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Original article

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

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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.

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

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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, 0.4 mM 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-47,778), 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*.