Deficient p27 Phosphorylation at Serine 10 Increases Macrophage Foam Cell Formation and Aggravates Atherosclerosis Through a Proliferation-Independent Mechanism

José J. Fuster, Herminia González-Navarro, Angela Vinué, Pedro Molina, Maria J. Andrés-Manzano, Keichi I. Nakayama, Keiko Nakayama, Antonio Díez-Juan, Antonio Bernad, Cristina Rodríguez, José Martínez-González, Vicente Andrés

Objective—Genetic ablation of the growth suppressor p27Kip1 (p27) in the mouse aggravates atherosclerosis coinciding with enhanced arterial cell proliferation. However, it is unknown whether molecular mechanisms that limit p27’s protective function contribute to atherosclerosis development and whether p27 exerts proliferation-independent activities in the arterial wall. This study aims to provide insight into both questions by investigating the role in atherosclerosis of p27 phosphorylation at serine 10 (p27-phospho-Ser10), a major posttranslational modification of this protein.

Methods and Results—Immunoblotting studies revealed a marked reduction in p27-phospho-Ser10 in atherosclerotic arteries from apolipoprotein E–null mice, and expression of the nonphosphorylatable mutant p27Ser10Ala, either global or restricted to bone marrow, accelerated atherosclerosis. p27Ser10Ala expression did not affect cell proliferation in early and advanced atheroma but activated RhoA/ROCK signaling and promotes macrophage foam cell formation in a ROCK-dependent manner. Supporting the clinical relevance of these findings, human atherosclerotic coronary arteries exhibited a prominent reduction in p27-phospho-Ser10 and increased ezrin/radixin/moesin protein phosphorylation, a marker of RhoA/ROCK activation.

Conclusion—Scarce phosphorylation of p27 at Ser10 is a hallmark of human and mouse atherosclerosis and promotes disease progression in mice. This proatherogenic effect is mediated by a proliferation-independent mechanism that involves augmented foam cell formation owing to increased RhoA/ROCK activity. These findings unveil a new atheroprotective action of p27 and identify p27-phospho-Ser10 as an attractive target for the treatment of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ genetically altered mice ■ molecular biology ■ Rho ■ p27

Atherosclerosis and associated ischemic events are leading causes of morbidity and mortality in industrialized countries. Atherosclerotic plaque formation is a multifactorial chronic inflammatory process characterized by the interaction of oxidized low-density lipoproteins (oxLDLs), endothelial cells, macrophages, T cells, and vascular smooth muscle cells (VSMCs). Atherosclerosis is initially driven by deposition in the arterial wall of oxLDLs, which provoke a chronic inflammatory response. Macrophages in the atherosclerotic plaque avidly internalize oxLDLs and become foam cells that critically contribute to plaque development by secreting a plethora of inflammatory mediators.1–3 Human and animal studies have demonstrated that excessive cell proliferation in the arterial wall is a hallmark of atherosclerosis.1–5 Mammalian cell proliferation is controlled by a large number of proteins that regulate the cell cycle. Major positive regulators of cell proliferation are holoenzymes composed of a catalytic cyclin-dependent protein kinase (CDK) and a regulatory cyclin. Cell cycle progression is negatively regulated by CDK inhibitory proteins of the CDK interacting protein/kinase inhibitory protein (Cip/Kip; p21Cip1, p27Kip1, p57Kip2) and inhibitor of CDK4 (Ink4; p16Ink4a, p15Ink4b, p18Ink4c, p19Ink4d) families.5 Mitogen-induced downregulation of p27Kip1 (p27) is critical for the activation of specific CDK-cyclin complexes, the...
subsequent transcriptional activation of genes required for the G_{1}/S-phase transition, and the initiation of DNA replication.\textsuperscript{7} In most cell types, the activity of p27 is regulated by posttranslational modifications that affect its stability and subcellular localization.\textsuperscript{2,3,8,9} Phosphorylation of p27 at serine 10 (p27-phospho-Ser10) is a major mechanism of p27 regulation in different scenarios,\textsuperscript{9,10-15} and it has been estimated to be the most abundant posttranslational modification of p27 in cultured cells, accounting for 70 to 75% of its phosphate incorporation.\textsuperscript{9} This phosphorylation event contributes importantly to p27 stabilization in the G\textsubscript{0} phase of the cell cycle\textsuperscript{9,11,13-15} and promotes p27 exit from the nucleus in certain settings.\textsuperscript{10,12,15,16}

p27 is a major negative regulator of cell proliferation in various pathophysiological settings, including cancer\textsuperscript{17,18} and vasculoproliferative disease (eg, atherosclerosis and restenosis).\textsuperscript{4,18,19} We have previously demonstrated that both global and hematopoietic cell–restricted inactivation of p27 accelerates diet-induced atherosclerosis in apolipoprotein E–deficient (apoE\textsuperscript{−/−}) mice, coinciding with increased cell proliferation within the atheroma.\textsuperscript{20,21} The atheroprotective action of p27 in this animal model has been corroborated by others.\textsuperscript{2,22} However, it remains unknown whether molecular mechanisms that limit p27's protective function indeed occur during human or experimental atherosclerosis. Moreover, although compelling in vitro evidence has emerged suggesting that p27 has cell cycle–independent activities,\textsuperscript{23} the pathophysiological relevance of these activities, and in particular their possible atheroprotective role, remains unexplored. In the present study, we provide insight into these questions by demonstrating that p27-phospho-Ser10 is markedly reduced in human and mouse atherosclerosis and that expression of the nonphosphorylatable p27Ser10Ala mutant aggravates disease progression through a cell cycle–independent mechanism that involves increased RhoA/ROCK signaling and augmented foam cell formation.

**Methods**

See additional Methods in the supplemental material, available online at http://atvb.ahajournals.org.

**Mice**

Care of animals was in accordance with institutional guidelines and regulations. p27Ser10Ala knock-in mice\textsuperscript{14} were backcrossed for more than 8 generations in a C57BL/6J background and then interbred with apoE\textsuperscript{−/−} mice (C57BL/6J, Charles River) to generate apoE\textsuperscript{−/−} p27Ser10Ala mice and apoE\textsuperscript{−/−} littermates. After weaning, mice were maintained on a low-fat standard diet (2.8% fat, Panlab, Barcelona, Spain). For diet-induced atherosclerotic studies, 2-month-old mice were placed on atherogenic diet (10.8% total fat, 0.75% cholesterol, S4892-E010, Sniff, Germany) for the indicated periods of time.

**Quantification of Atherosclerosis Burden**

Mice were euthanized and aortas were removed after in situ perfusion with PBS followed by 4% paraformaldehyde/PBS. Fixation was continued overnight at 4°C. An operator who was blinded to genotype quantified the extent of atherosclerosis by computer-assisted morphometric analysis (SigmaScan Pro 5) of both whole-mounted aorta stained with Oil Red O (O0625, Sigma, 0.2% Oil Red O in 80% MeOH) and hematoxylin/eosin-stained cross-sections of aortic tissue as previously described.\textsuperscript{24,25}

**RhoA Activity Assay**

RhoA activity in peritoneal macrophages obtained from 5-month-old mice fed standard chow (pool of 3 mice) was measured as the amount of RhoA-GTP using the absorbance-based RhoA G-LISA activation assay according to the manufacturer’s instructions (BK-124, Cytoskeleton).

**Analysis of Modified Low-Density Lipoprotein Uptake and Macrophage Foam Cell Formation**

For modified acetylated low-density lipoprotein (acLDL) uptake studies, peritoneal macrophages from female fat-fed apoE\textsuperscript{−/−} and apoE\textsuperscript{−/−} p27Ser10Ala mice were incubated for 3 hours with Alexa Fluor 488–labeled acLDL (1 \mu g/mL, Invitrogen) in serum-free media. Cells were then collected and acLDL uptake was quantified by flow cytometry as the relative median fluorescence intensity. In vivo oxLDL uptake by arterial macrophages, fat-fed mice received an intravenous injection of 20 \mu g of dil-labeled oxLDLs (Biomedical Technologies). One day postadministration, mice were euthanized, and the aortas were digested as described.\textsuperscript{26} Cells were then collected and stained with an Alexa 647–conjugated antibody against the macrophage-specific antigen F4/80 (Serotec). oxLDL uptake was quantified by flow cytometry as the relative median fluorescence intensity in F4/80-positive cells from aortic cell suspensions. For in vivo foam cell counting, freshly isolated resident peritoneal macrophages were plated on coverslips for 60 minutes and extensively washed to remove nonadherent cells. After fixation with 4% paraformaldehyde, cells were stained with Oil Red O and counterstained with hematoxylin.

**Statistical Analysis**

Data are presented as mean±SEM. In experiments with 2 groups, statistical significance was evaluated using a 2-tailed, unpaired Student t test. Otherwise, a 2-way ANOVA with the Bonferroni post hoc test was performed (GraphPad Prism software). Differences were considered statistically significant at \( P<0.05 \).

**Results**

**Reduced Level of p27-Phospho-Ser10 Is Associated With Atherosclerosis and Aggravates Disease Progression in apoE\textsuperscript{−/−} Mice**

To gain insight into the potential role in atherosclerosis of p27 phosphorylation at Ser10, we first performed Western blot analysis in murine atherosclerotic arteries using a phosphospecific antibody. We found that p27-phospho-Ser10 is markedly downregulated in the atherosclerotic aortic arch of fat-fed apoE\textsuperscript{−/−} mice (which exhibited prominent atherosclerotic lesions) compared with nonatherosclerotic tissue from age-matched controls fed standard chow (Figure 1A). To assess whether impaired p27-phospho-Ser10 is causally linked to atherogenesis, we interbred apoE\textsuperscript{−/−} mice with p27Ser10Ala knock-in mice, which have both p27 alleles replaced by a version carrying a Ser→Ala mutation at position 10 that blocks phosphorylation at this residue.\textsuperscript{14} On both standard chow and high-fat diet, circulating lipids levels (Figure 1B and Supplemental Figure 1A), body weight (Supplemental Figure 1B and 1C), and blood cell populations (Supplemental Figure 1D) were similar in apoE\textsuperscript{−/−} and apoE\textsuperscript{−/−} p27Ser10Ala mice. However, Oil Red O staining revealed increased atheroma size in the aortic arches and thoracic aortas of fat-fed male apoE\textsuperscript{−/−} p27Ser10Ala compared with apoE\textsuperscript{−/−} mice (Figure 1C). Likewise, we observed augmented atherosclerosis in cross-sections from the aortic sinus and 3 different regions of the ascending aorta separated by approximately 30 \mu m (Figure 1D). Expression...
of p27Ser10Ala also increased atherosclerosis burden in fat-fed female apoE−/− mice (Supplemental Figure II) and in mice of both genders fed standard chow (Supplemental Figure III). Overall, the relative content of macrophages and VSMC in the atherosclerotic plaque was comparable in both genotypes, with the exception of a significant but very modest decrease in VSMC content in the ascending aortas of apoE−/− p27Ser10Ala mice (Figure 2). Similarly, no significant differences were observed in the percentage of Ki67-immunoreactive Mac3-positive cells in early fatty-streaks consisting mostly of macrophages (Figure 3D). Moreover, cultured bone marrow (BM)–derived macrophages of both genotypes exhibited similar cell-cycle kinetics (Supplemental Figure VA) in spite of lower p27 levels in apoE−/− p27Ser10Ala macrophages at all time-points analyzed (Supplemental Figure VB). These findings suggest that expression of the nonphosphorylatable p27Ser10Ala mutant promotes atherosclerosis by a proliferation-independent mechanism.

**Proatherogenic Effect of p27Ser10Ala Expression Is Cell Proliferation Independent**

We have previously shown that genetic inactivation of p27 in apoE-null mice increases cell proliferation within atherosclerotic plaques and aggravates disease progression.20,21 To assess whether p27Ser10Ala expression similarly affects neointimal cell proliferation, we performed immunofluorescence assays in the aortic sinus of fat-fed mice to detect expression of the proliferation marker Ki67 together with different cell-type specific antigens (smooth muscle actin for VSMCs, CD3 for T cells, Mac3 for macrophages). We noted similar percentage of neointimal Ki67-positive VSMCs (Figure 3A), T cells (Figure 3B), and macrophages (Figure 3C) in advanced plaques of both genotypes. Likewise, no significant differences were observed in the percentage of Ki67-positive VSMCs (Figure 3A), T cells (Figure 3B), and macrophages (Figure 3C) in advanced plaques of both genotypes. Collectively, these findings demonstrate that expression of the nonphosphorylatable p27Ser10Ala mutant hastens both native and diet-induced atherosclerosis development in male and female hypercholesterolemic apoE−/− mice.

**Figure 1.** Reduced p27 phosphorylation at Ser10 is associated with murine atherosclerosis and accelerates disease progression in hypercholesterolemic mice. A, Western blot analysis of a pool of 6 aortic arches obtained from male apolipoprotein E–deficient (apoE−/−) mice fed either control chow (tissue essentially free of atheroma) or a high-fat atherogenic diet for 12 weeks (tissue with prominent atherosclerotic lesions). The p27-phospho-Ser10/p27 ratio was normalized to actin as loading control, and the average result of 3 different blots is shown (p27-pS10: p27 phosphorylated at serine 10). B to D, Male apoE−/− and apoE−/− p27Ser10Ala mice were fed a high-fat diet for 12 weeks. B, Plasma lipid levels before and after fat feeding. C, Atheroma size in the aortic arch and the thoracic aorta quantified by en face Oil Red O staining. Representative images are shown. D, Atheroma size quantified in histological sections from the aortic sinus and from 3 different regions of the ascending aorta (I, II, and III). Representative images of hematoxylin/eosin-stained sections are shown below (atherosclerotic plaques delineated by discontinuous lines).
Expression of p27Ser10Ala in BM-Derived Cells Accelerates Atherosclerosis in apoE−/− Mice

Given that macrophages are the most abundant cell type in atheromas of apoE−/− mice (cf Figure 2) and play key roles in atherosclerosis,1–3 we performed BM transplants to assess whether expression of the p27Ser10Ala mutant restricted to BM-derived cells affects the development of atherosclerosis. Lethally irradiated female apoE−/− mice were transplanted with male apoE−/− or apoE−/− p27Ser10Ala BM and then fed a high-fat diet for 8 weeks. Transplant efficiency in the BM was similar in both groups (apoE−/−BM: 88±12%; apoE−/− p27Ser10Ala-BM: 83±10%). Remarkably, atheroma size in the aortic arch and the thoracic aorta was increased in apoE−/− mice transplanted with apoE−/− p27Ser10Ala BM compared with apoE−/− BM (Figure 4). These results demonstrate that abrogating p27-phosphoSer10 specifically in hematopoietic cells is sufficient to aggravate atherosclerosis and suggest that this posttranslational modification plays an important role in the regulation of the atheroprotective actions that p27 exerts in macrophages, the most abundant BM-derived cell in the atherosclerotic plaque.

Expression of p27Ser10Ala in Macrophages Reduces p27 Total Levels But Increases Its Nuclear Localization

Previous studies have reported that p27Ser10Ala expression leads to decreased p27 protein levels by reducing its stability in quiescent fibroblasts and lymphocytes,14,15 and may also alter p27 subcellular localization by blocking its nuclear export.10,12,15,16 Consistent with these findings, we found a 2-fold reduction in total p27 protein levels (Figure 5A) and increased relative nuclear accumulation of p27 in apoE−/− p27Ser10Ala macrophages (Figure 5B).

Expression of p27Ser10Ala Increases RhoA/ROCK Signaling

The results presented thus far suggest that expression of p27Ser10Ala aggravates atherosclerosis via a cell proliferation-independent mechanism that operates in macrophages. Previous studies have shown that p27 can interact in the cytoplasm with the small GTPase RhoA, blocking its activation by RhoGEFs and thereby restricting signaling through the RhoA/ROCK pathway.27 A major contributor to cardiovascular disease.28,29 To address whether p27Ser10Ala expression affects RhoA/ROCK signaling, we first analyzed...
the degree of phosphorylation of the ezrin/radixin/moesin proteins (ERM), a reliable marker of the activity of this pathway.30 Consistent with a previous study,30 phospho-ERM was abundant in neointimal macrophages but absent in medial VSMCs (Figure 6A). Importantly, we found increased phospho-ERM in the atherosclerotic aortic arch of fat-fed apoE−/−p27Ser10Ala compared with apoE−/− mice (Figure 6B). Likewise, peritoneal macrophages obtained from fat-fed apoE−/−p27Ser10Ala mice exhibited increased phospho-ERM, as well as higher levels of GTP-bound-RhoA and cofilin phosphorylation, 2 additional markers of RhoA/ROCK activation (Figure 6C).

Expression of p27Ser10Ala Augments Modified Lipoprotein Uptake and Foam Cell Formation in a ROCK-Dependent Manner

We next investigated the internalization of modified lipoproteins by macrophages, an essential proatherogenic process that is facilitated by RhoA/ROCK signaling.31 To this end, we analyzed in vitro the uptake of fluorescently labeled acLDL by resident peritoneal macrophages. We found increased uptake of acLDL in apoE−/−p27Ser10Ala macrophages, and this was blunted by pharmacological inhibition of ROCK with either Y-27632 or hydroxyfasudil (Figure 7A). We also observed increased 3H-cholesterol accumulation in acLDL-loaded p27Ser10Ala macrophages (Figure 7B) without significant effects on cholesterol efflux (Figure 7C). Supporting the in vivo relevance of these findings, we found increased uptake of diH-labeled-oxLDLs by macrophages within the atheroma of apoE−/− p27Ser10Ala mice, which almost reached statistical significance ($P=0.08$) (Figure 7D), and a higher percentage of foam cells in peritoneal macrophages of fat-fed apoE−/− p27Ser10Ala mice (Figure 7E). The latter occurred without changes in the expression of either the prototypical scavenger receptors CD36 and SRA or other membrane proteins that may mediate lipoprotein uptake, such as very-low-density lipoprotein receptor, TLR2, TLR4, or LOX1 (Supplemental Figure VI), but coincided with increased bead phagocytosis by apoE−/− p27Ser10Ala macrophages, which was blunted by pharmacological inhibition of ROCK (Figure 7F).

Human Atherosclerotic Arteries Exhibit Reduced p27-Phospho-Ser10 and Increased ERM Phosphorylation

To address the clinical relevance of our findings, we examined the degree of phosphorylation of p27 and ERM in human coronary arteries (Figure 8). These studies revealed abundant...
p27-phospho-Ser10 in nonatherosclerotic vessels but undetectable levels in atherosclerotic specimens, which correlated with increased phospho-ERM in atherosclerotic arteries. These findings support the notion that p27-phospho-Ser10 regulates the activity of the RhoA/ROCK signaling pathway in human arteries.

Discussion

Animal studies have demonstrated an important atheroprotective role of the tumor suppressor p27, which has been attributed to its function as a negative regulator of cell proliferation. Consistent with this idea, human studies revealed frequent colocalization of p27 and transforming growth factor-β receptors in atherosclerotic coronary arteries and abundant expression of p27 in nonproliferating cells within both normal and atherosclerotic arteries. However, the possibility that p27 exerts proliferation-independent activities in the arterial wall has not been analyzed to date, in spite of an increasing body of evidence suggesting that p27 modulates the activity of various signaling proteins other than CDKs and cyclins. It also remains largely unexplored whether changes in the expression or function of p27 occur during human or experimental atherosclerosis and are causally linked to disease progression. In this study, we address these questions by combining cell culture experiments and studies with atherosclerosis-prone apoE−/− mice and human specimens. To the best of our knowledge, we demonstrate for the first time the impairment of a posttranslational modification of p27 during atherosclerosis that might be causally linked to disease progression by limiting a proliferation-independent atheroprotective function of p27 in macrophages.

In most cell types, the activity of p27 is regulated by posttranslational modifications, predominantly phosphorylation at different residues. We previously found that atherosclerosis development is not affected in apoE−/− mice unable to phosphorylate p27 at threonine 187, a posttranslational modification of p27 that controls its stability and function in several tissues and cell types. In this study, we assessed the role in atherosclerosis of p27 phosphorylation at Ser10, which appears to be the most abundant posttranslational modification of p27 and modulates its stability in different scenarios. Using a phospho-specific antibody that exhibits high specificity in Western blot (confer Figure 5A), we found a marked downregulation of p27-phospho-Ser10 in atherosclerotic specimens that might be causally linked to disease progression by limiting a proliferation-independent atheroprotective function of p27 in macrophages.

Figure 5. Effects of p27Ser10Ala expression on p27 protein levels and subcellular localization. A, Representative Western blot analysis of bone marrow (BM)–derived and resident peritoneal macrophages from apolipoprotein E–deficient (apoE−/−) and apoE−/− p27Ser10Ala mice. The relative level of p27 was determined as the p27/tubulin or p27/actin ratio, and the average result of 2 different experiments is shown. B, Representative Western blot analysis of cytoplasmic and nuclear fractions from apoE−/− and apoE−/− p27Ser10Ala BM-derived macrophages. The relative level of p27 was determined as the ratio p27/GAPDH (cytoplasmic extracts) or p27/lamin (nuclear extracts). The graph shows the relative nuclear/cytoplasmic p27 distribution (n=2 experiments).

Figure 6. p27Ser10Ala expression increases RhoA/ROCK signaling in atheromas and cultured macrophages. A, Immunofluorescence staining to simultaneously detect Mac3, smooth muscle actin (SMA), and phospho–ezrin/radixin/moesin protein (pERM) (marker of RhoA/ROCK signaling) in atherosclerotic aorta of fat-fed apoE−/− mice. B, RhoA/ROCK activity in atherosclerotic aorta of fat-fed apoE−/− and apoE−/− p27Ser10Ala mice measured as the degree of ERM phosphorylation. The average quantification of 2 different experiments is shown. C, RhoA/ROCK activity in apoE−/− and apoE−/− p27Ser10Ala peritoneal macrophages measured as the amount of RhoA bound to GTP (top), phospho-cofilin (pCofilin) (middle), and pERM (bottom).
Ser10 in the atherosclerotic aortic arch of fat-fed apoE knockout mice versus nonatherosclerotic arteries of controls fed with standard diet. Supporting a cause-and-effect relationship between impaired p27-phospho-Ser10 and atherosclerosis, we found that expression of the nonphosphorylatable p27Ser10Ala mutant accelerates atherosclerosis in different vascular beds in apoE knockout mice of both genders fed either standard chow or high-fat diet. Our BM transplantation studies demonstrate that expression of the p27Ser10Ala mutant restricted to hematopoietic cells is sufficient to accelerate atherosclerosis development in apoE knockout mice, suggesting that lack of p27-phospho-Ser10 critically affects the function of macrophages in the vascular wall. Supporting this notion, we found reduced total p27 protein levels and increased nuclear p27 localization in cultured macrophages expressing p27Ser10Ala. These results are consistent with previous studies showing that expression of this nonphosphorylatable mutant reduces total p27 protein levels in fibroblasts and lymphocytes and affects p27’s subcellular localization in some cell types by restraining its exit from the nucleus to the cytoplasm.

We have previously shown that genetic disruption of p27 aggravates atherosclerosis in apoE knockout mice coinciding with increased VSMC and macrophage proliferation in the vessel wall. Moreover, mice lacking p27 exhibit increased body size and organomegaly, which have been attributed to increased cell proliferation. Therefore, we hypothesized that expression of p27Ser10Ala promotes atherogenesis by increasing neointimal cell proliferation. However, our studies revealed comparable amounts of proliferating VSMCs, T cells, and macrophages in advanced atherosclerotic lesions of fat-fed apoE knockout and apoE knockout p27Ser10Ala mice. Similarly, we found no differences in the proliferation of macrophages in early fatty streaks and similar kinetics of cell-cycle progression in cultured macrophages obtained from apoE knockout and apoE knockout p27Ser10Ala mice. Moreover, unlike p27-null mice, apoE knockout p27Ser10Ala mice have normal body weight. These results are consistent with previous studies showing that p27Ser10Ala expression does not affect body size or cell-cycle progression of lymphocytes and fibroblasts despite lowering p27 levels, suggesting that impaired p27-phospho-Ser10 does not affect cell proliferation. It is plausible that higher nuclear localization of p27 in cells expressing p27Ser10Ala compensates for the overall lower level of p27, thus allowing normal cell cycle progression. Supporting this notion, Besson et al reported that the p27Ser10Ala mutant exhibits increased interaction with...
than CDKs and cyclins. We focused our attention on the regulation of a number of regulatory proteins other than CDK/cyclin complexes because of its nuclear accumulation.15 Taken together, our previous results in apoE−/− mice lacking p27 and the findings in apoE−/− p27Ser10Ala presented herein strongly support that p27 exerts both cell cycle–dependent and cell cycle–independent atheroprotective functions.

Having discarded abnormal cell proliferation as the mechanism underlying the aggravation of atherosclerosis in apoE−/− p27Ser10Ala mice, we examined whether p27Ser10Ala expression promotes atherogenesis by limiting a yet unidentified atheroprotective activity of p27 independent of its growth suppressive function. Indeed, p27 can regulate the activity of a number of regulatory proteins other than CDKs and cyclins.27,37,38 We focused our attention on the small GTPase RhoA, which is inhibited through interaction with p27.27 RhoA regulates a plethora of cellular processes via its downstream kinases ROCK1/2,39 and strong evidence exists that activation of this pathway contributes to neointimal thickening in the setting of atherosclerosis, vessel ligation, and balloon angioplasty in different murine models.28,29,31,40–44 Our mechanistic studies provide the first evidence supporting a pathophysiologically relevant link between p27 and RhoA. We have shown that p27Ser10Ala expression augments RhoA/ROCK signaling in both atherosclerotic plaques of apoE−/− and macrophages. Moreover, apoE−/− p27Ser10Ala macrophages exhibit RhoA-mediated cytoskeletal alterations that favor lipoprotein endocytosis/phagocytosis, leading to increased foam cell formation.

Supporting the clinical relevance of our findings, we found a marked reduction in p27-phospho-Ser10 level in atherosclerotic human coronary arteries. Human atherosclerosis was also accompanied by increased ERK phosphorylation in the arterial wall, suggesting augmented RhoA/ROCK signaling. On the basis of the results presented herein, we propose that scarce phosphorylation of p27 at Ser10 in the atherosclerotic plaque contributes to disease progression in a proliferation-independent manner, at least in part because of reduced p27 levels in macrophages, which lead to increased foam cell formation through RhoA/ROCK activation. When taken together with our previous studies with p27-null mice,20,21 these findings indicate that p27 exerts both cell cycle–dependent and cell cycle–independent atheroprotective functions that could be potentiated by overexpressing p27 and preventing the loss of p27-phospho-Ser10, respectively. However, given that p27 overexpression may be expected to indiscriminately block cell proliferation and thus compromise plaque stability (eg, by decreasing the thickness of VSMC-containing fibrous caps), the development of drug-based therapies preventing the loss of p27-phospho-Ser10 merits further investigation.

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**Disclosures**

None.

**References**

20. Diez-Juan A, Andres V. The growth suppressor p27Kip1 protects against

JK, Boisvert WA. Deficiency of ROCK1 in bone marrow-derived cells
protects against atherosclerosis in LDLR−/− mice. FASEB J. 2008;22:
2026–2035.

10. Kawauchi T, Chihama K, Nabeshima Y-i, Hoshino M. Cdk5 phosphor-
ylation of p27 KIP1.

9. Boime M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ,
Nabel EG. A growth factor-dependent nuclear kinase phosphorylates
p27Kip1 and regulates cell cycle progression. EMBO J. 2002;21:
3390–3401.

8. Boehm M, Yoshimura T, Crook MF, Nallamshetty S, True A, Nabel GJ,
Nabel EG. A growth factor-dependent nuclear kinase phosphorylates
p27Kip1 and regulates cell cycle progression. EMBO J. 2002;21:
3390–3401.

7. Koff A. Cell-cycle inhibitors: three families united by a common
mechanism.

6. Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common

5. Sanz-González SM, Molero-Fernández-de-Mera R, Malek NP, Andris V.
Atheroma development in apolipoprotein E-null mice is not regulated by
p27Kip1 phosphorylation at Ser10.

Yin, Andres V. Control of cell proliferation in atherosclerosis: insights
from animal models and human studies. Cardiovasc Res. 2010;86:
258–264.

503–516.

2. Fuster JJ, Fernandez P, Gonzalez-Navarro H, Silvestre C, Abu Nabah
Yin, Andres V. Control of cell proliferation in atherosclerosis: insights
from animal models and human studies. Cardiovasc Res. 2010;86:
258–264.

1. Diez-Juan A, Andres V. The growth suppressor p27Kip1 protects against
atherosclerosis in LDLR−/− mice. FASEB J. 2008;22:
2026–2035.

0. Koff A. Cell-cycle inhibitors: three families united by a common

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SUPPLEMENTAL MATERIAL

Deficient p27 Phosphorylation at Serine 10 Increases Macrophage Foam Cell Formation and Aggravates Atherosclerosis Through a Proliferation-Independent Mechanism

J. J. Fuster¹; H. González-Navarro², *; A. Vinuez³; P. Molina¹; M.J. Andrés-Manzano¹, KI. Nakayama³; K. Nakayama⁴; A. Díez-Juan⁵, **; A. Bernad⁵; C. Rodríguez⁶; J. Martínez-González⁶; V. Andrés¹,7

(1) Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Department of Epidemiology, Atherothrombosis and Imaging, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.

(2) Laboratory of Vascular Biology, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia, Consejo Superior de Investigaciones Científicas (IBV-CSIC), Valencia, Spain.

(3) Medical Institute of Bioregulation - Kyushu University, Fukuoka, Japan

(4) Tohoku University Graduate School of Medicine, Miyagi, Japan

(5) Department of Regenerative Cardiology, CNIC, Madrid, Spain

(6) Centro de Investigación Cardiovascular, Institut Català de Ciències Cardiovasculars, Instituto de Investigaciones Biomédicas Sant Pau, Barcelona, Spain

(7) Corresponding author.

CNIC, Melchor Fernández Almagro 3, 28029 Madrid, Spain.

Telephone: +34-914531200  FAX: +34-914531262

e-mail: vandres@cnic.es

* Present address: Fundación Investigación Hospital Clínico de Valencia, Valencia, Spain

** Present address: Centro de Investigaciones Príncipe Felipe, Valencia, Spain
METHODS

Metabolic measurements. Plasma lipid levels in mice fasted overnight were measured using enzymatic procedures (WAKO). HDL-cholesterol (HDL-C) was determined after precipitation of the apolipoprotein B-containing lipoproteins with dextran-sulphate/MgCl₂ (SIGMA) as previously described.¹

Immunohistochemical analysis of atherosclerotic plaque composition. Plaque composition was examined by immunohistochemical techniques performed by a researcher blinded to genotype. Vascular smooth muscle cells (VSMCs) were identified with mouse anti-smooth muscle α-actin (SMA) monoclonal alkaline phosphatase-conjugated antibody (1/20 dilution, clone 1A4) and Fast Red substrate (both from Sigma). Macrophages were detected with a rat anti-Mac3 monoclonal antibody (1/200 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology), followed by biotin-conjugated goat anti-rat secondary antibody (1/300 dilution, sc-2041, Santa Cruz Biotechnology), streptavidin-HRP (Ref. TS-060-HR, LabVision Corporation) and DAB substrate (BUF021A, AbD SEROTEC). Specimens were counterstained with hematoxylin. Collagen content was determined by Masson’s trichrome staining.

Assessment of cell proliferation in the atherosclerotic plaque. Identification of proliferating macrophages, VSMCs and T-cells within the atherosclerotic lesions was achieved by double immunofluorescence assays to detect expression of the proliferation marker Ki67 together with the cell-type-specific antigens Mac3 (macrophages) CD3 (T-cells) and SMA (VSMCs). After deparaffinization, antigen retrieval and blocking of non-specific interactions (5% horse serum in PBS, 45 minutes), histological sections were incubated overnight at 4ºC with anti-Ki67 antibody (prediluted, Clone SP6; Vitro) together with anti-Mac3 (1/500 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology), anti-SMA (1/75 dilution, Cy3-conjugated, C6198, SIGMA) or anti-CD3 (1/75 dilution, A0452, DAKO). Mac3 and CD3 were visualized with Alexa488-conjugated secondary antibodies (A11006, A-11034, Invitrogen) and Ki67 with an Alexa555-conjugated anti-rabbit IgG antibody (A21429, Invitrogen). Cell nuclei were stained with TOPRO-3 (T3605, Invitrogen). Slides were mounted with Slow-Fade Gold Antifade reagent (S36936, Invitrogen) and images were acquired on a Leica TCS/SP2 confocal microscope with a 40X oil immersion objective. Settings were adjusted to produce the optimum signal-to-noise ratio. Moreover, the sequential mode was used for image acquisition in order to avoid any interference from overlapping fluorescence. Quantification was done using the Metamorph software (Molecular Devices).

Blood cells counting. Circulating blood cells were counted and identified using an Abacus junior automated cell counter (Diatron).

Western blot analysis. Protein extracts from cultured macrophages and mouse aortic tissue were obtained using an ice-cold lysis buffer containing 50 mM Tris-Cl, pH 7.2, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 500 mM NaCl and 10 mM MgCl₂, supplemented with phosphatase and protease inhibitors (Roche). Protein extracts from human aortic samples were obtained using an ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 1% (w/v) Triton X-100, 150 mM NaCl and 1 mM DTT, supplemented with phosphatase and protease inhibitors. Polyacrylamide gel-electrophoresis and Western blot analysis were done as reported¹ and detection of proteins was performed with the following antibodies: anti-p27 (610242), from BD Transduction Laboratories; anti-actin (A2066), from SIGMA; anti-tubulin
(sc-8035) and anti-cofilin (sc12549), from Santa Cruz Biotechnologies; anti-phospho-cofilin (Ab3831), from Chemicon-Millipore; anti-α-actin (M0851), from Dako; anti-ERM (3142) and anti-phospho-ERM (3149), from Cell Signalling; and anti-β-actin (ab8227) and anti-p27-phospho-S10 (ab62364), from Abcam. HRP-conjugated secondary antibodies were from Santa Cruz Biotechnologies. Immunocomplexes were detected with the ECL Plus detection kit (Amersham Biosciences).

Macrophage culture. Bone marrow-derived macrophages (BMDMs) were obtained from suspensions of femoral BM and differentiated for 7 days in the presence of Dulbecco’s Modified Eagle Medium supplemented with antibiotics, 10% fetal bovine serum and 10% L929-cell conditioned medium as a source of macrophage colony-stimulating factor (MCSF). Peritoneal macrophages were obtained from the peritoneal cavity of mice, plated in standard cell culture dishes for 2 h and then extensively washed to eliminate non-attached cells.

Macrophage cell cycle analysis. For in vitro cell-cycle analysis, BMDM were synchronized in G0/G1 by 36 h of MCSF deprivation (DMEM, 10% FBS, 0.5% L929-cell-conditioned medium) and then stimulated for different times with complete medium (DMEM, 10% FBS, 10% L929-cell-conditioned medium). Macrophages were trypsinized and collected by centrifugation for 5 min at 300g. After fixation in 80% ethanol for 1 h at -20ºC, cells were incubated for at least 30 min with 50 μg/mL propidium iodide (P4170, SIGMA) containing 0.25 mg/mL RNAse A (R4642, SIGMA). Labelled cells were analyzed in a FACSCanto flow cytometer (Becton Dickinson) and DNA histograms were fitted into cell cycle distributions using the ModFit 3.0 software (Verity Software House).

BM transplantation. BM transplantation was carried out essentially as we have previously described. Briefly, female apoE-/- mice were irradiated with 2 doses of 4 Gy and transplanted with BM cells (2 x 10⁶) obtained from five pooled femurs of male apoE-/- or apoE-/-p27S10A mice. After 4 weeks on standard diet, transplanted mice were placed on atherogenic diet for 2 months. Transplant efficiency was assessed as the relative proportion of donor cells in the BM of recipient mice by real-time quantitative DNA amplification of Y-chromosome sequences essentially as previously described.

Phospho-ERM immunofluorescence. After deparaffinization, antigen retrieval and blocking of non-specific interactions (1% BSA in PBS, 45 minutes), aortic cross-sections were incubated overnight at 4ºC with anti-phospho-ERM antibody (1/500, clone 41A3, 3149, Cell Signalling) together with anti-Mac3 (1/500 dilution, clone M3/84, sc-19991, Santa Cruz Biotechnology) and anti-SMA (1/75 dilution, Cy3-conjugated, C6198, SIGMA). Phospho-ERM and Mac3 were visualized with an Alexa635-conjugated anti-rabbit-IgG antibody (A31577, Invitrogen), and an Alexa488-conjugated anti-rat IgG antibody (A11006, Invitrogen), respectively. Cell nuclei were stained with DAPI (D3571, Invitrogen). Slides were processed and images were acquired as described above (see Assessment of cell proliferation in the atherosclerotic plaque).

Gene expression analysis by quantitative real-time PCR (qPCR). RNA from peritoneal macrophages was obtained using TRIzol Reagent (Invitrogen). After verification of purity and concentration by the A₂₆₀/₂₈₀ ratio, RNA (0.5-1μg) was retro-transcribed and amplified with Superscript III First Strand Synthesis Supermix and Platinum Quantitative PCR Supermix-UDG with Rox dye (both from Invitrogen). The following primers (Forward: Fw;
Reverse: Rv) designed with the Primer Express software (Applied Biosystems) were used (mouse sequences):

**Cd36:**
- Fw 5’-TCGGAACTGTGGGCTCATTG-3’
- Rv 5’-CCTCGGGGTCTGTAGTTATATTTTC-3’

**Sra:**
- Fw 5’-CATGAACGAGAGGATGCTGACT-3’
- Rv 5’-GGAAGGGATGCTGCTATTGAA-3’

**Lox-1:**
- Fw: 5’-TGCGAATGACGAGCCTGAT-3’
- Rv: 5’-AGAAAGCAAATGCAGACCTTTAGG-3’

**Vldlr:**
- Fw: 5’-GAAGGAATGCCATATCAACGAAT-3’
- Rv: 5’-AGGTCTTTTGACATATGGAACA-3’

**Tlr2:**
- Fw: 5’-CCCTGTGCCACCATTTCC-3’
- Rv: 5’-GCCACGCCCACATCATTCC-3’

**Tlr4:**
- Fw: 5’-CCTGACACCAGGAAGCTTGAA-3’
- Rv: 5’-TCTGATCCATGCATTGGTAGGT-3’

**Cyclophilin:**
- Fw 5’-TGGAGAGCACTGTGGGCTCATTG-3’
- Rv 5’-TGCCGGAGTCCTGTAGTTATATTTTC-3’

Reactions were run on a thermal Cycler 7500 Fast System and results were analyzed with the software provided by the manufacturer (Applied Biosystems). mRNA levels in apoE/−/−p27S10A mice were expressed relative to those in apoE/−/− mice. Gene expression in both genotypes was normalized to the expression of the endogenous cyclophilin control.

**Cholesterol accumulation and efflux assays.** For cholesterol accumulation assays, peritoneal macrophages were plated at a density of 5 × 10^5 cells/well in 24-well plates. After 24 h, triplicate wells were cultured in DMEM with 10% human lipoprotein deficient serum, in the presence of 2 μCi/ml of 3H-cholesterol (Perkin Elmer) and 50 μg/ml of acLDL (Biomedical Technologies). 3H-cholesterol accumulation in macrophages was measured by scintillation counting after extraction of cellular lipids with hexane/isopropanol (2:1). For cholesterol efflux assays, peritoneal macrophages were subjected to 3H-cholesterol loading as described above for 36 h. Next, cells were extensively washed in PBS, and then incubated in DMEM plus either 100 μg/ml HDL (Biomedical Techoonlogies) or 0.1 % BSA for 24 h. Radioactivity was measured by scintillation counting in the medium and in cellular lipids.
after extraction with hexane/isopropanol (2:1). HDL-induced cholesterol efflux was calculated using the following equation:

$$\text{% efflux} = \left( \frac{\text{cpm in medium}}{\text{cpm in medium} + \text{cpm in cells}} \cdot 100 \right)^{\text{HDL}} \text{ - } \left( \frac{\text{cpm in medium}}{\text{cpm in medium} + \text{cpm in cells}} \cdot 100 \right)^{\text{BSA}}$$

**Beads phagocytosis assays.** Peritoneal macrophages were plated at a density of $1 \times 10^6$ cells/well in 6-well plates and incubated for 90 minutes in the presence of 1 μm carboxilate-coated fluorescent beads (Sigma). After extensive washes in PBS, macrophages were collected by trypsinization and the internalization of beads was examined by flow cytometry.

**Human artery sampling and preservation.** Human coronary artery samples were collected from freshly excised hearts removed during transplant operations at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The study was approved by the local ethics committee of and was conducted according to the Declaration of Helsinki (revised in 2000). Atherosclerotic samples were obtained from coronary artery disease patients aged 41-63 years, while non-atherosclerotic samples were from patients aged 14-48 years who had healthy coronary arteries but with serious dysfunctions affecting myocardium or great vessels that require heart transplantation (i.e. dilated myocardopathy, great vessel transposition). Immediately after surgical excision, the common trunk of the left coronary artery was dissected, cut in rings of approximately 0.5 cm, immersed in cell-maintenance media, and cleaned of connective tissue and fat under low magnification with a zoom stereo microscope. This examination permitted the classification of tissue as either atherosclerotic, assessed by the presence of evident atherosclerotic lesions, or non-atherosclerotic, as deduced from absence of fibro-fatty tissue or visible plaques. Specimens were frozen in liquid nitrogen and stored at -80º C until protein extraction.

**REFERENCES**


Supplemental Figure I. Effects of p27Ser10Ala mutant expression on plasma lipid levels, body weight, and circulating blood cell counts. Mice were fed standard chow (A, C) or high-fat diet for 12 weeks (B, D). Total chol.: Total cholesterol; HDL-chol.: HDL-cholesterol.
Supplemental Figure II. Expression of p27Ser10Ala mutant hastens atherosclerosis in female apoE−/− mice. Atherosclerosis burden was quantified in female mice fed a high-fat diet for 8 weeks. (A) Atheroma size in the aortic arch and the thoracic aorta quantified by en-face Oil Red-O staining. Representative images are shown. (B). Atheroma size quantified in histological sections from the aortic sinus and from three different regions of the ascending aorta separated by approximately 30 µm (Ascending Aorta I, II and III, starting at the end of the aortic valve). Representative images of hematoxylin/eosin-stained sections are shown below. Atherosclerotic plaques are delineated by discontinuous lines.
Supplemental Figure III. Expression of p27Ser10Ala mutant accelerates native atherosclerosis in apoE-/- mice fed standard chow. Atheroma size in the aortic arch of 9-month-old mice was quantified by en-face Oil Red-O staining. Representative images are shown and the number of mice analyzed is indicated.
Supplemental Figure IV. Effects of p27Ser10Ala mutant expression on collagen content in atheromata. Collagen content was determined by Masson’s trichrome staining of histological sections from apoE-/- and apoE-/-p27Ser10Ala mice fed a high-fat diet for 12 weeks. No statistically-significant differences were observed.
Supplemental Figure V: Expression of p27Ser10Ala mutant in macrophages reduces p27 protein levels but does not affect cell-cycle progression. Bone-marrow-derived macrophages were synchronized in G0 by 36 h of MCSF deprivation and then stimulated for the indicated times with 10% L929-cell conditioned medium as a source of MSCF. (A) Cell cycle kinetics were analyzed by propidium iodide staining and flow cytometry. (B) p27 levels were analyzed by Western blot.
Supplemental Figure VI: Expression of p27Ser10Ala mutant in macrophages does not affect the expression of receptors involved in the uptake of modified LDL. Transcript levels in peritoneal macrophages were analyzed by quantitative real-time PCR (n=4). No statistically-significant differences were observed. SRA: Scavenger receptor A; LOX-1: lectin-like oxidized low-density lipoprotein receptor-1; TLR2/4: Toll-like receptor 2/4; VLDLR: very low-density lipoprotein receptor.