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C/EBPβ and Nuclear Factor of Activated T Cells Differentially Regulate Adamts-1 Induction by Stimuli Associated with Vascular Remodeling

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Emerging evidence indicates that the metalloproteinase Adamts-1 plays a significant role in the pathophysiology of vessel remodeling, but little is known about the signaling pathways that control Adamts-1 expression. We show that vascular endothelial growth factor (VEGF), angiotensin-II, interleukin-1β, and tumor necrosis factor α, stimuli implicated in pathological vascular remodeling, increase Adamts-1 expression in endothelial and vascular smooth muscle cells. Analysis of the intracellular signaling pathways implicated in this process revealed that VEGF and angiotensin-II upregulate Adamts-1 expression via activation of differential signaling pathways that ultimately promote functional binding of the NFAT or C/EBPβ transcription factors, respectively, to the *Adamts-1* promoter. Infusion of mice with angiotensin-II triggered phosphorylation and nuclear translocation of C/EBPβ proteins in aortic cells concomitantly with an increase in the expression of Adamts-1, further underscoring the importance of C/EBPβ signaling in angiotensin-II-induced upregulation of Adamts-1. Similarly, VEGF promoted NFAT activation and subsequent Adamts-1 induction in aortic wall in a calcineurin-dependent manner. Our results demonstrate that Adamts-1 upregulation by inducers of pathological vascular remodeling is mediated by specific signal transduction pathways involving NFAT or C/EBPβ transcription factors. Targeting of these pathways may prove useful in the treatment of vascular disease.

Hemodynamic mechanical forces, endocrine or paracrine cellular factors such as vascular endothelial growth factor (VEGF) or angiotensin-II (Ang-II), and the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are strongly implicated in vascular remodeling, including vascular smooth muscle cell (VSMC) proliferation and migration, neovascularization, endothelial cell (EC) dysfunction, and/or inflammatory cell infiltration (1–4). Furthermore, profound changes in the extracellular matrix of the aortic wall, mediated by cellular secretion of extracellular matrix components and protein metalloproteinases, represent a critical hallmark of this process (5, 6).

Emerging evidence suggests that increased expression of the metalloproteinase Adamts-1 is associated with remodeling of the extracellular matrix in the aortic wall (7–10). Adamts-1 is a member of the ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs type I) family of proteases, which degrades the proteoglycans nidogen, aggrecan, syndecan, versican, and brevican (11). The expression of *Adamts-1* mRNA has been reported to increase in proliferating/migrating VSMCs (7) and in ECs treated with VEGF (12) and high wall shear stress (13, 14). However, the intracellular signaling pathways involved in its upregulation by these stimuli are poorly understood.

Here, we report that the expression of Adamts-1 in endothelial and vascular smooth muscle cells is induced by a broad range of stimuli associated with vascular remodeling, including VEGF, Ang-II, IL-1β, and TNF-α. We provide evidence supporting the alternative involvement of either NFAT or C/EBPβ in *Adamts-1* transcriptional activation by these stimuli and show *in vivo* activation of these transcription factors by VEGF and Ang-II, respectively, which might be involved in Adamts-1 induction in the aorta.

MATERIALS AND METHODS

Cell culture and reagents. Human umbilical vein ECs (HUVECs) were isolated from umbilical veins (15). Cells were cultured in 0.5% gelatin-coated plates in medium 199 supplemented with 20% fetal calf serum (FCS), 50 μg/ml bovine brain extract, 100 μg/ml heparin, and 1% penicillin-streptomycin. Cells were used between passages 4 and 6. Murine VSMCs were isolated and grown as described previously (16). Murine lung ECs (MLECs) were purified and cultured by previously published methods (17).

Cells were stimulated with 50 ng/ml recombinant human $VEGF_{165}$, 100 ng/ml IL-1β, or 50 ng/ml TNF-α (all from Peprotech). Ang-II (10^{-6} M for MLECs or VSMCs or 10^{-5} M for HUVECs) and phorbol myristate

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acetate (10 ng/ml) were purchased from Sigma-Aldrich. Calcium ionophore A23187 (1 μM) was obtained from EMD, Tocris Bioscience. Where indicated, cells were treated with 200 ng/ml cyclosporine (CsA) for 30 min prior to stimulation.

Western blot analysis. Cell extracts were obtained as described previously (16). Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with the corresponding primary antibody. Primary antibodies were detected by incubation with either a 1:5,000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin antibody (Sigma) or a 1:5,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (GE Healthcare), depending on the origin of the primary antibody. All antibody dilutions were carried out in 1% bovine serum albumin (BSA) in Tris-buffered saline–Tween 20. Bound antibodies were detected by enhanced chemiluminescence detection (Millipore).

The antibodies used in this study were mouse anti-ADAMTS1 monoclonal antibody (1:1,000), mouse anti-NFATc1 monoclonal antibody (1:500), rabbit anti-NFATc3 polyclonal antibody (1:1,000), and rabbit anti-NFATc4 polyclonal antibody (1:1,000) (all from Santa Cruz), mouse anti-human Cox-2 monoclonal antibody (Cayman; 1:4,000), rabbit anti-mouse Cox-2 polyclonal antibody (Cayman; 1:1,000), rabbit anti-C/EBPβ polyclonal antibody (Santa Cruz; 1:1,000), rabbit phospho-specific anti-C/EBPβ polyclonal antibody (Cell Signaling; 1:1,000), rabbit anti-CNA polyclonal antibody (Chemicon, Ab-1695; 1:1,000), mouse anti-CNB monoclonal antibody (Upstate; 1:4,000), rabbit anti-RCAN1 polyclonal antibody (Sigma; 1:500), mouse antitubulin monoclonal antibody (Sigma; 1:40,000), mouse anti-glyceraldehyde phosphate dehydrogenase monoclonal antibody (Abcam; 1:10,000), and mouse anti-polypyrimidine tract-binding protein-associated splicing factor (anti-PSF) monoclonal antibody (Sigma; 1:1,000).

Reverse transcription and RT-qPCR analysis. RNA was extracted with the TriPure kit (Roche), and cDNA synthesis was performed with 2 μg of total RNA (16). Quantification of RNA levels was carried out by real-time quantitative PCR (RT-qPCR) with the following TaqMan assays (Applied Biosystems): human *ADAMTS-1* (Hs00199608_m1), human *TFCR* (Hs00951083_m1), mouse *Adamts-1* (Mm00607939_s1), and mouse *Hprt1* (Mm00446968_m1). RT-qPCR assays were performed as described previously (16). Analysis of RT-qPCR data was carried out by the comparative $2^{-\Delta\Delta CT}$ method.

Plasmids. Luciferase-based reporter vectors containing the human *ADAMTS-1* proximal promoter region −706/+207 and −342/+207 (18) were generously provided by Y. Ninomiya (Okayama University, Okayama, Japan). To generate pMetLuc(−274/+207), the corresponding region of the *ADAMTS-1* promoter was amplified by PCR with Adamts-1 hPromoter−274 sense and Adamts-1 hPromoter+207 antisense oligonucleotides and cloned into pMet-Luc (Clontech). To generate luciferase-based reporter vectors containing the −539/+41 and −269/+41 regions of the murine *Adamts-1* promoter, the corresponding regions were amplified by PCR from mouse genomic DNA isolated from MLECs as a template with either Adamts-1 mPromoter−539 sense or Adamts-1 mPromoter−269 sense oligonucleotides in combination with the Adamts-1 mPromoter+41 antisense primer. Amplified products were cloned into pGL3-Basic (Promega), generating plasmids pGL3(−539/+41) and pGL3(−269/+41), respectively. Construct pGL3(−325/+41) was generated by digestion of pGL3(−539/+41) with PstI and XhoI and subcloning into pGL3-Basic. The oligonucleotides used for cloning were Adamts-1 hPromoter+207 antisense (5′-GCAGGCAGAGTGGCTC-3′), Adamts-1 hPromoter−274 sense (5′-CGGTGGAAGGGAGAGTC-3′), Adamts-1 mPromoter−539 sense (5′-GGTACCGAGAAGCGCAGAATCAAC-3′), Adamts-1 mPromoter−269 sense (5′-GGTACCCCGAGAGATGAAGTT AAAGG-3′), and Adamts-1 mPromoter+41 antisense (5′-CTCGAGCT TGCGATAGCACCTAG-3′).

Site-directed mutagenesis was performed with the QuikChange mutagenesis kit (Stratagene). The fidelity of all amplified products was confirmed by sequencing. The oligonucleotides used for site-directed mutagenesis were (the mutated positions are underlined) GGGGAGAAAAG GAATGTCGAGGTTTCTTTTCAATTCGACC (to mutate the C/EBPβ binding site at position −365 of the murine promoter region), CAGG ATAGGGAAATGTCGAAGTTGGGACTGCGTTCTCC (to mutate the C/EBPβ binding site at position −410 of the human promoter region), GTGGGGAGGAAGGGTGTTGTAGGAAACCGGCGAGG (to mutate the binding site for NFAT at position −302 of the human promoter region), GGGGAGGAAGGGTGGTCCATTACACCGGCGAGG (to mutate the binding site for NFAT at position −294 of the human promoter region), and GAGGAAGGGTGTTGTATTACACCGGCGAGGGAGA AAAG (to mutate both binding sites for NFAT at positions −302 and −294 of the human promoter region).

Transient transfection and luciferase assay. HUVECs (5×10^4) were transfected with 0.6 μg of luciferase reporter vector by the calcium phosphate method (15). Murine VSMCs (10^4) were transfected with 0.6 μg of luciferase reporter vector with Lipofectamine (16). In cotransfection experiments, pcDNA3.1-LIP or the pCDNA3.1-Empty control expression vector was transfected together with the corresponding luciferase reporter plasmid at a ratio of 3:1. Differences in cell survival or changes in global translation rate due to different treatments were ruled out through the parallel transfection of the corresponding empty luciferase vector (basal activity, 10,000 to 30,000 relative light units). Luciferase activity was measured with the luciferase assay kit (Promega) or the Ready to Glow kit (Clontech) for pGL3- or pMetLuc-derived plasmids, respectively.

Formaldehyde cross-linking and ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (19). HUVECs (1.5×10^7) were stimulated with 50 ng/ml VEGF or 10^{-5} M Ang-II for 30 min and then fixed with 1% formaldehyde for 5 min at 4°C. Fixation was quenched by the addition of 0.125 M glycine and incubation for 10 min at 4°C. Cells were washed three times with cold phosphate-buffered saline (PBS) and scraped into PBS containing proteinase inhibitors. After a centrifugation step, cells were suspended in lysis buffer 1 (50 mM HEPES-KOH [pH 7.5], 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 140 mM NaCl, proteinase inhibitors) for 10 min at 4°C. Nuclear pellets were washed in lysis buffer 2 (10 mM Tris-HCl [pH 8], 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), lysed in lysis buffer 3 (10 mM Tris-HCl [pH 8], 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.5% SDS), and sonicated with a Bioruptor sonicator (Diagenode). Before immunoprecipitation, the size of DNA fragments was confirmed to be 200 to 600 bp by reverse cross-linking (3 volumes of Tris-EDTA [TE] buffer and 0.3 M NaCl at 65°C overnight) of an aliquot of chromatin sample and subsequent electrophoresis in a 2% agarose gel. A portion (5%) of the total chromatin sample was saved for input detection. For each immunoprecipitation, 50 μl of protein A Dynabeads (Invitrogen) was blocked with 0.5% BSA-PBS for 1 h and incubated overnight in 0.5% BSA-PBS with 6 μg of control mouse or rabbit IgG (Santa Cruz), a rabbit anti-C/EBPβ polyclonal antibody (Santa Cruz), or an anti-NFATc1 monoclonal antibody (kindly provided by T. Minami, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan). Chromatin was diluted 1/6 in TE buffer containing 50 mM NaCl and 1% Triton X-100 (Sigma) and incubated with rocking overnight at 4°C with antibody-coupled Dynabeads. The beads were washed three times with low-salt washing buffer (20 mM Tris-HCl [pH 8], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.3% sarcosyl, 0.5% sodium deoxycholate), three times with high-salt buffer (20 mM Tris-HCl [pH 8], 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), three times with high-salt radioimmunoprecipitation assay buffer (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate), and twice with TE buffer containing 150 mM NaCl. Finally, chromatin bound to Dynabeads was eluted in 200 μl of 10 mM Tris-HCl [pH 8]–10 mM EDTA–2% SDS buffer by rocking at 65°C for 45 min, followed by vortexing for 5 min. Reverse cross-linking was performed by incubation with 600 μl of TE buffer containing 300 mM NaCl at 65°C overnight. DNA samples were treated with 0.2 μg/ml RNase (Sigma-Aldrich) for 1 h at 37°C and 0.2 μg/ml proteinase K (Sigma-Aldrich) for 1 h

at 50°C. DNA was extracted with phenol-chloroform, precipitated, and resuspended in 50 μl of water. One-third of the final volume was analyzed by Sybr green real-time PCR with the oligonucleotides ADAMTS-1 promoter C/EBPβ site (sense, CCTAGCTCATCTCCTAGATTG; antisense, GGTTCGGTTGGAGAACGC), ADAMTS-1 NFATs sites (sense, GGAAG GGTGGTCCAGGAAA; antisense, CGCCACTGCTCGTCAATC), ADAMTS-1 3′ UTR (sense, GACAAGTGATCTCAATGTCCCC; antisense, GAGCTTAGTACCTCCAACTTCCT), and COX-2 promoter (sense, GG TTTCCGATTTTCTCATTTCCG; antisense, CACTGCAAGTCGTATGA CAATTG).

Animals. Animal studies were performed in accordance with the guidelines of the European Union on animal care and approved by the Animal Care and Ethics committee at the Centro Nacional de Investigaciones Cardiovasculares. Calcineurin (CN) B1 (*Cnb1*$^{\Delta/fl}$) conditional knockout mice (20) were kindly provided by Gerald R. Crabtree (Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA). Mice were administered VEGF or Ang-II by subcutaneous osmotic minipump (Alzet Corp) infusion at 25 μg/kg/day and 1 μg/kg/min, respectively. Where indicated, minipumps loaded with CsA (Novartis; 5 mg/kg/day) were implanted 24 h before VEGF or Ang-II administration. The AT_1R blocker losartan (Fluka) was administered at 10 mg/kg/day by minipumps implanted 24 h before the administration of Ang-II.

Lentivirus production and infection. Lentivirus production was performed as described previously (16). Lentiviruses encoding Cre recombinase, green fluorescent protein (GFP)-LXVP, and GFP-LXVP Mutant have been previously described (21, 22). Lenti-LIP, a lentivirus encoding LIP (liver inhibitor protein), was generated by subcloning mouse C/EBPβ (LIP) from pcDNA3.1-C/EBPβ (LIP) (a gift from Peter Johnson; Addgene plasmid 12561) into lentiviral plasmid pHRSIN-IRES-GFP. VSMCs, HUVECs, and MLECs were infected at a multiplicity of infection of 3.

Histological analysis. Aortas were fixed in 4% paraformaldehyde overnight at 4°C. Paraffin cross sections (5 μm) from fixed aortas were used for immunohistochemistry analysis. Heat-induced epitope retrieval was performed in citrate buffer (10 mM sodium citrate [pH 6], 0.05% Tween 20) at 125°C for 5 min. The antibodies used for immunohistochemistry analysis were anti-C/EBPβ (1/100; Santa Cruz Biotechnology), anti-p-C/EBPβ (1/25; Cell Signaling), rabbit polyclonal anti-Adamts-1 (1/100; Santa Cruz), and anti-NFATc4 (1/100; Santa Cruz). Substrate staining was developed with diaminobenzidine (Vector Laboratories), and sections were counterstained with hematoxylin, dehydrated, and mounted in DPX (Fluka).

Statistical analysis. Results are expressed as mean values ± standard deviations (SD). Differences were evaluated by one-way analysis of variance with the *post hoc* Newman-Keuls comparison test. Statistical significance was assigned at P values of <0.05.

RESULTS

Different signal transduction pathways regulate VEGF- and Ang-II-induced Adamts1 gene expression in vascular cells. Because Adamts-1 expression increases in response to the activation of ECs with VEGF (12) and shear stress (13, 14), we hypothesized that other extracellular inducers of vascular remodeling may also regulate Adamts-1 expression. We used Ang-II to test this hypothesis. Treatment of HUVECs, murine VSMCs, and MLECs with Ang-II resulted in a strong increase in Adamts-1 protein and mRNA levels (Fig. 1A and B), with maximal expression occurring at approximately 6 h poststimulation.

Because VEGF and Ang-II activate the CN/nuclear factor of activated T cells (NFAT) signaling pathway in ECs and VSMCs (16, 23, 24), we investigated the involvement of this pathway in the upregulation of the *Adamts-1* gene by these stimuli. Vascular cells were preincubated with the CN inhibitor CsA (25), and Adamts-1 expression was analyzed by Western blot and RT-qPCR analyses. VEGF-dependent upregulation of Adamts-1 expression at the

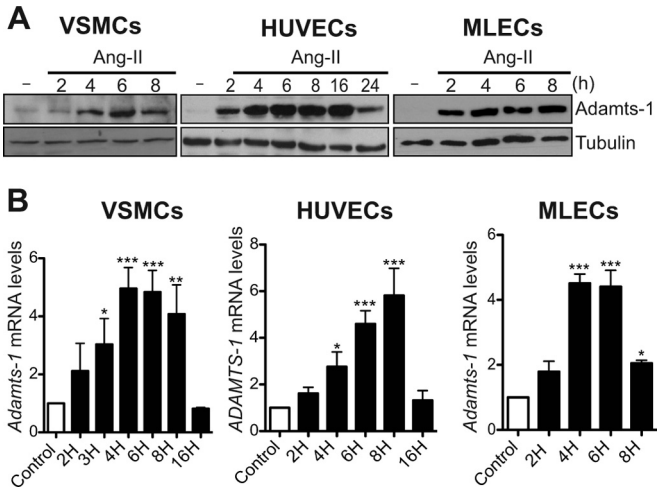

FIG 1 Ang-II induces the expression of Adamts-1 in vascular cells. (A) Western blot analysis of Adamts-1 expression in protein extracts from primary VSMCs, HUVECs, and MLECs stimulated with Ang-II. (B) *Adamts-1* gene expression was measured by qPCR in VSMCs, HUVECs, and MLECs treated with Ang-II as indicated. Values are expressed as fold increases relative to nonstimulated cells (Control). Histograms represent means ± SD of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus the control).

mRNA and protein levels was markedly reduced by inhibition of the CN/NFAT pathway with CsA (Fig. 2A). In contrast, induction of Adamts-1 expression in response to Ang-II was unchanged in the presence of CsA (Fig. 2B), despite efficient blockade of CN signaling by CsA, as confirmed by CsA-mediated inhibition of the genes for cyclooxygenase 2 (Cox-2) and RCAN1.4 (Fig. 2), two known CN/NFAT targets (19, 24). These results suggest that CN signaling is implicated in VEGF-dependent induction of Adamts-1 expression but not in Ang-II-promoted Adamts-1 upregulation.

In addition to inhibiting CN, CsA can exert additional effects on the cell, such as activation of the transforming growth factor β1 pathway (26) or stabilization of the RNA encoding VEGF (27). To confirm that the CsA-mediated reduction of Adamts-1 expression by VEGF was not the result of off-target effects of CsA, we transduced ECs and VSMCs with lentiviral vectors encoding LXVP, a peptide that inhibits CN phosphatase activity and blocks its docking site for NFAT (28). Consistent with the data obtained with CsA, LXVP expression in vascular cells abrogated *Adamts-1* gene upregulation in response to VEGF, but not in response to Ang-II (Fig. 3A to D). As expected, the LXVP peptide inhibited Cox-2 upregulation induced by both stimuli (Fig. 3A, B, E, and F), demonstrating efficient impairment of CN signaling in all cases, whereas an LXVP mutant had no effect on either Adamts-1 or Cox-2 upregulation by these stimuli (Fig. 3A to F). The participation of CN-independent signal transduction pathways in the regulation of Adamts-1 expression by Ang-II led us to investigate whether other stimuli that operate in a CN-independent fashion, such as the proinflammatory cytokines TNF-α and IL-1β, might also induce Adamts-1. Stimulation of HUVECs and VSMCs with TNF-α or IL-1β led to a time-dependent increase in Adamts-1 levels in both cell types, peaking at 6 to 8 h poststimulation (Fig. 4A and B). CN inhibition with CsA or the LXVP peptide failed to block TNF-α- or IL-1β-induced upregulation of Adamts-1 ex-

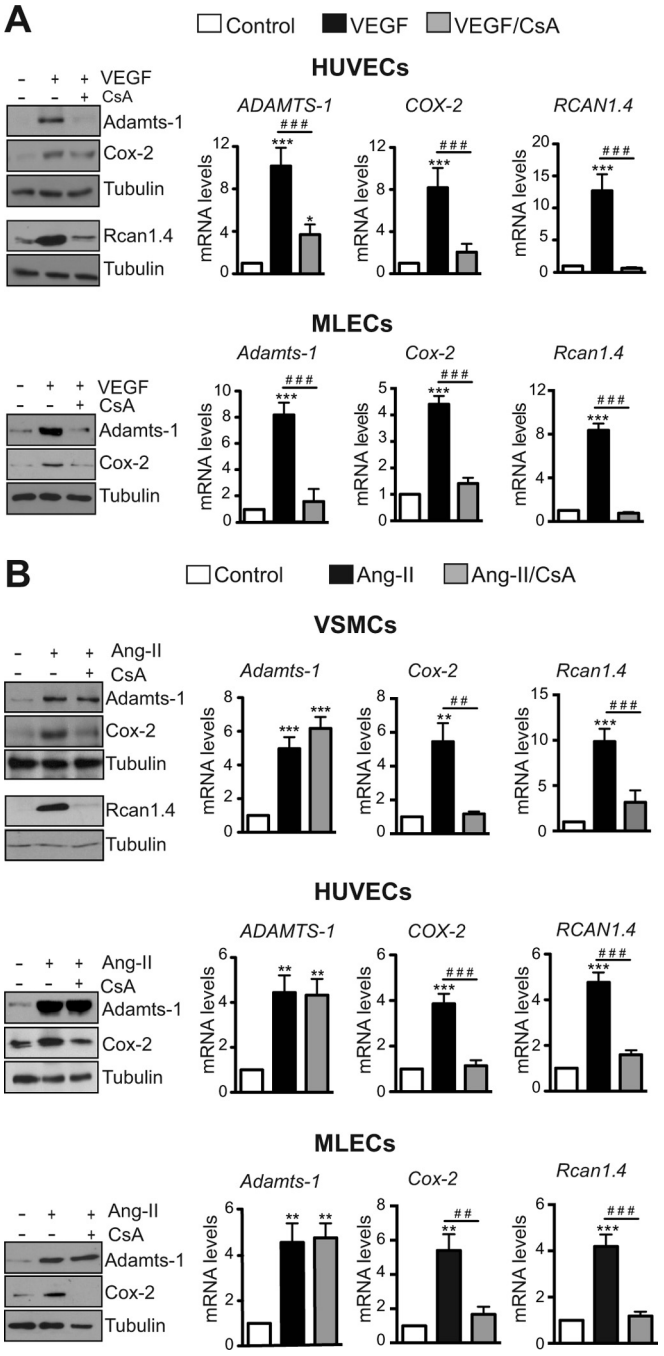

FIG 2 Ang-II and VEGF upregulate Adamts-1 expression in vascular cells via differential signaling mechanisms. Western blot and qPCR analyses of *Adamts-1*, *Cox-2*, and *RCAN1.4* expression in HUVECs and MLECs treated with CsA or the vehicle 1 h before stimulation with VEGF for 4 h (A) and VSMCs, HUVECs, and MLECs treated with CsA or the vehicle 1 h before stimulation with Ang-II for 4 h (B). In all Western blot assays, the expression of tubulin was analyzed as a loading control. The immunoblot assays shown are representative of three independent experiments. In qPCR analyses, mRNA levels were normalized to hypoxanthine phosphoribosyltransferase expression. Data are expressed as fold increases relative to nonstimulated cells (Control) and shown as means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus control); ##, $P < 0.01$; ###, $P < 0.001$ (versus CsA-pretreated cells).

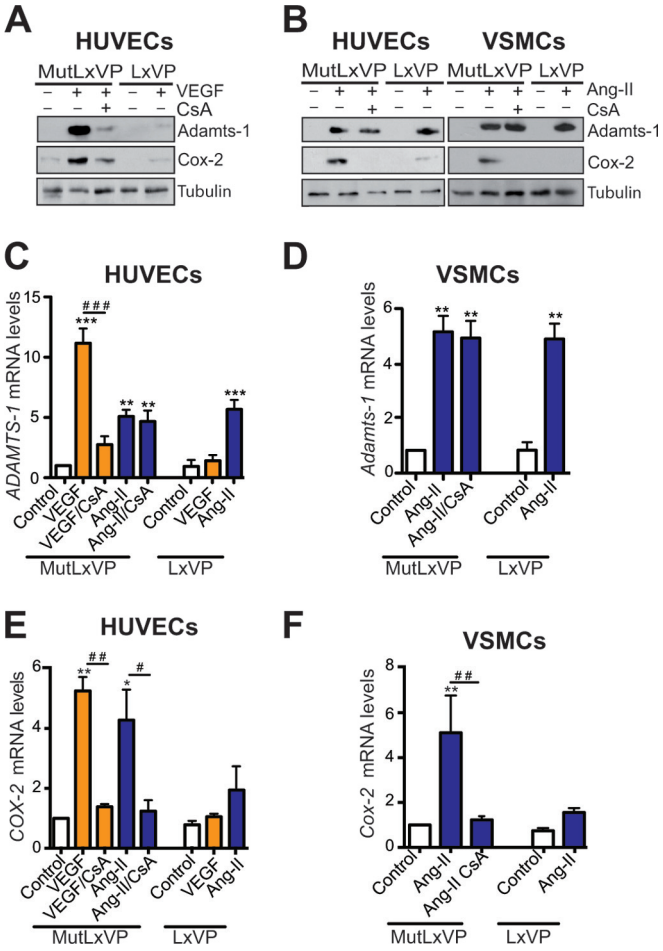

FIG 3 Blockade of CN activity with the NFAT-derived peptide LXVP inhibits Adamts-1 upregulation by VEGF but not Ang-II in vascular cells. (A and B) Western blot analysis of Adamts-1 and Cox-2 expression in the indicated vascular cells transduced with lentiviral particles expressing GFP tagged with the CN inhibitory peptide LXVP (LxVP) or a mutant version unable to inhibit CN (MutLxVP). Infected cells were stimulated with VEGF (A) or Ang-II (B) for 4 and 6 h, respectively, in the absence or presence of CsA as indicated. Tubulin expression was analyzed as a loading control. The immunoblot assays shown are representative of at least two independent experiments. (C to F) *Adamts-1* (C and D) and *Cox-2* (E and F) mRNA expression was measured by qPCR in cells infected with lentiviruses as described above and stimulated as indicated. Values are expressed as fold increases relative to nonstimulated cells (Control). Data are shown as means ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus the control); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (versus CsA-pretreated cells).

pression (Fig. 4C to F), suggesting that activation of the CN/NFAT pathway is not involved in this regulation. In parallel, we measured Cox-2 expression as a positive control for CN-independent TNF-α and IL-1β stimulation (24). As anticipated, Cox-2 induction by TNF-α and IL-1β was unaltered by inhibition of the CN/NFAT pathway (Fig. 4A to D, G, and H).

Finally, to further confirm the involvement of CN-dependent and -independent transduction mechanisms in the regulation of *Adamts-1* gene expression, we employed cells lacking CN. MLECs and VSMCs isolated from a conditional CN knockout mouse (20) were transduced with a lentivirus expressing Cre recombinase (Lenti-Cre) to promote deletion of the *Cnb1* gene. As shown in Fig. 5, Cnb1 deletion led to the destabilization of CN A in both cell

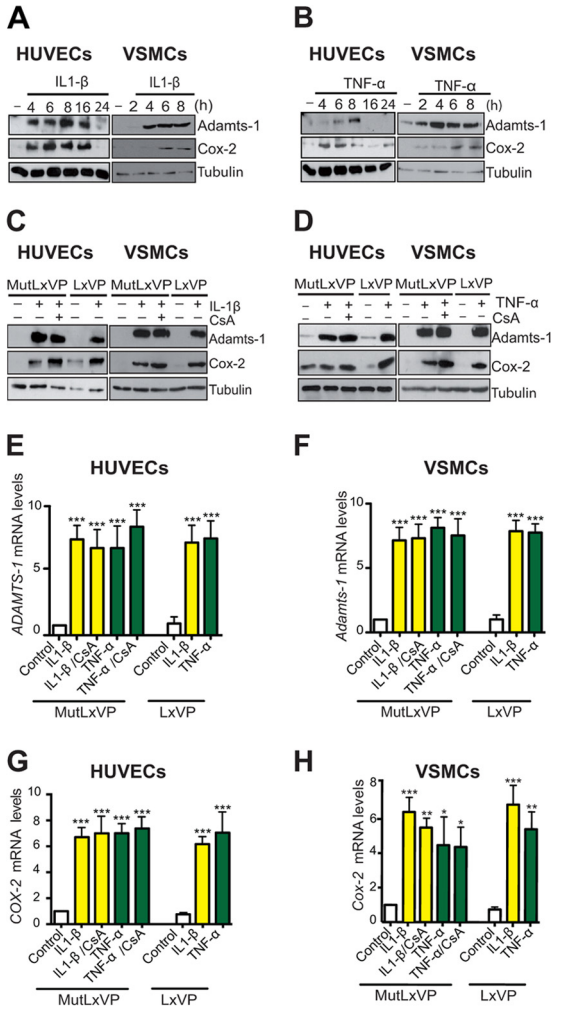

FIG 4 Upregulation of *Adamts-1* gene expression by the proinflammatory stimuli TNF-α and IL-1β does not require CN activation. (A to D) Western blot analysis of Adamts-1 and Cox-2 expression in vascular cells treated with IL-1β (A) or TNF-α (B) for the times indicated. (C and D) HUVECs and VSMCs were transduced with lentiviral particles expressing GFP tagged with the CN inhibitory peptide LXVP (LxVP) or a mutant version unable to inhibit CN (MutLxVP). Cells were stimulated with IL-1-β (C) or TNF-α (D) for 6 h in the absence or presence of CsA as indicated. In all cases, tubulin expression was analyzed as a loading control. The immunoblot assays shown are representative of at least two independent experiments. (E to H) *Adamts-1* and *Cox2* mRNA expression was measured by qPCR in HUVECs (E and G) or VSMCs (F and H) infected with lentiviruses as described above and stimulated as indicated. Values are expressed as fold increases relative to nonstimulated cells (Control). Data are shown as means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus the control).

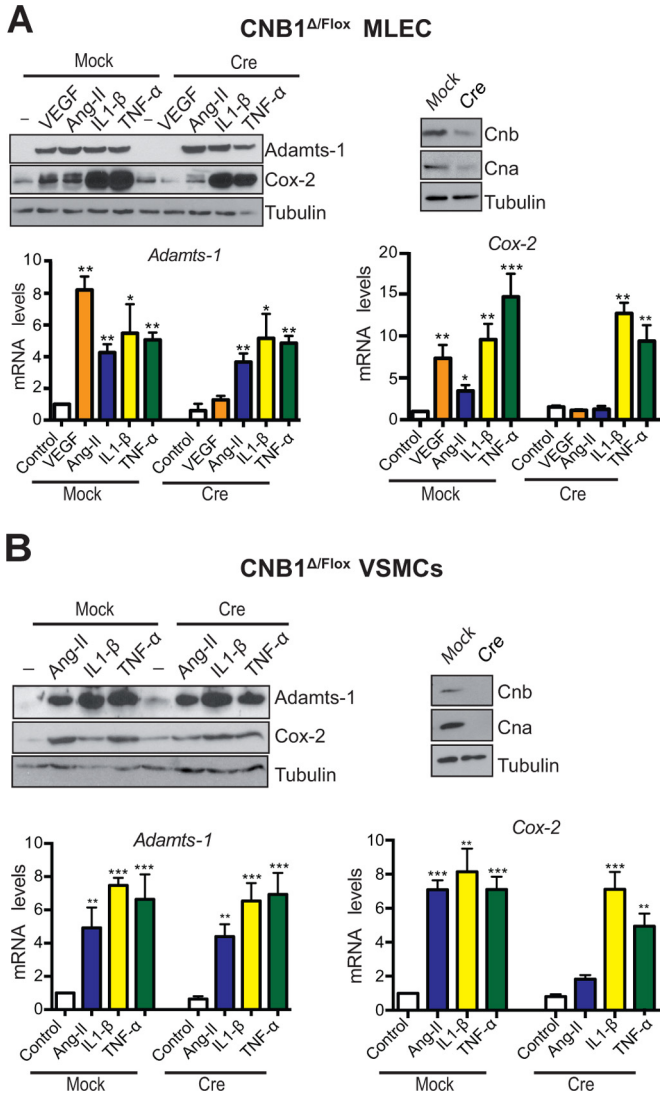

FIG 5 CN deletion impairs Adamts-1 upregulation by VEGF but not Ang-II, TNF-α, or IL-1β. To suppress CN expression in primary vascular cells, MLECs (A) and VSMCs (B) isolated from *Cnb1*$^{\Delta/fl}$ mice were infected with lentiviruses expressing the Cre recombinase (Cre) or GFP as a control and then stimulated as indicated. CN deficiency was assessed by Western blot analysis with antibodies specific for the CN B (Cnb) or A (Cna) subunit. Adamts-1 and Cox-2 protein levels were determined by Western blotting. In all cases, the expression of tubulin was analyzed as a loading control. Immunoblot assays are representative of at least three independent experiments. *Adamts-1* and *Cox2* mRNA expression was measured by qPCR in CN-deficient MLECs (A) or VSMCs (B) stimulated as indicated. Values are expressed as fold increases relative to nonstimulated cells (Control). Data are shown as means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (versus the control).

types. VEGF stimulation was no longer able to induce Adamts-1 or Cox-2 gene expression in CN-deficient MLECs (Fig. 5A). In contrast, Adamts-1 upregulation by Ang-II, TNF-α, or IL-1β was unaffected by disruption of CN expression (Fig. 5). Consistent with the data obtained with CN inhibitors (Fig. 2 to 4), whereas Cox-2 induction by TNF-α or IL-1β was unaltered by CN deletion, Cox-2 upregulation by Ang-II was largely abolished in CN-deficient vascular cells (Fig. 5).

Collectively, these results indicate that stimulation of vascular cells induces *Adamts-1* gene expression via the activation of at least two different intracellular signaling pathways. Whereas VEGF-dependent upregulation of Adamts-1 requires CN activity, Ang-II, TNF-α, and IL-1β operate through the activation of an alternative signaling mechanism.

***Adamts-1* gene upregulation requires the binding of either NFAT or C/EBPβ to specific sequences located in its promoter region.** To identify the transcription factors that link signal transduction processes in vascular cells with upregulation of *Adamts-1* gene expression, we transfected HUVECs and VSMCs with luciferase-based reporter constructs containing the proximal

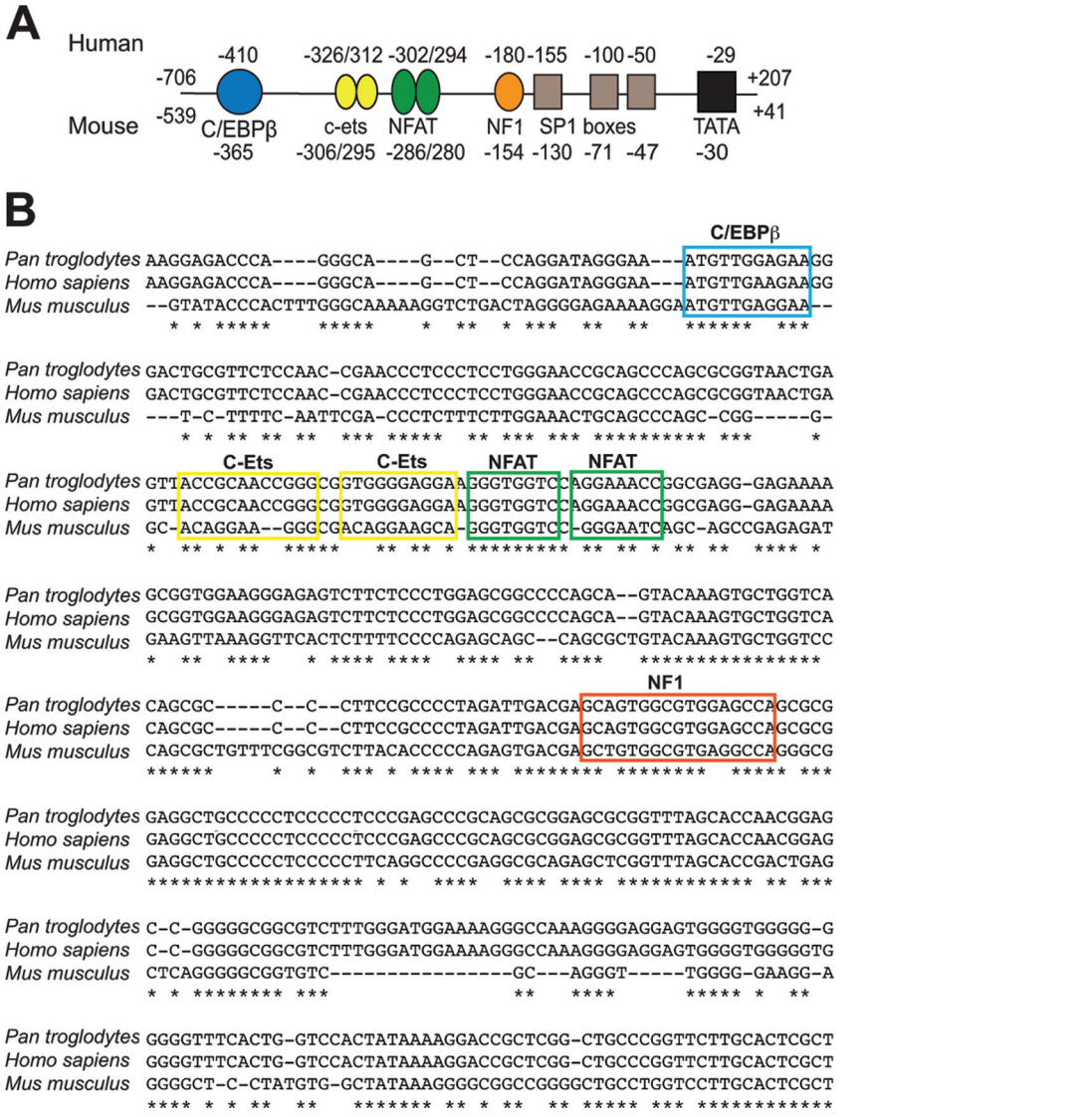

FIG 6 Sequence and putative regulatory elements of the *Adamts-1* proximal promoter region. (A) Diagram depicting putative *cis*-acting regulatory elements in the human and mouse promoter regions of *Adamts-1*. (B) Alignment of the proximal regions of the human (*Homo sapiens*), mouse (*Mus musculus*), and chimpanzee (*Pan troglodytes*) *Adamts-1* gene promoters. Nucleotides identical in the three species are indicated by stars. Putative transcription factor binding sites are boxed.

promoter regions of *Adamts-1*, pMetLuc(−706/+207) and pGL3(−539/+41), for the human and murine genes, respectively (Fig. 6). The presence of binding motifs for the transcription factors C/EBPβ, c-ets, NF-1, and SP1 in the *Adamts-1* proximal promoter (Fig. 6) has been previously reported (29), but no binding sites for NFAT have yet been identified in this region. A detailed *in silico* analysis of the human *ADAMTS-1* proximal promoter sequence revealed two potential NFAT binding sites at positions −302 and −294, corresponding to nucleotides −286 and −280 in the murine promoter (Fig. 6).

Stimulation of the transfected cells with VEGF, Ang-II, IL-1β, or TNF-α resulted in a significant increase in luciferase activity of the *Adamts-1* reporter vectors (Fig. 7), suggesting that this promoter region contains regulatory elements that mediate its transcriptional activation by these stimuli. In agreement with our results on protein and mRNA induction (Fig. 2), pretreatment of the transfected cells with CsA abolished the activation of the reporter in response to VEGF but not that in response to Ang-II, IL-1β, or TNF-α (Fig. 7). These results suggest that the −706/+207 region of human *ADAMTS-1* (−539/+41 in the mouse) contains CN-dependent and -independent regulatory elements that respond to stimulation by VEGF and Ang-II, IL-1β, or TNF-α, respectively.

We generated a series of deletion-containing versions of the promoter constructs to determine the functional relevance of the transcription factor-binding motifs identified in the *ADAMTS-1* proximal promoter. Deletion of the region encompassing the C/EBPβ-binding motif failed to alter the luciferase activity of the reporter vector in response to VEGF stimulation in HUVECs (Fig. 7A). Accordingly, preincubation of the cells with CsA prior to VEGF stimulation substantially inhibited the reporter activity of this construct (Fig. 7A). Conversely, loss of this region completely

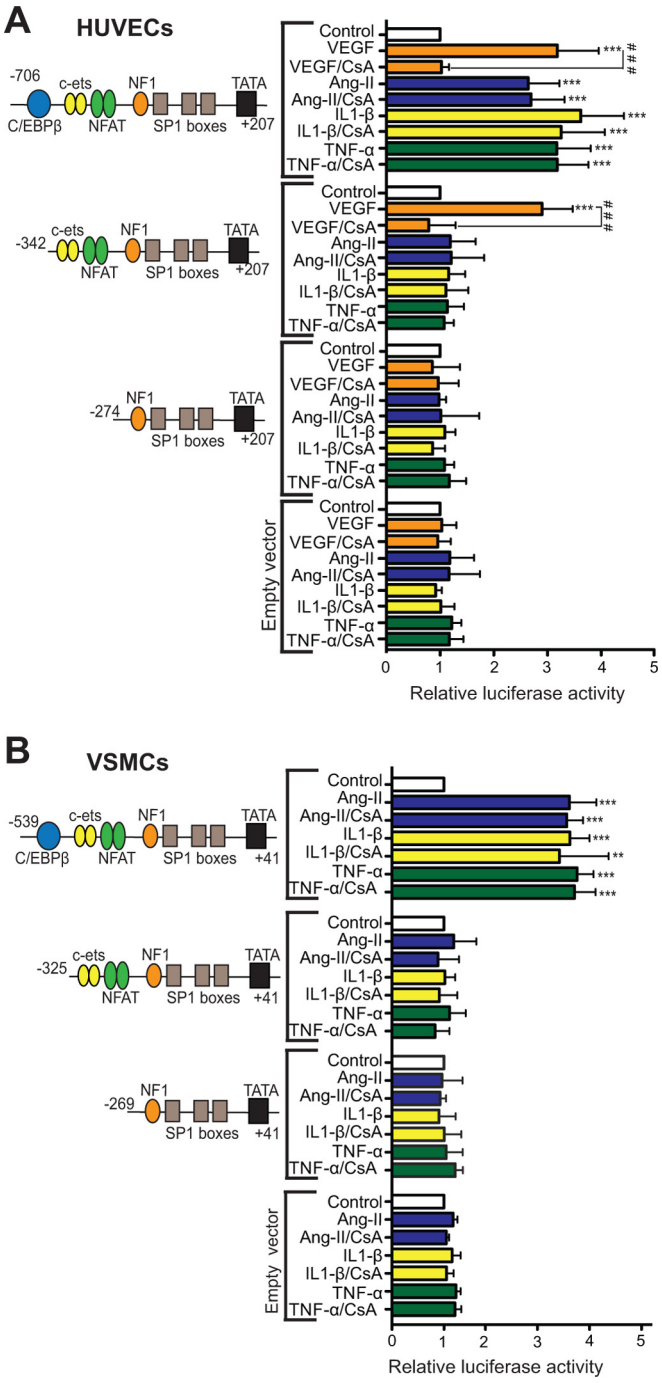

FIG 7 Functional analysis of *Adamts-1* promoter activity in response to VEGF, Ang-II, TNF-α, and IL-1β. HUVECs (A) or murine VSMCs (B) were transfected with luciferase-based reporter plasmids containing serial deletions of the human (A) or mouse (B) *Adamts-1* promoter region. Transfected cells were left unstimulated (Control) or stimulated with VEGF, Ang-II, IL-1-β, and TNF-α for 8 h. Where indicated, cells were treated with CsA for 1 h prior to stimulation. Luciferase activity is expressed as fold increases relative to the activity of the reporter in nonstimulated cells. Empty plasmids pMetLuc and pGL3 were used as negative controls. Histograms show data as means ± SD of at least five independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus nonstimulated cells); ###, $P < 0.001$ (VEGF versus VEGF/CsA-stimulated cells).

blunted the upregulation of reporter gene activity in cells treated with Ang-II, IL-1β, or TNF-α (Fig. 7).

Further removal of the region containing the two potential binding sites for NFAT completely abrogated the VEGF-stimulated increase in reporter activity (Fig. 7A). As expected, this construct and the equivalent murine construct did not respond to Ang-II, TNF-α, or IL-1β treatment (Fig. 7). These results demonstrate that the −706/−343 region of the human *ADAMTS-1* promoter (−539/−326 in the mouse) contains CN-unrelated regulatory elements that control the transcriptional activation of the gene in response to Ang-II, TNF-α, and IL-1β, whereas the −342/−275 fragment (−325/−270 in the mouse) regulates VEGF-induced Adamts-1 expression in a CN-dependent fashion.

To test the possibility that the C/EBPβ and NFAT putative binding sites were implicated in *Adamts-1* promoter activation, we mutated these sequences and analyzed their transcriptional contribution by using luciferase reporter assays. Mutation of the C/EBPβ-binding site blunted the response of the human and mouse *Adamts-1* promoters to Ang-II, TNF-α, and IL-1β stimulation but did not affect their activation by VEGF (Fig. 8). Conversely, mutation of the two putative NFAT binding sites (either individually or together) blocked the transcriptional response to VEGF but not that to Ang-II, TNF-α, or IL-1β (Fig. 8A).

We next performed ChIP analysis to evaluate the functional occupancy of these motifs by the transcription factors C/EBPβ and NFAT in the endogenous *ADAMTS-1* promoter. ChIP assays of Ang-II-stimulated HUVECs with an antibody specific for C/EBPβ confirmed the recruitment of this factor (Fig. 9A). An identical experiment with an antibody specific for NFATc1 did not result in coprecipitation of the promoter region encompassing the NFAT binding sites (Fig. 9B), indicating that stimulation with Ang-II induces binding of C/EBPβ but not NFATc1 to the *ADAMTS-1* promoter. In contrast, ChIP assays of HUVECs stimulated with VEGF revealed the interaction of NFATc1 but not C/EBPβ with the corresponding promoter binding sites (Fig. 9A and B). Importantly, negative-control ChIP assays with anti-C/EBPβ or anti-NFATc1 antibodies on the 3′ untranslated region (UTR) of the *Adamts-1* mRNA excluded the possibility of coprecipitation of nonspecific DNA sequences (Fig. 9A and B). As a positive control, the *COX-2* promoter region was amplified in parallel in a ChIP assay to demonstrate C/EBPβ and NFATc1 recruitment upon Ang-II and/or VEGF treatment (Fig. 9C).

Taken together, these results establish the presence of functional binding sites for the transcription factors C/EBPβ and NFAT in the *ADAMTS-1* promoter region that play an essential role in the differential transcriptional activation of the gene in response to Ang-II/IL-1β/TNF-α or VEGF, respectively.

Ang-II and proinflammatory cytokines regulate *Adamts-1* gene expression through C/EBPβ. Alternative translation initiation sites in the C/EBPβ mRNA generate different protein isoforms designated LAP (liver activator protein) or LIP. LAP contains a conserved bZIP DNA-binding domain and a transactivation domain (and therefore is fully functional), whereas LIP lacks the transactivation domain and functions as a repressor (30). Phosphorylation of human C/EBPβ-LAP at residue Thr235 (Thr188 in the mouse) efficiently increases its transactivation activity (30). VSMCs stimulated with Ang-II resulted in a substantial increase in C/EBPβ-LAP phosphorylation at Thr235/188 after only 10 min of treatment, which persisted for 2 h (Fig. 10A). IL-1β

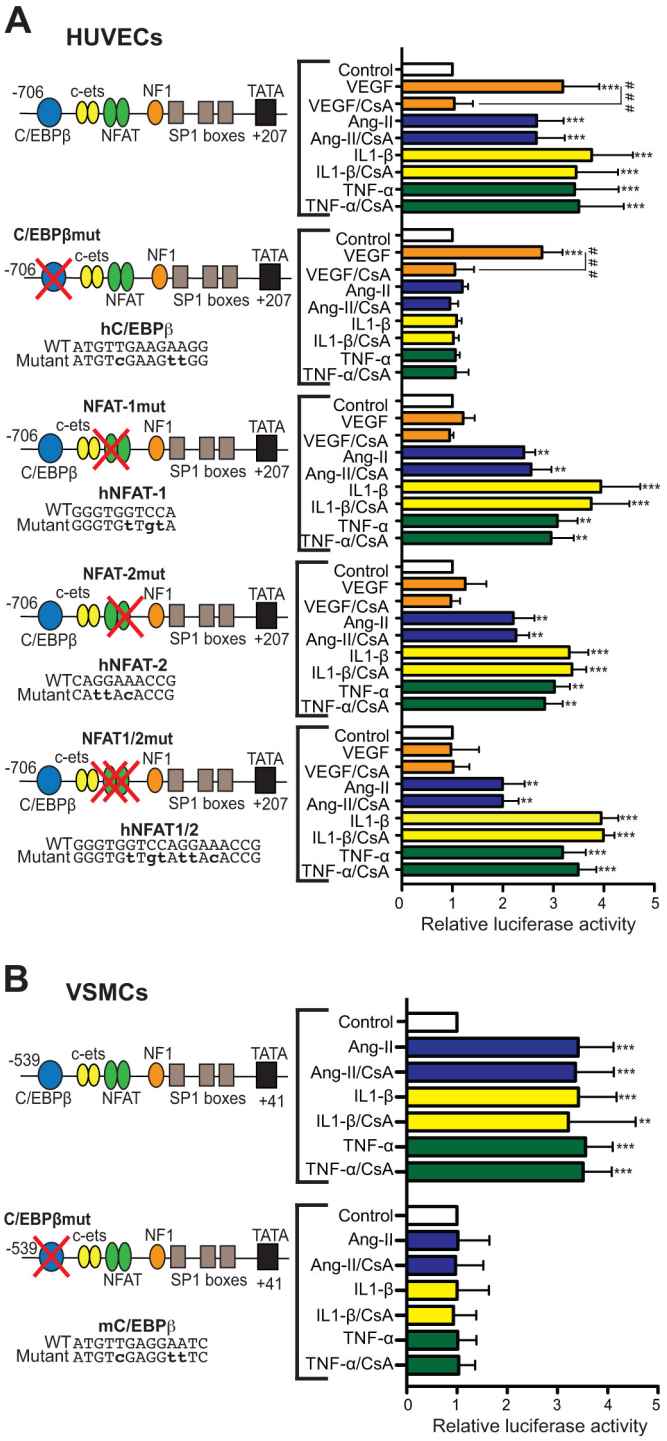

FIG 8 Integrity of NFAT- and C/EBPβ-binding sites is essential for *Adamts1* promoter activity in response to VEGF and Ang-II, TNF-α, or IL-1β, respectively. Mutant versions of the reporter vectors pMetLuc(−706/+207) (A) and pGL3(−539/+41) (B), where the putative binding sites for C/EBPβ and NFAT were abolished by site-directed mutagenesis (base substitutions are in bold), were transfected in HUVECs (A) or murine VSMCs (B). Transfected cells were left untreated (Control) or stimulated with VEGF, Ang-II, IL-1-β, or TNF-α for 8 h. Where indicated, cells were treated with CsA for 1 h prior to stimulation. Luciferase activity was expressed as fold increases relative to the activity of the reporter in nonstimulated cells. Histograms show data as means ± SD of at least three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus untreated cells); ###, $P < 0.001$ (VEGF versus VEGF/CsA-stimulated cells).

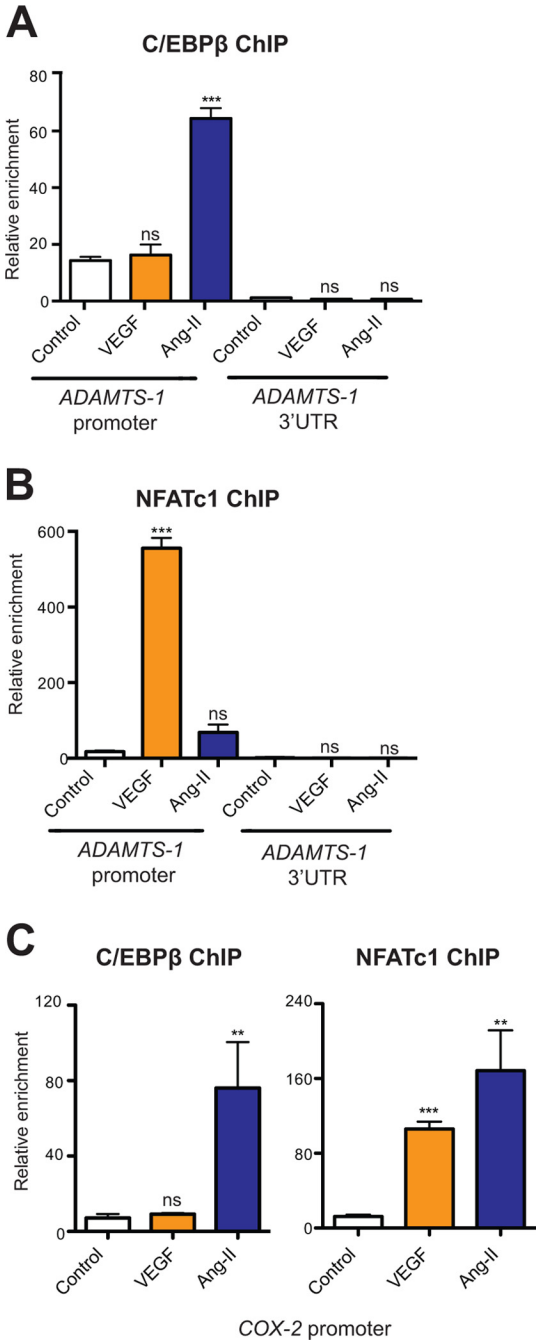

FIG 9 Recruitment of C/EBPβ and NFAT to the *Adamts-1* and *Cox-2* promoters is differentially induced by Ang-II and VEGF. ChIP assays of the promoter and 3′ UTR of *Adamts-1* (A and B) and the *Cox-2* promoter (C) performed with HUVECs left unstimulated (Control) or treated with either VEGF or Ang-II for 30 min, with antibodies specific for C/EBPβ or NFATc1 as indicated. Data are shown as enrichment of the amount of chromatin precipitated with anti-C/EBPβ or anti-NFATc1 antibody relative to the values obtained with an IgG control antibody. Histograms show means ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus the control); ns, nonsignificant.

and TNF-α, but not VEGF, induced a similar increase in C/EBPβ-LAP phosphorylation (Fig. 10B and C). Analysis of the protein extracts with an antibody recognizing total C/EBPβ-LAP showed equivalent C/EBPβ-LAP expression levels in all cases (Fig. 10A to

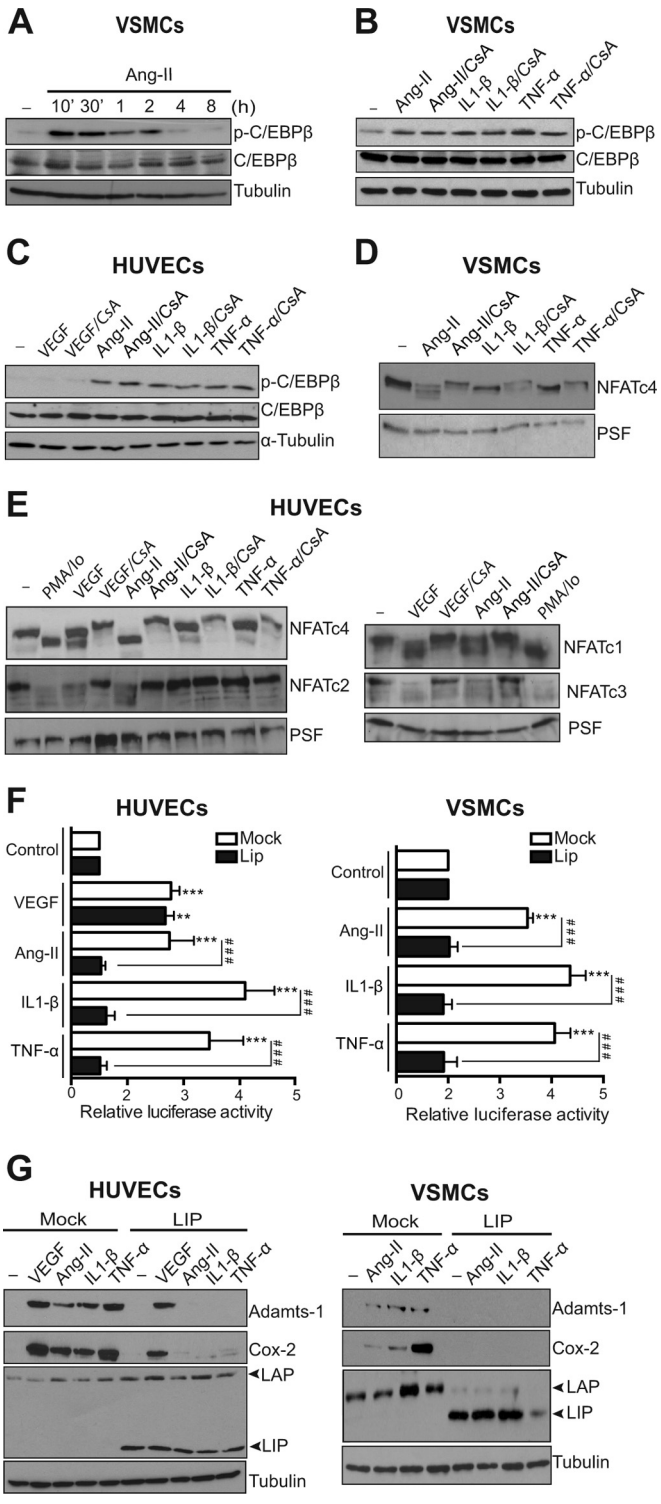

FIG 10 Ang-II, IL-1β, and TNF-α upregulate Adamts-1 in a C/EBPβ-dependent manner. (A to C) Immunoblot analysis of phospho-C/EBPβ and total C/EBPβ in protein extracts of VSMCs treated with Ang-II for the times indicated (A) and VSMCs and HUVECs treated with Ang-II, IL-1-β, TNF-α or VEGF for 15 min (B and C). Where indicated, cells were treated with CsA for 1 h prior to stimulation. Tubulin expression was analyzed as a loading control. (D and E) VSMCs and HUVECs were treated with the indicated stimuli for 30 min, and the activation (dephosphorylation) status of NFATc1 to -c4 transcription factors was analyzed by Western blotting. Where indicated, cells were treated with CsA for 1 h prior to addition of the stimulus. PSF expression was analyzed as a loading control. Immunoblot assays are representative of three independent experiments. (F) HUVECs and VSMCs were cotransfected with an expression vector encoding the C/EBPβ inhibitory isoform (LIP) or the empty plasmid (Mock), together with a luciferase-based reporter vector containing the human or mouse *Adamts-1* proximal promoter, pMetLucAdamts-1(−706/+207), or pGL3mAdamts-1(−539/+41), respectively. Transfected cells were left untreated (Control) or stimulated as indicated for 8 h. Luciferase activity was expressed as fold increases relative to the activity of the reporter in untreated cells. Histograms show data as means ± SD of four independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus untreated cells); ###, $P < 0.001$ (versus CsA-pretreated cells). (G) HUVECs or VSMCs were infected with lentiviral particles expressing GFP or C/EBPβ-LIP (LIP). Transduced cells were stimulated with Ang-II, IL-1-β, or TNF-α for 6 h or with VEGF for 4 h, and Adamts-1 expression was analyzed by Western blotting. Successful expression of exogenous LIP was confirmed by Western blotting with an anti-C/EBP antibody that recognizes both the LAP and LIP isoforms of C/EBPβ. Cox-2 expression is shown as a positive control for stimulation, and tubulin is shown as a loading control. The immunoblot assays shown are representative of three independent experiments.

C). Whereas both VEGF and Ang-II induced significant dephosphorylation of all NFAT family members, neither IL-1β nor TNF-α activated these transcription factors (Fig. 10D and E). As expected, the observed increase in C/EBPβ-LAP phosphorylation was not affected by preincubation of the cells with CsA (Fig. 10B and C), which efficiently inhibited VEGF- and Ang-II-induced dephosphorylation of NFAT family members (Fig. 10D and E). These results indicate that Ang-II, IL-1β, or TNF-α, but not VEGF, triggers the activation of the C/EBPβ signal transduction pathway in vascular cells.

Given these results, we hypothesized that Ang-II-, IL-1β-, or TNF-α-dependent activation of the C/EBPβ pathway is implicated in the transcriptional activation of the *Adamts-1* gene. To explore this possibility, we cotransfected HUVECs or VSMCs with a luciferase reporter vector containing the *Adamts-1* proximal promoter region together with an expression plasmid encoding the C/EBPβ repressor C/EBPβ-LIP (pcDNA3.1-LIP) or the corresponding empty vector and measured promoter activation after stimulation. Whereas ectopic expression of C/EBPβ-LIP robustly inhibited the induction of luciferase activity by Ang-II, IL-1β, or TNF-α stimulation, induction by VEGF was not significantly affected by C/EBPβ-LIP (Fig. 10F). Confirming these observations, upregulation of endogenous Adamts-1 protein expression in vascular cells stimulated with Ang-II, IL-1β, or TNF-α was impaired with a lentivirus encoding LIP (Fig. 10G), whereas LIP ectopic expression did not affect the levels of Adamts-1 expressed in VEGF-treated cells (Fig. 10G).

These results support a selective participation of C/EBPβ signaling in the upregulation of Adamts-1 expression in cells treated with Ang-II, IL-1β, or TNF-α but not in cells treated with VEGF.

Regulation of Adamts-1 expression by VEGF and Ang-II in the aorta. Given the importance of NFAT and C/EBPβ activation in the regulation of Adamts-1 expression by VEGF and Ang-II *in vitro*, we studied whether these pathways are also operative *in vivo*. To this end, we infused VEGF or Ang-II into C57BL/6 mice and analyzed Adamts-1 expression in the aortas of control and treated animals. VEGF treatment promoted NFAT nuclear translocation and subsequent induction of Adamts-1 expression in the aorta, which was sensitive to CsA pretreatment (Fig. 11A). In contrast, the phosphorylation status of C/EBPβ was not modified by VEGF infusion into the aortic wall (Fig. 11B). Similarly, the expression of Adamts-1 in protein lysates of aortas isolated from animals in-

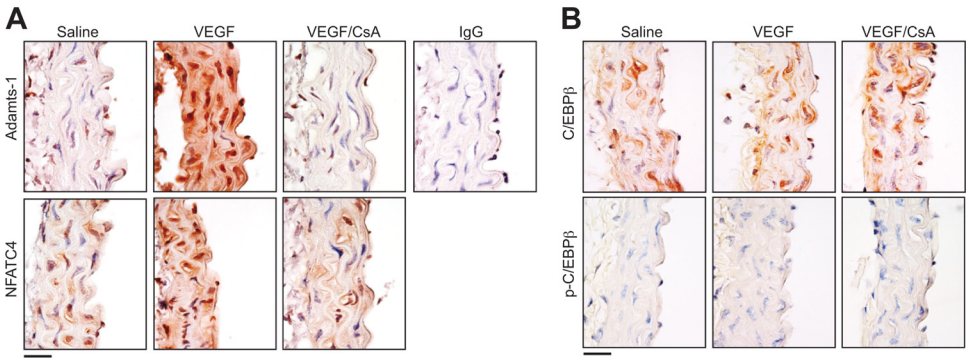

FIG 11 VEGF induces Adamts-1 expression and NFAT activation in the murine aorta. Representative immunostaining ($n = 3$) for Adamts-1 and NFATc4 (A) or total (C/EBPβ) and phosphorylated (p-C/EBPβ) C/EBPβ (B) proteins in aortic sections of mice treated with saline or VEGF at 25 μg/kg/day for 3 days or 1 day, respectively. Where indicated, mice were treated with CsA at 10 mg/kg/day for 1 day prior to VEGF administration. Bars, 20 μm. Staining of aortic sections with an IgG antibody was performed as a negative control.

fused with Ang-II for 1 to 21 days increased markedly (Fig. 12A). Correspondingly, *Adamts-1* mRNA levels increased significantly after only 5 h of Ang-II infusion and remained elevated for 21 days (Fig. 12A). Consistent with our *in vitro* findings, CsA did not inhibit Adamts-1 upregulation by Ang-II in the aorta (Fig. 12B). Moreover, immunostaining of Adamts-1 in murine aortic tissue sections revealed a significant induction of its expression by Ang-II (Fig. 12C), further supporting our observations.

Immunohistochemistry analysis of aortic tissue sections revealed that C/EBP-LAP staining was mainly cytoplasmic in the vasculature of saline-infused control animals but partially translocated to the nucleus upon activation by Ang-II (Fig. 12D). Concomitant with nuclear translocation, Ang-II induced a notable increase in C/EBP-LAP phosphorylation at Thr188 (Fig. 12D). Both Ang-II-induced Adamts-1 upregulation and C/EBPβ activation were prevented by the AT_1 receptor inhibitor losartan (Fig. 12C and D). These results demonstrate that aortic Adamts-1 expression increases after *in vivo* VEGF and Ang-II treatment and suggest that NFAT and C/EBPβ may mediate Adamts-1 upregulation induced by these stimuli in the murine aorta.

Altogether, our data show differential usage of signaling pathways in the regulation of Adamts-1 expression by stimuli associated with vascular remodeling and that Ang-II induces *in vivo* C/EBPβ phosphorylation and nuclear translocation, which could be involved in Ang-II-mediated Adamts-1 induction *in vivo*.

DISCUSSION

Emerging evidence indicates that the metalloproteinase Adamts-1 plays an important role in the pathophysiology of vascular disorders (7, 8, 10), but the intracellular mechanisms that control Adamts-1 expression during these processes remain poorly understood. In this work, we show that vascular expression of Adamts-1 is induced by mediators of vascular remodeling, such as VEGF, Ang-II, and the proinflammatory cytokines IL-1β and TNF-α. We have also characterized the coupling of specific signaling pathways and transcription factor activation in the regulation of Adamts-1 expression by these stimuli.

Using different approaches to block the CN/NFAT signal transduction pathway, we have identified this pathway as a critical mediator of the transcriptional activation of *Adamts-1* induced by VEGF in ECs. Previous reports have shown that VEGF upregulates *Adamts-1* mRNA (12, 31) and that NFATc1 binds the *Adamts-1* promoter upon VEGF treatment in ECs (32). Nevertheless, a detailed analysis of the transcriptional mechanisms involved in *Adamts-1* induction by VEGF had not been performed. Through our analysis, we have identified two novel NFAT binding sequences responsible for driving *Adamts-1* expression in response to VEGF. NFAT is known to activate transcription through functional cooperation with a variety of transcription factors, including AP1 (33, 34), CREB (35), FoxP3 (36), MyoD (37), and C/EBPβ (38); however, NFAT proteins may also bind DNA as monomers or dimers (39, 40). Thus, NFAT could be operating in our system without a heterologous partner, as is the case for TNF-α and IL-13 promoter activation (39). The possible requirement of NFAT dimers to mediate VEGF-induced *Adamts-1* expression would be in line with the abrogation of transcription upon the mutation of either NFAT site in the promoter. On the other hand, previous work has shown that VEGF upregulates endothelial Adamts-1 expression in a protein kinase C (PKC)-dependent manner (12). It is feasible that the increased expression of endothelial Adamts-1 triggered by VEGF requires the activation of both pathways. Accordingly, concomitant activation of CN and PKC signaling has been described during upregulation of the endothelial proteins RCAN1 to -4 (41, 42) and tissue factor (43) in activated ECs. Moreover, concerted activation of these two signaling pathways is a regulatory mechanism widely described for the regulation of gene expression in other cell types (44–46). Our data indicate that the CN/NFAT pathway not only participates in Adamts-1 induction by VEGF *in vitro* but is also required for VEGF-mediated Adamts-1 upregulation in the aortic wall (Fig. 11). In this context, we detected NFAT nuclear translocation and Adamts-1 induction in response to VEGF both in the endothelial layer and in VSMCs present in the tunica media. We cannot discard a paracrine effect exerted by an unidentified factor secreted by ECs, which activates the CN/NFAT pathway and Adamts-1 expression in VSMC in a secondary manner. Nonetheless, VEGF receptor expression in VMSCs *in vitro* and *in vivo* has been reported by several authors (47–50). VEGF might be therefore acting directly also on this cell type to promote Adamts-1 expression through the CN/NFAT pathway.

Stimulation of vascular cells *in vitro* and *in vivo* with Ang-II also induced a robust increase in the expression of Adamts-1, suggesting that this metalloproteinase is a molecular effector of Ang-II. Analogous to VEGF, Ang-II has been reported to trigger acti-

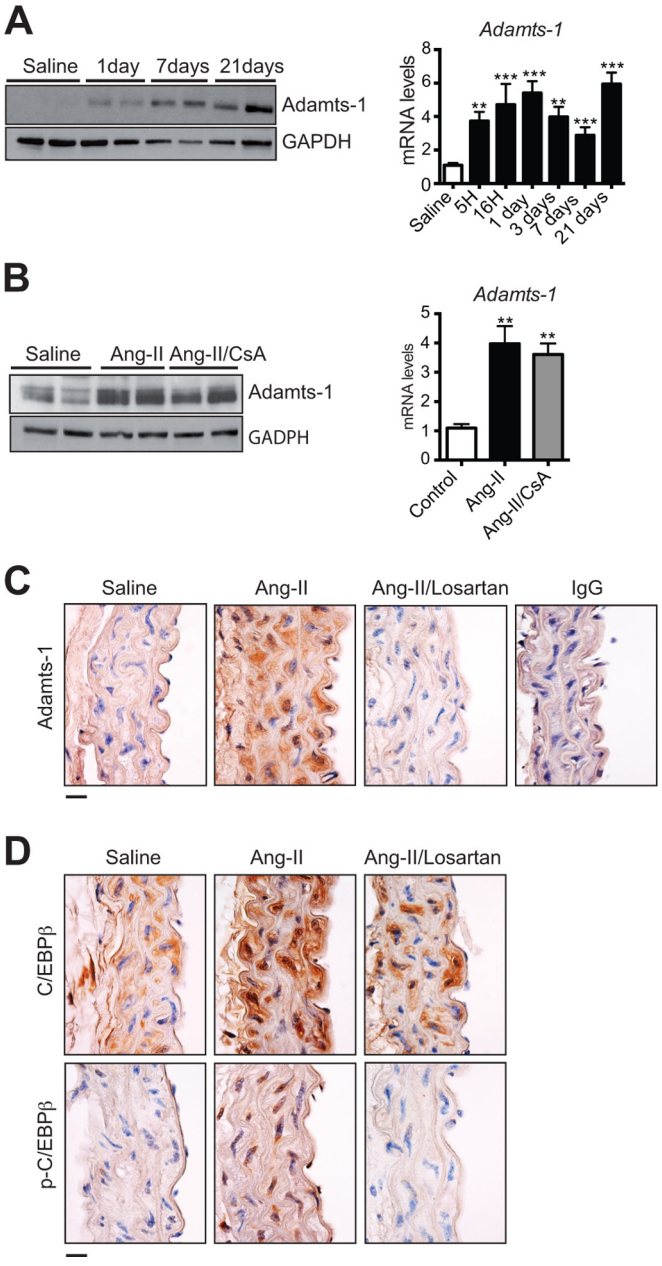

FIG 12 Ang-II induces Adamts-1 expression and C/EBPβ activation in the murine aorta. (A) Representative immunoblot ($n = 5$) and qPCR analyses of Adamts-1 expression in aortic extracts isolated from mice infused with saline or Ang-II (1 μg/kg/min) for the times indicated. Histograms show means ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (versus the control). (B) Adamts-1 protein and mRNA expression was analyzed as described above in aortic samples isolated from mice infused with Ang-II for 3 days. Where indicated, mice were treated with CsA at 10 mg/kg/day for 1 day prior to Ang-II administration. Images are representative of four independent experiments. (C) Representative Adamts-1 immunostaining ($n = 3$) of aortic sections from mice treated with saline or Ang-II for 3 days. Where indicated, animals were infused with losartan (10 mg/kg/day) for 1 day before Ang-II administration. Bar, 20 μm. (D) Representative immunostaining ($n = 3$) of total (C/EBPβ) or phosphorylated (p-C/EBPβ) C/EBPβ proteins in aortic sections isolated from mice infused with Ang-II or saline for 1 day. Where indicated, animals were infused with losartan (10 mg/kg/day) for 1 day before Ang-II administration. Bar, 20 μm.

vation of the CN/NFAT pathway in vascular cells (16). Thus, we initially inferred that activation of CN/NFAT signaling would drive the transcriptional upregulation of *Adamts-1* by Ang-II. However, characterization of the intracellular pathways governing this process revealed a role for C/EBPβ proteins, and not for NFAT transcription factors. The C/EBPβ-binding sites in the *Adamts1* promoter have also been involved in its induction by progesterone receptor-mediated activation of granulosa cells (29), further supporting the relevance of C/EBPβ proteins in the regulation of Adamts-1 expression. Furthermore, active C/EBPβ proteins have been linked to the expression of other metalloproteinase proteins, including MMP-3, MMP-13, and Adamts-5, during arthritic tissue remodeling (51, 52).

ChIP analysis revealed the differential binding of NFATc1 to the *Adamts-1* promoter upon stimulation with VEGF or Ang-II (Fig. 9). The involvement of specific NFAT family members in the regulation of diverse target genes has been reported (19) and could underlie our data on the disparate role of NFAT on *Adamts-1* transcriptional regulation by VEGF and Ang-II. Alternatively, differential activation kinetics or selective activation of NFATs might also account for the differential response mediated by VEGF and Ang-II. However, both factors activate all of the NFAT family members (Fig. 10D and E), and we cannot conclude that VEGF and Ang-II activate NFAT proteins to different extents because we have observed variability in the response to the treatments. This is probably due to variations in receptor expression among batches or passages of HUVECs. Another possibility is that VEGF and Ang-II trigger stimulus-dependent epigenetic modifications of NFAT family members, further influencing their transcriptional activity (53, 54).

The *Cox-2* promoter region also contains C/EBPβ- and NFAT-binding sites (24). Unlike VEGF, C/EBPβ activation is essential not only for *Adamts-1* but also for *Cox-2* transcription induced by Ang-II, which is associated with functional occupancy of both C/EBPβ- and NFAT-binding motifs (Fig. 9C). It is well established that the C/EBPβ and NFAT transcription factors cooperate synergistically for DNA binding and gene activation (30, 55). Because the C/EBPβ and NFAT binding sites are in close proximity in the *Cox-2* promoter (24), both factors could hypothetically cooperate in Cox-2 induction by Ang-II, as suggested by our functional data. Conversely, C/EBPβ could drive *Adamts-1* transcription in response to Ang-II without functional cooperation with NFAT, which, as discussed above, would operate in a partner-independent manner upon VEGF activation. Hence, we hypothesize that cooperation with different partners might be responsible for the differential regulation of the *Adamts-1* and *Cox-2* promoters by the NFAT and C/EBPβ transcription factors (Fig. 13).

Unlike ECs, LIP overexpression in VSMCs leads to a decrease in endogenous LAP expression. LIP lacks a transactivation domain and is known to impair C/EBP function either through competition for C/EBP DNA binding sites or by forming inactive heterodimers with other C/EBPs. In addition, cross talk between tyrosine kinase receptors and LAP/LIP isoforms has been described, which could act in some cases as a regulatory loop of LAP-LIP equilibrium (55). Accordingly, LIP overexpression might initiate a cell-type-dependent regulatory circuit that directly or indirectly modulates LAP expression.

Metalloproteinases regulate diverse aspects of inflammatory processes and immune responses, either through the release and

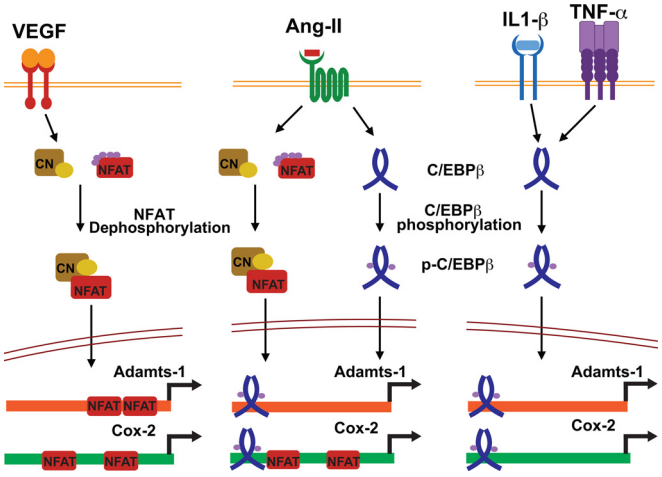

FIG 13 Differential transcriptional activation of the *Adamts-1* and *Cox-2* genes in response to VEGF, Ang-II, TNF-α, and IL-1β. The model shown represents the differential regulation of the *Adamts-1* and *Cox-2* promoters by the NFAT and C/EBPβ transcription factors during the stimulation of vascular cells by these factors. VEGF-induced upregulation of *Adamts-1* and *Cox-2* gene expression requires activation of the CN/NFAT signal transduction pathway. Although Ang-II also triggers the activation of CN-dependent signaling, Adamts-1 expression in response to Ang-II is mediated not by this pathway but instead by activation of C/EBPβ signaling. However, Ang-II-induced upregulation of Cox-2 requires activation of both the NFAT and C/EBPβ signaling pathways. Inflammatory stimuli induce Adamts-1 and Cox-2 upregulation via a molecular mechanisms that involves activation of C/EBPβ but not CN signaling.

activation of immune mediators (e.g., members of the TNF superfamily, epidermal growth factor, and Notch signaling pathways) or through a direct action on immune cells. Thus, Adamts-12 function is associated with neutrophil apoptosis and participates in inflammatory response control (56). Illustrated by atherosclerosis, many inflammatory diseases comprise extracellular matrix remodeling processes that are regulated at different levels by proinflammatory cytokines such as IL-1β and TNF-α and require metalloproteinase activity. IL-1β and TNF-α are known to induce *Adamts-1* mRNA in different cell types (57, 58); however, the precise transcriptional mechanism involved awaits identification. Similar to Ang-II, we have identified C/EBPβ as the chief mediator of Adamts-1 regulation by IL-1β and TNF-α in vascular cells. C/EBPβ is also implicated in the induction of Cox-2 expression by IL-1β and TNF-α. The convergence of signaling pathways triggered by disparate stimuli such as Ang-II and proinflammatory cytokines in C/EBPβ activation (Fig. 13) suggests a decisive role for this transcription factor in a broad range of pathophysiological processes. The role of matrix metalloproteinases in diseases that involve remodeling of the vessel wall, such as atherosclerosis, aortic aneurysm, and neointima formation in vascular restenosis, or genetic conditions, including familiar aortic diseases, indicates that Adamts-1 is a promising candidate for the development of novel therapeutic strategies for these disorders. Moreover, the prominent role of C/EBPβ in the induction of Adamts-1 by an array of stimuli, including Ang-II, IL-1, and TNF-α, suggests that pharmacological targeting of C/EBPβ may have utility in the treatment of vascular diseases of different origins.

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We have no competing interests to declare.

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