Cellular Repressor of E1A-Stimulated Genes Is a Critical Determinant of Vascular Remodeling in Response to Angiotensin II

Yang Li, Yanxia Liu, Xiaoxiang Tian, Yan Zhang, Haixu Song, Meili Liu, Xiaolin Zhang, Haiwei Liu, Jian Zhang, Quanyu Zhang, Dan Liu, Chengfei Peng, Chenghui Yan, Yaling Han

Objective—Cellular repressor of E1A-stimulated genes (CREG) is a lysosomal glycoprotein implicated in maintaining vascular homeostasis. Here, we have hypothesized that CREG is a critical target of intervention for the prevention of hypertensive vascular remodeling.

Approach and Results—CREG gene expression was significantly decreased accompanied by an upregulated expression of angiotensin II (Ang II) in remodeled vascular tissues of high salt–induced Dahl salt-sensitive rats and Ang II–induced mice. In particular, the downregulation of CREG gene was Ang II specific and independent from blood pressure. Prominent medial hypertrophy and vascular fibrosis in both thoracic aortas and mesenteric arteries were observed in CREG−/− mice infused with Ang II than in CREG+/+ mice, but blunted response in CREG+/+ mice received recombinant human CREG protein, suggesting that changes in CREG expression account for the different phenotype between genotypes. Within a tiled promoter array, E26 transformation-specific-1 led to restoration of CREG expression in aortas and rescue of experimental vascular remodeling by systemic administration of dominant negative E26 transformation-specific-1 membrane-permeable peptides.

Conclusions—CREG is a novel mediator of vascular remodeling in response to Ang II and may be an attractive therapeutic target for prevention of vascular diseases.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:485-494. DOI: 10.1161/ATVBAHA.116.308794.)

Key Words: Ang II ■ CREG ■ Ets1 ■ vascular remodeling ■ VSMCs

Reducing the high incidence of hypertension-related cardiovascular diseases is an important strategy for the optimal treatment of hypertension. Vascular remodeling, characterized by increased lumen size and thickened media with increased collagen deposition, is a typical feature of end-organ damage from hypertension, contributing to clinical morbidity and mortality.1–3 One of the causal factors for vascular remodeling is angiotensin II (Ang II), a key effector of the renin–angiotensin system. Studies have indicated that in addition to its roles in vasoconstriction and retention of sodium and water, causing hypertension, Ang II is also involved in stimulating vascular smooth muscle cell (VSMC) contraction, augmenting cell growth, increasing deposition of extracellular matrix, inducing cell migration, and promoting inflammation and oxidative stress, ultimately leading to vascular hypertrophy, fibrosis, and remodeling.4–6 Increased knowledge of the downstream signaling cascades of Ang II–induced hypertension and vascular remodeling is needed if effective clinical interventions are to be developed. The cellular repressor of E1A-stimulated genes (CREG) is a novel secreted glycoprotein associated broadly with diverse cellular processes.7,8 CREG is ubiquitously expressed in mature tissues and cells in mammals and is expressed at low levels in immature cells, such as pluripotent mouse embryonic stem cells, human embryonic carcinoma cells, and synthetic transformation-specific-1 led to restoration of CREG expression in aortas and rescue of experimental vascular remodeling. Within a tiled promoter array, E26 transformation-specific-1 binds to CREG promoter at high stringency with the stimulation of Ang II. Moreover, the Ang II–induced E26 transformation-specific-1 directly interacted with the CREG promoter (∼1179 and ∼271 bp) and inhibited its transcription in vascular smooth muscle cells. Selective, pharmacological inhibition of E26 transformation-specific-1 led to restoration of CREG expression in aortas and rescue of experimental vascular remodeling by systemic administration of dominant negative E26 transformation-specific-1 membrane-permeable peptides.

Modulation of CREG levels may, therefore, determine the processes of vascular (patho)biology. Previous studies...
have demonstrated that transcription factor (TF) E2F1 and microRNA-31 binds directly to CREG promoter and 3′UTR (untranslated region) to repress expression of CREG gene, respectively, and then modulates the VSMC phenotype through the interactions. However, no information is available regarding a role of CREG in Ang II–mediated vascular remodeling. We report here that CREG is a determining factor in the development of vascular remodeling in which Ang II–induced Ets1 activation plays a critical role in the downregulation of CREG gene expression.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Involvement of CREG in Ang II–Mediated Vascular Remodeling
For evaluation of the potential role of CREG as a mediator of vascular remodeling in vivo, Dahl salt-sensitive rats fed a high salt (4% NaCl) diet for 8 weeks developed an increase in medial to lumen ratio and medial area (Figure I in the online-only Data Supplement), a decrease in CREG gene expression, and an increase in vascular local Ang II and aldosterone content as compared with those fed with a normal salt (0.5% NaCl) diet (Figure 1). The administration of the ARB (angiotensin II receptor blocker) candesartan (10 mg/kg per day) significantly inhibited the downregulation of CREG expression while on high salt diet (Figure 1A and 1B), indicating that high salt–induced increase in local Ang II level might cause CREG downregulation and vascular remodeling in artery tissues.

In Ang II–induced hypertension model, Ang II infusion (400 ng/kg per minute) led to a predictable increase in plasma and vascular Ang II levels (Figure 2A and 2B). CREG was markedly detectable in mouse normal aorta. The administration of Ang II resulted in a significant decrease in the level of CREG mRNA from the second day and CREG protein from the third day compared with saline infusion groups (Figure 2C and 2D). Increased medial to lumen ratio and medial area and deposition of collagen content in the medial layer of thoracic and mesenteric aortas were seen at 7 days and gradually increased in quantity ≤28 days (Figure 2E through 2G; Figures II and III in the online-only Data Supplement). Of interest, CREG expression was found to be reduced prior to the development of hypertensive vascular remodeling.

A low dose of Ang II (100 ng/kg per minute for 28 days) did not modify blood pressure levels in mice. However, the subpressure dose of Ang II also induced the downregulation of CREG gene expression, as well as medial hypertrophy and vascular fibrosis in thoracic aortas (Figure IV in the online-only Data Supplement). By contrast, although norepinephrine (5.6 mg/kg per day for 28 days) produced a similar increase in blood pressure like high dose of Ang II (400 ng/kg per minute), norepinephrine did not change CREG gene expression and vascular morphology (Figure V in the online-only Data Supplement). Thus, downregulation of CREG expression during vascular remodeling was not the result of hypertension alone, but of specific effect of Ang II.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
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<tr>
<td>Ang II</td>
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<tr>
<td>CREG</td>
<td>cellular repressor of E1A-stimulated genes</td>
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<td>Ets1</td>
<td>E26 transformation-specific-1</td>
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<td>rhCREG</td>
<td>recombinant human cellular repressor of E1A-stimulated genes</td>
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<td>TF</td>
<td>transcription factor</td>
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<td>VSMCs</td>
<td>vascular smooth muscle cells</td>
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Figure 1. High salt (HS) induced increase in downregulation of cellular repressor of E1A-stimulated genes (CREG) gene expression in aortas and local angiotensin II (Ang II) and aldosterone levels of Dahl salt-sensitive (Dahl SS) rats. A and B, mRNA and protein content of CREG in the thoracic aortas were analyzed using real-time polymerase chain reaction (PCR) and Western blot analysis in normal salt (NS, 0.5% NaCl), HS (4% NaCl), and HS+ARB (angiotensin II receptor blocker; candesartan, 10 mg/kg per day), respectively. C and D, Plasma and vascular tissue Ang II and aldosterone levels were detected. Values are means±SEM, n=10 per group; *P<0.05, **P<0.01 vs NS; ##P<0.01 vs HS.
CREG Deficiency Aggravates Ang II–Induced Vascular Dysfunction

Heterozygous deletion of the CREG gene was found to significantly reduce CREG expression in the aorta. Meanwhile, a reduced expression of CREG was observed in both the CREG<sup>+/−</sup> and CREG<sup>−/−</sup> mouse aortas after Ang II infusion, with a significant reduction from that of CREG<sup>+/−</sup> mouse aortas (Figure 3A and 3B). Vascular morphometry in CREG<sup>+/−</sup> showed a more severe phenotype than that in CREG<sup>+/+</sup> mice 28 days after Ang II infusion, including media hypertrophy and vascular fibrosis (Figure 3C through 3E; Figures VI and VII in the online-only Data Supplement). Taken together, these results indicate that CREG deficiency exacerbates the Ang II–induced vascular remodeling.

Rescue With Exogenous Recombinant Human CREG Protein Reverses Ang II–Induced Vascular Remodeling

We next evaluated whether exogenous rhCREG (recombinant human CREG) protein could prevent Ang II–mediated pathological remodeling in the aortas implanted with a drug delivery system connected to a mini-osmotic pump. Application of rhCREG protein prevented downregulation of CREG protein expression induced by Ang II in the aortas (Figure 4B), although that had no effect on CREG mRNA level (Figure 4A). Morphometric analyses confirmed the marked protective effects of rhCREG protein against Ang II–induced vascular remodeling (Figure 4C and 4D; Figures VIII through XA in the online-only Data Supplement), with reduced media to lumen ratio, medial area, and vascular fibrosis in a dose-dependent manner. These findings clearly indicate that rhCREG protein rescued the adverse effects of Ang II on the arteries.

Transcription Factor Ets1-Mediated Repression of CREG Gene Expression by Ang II

Based on the above data demonstrating that CREG is a critical regulator of vascular remodeling in response to Ang II, and Ang II–induced downregulation of CREG gene in arteries plays an important role in regulating vascular remodeling,
we, therefore, explored the regulative mechanism of Ang II–mediated downregulation of CREG gene expression both in arteries and in VSMCs. First, the promoter-binding TFs profiling plate array was performed in VSMCs with or without Ang II stimulation. Out of 48 TFs, Ets1 and Stat1 were chosen as the TFs most likely to bind to the CREG promoter according to the bio-information analysis (PROMO database, version 8.3 of TRANSFAC; Table).

Then, a ChIP assay was used to confirm binding of Ets1 and Stat1 to specific binding sites within the CREG promoter using cellular extracts from VSMCs stimulated with Ang II. PCR primers were made for 6 regions of the CREG promoter, denoted ChIP1 through ChIP6. Immunoprecipitation of the protein–DNA cross-linked fragments using Ets1-specific antibody confirmed binding of Ets1 to regions containing the -1179 and -271 Ets1 sites (ChIP3 and ChIP5; Figure 5). We also performed a similar ChIP for the TF Stat1 that indicated no binding site of Stat1 in CREG promoter (Figure XB in the online-only Data Supplement). The data support Ets1 binding to the CREG promoter in response to Ang II stimulation.

Furthermore, Ets1 expression was detected to increase in a concentration- and time-dependent manner when VSMCs were treated with Ang II (Figure 6A and 6B). The presence of candesartan blocked the Ang II–induced changes of CREG and Ets1 protein expressions in VSMCs. In contrast, inhibition of the Ang II type 2 receptor did not significantly alter the changes of CREG and Ets1 protein expressions.
Li et al  CREG Is a Determinant of Vascular Remodeling

Figure 4. Exogenous rhCREG (recombinant human cellular repressor of E1A-stimulated genes) protein rescues vascular remodeling in response to angiotensin II (Ang II). A and B, mRNA and protein contents of CREG in the aortas of CREG+/+ mice after infusion of Ang II or Ang II plus rhCREG protein for 4 weeks were analyzed using real-time polymerase chain reaction (PCR) and Western blot analysis, respectively. C, Representative images of vessel sections stained with hematoxylin & eosin (HE) and Masson. Bar graphs show the media/lumen ratio, total media area, and collagen content in the media. D, Medial thickness was determined in the ascending aorta and in the brachiocephalic artery (BCA) by ultrasound biomicroscopy. Values are means±SEM, n=15 to 16 per group, *P<0.05, **P<0.01 vs saline; #P<0.05, ##P<0.01 vs Ang II.

In response to Ang II (Figure 6C), Confocal microscopy showed that Ets1 was mainly localized in the cytoplasm under basal conditions, and nucleus translocation of Ets1 was noted with Ang II treatment for 2 hours in VSMCs (Figure 6D), an earlier time point than the downregulation of the CREG gene. We then examined whether Ets1 is directly involved in the regulation of CREG gene transcription. VSMCs treated with siRNA directed against Ets1 led to a marked suppression in the decrease of the activity of the CREG luciferase reporter (Figure 6E) and CREG gene expression by Ang II (Figure 6F and 6G). Additionally, transient transfection in VSMCs with the plasmid encoding Ets1 resulted in a decrease in the activity of the CREG luciferase reporter (Figure 6E) and CREG gene expression by Ang II (Figure 6F and 6G). The dominant negative Ets1 peptide did not have this effect (Figure 7A). Similarly, the dominant negative Ets1 peptide blunted the increase in aortic medial area, media to lumen ratio, and collagen deposition compared with those treated with saline or the mutant Ets1 peptide (Figure 7B; Figure XC in the online-only Data Supplement). These results indicate that the Ets1-mediated decrease in CREG expression observed in the aorta in response to Ang II is attributable, at least in part, to the induction of hypertensive vascular remodeling (Figure XD in the online-only Data Supplement).

Discussion

The CREG gene, discovered in 1998, is located at Chromosome 1 locus 1q24.7 Mammalian CREG is a small, 220 amino acid, endosomal/lysosomal glycoprotein implicated in cellular growth and differentiation.5,8-10,15,17-19 Human CREG is 77% identical to mouse CREG and 31% identical to the Drosophila homolog. Previous studies showed that CREG resides in lysosomal compartments and is proteolytically processed by the action of lysosomal cysteine proteinases.7 In the present study, the results demonstrate, for the first time, an important role of CREG in controlling vascular remodeling in response to Ang II.

Systemic administration of Ang II via continuous infusion is associated with significant vascular remodeling over a period of several weeks.5,20,21 In our study, we demonstrated that Ang II leads to structural changes in the thoracic and mesenteric aorta that are similar to those observed previously,
including medial hypertrophy and collagen deposition. Moreover, we also found that CREG expression was downregulated in the arteries of mice with vascular remodeling.

Figure 5. Transcription factor E26 transformation-specific-1 (Ets1) binds to the cellular repressor of E1A-stimulated genes (CREG) promoter in response to angiotensin II (Ang II) stimulation in vascular smooth muscle cells (VSMCs). ChIP analysis of CREG promoter in VSMCs stimulated with Ang II (100 nmol/L) for 2 and 6 hours. Cross-linked chromatin was immunoprecipitated with an antibody to Ets1, in the absence of antibody (input), or an isotype-matched control (IgG). Isolated DNA was purified and analyzed by polymerase chain reaction (PCR) using primers shown at top. Data are representative of 5 independent experiments.

Table. Transcription Factors Binding to the Mouse CREG Promoter

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CREG indicates cellular repressor of E1A-stimulated genes; Ets1, E26 transformation-specific-1; and RLU, relative light unit.

Table. Continued

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*TFs most likely to bind to the CREG promoter according to the promoter-binding TF profiling array and the bio-information analysis.
compared with saline-treated mice. Changes in CREG expression in arteries, prior to initiation of vascular remodeling, may provide information about the pathological status of the artery. In earlier studies, we reported that the expression of CREG in an injured carotid wall was decreased, and injury-induced CREG downregulation facilitated VSMC dedifferentiation and adventitial fibroblast transformation, as well as cell proliferation, migration, and lesion formation. 8,12–15 In this context, CREG may serve as a critical mediator in the maintenance of vascular homeostasis in the process of vascular remodeling.

Figure 6. E26 transformation-specific-1 (Ets1) inhibits cellular repressor of E1A-stimulated genes (CREG) gene expression in response to angiotensin II (Ang II) simulation. A and B, Ang II regulates the expression of CREG and Ets1. Vascular smooth muscle cells (VSMCs) were stimulated for 24 h with increasing concentrations of Ang II or 100 nmol/L Ang II for the indicated time. C, Effect of Ang II on CREG and Ets1 protein expressions was mediated by angiotensin II receptor type 1 (AT1R) but not by angiotensin II receptor type 2 (AT2R). Changes of CREG and Ets1 protein expressions by Ang II were blocked by pretreatment with candesartan (10 μM), an AT1R antagonist, but not with PD123319 (10 μM), an AT2R antagonist. D, Immunofluorescence analysis of nuclear translocation of Ets1 induced by Ang II for 0, 2, 6, and 12 h. Green fluorescence shows Ets1 localization; blue shows DAPI nuclear stain; the merged images show colocalization of activated Ets1 in the nuclei. E, VSMCs were transfected with firefly luciferase (Luc) expression plasmids containing the promoter region of CREG. In addition, control siRNA, Ets1 siRNA, empty plasmid, or Ets1 expression plasmid were transfected with or without application of Ang II (12 h). After 36 hours, cell lysates were assayed for luciferase activity.

Ets1 siRNA transfection on Ang II–induced downregulation of CREG expression. VSMCs were transfected with Ets1 siRNA (20–40 nmol/L) or a control siRNA (40 nmol/L). mRNA and protein level of CREG in VSMCs in response to Ang II for 12 or 24 h were analyzed using real-time PCR and Western blot analysis, respectively. H, Effect of Ets1 expression plasmid on CREG expression. VSMCs were transfected with Ets1 expression plasmid (3 μg) or an empty plasmid (3 μg) for 72 h. mRNA and protein level of CREG in VSMCs were analyzed using real-time PCR and Western blot analysis, respectively. Real-time PCR (I) and Western blot analysis (J) of CREG in untreated control or Ang II–stimulated VSMCs after pretreatment with dominant negative-Ets1 (DN-Ets1; 10 and 20 μmol/L) or mutant Ets1 (Mut-Ets1) peptide (20 μmol/L) for 6 h. Data are summaries of 5 to 7 separate experiments. Values are means±SEM. **P<0.01 vs control; #P<0.05, ##P<0.01 vs Ang II.
CREG gene ablation in mice causes early embryo lethality. The heterozygous CREG mice with loss of only a single copy of the CREG gene are born at the expected Mendelian ratio and develop into adulthood. CREG+/− mice, of which levels of CREG are 50% to 55% lower than those of wild-type mice, show normal vascular function under basal conditions. However, they are more prone to stress than wild-type mice. We showed an exaggerated hypertensive vascular remodeling response to Ang II in mice with lower levels of CREG, which could be the cumulative outcome of drastically reduced expression of CREG in arteries. In contrast, rescue with exogenous rhCREG protein protected against Ang II–induced vascular remodeling in a dose-dependent manner, which agrees with the hypothesis that protective effects may act through the regulation of CREG expression in vascular cells, in particular with regard to the possible direct positive or negative regulation of CREG as a promoter-bound factor.

Available data indicate that several mechanisms could be involved in the regulation of CREG gene. TF E2F1, positioned on the human CREG promoter at −446 and −97 bp from the transcription initiation site, results in repression of CREG promoter activity and mRNA/protein levels. MicroRNA is a type of posttranscriptional regulation of gene expression and may regulate at least 30% of the genes in a cell. We previously showed that miRNA-31 directly binds to CREG 3′UTR and modulates the VSMC phenotype through this interaction. The main purpose of this study was to elucidate the detailed molecular mechanism, whereby Ang II downregulates the expression of the CREG gene in VSMCs. This led to the identification of Ets1 as the major and direct mediator of Ang II–induced inhibitory effect on mouse CREG transcription.

The Ets1 is the founding member of the Ets gene family, whose members share a highly conserved DNA-binding domain GGAA/T sequence and are commonly involved in the regulation of development, inflammation, immunity, differentiation, and proliferation. It has been reported that Ets1 is expressed in endothelial cells and VSMCs in blood vessels and is a critical regulator of Ang II–mediated vascular inflammation and remodeling. In agreement with previous observations, our study shows that Ang II increases Ets1 expression in a time- and dose-dependent manner in mouse VSMCs, which inversely correlates with the changes seen in CREG, and it also induces Ets1 nucleus translocation, delineating the critical role of Ets1 in the mechanism by which Ang II controls CREG gene expression. Moreover, this notion was confirmed by siRNA-mediated silencing of Ets1, which led to inhibition of Ang II–induced downregulation of CREG.

Figure 7. Effects of DN-Ets1 (dominant negative E26 transformation-specific-1) peptide on cellular repressor of E1A-stimulated genes (CREG) expression and vascular remodeling in response to angiotensin II (Ang II). A, Protein content of CREG in the aortas of CREG+/+ mice after infusion of Ang II with or without coinfusion DN-Ets1 peptide (20 mg/kg-per d) or mutant Ets1 (Mut-Ets1) peptide (20 mg/kg-per d) for 0, 7, and 28 days was analyzed using Western blot analysis. B, Representative images of vessel sections stained with hematoxylin & eosin (HE) and Masson showed the effect of DN-Ets1 peptide to Ang II–induced vascular remodeling. Quantization of media/lumen ratio, total media area, and collagen content in the media after infusion with saline or Ang II with coinfusion of DN-Ets1 or Mut-Ets1 peptide for 4 weeks. Values are means±SEM, n=12 to 13 per group, *P<0.05, **P<0.01 vs pre group, #P<0.05 vs Ang II infusion group.
levels. Additionally, the involvement of Ets1 in regulating CREG expression was further examined by upregulating Ets1 expression in VSMCs. Increasing the expression level of Ets1 resulted in marked downregulation of CREG mRNA and protein. Taken together, these data demonstrate that the low level of expression of CREG in VSMCs is maintained by Ang II–induced Ets1, providing a rationale for understanding the occurrence and development of vascular remodeling in response to Ang II.

Ets1 has a dual function, acting as both a transcriptional activator and a repressor via association with specific cofactors and in combination with other TFs, depending on promoter context. In most cases, Ets1 positively regulates the expression of its target genes. However, Ets1 also functions as repressor of its target genes, including interleukin-10, cyclin D3, retinoblastoma-associated factor 600, AXL receptor tyrosine kinase, MET tyrosine kinase, β2-adrenergic receptor, stromal cell–derived factor receptor 1, ATP5G3, 5-tubulin, serine–threonine–kinase 6, and anilin/actin-binding protein. In addition, Ets1 can also suppress gene expression through interacting with repressive factors like EAP1/Daxx, HDAC1, mSin3a, and DNA methyltransferase 3a and 3b.

These molecules could form an Ets1 repressor complex and affect the epigenetic state of DNA methylation and histone modifications, resulting in an inactive chromatin configuration at Ets1 target gene loci.

Understanding the mechanisms of transcriptional gene regulation involved in vascular homeostasis can provide key insights into potential therapeutic strategies. Our findings demonstrate that CREG is a novel modulator that is involved in the process of Ang II–induced vascular remodeling and further elucidate that the activation of Ets1 activity by Ang II contributes to decreased expression of the CREG gene (Figure 7G). These effects, in turn, lead to medial hypertrophy and collagen deposition. This newly discovered relationship between vascular CREG and Ets1 may be informative for the treatment of patients with vascular remodeling associated with Ang II.

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Disclosures

None.

References


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**Highlights**

- The downregulating gene expression of cellular repressor of E1A-stimulated genes in remodeled arteries is angiotensin II specific and blood pressure independent.
- Cellular repressor of E1A-stimulated genes is key regulator of angiotensin II–induced vascular remodeling.
- Angiotensin II–induced cellular repressor of E1A-stimulated genes downregulation is most likely mediated by the activation of the transcription factor E26 transformation-specific-1.
Cellular Repressor of E1A-Stimulated Genes Is a Critical Determinant of Vascular Remodeling in Response to Angiotensin II

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Materials and Methods

Animal models

All experimental procedures were performed in accordance with the guidelines and approval from the Animal Research Committee of General Hospital of Shenyang Military Region. Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The male Dahl salt-sensitive (Dahl SS) rats and mice were housed at 18 to 22 °C with bedding and nest material and purified water (Milli-Q system; Millipore, Glostrup, Denmark) ad libitum. Rats and Mice were kept under conditions of a 12-h light: dark cycle. Experiments began after 1 week of acclimatization.

8-week-old Dahl SS rats were divided into 3 groups (n=10 per group) and treated for 8 weeks as follows: normal salt (NS), fed 0.5% NaCl diet; high salt (HS), fed 4% NaCl diet; HS+angiotensin II receptor I blocker (ARB), fed 4% NaCl diet plus the ARB candesartan (10 mg/kg·d in the drinking water).

12-week-old CREG heterozygous (CREG+/-) mice and age-matched littermate male wild-type (CREG+/-) mice backcrossed for 10 generations with the C57BL/6 strain were used. The methods for generating the CREG+/- mice and characteristics of the strain were as previously described.

Saline, Ang II (400 ng/kg·min, A-9525, Sigma), rhCREG protein (15-300 μg/kg·d, TP750059, Origene), DN-Ets1 peptide (20 mg/kg·d) or Mut-Ets1 peptide (20 mg/kg·d) loaded micro-osmotic pumps (Alzet model 1004; AlzaCorp., Mountain View, CA) were implanted ≤ 28 days subcutaneously (intrascapular region) under isoflurane anesthesia using sterile procedures.

VSMC cell culture and gene transfer

Mouse VSMCs were isolated from thoracic aortas and cultured in fresh DMEM containing 20% FBS (Biochrom AG, Germany) as previously described. Scrambled (sc-36869), mouse CREG (sc-142565) siRNA (Santa Cruz Biotechnology), or mouse Ets1 siRNA (Origene, SR414467), or mammalian expression plasmid encoding Ets1 (Origene, SR302346) were transfected into VSMCs using Fugene HD reagent according to the manufacturer’s protocol (Promega, Madison, WI, United States).

Blood pressure measurement

The PhysioTel telemetry system with PA-C10 telemetry transmitters (Data Sciences International) was used to quantify changes in blood pressure in the rats and mice as described previously.

Morphometric analysis

On 0, 1, 2, 3, 7, 14, or 28 days after Ang II infusion, all mice were perfuse-fixed at 80 mmHg with paraformaldehyde after a PBS wash. Thoracic aortas and mesenteric arteries were dissected out, further fixed in 4% buffered paraformaldehyde for 48 h. After fixation, tissues were paraffin embedded, sectioned (5-μm thickness), and stained with Hematoxylin and Eosin (HE), Masson’s trichrome (Sigma, HT15), Sirus red, and Verhoeff van Gieson Elastic (H Sigma, T25A) stain. For the area calculations,
four measurements for each artery section were made using the Image Pro Plus software program (Media Cybernetics Inc; Silver Spring, MD): circumference of the inner/outer elastic lamina, and area inside the inner/outer elastic lamina. The mean vascular diameter was calculated as the circumference of the inner elastic lamina/π. The index of arterial medial thickening was determined by the ratio of media to lumen, which was calculated-[(circumference of the outer elastic lamina-circumference of the inner elastic lamina)/2m]/(circumference of the inner elastic lamina/π), and medial area, which was calculated-(area inside the outer elastic lamina-area inside the inner elastic lamina); the index of vascular fibrosis was calculated as the ratio of fibrosis area within the vessel (collagen deposition stained with blue in Masson’s trichrome staining or red in Sirus red staining) to the total vessel area. Eight to ten sequential sections per thoracic aorta were measured for each mouse and used for analysis.

Immunohistochemistry/immunofluorescence staining
To directly visualize the endocytosis of CREG, purified rhCREG protein was labeled with Alexa 488 in vitro using the Alexa Fluor 488 Protein Labeling Kit (Life Technologies), following the instructions from the manufacturer. Immunohistochemistry and immunofluorescence analysis were performed as described previously.

Ang II and aldosterone measurement
Ang II concentrations in the thoracic aorta and plasma samples were measured by HPLC and radioimmunoassay as previously described. Plasma and aortic aldosterone concentrations were determined using a commercial EIA kit (EnZo Life Science, Plymouth Meeting, PA) according to the manufacturer’s instructions.

Gene Expression Analysis
Total RNA extraction and real-time PCR were performed as described previously. The following primers were used: for mouse CREG, 5’-TGTACCTGAGTCCACTGCAG-3’ (forward) and 5’-TCGAACAAACAGCGAATCCC-3’ (reverse); for rat CREG, 5’-TTGGACTACTTTTGGTGACC-3’ (forward) and 5’-TCGAAACAGAGGGTGGCA-3’ (reverse); for mouse GAPDH, 5’-ACATCATCCCTGCATCCACT-3’ (forward) and 5’-CCTGCTTCACCACCTTCTTG-3’ (reverse); and for rat GAPDH, 5’-GCATCTTCTTGTGCAGTC-3’ (forward) and 5’-CTTGCCGTGGGTAGAGTCAT-3’ (reverse).

Western-blot analysis
The thoracic aortas of each rat or mouse were dissected, combined, and homogenized. The homogenized tissue or cells were lysed in RIPA buffer (Thermo Scientific, USA). The cleared supernatant was collected and protein concentration was determined by the BCA Protein Assay Kit (Thermo Scientific). Western blot was performed as described previously.
Ultrasound biomicroscopy

On the day of euthanasia, medial thickness was determined by B-mode ultrasonography with a Vevo 2100 apparatus (Visual Sonics, Toronto, Canada) equipped with a 30-MHz probe, with a depth focus at 12.7 mm. A left parasternal long-axis view was used to visualize the ascending aorta. A right parasternal short-axis view was used to visualize the brachiocephalic artery. All measurements were performed at systole by an operator blinded for genotypes. For the ascending aorta, measurements were taken ≈ 1 mm away from the beginning of the aortic root; and for the brachiocephalic artery, the measurement was taken ≈ 0.5 mm away from the branching from the aortic arch. Medial thickness was defined as follows: (outer diameter-inner diameter)/2.

Promoter-binding transcription factor profiling array

To characterize transcription factors (TFs) that bind to the CREG promoter or that regulate the expression of the CREG gene via its upstream promoter, the activity of 48 TFs in mouse VSMCs was assayed using a promoter-binding TF profiling plate array according to the manufacturer’s instructions (Signosis Inc.).

Expression vector and luciferase reporter gene constructs

A 2,000-bp fragment corresponding to nucleotides -2000 to 0 of the mouse CREG promoter was inserted into pGL3 (Promega) at the XhoI/BglII multiple cloning site upstream of the luciferase gene of pGL3 vector, resulting in a pGL3-CREG promoter region. In reporter assays, 60 ng pGL3-CREG promoter was transfected together with 720 ng Ets1 expression plasmid, 20 nmol/L Ets1 siRNA and 6 ng internal control plasmid (pCMV-Renilla luciferase) using Fugene HD reagent (Promega). Transfected cells were cultured for 72 h, and a Dual Luciferase Assay (Promega) was performed. For this assay, relative activity was defined by the ratio of firefly to renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a ChIP assay kit (Millipore). PCR was performed using the following primer sets: for ChIP1 (-1773 ~ -1630 bp), 5’-TATTGGTGACCCACAGGAC-3’ (forward) and 5’-AGGAGGAGGAAGAAGAAG-3’ (reverse); for ChIP2 (-1692 ~ -1592 bp), 5’-ACTCCTCCTCTGCTTCTT-3’ (forward) and 5’-TAGGTGAGACGGGGAAAG-3’ (reverse); for ChIP3 (-1292 ~ -1139 bp), 5’-AGGGAGCACCTCAG-3’ (forward) and 5’-TAGGCCAGATGAACTAA-3’ (reverse); for ChIP4 (-793 ~ -663 bp), 5’-GCACGGACTGCTCTT-3’ (forward) and 5’-CTCGTTTGCCTCGTTTTA-3’ (reverse); for ChIP5 (-346 ~ -246 bp), 5’-AGGGAGGAGGATGACCTAAAG-3’ (forward) and 5’-CTAGAAGATTGAACTCTAAT-3’ (reverse); for ChIP6 (-197 ~ -87 bp), 5’-AGGGCAATTCGCACTCT-3’ (forward) and 5’-GAGATGACGAGGGAAG-3’ (reverse).

Statistical analysis
All values are expressed as mean ± SEM. Data were evaluated using SPSS version 19.0 (SPSS, Inc., Chicago, Illinois). Differences between the two groups were compared using unpaired Student’s t-tests. Differences among three or more than three groups were compared using one-way ANOVA followed Tukey’s post-hoc test. A p value < 0.05 was considered statistically significant.

Reference


Supplemental Figure I. Effect of ARB on high salt (HS)-induced vascular remodeling in aortas of Dahl salt-sensitive (Dahl SS) rats. Representative images showing the effect of NS diet (0.5% NaCl), HS diet (4% NaCl) or HS diet +ARB (candesartan, 10 mg/kg·d) on vascular remodeling. Vessel sections were stained with HE. Bar graphs showed the media/lumen ratio and total media area. Values are means ± SEM, n = 10 per group, **p < 0.01 vs. NS; ##p < 0.01 vs. HS.
Supplemental Figure II. Ang II induced hypertension and vascular remodeling in CREG\textsuperscript{+\,+} mice. (A) The time courses of changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rates (HR) in response to chronic infusion of saline and Ang II (400 ng/kg·min) as determined by telemetry. (B) Representative images of vessel sections stained with HE, Masson, Sirius red, elastin or α-SMA. Bar show showed the media/lumen ratio, total media area, and collagen content in the media. Values are means ± SEM, n = 12-15 per group, *p < 0.05, **p < 0.01.
Supplemental Figure III. Structural changes of mesenteric arteries in response to Ang II. Representative images of vessel sections stained with HE, Masson, Sirus red, elastin or α-SMA. Bar graphs showed the media/lumen ratio, total media area, collagen and elastin content in the medial. Values are means ± SEM, n = 12-15 per group, *p < 0.05, **p < 0.01.
Supplemental Figure IV. Subpressor Ang II treatment induces the downregulation of CREG gene and vascular remodeling. The time courses of changes in SBP, DBP, MAP and HR in response to chronic infusion of saline and subpressor Ang II (100 ng/kg·min) as determined by telemetry. (B and C) mRNA and protein content of CREG in the thoracic aortas of CREG+/+ mice after infusion of Ang II for 28 days were analyzed using real-time PCR and western blot analysis, respectively. (D) Representative images of vessel sections stained with HE, Masson, Sirius red, elastin or α-SMA. Bar showed showed the media/lumen ratio, total media area, and collagen content in the media. Values are means ± SEM, n = 12-14 per group, *p < 0.05, **p < 0.01.
Supplemental Figure V. Effect of norepinephrine infusion on blood pressure, aterial CREG gene expression and vascular remodeling in mice. (A) The time courses of changes in SBP, DBP, MAP and HR in response to chronic infusion of saline and norepinephrine (NE, 5.6 mg/kg·d) as determined by telemetry. (B and C) mRNA and protein content of CREG in the thoracic aortas of CREG+/+ mice after infusion of NE for 28 days were analyzed using real-time PCR and western blot analysis, respectively. (D) Representative images of vessel sections stained with HE, Masson, Sirus red, elastin or α-SMA. Bar show showed the media/lumen ratio, total media area, and collagen content in the media. Values are means ± SEM, n = 8-10 per group, **p < 0.01.
Supplemental Figure VI. Exacerbated hypertension and thoracic aorta remodeling in CREG\textsuperscript{+/−} mice in response to Ang II infusion. Representative images of vessel sections stained with Sirus red, elastin or α-SMA. n = 10-15 per group.
Supplemental Figure VII. Exacerbated mesenteric aorta remodeling in CREG\(^{+/−}\) mice in response to Ang II infusion. Representative images of vessel sections stained with HE, Masson, Sirius red, elastin or α-SMA. Bar graphs show the media/lumen ratio, total media area, collagen and elastin content in the medial. Values are means ± SEM, n = 10-15 per group, *\(p < 0.05\), **\(p < 0.01\).
Supplemental Figure VII. Location of exogenous rhCREG protein. (A) Confocal microscopy detects Alexa-488 labeled rhCREG protein in VSMCs. Expression of CREG (red) was examined by immunofluorescence staining after incubation with rhCREG protein (5 μg/ml, green) for 12 h. Nuclei were counterstained with DAPI (blue). (B) The rhCREG protein contains a C-terminal His tag. Expression of the His tag was examined by immunohistochemistry in heart, liver, lung, kidney, aorta, and fat tissue 2 weeks after exogenous rhCREG protein (300 μg/kg·d) infusion. Data are representative of 4-5 independent experiments.
Supplemental Figure IX. Location of exogenous rhCREG protein. Confocal microscopy detects Alexa-488 labeled rhCREG protein in VSMCs. Expression of lysosome markers (cathepsin D, cathepsin S and LAMP1, red) was examined by immunofluorescence staining after incubation with rhCREG protein (5 μg/ml, green) for 12 h. Nuclei were counterstained with DAPI (blue). The data indicate that exogenous rhCREG protein is located in lysosomes. Data are representative of 5-7 independent experiments.
Supplemental Figure X. Exogenous rhCREG protein rescues vascular remodeling in response to Ang II. Representative images of vessel sections stained with Sirius red, elastin or α-SMA. n = 15-16 per group.
Supplemental Figure XA. Exogenous rhCREG protein rescues mesenteric aortic remodeling in response to Ang II. Representative images of vessel sections stained with HE, Masson, Sirius red, elastin or α-SMA. Bar graphs showed the media/lumen ratio, total media area, collagen and elastin content in the medial. Values are means ± SEM, n = 15-16 per group, **p < 0.01.
Supplemental Figure XB. Transcription factor Stat1 does not bind to the CREG promoter in response to Ang II stimulation in VSMCs. ChIP analysis of CREG promoter in VSMCs stimulated with Ang II (100 nM) for 2 and 6 hours. Cross-linked chromatin was immunoprecipitated with an antibody to Stat1, in the absence of antibody (input), or an isotype-matched control (IgG). Isolated DNA was purified and analyzed by PCR using primers shown at top. Data are representative of five independent experiments.
Supplemental Figure X. Localization of DN-Ets1 peptide. (A) Expression of biotinylated DN-Ets1 (green) in VSMCs were examined by immunofluorescence staining after incubation with DN-Ets1 peptide (10 μmol/L and 20 μmol/L) for 6 h. (B) Detection of the DN-Ets1 peptide within the vessel wall was evaluated using an anti-biotin-FITC antibody (green) by confocal microscopy 2 weeks after infusion. Control aortic segments were obtained from the saline infused mice. Nuclei were counterstained with DAPI (blue), and evidence for colocalization was demonstrated by merging the two images. Data are representative of 6 to 8 independent experiments.
Supplemental Figure XD. Proposed model. Ets1-mediated CREG down-regulation induced by Ang II promotes vascular remodeling.
Supplemental Figure XE. Ang II inhibits CREG expression in primary mouse VSMCs. (A) Representative micrographs of α-SMA, desmin, smoothlin and vimentin immunofluorescent staining in primary cultured cells. (B) Ang II treatment (100 nM, 24 h) induced a decrease in CREG expression in VSMCs. Data are representative of five independent experiments. Nuclei are stained using DAPI (blue).
Supplemental Figure XF. Physiological assessment of the CREG$^{++}$ mice. Bar graphs showed systolic blood pressure, heart rate, heart weight, body weight, levels of plasma and aortic Ang II and aldosterone with Pre, saline post 28 d and Ang II post 28 d.
Supplemental Figure XG. Physiological assessment of the CREG$^{+/+}$ mice infused with rhCREG protein. Bar graphs showed systolic blood pressure, heart rate, heart weight, body weight, levels of plasma and aortic Ang II and aldosterone with Pre, saline post 28 d and Ang II post 28 d.