Defective p27 phosphorylation at serine 10 affects vascular reactivity and increases abdominal aortic aneurysm development via Cox-2 activation

Pedro Molina-Sánchez[1,*], Lara Del Campo[1,2,*], Vanesa Esteban[1,†], Cristina Rius[1,2], Raphael Chèvre [1,†], José J. Fuster[1,†], Mercedes Ferrer[3,4], Juan Miguel Redondo[1,2] and Vicente Andrés[1,2,‡]

[1] Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain

[2] Centro de Investigaciones Biomédicas en Red en Enfermedades Cardiovasculares (CIBER-CV), Spain

[3] Department of Physiology, Universidad Autónoma de Madrid, Spain

[4] Cardiovascular Area, Hospital La Paz Institute for Health Research (IdiPAZ), Madrid, Spain

* These authors contributed equally

† Current address:

P. Molina: The Tisch Cancer Institute at Mount Sinai, New York, US

V. Esteban: IIS-Fundación Jiménez Díaz, Madrid, Spain

Cristina Rius: School of Biomedical and Health Sciences, Universidad Europea de Madrid, Spain

R. Chèvre: Translational Laboratory in Genetic Medicine, Singapore

J.J. Fuster: Robert M. Berne Cardiovascular Research Center. University of Virginia School of Medicine. Charlottesville, VA. USA

‡ Corresponding author:

Vicente Andrés
CNIC
Melchor Fernández Almagro 3, 28029 Madrid (Spain)
Phone: +34-91 453 12 00 (Ext. 1502)
Fax: +34-91 453 12 65
E-mail: vandres@cnic.es

ABSTRACT

Phosphorylation at serine 10 (S10) is the major posttranslational modification of the tumor suppressor p27, and is reduced in both human and mouse atherosclerosis. Moreover, a lack of p27-phospho-S10 in apolipoprotein E-null mice (apoE-/-) leads to increased high-fat diet-induced atherosclerosis associated with endothelial dysfunction and augmented leukocyte recruitment. In this study, we analyzed whether p27-phospho-S10 modulates additional endothelial functions and associated pathologies. Defective p27-phospho-S10 increases COX-2 activity in mouse aortic endothelial cells without affecting other key regulators of vascular reactivity, reduces endothelium-dependent dilation, and increases arterial contractility. Lack of p27-phospho-S10 also elevates aortic COX-2 expression and thromboxane A_2 production, increases aortic lumen diameter, and aggravates angiotensin II-induced abdominal aortic aneurysm development in apoE-/- mice. All these abnormal responses linked to defective p27-phospho-S10 are blunted by pharmacological inhibition of COX-2. These results demonstrate that defective p27-phospho-S10 modifies endothelial behavior and promotes aneurysm formation via COX-2 activation.

KEY WORDS: p27, p27 phosphorylation at serine 10, COX-2, endothelial cell, aneurysm, vascular contractility

1. INTRODUCTION

The endothelium is a key player in the maintenance of vascular homeostasis. Among other functions, endothelial cells (ECs) regulate leukocyte trafficking [1], angiogenesis [2], coagulation [3], vascular tone and arterial blood pressure [4]. Endothelial dysfunction leads to local and systemic alterations that contribute to cardiovascular diseases, and is strongly associated with hypertension [5, 6], a common cardiovascular risk factor. ECs modulate the behavior of vascular smooth muscle cells (VSMCs), affecting their contractile capacity through the release of a wide variety of vasoactive factors, such as nitric oxide (NO), prostaglandins (PGI_2, PGE_2, etc), and thromboxane (TX). An imbalance in the synthesis of these agents, caused by dysfunction of their main enzymatic producers (NO synthases, cyclooxygenases, and PG and TX synthases) can lead to hypertension [6] and generate or aggravate vascular pathological manifestations. Endothelial dysfunction also promotes other vascular disorders, such as atherosclerosis or some types of aneurysm, at least in part through overexpression of adhesion molecules that promote leukocyte extravasation and accumulation within the inflamed arterial wall [7]. These events can alter the blood flow and induce medial degeneration [8], an early event in both atherosclerosis and aneurysm.

Cell cycle inhibitors have emerged as important protective agents against vascular disease and cardiovascular risk. $p27^{kip1}$ is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs) that inhibits VSMC proliferation and migration in vitro [9, 10]. Studies in the apolipoprotein E-null mouse model (apoE-/-) demonstrated that loss of p27 increases the proliferation of VSMCs and macrophages in atherosclerotic vessels and exacerbates atherosclerosis progression [11, 12]. Similarly, studies of angiotensin II-

induced vascular injury in p27-null mice have shown increased cellular proliferation in aorta and arterial thickening [13].

The protective effect of p27 on the cardiovascular system is not only related to its classical proliferation-dependent activity. p27 has emerged as a versatile protein able to mediate a wide range of cellular processes, including cytoskeletal organization [14], cellular migration [15], and gene transcription [16]. Some of these properties are modulated by posttranslational modification, the most prominent being phosphorylation at serine 10 (S10) [17]. In previous studies, we demonstrated that loss of p27-phospho-S10 is a hallmark of human and mouse atherosclerosis [18]. By crossing athero-susceptible apoE-/- mice with p27S10A knock-in mice, which are defective for p27-phospho-S10 [19], we found that this phosphorylation protects against endothelial dysfunction and leukocyte recruitment, foam cell formation, and early and advanced states of high-fat diet-induced atherosclerosis development through cell-cycle-independent mechanisms [18, 20]. Whether defective p27-phospho-S10 regulates other aspects of vascular pathophysiology remains unknown. To address this question, we investigated here the role of p27-phospho-S10 in the modulation of vascular reactivity in thoracic aorta and arterioles of the cremaster muscle, blood pressure, and the formation of abdominal aortic aneurysms (AAA) in apoE-/- mice fed a standard non-atherogenic diet. Our results reveal that loss of p27-phospho-S10 promotes endothelial dysfunction and pathological vascular remodeling via COX-2 activation, identifying a new function of the multifaceted p27.

2. MATERIALS AND METHODS

2.1. Mice

Wild-type and mutant mice used in this study were males on the C57BL/6J genetic background. Double mutant apoE-/-p27S10A mice were obtained by crossing apoE-/- mice (The Jackson Laboratory, Madison, WI) with p27S10A mice defective for p27-phospho-S10 [19]. Mice were fed *ad libitum* a standard diet (LabDiet JL Rat and Mouse/Auto 6F 5K67). All mouse procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. Animal protocols were approved by the local ethics committees and the Animal Protection Area of the Comunidad Autónoma de Madrid (PROEX 135/14).

2.2. Anesthesia and euthanasia

For intravital microscopy experiments and osmotic minipump implantation, animals were anesthetized with an intraperitoneal injection of a mixture of medetomidine and ketamine (0.5 mg/kg and 50 mg/kg, respectively). For ultrasound aorta visualization, animals were anesthetized by inhalation of vaporized 2% isoflurane. Mice were sacrificed by cervical dislocation or CO_2 inhalation.

2.3. Isolation and culture of mouse aortic ECs (mAECs)

mAECs were isolated as previously described [21]. Briefly, 7 adult mice were sacrificed, the aortas were harvested, and adipose tissue and the adventitia were removed. Tissue was then cut into ~1 mm rings and placed on 0.5% gelatin coated plates. The rings were incubated for 1 week in EC-specific medium (DMEM:F12, Lonza) containing 1% penicillin/streptomycin, 0.4mM L-glutamine, 10 mM Hepes, 1 μg/ml fungizone, 10% fetal

bovine serum (FBS), 0.1 mg/ml heparin (Sigma-Aldrich), and 50 µg/ml EC growth supplement (Becton Dickinson). mAECs that had migrated from the rings onto the plate were selected after incubation with CD102 antibody (Purified Rat Anti-Mouse CD102 - ICAM-2 Monoclonal Antibody, BD Pharmigen) followed by incubation with a secondary antibody linked to magnetic beads (Dynabeads Sheep anti-Rat IgG, Invitrogen). Cells were collected using a DynaMag-15 Magnet platform (Life Technologies), and were expanded at passage 0 in gelatin-coated plates containing mAEC medium. All cells used for assays were between passages 4 and 7.

2.4. Western blot

Proteins from mouse abdominal aorta and mAECs were extracted with cold lysis buffer (50 mM Tris·Cl, pH 7.2, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 500 mM NaCl and 10 mM $MgCl_2$) supplemented with protease and phosphatase inhibitors (Roche). Polyacrylamide gel-electrophoresis and western blot were performed as described [22]. The following primary antibodies were used at the indicated dilutions: anti-β-actin 1/2000 (sc-47778), anti ERK-2 1/1000 (sc-1647), anti-iNOS 1/1000 (sc-650), and anti-eNOS 1/1500 (sc-654) from Santa Cruz Biotechnology; anti-COX-1 1/1000 (160109) and anti-COX-2 1/1000 (160112) from Cayman Chemical. Secondary HRP-conjugated antibodies were from Santa Cruz Biotechnologies: anti-IgG-rabbit 1/5000 (sc-2004) and anti-IgG-mouse 1/5000 (sc-2005). Immunocomplexes were detected by incubation with Luminata Forte Western HRP substrate (Millipore).

2.5. Gene expression studies

Total RNA from mAECs and mouse whole aortas was purified using TRIzol Reagent (Invitrogen). RNA was retrotranscribed with Superscript III First Strand Synthesis Supermix

(Invitrogen) and amplified with Power Syber Green PCR Master Mix (Applied Biosystems) using the following primers (Fw: Forward; RV: Reverse; 5´to 3´):

Gene (protein)	
Nos2 (iNOS)	Fw: GGCAGCCTGTGAGACCTTTG
	Rv: GCATTGGAAGTGAAGCGTTTC
Nos3 (eNOS)	Fw: TCAGCCATCACAGTGTTCCC
	Rv: ATAGCCCGCATAGCGTATCAG
COX1 (COX-1)	Fw: TGGCCAAGGTCTACCCCG
	Rv: CTCTGTACCCAAAGACTGCC
COX2 (COX-2)	Fw: AGTCTCTCAATGAGTACCGGAAA
	Rv: AAGTTCTTCAAATGATGTGTACGG
mPges1 (mPGES1)	Fw: AGCACACTGCTGGTCATCAA
	Rv: TCCACATCTGGGTCACTCCT
mPges2 (mPGES2)	Fw: ACTTCCACTCCCTGCCCTAT
	Rv: GTTGCAAGCTGTCTCCTTCC
cPges3 (cPGES)	Fw: GGCAAAGCTTAATTGGCTCA
	Rv: ATCCTCATCACCACCCATGT
Ptgis (PTGIS)	Fw: TCCATCCCTATGCCATCTTC
	Rv: ACTGCCTGCTTCTGTGGAGT
Tbxas1 (TBXAS1)	Fw: GAGGTGCTGGGACAACGTAT
	Rv: GCCTCTGCTGTGAACCTTTC
Gapdh (GAPDH)	Fw: TGTGTCCGTCGTGGATCTGA
	Rv: CCTGCTTCACCACCTTCTTGAT
Hprt1 (HGPRT)	Fw: CCTAAGATGAGCGCAAGTTGAA
	Rv: CCACAGGACTAGAACACCTGCTAA

36b4 (RPLP0)	Fw: ACTGGTCTAGGACCCGAGAAG
	Rv: TCCCACCTTGTCTCCAGTCT

Reactions were conducted in an ABI Prism 7500 Fast System and were analyzed with SDS 2.3 software (Applied Biosystems). Gene expression was normalized to the housekeeping genes *36b4*, *Gapdh*, and *Hprt1*.

2.6. COX activity in mAECs

mAECs (10^6 cells) were homogenized in cold buffer (0.1 M Tris-HCl, pH 7.8 containing 1mM EDTA). COX peroxidase activity was determined using the COX Activity Assay Kit (760151, Cayman Chemical). When indicated, COX-1 and COX-2 activities were distinguished by incubating the samples with the COX-2 inhibitor Dup-697 (provided in the kit).

2.7. Wire myography

Wire myography was performed as previously described [23]. Briefly, thoracic aortas were obtained from 11-13-week-old mice and cleaned of fat and connective tissue. Aortic tissue was cut into four ~2-mm long rings, which were mounted on two tungsten wires in a wire myograph system (620M, DMT) and immersed in 37ºC Krebs Henseleit Solution (KHS: 115 mM NaCl, 2.5 mM $CaCl_2$, 4.6 mM KCl, 1.2 mM KH_2PO4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose, and 0.01 mM EDTA) with constant gassing (95% O_2 and 5% CO_2). Vessel rings were mounted on the wire myograph, and diameter-tension relations were determined by stepwise stretching the tissue, increasing its passive diameter by augmenting the distance between the wires. Force and distance between the wires were recorded in each step. The diameter of vessel segments was calculated from these data

when the force is equivalent to 100 mmHg (L100) using the Laplace Equation (Tension=[pressure*radius]/thickness) [23]. Arterial segments were then set up at their optimal distension (0.9 of L100), which was maintained for the rest of the experiment. After stabilization for 30 minutes, arteries were exposed to 120 mM KCl to check their functional integrity. Optimal removal of the endothelium was assessed by the inability of acetylcholine (10 μM) to induce vasodilation in vessels precontracted with 1 μM phenylephrine. Endothelial function was examined by recording dose-dependent vasodilation induced by acetylcholine (from 0.1 nM to 10 μM) in aortic rings precontracted with 1 μM phenylephrine, and data are presented as a percentage of the initial precontraction. Vasoconstriction was studied by exposing the segments to increasing doses of phenylephrine (from 1nM to 10μM), and data are presented as a percentage of the contraction induced by 120 mM KCl. To determine the role of the endothelium in phenylephrine-induced vasoconstriction, experiments were performed with endothelium-denuded tissue, which was obtained by placing the aortic segment on absorbent laboratory countertop paper, letting the inner walls touch, and very carefully rolling the segment.

To evaluate the participation of COX-2 in responses to acetylcholine or phenylephrine, dose-response curves were also performed in the presence of 1 μM of the specific COX-2 inhibitor NS-398 (Abcam).

2.8. Intravital microscopy

Mouse cremaster muscle was dissected, exteriorized and visualized as described [24]. The muscle was cut longitudinally by thermocautery, and the extended muscle was positioned on a clear viewing pedestal. During the procedure, the muscle was perfused continuously with 37ºC Tyrode's buffer to maintain physiological conditions. Cremaster

arterioles were recorded using a Leica DM6000-FS intravital microscope equipped with an Apo 40x NA 1.0 water-immersion objective and a DFC350-FX camera. Vascular contraction was induced by intravenous administration of phenylephrine (100 µl, 1 mM). Arterioles were recorded from 10 seconds before bolus administration until they recovered the basal state. Arteriolar diameters were measured off-line from videotapes using SlideBook 5.5 digital microscopy imaging software. The mean diameter during the 10 seconds before stimulation was considered the baseline state. To inhibit COX-2 activity, animals were treated orally with 0.12 mg/day of NS-398 (Abcam) for 3 days prior to surgery.

2.9. Prostanoid release

Thoracic aortas were cut into four ~2-mm long segments, which were immersed in 37ºC HEPES buffer (119 mM NaCl, 20 mM HEPES, 4.6 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 0.15 mM $Na_2HPO_4 \cdot 12\ H_2O$, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $CaCl_2 \cdot 2H_2O$, 5.5 mM glucose). After a 30 min stabilization period, segments were washed twice with 0.2 ml HEPES (10 min each). The baseline levels of the prostanoids PGI_2, PGE_2, and TXA_2 were quantified in the medium using ELISA kits (Caiman Chemical) to measure their stable metabolites 6-keto-PGF1α, 13,14-dihydro-15-keto PGE_2, and TXB_2, respectively. Fresh medium was then added and the arteries were incubated for 2 min with phenylephrine (1 µM), followed by addition of acetylcholine (10 µM) and incubation for another 8 min. The medium was then collected for quantification of stimulated prostanoid release. Collected media were stored at -80ºC until ELISA.

2.10. Blood pressure analysis

Blood pressure was determined in conscious mice by the tail-cuff method using the BP-2000 Blood Pressure Analysis System (Visitech). Animals were trained for one week before blood pressure measurements. Values were calculated as the mean of 10 single measurements per animal at each time point. Mice treated with 50 μl of 1 mM phenylephrine were previously anesthetized with a mixture of medetomidine and ketamine (0.5 mg/kg and 50 mg/kg, respectively).

2.11. Histological and immunofluorescence analysis of thoracic aorta

Thoracic aortas from male wild-type and p27S10A mice were divided into four ~2-mm-long segments (numbered 1-4, #1 being the segment more proximal to the heart). For histological studies and elastin visualization, the second segment was fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 4-μm thick cross-sections. Sections were either stained with hematoxylin/eosin for structural studies or analyzed with a Leica TCS/SP5 confocal microscope equipped with a 40X objective to visualize the autofluorescence emitted by elastin layers upon excitation with an Argon 488 laser.

To quantify VSMC content, the third thoracic aorta segment was cryoprotected with 15% and 30% sucrose (in PBS), embedded in OCT (Tissue-Tek) and stored at -80°C until sectioning with a cryostat. Six-μm thick sections were defrosted, fixed with 4% paraformaldehyde in PBS, and permeabilized during 1h at room temperature with PBS containing 0.3% Triton X100, 5% BSA and 5% normal goat serum. Sections were then incubated with Cy3-conjugated anti-α-SMC actin (SMA-Cy3 antibody, 1/200, SIGMA) and DAPI (1/500, Invitrogen) during 2h at room temperature. Sections were washed, mounted with Fluoromount (SIGMA), and stored at 4ºC until image acquisition with a Leica TCS/SP5 confocal microscope. All images were acquired using the same laser and

photomultiplier settings to obtain maximal projection images of z-stacks covering the whole section thickness. Images were analyzed with ImageJ/Fiji software (National Institutes of Health) to automatically adjust the threshold for SMA-Cy3 fluorescence signal by using the IsoData algorithm. The mean intensity of the SMA-Cy3 signal above threshold was used to quantify the relative VSMC content for each genotype.

2.12. Abdominal aortic aneurysm model

AAA was induced in 8-week-old male apoE-/- and apoE-/-p27S10A mice fed standard diet according to the method developed by Daugherty et al [25]. Briefly, osmotic minipumps releasing angiotensin II (1 μg/kg/min, Alzet Corp) were implanted subcutaneously and maintained for 28 days. Aortic diameter was quantified in live mice at scheduled times after minipump implantation with a high-frequency ultrasound VEVO 770 system [26]. Aortic dilations were considered AAA when vessel diameter exceeded the baseline diameter by more than 50%. For COX-2 inhibition, mice were treated orally with 0.12 mg/day of NS-398 (Abcam) throughout angiotensin II infusion.

To characterize aneurysms, abdominal aorta was fixed with 4% PFA/PBS, embedded in paraffin, and sectioned for histological and immunofluorescence analysis. Collagen content was determined by Masson trichrome staining. Macrophage content was determined by immunochemistry with a rat anti-Mac3 monoclonal antibody (clone M3/84, sc-19991, Santa Cruz Biotechnology) followed by incubation with biotin-conjugated goat anti-rat secondary antibody (1/300), sc-2041, Santa Cruz Biotechnology). Mac3 immunocomplexes were visualized by incubating with streptavidin-HRP (Ref. TS-060-HR, LabVision Corporation) and DAB substrate (BUF 021A, AbD SEROTEC). VSMC content was determined by immunochemistry with a mouse anti-smooth muscle α-actin (SMA,

1/50) monoclonal antibody conjugated to alkaline phosphatase (clone 1A4) followed by reaction with Fast Red substrate (both from Sigma). Elastin was visualized using van Gieson staining, and elastin breakage was quantified by averaging the number of elastin breaks in 3 different sections of each AAA. The extent of AAA lesions and areas positive for all components was quantified in at least two sections per aorta by an operator blinded to genotype using ImageJ software. The mean value of all sections analyzed were represented for each mouse (n=5 mice of each genotype).

2.13. Statistical analysis

Results are shown as mean ± SEM. Student's t tests were used to compare one variable data, followed by Bonferroni corrections as appropriate. Dose-response curves were compared using repeated-measures two-way ANOVA with Bonferroni´s multiple comparison test. Diameter-tension relations were analyzed by comparing exponential growth equation fitting curves with the extra sum-of-squares F test. Differences were considered statistically significant at $P<0.05$. Statistical analysis was performed with GraphPad Prism 5.

3. RESULTS

3.1. Lack of p27-phospho-S10 activates COX-2 in mAECs. We first investigated a possible role of p27-phospho-S10 in regulating vasoreactivity by analyzing the expression of the synthases of key endothelium-derived vasomodulators. Specifically, we compared the expression of eNOS, iNOS, COX-1, and COX-2 in mAECs cultured from wild-type mice and p27S10A mice, which are unable to phosphorylate p27 at S10 [19]. Analysis by qPCR and western blot revealed no between-genotype differences in mRNA or protein expression of eNOS, iNOS, and COX-1; however, p27S10A mAECs showed higher COX-2 mRNA and protein levels (Fig. 1A,B). Accordingly, p27S10A mAECs showed a 4-fold higher COX enzyme activity, which was blunted by treatment with the COX-2-specific inhibitor DUP-697 (Fig. 1C). These results demonstrate that the absence of p27-phospho-S10 promotes COX-2 expression and activity in mAECs.

3.2. Lack of p27-phospho-S10 impairs vasodilation in an endothelial- and COX-2-dependent manner. We next performed ex vivo wire myography experiments using thoracic aorta rings from wild-type and p27S10A mice to assess endothelial-dependent vasodilation measured by the ability of the vessel to dilate upon stimulation with acetylcholine. This factor activates specific nicotinic receptors on ECs that further induce the release of different vasomodulators, such as nitric oxide or COX-2. Acetylcholine-induced dilation was impaired in aortic segments of p27S10A mice compared with wild-type controls (Fig. 2A, *left*). This differential response was endothelium-dependent, since endothelial denudation completely abolished acetylcholine-induced dilation of aortic segments of both genotypes (Fig. 2A, *right*). We also found improved vasodilator response in aortic segments from p27S10A mice treated with the COX-2-specific inhibitor NS-398,

which did not affect the response in wild-type aorta (Fig. 2B). These results indicate that defective p27-phospho-S10 impairs endothelium-dependent vasodilation in thoracic aorta through COX-2 activation.

3.3. Lack of p27-phospho-S10 increases phenylephrine-induced vascular contraction in an endothelial- and COX-2-dependent manner. A major function of COX-2 is the modulation of vascular contractility through the production of prostanoids [27], and we therefore next assessed the effect of defective p27-phospho-S10 on vascular contractility in *ex vivo* wire myography assays with thoracic aorta rings. Phenylephrine-induced contractions were stronger in thoracic aortas from p27S10A mice than in those from wild-type controls (Fig. 3A, *left*); however, no between-genotype differences in vessel contraction were observed when the experiments were performed with de-endothelialized aortic segments (Fig. 3A, *right*) or in the presence of the COX-2 inhibitor NS-398 (Fig. 3B). Higher phenylephrine-induced contractility in arterioles of p27S10A mice was also evident from *in vivo* intravital microscopy of the cremaster muscle (Fig. 3C), and this difference was also abolished by NS-398 (Fig. 3D). These results indicate that lack of p27-phospho-S10 increases arterial contractility due to COX-2-induced endothelial dysfunction.

3.4. Lack of p27-phospho-S10 increases the release of arterial thromboxane. To uncover potential mechanisms underlying the contractile hypersensitivity caused by the absence of p27-phospho-S10, we carried out ELISAs to determine in thoracic aorta the release of PGE_2, PGI_2, and TXA_2, the main vasomodulators produced by vascular COX-2 activity. We found no between-genotype significant differences in PGE_2 and PGI_2 release (Fig. 3E). Moreover, defective p27-phospho-S10 had no effect on mRNA expression of prostaglandin E synthase (PGES) and prostaglandin I_2 synthase (PGIS) in aorta (Fig. 3F).

In contrast, aortic release of TXA_2 was significantly increased in p27S10A mice, this effect was blunted by NS-398, and thromboxane A2 synthase 1 (TXAS1) mRNA expression was higher in p27S10A aorta (Fig. 3E,F).

3.5. Lack of p27-phospho-S10 does not modify blood pressure but promotes outward remodeling in mouse thoracic aorta. We next investigated possible pathological consequences of the observed vascular abnormalities in p27S10A mice. We first measured blood pressure in wild-type and p27S10A animals, both at baseline and upon treatment with increasing doses of the vasoconstrictor phenylephrine. As expected, phenylephrine strongly increased systolic and diastolic blood pressure (Fig. 4A). Surprisingly, however, lack of p27-phospho-S10 did not significantly affect blood pressure in any experimental condition (Fig. 4A). These results suggested that compensatory mechanisms prevent the predicted increase in blood pressure in p27S10 mice. To assess this possibility, we analyzed the thoracic aorta by microscopy and wire myography. Comparison of specimens from wild-type and p27S10A mice revealed no differences in the number of elastin layers in the media (an average of 5.1 layers in both genotypes) (Fig. 4B), media-to-lumen ratio (Fig. 4C), or in the relative amount of VSMCs (Fig. 4B,D). However, wire myography studies revealed a right-shift in the diameter-tension relations and significantly larger diameter under physiological conditions (100 mm Hg) in p27S10A mice (Fig. 4E). These results indicate that lack of p27-phospho-S10 induces outward remodeling in the thoracic aorta.

3.6. Lack of p27-phospho-S10 promotes abdominal aortic aneurysm formation via COX-2 activation. AAA is characterized by excessive aortic dilation caused by structural defects in the arterial wall [28]. In light of our findings and previous studies revealing

protective effects of p27-phospho-S10 against pathological vascular remodeling [18, 20], we investigated the potential involvement of p27-phospho-S10 in AAA initiation and development, using the angiotensin II infusion model for AAA generation in 8-week-old apoE-/- and apoE-/-p27S10A mice fed normal diet. Ultrasound visualization of abdominal aortas revealed more pronounced aortic dilation over time in apoE-/-p27S10A mice than in apoE-/- controls (Fig. 5A). In addition, the incidence of AAA and associated mortality in the first days were higher in apoE-/-p27S10A mice (Fig. 5B). These results demonstrate accelerated AAA development and worse disease prognosis in apoE-/- mice unable to phosphorylate p27 at S10A.

Histological analysis of AAA lesions in apoE-/- and apoE-/-p27S10A mice showed that lack of p27-phospho-S10 does not significantly alter key parameters in aneurysmal lesions, including the percentage of total area occupied by collagen, macrophage, and VSMCs, as well as elastin breakage (Fig. 6A). Because hypertension is associated with AAA development [29] and p27-phospho-S10 modulates vascular reactivity (Fig. 2 and 3), we also evaluated blood pressure. As expected, angiotensin II administration increased blood pressure in both apoE-/- and apoE-/-p27S10A mice; however, the increase did not differ between the two groups, indicating that it was independent of p27 phosphorylation status (Fig. 6B).

Increased COX-2 activity, as observed in the absence of p27-phospho-S10 (Fig. 1), correlates with AAA formation in humans [30] and mouse models [31, 32]. We therefore analyzed COX-2 expression in the abdominal aorta of angiotensin II-treated apoE-/- and apoE-/-p27S10A mice. Western blot analysis demonstrated above-normal COX-2 expression in the abdominal aorta of both control and angiotensin II-treated apoE-/-

p27S10A mice (Fig. 7A), as well as in cultures of angiotensin II-treated mAECs (Fig. 7B). Accordingly, treatment of apoE-/-p27S10A mice with the COX-2 inhibitor NS-398 prevented the angiotensin II-dependent increase in aortic diameter, AAA incidence, and associated mortality (Fig. 7C).

4. DISCUSSION

Previous studies have demonstrated a protective role of p27 in the cardiovascular system. As a growth suppressor, p27 limits high-fat diet-induced atherosclerosis progression in apoE-/- mice by reducing hyperproliferation of both VSMCs and macrophages [11, 12]. p27 also inhibits atherosclerosis in fat-fed apoE-/- mice through cell cycle-independent mechanisms mediated by its phosphorylation at S10, which regulates macrophage foam cell formation, endothelial function, and arterial leukocyte infiltration in a RhoA/ROCK-dependent manner [18, 20]. To explore new functions of p27-phospho-S10 in the vascular system, we investigated here its possible role in the regulation of vascular tone, blood pressure, and AAA formation in mice maintained on a control low-fat diet to minimize the potential interference of atherosclerosis development. Our results establish a link between p27-phospho-S10 status and COX-2 expression and activity that has pathophysiological implications. In pilot experiments assessing the expression of the synthases of key endothelium-derived vasomodulators, we found COX-2 hyperactivation in mAECs from mice unable to phosphorylate p27 at S10, which exhibited impaired endothelial-dependent dilation and elevated contractility in thoracic aorta and arterioles of the cremaster muscle. Both reduced vasodilation and increased vasoconstriction in p27S10A mice were reversed upon treatment with the specific COX-2 inhibitor NS-398. A link between the endothelium and the elevated vasoconstriction in p27S10A mice was further supported by the restoration of normal contraction in de-endothelialized vessels. Mice lacking p27-phospho-S10 had normal blood pressure, but exhibited higher susceptibility to AAA formation via COX-2 hyperactivation.

In most tissues and cell types, COX-2 is usually expressed at very low levels, with expression increasing rapidly in response to inflammatory signals [33]. However, COX-2 is

constitutively expressed in the endothelium and regulates vascular tone as a major producer of the vasodilator PGI_2. In diseases such as diabetes and hypertension, COX-2 activity can compensate endothelial dysfunction by increasing PGI_2 production [34, 35]. COX-2 inhibition increases vasoconstriction by reducing PGI_2 and PGE_2 production [36]. Other studies reported that COX-2 induction in mouse ECs is linked to increased production of vasocontractile prostanoids [37-41]. Moreover, COX-2 increases the production of the vasoconstrictor TXA_2 in inflammatory conditions [42]. We therefore hypothesized that COX-2 overexpression caused by loss of p27-phospho-S10 would affect vasomotor responses. We found that COX-2 activity contributes to the below-normal endothelium-dependent vasodilation in thoracic aortas from p27S10A mice. We also observed an association between the elevated COX-2 activity in p27S10A mAECs and above-normal contractility in thoracic aorta and arterioles of the cremaster muscle, detected by *ex vivo* wire myography and *in vivo* intravital microscopy, respectively. The altered vasomotor responses in p27S10A mice were also associated with above-normal TXAS1 expression and TXA_2 release in aorta, without significant changes in other COX-2-derived prostanoids, including PGE_2 and PGI_2 and their respective synthases PGES and PGIS.

Our results show that vasomotor alterations in p27S10A mice are not associated to increased blood pressure, either without stimulation or after induction of hypertension with phenylephrine. Importantly, blood pressure is modulated by vascular tone, but also by vascular structure. Adaptive changes in the vessel wall can occur to maintain the vessel under optimal mechanical conditions, thus preventing increased blood pressure [43]. We hypothesize that the outward remodeling observed in p27S10A might be such a mechanism that prevents high blood pressure. Our wire myography studies revealed an

association between defective p27-phospho-S10 and increased passive thoracic aortic diameter, without changing the media-to-lumen ratio or relative VSMC content. This vessel remodeling could be an adaptive response to the dysfunctional endothelium, which could in part, prevent hypertension in p27S10A animals but might predispose to other vascular pathologies. Further studies are required to investigate in detail other possible vascular alterations induced by defective p27-phospho-S10.

The vascular alterations in p27S10A mice concomitant to COX-2 hyperactivation, together with the association of COX-2 with aneurysm formation [31, 44, 45], prompted us to investigate the involvement of defective p27-phospho-S10 in COX-2-mediated AAA development in apoE-/- mice fed a non-atherogenic control diet. We found higher aortic diameter, AAA incidence, and associated mortality in angiotensin II-treated apoE-/-p27S10A mice than in apoE-/- controls. This phenotype was not accompanied by elevated blood pressure or increased leukocyte infiltration of the aneurysms, but was associated with increased expression of COX-2, as revealed by western blot analysis of abdominal aorta. Remarkably, treatment with NS-398 inhibited AAA formation and blocked the associated increased mortality in angiotensin II-treated apoE-/-p27S10A mice. We therefore conclude that defective p27-phospho-S10 accelerates AAA development in apoE-/- mice via COX-2 hyperactivation. Of note, we observed a minor increase in mortality in apoE-/- mice treated with NS-398 compared with untreated controls (Fig. 5B versus Fig. 7C), consistent with previous studies suggesting side effects of long term treatment with COX inhibitors [46].

5. CONCLUSIONS

Our findings demonstrate new vascular implications of loss of p27-phospho-S10, which leads to impaired endothelial-dependent vasodilation, augmented vasoconstrictor responses, outward remodeling in thoracic aorta, and increased AAA susceptibility via COX-2 hyperactivation, thus identifying new functions of the multifaceted p27. Together with our earlier demonstration of increased atherosclerosis in fat-fed apoE-/-p27S10A mice [18, 20], these findings suggest that drugs developed to increase p27-phospho-S10 might provide a therapeutic benefit in the setting of endothelium-derived vasculopathies.

ACKNOWLEDGEMENTS

We thank Simon Bartlett for English editing, Fátima Sánchez-Cabo for help with statistical analysis, and the CNIC Animal Facility for animal care. This study was supported by the Spanish Ministerio de Economía, Industria y Competitividad (MEIC, grants SAF2013-46663-R and SAF2016-79490-R), and by the Instituto de Salud Carlos III (grants RD12/0042/0028, RD12/0042/22 and PI1100406), with co-funding from the Fondo Europeo de Desarrollo Regional (FEDER). P.M-S. was supported by a FPU predoctoral fellowship from the Spanish Ministerio de Educación (REF. AP2009-01833), C.R. by a MEIC postdoctoral contract (REF. FPDI-2013-17423), and L.d.C. by a Jordi Soler postdoctoral grant from the Red de Investigación Cardiovascular (RETIC Program, Instituto de Salud Carlos III). The CNIC is supported by the MEIC and the Pro-CNIC Foundation, and is a Severo Ochoa Center of Excellence (MEIC award SEV-2015-0505).

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

[1] Pober JS, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 2007; 7:803-15.

[2] Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circ Res 2007; 100:782-94.

[3] van Hinsbergh VW. Endothelium--role in regulation of coagulation and inflammation. Semin Immunopathol 2012; 34:93-106.

[4] Feletou M, The Endothelium: Part 1: Multiple functions of the endothelial cells-Focus on endothelium-derived vasoactive mediators, San Rafael (CA), 2011.

[5] Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. Acta Cardiol 2000; 55:221-32.

[6] Dharmashankar K, Widlansky ME. Vascular endothelial function and hypertension: insights and directions. Curr Hypertens Rep 2010; 12:448-55.

[7] Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473:317-25.

[8] O'Rourke M. Mechanical principles in arterial disease. Hypertension 1995; 26:2-9.

[9] Diez-Juan A, Andres V. Coordinate control of proliferation and migration by the p27Kip1/cyclin-dependent kinase/retinoblastoma pathway in vascular smooth muscle cells and fibroblasts. Circ Res 2003; 92:402-10.

[10] Castro C, Diez-Juan A, Cortes MJ, Andres V. Distinct regulation of mitogen-activated protein kinases and p27Kip1 in smooth muscle cells from different vascular beds. A potential role in establishing regional phenotypic variance. J Biol Chem 2003; 278:4482-90.

[11] Diez-Juan A, Andres V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. Faseb J 2001; 15:1989-95.

[12] Diez-Juan A, Perez P, Aracil M, Sancho D, Bernad A, Sanchez-Madrid F, et al. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. Blood 2004; 103:158-61.

[13] Kanda T, Hayashi K, Wakino S, Homma K, Yoshioka K, Hasegawa K, et al. Role of Rho-kinase and p27 in angiotensin II-induced vascular injury. Hypertension 2005; 45:724-9.

[14] Belletti B, Pellizzari I, Berton S, Fabris L, Wolf K, Lovat F, et al. p27kip1 controls cell morphology and motility by regulating microtubule-dependent lipid raft recycling. Mol Cell Biol 2010; 30:2229-40.

[15] Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. p27Kip1 modulates cell migration through the regulation of RhoA activation. Genes Dev 2004; 18:862-76.

[16] Pippa R, Espinosa L, Gundem G, Garcia-Escudero R, Dominguez A, Orlando S, et al. p27Kip1 represses transcription by direct interaction with p130/E2F4 at the promoters of target genes. Oncogene 2012; 31:4207-20.

[17] Ishida N, Kitagawa M, Hatakeyama S, Nakayama K. Phosphorylation at serine 10, a major phosphorylation site of p27(Kip1), increases its protein stability. J Biol Chem 2000; 275:25146-54.

[18] Fuster JJ, Gonzalez-Navarro H, Vinue A, Molina-Sanchez P, Andres-Manzano MJ, Nakayama KI, et al. Deficient p27 phosphorylation at serine 10 increases macrophage foam cell formation and aggravates atherosclerosis through a proliferation-independent mechanism. Arterioscler Thromb Vasc Biol 2011; 31:2455-63.

[19] Kotake Y, Nakayama K, Ishida N, Nakayama KI. Role of serine 10 phosphorylation in p27 stabilization revealed by analysis of p27 knock-in mice harboring a serine 10 mutation. J Biol Chem 2005; 280:1095-102.

[20] Molina-Sanchez P, Chevre R, Rius C, Fuster JJ, Andres V. Loss of p27 phosphorylation at Ser10 accelerates early atherogenesis by promoting leukocyte recruitment via RhoA/ROCK. J Mol Cell Cardiol 2015; 84:84-94.

[21] Molina-Sanchez P, Andres V. Isolation of mouse primary aortic endothelial cells by selection with specific antibodies. Methods Mol Biol 2015; 1339:111-7.

[22] Gonzalez-Navarro H, Vinue A, Vila-Caballer M, Fortuno A, Beloqui O, Zalba G, et al. Molecular mechanisms of atherosclerosis in metabolic syndrome: role of reduced IRS2-dependent signaling. Arterioscler Thromb Vasc Biol 2008; 28:2187-94.

[23] del Campo L, Ferrer M. Wire myography to study vascular tone and vascular structure of isolated mouse arteries. Methods Mol Biol 2015; 1339:255-76.

[24] Rius C, Sanz MJ. Intravital microscopy in the cremaster muscle microcirculation for endothelial dysfunction studies. Methods Mol Biol 2015; 1339:357-66.

[25] Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. J Clin Invest 2000; 105:1605-12.

[26] Martin-McNulty B, Vincelette J, Vergona R, Sullivan ME, Wang YX. Noninvasive measurement of abdominal aortic aneurysms in intact mice by a high-frequency ultrasound imaging system. Ultrasound Med Biol 2005; 31:745-9.

[27] Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. J Immunol 2001; 167:2831-8.

[28] Curci JA, Thompson RW. Adaptive cellular immunity in aortic aneurysms: cause, consequence, or context? J Clin Invest 2004; 114:168-71.

[29] Forsdahl SH, Singh K, Solberg S, Jacobsen BK. Risk factors for abdominal aortic aneurysms: a 7-year prospective study: the Tromso Study, 1994-2001. Circulation 2009; 119:2202-8.

[30] Holmes DR, Wester W, Thompson RW, Reilly JM. Prostaglandin E2 synthesis and cyclooxygenase expression in abdominal aortic aneurysms. J Vasc Surg 1997; 25:810-5.

[31] Ghoshal S, Loftin CD. Cyclooxygenase-2 inhibition attenuates abdominal aortic aneurysm progression in hyperlipidemic mice. PLoS One 2012; 7:e44369.

[32] King VL, Trivedi DB, Gitlin JM, Loftin CD. Selective cyclooxygenase-2 inhibition with celecoxib decreases angiotensin II-induced abdominal aortic aneurysm formation in mice. Arterioscler Thromb Vasc Biol 2006; 26:1137-43.

[33] Seibert K, Masferrer JL. Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor 1994; 4:17-23.

[34] Nacci C, Tarquinio M, De Benedictis L, Mauro A, Zigrino A, Carratu MR, et al. Endothelial dysfunction in mice with streptozotocin-induced type 1 diabetes is opposed by compensatory overexpression of cyclooxygenase-2 in the vasculature. Endocrinology 2009; 150:849-61.

[35] Meeking DR, Browne DL, Allard S, Munday J, Chowienczyck PJ, Shaw KM, et al. Effects of cyclo-oxygenase inhibition on vasodilatory response to acetylcholine in patients with type 1 diabetes and nondiabetic subjects. Diabetes care 2000; 23:1840-3.

[36] Foudi N, Louedec L, Cachina T, Brink C, Norel X. Selective cyclooxygenase-2 inhibition directly increases human vascular reactivity to norepinephrine during acute inflammation. Cardiovasc Res 2009; 81:269-77.

[37] Alvarez Y, Briones AM, Balfagon G, Alonso MJ, Salaices M. Hypertension increases the participation of vasoconstrictor prostanoids from cyclooxygenase-2 in phenylephrine responses. J Hypert 2005; 23:767-77.

[38] Blanco-Rivero J, Cachofeiro V, Lahera V, Aras-Lopez R, Marquez-Rodas I, Salaices M, et al. Participation of prostacyclin in endothelial dysfunction induced by aldosterone in normotensive and hypertensive rats. Hypertension 2005; 46:107-12.

[39] Heymes C, Habib A, Yang D, Mathieu E, Marotte F, Samuel J, et al. Cyclo-oxygenase-1 and -2 contribution to endothelial dysfunction in ageing. Br J Pharmacol 2000; 131:804-10.

[40] Shi Y, Vanhoutte PM. Oxidative stress and COX cause hyper-responsiveness in vascular smooth muscle of the femoral artery from diabetic rats. Br J Pharmacol 2008; 154:639-51.

[41] Virdis A, Colucci R, Versari D, Ghisu N, Fornai M, Antonioli L, et al. Atorvastatin prevents endothelial dysfunction in mesenteric arteries from spontaneously hypertensive rats: role of cyclooxygenase 2-derived contracting prostanoids. Hypertension 2009; 53:1008-16.

[42] Grandel U, Fink L, Blum A, Heep M, Buerke M, Kraemer HJ, et al. Endotoxin-induced myocardial tumor necrosis factor-alpha synthesis depresses contractility of isolated rat hearts: evidence for a role of sphingosine and cyclooxygenase-2-derived thromboxane production. Circulation 2000; 102:2758-64.

[43] Martinez-Lemus LA, Hill MA, Meininger GA. The plastic nature of the vascular wall: a continuum of remodeling events contributing to control of arteriolar diameter and structure. Physiology 2009; 24:45-57.

[44] Hasan D, Hashimoto T, Kung D, Macdonald RL, Winn HR, Heistad D. Upregulation of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E2 synthase-1 (mPGES-1) in wall of ruptured human cerebral aneurysms: preliminary results. Stroke 2012; 43:1964-7.

[45] Miralles M, Wester W, Sicard GA, Thompson R, Reilly JM. Indomethacin inhibits expansion of experimental aortic aneurysms via inhibition of the cox2 isoform of cyclooxygenase. J Vasc Surg 1999; 29(5):884-92; discussion 892-3.

[46] Cannon CP, Cannon PJ. Physiology. COX-2 inhibitors and cardiovascular risk. Science 2012; 336:1386-7.

Figure 1: **Lack of p27-phospho-S10 causes endothelial COX-2 hyperactivation. (A)** qPCR analysis of iNOS (Nos2), eNOS (Nos3), COX-1, and COX-2 in mAECs from wild-type and p27S10A mice (n=3 per genotype). **(B)** Representative western blot and quantification of vasomodulator synthases in mAECs from wild-type and p27S10A mice (n=2 per genotype, 2 duplicates). **(C)** COX activity in wild-type and p27S10A mAECs without treatment and upon treatment with the COX-2 inhibitor DUP 697 (n=2 per genotype, 2 technical replicates performed). Results are expressed relative to untreated wild-type cells.

Figure 2: **Lack of p27-phospho-S10 impairs endothelium-dependent acetylcholine-induced vasodilation in a COX-2-dependent manner.** Thoracic aorta segments from wild-type and p27S10A mice were mounted on a wire myograph to examine acetylcholine-induced dilation. Data are presented as a percentage of the contraction induced by 1μM phenylephrine. **(A)** Vessel segments were intact (n=7 per genotype) or denuded (n=7 wild-type; n=8 p27S10A). **(B)** Effect of COX-2 inhibition with 1 μM NS-398 on acetylcholine-induced vasodilator response (n=6 wild-type; n=7 p27S10A).

Figure 3: **Lack of p27-phospho-S10 increases arterial contractility and TXA_2 release. (A, B)** Wire myography analysis of phenylephrine-induced contraction of thoracic aorta segments from wild-type and p27S10A mice. Vessels examined in A had an intact endothelium (left: n=5 wild-type; n=7 p27S10A) or had the endothelium removed (right: n=7 wild-type; n=6 p27S10A). Experiments in B were performed with vessels with intact endothelium that were incubated with 1 μM NS-398 to inhibit COX-2 (n=6 wild-type; n=6 p27S10A). Data are presented as a percentage of the contraction induced by 120 mM KCl.

(C, D) Intravital microscopy of cremaster arteriole contraction in wild-type and p27S10A mice in response to 100 μM phenylephrine (C: n=7 per genotype) and after treatment with NS-398 (D: n=8 wild-type; n=6 p27S10A). Results in A-D are presented as the percentage of the arterial diameter without treatment. **(E)** Relative release of PGE_2, PGI_2, and TXA_2 induced by 1μM phenylephrine/10μM acetylcholine in thoracic aorta from wild-type and p27S10A mice. Results are expressed as the ratio of induced to baseline release (=1). PGE_2: n=5 wild-type, n=6, p27S10A; PGI_2: n=17 wild-type, n=17 p27S10A; TXA_2: n=8 wild-type, n=9 p27S10A; TXA_2 + NS-398: n=10 wild-type, n=10 p27S10A. **(F)** qPCR analysis of major prostanoid production enzymes in aortas from wild-type (n=5) and p27S10A (n=4) mice. Results are expressed relative to wild-type mice.

Figure 4: **Lack of p27-phospho-S10 does not alter blood pressure but induces outward remodeling in thoracic aorta. (A)** Systolic, diastolic, and mean blood pressure (BP) in wild-type mice (n=3) and p27S10A mice (n=3) without treatment and after phenylephrine administration (50 μl of 0.1 mM and 1 mM stock). **(B)** Representative images of thoracic aorta sections from wild-type and p27S10A mice showing hematoxylin/eosin staining, and confocal microscopy for autofluorescence of elastin fibers and SMA+DAPI staining. **(C)** Media-to-lumen ratio in thoracic aorta expressed as medial area/luminal perimeter (n=5 wild-type, n=4 p27S10A). **(D)** Relative VSMC content in SMA-Cy3-immunostained sections of thoracic aorta from wild-type and p27S10A mice (n=5 per genotype), calculated as mean intensity of the fluorescent signal (A.U.: arbitrary units). **(E)** Diameter–force curves and aortic diameter determined by wire myography at 100 mmHg in thoracic aorta segments from wild-type and p27S10A mice (n=7 per genotype).

Figure 5: **Lack of p27-phospho-S10 aggravates angiotensin II-induced AAA in mice.** apoE-/- mice and apoE-/-p27S10A mice fed control diet. **(A)** ***Left,*** Ultrasound-determined abdominal aorta diameter at baseline, and after implantation of angiotensin II (AngII) minipumps for the indicated time. Results are shown for the 9 mice of each genotype that survived throughout the 28 days of AngII infusion. ***Right,*** Representative ultrasound images of abdominal aorta at baseline and after 24 days of AngII treatment. **(B)** AAA incidence and survival curve (n=12 per genotype).

Figure 6: **Lack of p27-phospho-S10 does not modify AAA composition or blood pressure in angiotensin II-treated apoE-/- mice.** apoE-/- and apoE-/-p27S10A mice fed control diet. **(A)** Relative content of collagen, macrophages, and VSMCs, and number of elastin breaks in AAA lesions in apoE-/- and apoE-/-p27S10A mice 28 days after angiotensin II infusion (n=5 per genotype). Representative section stainings are shown in the lower images. Asterisks mark luminal areas. **(B)** Systolic, diastolic, and mean blood pressure in apoE-/- and apoE-/-p27S10A mice at baseline and during angiotensin II treatment (n=6 per genotype).

Figure 7: **Lack of p27-phospho-S10 promotes AAA formation through COX-2 overexpression. (A)** Western blot analysis of COX-2 protein content in abdominal aortas of control diet-fed apoE-/- and apoE-/-p27S10A mice at baseline and after 28 days of angiotensin II (AngII) treatment. Two samples were analyzed for each experimental condition and two western blot were performed. A representative blot is shown and the chart shows relative quantification. **(B)** Western blot analysis of COX-2 in wild-type mAECs without treatment (control) or treated with 1 mM AngII for 2 h. Two different preparations of mAECs were analyzed and a representative western blot is shown. **(C)** ***Left,*** Maximal

aortic diameter in apoE-/- and apoE-/-p27S10A mice treated with NS-398 at baseline and after AngII infusion for the indicated time. Results are shown for the mice of each genotype that survived throughout the 28 days of AngII infusion (n=8 apoE-/-, n=7 apoE-/-p27S10A). ***Middle,*** AAA incidence in NS-398-treated apoE-/- and apoE-/- mice infused with AngII for the indicated times. ***Right,*** Survival curves for NS-398-treated apoE-/- and apoE-/-p27S10A mice infused with AngII for 28 days (n=15 apoE-/-, n=11 apoE-/-p27S10A).