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

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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

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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