Supplemental Data

Materials and Methods

Materials

Human AngII and Rho kinase inhibitor Y-27632 were purchased from Calbiochem. Trichostatin A and Acetyl-CoA were purchased from Sigma-Aldrich. Recombinant human cyclophilin A and rabbit anti-cyclophilin A were purchased from Enzo Life Science. Recombinant p300 HAT domain and anti-GAPDH antibody were from EMD Millipore. 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Invitrogen. Protein A- and Protein G- agarose were from Roche. Mouse anti-FlagM2 antibody was from Agilent Technologies. Rabbit anti-acetyl lysine (#9441) and phospho-ERK1/2 were from Cell Signaling Technology. Mouse anti-ERK2, goat anti-VCAM1, mouse anti-ICAM1 and mouse anti-CyPA were purchased from Santa Cruz Biotechnology.

Cell Isolation and Culture

Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Rochester published by the United States National Institutes of Health. Animals (8-12 weeks old) were anesthetized with ketamine (130mg/kg) and xylazine (8.8mg/kg). Aortic smooth muscle cells from rats (RASMC) or mice (MASMC) from WT, *Ppia*-/- or overexpressed Flag-CyPA in Smooth Muscle Cell were isolated by enzymatic digestion and maintained in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% fetal bovine serum (FBS) as described previously[1]. RASMC at passages 6-12 or MASMC at passage 4-6 at 70% to 80% confluence were growth arrested by incubation in DMEM containing 0.3% FBS for 24hr and stimulated with Angiotensin II (AngII) for the indicated times. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described [2] and

seeded onto 0.2% gelatin pre-coated dishes maintained in Medium 200 (Cascade Biologic) with low serum growth supplement (LSGS; Invitrogen), 5% FBS, 100µg/ml streptomycin and 100IU/ml penicillin. U937 monocytes were maintained in RPMI-1460 medium containing 10% FBS, 100ug/ml streptomycin and 100IU/ml penicillin. HEK293 cell lines were maintained in 10% FBS, 100µg/ml streptomycin and 100IU/ml penicillin. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Plasmid

Mutants of CyPA (lysine to arginine) were generated by site directed mutagenesis according to the manufacturer's protocol (QuikChange Site-Directed Mutagenesis; Agilent Technologies). The primers used were:

K44R F: 5`- GCA CTG GGG AGA GAG GAT TTG GCT ATA AG – 3`

K44R R: 5` - CTT ATA GCC AAA TCC TCT CTC CCC AGT GC – 3`

K82R F: 5` - CCA TCT ACG GAG AGA GAT TTG AGG ATG AGA AC-3`

K82R R: 5` - GTT CTC ATC CTC AAA TCT CTC TCC GTA GAT GG-3`

K125R F: 5` - GTG GCT TGG CAG GCA TGT GGT CTT TGG – 3`

K125R R: 5` - CCA AAG ACC ACA TGC CTG CCA TCC AGC CAC -3`

DNA sequences were verified at the University of Rochester Genomics Research Center.

Construction of recombinant lentivirus and VSMC transduction

pLV-CMV-IRES-GFP is an HIV-1 based lentiviral expression vector that allows simultaneous expression of CyPA cDNA (and mutants thereof) from the CMV promoter and EGFP by means of an IRES element. Infectious viral particles were generated by co-transfection of the

transgene, with plasmids expressing viral gag/pol genes (psPAX2) and VSV-G coat protein (pMD2.G) into HEK293T cells using Fugene6 (Promega). 48 hr post-transfection viral containing supernatant were collected, filtered through 0.45µm cellulose acetate filters and stored in aliquots at -80^{o}C. For transduction, VSMC were plated at subconfluence in 6 well culture plates and spin-oculated (1500 x g for 1.5 hours at room temperature) with virus in the presence of polybrene (8µg/ml). Cells were incubated for 36 hours and changed the media to serum free DMEM 1hr before AngII stimulation.

In vitro acetylation assay

Reactions (20µl) containing rhCyPA 50 nM, 1.2 mM acetyl-CoA and 1µg p300 protein in HAT buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 10 mM sodium Butyrate and 10% Glycerol) were incubated at 30^{o}C for 45min and reaction was stopped by incubation on ice.

Measurement of reactive oxygen species (ROS) by flow cytometry

Growth arrested VSMC were collected by trypsinization and incubated with 2 mM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) in 3% FBS/PBS at 37°C for 30min. Cells were centrifuged, washed and incubated with CM or rhCyPA at 37^{o}C. After, 4 hr incubation, cells were centrifuged, washed and resuspended in 3% FBS/PBS followed by flow cytometry measurement (Accuri® C6). The data were analyzed using FlowJo software (TreeStar Inc.).

Gelatin zymography for MMP2 activity

Gelatin zymography for the detection of MMP2 activity in CM was performed as follows. Samples were mixed in loading buffer (125 mM Tris pH6.8, 5% SDS, 20% glycerol, 0.03% bromophenol blue) and incubated at room temperature for 5min. Samples were resolved through 8% non-denaturing PAGE gels containing 0.1% gelatin in Tris/Glycine/SDS running buffer. Following electrophoresis, gels were immersed in renaturation buffer (2.5% Triton X-100 in 50 mM Tris pH 7.5) for 1hr at room temperature. Gels were then immersed in digestion buffer (50 mM Tris pH7.5, 4 mM $CaCl_2$, 200 mM NaCl, 0.02% Brij35) for 48hr at 37^{o}C. MMP2 activation was detected as clear areas on a blue background by staining in 0.1% Coomassie Blue R250 (in 40% methanol/10% acetic acid) and destaining in 40% methanol/10% acetic acid).

Immunoprecipitation and Western blotting

VSMC were lysed in NP-40 buffer (1% NP-40, 50 mM Tris HCl; pH 7.5, 150 mM NaCl, 10 mM sodium fluoride, 1 mM PMSF, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate and 1:1000 protease inhibitor cocktail (Sigma) and protein concentrations were determined by Bradford protein assay (Bio-Rad). Lysates containing equal amounts of soluble proteins were incubated with antibody overnight at 4°C. Antibody complexes were collected by incubation with protein A agarose for 2 hours at 4^{o}C. Precipitates were washed 3 times in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and analyzed by Western blot. Protein reactivity was detected using ECL (GE Healthcare).

Endothelial to monocytes adhesion assay

HUVEC cultured in 35mm dish were growth arrested for 2hr with serum free medium and stimulated with CM or 50nM rhCyPA for 6hr. Then, medium was removed and U937 monocytes (1x 10^4 cells / 2ml RPMI) were added and incubated for 30min at 37°C. Unbound cells were removed by washing 3 times with PBS. Adherent cells were counted in 5 randomly selected optical fields in each well. Phase-contrast microphotographs of cells were obtained using an inverted fluorescent microscope (IX50, Olympus) with 20x lens.

Supplementary references

1. Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, Yan C, Abe J, Berk BC. Cyclophilin a mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation*. 2008;**117**:3088-3098
2. Kim GY, Nigro P, Fujiwara K, Abe J, Berk BC. P62 binding to protein kinase c zeta regulates tumor necrosis factor alpha-induced apoptotic pathway in endothelial cells. *Arterioscler Thromb Vasc Biol*. 2012;**32**:2974-2980
3. Suzuki J, Jin ZG, Meoli DF, Matoba T, Berk BC. Cyclophilin a is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res*. 2006;**98**:811-817

Figure Legends

Supplemental Figure 1. A and B, AngII-induced CyPA acetylation was measured in Flag-CyPA over expressed mouse aortic smooth muscle cells (MASMC- FlagCyPA) using immunoprecipitation and immunoblotting method. The results are normalized to the fluorescence intensity at the 0 time point, which was set to 1.0. C and D, TCL from WT or CyPA knockout mouse aortic smooth muscle cell (*Ppia*-/- MASMC) were analyzed by western blot to detect 17kDa protein acetylation. All experiments are performed three independent times and data are shown as mean±SEM. (*$p<0.05$ versus untreated cells).

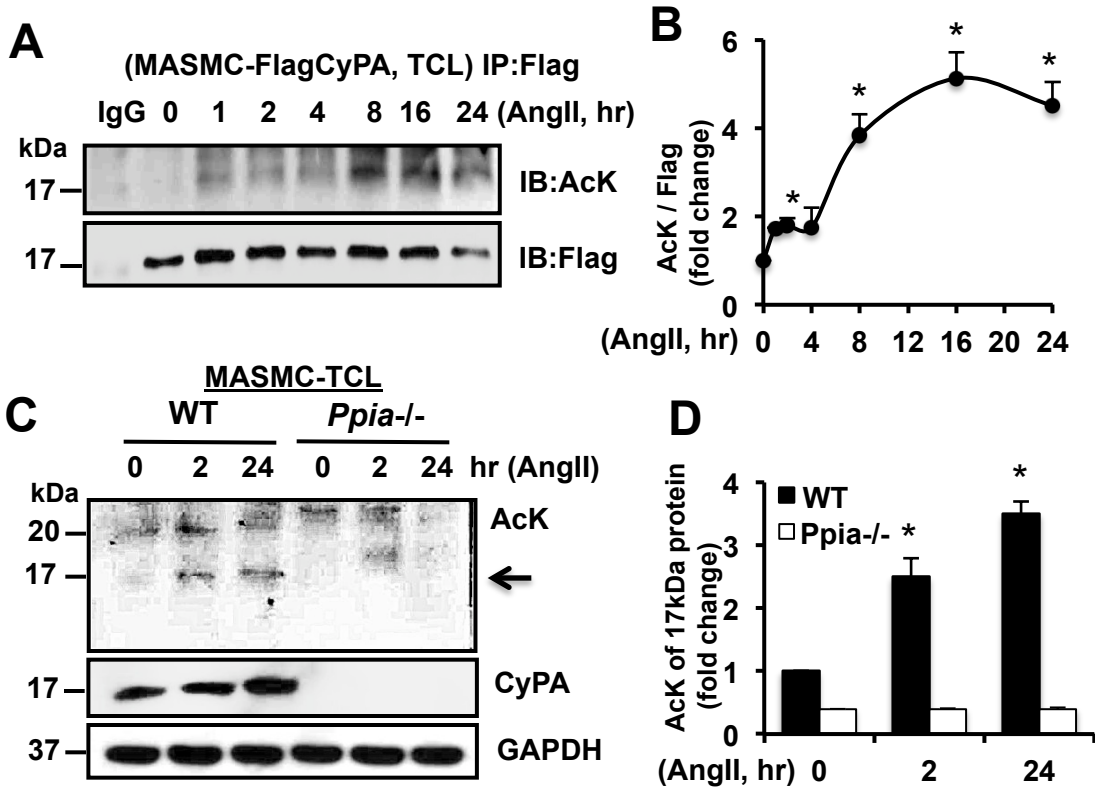
Supplemental Figure 2. Rho kinase inhibitor and AcK-CyPA secretion. A, RASMC were pretreated with Rho kinase inhibitor Y-27632 (30μM) for 30min and stimulated with AngII (300nM) for 24hr. CM were immunoprecipitated with anti-CyPA antibody and immunoblotted with anti-AcK or CyPA, respectively. B and C, The quantitative analysis of AcK-CyPA using Image J (NIH). Data are representative of three independent experiments and values are mean ± SEM. (*$p<0.05$ versus vehicle, # $p<0.05$ versus AngII). D. Equal amounts of Total cell lysates from RASMC treated as described were immunoprecipitated with anti-CyPA and the complexes western blotted for CyPA and acetylation. Y27632, in the presence or absence of AngII did not affect CyPA expression. E. Quantitation of ratio of AcK-CyPa to CyPA in the presence of Y27632.

Supplemental Figure 3. Confirmation of the total amount of Flag-CyPA in mixtures of CM, which is used for pERK1/2, ROS production and MMP2 activity experiments. CyPA in the CM from *Ppia-/-* MASMC transduced with WT or mutant lentiviral particles was determined from quantitation of the western blot reactivity (Figure 4) and the volumes were normalized using conditioned medium from *Ppia-/-* MASMC. Aliquots of the mixtures used in zymography and ROS experiments and pErk activation were subjected to Western blot and immunoblotted with anti-Flag antibody.

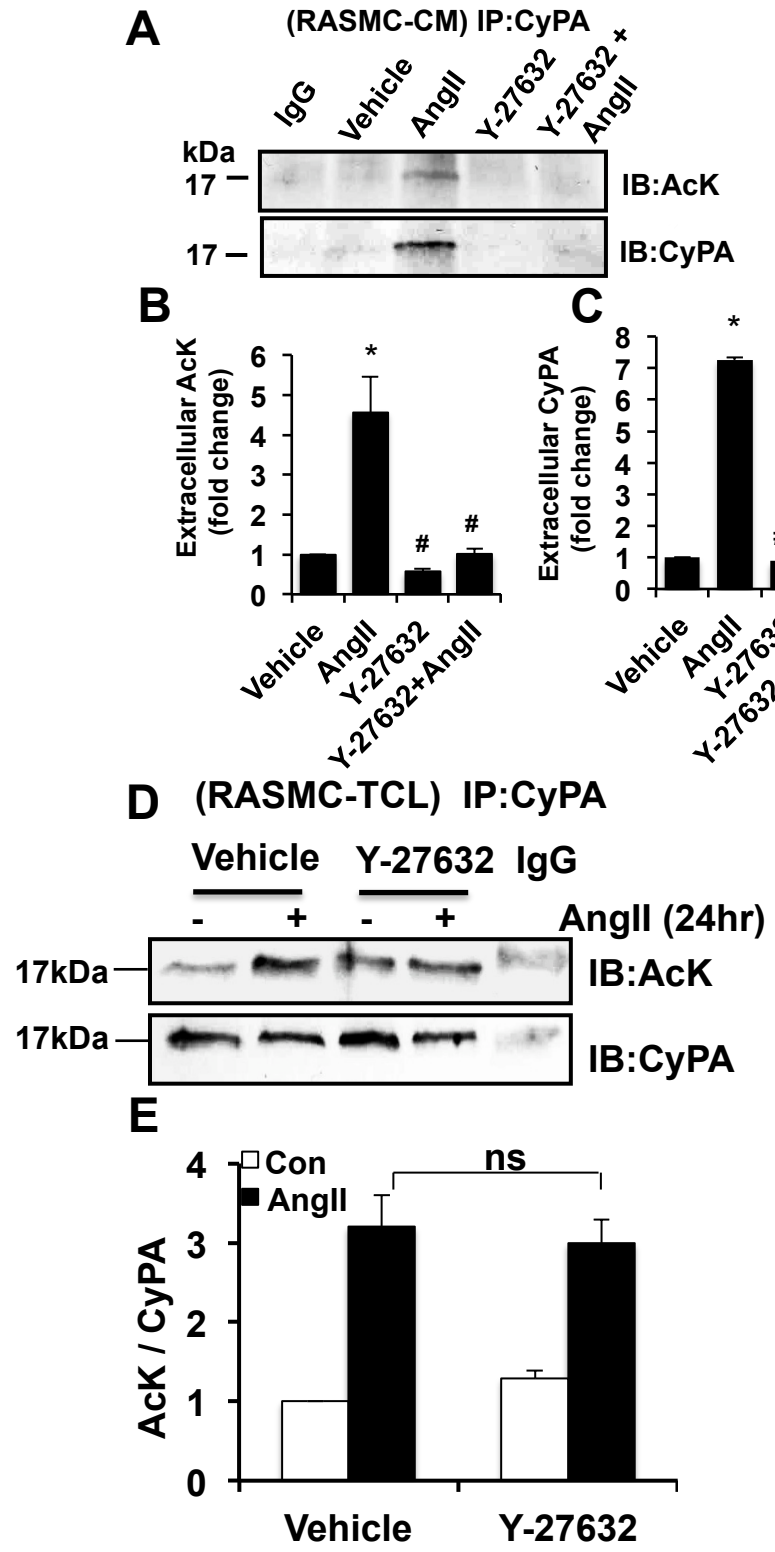
Supplemental Figure 4. In vitro acetylation confirmation. rhCyPA was acetylated in vitro in HAT buffer containing acetyl CoA and p300 acetyltransferase. In vitro acetylated rhCyPA (AcK-rhCyPA) and native rhCyPA (in HAT buffer lacking p300) were subjected to Western analysis to detect acetylation using anti-AcK or CyPA antibody.

Supplemental Figure 5. A. The schematic model shows the role of AngII-induced acetylated CyPA in secretion and regulation of vascular cells activation. **B**. Angiotensin II (AngII)-induced oxidative stress regulates lysine acetylase (KAT) and/or deacetylase (KDAC) activity, which alters the cellular equilibrium of the two enzyme activities. CyPA is one of the substrates whose acetylation is affected by the alteration of this equilibrium. Acyl-CyPA is preferentially secreted and is a more potent agonist than CyPA as measured by ERK1/2 and MMP2 activation.

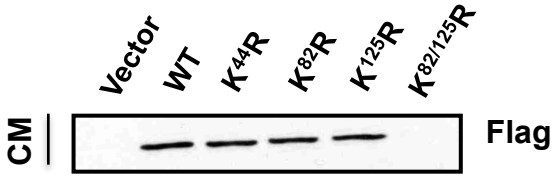
Supplemental Figure 1



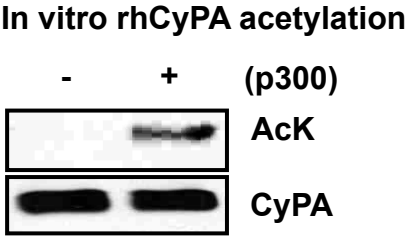
Supplemental Figure 2



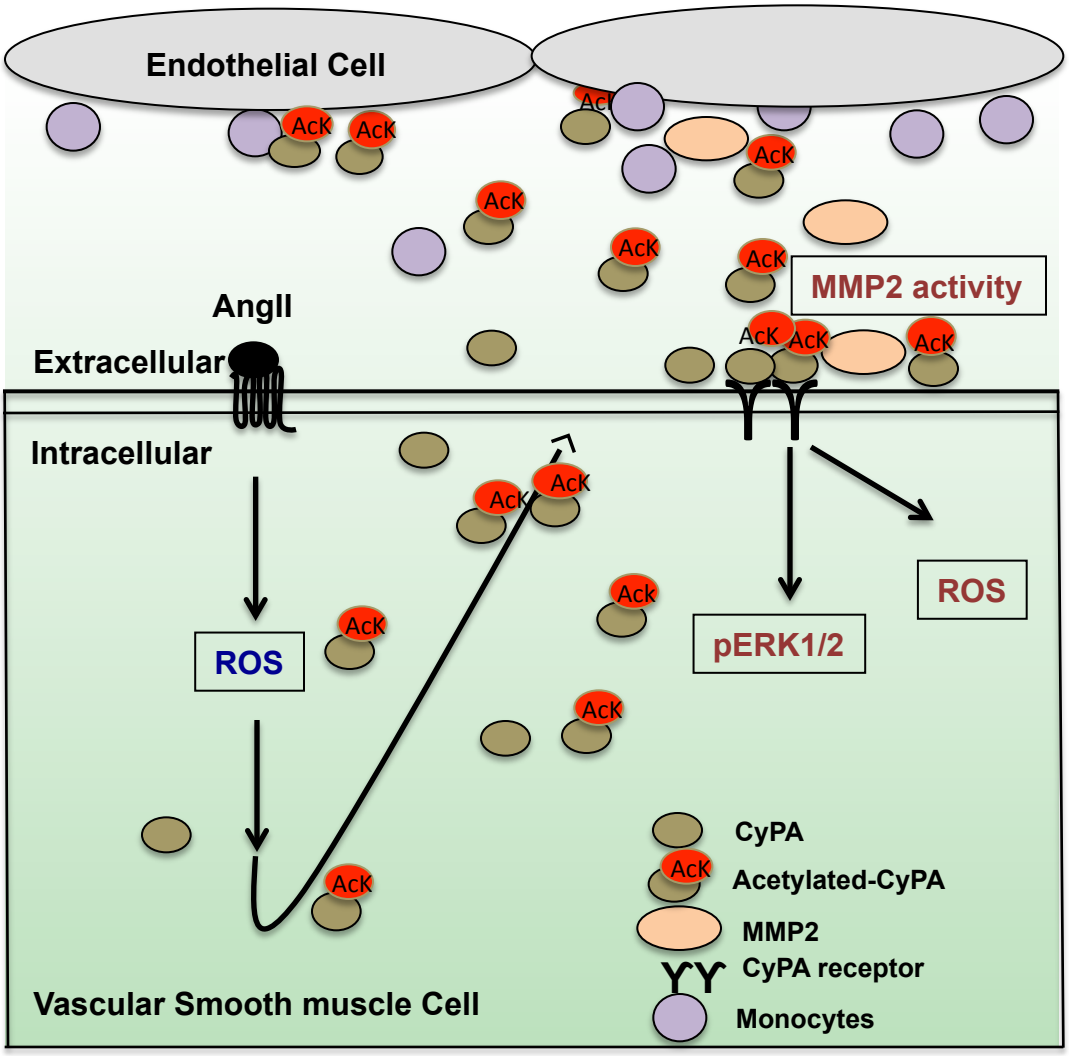
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5A



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