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Role of PTEN in modulation of ADP-dependent signaling pathways in vascular endothelial cells

Rosa Bretón-Romero [a,b], Hermann Kalwa [a], Santiago Lamas [b,*], Thomas Michel [a,**]

[a] Cardiovascular Medicine Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA
[b] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM). c/ Nicolás Cabrera 1, Universidad Autónoma de Madrid, 28049 Madrid, Spain.

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ABSTRACT

ADP plays critical signaling roles in the vascular endothelium. ADP receptors are targeted by several cardiovascular drugs, yet the intracellular pathways modulated by ADP are incompletely understood. These studies have identified important roles for the phosphatase PTEN in ADP-dependent modulation of the endothelial isoform of nitric oxide synthase (eNOS) as well as of lipid and protein kinase pathways in endothelial cells. We find that ADP-promoted eNOS activation as well as phosphorylation of p38 MAPK are enhanced by siRNA-mediated PTEN knockdown. However, the increase in ADP-dependent eNOS activation promoted by PTEN knockdown is abrogated by siRNA-mediated knockdown of p38 MAPK. These findings indicate that PTEN tonically suppresses both p38 phosphorylation as well as ADP-stimulated eNOS activity. A key enzymatic activity of PTEN is its role as a lipid phosphatase, catalyzing the dephosphorylation of phosphoinositol-3,4,5-trisphosphate (PIP_3) to phosphoinositol-4,5-bisphosphate (PIP_2). We performed biochemical analyses of cellular phospholipids in endothelial cells to show that siRNA-mediated PTEN knockdown leads to a marked increase in PIP_3. Because these complex lipids activate the small GTPase Rac1, we explored the role of PTEN in ADP-modulated Rac1 activation. We used a FRET biosensor for Rac1 to show that ADP-dependent Rac1 activation is blocked by siRNA-mediated PTEN knockdown. We then exploited a FRET biosensor for PIP_3 to show that the striking ADP-dependent increase in intracellular PIP_3 is entirely blocked by PTEN knockdown. These studies identify a key role for PTEN in the modulation of lipid mediators involved in ADP receptor-regulated endothelial signaling pathways involving eNOS activation in vascular endothelial cells.

1. Introduction

The nucleotide ADP plays a key role as an extracellular signaling molecule and binds to a family of G protein-coupled receptors that modulate diverse biological processes including platelet aggregation, vascular tone, and neurotransmission. ADP is released by red blood cells and by activated platelets in response to both physiological and pathophysiological stimuli [1]. ADP receptor antagonists are widely used in cardiovascular therapeutics [2], yet the pathways whereby extracellular ADP modulate endothelial function are incompletely understood. We recently identified some of the signaling pathways connecting P2Y1 ADP receptors with the endothelial isoform of nitric oxide synthase (eNOS). Studies in bovine aortic endothelial cells (BAEC) showed that P2Y1 is the principal purinergic receptor subtype that mediates ADP signaling responses [3]. eNOS is a key determinant of vascular tone, and is dynamically regulated by a broad range of cell surface receptors that elicit increases in intracellular calcium levels. The enzymatic activity of eNOS is critically influenced by enzyme phosphorylation at multiple serine, threonine, and tyrosine residues [4–6]. The protein kinase and phosphoprotein phosphatase pathways that regulate eNOS phosphorylation are complexly regulated; the phosphatidylinositol 3-kinase (PI3K)/kinase Akt pathway has been clearly implicated in eNOS signaling responses. ADP treatment of endothelial cells promotes the receptor-dependent activation of numerous protein kinases, including the AMP-activated protein kinase. The eNOS phosphorylation pathways that are modulated by endothelial P2Y1 receptors for ADP are particularly enigmatic: ADP-dependent eNOS activation requires the expression but not the activity of several protein kinases in endothelial cells [3]. Our search for other signaling pathways that might dynamically regulate ADP-modulate eNOS phosphorylation led us to study the lipid and protein phosphatase PTEN.

PTEN ("phosphatase and tensin homolog on chromosome 10") catalyzes the dephosphorylation of phospholipids as well as phosphoproteins (for reviews see refs. [7,8]). PTEN is the protein most commonly mutated in human malignancies, and the regulation of this key enzyme has been extensively analyzed in studies of cell transformation. PTEN contains the signature sequence motif of the

* Corresponding author. Tel.: +34 911964455.
** Corresponding author. Tel.: +1 6177327376.
E-mail addresses: slamas@cbm.uam.es (S. Lamas), thomas_michel@harvard.edu (T. Michel).

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protein tyrosine phosphatase family, but the major targets of PTEN in most cells are not phosphoproteins. Instead, the principal PTEN substrate is the phospholipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is dephosphorylated by PTEN to yield phosphatidylinositol 4,5-bisphosphate (PIP_2). Changes in the levels of these complex lipids second messengers influence diverse cellular pathways, including alterations in cell growth, differentiation, apoptosis, metabolism, and motility [9,10]. These responses are initiated by the binding of phosphoinositides to proteins that possess highly specific phosphoinositide-binding pleckstrin homology domains that selectively bind PIP_3, PIP_2, and other structurally related bioactive complex lipids [11]. PTEN-mediated catabolism of PIP_3 to PIP_2 antagonizes signaling through the PI3K pathway, with important consequences for the phosphorylation of kinase Akt and the regulation of eNOS [12]. PTEN modulates not only the abundance of PIP_3 but also the temporal and spatial distribution of this lipid and related biologically active phosphoinositides [13]. PTEN is widely distributed in mammalian tissues, and its enzymatic activity is controlled both by reversible phosphorylation and by cellular redox state [10,14,15]. An active interplay between phosphorylation and redox pathways permits PTEN activity and targeting to be precisely regulated within the cell. PTEN phosphorylation occurs on several threonine and serine residues located in a polybasic domain in the protein's C terminus, leading both to PTEN translocation as well as a decrease in enzymatic activity. The protein kinase pathways that modulate PTEN phosphorylation are less well understood and a connection between ADP signaling and PTEN has not previously been established. The phosphoprotein phosphatase substrates of PTEN include diverse protein kinases, including the focal adhesion kinase, Shc [16–19], as well as p38 MAP kinase [20]. The reversible phosphorylation of p38 MAPK has been implicated in a broad range of intracellular responses [21–23], including key roles in endothelial signaling pathways [24].

These studies have explored the role of PTEN in ADP-modulated eNOS activation in vascular endothelial cells. We exploited novel biosensors and used both RNA interference and biochemical approaches to provide evidence showing that PTEN is dynamically regulated by ADP pathways in vascular endothelial cells. These studies help to establish a critical role for lipid second messengers in ADP-modulated signaling to eNOS in the endothelium.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). All other cell culture reagents, media, and Lipofectamine 2000 were from Invitrogen (San Diego, CA). PD169316 was from Calbiochem (San Diego, CA). PIP3 Grip was from Echelon Bioscience (Salt Lake City, UT). Protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Polyclonal antibodies against phospho-eNOS (Ser1177), p38 MAPK, PTEN, phospho-PTEN (Ser380/Thr382/383), Akt, actin, and phospho-Akt (Ser473) were from Cell Signaling (Danvers, MA). Monoclonal antibody phospho-p38 MAPK (Thr180/Tyr182) was from Cell Signaling, monoclonal antibody against eNOS was from BD Transduction Laboratories (San Jose, CA), and GST monoclonal antibody from GenScript (Piscataway, NJ). SuperSignal chemiluminescence detection reagents and secondary antibodies conjugated with horseradish peroxidase were from Pierce Biotechnology. All other reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and transfection

BAEC were obtained from Genlantis (San Diego, CA) and maintained in culture in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v) as described [25]. Cells were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passages 6 and 8. siRNA transfections were performed as described previously [26]. BAEC were transfected with 30 nM siRNA 24 h after cells were split at a 1:8 ratio using Lipofectamine 2000 (0.15%, v/v), following the protocol provided by the manufacturer. Lipofectamine 2000 was then removed by changing into fresh medium containing 10% FBS 5 h after transfection.

2.3. siRNA preparation

We designed small interfering RNA duplex oligonucleotides targeting constructs, which were purchased from Ambion. The following sense sequences were used: siRNA-p38, 5′-GGUCUCUGGAGGAAUUCAAtt-3′; siRNA-PTEN, 5′-GUAUAGAGCGUGCAGAUAAtt-3′; and siRNA-Akt1, 5′-G GA CGU GUA CGA GAA GAA GdTdT-3′.

2.4. Cell treatments and immunoblot analysis

Culture medium was changed to serum-free medium overnight prior to all experiments. ADP was dissolved in water and stored at −20 °C. PD169316 was solubilized in dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. Where indicated, dimethyl sulfoxide 0.1% (v/v) was used as vehicle control. After drug treatments, BAEC were washed with PBS and incubated for 20 min in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail tablets (following the instructions provided by the supplier). After cells were harvested and lysed, immunoblot analyses of protein abundance and phosphorylation were processed as previously described [27].

2.5. Lipid extraction

Cells cultured in 6-well plates were transfected as described above; 48 h after transfection, cells were scraped into 0.3 ml of 0.5 M sodium carbonate, pH 11 and homogenized in a Dounce homogenizer followed by sonication. After sonication, triplicate 50 μl aliquots were spotted on a nitrocellulose membrane and dried; the membranes were washed twice with PBS-5% Tween, blocked with PBS-3% BSA for 1 h at room temperature, incubated with the GST-tagged "PIP3-Grip", washed extensively, and detected using the GST antibody.

2.6. eNOS activity assay

eNOS activity was quantitated by measuring the formation of L-[^{3}H] citrulline from L-[^{3}H]arginine as described previously [28]. The basal eNOS activity in these experiments ranged from 0.3 to 0.7 pmol/mg protein/min. Briefly, the reaction was initiated in cultured BAEC by adding L-[^{3}H]arginine (10 μCi/ml, diluted with unlabeled L-arginine to give a final concentration of 10 μM) plus various drug treatments. Each treatment was performed in triplicate cultures, which were analyzed in duplicate.

2.7. Fluorescent resonance energy transfer (FRET) measurement

Monitoring of PIP_3 production and Rac1 activation were performed by the use of specific FRET biosensor probes, as described in detail [29]. Briefly, the FRET biosensor for PIP3 consists of the PIP3 binding domain flanked by CFP and YFP sequences in a conformation that does not allow FRET between the fluorophores. When PIP3 accumulates in the plasma membrane, the biosensor binds to PIP3, resulting in a conformational change that leads to an increase in FRET efficiency. BAEC were transfected with duplex siRNA targeting constructs along with either the PIP_3 or Rac1 biosensor plasmids. 48 h after transfection, living cells were imaged in an Olympus DSU fluorescence microscope. Agonists were added after 5 min, and fluorescence images were captured every

minute for 1 h following agonist addition. Analysis was performed using MetaMorph software (Universal Imaging, Downingtown, PA).

2.8. Confocal fluorescence microscopy

BAEC were grown on glass Lab-Tek Chamber Slides and transfected with control or PTEN siRNA. 48 h after transfection, cells were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized in 0.1% Triton X-100 for 15 min and blocked for 1 h with 10% goat serum. After incubation with vinculin antibody at 4 °C overnight (1:400), cells were washed three times in PBS and incubated for 2 h with an Alexa Fluor 568 antibody (1:2000). For staining of F-actin, Alexa Fluor 488 phalloidin was incubated together with the secondary antibody. Fixed cells were washed to remove excess antibody and mounted on slides, which were examined using an Olympus DSU microscope; images were analyzed using Metamorph software.

2.9. Scratch wound cell migration assay

BAEC were transfected with siRNA-targeting constructs and grown for 24 h. Cells were added to each chamber of a 8-well cell culture chamber slide (IBIDI, Munich, Germany) to adhere for 24 h in Dulbecco's modified Eagle's medium with 1.0% FBS. The cell monolayer was scratched by a pipette tip and migration was recorded using a 10X DIC objective on an inverted Olympus DSU microscope for 30 h.

2.10. Statistical analysis

In the experiments involving multiple comparisons, ANOVA was used; a p value of less than 0.05 was considered statistically significant.

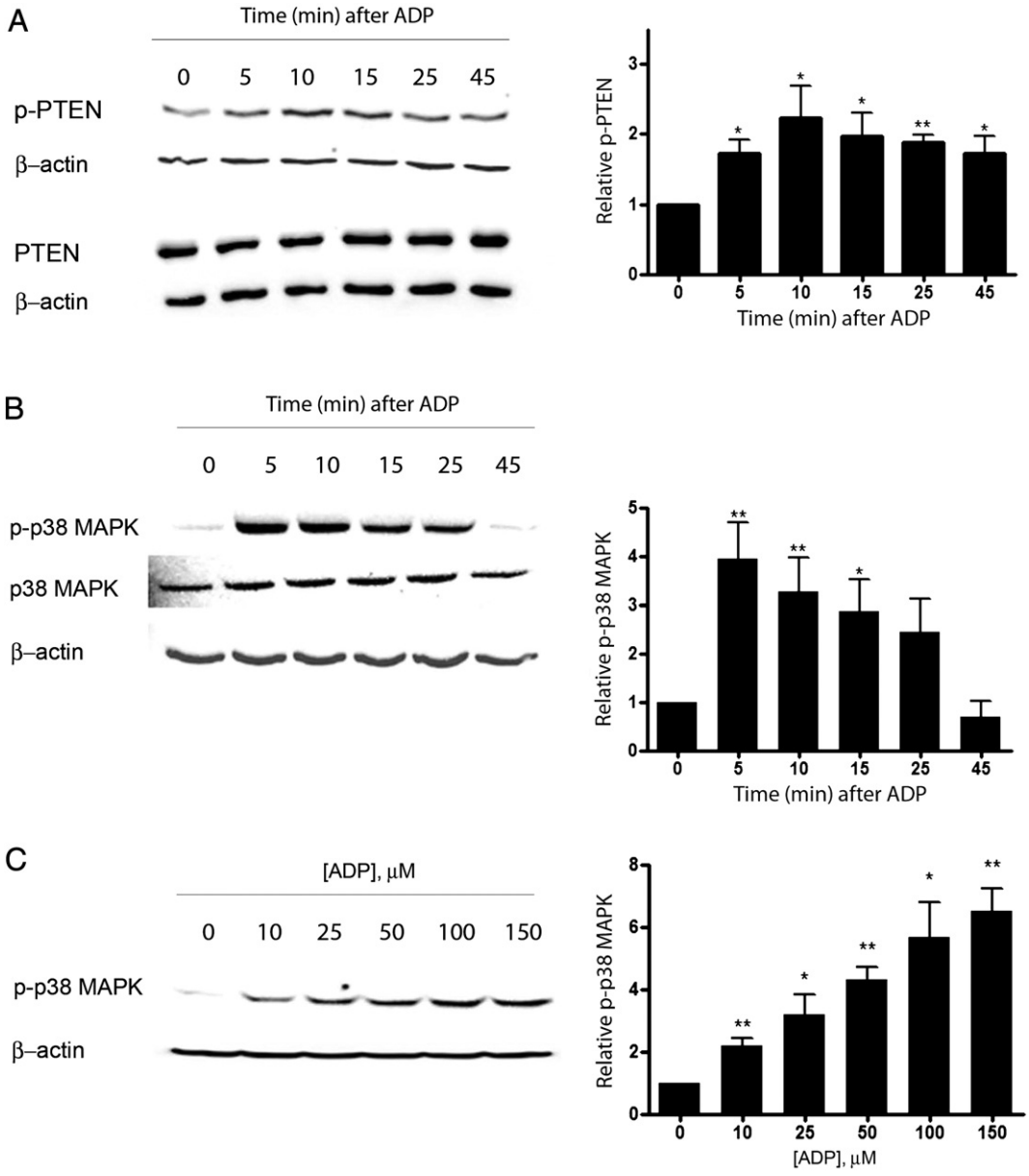

Fig. 1. ADP-stimulated phosphorylation of PTEN and p38 MAPK. Lysates prepared from ADP-treated endothelial cells were analyzed in immunoblots probed with antibodies as shown. Panel A, time course of PTEN phosphorylation in endothelial cells treated with ADP (25 μM); panels B and C (respectively), show time course and dose response (determined at t = 20 min) for ADP-stimulated p38 MAPK phosphorylation (pp38 MAPK). The blots shown are representative of at least three independent experiments that yielded equivalent results; the graphs next to each blot represent quantification of pooled data.

3. Results

3.1. ADP promotes reversible phosphorylation of PTEN and p38 MAPK in endothelial cells

We first explored the effect of ADP on the phosphorylation of PTEN and related signaling proteins in cultured endothelial cells. As shown in Fig. 1A, physiological concentrations of ADP promote the robust phosphorylation of PTEN; phosphorylation of PTEN is associated with inactivation of its lipid phosphatase activity [9,15]. We next explored the effects of ADP on phosphorylation of p38 MAP kinase (p38 MAPK) (Fig. 1B and C), a phosphoprotein that modulates multiple signaling responses in these cells [30]. As we found for PTEN, ADP also induces a reversible phosphorylation of p38 at its activation site (Thr180/Tyr182).

3.2. ADP-mediated eNOS and p38 MAPK activation are modulated by PTEN

PTEN is the principal negative regulator of PI3K/Akt pathway [31,32], which in turn plays a key role in eNOS regulation [33–35]. We designed and validated highly specific and potent duplex siRNA targeting constructs for PTEN that yielded ~90% PTEN knockdown in transfected endothelial cells, without affecting the abundance of eNOS (Fig. 2A). As shown in Fig. 2A and B, siRNA-mediated PTEN knockdown markedly increased basal as well as ADP-stimulated eNOS phosphorylation at serine 1179 and led to an increase in basal and ADP-stimulated eNOS activity. siRNA-mediated PTEN knockdown also led to a marked increase in basal and ADP-stimulated p38 MAPK phosphorylation (Fig. 2C).

3.3. p38 MAPK inhibition blocks ADP-dependent eNOS activation

We developed and validated a duplex siRNA targeting construct to knock down p38 MAPK; this targeting construct promoted the potent and specific knockdown of p38 MAPK in endothelial cells (Fig. 3A). Although four different isoforms of p38 MAPK have been described in mammals (α, β, γ and δ), the cardiovascular importance of p38α is the best characterized [36–38]. As shown in Fig. 3A (lower panel), siRNA-mediated p38 MAPK-knockdown markedly suppressed ADP-

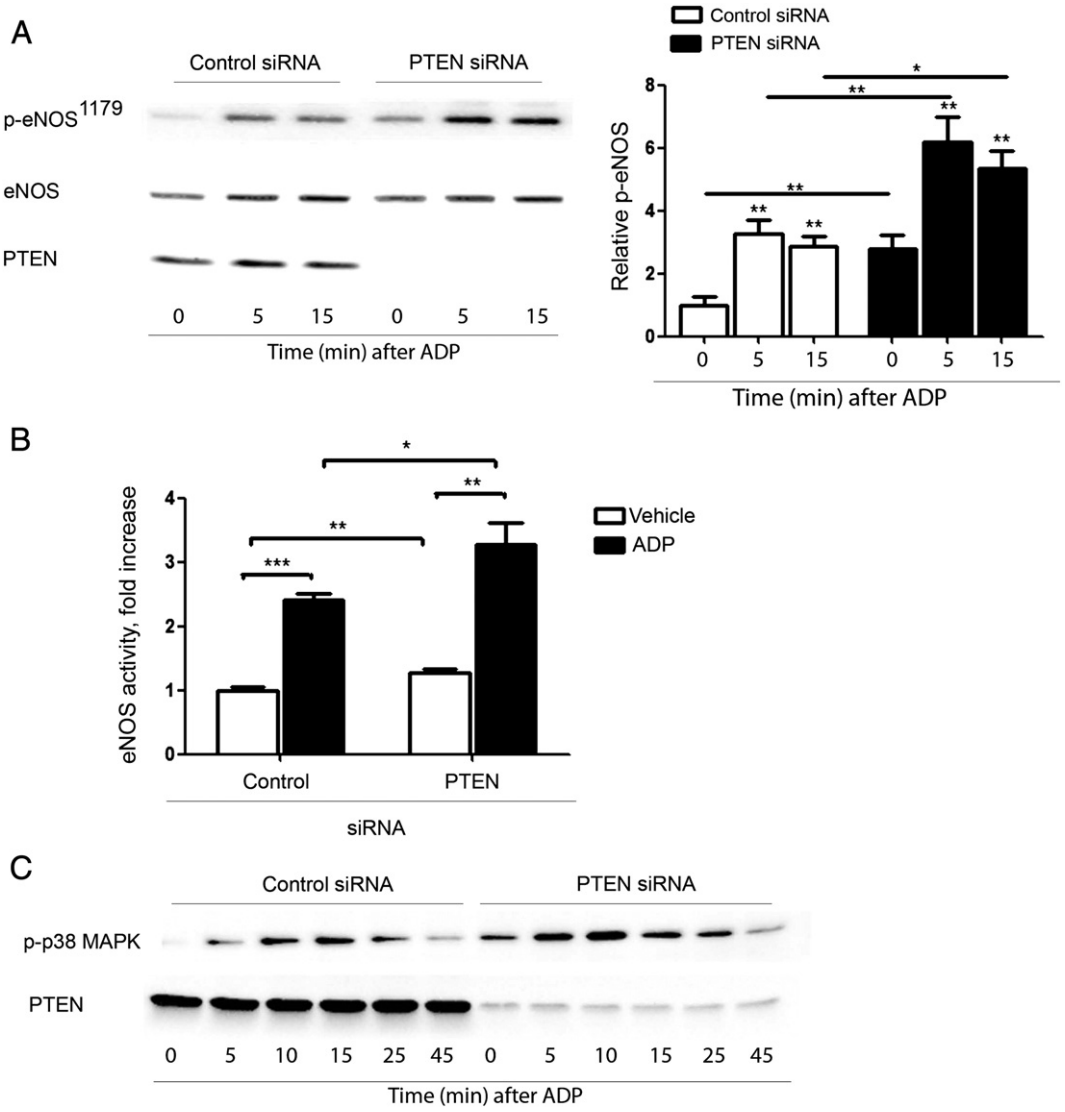

Fig. 2. Effects of siRNA-mediated PTEN knockdown on eNOS phosphorylation and enzyme activity. Immunoblots in panels A and panel C show representative time course experiments in which endothelial lysates were prepared from control or PTEN siRNA-transfected cells that were treated with ADP (25 μM) for the indicated times, and then probed with antibodies in immunoblots as shown. Bar graph shows the mean ± SEM of four independent experiments. Panel B shows pooled data from four experiments measuring eNOS activity using the [^{3}H]-arginine-[^{3}H]-citrulline assay (as described in Materials and methods), as analyzed in PTEN and control siRNA-transfected endothelial cells and treated with ADP or vehicle for 20 min. Since the basal eNOS activity varied slightly from experiment to experiment, the data are normalized to the enzyme activity seen in control siRNA-transfected cells in the absence of ADP. The graph shows the mean ± s.e.m. of four independent experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

induced eNOS activity; similar results were obtained with the cell permeable, competitive and selective p38 MAPK kinase inhibitor PD169316 (Fig. 3B). The concordant effects of siRNA-mediated p38 MAPK knockdown and a p38 MAPK kinase inhibitor help to establish p38 MAPK as an upstream kinase critically involved in ADP-mediated eNOS activation.

3.4. Interactions between PTEN, p38 MAPK and kinase Akt in eNOS activation

In order to explore the interplay between PTEN and p38 MAPK in eNOS regulation, we performed "double knockdown" experiments using two separate siRNA constructs to target PTEN and p38 MAPK. As shown in Fig. 4A, siRNA-mediated p38 MAPK knockdown attenuated the increase in eNOS enzyme activity and eNOS phosphorylation that was observed following PTEN knockdown. Interestingly, the increase in Akt phosphorylation that was seen after PTEN knockdown was further enhanced by also knocking down p38 MAPK (Fig. 4A, right panel), indicating possible interactions between these two kinase pathways. We next explored the effect of siRNA-mediated knockdown of p38 MAPK on PTEN inactivation by ADP. As shown in Fig. 4B, p38 MAPK knockdown led to an increase in PTEN inhibition, which correlated with an increase in Akt activation mediated by ADP. These events might explain the residual eNOS activity that is observed following p38 MAPK knockdown (Fig. 3). We next performed double knockdown experiments with targeting constructs for both PTEN and Akt. As shown in Fig. 4C, siRNA-mediated Akt knockdown partially blocked ADP-stimulated eNOS activity, yet additional siRNA-mediated knockdown of PTEN partially recovered the eNOS response to ADP.

3.5. PTEN knockdown blocks ADP-mediated PIP_3 generation

To study the inhibitory effect of ADP on PTEN activity and PIP_3 levels, we performed live cell imaging experiments. We transfected endothelial cells with control or PTEN siRNA, and determined cellular PIP_3 content using a biochemical assay for PIP_3. As expected, since PTEN is the main phosphoinositide phosphatase that metabolizes PIP_3 [13], siRNA-mediated PTEN knockdown led to a significant increase in cellular PIP_3 levels (Fig. 5A). We next performed live cell imaging experiments using a highly specific FRET biosensor [29] to measure the dynamics of ADP-modulated changes in PIP_3 following siRNA-mediated PTEN knockdown. We co-transfected endothelial cells with the PIP_3 biosensor along with either control or PTEN siRNA, and then analyzed dynamic FRET responses in live cells following addition of ADP. In control siRNA-treated cells, ADP treatment stimulated a marked rise in the FRET signal from the PIP_3 biosensor, reflecting a dynamic increase in PIP_3 levels along endothelial cell plasmatic membranes following agonist treatment. By contrast, following siRNA-mediated PTEN knockdown, the ADP-promoted increase in FRET from the PIP_3 biosensor was no longer observed (Fig. 5B). Since live cell imaging using the FRET biosensor can only detect *changes* in PIP_3 abundance but not the absolute levels of intracellular PIP_3, FRET data were normalized to the basal CFP/YFP ratio in each condition (Fig. 5C), indicating that PTEN knockdown effectively suppresses any *further* increase in PIP_3 after addition of ADP, consistent with the effect of ADP on inactivation of PTEN.

3.6. PTEN regulates endothelial cell migration, actin organization and Rac1 activation

Given the involvement of ADP in PTEN regulation, we next analyzed the functional consequences of PTEN inactivation on endothelial function. It has been previously described that the complex lipid PIP_3 is a regulator of cytoskeletal reorganization and cell motility [39]. We therefore studied whether the deficit of PTEN could modify endothelial cell migration, in a wound scratch assay. As shown in Fig. 6A, siRNA-mediated PTEN knock-down markedly suppressed directional migration of endothelial cells.

Increases in intracellular PIP_3 may stimulate F-actin polarization [40], and we therefore tested whether PTEN knockdown would affect actin polymerization in endothelial cells. We used cellular imaging approaches to analyze the effects of siRNA-mediated PTEN knockdown on actin cytoskeleton structure, using phalloidin to label F actin in these cells. As shown in Fig. 6B, siRNA-mediated PTEN knockdown led to a marked increase in actin polymerization compared with cells transfected with control siRNA. We next studied the effects of PTEN knockdown on activation of the small GTPase Rac1, a member of the Rho GTPase family that has been implicated in cortical actin filament reorganization [41,42] and Akt phosphorylation [26], as well as in ADP-mediated eNOS activation [3]. Rac1 activity was analyzed in live cells using a Rac1 FRET biosensor [29]. Endothelial cells were co-transfected with the Rac1 biosensor plus either control or PTEN siRNA, and then stimulated with ADP and imaged in real time.

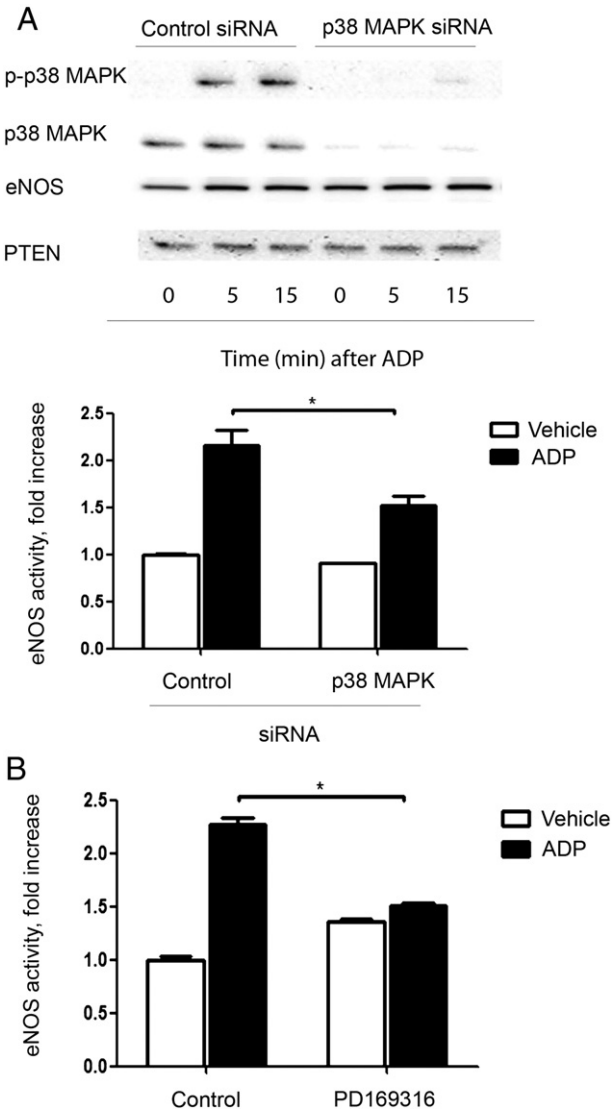

Fig. 3. Effects of siRNA-mediated knockdown or pharmacological inhibition of p38 MAPK on eNOS activity. In Panel A, endothelial cells transfected with control or p38 MAPK siRNA targeting constructs were treated with ADP (25 μM) for the indicated times. Equal loading was assessed by immunoblotting with antibodies directed against total eNOS, p38 MAPK and PTEN, and eNOS activity was measured using the [^{3}H]-arginine-[^{3}H]-citrulline assay. Panel B shows the pooled results of eNOS activity assays analyzed in endothelial cells treated with the p38 MAPK inhibitor PD169316 (10 μM) for 30 min and then treated with ADP (25 μM) for 20 min. * indicates $p < 0.05$.

As shown in Fig. 6C, ADP-stimulated Rac1 activity is completely blocked following siRNA-mediated PTEN-knockdown.

4. Discussion

These studies have explored the role of the lipid–protein phosphatase PTEN in ADP-modulated regulation of eNOS and related signaling pathways in vascular endothelial cells. Although the involvement of PTEN in eNOS regulation has been recently characterized [12], the present studies provide novel information on the role of the purine nucleotide ADP in this signaling pathway. The present study, uses physiological ADP concentrations [3,43,44], to show that ADP is able to promote the dynamic phosphorylation of PTEN in vascular endothelial cells (Fig. 1). One principal enzymatic activity of PTEN is the dephosphorylation of the signaling phospholipid PIP_3, forming PIP_2; the resulting depletion of PIP_3 levels serves to inhibit activation of the phospholipid-dependent kinase-1 (PDK1), thereby attenuating the PI3K/Akt pathway. Phosphorylation of PTEN is associated with inactivation of its lipid phosphatase activity as a consequence of the phosphoenzyme's translocation away from the plasma membrane, where the enzyme's hydrophobic lipid substrates are sequestered [9]. Conversely, phosphorylated PTEN is sequestered in the cytosol, where the phospho-enzyme apparently may still exert its phosphoprotein phosphatase activity despite its lack of access to lipid substrates in the cell

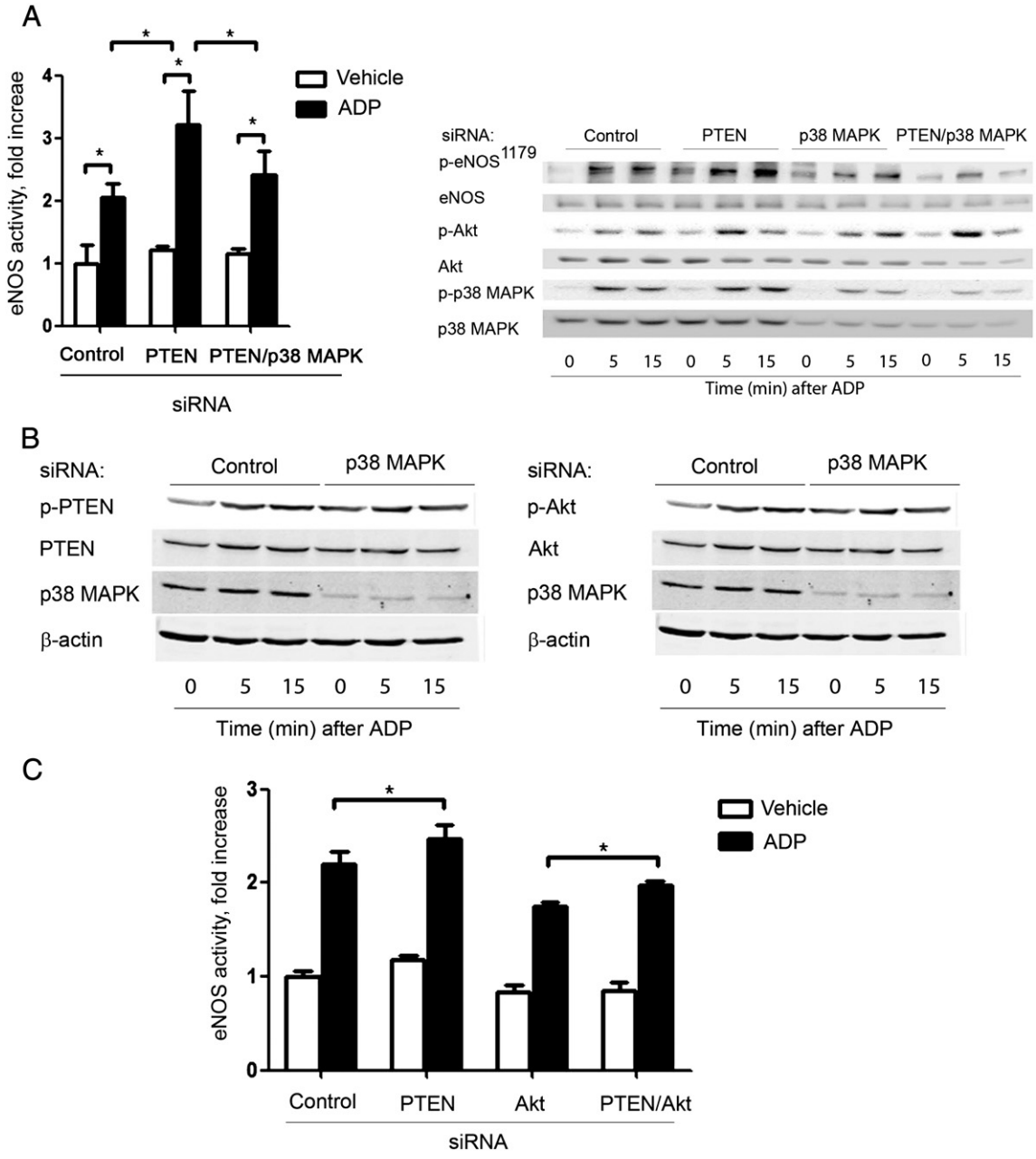

Fig. 4. siRNA-mediated "double knockdown" of p38 MAPK and PTEN. Endothelial cells were transfected at a final siRNA concentration of 60 nM, comprised of 30 nM PTEN and/or p38 MAPK targeting constructs as indicated; for each experiment the balance was made up by control siRNA to yield a final concentration of 60 nM for all experiments. 48 h following transfection, endothelial cells were treated with ADP (25 μM) and analyzed in eNOS activity assays or in immunoblots probed with antibodies as shown (panel A). In Panel B, endothelial cells transfected with control or p38 MAPK siRNA targeting constructs were treated with ADP (25 μM) for the indicated times, and then probed with antibodies in immunoblots as shown. Panel C shows the effect on eNOS activity of the siRNA mediated double knockdown of Akt and PTEN. For each condition siRNA final concentration of 60 nM was used. The results shown for the activity assays represent data pooled from 3 experiments, each performed in duplicate. The immunoblot shown is representative of three independent experiments with equivalent results. * indicates $p < 0.05$.

membrane [15]. PTEN is a critical negative regulator of the PI3K/Akt pathway in several cell types [31,45–47], yet the enzyme also modulates many other cellular responses independent of its effects on PI3K/Akt [48]. Our new findings implicate PTEN in a broad range of endothelial cell signaling responses involving eNOS.

We found that PTEN knockdown led to a marked increase in both basal and ADP-dependent eNOS phosphorylation at its Ser1179 activation site, associated with an increase in the phosphorylation of kinase Akt. Ser1179 is the most thoroughly studied eNOS phosphorylation site, and has been shown to be phosphorylated not only by kinase Akt, but also by AMPK, PKG, PKA, among other protein kinases [49]. Moreover, numerous distinct protein kinases have been found to phosphorylate eNOS at other serine, threonine, and tyrosine residues in the protein. Despite a central role for PTEN in modulating PI3K/Akt responses, we have previously shown that the inhibition of the PI3K/Akt pathway does *not* affect ADP-promoted eNOS phosphorylation [3]. Since PTEN may modulate a broad range of PI3K/Akt-independent signaling pathways in different cell types, the enhancement of eNOS phosphorylation following PTEN knockdown most plausibly reflects the effects of PTEN on a phosphorylation pathway distinct from PI3K/Akt. Indeed, PTEN has an established role as a phosphoprotein phosphatase, and the phosphoprotein phosphatase substrates for PTEN include

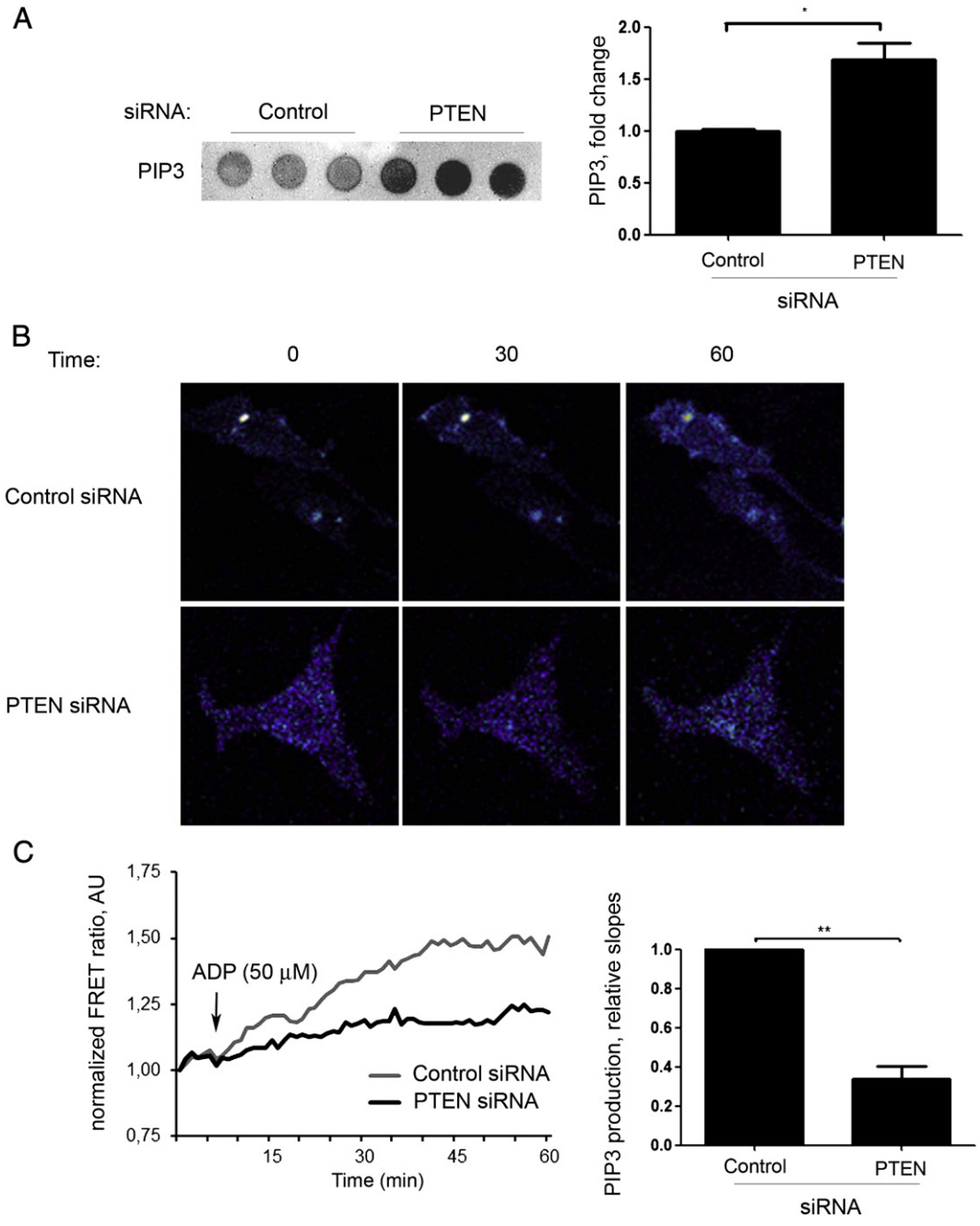

Fig. 5. Analyses of PIP_3 metabolism in endothelial cells following siRNA-mediated PTEN knockdown. Panel A, endothelial cells transfected with control or PTEN siRNA were harvested and lysed, and total cellular PIP_3 levels were measured in a dot-blot assay that was probed with a GST-tagged PIP_3-Grip construct, which was then developed with anti-GST antibody, as described in the text. A representative dot-blot is shown, as well as a bar graph showing pooled data from three experiments, each performed in triplicate. * indicates $p < 0.05$. Panel B shows representative images of endothelial cells transfected with PIP_3 biosensor into control or PTEN siRNA-transfected cells. Cells were then treated with ADP and analyzed for PIP_3-accumulation by FRET microscopy in live cells for 60 min. Panel C, tracings shown are representative of three independent experiments. FRET data were normalized to the basal CFP/YFP ratio at $t = 0$ min. The *bar* graph represents the mean values ± s.e.m. of three independent experiments analyzing ADP-stimulated PIP_3 accumulation at the plasma membrane in control vs. PTEN siRNA-transfected cells. ** indicates $p < 0.01$.

different MAPKs, such as p38 MAPK [20]. Importantly, in our current study, we found that ADP promotes the robust phosphorylation of p38 MAPK, and we also found that ADP-promoted p38 MAPK phosphorylation was significantly increased after siRNA-mediated PTEN knockdown (Fig. 1 and 2). The fact that basal eNOS activity increases following PTEN knockdown may suggest a role for PTEN in the maintenance of basal vascular tone, yet the fact that ADP treatment further augments eNOS phosphorylation and activation suggests that ADP-mediated eNOS activation also is modulated by PTEN. Our studies provide evidence indicating that the inhibition of PTEN may be a

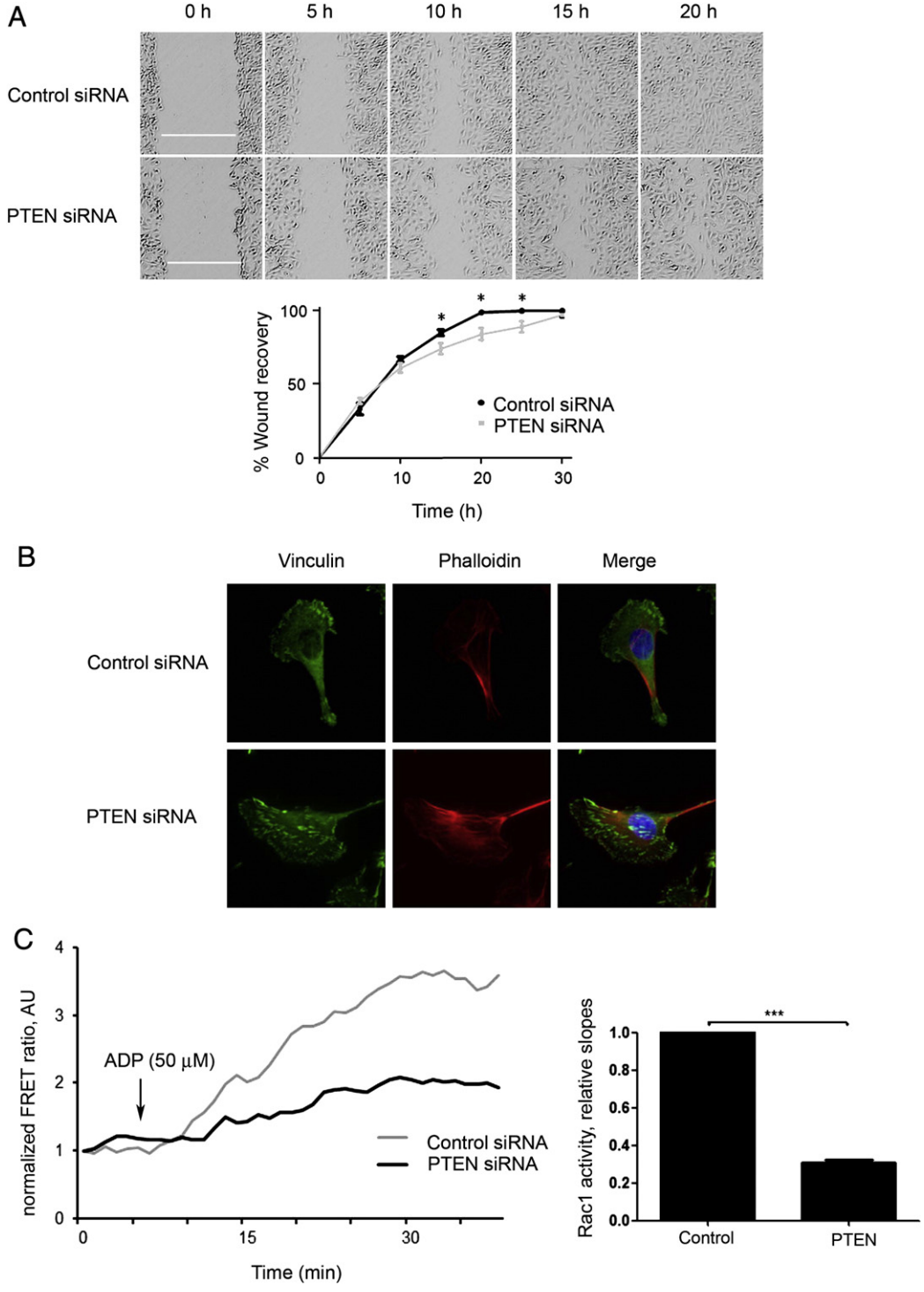

Fig. 6. PTEN modulates actin organization and Rac1 activity in endothelial cells. Cell migration was recorded by phase contrast microscopy over a 20 h time course following wound scratch in endothelial cells transfected with either control or PTEN siRNA. Pooled data of the wound recovery from three independent experiments is quantified as a function of time (Panel A). Panel B, confocal micrographic images of endothelial cells that were transfected with control or PTEN siRNA, and then fixed and stained with phalloidin to reveal actin (red) in the left panels or with vinculin antibodies to show focal adhesion contacts (green) in the middle panels; the merged images in the right-hand panels show the overlap between actin and vinculin staining in yellow. Panel C shows the results of a Rac1 activity assay analyzed using a FRET-based Rac1 biosensor. Cells transfected with control or PTEN siRNA were co-transfected with a FRET-based active Rac1 biosensor, and then treated with ADP or vehicle. FRET was recorded in live cells for 60 min following ADP treatment, as described in the text. The FRET values were normalized to the basal CFP/YFP ratio at 0 min; a representative experiment is shown. The bar graph represents the mean values ± SEM of three independent experiments analyzing Rac1 activity in control vs. PTEN siRNA-transfected cells after stimulation with ADP for 40 min. *** indicates $p < 0.0001$.

determinant of eNOS-dependent endothelium relaxation responses [50]. p38 MAPK and eNOS crosstalk have been previously observed in various endothelial models [24]. The current studies have established that ADP-stimulated eNOS activation is markedly attenuated by the inhibition of p38 MAPK. These findings support a model in which p38 MAPK acts as a key modulator of ADP signaling to eNOS (Fig. 3). Our "double knockdown" experiments targeting both p38 MAPK and PTEN (Fig. 4) indicated that the increased eNOS activation promoted by siRNA-mediated PTEN knockdown was blocked by also knocking down p38 MAPK. Taken together, these findings suggest that ADP-promoted NO production is enhanced by PTEN phosphorylation and inactivation, which then permits the sequential activation of p38 MAPK and NOS in endothelial cells. From the pathophysiological point of view, it is enticing to suggest that a feedback loop may exist between platelet-derived ADP and endothelium-derived nitric oxide that modulates the prothrombotic/proinflammatory consequences of platelet aggregation. Indeed, ADP released by platelets may interact with endothelial P2Y1 receptors and inactivate PTEN leading to eNOS activation and NO synthesis with subsequent inhibition of platelet aggregation. These may have important clinical implications when considering the effects of ADP receptor antagonists in the treatment of cardiovascular events.

A key enzymatic activity of PTEN is its role as a lipid phosphatase able to catalyze dephosphorylation of PIP_3 to PIP_2 [51,52]. As expected, we found that PTEN knockdown yielded a marked increase in steady state PIP_3 levels in endothelial cells (Fig. 5A). Analyses in living cells revealed that ADP-dependent increases in PIP_3 were no longer seen following siRNA-mediated PTEN knockdown, consistent with our finding that ADP promotes PTEN inhibition, such that when PTEN is knocked down, further ADP-stimulated PTEN-mediated PIP_3 response is no longer observed (Fig. 5B). Through its role in phosphatidylinositol homeostasis, PTEN has been described to be implicated in cell polarity and migration, leading to cytoskeleton reorganization [53,39]. siRNA-mediated PTEN knockdown markedly suppressed directional migration of endothelial cells, while PIP_3 increase into the cells is enough to stimulate polarized accumulation of F-actin [40] (Fig. 6). Upon PTEN inhibition, PIP_3 accumulates and promotes the activation of a subset of proteins involved in actin remodeling and cell motility, including Rac1 [13,14,54,55]. We analyzed Rac1 activity in living cells using a Rac1 FRET biosensor, and found that Rac1 activation in response to ADP was decreased following siRNA-mediated PTEN. These results implicate PTEN inactivation in the pathway leading from ADP receptor stimulation to activation of Rac1.

5. Conclusion

These studies demonstrate a role for PTEN in ADP receptor-dependent eNOS activation. Our results support a model (Fig. 7) in which ADP promotes the transient phosphorylation and inactivation of PTEN, leading to an increase in PIP_3 levels, which then triggers the activation of PI3K/Akt, p38 MAPK, and an enhanced eNOS activity. Furthermore we establish PTEN and PIP_3 levels as critical mediators for Rac1 activation and ADP-induced cell migration. These studies add new insight into the response of the endothelium to extracellular ADP, potentially identifying novel targets for the pharmacological regulation of NO signaling.

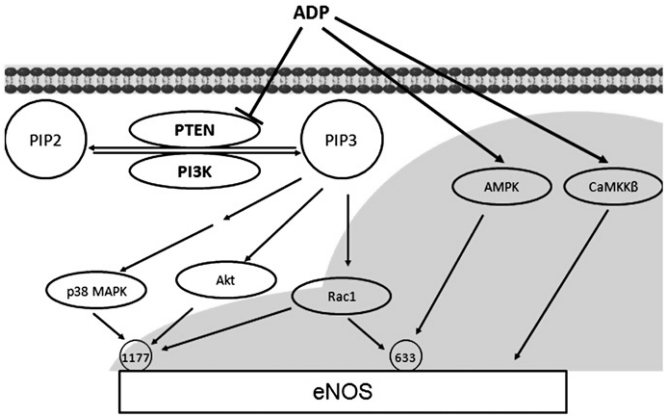

Fig. 7. Proposed model for the role of PTEN in ADP-dependent nitric oxide production. This figure shows a model portraying some of the key interactions between signaling pathways that are modulated by ADP and lead to the activation of eNOS and the production of nitric oxide. Activation by extracellular ADP of cell surface P2Y1 receptors leads to the phosphorylation and subsequent inactivation of PTEN in endothelial cells, which in turn attenuates PTEN-catalyzed PIP_3 hydrolysis and thereby increases intracellular PIP_3. The resulting increase in PIP_3 levels leads to the activation of kinases Akt and p38 MAPK and promotes eNOS and Rac1 activation.

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