fold increase in SM-Calp promoter activity when the first intron of SM-Calp is present. Second, steady-state mRNA levels were consistently elevated > 2-fold (in agreement with the transfection results) upon AII stimulation. Finally, exposure to the RNA polymerase II inhibitor, actinomycin D, revealed complete suppression of AII-induced SM-Calp protein as early as 3 h after stimulation.

It is important to point out that the activation of SM-Calp by AII was not seen with respect to the smooth muscle α -actin protein (Figure 1B). A previous study by Hautmann et al. [23] showed that AII-activated transcription of the smooth muscle α -actin gene through the recruitment of the homeodomain-containing protein Mhox (mesodermal homeobox) to a serum-response factor-binding CARG box. The latter finding is in agreement with our transient transfection results showing some attenuation of AII-induced SM-Calp promoter activity when the intronic CARG box is mutated (Figure 4). It is possible that there are elevations in smooth muscle α -actin mRNA in our system that do not substantively contribute to the overall pool of smooth muscle α -actin protein given the already high-level expression of this protein in our cells. In contrast, SM-Calp (and other more stringent markers of this lineage such as smooth muscle myosin heavy chain [24]) are notoriously low in cultured SMCs so that any activation of these genes might easily reveal elevations in steady-state protein levels. It will be of some interest to define the localization of SM-Calp in AII-treated SMCs and begin assessing its possible function in this cellular context. It will be important to ascertain whether other contractile genes that are CARG-dependent, such as smooth muscle myosin heavy chain, show similar elevations in protein expression. A careful examination of the contractile competence of such cells should also be made. The latter would be of great utility since the de-differentiation of SMC *in vitro* has complicated efforts to fully grasp the relative importance of SM-Calp and other marker genes (e.g. smoothelin [25]) in SMC contraction.

In addition to providing evidence supporting a transcriptional basis for the increase in SM-Calp protein with AII stimulation,

we have begun to dissect out the signalling pathways that may be involved. First, we have shown that the activation of SM-Calp occurs with the GPCR for AII, but not the tyrosine kinase receptor for EGF (Figure 1). It should be noted, however, that transforming growth factor- β 1, which binds a serine-threonine kinase receptor, has also been shown to activate several SMC markers including smooth muscle α -actin, smooth muscle myosin heavy chain, and SM-Calp [17,26]. Second, studies in this report show that while AII activates several prominent signalling pathways involved with gene expression (Erk1/2, PKB/Akt, p38 MAP kinase and PKC), none of them seems to be crucial for AII-induced SM-Calp protein, although Erk1/2 may be partially involved. Thus there are likely to be other, as yet undefined, pathways involved with both the transcriptional and translational activation of SM-Calp by AII.

The AT₁ and AT₂ receptors have opposing actions in SMCs. The AT₂ receptor is expressed during development and after balloon injury of the vessel wall and appears to be involved with anti-mitogenesis whilst the AT₁ receptor is expressed predominantly in postnatal vessels and mediates much of the growth-promoting effects of AII [27]. The fact that SM-Calp protein levels can be dramatically elevated with AII points to a possible role for this hormone in the stimulation of an SMC-differentiated phenotype. In this regard, Yamada et al. [28] found a significant decrease in SM-Calp mRNA and protein in mice null for the AT₂ receptor. The AT₂ receptor does not signal increases in SM-Calp in adult SMCs ([18] and this study), because levels of this receptor are restricted to embryonic development [27]. Thus, while the AT₁ and AT₂ receptors are often considered to have opposing biological actions, the results, with respect to SMC-differentiation gene expression, would suggest that both receptors are competent to activate SM-Calp expression.

In summary, we have shown that AII is a potent inducer of SM-Calp protein and that such stimulation requires some level of SM-Calp transcriptional activation, possibly through an Erk1/2-dependent pathway. Future work should be directed towards elucidating the functional role of SM-Calp in AII-induced SMC responses.

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