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Original article

Inhibition of Egr1 expression underlies the anti-mitogenic effects of cAMP in vascular smooth muscle cells

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ABSTRACT

Aims: Cyclic AMP inhibits vascular smooth muscle cell (VSMC) proliferation which is important in the aetiology of numerous vascular diseases. The anti-mitogenic properties of cAMP in VSMC are dependent on activation of protein kinase A (PKA) and exchange protein activated by cAMP (EPAC), but the mechanisms are unclear.
Methods and results: Selective agonists of PKA and EPAC synergistically inhibited Egr1 expression, which was essential for VSMC proliferation. Forskolin, adenosine, A2B receptor agonist BAY60-6583 and Cicaprost also inhibited Egr1 expression in VSMC but not in endothelial cells. Inhibition of Egr1 by cAMP was independent of cAMP response element binding protein (CREB) activity but dependent on inhibition of serum response element (SRE) activity. SRF binding to the Egr1 promoter was not modulated by cAMP stimulation. However, Egr1 expression was dependent on the SRF co-factors Elk1 and 4 but independent of MAL. Inhibition of SRE-dependent Egr1 expression was due to synergistic inhibition of Rac1 activity by PKA and EPAC, resulting in rapid cytoskeleton remodelling and nuclear export of ERK1/2. This was associated with de-phosphorylation of the SRF co-factor Elk1.
Conclusion: cAMP inhibits VSMC proliferation by rapidly inhibiting Egr1 expression. This occurs, at least in part, via inhibition of Rac1 activity leading to rapid actin-cytoskeleton remodelling, nuclear export of ERK1/2, impaired Elk1-phosphorylation and inhibition of SRE activity. This identifies one of the earliest mechanisms underlying the anti-mitogenic effects of cAMP in VSMC but not in endothelial cells, making it an attractive target for selective inhibition of VSMC proliferation.

1.0. Introduction

Vascular smooth muscle cell (VSMC) proliferation contributes towards the development of various vascular diseases characterised by pathological intima formation, including atherosclerosis [1], transplant vasculopathy and pulmonary hypertension. Increased VSMC proliferation also contributes towards neointima formation after balloon angioplasty with and without stenting and vein grafting, limiting long-term success of clinical interventions designed to treat atherosclerosis. A complete understanding of the mechanisms regulating VSMC proliferation is therefore essential for the development of new therapies.

In healthy vessels, VSMCs have extremely low rates of proliferation but this is elevated by injury or insult to the vessel wall. Pathological loss of quiescence is triggered by release of mitogens from platelets and VSMCs that activate signalling pathways that stimulate expression of cell-cycle genes. Inactivation of negative signals that normally repress VSMC proliferation is also required. The second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) is a well characterised inhibitor of VSMC proliferation implicated in maintaining VSMC quiescence and promoting healing after vessel injury [2–4]. Elevated cAMP inhibits VSMC proliferation in vitro and in vivo after vascular injury, ultimately leading to a reduction in intima formation [4]. Although this phenomenon has been recognised for many years, the underlying mechanisms have remained incompletely understood. Immediate events include activation of PKA which for many years was believed to be responsible for all cAMP-mediated effects. PKA inhibition reverses anti-mitogenic effect in VSMCs [5] but selective activation of PKA does not inhibit VSMC proliferation, implicating a second cAMP-sensitive pathway [5]. EPAC1 is a cAMP-sensitive protein with intrinsic GEF activity that couples cAMP to activation of members of the Ras-like family of GTPases, such as Rap1. Using EPAC-selective cAMP-analogues, we demonstrated that PKA and EPAC pathways act synergistically to repress VSMC proliferation [5]. However, the mechanisms underlying this synergy remained elusive. cAMP blocks progression through the G1-phase of the cell-cycle, at

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least in part by inhibiting expression of multiple cell-cycle genes needed for S-phase entry (e.g. Skp2, Cyclin D, c-myc) but these are relatively late events following cAMP elevation [4,6,7]. Although cAMP inhibits ERK phosphorylation in VSMC, prompting speculation that this underlies the anti-mitogenic effects of cAMP, other studies show that cAMP-anti-mitogenesis can be dissociated from ERK inhibition, implying the involvement of alternative mechanisms [3].

We characterised the early transcriptomic response in VSMCs after selective activation of PKA, EPAC or both to identify novel mechanisms underlying their ability to synergistically inhibit VSMC proliferation. We identified, for the first time, coordinated inhibition of the immediate response gene Early growth response 1 (Egr1) by PKA and EPAC and showed this to be essential for cell-cycle control. cAMP-mediated repression of Egr1 expression resulted from inhibition of serum-response element activity via a mechanism involving the rapid inhibition of Rac1-mediated actin-cytoskeleton remodelling, nuclear export of ERK1/2 and de-phosphorylation of the SRF co-factor, Elk1. Furthermore, we provide evidence that this mechanism is cell-type specific, accounting at least in part for the divergent effects of cAMP on VSMC and Endothelial cell proliferation,

2. Methods

Detailed materials and methods are outlined in the supplement.

2.1. Smooth muscle cell culture and HUVEC and bromo-deoxyuridine (BrdU) labelling

Male Sprague Dawley rats were killed by cervical dislocation in accordance with the Directive 2010/63/EU of the European Parliament. Approval was granted by the University of Bristol ethical review board. Medial tissue was carefully dissected from the thoracic aorta and cut into 1 mm^2 pieces for explant culture, essentially as described previously [6]. Pooled donor HUVECs were purchased from Promocell and cultured in Promocell endothelial cell culture media (C-22210) with 2% FCS unless otherwise stated. Stimulations were performed in 5% foetal calf serum/DMEM unless otherwise stated. Proliferation was measured by culture in the presence of 10 μM bromo-deoxyuridine. Following fixation in 70% ethanol, incorporated bromo-deoxyuridine was detected by immune-histochemical staining as previously described [5].

2.2. Quantitative RT-PCR and western blotting

Quantification of mRNA and protein levels was performed by qRT-PCR and western blotting essentially as described previously [5].

2.3. Transfection and recombinant adenoviruses transduction

Plasmid transfection was performed by using nucleofection as previously described [5].

2.4. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed essentially as described previously [8].

3. Results

3.1. Selective activation of EPAC and PKA using selective cAMP-analogues

We used the cAMP analogues 8-CPT-2′-O-Me-cAMP (CPT) and N^6-Benzoyl-cAMP (BNZ) to selectively probe the biological function of EPAC and PKA, respectively. Increasing concentrations of CPT dose-dependently activated the EPAC-effector protein, Rap1 (Supplement Fig. 1A). BNZ stimulation dose-dependently activated PKA (Supplement Fig. 1B). Activation of both EPAC-Rap1 and PKA was detected after 30 min and persisted for at least 2 h (Supplement Fig. 1C). Importantly, CPT did not stimulate any detectable PKA activation or phosphorylation of the PKA/PKG substrate VASP at any time-point, confirming highly selective EPAC activation. Likewise, BNZ did not stimulate EPAC-Rap1 signalling, confirming selective PKA activation (Supplement Fig. 1C). The effects are consistent with our previous observations [5].

3.2. Characterisation of EPAC and PKA-dependent transcriptomes in VSMC

We characterised the transcriptome of VSMC stimulated with EPAC or PKA agonists to gain a mechanistic insight into the anti-mitogenic action of cAMP. Stimulation with 200 μM of each agonist, alone or in combination, for 8 h identified 86 significantly regulated transcripts; the twenty most changed are shown in Table 1. CPT stimulation alone regulated only one gene compared to control (Zfp365; 1.28-fold down-regulated), despite efficient activation of EPAC-Rap1 signalling in these cultures (Supplement Fig. 2). Gene ontology analysis of all 86 genes identified functions associated with ERK1/2 signalling, signal transduction and cardiovascular disease (Supplement Table 3). Validation of selected genes by qRT-PCR confirmed the array data (Supplement Fig. 3) and identified an additional small but significant inhibition of EGR1 and CNKSR3 in response to CPT. Furthermore, CPT significantly amplified the BNZ-dependent suppression of Egr1 expression.

3.3. PKA and EPAC synergise to inhibit Egr1 expression

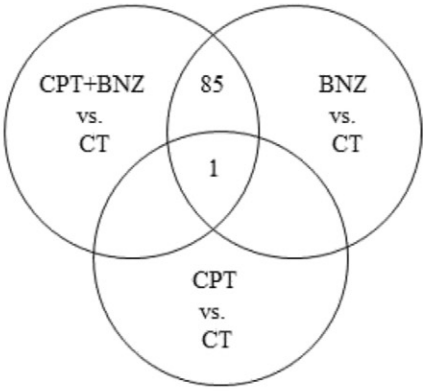
Based on the transcriptomic and qRT-PCR data, we focussed on Egr1, given its role in cell-cycle regulation [9] and neointima formation [10]. Time course analysis demonstrated transient inhibition of Egr1 mRNA by BNZ from 2 to 8 h, returning to control levels after 18 h (Fig. 1A). Importantly, CPT significantly deepened Egr1 mRNA inhibition by BNZ. In this experiment, CPT had no significant effect on Egr1 mRNA, demonstrating a synergistic action of PKA and EPAC pathways. To further confirm a role of EPAC, cells were infected with recombinant adenovirus expressing constitutively active EPAC (Ad:active-EPAC) [5]. BNZ stimulation of Ad:active-EPAC infected cells resulted in a significantly greater inhibition of Egr1 mRNA levels compared to Ad:Control infected cells (Supplement Fig. 4). Stimulation of uninfected cells with neither BNZ nor CPT alone for either 4 or 8 h significantly reduced Egr1 protein levels (Fig. 1B). However, co-stimulation with BNZ plus CPT for 8 h dramatically reduced Egr1 protein to levels significantly lower than BNZ alone, further demonstrating synergistic inhibition of Egr1 by PKA and EPAC.

3.4. Species and cell-type dependent inhibition of Egr1 expression by cAMP

The adenylate–cyclase activator, Forskolin, which activated both PKA and EPAC pathways (Supplement Fig. 1C), rapidly inhibited Egr1 mRNA and Egr1 protein levels after 8 h (Fig. 1C) in cells cultured continuously in the presence of 5% serum mitogens. This effect occurred in rat (Fig. 1C) or human aortic VSMC (Fig. 1D). Furthermore, stimulation with Forskolin or BNZ plus CPT inhibited $PDGF_{BB}$ stimulated Egr1 mRNA and protein expression in rat VSMC (Supplement Fig. 5). Forskolin stimulation did not however inhibit Egr1 mRNA or protein expression in human umbilical vein endothelial cells (HUVEC) cultured asynchronously in 5% serum mitogens (Fig. 1E). Forskolin stimulation significantly stimulated Egr1 mRNA levels in serum-starved HUVEC and significantly enhanced VEGF-A stimulated Egr1 mRNA (Supplement Fig. 6) indicating that cAMP-mediated inhibition of Egr1 is cell-type specific. Co-incubation of VSMC with a pharmacological PKA inhibitor completely prevented Forskolin-mediated inhibition of Egr1 (Fig. 1F), consistent with an essential role of PKA. Furthermore, siRNA-mediated silencing of EPAC (Fig. 1G) also prevented the Forskolin inhibitor of Egr1 (Fig. 1H), consistent with a role of EPAC.

Table 1
List of highest and lowest regulated genes (fold change) following stimulation with BNZ, CPT or both in combination for 8 h. Venn diagram shows distribution of all 86 significantly regulated genes.

Gene symbol	BNZ	CPT	CPT+BNZ
A_64_P083309	90.78269		85.85053
Cxcl13	10.044527		8.67422
Cd55	9.209881		7.38491
Has1	8.071952		6.3642187
Nr4a1	7.979323		5.717209
Mall	7.402537		6.8510246
Crem	7.2448845		7.1704664
Sik1	6.8623953		6.3758006
Ypel4	6.718018		7.727832
Pde4b	5.9444585		6.0033617
Zfp365	−1.762975	−1.2890157	−1.7610738
LOC690460	−2.5759995		−2.43896
Egr1	−2.6028636		−4.0659876
Osr1	−2.7095006		−2.85619
Bmp3	−3.0719805		−3.1082225
Cnksr3	−3.1754086		−3.4565294
Cnksr3_1	−3.5447962		−3.407171
LOC690276	−5.0445037		−4.5350156
Adcy10	−5.5453434		−7.5765357
Pln	−6.3946085		−6.6119823



3.5. Adenosine and Cicaprost inhibit Egr1 expression in VSMC dependent on PKA and EPAC

Vasoactive agents, including adenosine and prostacyclin mediate cAMP production and inhibition of VSMC proliferation by activation of G-protein coupled receptors (GPCRs). To test if activation of these GPCRs inhibits Egr1 expression we stimulated VSMC either with adenosine, the specific adenosine A2B-receptor agonist BAY60-6583 or the prostacyclin mimetic, Cicaprost. These agonists increased phosphorylation of the PKA substrate VASP and activated the EPAC effector, Rap1 (Supplement Fig. 7), confirming activation of both pathways. Adenosine and BAY60-6583 significantly inhibited Egr1 mRNA levels after 2 and 4 h (Fig. 2A) and strongly inhibited Egr1 protein levels after 8 h (Fig. 2A inset). Cicaprost stimulation also transiently inhibited Egr1 mRNA expression after 2 h, returning to control levels after 4 h (Fig. 2B), although Egr1 protein levels were still reduced after 8 h. The PKA inhibitor Rp-cAMPS significantly attenuated BAY60-6583 (Fig. 2C) and Cicaprost (Fig. 2D) mediated inhibition of Egr1. EPAC silencing also rendered cells insensitive to BAY60-6583 or Cicaprost-mediated inhibition of Egr1 expression (Fig. 2E).

3.6. Egr1 is required for VSMC proliferation and its inhibition contributes towards cAMP-induced growth arrest

Egr1 is essential for proliferation in many but not all [11] cell types. To test if Egr1 inhibition contributes to VSMC proliferation we used overexpression of a dominant-negative (DN) Egr1 or NAB2, an Egr1 repressor, to inhibit Egr1 transcriptional activity (Supplement Fig. 8). DN-Egr1 and NAB2 expression both significantly inhibited VSMC proliferation (Fig. 3A, B, C), demonstrating a requirement for Egr1 activity for efficient cell-cycle progression in VSMC. To test the importance of Egr1 inhibition for the anti-mitogenic effects of cAMP we quantified the ability of Forskolin to inhibit proliferation in cells lacking Egr1 activity (achieved via NAB2 overexpression). Forskolin stimulation inhibited proliferation in control adenovirus infected cells (Fig. 3C). Infection with Ad:NAB2 also significantly inhibited proliferation (Fig. 3B, C). However, Forskolin no longer significantly inhibited VSMC proliferation in NAB2 overexpressing cells compared to untreated NAB2 overexpressing cells (Fig. 3C), which lack Egr1 activity (Supplement Fig. 8). Proliferation in Ad:Control plus Forskolin and Ad:NAB2 plus Forskolin cells was not significantly different.

3.7. Elevation of cAMP inhibits Egr1 transcription

To test if cAMP reduces Egr1 mRNA levels by inhibiting Egr1 gene transcription we quantified Egr1 hnRNA (pre-spliced RNA), a surrogate measure of transcriptional rate, as previously described [12], and activity of a −600 bp Egr1 promoter luciferase reporter (Egr1-luc). Forskolin treatment potently inhibited levels of Egr1 hnRNA within 1 h, with maximal inhibition after 4 h (Fig. 4A) and also significantly inhibited Egr1-luc promoter activity after 4 and 8 h (Fig. 4B). Inhibition of Egr1-luc reporter activity was slower than endogenous hnRNA presumably due to inherent differences in turnover rates of luciferase and Egr1 protein and mRNA. To test if PKA and EPAC cooperate to inhibit Egr1 transcription, cells were stimulated with BNZ, CPT or BNZ plus CPT (Fig. 4C). BNZ or CPT stimulation alone resulted in modest but significant inhibition of Egr1 promoter activity that was enhanced by co-stimulation with BNZ and CPT to levels that were significantly less than BNZ or CPT alone. Taken together, this data demonstrates that cAMP mediates inhibition of Egr1 gene transcription in VSMC through PKA and EPAC.

3.8. Elevation of cAMP inhibits Egr1 expression via a CREB-independent but SRF-dependent pathway

The proximal Egr1 promoter contains two binding elements for CREB. CREB activation by cAMP has been linked to regulation of gene transcription implicated in inhibition of VSMC proliferation [13]. We therefore tested if CREB activation underlies the cAMP-mediated

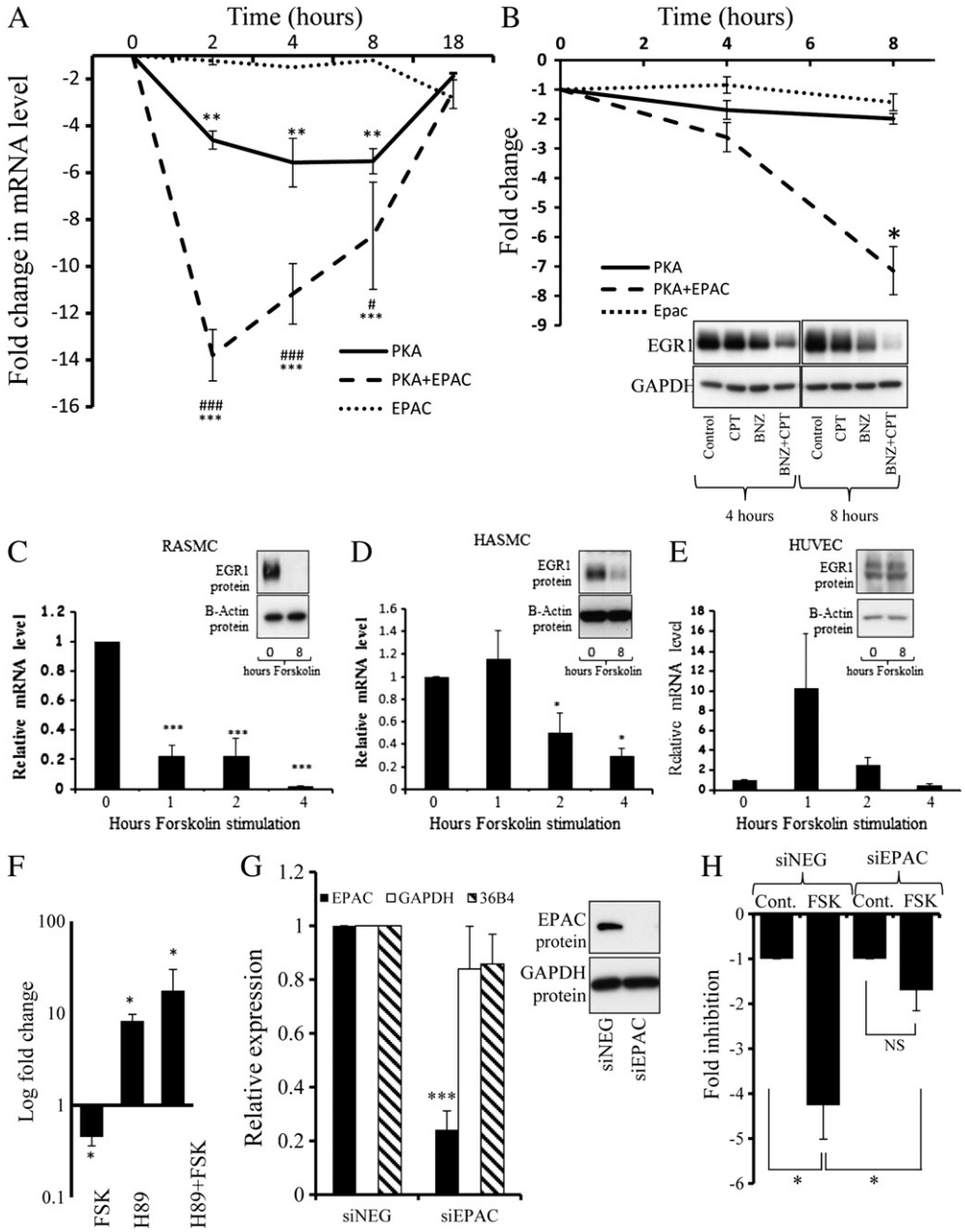


Fig. 1. cAMP-elevating stimuli inhibit Egr1 expression via PKA and Epac in VSMC but not EC. VSMCs were stimulated with 200 μM BNZ, CPT or both for times indicated. Egr1 mRNA was quantified by qRT-PCR (A) and protein by western blotting (B). Rat (C) or Human (D) aortic VSMC or HUVEC (E) were stimulated with 25 μM Forskolin for the indicated times and Egr1 mRNA and protein was measured by qRT-PCR (graphs) and western blotting (inset). Cells pre-treated with 10 μM H89 for 10 min were stimulated with Forskolin for 2 h (F). Cells were transfected with control or EPAC-targeting siRNA and EPAC mRNA and protein analysed after 48 h (G) or cells stimulated with Forskolin for 2 h and Egr1 mRNA quantified (H). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ relative to control. # symbols indicate the same p values but versus BNZ alone samples.

inhibition of Egr1. BNZ stimulation increased CREB-dependent reporter gene activity, consistent with CREB activation (Fig. 5A) which was completely blocked by adenovirus-mediated expression of dominant-negative A-CREB (Ad:A-CREB) (Fig. 5B). Ad:A-CREB infection also completely blocked BNZ stimulated expression of classical CREB-dependent genes, CREM and HAS (Fig. 5C), confirming efficient inhibition of CREB activity. Importantly, Ad:A-CREB had no effect on BNZ-mediated inhibition of Egr1 mRNA expression (Fig. 5D), indicating that this operates via a CREB-independent mechanism.

The Egr1 promoter also contains multiple serum response elements (SREs) that bind serum response factor (SRF) and members of the Ternary Complex Factor (TCF) family, including Elk1 that function as SRF co-factors. Given the central role of SRF and its co-factors in controlling proliferation, we asked if modulation of SRE activity could explain cAMP-mediated inhibition of Egr1 expression in VSMC. Consistent with this, Forskolin inhibited SRE-dependent reporter activity 4 and 8 h after stimulation (Fig. 5E). CPT stimulation did not affect SRE-luc activity, whereas BNZ resulted in a small but significant inhibition (Fig. 5F). Furthermore, BNZ inhibition of SRE-luc activity was further enhanced by co-stimulation with CPT (Fig. 5F). This data indicates that cAMP mediates suppression of Egr1 expression via PKA and EPAC inhibition of SRE activity. Consistent with this, Egr1 promoter activity was inhibited by truncation of the distal SRE elements and abolished by mutation of the proximal pair of SREs (Fig. 5G). We next asked if cAMP-mediated inhibition of Egr1 was mediated by reduced SRF binding to the Egr1 promoter. Surprisingly, despite significant inhibition of SRE-dependent transcriptional activity, Forskolin stimulation had no effect on SRF binding, measured by ChIP, to either the proximal or distal

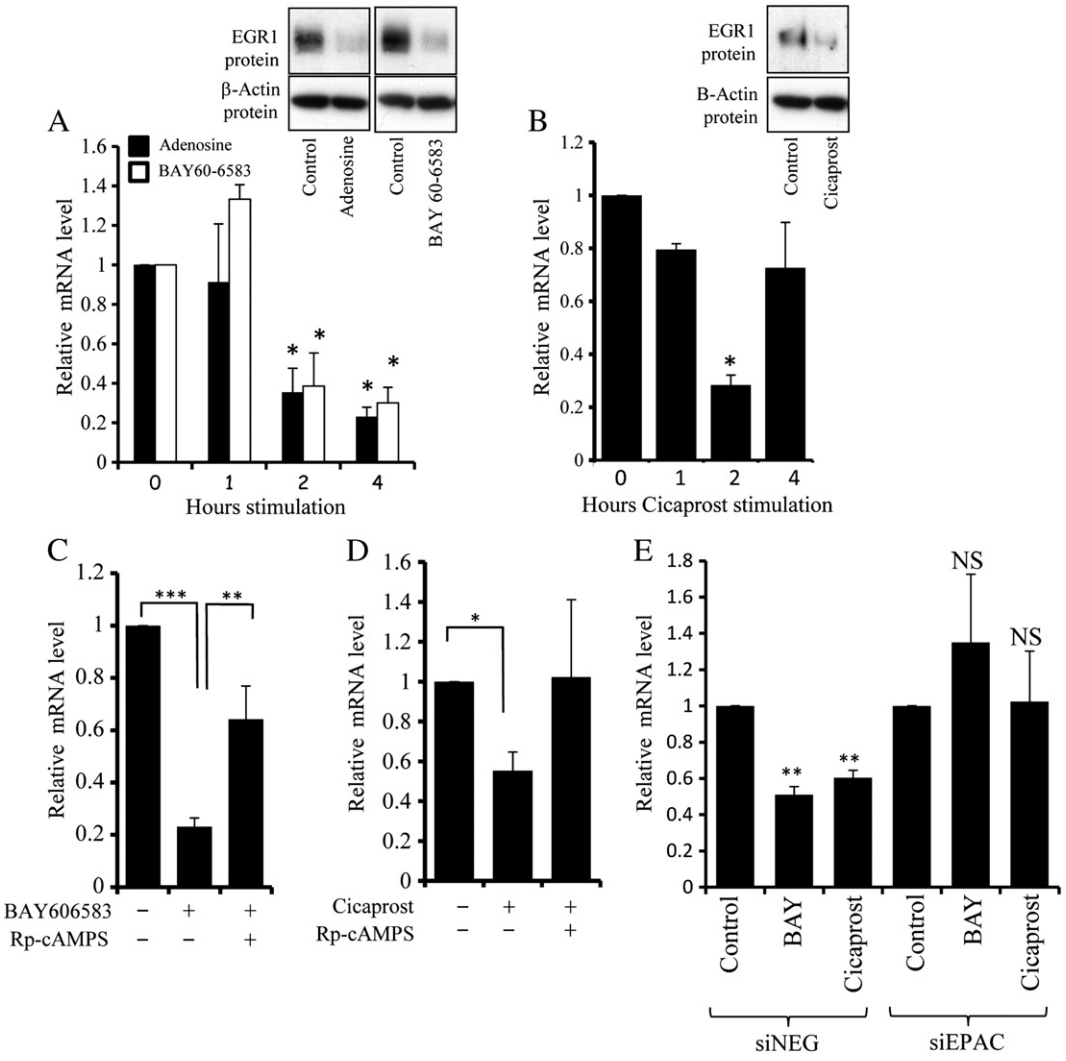


Fig. 2. Inhibition of Egr1 expression by Adenosine, BAY60-6582 and Cicaprost via PKA and EPAC. Rat VSMC stimulated with 100 μM adenosine or 1 μg/ml BAY-60-6583 (A, C and E) or 1 μM Cicaprost (B,D and E) for indicated times were analysed for Egr1 mRNA and protein expression qRT-PCR (graphs) and western blotting (insets). Where indicated, cells were pre-treated with 200 μM Rp-cAMPS (C and D) or transfected with siRNA targeting EPAC (E). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

SRE clusters in the Egr1 promoter (Fig. 5H). To confirm a functional role for SRF in cAMP-mediated inhibition of Egr1, we attempted to rescue Egr1 expression by expressing constitutively-active SRF (SRF-VP16). In control vector transfected cells, Forskolin significantly inhibited Egr1 expression (Fig. 5I). Importantly, SRF-VP16 expression significantly elevated basal Egr1 mRNA levels and completely prevented Forskolin-mediated inhibition (Fig. 5I).

3.9. Elevation of cAMP suppresses Egr1 via inhibition of Rac1 and disruption of the actin-cytoskeleton

cAMP-mediated growth arrest in VSMC is associated with a dramatic change in cell morphology, characterised by a condensed cell body with extended membrane processes (often referred to as stellate morphology) together with loss of F-actin stress fibres [14]. Since PKA and EPAC synergise to induce these morphological and cytoskeletal changes [15], and actin-cytoskeleton remodelling has been implicated in the regulation of SRE activity [16], we asked if this mechanism underlies Egr1 inhibition. Consistent with our previous observations [15], CPT plus BNZ stimulation, but not BNZ or CPT alone, strongly induced stellate morphology and F-actin loss (data not shown). Importantly, stellate morphology is evident within 20 min in response to a range of cAMP-elevating stimuli (Supplement Fig. 9), preceding inhibition of Egr1 expression. Disruption of actin polymerisation with either cytochalasin-D or latrunculin-B potently inhibited Egr1 mRNA expression (Fig. 6A), consistent with actin-dependent regulation of Egr1. However, it is interesting to note that in endothelial cells, cAMP stimulation does not cause a loss of F-actin stress fibres (Supplement Fig. 10) and does not inhibit Egr1 expression (Fig. 1E and Supplement Fig. 6), consistent with a role of actin-remodelling in the cAMP-dependent regulation of Egr1.

RhoA and Rac1 GTPases have been implicated in cAMP-mediated actin-cytoskeleton remodelling, morphological changes and mitogenic responses in VSMC [14,17]. Forskolin stimulation potently suppressed RhoA activity in VSMC (Fig. 6B) and inhibition of RhoA or its effector ROCK using C3 transferase or Y27632 respectively, mimicked cAMP-induced morphological changes (Fig. 6C). However, RhoA or ROCK inhibition did not significantly affect Egr1 mRNA expression (Fig. 6D). Furthermore, expression of a constitutively-active mutant of RhoA (RhoA G14V) (Supplement Fig. 11A) did not rescue Egr1 mRNA (Fig. 6E) or protein (Fig. 6F) expression after Forskolin stimulation, despite a partial reversal of stellate morphology (Supplement Fig. 11C). Together, this data implies RhoA-independent regulation of Egr1 by cAMP. We therefore investigated the role of Rac1. CPT plus BNZ or Forskolin stimulation, but not CPT or BNZ alone, significantly inhibited Rac1 activity (Fig. 6G). Importantly, adenovirus-mediated expression of a constitutively-active mutant of Rac1 (Rac1-G12V) (Supplement Fig. 11B) prevented Forskolin-induced stellate morphology

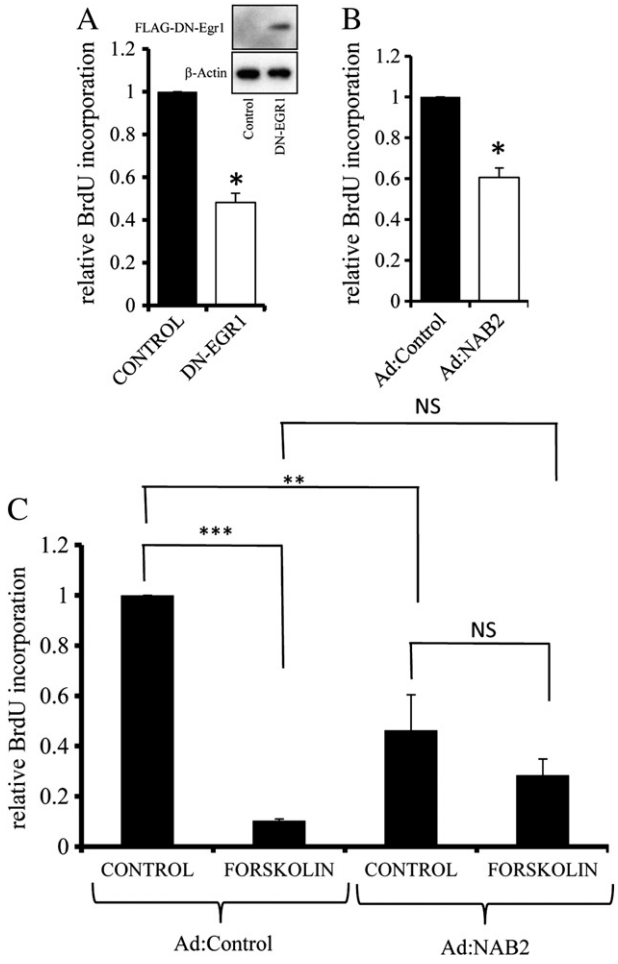


Fig. 3. Inhibition of Egr1 contributes toward cAMP anti-mitogenesis. BrDU incorporation was quantified 42 h after inhibition of Egr1 activity by transfection with dominant-negative Egr1 (A) or adenovirus encoding the Egr1 repressor NAB2 (B). Cells infected with either control or NAB2 adenovirus were stimulated with 25 μM Forskolin for 16 h followed by 4-h incubation with BrDU (C). Data is mean fold regulation ± SEM from at least three experiments. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$; ANOVA with Student–Newman–Keuls post-test.

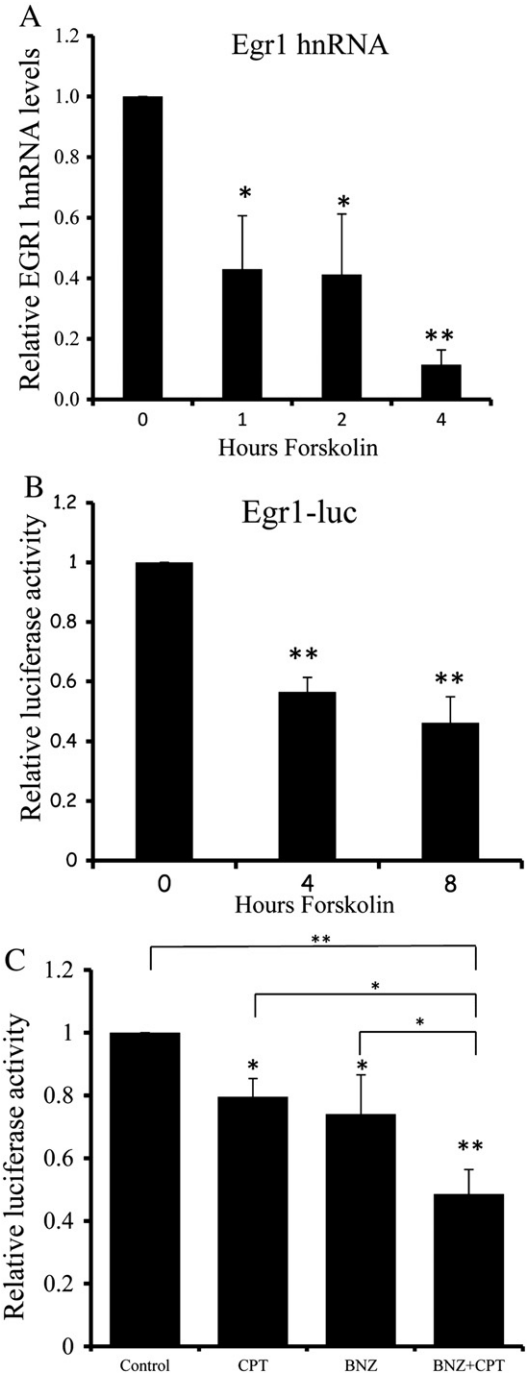


Fig. 4. cAMP inhibits Egr1 gene transcription. VSMC were stimulated with 25 μM Forskolin for indicated times. Egr1 pre-spliced hnRNA quantified by qRT-PCR, $n = 4$ (A) Effect on Egr1-luc reporter ($n = 5$) gene activity was quantified after stimulation with 25 μM Forskolin (B) or 200 μM BNZ and/or CPT for 4 h (C). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ vs Control; # indicates $p < 0.05$ vs BNZ by ANOVA.

(Supplement Fig. 11C), prevented the Forskolin-mediated reduction in SRE-luc (Fig. 6H) and significantly attenuated the Forskolin-mediated inhibition of Egr1 mRNA (Fig. 6I) and protein (Fig. 6J).

3.10. cAMP represses Egr1 expression via an Elk-dependent but MAL independent mechanism

Inhibition of Rac1 activity, impaired actin polymerisation and the resulting increase in actin monomer have all been linked to inhibition of SRE activity in other cell types [18]. This occurs, in part, via cytoplasmic sequestration of the SRF co-factor MAL (MAL/MKL1/MRTF-A) by monomeric-actin. Consistent with this, elevation of monomeric-actin, via expression of a non-polymerisable actin-mutant (Actin-R62D), potently and significantly inhibited SRE-luc activity (Fig. 7A). Actin-R62D also inhibited Egr1 mRNA (Fig. 7B). We therefore asked if cAMP-elevation inhibits Egr1 expression by sequestering MAL in the cytoplasm. GFP-MAL was localised exclusively in the cytoplasm in serum-starved cells but translocated to the nucleus within 1 h in response to serum stimulation (Fig. 7C). Forskolin stimulation blocked serum-induced nuclear localisation of MAL, consistent with cAMP inhibition of MAL-SRF signalling. However, siRNA-mediated silencing of MAL did not affect Egr1 mRNA expression (Fig. 7D), implying that cAMP-dependent regulation of Egr1 is independent of the MAL-SRF pathway. We therefore investigated the role of the other SRF cofactors, Elk1 and Elk4. Silencing of Elk1 or Elk4 alone had no effect on Egr1 mRNA expression (data not shown) whereas simultaneous silencing of both potently inhibited Egr1 mRNA expression (Fig. 7E). This is consistent with previously reported functional redundancy between these co-factors [19]. Forced expression of constitutively-active Elk1 (Elk1-VP16) resulted in a partial but significant reversal of Forskolin-inhibited Egr1 expression (Fig. 7F). Taken together, this data demonstrates a role for Elk in the cAMP-dependent regulation of Egr1 in VSMC. To gain an insight into the underlying mechanism we measured changes in Elk1 binding to the Egr1 promoter and Elk-phosphorylation

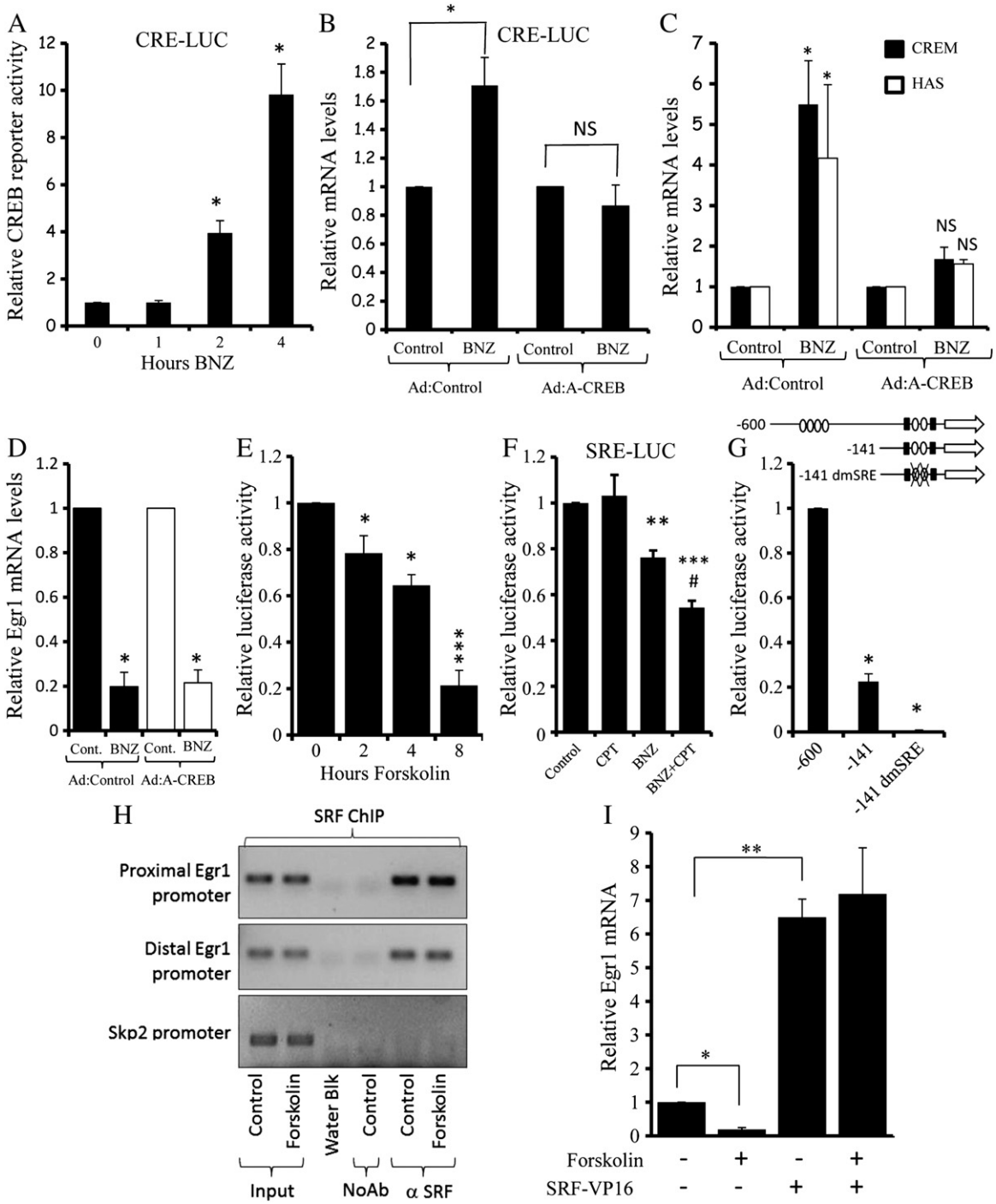


Fig. 5. cAMP inhibition of Egr1 expression is CREB-independent but SRE-dependent. CRE-Luc transfected VSMC were infected with either Ad:Control or Ad:A-CREB (dominant-negative CREB). Cells were stimulated with 200 μM BNZ and luciferase activity, (A and B) or mRNA expression of CREM and HAS (C) or Egr1 (D) quantified after 4 h for luciferase or 8 h for mRNA. Luciferase activity was quantified in cells transfected with SRE-luc and stimulated with 25 μM forskolin (E) or 200 μM BNZ, CPT or both for 4 h (F). Luciferase activity was quantified in cells transfected with either −600 Egr1-luc, -141 Egr1-luc of −141 double SRE mutated Egr1-luc (G). SRF binding to the proximal and distal SRE clusters in the Egr1 promoter was quantified by ChIP (H). Proximal Skp2 promoter was used as a non-SRE control. VSMC were transfected with SRF-VP16 and stimulated with Forskolin for 90 min 24 h post-transfection (I) Egr1 mRNA was quantified by qRT-PCR. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, # indicates $p < 0.05$ vs BNZ alone.

in response to cAMP-elevation. ChiP analysis detected basal binding of Elk1 to the proximal and distal Egr1 promoter but this was not modulated by Forskolin stimulation (Fig. 7H). However, Forskolin stimulation rapidly resulted in a loss of Elk1-phosphorylation (Fig. 7I). Since Elk1 is an ERK1/2 substrate and cAMP inhibits ERK1/2 activity in VSMC [2], we investigated the role of ERK1/2 in cAMP-dependent Egr1 regulation. ERK1/2 inhibition with PD98059 potently repressed Egr1 mRNA expression to similar levels achieved by forskolin (Fig. 7J). However, cAMP stimulation inhibited Egr1 mRNA expression as early as 60 min (Fig. 7K) but did not inhibit ERK1/2 phosphorylation at this timepoint (Fig. 7L). Although Egr1 expression is ERK1/2 dependent, its inhibition by cAMP preceded the loss of ERK1/2 phosphorylation, implicating an alternative cAMP-regulated mechanism. Interestingly, cAMP-elevation rapidly (within 20 min) blocked mitogen-stimulated nuclear localisation of ERK1/2 (Fig. 7M). Actin-cytoskeleton disruption with cytochalasin-D also blocked ERK1/2 nuclear localisation, consistent with our observed effects of cAMP on cytoskeleton remodelling in these cells (Fig. 7M).

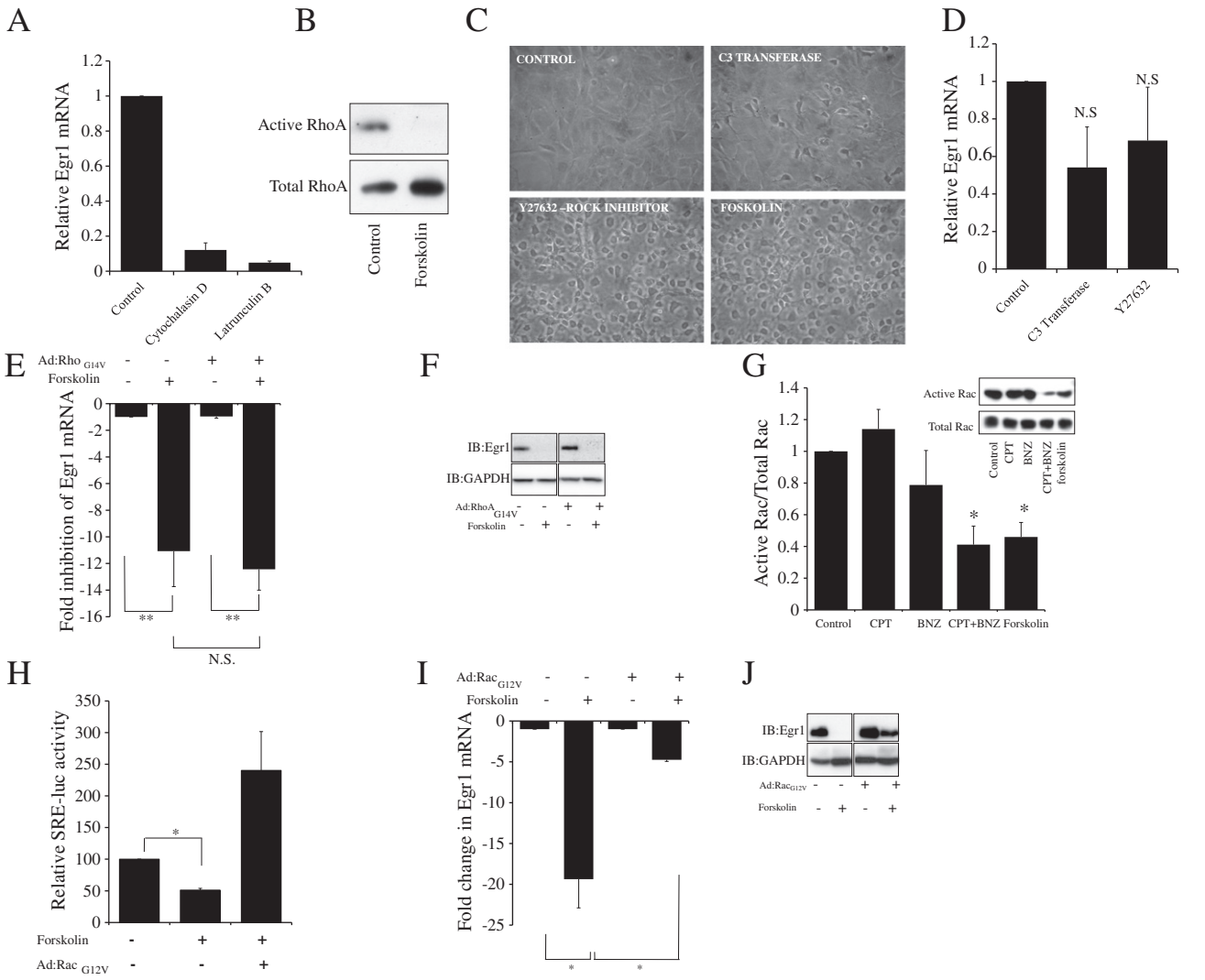


Fig. 6. cAMP suppresses SRE-activity and Egr1 expression via inhibition of Rac1 and impaired actin polymerisation. VSMC were stimulated with 2 μM cytochalasin-D or 5 μg/μl latrunculin-B for 4 h and Egr1 mRNA levels quantified by qRT-PCR (A). RhoA activity was quantified in VSMC stimulated with 25 μM Forskolin (B). VSMC were stimulated with 2 μg/ml C3 Transferases, 30 μM Y27632 or 25 μM forskolin for 1 h (C) or 4 h (D). Morphology was analysed by phase contrast microscopy (C) and Egr1 mRNA by qRT-PCR (D). Cells infected with constitutively-active RhoA (Ad:$RhoA_{G14V}$) adenovirus were stimulated with 25 μM forskolin for either 2 h (E) or 8 h (F) and Egr1 mRNA (E) and protein (F) levels quantified. Rac1 activity was quantified in cells stimulated with 200 μM BNZ, CPT, BNZ plus CPT or 25 μM Forskolin for 1 h (G). Cells were infected with constitutively-active Rac1 (Ad:Rac_{G12V}) adenovirus and stimulated with 25 μM forskolin (H, I and J). SRE-luc activity was quantified after 4 h (H) and Egr1 mRNA (I) and protein (J) after 2 and 8 h respectively. * indicates $p < 0.05$; ** indicates $p < 0.01$.

4. Discussion

Elevated levels of cAMP potently inhibit VSMC proliferation by inhibiting expression of multiple G_1 cell-cycle proteins, including Cyclin-D_1, Skp2 and c-myc [4]. However, the early upstream signalling mechanisms are incompletely characterised. For many years, PKA was thought to be solely responsible for the action of cAMP. However, we recently demonstrated that selective PKA activation is insufficient to inhibit VSMC proliferation, implicating the involvement of a second cAMP-sensitive pathway, which we identified as EPAC [5]. EPAC is a cAMP-sensitive protein with GEF activity coupling cAMP levels to downstream effectors, such as Rap1. PKA and EPAC pathways act synergistically to repress VSMC proliferation [5]. However, the mechanisms remained elusive. In this study, we demonstrate that PKA and EPAC synergistically inhibit expression of Egr1, which we show is an essential factor initiating cell-cycle progression [20]. Our data indicates that suppression of Egr1 occurs in response to inhibition of Rac1-dependent cytoskeleton-remodelling, which triggers rapid nuclear export of ERK1/2 and dephosphorylation of Elk1. This represents one of the earliest mechanisms underlying the anti-mitogenic effects of cAMP in VSMC.

Egr1 is a zinc-finger transcription factor that is rapidly and transiently induced in many different cell types, including VSMC, in response to growth factors, cytokines and tissue injury [21]. Increased Egr1 expression is found in various vascular pathophysiological processes associated with increased vascular cell proliferation, including atherosclerosis, restenosis [22], diabetes [23], hypoxia induced pulmonary hypertension [24] and ischemia induced arteriogenesis [25] where it is associated with elevated expression of multiple Egr1 target genes involved in the proliferative response [26]. Interventions to block Egr1 expression or function, including antisense ODNs [27], decoy ODNs [28], DNAzymes [20], and siRNA [29] effectively inhibit VSMC proliferation in vitro and in vivo, ultimately reducing neointimal lesion formation, identifying Egr1 as a potential therapeutic target. Our data showing that inhibition of Egr1 activity effectively blocks VSMC proliferation confirms the essential role for Egr1 in controlling G_1-S phase progression in VSMC. Our data also demonstrates for the first time that cAMP rapidly and potently inhibits Egr1 expression in VSMC and that cAMP induced growth arrest is attenuated in cells lacking Egr1 activity. We therefore suggest that this represents one of the earliest mechanisms underlying the anti-mitogenic effects of cAMP in these cells. We provide evidence

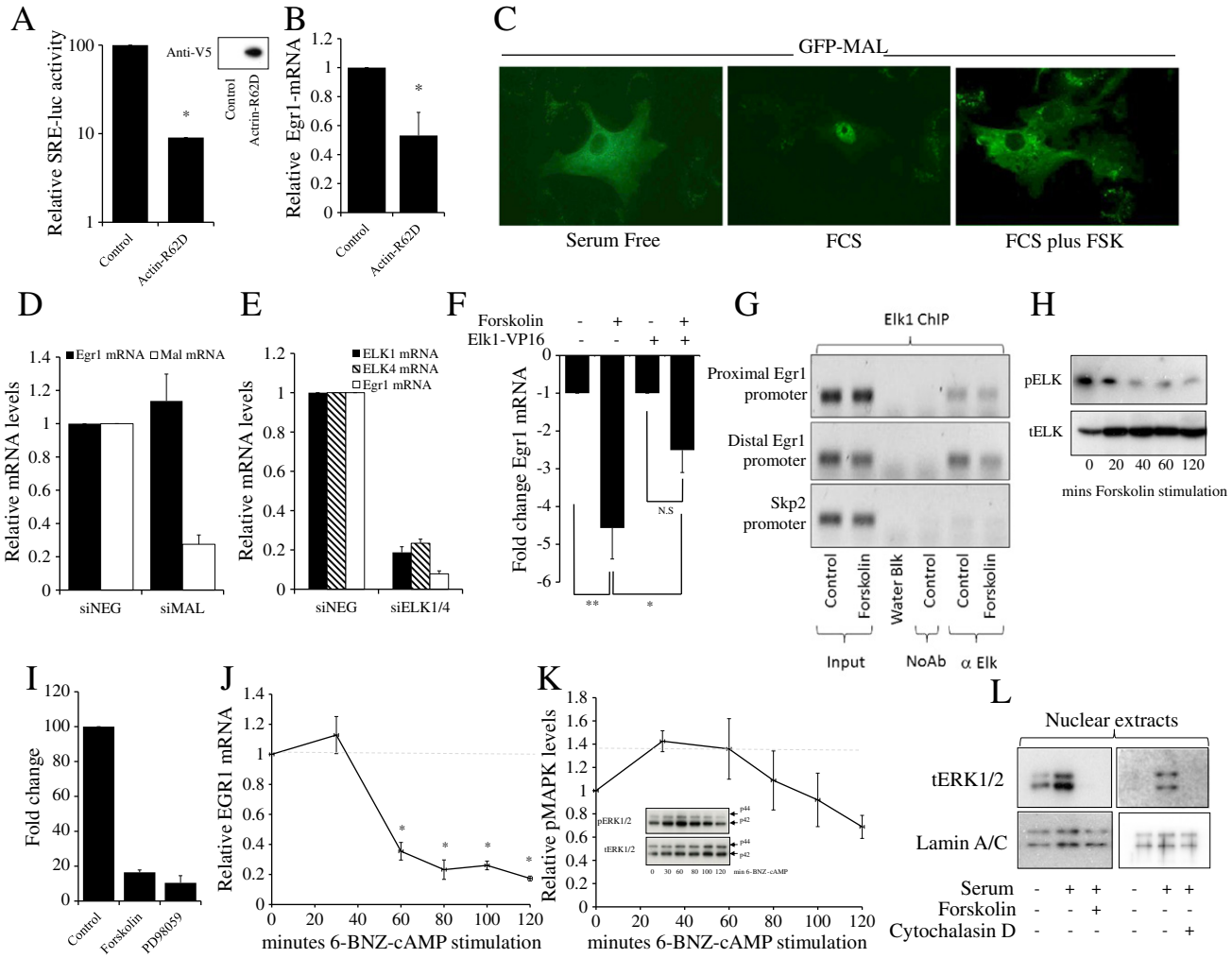


Fig. 7. cAMP-mediated inhibition of Egr1 is Elk-dependent and MAL-independent. VSMC were infected with recombinant adenovirus expressing V5-tagged Actin-R62D (A and B). SRE-luc (A) and Egr1 mRNA (B) was quantified 24 h post infection. Cells transfected with GFP-MAL were pre-treated with 25 μM forskolin for 20 min before stimulation with 5% FCS and MAL localisation assessed by fluorescence microscopy (C). Cells were transfected with control siRNA (siNEG) or siRNAs targeting MAL or Elk1 plus Elk4 (D and E). MAL and Egr1 mRNA (D) or Elk1 and Elk4 (E) was quantified 48 h post transfection. Egr1 mRNA was quantified in cells transfected with empty vector or Elk1-VP16 and stimulated with Forksokin for 90 min, 48 h post transfection (F). Elk1 binding to the proximal and distal SRE clusters in the Egr1 promoter (Skp2 promoter used as negative control) was quantified by ChIP (G). Quantification of total and phosphorylated Elk1 levels in 25 μM Forskolin stimulated cells (H). Effect of 25 μM forskolin or 20μM PD08959 on Egr1 mRNA (I). Time course of Egr1 mRNA levels in response to 200 μM BNZ stimulation (J). Time course of ERK1/2 phosphorylation in response to 200 μM BNZ stimulation (K). Cells were pre-treated with either 25 μM Forskolin or 2 μM Cytochalasin D followed by a 5 min stimulation with 5% FCS. ERK1/2 and Lamin A/C levels were quantified in nuclear extracts by western blotting (L). * indicates $p < 0.05$; ** indicated $p < 0.01$.

that this mechanism is triggered by physiological vasoactive agents, such as prostacylin and extracellular adenosine that are known to play a role in maintaining a quiescent VSMC phenotype and restraining proliferation after injury by stimulating cAMP synthesis, suggesting that it may play a role in regulating vascular homeostasis. It is interesting to note that Egr1 is also a critical positive regulator of endothelial cell proliferation [30]. However, our data demonstrates that cAMP-dependent inhibition of Egr1 expression is cell-type specific, occurring in VSMC but not in endothelial cells, where cAMP actually stimulates Egr1 expression. This raises the possibility that these properties might be exploited pharmacologically to specifically inhibit VSMC proliferation to combat restenosis.

We show that the early down-regulation of Egr1 expression by cAMP elevation in VSMC is largely due to transcriptional inhibition. Although the Egr1 promoter contains two conserved CREB binding elements our data shows that its inhibition by cAMP is CREB-independent. This is consistent with previous studies showing that CREB positively regulates Egr1 transcription in VSMC [31] and pancreatic β-cells [32] in response to other stimuli. Instead, our data implicates the SRE elements in the Egr1 promoter as the target for repression by cAMP. Egr1 transcription is dependent on these SRE elements and cAMP signalling, via PKA and EPAC, inhibits SRE-dependent transcriptional activity. Furthermore, expression of constitutively-active SRF rescues Egr1 expression after cAMP-stimulation, consistent with SRE being the target for cAMP-mediated repression. Interestingly, this is not associated with loss of binding of SRF to these elements. Instead our data demonstrates that inhibition of SRE activity and Egr1 expression is triggered by actin-cytoskeleton remodelling induced by cAMP. Elevated cAMP induces profound and rapid morphological changes due to disruption of actin stress fibres in VSMC [5,14] but not in endothelial cells where we show that cAMP does not inhibit Egr1. cAMP, via the synergistic action of PKA and EPAC, induces this cytoskeleton reorganisation by inhibiting RhoA and Rac1 GTPases [14,17]. However, our data demonstrates that although inhibition of the RhoA/ROCK pathway mimicked cAMP-induced morphological changes and this was at least partially reversed by constitutively-active RhoA, neither affected Egr1 expression. Hence Rac1, rather than RhoA, is the primary mediator of cAMP-dependent inhibition of Egr1 expression in VSMC. This is consistent with previous work demonstrating distinct functional roles of these GTPases on gene expression [33] and an essential role for Rac1 for VSMC

proliferation and intima formation in vivo [14]. We demonstrate that expression of constitutively-active Rac1 rescues SRE-activity and Egr1 expression after cAMP-stimulation, consistent with previous work linking Rac1 activity to SRF-dependent gene expression in fibroblasts [34].

Increased levels of monomeric-actin, resulting from impaired actin polymerisation, have been implicated in mediating SRF inhibition and, as we now show, also inhibit Egr1 expression in VSMC. One previously described mechanism is via cytoplasmic sequestration of the SRF co-factor MAL [16] and we show, for the first time, that cAMP-mediated actin-remodelling regulates nuclear localisation of MAL in VSMC, which could be functionally important. However, siRNA-silencing of MAL clearly demonstrated that Egr1 is not a MAL target in VSMC, implicating alternative mechanisms. Instead our data demonstrates that Egr1 expression is absolutely dependent on the SRF co-factors Elk1 and Elk4. cAMP stimulation inhibits Elk1 phosphorylation and cAMP-mediated inhibition of Egr1 reversed in cells expressing constitutively-active Elk1, strongly suggesting that Elk1, at least in part, is a target for cAMP-mediated inhibition of SRE-activity and Egr1 expression in VSMC. Given that Elk1 is an ERK1/2 target and cAMP is known to inhibit ERK1/2 activity in VSMC it is likely that cAMP-mediated ERK1/2 inhibition is involved in Egr1 regulation. Our data demonstrates that cAMP elevation rapidly (within 20 min) blocks nuclear targeting of ERK1/2, preceding Egr1 inhibition, which implies that nuclear export of ERK1/2 rather than inhibition of phosphorylation underlies the inhibition of Elk1 phosphorylation and Egr1 expression. The fact that direct cytoskeleton disruption with CytochalasinD also rapidly blocks ERK1/2 nuclear targeting strongly suggests that cAMP-induced cytoskeleton remodelling is responsible for cAMPs' effects on ERK1/2 localisation, Elk1-phosphoryaltion, Egr1 expression and ultimately growth arrest. Why Egr1 expression is divergently regulated by cAMP in VSMC and EC is currently unknown but clearly an important area of future research. Our data points to the striking differences in cytoskeleton remodelling in response to cAMP between these cell types. Whether this reflects differences in the regulation of upstream Rho-family GTPasess or the way the cytoskeleton organised in these cells is currently unknown.

5. Conclusions

In summary, we demonstrate that PKA and EPAC synergise to inhibit VSMC proliferation by rapidly inhibiting EGR1 expression. This occurs, at least in part, via inhibition of Rac1 activity leading to rapid actin-cytoskeleton remodelling, nuclear export of ERK1/2, impaired Elk1-phosphorylation and inhibition of SRE activity (see supplement Fig. 12). This likely represents one of the earliest mechanisms underlying the anti-mitogenic effects of cAMP and highlights the previously underappreciated importance of actin-cytoskeleton remodelling mediating the biological effects of cAMP in VSMC. Importantly, this mechanism is cell-type specific, operating in VSMC but not in EC and may have potential for the development of VSMC-specific anti-proliferative therapies for the treatment of restenosis.

Disclosures

Conflict of Interest: None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.02.001.

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Glossary

BrdU: 5-Bromo-2-deoxyuridine
cAMP: 3′-5′-cyclic adenosine monophosphate
CREB: cAMP response element binding protein
Egr1: early growth response 1
EPAC: exchange protein activated by cAMP
ERK1/2: extracellular signal-regulated kinase 1 and 2
ODNs: oligodeoxynucleotide
PKA: protein kinase A
Rap1: Ras-related protein 1
SRE: serum response element
SRF: serum response factor
VSMC: vascular smooth muscle cell
Elk: E26-like kinase 1
Rho: ras homolog family member A
Rac: ras-related C3 botulinum toxin substrate 1