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Differential regulation of Adamts-1 gene expression by VEGF and Angiotensin-II in endothelial and vascular smooth muscle cells.

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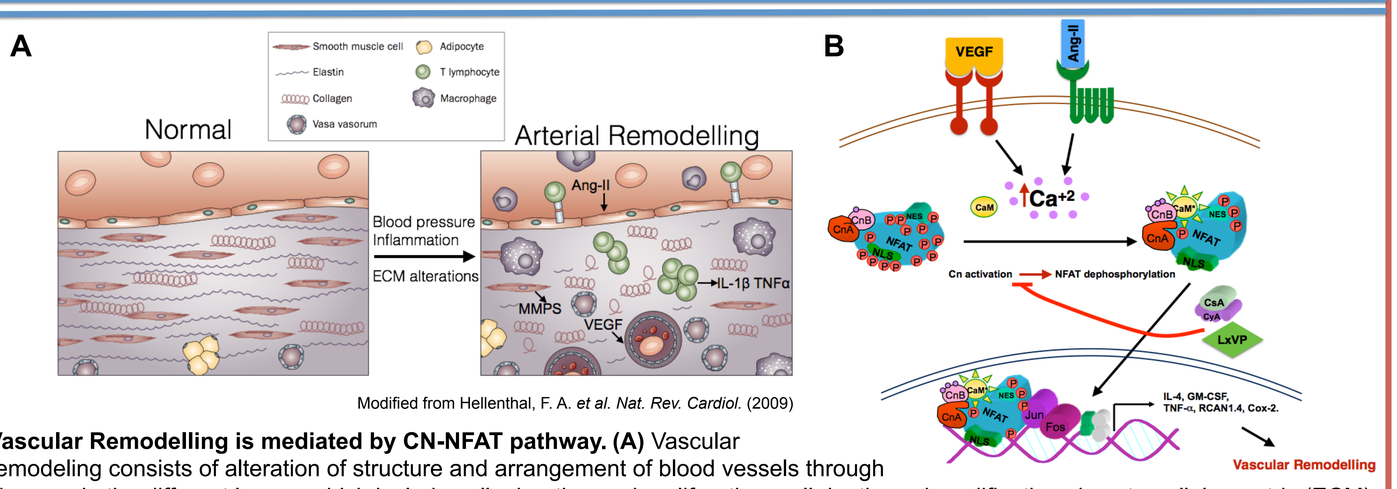
Introduction:

Vascular remodeling consists of the structural alteration and arrangement of blood vessels. During this process cell migration, proliferation, cell death, and modifications in extracellular matrix (ECM) components are produced. It is present in major vascular diseases such as hypertension, aneurysm, vascular stenosis, and atherosclerosis. Vascular Endothelial Growth Factor (VEGF) and Angiotensin-II (Ang-II) major regulatory factors in vascular biology. VEGF induce endothelial cell migration and proliferation and contribute to pathophysiological angiogenesis. Ang-II is a potent vasoconstrictor agent, although it is also involved in inflammation, cell growth, vascular permeability and matrix deposition. *In vivo*, Ang-II administration induces aortic abdominal aneurysm (AAA) in ApoE KO mice, and increases restenosis and atherosclerosis. These processes are blocked by inhibition of the the calcium-calcineurin-NFAT signaling pathway. in response to increases in intracellular calcium, Calcineurin (CN) dephosphorylates NFAT transcription factors, thus, they become transcriptionally active. Some metalloproteinases are among VEGF/Ang-II-regulated genes, which participate in vascular remodeling, modifying ECM. Adamts-1 (A Disintegrin and Metalloproteinase with Thrombospondin motifs type I), mainly degrades proteoglycans. Mechanisms which regulate Adamts-1 gene expression during vascular remodeling have not been fully elucidated. Our findings indicate that VEGF and Ang-II increase Adamts-1 expression through differential signal transduction pathways, which specifically switch on the activity of NFAT or C/EBPβ transcription factors respectively, in both endothelial (EC) and smooth muscle cells (VSMCs).



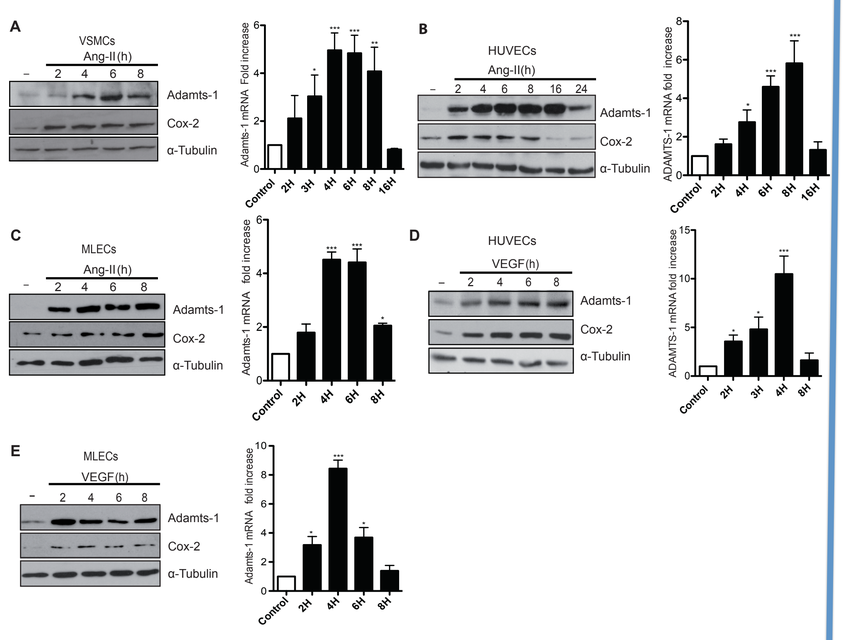
Modified from Hellenthal, F. A. *et al. Nat. Rev. Cardiol.* (2009)

Vascular Remodelling is mediated by CN-NFAT pathway. (A) Vascular remodeling consists of alteration of structure and arrangement of blood vessels through changes in the different layers, which include cell migration and proliferation, cell death, and modifications in extracellular matrix (ECM). This process is present in major vascular diseases such as hypertension, aneurysm, vascular stenosis, and atherosclerosis. **(B)** The major vascular regulator factors, Ang-II and VEGF increases intracellular Calcium in VSMCs and ECs, triggering Calcineurin phosphatase (CN) activation. Dephosphorylated NFAT translocate into the nucleus where they are transcriptionally active. CsA and LxVP peptide inhibit vascular remodeling through CN inhibition.

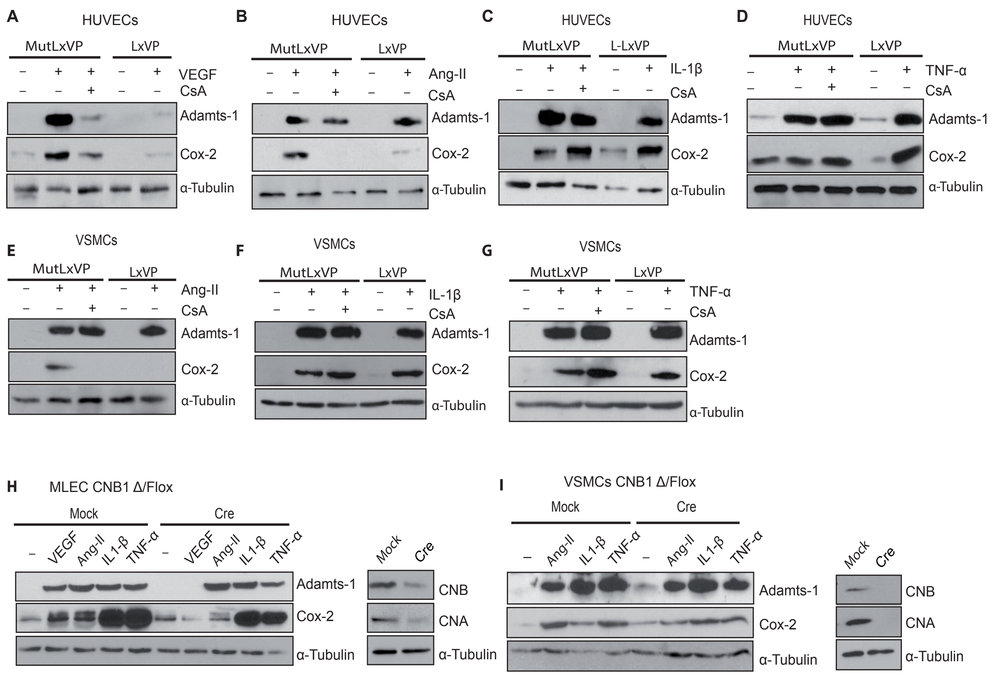
Results: 1.- Adamts-1 is up-regulated by Ang-II in VSMCs and ECs

The key vascular remodelling factors (Ang-II and VEGF) up-regulate Adamts-1 in Vascular cells.

(A) Representative Immunoblots and qPCRs of Adamts-1 in VSMCs treated with Ang-II, **(B)** Human Umbilical Vein ECs (HUVECs) and Mouse lung ECs (MLECs) **(C)**. Representative Inmunoblots and qPCRs in HUVECs **(D)** and MLECs **(E)** stimulated with VEGF. VEGF and Ang-II activity was confirmed by immunoblot showing Cox-2 induction. (n=4; fold induction relative to controls. mean+-, One Way ANOVA test, ***p<0,01).

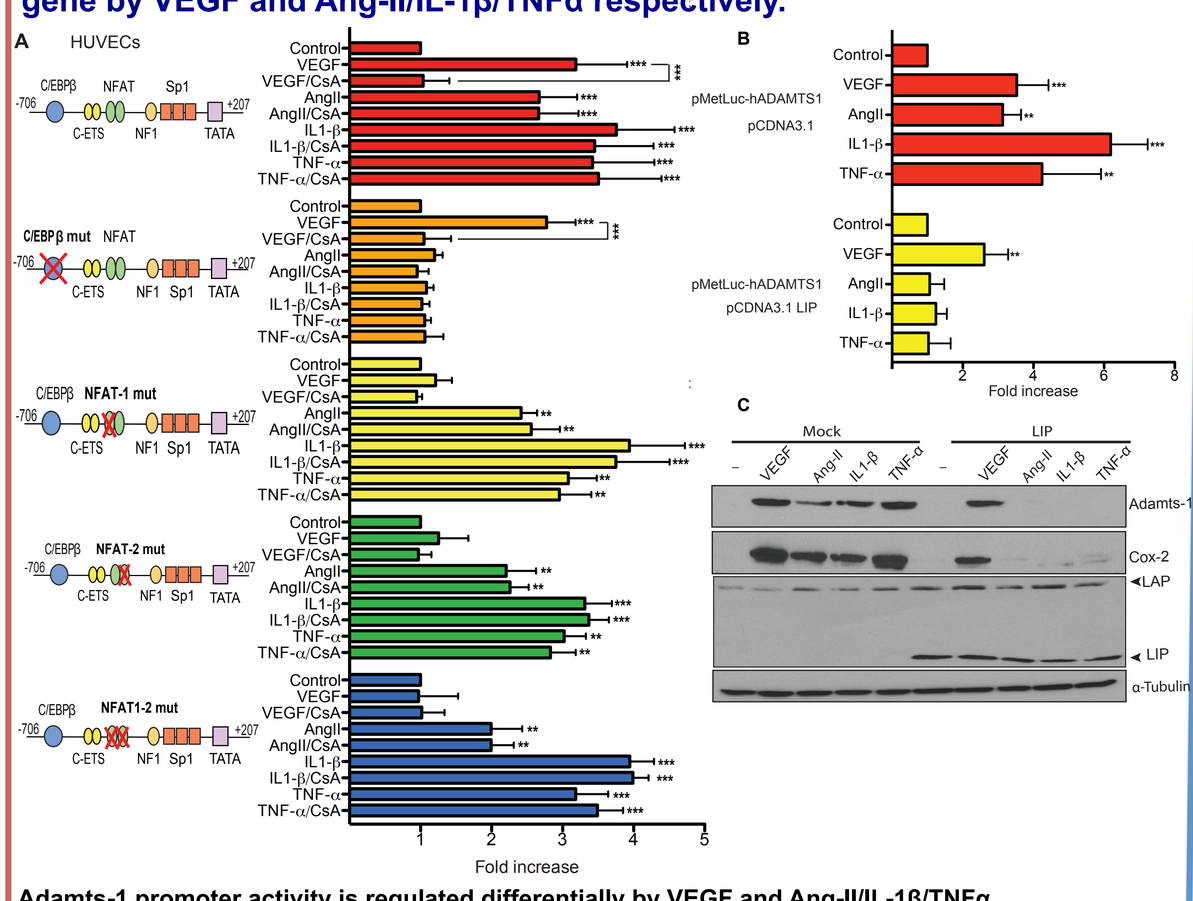


2.- Differential CN role in Adamts-1 regulation by Ang-II and VEGF



Specific CN-NFAT blockade, abrogates Adamts-1 up-regulation by VEGF but not by Ang-II or the pro-inflammatory cytokines /IL-1β/TNFα.
Representative Immunoblots of Adamts-1 in HUVECs **(A to D)** and VSMCs **(E to G)** were transduced by LXVP or mutated version (mutLxVP) lentivectors and stimulated with VEGF, Ang-II, IL-1β or TNFα. mutLXVP transduced cells were pre-treated with or without a CN inhibitory drug, Cyclosporine-A (CsA). Representative inmunoblots of Adamts-1 and CN from MLECs **(H)** and VSMCs **(I)** from conditional CN-deficient mice were transduced with mock or Cre-recombinase expressing lentivectors (n=3). Similar results were obtained by Adamts1 qPCR (data not shown).

3.-NFAT and C/EBPβ are essential for transcriptional regulation of Adamts-1 gene by VEGF and Ang-II/IL-1β/TNFα respectively.

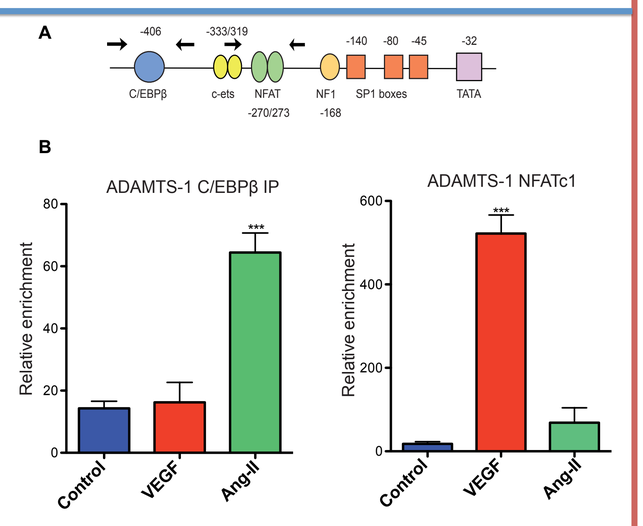


Adamts-1 promoter activity is regulated differentially by VEGF and Ang-II/IL-1β/TNFα.
In silico analysis of Adamts-1 promoter revealed two NFAT and C/EBPβ putative sites**.** Luciferase assay in HUVECs transfected with different mutants of Adamts1 promoter as indicated **(A)**, or the combination with an empty (pCDNA3.1) or with the C/EBPβ inhibitory isoform (LIP) expression vector **(B)** (n=5). Representative immunoblot of transduced HUVECs with mock or with LIP-expressing lentivirus **(C)** (n=3)

4.- C/EBPβ and NFAT are differentially recruited to Adamts-1 promoter

Ang-II and VEGF differentially increase C/EBPβ and NFAT binding to Adamts-1 promoter.

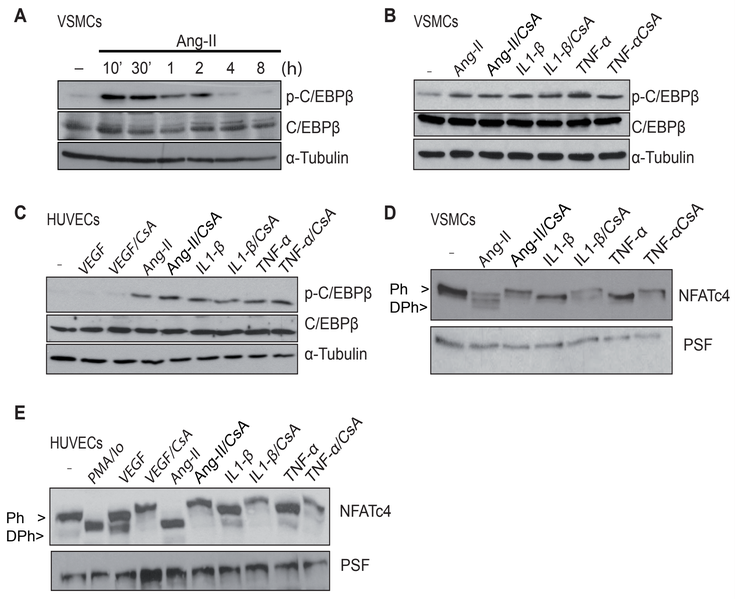
(A) Representation of the putative transcription sites of Adamts-1 promoter and the position of oligonucleotides used for qPCRs**. (B)** HUVECs were stimulated with Ang-II or VEGF for 30' and and ChiP was performed using specific C/EBPβ and NFAT antibodies. Relative enrichments are compared to control IgG.(n=3; mean+-SD, One Way Anova test, ***p<0,01).



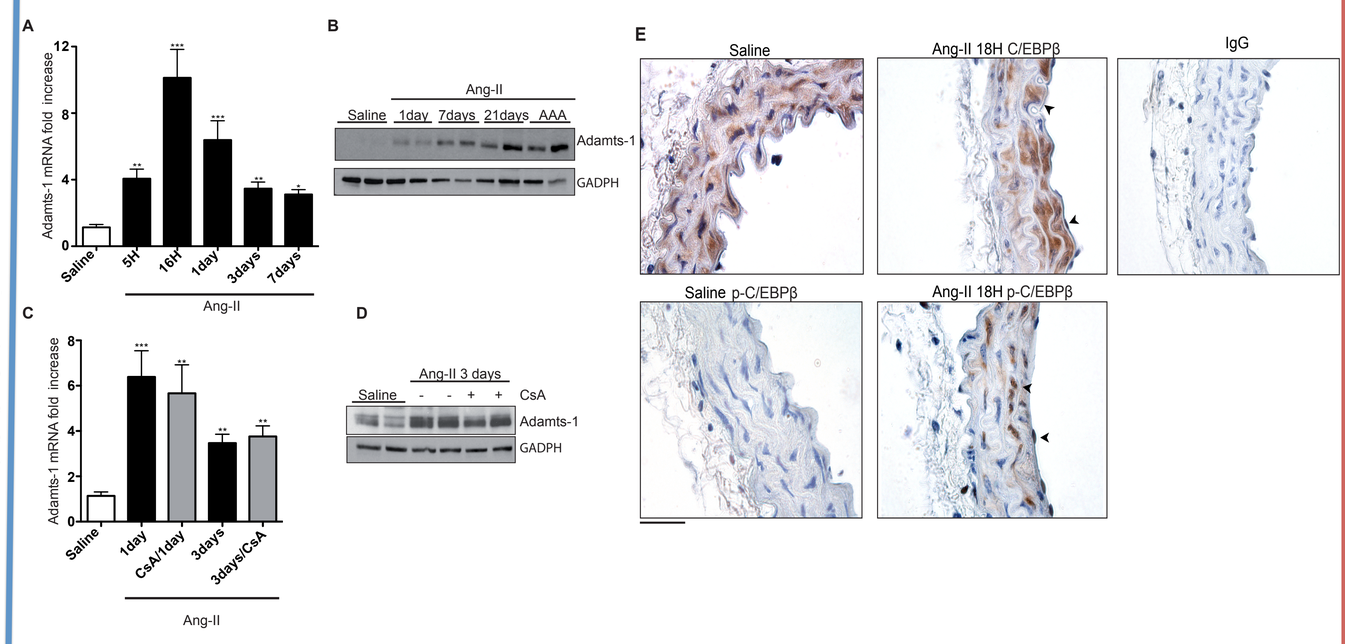
5.- C/EBPβ is selectively phosphorylated by Ang-II, IL-1β and TNFα.

C/EBPβ is phosphorylated upon Ang-II IL-1β and TNFα stimulation but not by VEGF.

Representative immunoblots of C/EBPβ and phosphorylated C/EBPβ (p-C/EBPβ) in VSMCs stimulated with Ang-II for different times **(A),** VSMCs with Ang-II, IL-1β and TNFα for 30 minutes in presence or not of CsA **(B),** or in HUVECs **(C)**. Activation of CN-NFAT were addressed by immunoblot of NFATC4 in VSMCs **(D)** or HUVECs **(E)**. NFAT dephosphorylated (DPh) band appears with more electrophoretic mobility than hyperphosphorylated (Ph) (n=3).



6.- Ang-II induces Adamts-1 up-regulation and C/EBPβ phosphorylation in vivo.



Adamts-1 and C/EBPβ activation are induced in murine aortas infused with Ang-II
Representative immunoblots and qPCRs of Adamts-1 from mice AORTAS infused with saline or with Ang-II (1μg/Kg/min). Ang-II infused animals were pre-infused with or without CsA (5mg/Kg/day). Adamts-1 RNA **(A)** and protein was up-regulated in wild type mice (WT) and in abdominal aortic aneurysm samples (AAA) from ApoE-KO mice **(B)**. Adamts-1 up-regulation in RNA **(C)** and protein **(D)** by Ang-II was not inhibited by CsA co-treatment. **(E)** Representative immunostainings for C/EBPβ or p-C/EBP in murine aortas infused with saline or Ang-II. One Way ANOVA, ***p<0,01, scale bar 5 μm).

Conclusions :

- Adamts-1 is up-regulated by Ang-II and VEGF, and by pro-inflammatory cytokines IL-1β and TNFα.
- Adamts-1 induction by Ang-II/IL-1β and TNFα requires C/EBPβ site. It is blocked by the inhibitory C/EBPβ isoform (LIP) over-expression.
- Although, both, Ang-II and VEGF activates CN-NFAT pathway, only VEGF up-regulates Adamts-1 in a CN-NFAT dependent manner. It is due to a differential C/EBPβ and NFAT recruitment to Adamts-1 promoter.
- C/EBPβ is phosphorylated by Ang-II response both, *in vivo* and *in vitro*.
- Ang-II up-regulates Adamts-1 expression and induces C/EBPβ activation in mouse aorta and in the murine Abdominal Aortic Aneurysm model (AAA).

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