Vessels

**Soluble Adenylyl Cyclase in Vascular Endothelium**

**Gene Expression Control of Epithelial Sodium Channel-α, Na+/K+-ATPase-α/β, and Mineralocorticoid Receptor**

Boris Schmitz,* Johanna Nedele,* Katrin Guske, Martina Maase, Malte Lenders, Michael Schelleckes, Kristina Kusche-Vihrog, Stefan-Martin Brand, Eva Brand

Abstract—The Ca²⁺- and bicarbonate-activated soluble adenylyl cyclase (sAC) has been identified recently as an important mediator of aldosterone signaling in the kidney. Nuclear sAC has been reported to stimulate cAMP response element–binding protein 1 phosphorylation via protein kinase A, suggesting an alternative cAMP pathway in the nucleus. In this study, we analyzed the sAC as a potential modulator of endothelial stiffness in the vascular endothelium. We determined the contribution of sAC to cAMP response element–mediated transcriptional activation in vascular endothelial cells and kidney collecting duct cells. Inhibition of sAC by the specific inhibitor KH7 significantly reduced cAMP response element–mediated promoter activity and affected cAMP response element–binding protein 1 phosphorylation. Furthermore, KH7 and anti-sAC small interfering RNA significantly decreased mRNA and protein levels of epithelial sodium channel-α and Na+/K⁺-ATPase-α. Using atomic force microscopy, a nano-technique that measures stiffness and deformability of living cells, we detected significant endothelial cell softening after sAC inhibition. Our results suggest that the sAC is a regulator of gene expression involved in aldosterone signaling and an important regulator of endothelial stiffness. Additional studies are warranted to investigate the protective action of sAC inhibitors in humans for potential clinical use. *(Hypertension. 2014;63:753-761.)* • **Online Data Supplement**

**Key Words:** adenylyl cyclase • aldosterone • cyclic AMP response element–binding protein • vascular stiffness

Arterial stiffening is associated with cardiovascular morbidity, such as myocardial infarction, heart failure, and stroke, but also with dementia and renal failure.¹ Central aortic elasticity can be measured by pulse wave velocity,² and increased pulse wave velocity is a strong and independent predictor of cardiovascular morbidity and all-cause mortality in patients with essential hypertension,³ end-stage renal disease,⁴ as well as the general population.⁵ The overall vascular elasticity reflects the mechanical property of the large arteries and is the result of individual genetic predisposition,⁶ lifestyle, other environmental factors, and their interaction.⁷

Vascular elasticity and compliance of the vascular wall depend to a great extent on the relative contribution of the scaffolding proteins elastin and collagen.⁸ Dysregulation of their expression, degradation, and protein cross-links may lead to alterations in the elastin/collagen ratio with subsequent arterial stiffening.⁹ The mineralocorticoid hormone aldosterone is a potent and blood pressure–independent inducer of both, elastin and collagen expression,¹⁰ pointing to the direct implication of aldosterone in vascular elasticity. Most recently, the involvement of the vascular endothelium has been discussed extensively as an important regulator of vascular elasticity.¹¹ Endothelial cells (ECs) contribute to the vascular tone and are sensitive to aldosterone and elevated sodium concentrations.¹² Regulation of endothelial stiffness involves the multi-subunit epithelial sodium channel (ENaC) and potentially the Na⁺/K⁺-ATPase.¹³,¹⁴ Gene expression of ENaC-α (SCNN1A), Na⁺/K⁺-ATPase-α/β (ATP1A1, ATP1B1), and the mineralocorticoid receptor (MR; NR3C2) has been suggested to depend on the second messenger cAMP.¹⁵,¹⁶ The human adenylyl cyclase 10 (ADCY10), also termed soluble adenylyl cyclase (sAC), generates cAMP in the cellular nucleus with subsequent protein kinase A (PKA) activation and cAMP response element–binding protein (CREB) phosphorylation. In the kidney, sAC has been reported to be involved in blood pressure homeostasis, whereas specific inhibition of sAC by its inhibitor KH7 resulted in abrogation of basic and agonist-stimulated Na⁺ reabsorption.¹⁷ ENaC, Na⁺/K⁺-ATPase, and MR expression may involve binding of phosphorylated CREB (CREB-p) to cAMP response elements (CRE) at the promoter level.⁴¹–⁴³ In

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the current work, we analyzed sAC-dependent CRE-mediated transcriptional activation through CREB-p in kidney and ECs and determined the impact of sAC on endothelial stiffness.

**Methods**

**Cell Culture**
The human vascular EC line EA.hy926 was maintained in DMEM (Sigma-Aldrich, Munich, Germany) with 10% fetal calf serum (PAA, Colbe, Germany), penicillin (100 U/mL), streptomycin (100 ng/mL), and l-glutamine (2 mmol/L; all Sigma-Aldrich). The human kidney epithelial cell line IHKE was maintained in DMEM/Ham’s F12, 1% fetal calf serum, penicillin (100 U/mL), streptomycin (100 ng/mL), and l-glutamine (2 mmol/L; all Sigma-Aldrich), and HEPES (15 mmol/L, Merck, Darmstadt, Germany). Human primary aortic ECs (PromoCell, Heidelberg, Germany) were maintained in endothelial cell growth medium MV (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 ng/mL), NaHCO3 (1.25 mmol/L), sodium pyruvate (55 μg/mL), epidermal growth factor (10 μg/mL; all Sigma-Aldrich), and hydrocortisone (1 μg/mL; all PromoCell). Cells were incubated with 500 μmol/L lipophilic 8-Br-cAMP (Biolog, Bremen, Germany) or 200 pmol/L aldosterone (Sigma-Aldrich) as indicated. The specific sAC inhibitor KH7 (Sigma-Aldrich) was administered at a concentration of 25 μmol/L, the MR antagonist spironolactone (Sigma-Aldrich) at 1 μmol/L.

**Preparation of Mice Aortae**
All animal experiments were approved by an institutional review committee. Mice aortae (n=4) for atomic force microscopy (AFM)-based cell stiffness measurements were dissected, freed from surrounding tissue, opened and fixed on glass coverslips coated with Cell-Tak (Becton Dickinson, Heidelberg, Germany) as described previously. Aortae were incubated for 24 hours in medium with KH7 (25 μmol/L) and aldosterone (1 nmol/L) or aldosterone alone.

**AFM-Based Cell Stiffness Measurements**
The stiffness (defined as Newton per meter) of EA.hy926 cells and in situ ECs of ex vivo mice aorta was measured at 37°C using a Multimode AFM (Bruker, Mannheim, Germany) equipped with a soft cantilever (MLC-contact microlever, spring constant: 0.018 N/m for ECs, 0.011 or 0.012 N/m for aortae; Bruker) and a spherical tip with a diameter of 1 μm (Novascan, Ames, IA) as described previously. A single-cell force-distance curves were recorded. During AFM measurements, cells were kept in HEPES-buffered solution (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 10 mmol/L HEPES, 5 mmol/L glucose, pH 7.4). AFM data were collected with the SPM-Explorer software (Bruker) and stiffness values were calculated using the Protein Unfolding and Nano-Indentation Analysis Software (http://punias.voila.net/).

**Transient Transfections and siRNA Experiments**
For details please see online-only Data Supplement.

**Real-Time Polymerase Chain Reaction**
For details please see online-only Data Supplement.

**Preparation of Nuclear Protein Extracts**
Nuclear protein extracts were generated using a modified protocol. For details please see online-only Data Supplement.

**Western Blot**
For details please see online-only Data Supplement.

**Chromatin Immunoprecipitation Assay**
Chromatin immunoprecipitation (ChIP) was performed as described previously. DNA/protein complexes were fixed using formaldehyde (1%, 30 minutes). DNA was sonicated using a Bioruptor (45 minutes, 0.5-second interval, 200 W; Diagenode, Liège, Belgium). Chromatin had an average size of 300 to 500 bp. Four microgram of anti-CREB-p or anti-specificity protein 1 (SP1; Millipore, Bedford, MA) or IgG (negative control; Millipore) were used. Oligonucleotide sequences are given in Table S1 in the online-only Data Supplement.

**Statistical Analysis**
P values were calculated using Graph Pad Prism 4.0 or OriginPro 8.5 (for AFM measurements) and unpaired Student t test or Mann–Whitney test for continuous variables. Significance was declared at P<0.05.

**Results**

**Cellular sAC Localization**
We initially determined expression levels of the cAMP-generating enzyme sAC in the vascular EC line EA.hy926 and the kidney proximal tubule epithelial cell line IHKE. EA.hy926 cells have been used commonly to investigate changes in endothelial function, whereas IHKE cells have been introduced to study the function of renal Ca2+-, Na+-, and K+-channels. We identified sAC protein in whole cell extract and, particularly, in the nucleus of EA.hy926 and IHKE cells (Figure 1), with 3 protein isoforms designated A (≈55 kDa), B (≈65 kDa), and C (≈80 kDa). The nuclear accumulation of all 3 detected isoforms in ECs was increased by cAMP stimulation. Isoform B was detected hardly in crude cellular extracts of kidney cells but was significantly enriched in nuclear extracts. Interestingly, we observed a significant increase in sAC transcript upon 24-hour cAMP stimulation, whereas stimulation with protein kinase C-activating PMA remained ineffective (data not shown), suggesting PKA signaling rather than protein kinase C signaling to be involved in sAC expression regulation.

**CRE-Mediated Transcriptional Activity Is Affected by sAC**
Because sAC has been reported to stimulate CREB phosphorylation, we investigated the impact of sAC on CRE-mediated
transcriptional activity. A CRE reporter gene vector was used to detect changes in cellular CRE-mediated transcriptional activity (Figure 2). We observed strong CRE-mediated transcriptional activity in ECs under basic conditions (Figure 2A), which was less prominent in IHKE cells (Figure 2B). In both cell lines, PKA stimulation via cAMP led to significant activation of CRE-mediated transcriptional activity (Figure 2A and 2B; \( P < 0.001 \)). Treatment with the specific sAC inhibitor KH7 significantly reduced CRE promoter activity in ECs (\( P = 0.0006 \); Figure 2A) and CRE-mediated transcriptional activity was abrogated completely in kidney cells (\( P = 0.0139 \); Figure 2B) and could not be restored by cAMP.

Our investigation of the impact of sAC inhibition on CREB phosphorylation (Ser\(_{133}\)-CREB-p) and total cellular CREB by Western blot analyses showed that application of KH7 significantly reduced CREB-p in endothelial (\( P < 0.05 \); Figure 2C) and kidney cells (\( P < 0.05 \); Figure 2D) compared with mock control. The effects of KH7 on CREB phosphorylation were prevented by cAMP. The level of unphosphorylated CREB remained unaffected by KH7 and cAMP in either cell line (Figure 2E and 2F). Notably, long-term treatment with KH7 (24 hours) resulted in an increase of Ser\(_{133}\)-CREB-p in both cell lines (Figure S1), suggesting a potential feedback mechanism.

**Expression of Genes Regulating Endothelial Stiffness Depends on sAC Activity**

To determine the regulatory effect of sAC on aldosterone-regulated genes, we analyzed the gene expression of Na\(^+\)/K\(^+\)-ATPase-\(\alpha\) (ATP1A1), Na\(^+\)/K\(^+\)-ATPase-\(\beta\) (ATP1B1), MR (NR3C2), ENaC-\(\alpha\) (SCNN1A), and sAC (ADCY10) via real-time polymerase chain reaction (Figure 3) in ECs expressing the MR (Figure S5). Compared with mock control, inhibition of sAC by KH7 resulted in significantly decreased expression of all genes tested (\( P < 0.05 \)), with the exception of NR3C2 (Figure 3A). Comparable results were obtained in human primary aortic ECs, in which treatment with KH7...
resulted in a significant downregulation of ATP1A1 and SCNN1A (Figure 3E). To validate the results obtained by sAC inhibition via KH7, we performed sAC-specific small interfering RNA (siRNA) transfection experiments (Figure 3B). Consistently, the siRNA approach resulted in a significant downregulation of the expression of all genes (P<0.001), again with the exception of NR3C2 (Figure 3B).

Furthermore, we assessed the regulative effect of sAC inhibition in ECs under stimulatory conditions including aldosterone and cAMP (Figure 3C and 3D). Application of 200 pmol/L aldosterone exerted a strong activating effect on the expression of ADCY10, ATP1A1, ATP1B1, and SCNN1A, whereas expression of NR3C2 was increased slightly (Figure 3C). The observed gene expression activation was spironolactone-sensitive and was prevented effectively by KH7. Validation experiments were performed in human primary aortic ECs, in which aldosterone stimulation resulted in a comparable increase of ATP1A1, ATP1B1, and SCNN1A expression (Figure 3F). This finding is consistent with data from a comparative gene expression analysis of human umbilical vein ECs and EA.hy926, which concluded that EA.hy926 cells have retained the gene expression profile of primary ECs. Short-term stimulation (2 and 4 hours) with cAMP significantly upregulated the expression of both genes coding for Na+/K+-ATPase subunits (ATP1A1, ATP1B1) and NR3C2 (P<0.05), whereas ADCY10 expression was not affected (Figure 3D). Notably, cAMP stimulation for 2 hours resulted in repression of NR3C2 and SCNN1A expression (P<0.01).

**Figure 3.** Aldosterone-sensitive genes are repressed by soluble adenylyl cyclase (sAC) inhibition. A and B, Treatment of endothelial cells (ECs) with KH7 and knockdown of sAC by small interfering RNA (siRNA) resulted in reduction of adenylyl cyclase 10 (ADCY10), Na+/K+-ATPase-α/β (ATP1A1, ATP1B1), and epithelial sodium channel-α (SCNN1A) mRNA, determined by real-time polymerase chain reaction (PCR). C, Treatment of ECs with aldosterone (200 pmol/L) resulted in an upregulation of ADCY10, ATP1A1, ATP1B1, and SCNN1A after 2 hours. Expression of mineralocorticoid receptor (NR3C2) was increased slightly after 4 hours. Upregulation was prevented by application of KH7 and the MR antagonist spironolactone. D, Treatment of ECs with cAMP increased expression of ATP1A1, ATP1B1, and NR3C2, which was prevented by KH7 with the exception of NR3C2. E and F, Comparable results were observed in human primary aortic ECs (HAoECs). Cells were treated with KH7/spironolactone or ethanol/DMSO (control). Cells were transfected with sAC siRNA or scrambled siRNA (5 μmol/L, 48 hours). Real-time PCR results include data of 3 independent experiments shown as mean±SEM. ***P<0.001, **P<0.01, and *P<0.05.
The activating effect of cAMP was prevented by sAC inhibition with the exception of NR3C2. The inhibitory effects of KH7 in the presence of aldosterone were also observed on isolated glucocorticoid response element- and CRE-mediated gene expression (Figure S2).

Because sAC inhibition led to a significant downregulation of ENaC-α and Na+/K+-ATPase-α gene expression (Figure 3) as well as a reduction in CREB-p and CRE-mediated promoter activity (Figure 2), we analyzed potential sAC-dependent changes of CREB-p binding at SCNN1A and ATP1A1 promoter regions in ChIP experiments. Because no reports existed that proved the binding of CREB-p to SCNN1A, we performed in silico analyses using PROMO 3.0.2.35 The analysis revealed a conserved CRE motive located at position −784 of SCNN1A. For the ATP1A1 promoter, we analyzed the well-characterized PUC-1 region, including the known CRE motive.34 Our ATP1A1 in silico analysis additionally suggested a SP1 binding site within the PUC-1 region. Our ChIP experiments in ECs revealed that CREB-p was bound to both promoters under basic conditions and binding of CREB-p was prevented by sAC inhibition (Figure 4). Notably, SP1 binding at the ATP1A1 CRE motive was also prevented by sAC inhibition.

With respect to the significant effects of sAC inhibition on EC gene expression, we investigated the consequences of sAC knockdown in ECs and renal cells on the protein level (Figure 5). Initially, we used an anti-sAC siRNA approach to confirm the identity of the detected sAC protein bands (Figure 5A and 5B). Expression of all 3 identified sAC isoforms was significantly reduced by application of anti-sAC siRNA in both cell lines compared with scrambled siRNA (P<0.05). sAC inhibition by siRNA also affected ENaC-α and Na+/K+-ATPase-α levels. We observed a significant reduction of active ENaC-α as well as lower levels for glycosylated and ubiquitylated ENaC-α46 compared with mock control (P<0.05; Figure 5C and 5D). The protein level of Na+/K+-ATPase-α was reduced significantly in ECs (P<0.05; Figure 5E), whereas no significant downregulation was observed in renal IHKE cells (Figure 5F). Comparable results were observed after 24-hour sAC inhibition by KH7, in that protein levels of active ENaC-α and Na+/K+-ATPase-α were reduced significantly in both cell lines compared with control (P<0.05; Figure S3).

**Discussion**

To the best of our knowledge, our study is the first to investigate the role of sAC in vascular ECs and the involvement of sAC in regulation of genes affecting endothelial stiffness. We were able to demonstrate the following: (1) inhibition of sAC impairs CRE-mediated promoter activity; (2) inhibition of sAC affects CREB phosphorylation; (3) sAC regulates the expression of genes involved in endothelial stiffness; and (4) inhibition of sAC leads to endothelial softening.

**Nuclear sAC as a Regulator of Active CRE Promoters**

We detected sAC transcript and protein in vascular ECs and in the kidney cell line IHKE. Two sAC isoforms (~55 and ~80 kDa), identified by Hallows et al10 in cell lysates of mpkCCDc14 kidney cells, were confirmed in IHKE cells, whereas we detected an additional isoform of ~65 kDa predominantly in ECs. We confirmed the identity of all 3 detected protein bands by an anti-sAC siRNA approach. sAC has been reported to be involved in transmembrane adenylyl cyclases–independent cAMP signaling inside the mammalian cell nucleus, affecting CREB phosphorylation via PKA.37,38 Our experiments, including a CRE reporter vector and the sAC-specific inhibitor KH7, revealed potent inhibition of CRE-dependent promoter activation on sAC depletion. Previous studies have demonstrated that KH7 effectively inhibits sAC enzyme activity at concentrations of 10 to 30 μmol/L, whereas transmembrane adenylyl cyclases and soluble guanylyl cyclases remained unaffected.59 The small molecule KH7 has been identified originally to display an IC50 between 3 and 10 μmol/L toward the recombinant human ~55 kDa sAC isoform.40 Until today, the exact molecular mechanisms underlying the inhibitory effects of KH7 are unknown. Notably, application of cAMP in our CRE reporter vector experiments in kidney cells could not overcome sAC inhibition by KH7, which...
caused total abrogation of CRE-mediated transcriptional activity. The above-mentioned effects may be explained, at least in part, by the inhibition of enzymatic sAC cAMP production by KH7, but also by the alteration of a putative sAC/PKA complex with subsequent inhibition of respective promoter activities.

Inhibition of sAC Affects CREB Phosphorylation
In CRE reporter vector experiments, we observed that inhibition of sAC in ECs resulted in a less prominent effect on CRE-mediated transcription compared with that in IHKE cells. Furthermore, CRE-mediated transcriptional activity could only be restored by cAMP in ECs but not in renal cells. Because sAC has been suggested to act predominantly via cAMP-dependent PKA, which phosphorylates CREB exclusively at Ser133,41 CRE-mediated transcriptional activation through CREB phosphorylation at Ser133 may be the major pathway in renal cells. Our analysis of cellular CREB revealed that the level of total CREB was unaffected by short-term application of KH7 (1 hour), whereas Ser133-phosphorylated CREB was significantly reduced. Long-term KH7 treatment (24 hours) resulted in an increase of cellular Ser133-phosphorylated CREB in both cell lines, indicating the initiation of an alternative back-up mechanism for CREB phosphorylation under long-term sAC inhibition. In ECs, additional kinases involved in CREB phosphorylation at alternative residues different from Ser133 might also be active.42 Because cGMP-generating soluble guanylyl cyclases are unaffected by KH7, and sAC has been reported to be the only AC in the mammalian cell nucleus,31 the endothelial soluble cGMP-dependent protein kinase I is a likely effector of alternative CRE-mediated transcription in ECs.43,44

An important function of cGMP in the vascular system has been stressed recently by the implication of phosphodiesterase 5 inhibitors, which increase intracellular cGMP concentration in disorders such as pulmonary and potentially essential hypertension.45 Taking our findings into account, it is conceivable that sAC inhibition may as well affect positively the cellular balance between cAMP and cGMP, resulting in a potential reduction of blood pressure.

Figure 5. Epithelial sodium channel-α (ENaC-α) and Na+/K+-ATPase-α protein is reduced by soluble adenylyl cyclase (sAC) knockdown. A and B. All 3 identified sAC protein isoforms (A, B, C) were reduced after transfection of anti-sAC small interfering RNA (siRNA) in EA.hy926 and IHKE cells compared with control. C and D. Knockdown of sAC resulted in reduced cellular levels of ENaC-α protein. The reduction was observed for active ENaC-α (a), glycosylated ENaC-α (g), as well as ubiquitylated ENaC-α (u). E and F, Na+/K+-ATPase-α protein levels were reduced in endothelial EA.hy926 cells. No significant reduction was observed in renal IHKE cells. Cells were transfected with sAC siRNA (125 or 250 nmol/L) or scrambled siRNA (250 nmol/L) for 72 hours. Western blot is representative for 3 independent experiments. Relative band intensities are shown as mean±SEM of densitometric measurements compared with β-actin loading control of 3 independent blots. **P<0.01 and *P<0.05.
NR3C2, ATP1A1/B1, and SCNN1A were not detected after 4 hours. The increased mRNA levels of sAC, ATP1A1/B1, and SCNN1A were not detected after 4 hours of aldosterone stimulation, suggesting a feedback inhibition including either short mRNA half-lives or active degradation. The activating effect of aldosterone was prevented completely by KH7 and spironolactone.

Individual reports have proposed a role for cAMP in gene expression regulation of SCNN1A, ATP1A1/B1, and NR3C2, which are targets of endothelial aldosterone signaling. These observations, together with our results, suggested that sAC is involved in the regulation of all 4 genes and could affect endothelial stiffness. Consistently, we detected decreased levels of ATP1A1 and ATP1B1, NR3C2, SCNN1A, and sAC mRNA after 24 hours of KH7 administration in EA.hy926 cells. The results obtained with the sAC-specific inhibitor KH7 were validated using an anti-sAC siRNA approach, which led to a comparable strong reduction of all transcripts tested. In contrast to another report,46 we detected aldosterone stimulation (200 pmol/L) of ECs to increase the expression of ADcy10, ATP1A1/B1, and SCNN1A after 2 hours, whereas NR3C2 increased after 4 hours. The increased mRNA levels of sAC, ATP1A1/B1, and SCNN1A were not detected after 4 hours of aldosterone stimulation, suggesting a feedback inhibition including either short mRNA half-lives or active degradation. The activating effect of aldosterone was prevented completely by KH7 and spironolactone. ATP1A1/B1, SCNN1A, and NR3C2 expression was also increased by cAMP stimulation, which was counterbalanced by sAC inhibition. Notably, NR3C2 expression remained activated in the presence of KH7. Our data obtained from ECs treated with anti-sAC siRNA and subsequent Western blot analyses suggest that the observed inhibition of ATP1A1 and SCNN1A gene expression may translate into a reduction of ENaC-α and Na+/K+-ATPase-α protein levels.

Moreover, our ChIP experiments on CREB-p and SP1 binding to CRE motives demonstrate that sAC inhibition alters the interaction between transcription factors and the ATP1A1 and SCNN1A promoter regions. These findings may explain the observed reduction in ATP1A1 and SCNN1A gene expression on sAC inhibition.

**Endothelium Is Softened by sAC Inhibition**

Extending our molecular functional analyses on sAC in ECs, we hypothesized that sAC inhibition translates into relevant physiological effects through alteration of the mechanical properties of ECs. Using AFM measurements of an EC line as well as ECs of mice ex vivo aorta, we detected a significant softening of cells after inhibition of sAC. The observed effect of ≈8% difference in EC stiffness may be explained by the inhibitory effect on mRNA and protein levels of the analyzed genes, resulting in a mild but significant cell softening. This result is of clinical relevance because chronic endothelial stiffness is accompanied by endothelial dysfunction as one of the first measurable features of arteriosclerosis.13 Future in vivo studies will be required to demonstrate the importance of sAC in the modulation of EC stiffness.

**Perspective**

In conclusion, we propose that sAC is involved in cAMP-mediated regulation of aldosterone-sensitive genes in renal epithelial and vascular ECs. sAC contributes differently to the CRE-mediated transcriptional activation in both cell types, suggesting a cell type-specific function together with other CREB-phosphorylating kinases. Vascular sAC may be a key regulator of long-term arterial elasticity because the amount of functional cAMP-producing sAC determines cellular transcript and protein levels of ENaC-α and the α subunit of Na+/K+-ATPase. Further analysis of sAC transcript and protein levels combined with specific sAC-dependent cAMP measurements in patients with elevated pulse wave velocity are warranted to address the impact of sAC on arterial stiffening. Additional studies are needed to investigate the potentially protective action of sAC inhibitors in humans with respect to possible clinical use.

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**Disclosures**

None.

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### What Is New?

- Soluble adenylyl cyclase (sAC) regulates expression of genes involved in endothelial stiffness.
- sAC inhibition results in endothelial softening.

### What Is Relevant?

- Our findings identify a potential mechanism by which endothelial sAC affects vascular elasticity.

### Summary

We illustrate a mechanism by which sAC affects endothelial stiffness. sAC is involved in expression regulation of aldosterone-sensitive genes *SCNN1A, ATP1A1, ATP1B1*, and *NR3C2*. Dysregulation of sAC seems to play a role in arterial stiffness.
Soluble Adenylyl Cyclase in Vascular Endothelium: Gene Expression Control of Epithelial Sodium Channel-α, Na+/K+-ATPase-α/β, and Mineralocorticoid Receptor
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Soluble adenylyl cyclase in vascular endothelium: gene expression control of ENaC-α, Na⁺/K⁺-ATPase-α/β and mineralocorticoid receptor

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Running title: Soluble adenylyl cyclase in endothelium

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Supplemental methods

Transient transfections and siRNA experiments

EA.hy926 and IHKE cells were transfected using Nanofectin (PAA) or jetPEI (PolyPlus-Transfection, Illkirch Cedex, France). The CRE reporter plasmid (pADneo2-C6-BGL) comprised six CRE promoter elements 5' to the firefly luciferase gene, the GRE reporter plasmid (TAT3-Luc) three GRE promoter elements 5' to the firefly luciferase gene. Transfection experiments were repeated at least two times in triplicates. sAC knockdown was performed by application of sAC-specific or scrambled siRNA (5 µM for 48h or 125 and 250 nM for 72h; Life Technologies, Darmstadt, Germany) using Oligofectamine (Invitrogen, Karlsruhe, Germany) in EA.hy926 and jetPRIME (PolyPlus) in IHKE cells. Transfection experiments were repeated at least three times in triplicates.

Real-time PCR

Total RNA was extracted using the NucleoSpin RNA II Extraction Kit (Macherey-Nagel, Düren, Germany). cDNA, generated from 1 µg of total RNA by Mul-V (Fermentas, St. Leon-Rot), was amplified in a 384-well format (standard real-time PCR conditions) in duplicates using Power SYBR Green (Applied Biosystem, Carlsbad, USA) on an Applied Biosystems 7500 Fast real-time PCR system. Relative quantification was calculated using the \( \Delta\Delta C_t \) method and GAPDH as endogenous control. The absence of non-specific amplification products was confirmed by agarose gel electrophoresis and generation of melting curves using the Applied Biosystems software. Oligonucleotides had an amplification efficiency of \( \geq 90\% \) (supplemental table S1).

Preparation of nuclear protein extracts

In brief, cells were resuspended in a “low salt” buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1 mM DTT, 1.5 mM MgCl₂, ‘Complete’ protease inhibitor cocktail; Roche, Mannheim,
Germany), lysed by addition of 0.05% NP40 and centrifuged. Intact nuclei were incubated in “high salt” buffer (20 mM HEPES pH 7.9, 0.2 mM EDTA, 1 mM DTT, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM PMSF, Roche ‘Complete’, 25% glycerol) and proteins were harvested by centrifugation.

**Western blot**

For crude extracts, cells were lysed in RIPA buffer containing 1% NP40 and 0.1% SDS supplemented with ‘PhosSTOP’ Phosphatase Inhibitor Cocktail (Roche). Immunodetection was performed using an anti-sAC (Deciphergen Biosystems, Fremont, USA; 1:1000), anti-CREB (Cell Signaling, Frankfurt, Germany; 1:1000), anti-phosphoCREB (Nanotools, Teningen, Germany; 1:1000), anti-ENaC-α (Santa Cruz, Heidelberg, Germany; 1:500), anti-Na⁺/K⁺-ATPase-α (Santa Cruz; 1:1000) and anti-mouse (GE Healthcare, Buckinghamshire, UK; 1:1000 - 1:10000), or anti-rabbit (GE Healthcare; 1:5000), or anti-goat (GE Healthcare; 1:20000) antibody, respectively. Sample loading was confirmed by β-actin detection (Cell Signaling; 1:5000; anti-rabbit secondary antibody; 1:10000).
Supplemental references


Table S1

Supplemental Table S1: Sequences and positions of oligonucleotides used in this study

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<tr>
<th>Description</th>
<th>Sequence 5'-3'</th>
<th>Position</th>
<th>Reference/Acc.#</th>
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Supplemental Figure S1: CREB phosphorylation is increased after 24h of sAC inhibition

Inhibition of sAC by KH7 for 24h resulted in increased CREB phosphorylation (CREB-p) in (A) EA.hy926 and (B) IHKE cells compared to control. Western blots are representative for three independent experiments. Band intensities were analyzed by densitometric quantification. Relative signal intensities are shown as standard deviation of the mean of three independent densitometric measurements compared to β-actin loading control. *** p<0.001, * p<0.05.
Supplemental Figure S2: sAC inhibition abrogates CRE- and GRE-mediated transcriptional activity in the presence of aldosterone

(A, B) CRE-mediated transcriptional activity was inhibited by KH7 in the presence of aldosterone in EA.hy926 and IHKE cells. Spironolactone (1 µM) did not affect CRE promoter activity. (C, D) GRE-mediated transcriptional activity was activated by application of aldosterone and dexamethasone in both cell lines. Treatment with KH7 and spironolactone (1 µM) prevented GRE promoter activation by aldosterone. Cell lines were treated with KH7/spironolactone and aldosterone (or dexamethasone, 50 µM) or DMSO/ethanol (control) starting 4h after reporter vector transfection for 24h. In combination with aldosterone (or dexamethasone, 50 µM), KH7/spironolactone was administered 15 min prior to aldosterone. Transfections are representative for three independent experiments. Transcriptional activity was assessed as relative light units (RLU). *** p<0.001, ** p<0.01, * p<0.05.
**Figure S3**

**Supplemental Figure S3: ENaC-α and Na⁺/K⁺-ATPase-α protein levels are reduced by sAC inhibition**

**A)** Inhibition of sAC by KH7 for 24h reduced cellular levels of active ENaC-α protein (band a) in EA.hy926 cells significantly compared to control (DMSO). No difference was observed for glycosylated ENaC-α (band g) or ubiquitinylated ENaC-α (band u). **B)** In IHKE cells, inhibition of sAC by KH7 for 24h resulted in a significant reduction of active ENaC-α (band a) and glycosylated ENaC-α (band g), while ubiquitinylated ENaC-α (band u) was not reduced. **C, D)** Na⁺/K⁺-ATPase-α protein levels were significantly reduced in both cell lines after inhibition of sAC by KH7 for 24h. Western blots are representative for three independent experiments. Relative band intensities are shown as mean ± SEM of densitometric measurements (24h KH7) compared to β-actin loading control of three independent blots. *** p<0.001, * p<0.05.
Supplemental Figure S4: Inhibition of sAC reduces endothelial stiffness

Treatment of ECs with KH7 for 24h resulted in a significant endothelial softening compared to control conditions (control: 0.98 ± 0.012 pN/nm, n=148; KH7: 0.90 ± 0.011 pN/nm, n=117). AFM measurements were repeated in three independent experimental settings. * p<0.05.
Supplemental Figure S5: Nuclear localization of the mineralocorticoid receptor (MR) in IHKE and EA.hy926 cells

The MR was detected by western blot analysis in nuclear extracts of IHKE and EA.hy926 cells under basic conditions. Western blots are representative for three independent experiments. β-actin served as gel loading control.