Angiotensin II stimulates phosphorylation of an ectodomain-truncated platelet-derived growth factor receptor-β and its binding to class IA PI3K in vascular smooth muscle cells

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PI3K (phosphoinositide 3-kinase) activity is involved in Ang (angiotensin) II-stimulated VSMC (vascular smooth muscle cell) growth and hypertrophy. In the present study, we demonstrate that the inhibition of PI3K in VSMCs by expression of a dominant-negative p85α mutant lacking the p110-binding domain (Δp85), or by treatment of cells with LY294002, inhibited Ang II-stimulated PAI-1 (plasminogen activator inhibitor-1) mRNA expression. Using a GST (glutathione S-transferase) fusion protein containing the p85 N-terminal SH2 (Src homology 2) domain as 'bait' followed by MS/MS (tandem MS), we identified a 70 kDa fragment of the p70 PDGFR-β (platelet-derived growth factor receptor-β) as a signalling adapter that is phosphorylated and recruits the p85 subunit of PI3K after Ang II stimulation of AT_1 (Ang II subtype 1) receptors on VSMCs. This fragment of the PDGFR-β, which has a truncation of its extracellular domain, accounted for approx. 15 % of the total PDGFR-β detected in VSMCs with an antibody against its cytoplasmic domain. Stimulation of VSMCs with Ang II increased tyrosine-phosphorylation of p70 PDGFR-β at Tyr^{751} and Tyr^{1021} and increased its binding to p85. PDGF also induced phosphorylation of p70 PDGFR-β, a response inhibited by the PDGF tyrosine kinase selective inhibitor, AG1296. By contrast, Ang II-induced phosphorylation of the 70 kDa receptor was not affected by AG1296. Ang II-stimulated phosphorylation of the p70 PDGFR-β was blocked by the AT_1 receptor antagonist, candesartan (CV 11974) and was partially inhibited by PP2 {4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine}, an Src family kinase inhibitor. Our result suggests that the p70 PDGFR-β functions as an adapter that recruits PI3K to the membrane upon AT_1 receptor stimulation.

Key words: angiotensin (Ang), phosphoinositide 3-kinase (PI3K), platelet-derived growth factor receptor-β (PDGFR-β), receptor stimulation, tyrosine phosphorylation, vascular smooth muscle cell (VSMC).

INTRODUCTION

The renin–Ang (angiotensin) system exerts a central role in blood pressure control and vascular homoeostasis. Activation of the AT_1 (Ang II subtype 1) receptor on vascular smooth muscle induces a plethora of responses that contribute to vascular hypertrophy, inflammation and fibrosis. AT_1 receptor signalling in VSMCs (vascular smooth muscle cells) is mediated, in part, by its activation of PI3K (phosphoinositide 3-kinase) [1]. Inhibition of PI3K activity by treatment of VSMCs with LY294002 or wortmannin decreases AT_1-receptor-stimulated cell proliferation, gene expression and activation of Akt/protein kinase B, mammalian target of rapamycin, and p70 S6 kinase [1–5]. These findings demonstrate that PI3K plays an important role in AT_1 signalling and hypertrophic reactions in VSMCs.

PI3K is a ubiquitous family of lipid kinases. Class I enzymes phosphorylate PtdIns(3,4)P_2 to generate PtdIns(3,4,5)P_3, a second messenger that mediates the recruitment of proteins with pleckstrin homology domains to the membrane. G-protein-coupled receptors, including the AT_1 receptor, can activate multiple isoforms of PI3K, including both class Ia (p110 α,β,δ) and class Ib (p110γ) enzymes [1,6]. PI3K class Ia enzymes consist of heterodimers with a regulatory subunit (p85, p55 and p50) and a 110 kDa catalytic subunit. Ang II has been shown to increase the level of tyrosine phosphorylation of p85 and induce its translocation from the cytosolic to the membraneous fraction in VSMCs [1,7]. Ang II-stimulated tyrosine phosphorylation in p85 is blocked by the AT_1 receptor antagonist losartan and inhibitors of the IGF-1 (insulin-like growth factor-1) receptor kinase [7,8]. Translocation of PI3K from the cytosol to the plasma membrane is mediated by an p85 SH2 (Src homology 2) domain binding to tyrosine-phosphorylated proteins at YXXM motifs. This SH2-binding of p85 decreases its inhibitory effect on the p110 catalytic domain and juxtaposes PI3K with the plasma membrane, which contains its substrate, PtdIns(4,5)P_2 [9]. Progress regarding the characterization of G-protein-coupled-receptor-stimulated tyrosine phosphorylation has led to the identification of p130 CAS (Crk-associated substrate) and SAM68 (68 kDa Src associated during mitosis) as AT_1-receptor-stimulated tyrosine-phosphorylated proteins that bind to the p85 subunit of PI3K [7,10].

Although PI3K has been implicated in Ang II-stimulated gene expression [2], information on the mechanisms involved in this Ang II-mediated action is limited. Previously, our group and others have shown that activation of the AT_1 receptor in VSMCs and in vascular tissue *in vivo* up-regulates expression of PAI-1 (plasminogen activator inhibitor-1) [11–15], the primary inhibitor of plasminogen activation [16]. Although there is considerable physiological evidence for a role for the AT_1 receptor in the regulation of PAI-1, much less is known regarding the signalling mechanisms that regulate its expression. AT_1-receptor-stimulated

Abbreviations used: Ang, angiotensin; AT_1, Ang II subtype 1; CAS, Crk-associated substrate; GST, glutathione S-transferase; IGF-1, insulin-like growth factor-1; MS/MS, tandem MS; PAI-1, plasminogen activator inhibitor-1; PDGFR-β, platelet-derived growth factor receptor-β; PI3K, phosphoinositide 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; PTP2, protein tyrosine phosphatase 2; SH2, Src homology 2; Shc, Src homology and collagen homology; VSMC, vascular smooth muscle cell.

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PAI-1 expression is mediated, in part, by the Rho kinase and MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase)]/ERK pathways [17,18]. Although tyrosine phosphorylation has been implicated in AT_1-receptor-stimulated PAI-1 expression [18], little is known regarding the role of PI3K in these pathways.

In the present study, we have investigated the role of PI3K in Ang II-stimulated PAI-1 expression in VSMCs. To characterize the mechanisms that mediate Ang II-stimulated recruitment of PI3K to the plasma membrane, we have used a proteomic approach to identify the Ang II/AT_1-receptor-stimulated tyrosine-phosphorylated proteins that bind to the p85 regulatory subunit of PI3K.

MATERIALS AND METHODS

Materials

Ang II and recombinant rat PDGF (platelet-derived growth factor)-BB were obtained from Sigma–Aldrich Co. (St. Louis, MO, U.S.A.). LY294002 and PP2 {4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine} were obtained from Calbiochem (San Diego, CA, U.S.A.). Antibodies against cortactin, p130 CAS, phospho-PDGFR (PDGF receptor-β) (p-Tyr^{1021}), PDGFR-β (C-terminus) and SH-PTP2 (protein tyrosine phosphatase 2) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). An anti-PAI-1 antibody was from American Diagnostica Inc. (Stamford, CT, U.S.A.). A polyclonal antibody against the phospho-PDGFR-β (p-Tyr^{751}), and a monoclonal anti-phosphotyrosine antibody (p-Tyr^{100}) were from Cell Signaling Technology (Beverly, MA, U.S.A.). Polyclonal anti-phosphotyrosine and anti-(PI3K p85) antibodies were from Upstate Biotechnology (Charlottesville, VA, U.S.A.). The GST (glutathione S-transferase)-p85 SH2 N-terminal construct, containing amino acid residues 330–436 of human p85, was provided by Dr Martin G. Myers Jr (Department of Medicine and Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI, U.S.A.). GST fusion proteins were expressed in bacteria and affinity-purified with glutathione–Sepharose beads (Amersham, Piscataway, NJ, U.S.A.). Candesartan (CV 11974) was provided by Astra Hassle AB (AstraZeneca R&D, Mölndal, Sweden).

Cell culture and adenoviral-mediated gene transfer

VSMCs were isolated from aortic explants from Sprague–Dawley rats and cultured in Dulbecco's modified Eagle's medium with 5 % BSA (Invitrogen, Carlsbad, CA, U.S.A.) as described previously [12]. In some studies, cultured VSMCs were transfected with adenoviral vectors that contained either a β-galactosidase control or dominant-negative p85α lacking the p110 binding site (Δp85), provided by Dr C. Ronald Kahn (Research Division, Joslin Diabetes Center, Boston, MA, U.S.A.) and Dr Masato Kasuga (Department of Clinical Molecular Medicine, Kobe University, Kobe, Japan) [19]. Adenoviruses were used at a multiplicity of infection of 10, which resulted in a transfection efficiency of VSMC cultures of approx. 80–90 %. Cells were cultured in 0.1 % (w/v) BSA for 18 h before stimulation with Ang II or PDGF.

RNA isolation and Northern blot analysis

VSMCs were pre-treated with the PI3K inhibitor LY294002 for 15 min or were transfected with a dominant-negative Δp85 PI3K regulatory subunit [19] for 2 days followed by an additional 3 h of incubation in the presence or absence of Ang II (100 nM). Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). RNA was separated by agarose gel electrophoresis and PAI-1 mRNA was probed using a cDNA probe against rat PAI-1, as described previously [11]. Expression of acidic ribosomal phosphoprotein (36B4) RNA levels was also determined by Northern blot analysis. Levels of mRNA expression were visualized and quantified by PhosphorImager analysis (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.).

Immunoprecipitation and immunoblot analysis

After experimental treatments, the VSMCs were washed twice with ice-cold PBS containing 1 mM Na_3VO_4, and solubilized in ice-cold lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 4 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 0.1 mg/ml aprotinin, 10 % glycerol and 1 % Triton X-100. Lysates were centrifuged at 20 000 ***g*** for 30 min. Protein from total cell lysates (1 mg) was precipitated with 5 μg of the GST–p85 fusion protein coupled glutathione–Sepharose beads or an anti-p85 antibody (1:1000). Precipitates were washed three times with lysis buffer, eluted with Laemmli sample buffer, and separated by SDS/4–12 % PAGE. Protein phosphorylation, phospho-PDGFR-β (p-Tyr^{1021}), phospho-PDGFR-β (p-Tyr^{1021}) and PDGFR-β were detected using anti-tyrosine, anti-phospho-PDGFR-β (p-Tyr^{1021}), anti-phospho-PDGFR-β (p-Tyr^{751}) or anti-PDGFR-β antibodies respectively. Results were visualized by enhanced chemiluminescence (Cell Signaling Technology) and quantified using ImageQuant 5.0 (Molecular Dynamics).

MS

Ang II-treated (100 nM, 5 min) VSMC lysates of 240 mg of protein were incubated with 100 μg of GST–p85 fusion protein coupled to glutathione–Sepharose beads at 4 °C overnight. Precipitates were washed three times with lysis buffer, and eluted using Laemmli sample buffer. Affinity purified proteins were separated by SDS/10 % PAGE, stained with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA, U.S.A.) and excess dye removed by overnight incubation in 7 % acetic acid. Gel slices were digested with 5 ng/μl sequencing-grade modified trypsin (Promega, Madison, WI, U.S.A.) in 25 mM ammonium bicarbonate containing 0.01 % n-octyl glucoside for 18 h at 37 °C. Peptides were eluted from the gel slices with 80 % acetonitrile/1 % formic acid. Tryptic digests were separated by capillary HPLC (C_{18}, 75 μm-internal-diameter Picofrit column; New Objective, Woburn, MA, U.S.A.) using a flow rate of 100 nl/min over a 2 h reverse phase gradient, and were analysed using a LTQ linear ion-trap LC/MS^n system (Thermo Electron, San Jose, CA, U.S.A.). Resultant MS/MS spectra were matched against a rat database using TurboSequest (BioWorks 3.1, Thermo Finnigan) with fragment-ion tolerance < 0.5 and amino acid modification variables, including phosphorylation (80 Da) of serine, threonine and tyrosine, oxidation (16 Da) of methionine, and methylation (14 Da) of lysine.

Statistical analyses

All statistical analyses were performed by a one-way ANOVA or an unpaired Student's *t* test (GraphPAD Software, San Diego, CA, U.S.A.). Results are expressed as the fold change (means $\pm$ S.E.M.) compared with controls in the absence of Ang II treatment. Values of $P < 0.05$ were considered significantly different.

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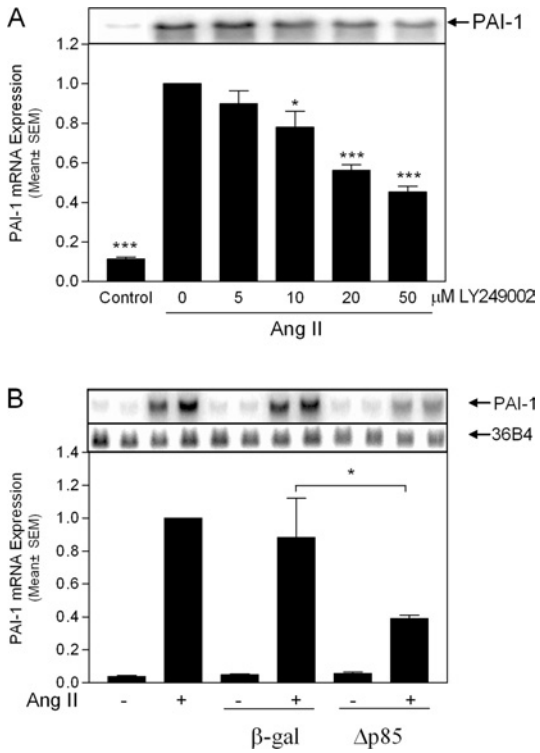

Figure 1 Role of PI3K on Ang II-induced PAI-1 mRNA expression

Northern blot analysis shows the effect of LY294002 on Ang II-stimulated PAI-1 mRNA expression. Cells were pre-treated for 15 min with LY294002 at the concentrations indicated and stimulated with 100 nM Ang II for 3 h. (**A**) Northern blot analysis shows the effect of adenoviral transfection with Δp85 and β-galactosidase on Ang II-stimulated PAI-1 mRNA expression. (**B**) Representative Northern blots and a bar graph show quantification from three independent experiments (means ± S.E.M.) with PAI-1. Significant effects on different treatment groups when compared with Ang II-stimulation alone or a control are indicated as *, $P < 0.05$ and ***, $P < 0.001$. 36B4, acidic ribosomal phosphoprotein.

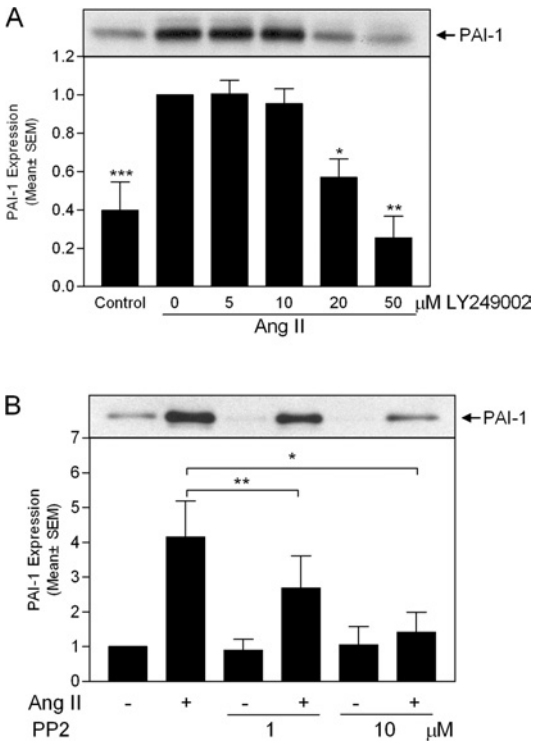

Figure 2 Effects of PI3K and Src-kinase-inhibition on Ang II-induced PAI-1 protein expression

Western blot analysis and a bar graph show quantification of the effect of LY294002 on Ang II-stimulated PAI-1 protein expression. Cells were pre-treated with LY294002, stimulated with Ang II for 18 h, and PAI-1 level in the culture medium was measured. (**A**) Western blot analysis and a bar graph show quantification of the effect of the Src kinase inhibitor, PP2, on Ang II-stimulated PAI-1 protein expression. Cells were pre-treated with PP2, stimulated with Ang II for 18 h, and PAI-1 levels in the culture medium were measured. (**B**) Values are expressed as the fold-change compared with unstimulated or treated controls. Significant effects on different treatment groups when compared with Ang II stimulation alone or a control are indicated as *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$. Values are expressed as the fold change compared with unstimulated or treated controls.

RESULTS

To examine the role of PI3K in Ang II-stimulated PAI-1 expression, quiescent VSMCs were pre-treated with the PI3K inhibitor LY294002 for 15 min, followed by an additional 3 h of incubation in the presence or absence of Ang II (100 nM). Cells were harvested and PAI-1 mRNA levels were quantified by Northern blot analysis. Stimulation of cells with Ang II increased PAI-1 mRNA expression by 9.1 ± 0.9-fold (Figure 1A). Pre-treatment of cells with LY294002 decreased Ang II-stimulated PAI-1 expression in a concentration-dependent manner by up to 55 % ± 3 % at 50 μM ($P < 0.001$) (Figure 1A). Similarly, pre-treatment of cells with the PI3K inhibitor, wortmannin, decreased Ang II-stimulated PAI-1 mRNA levels by 50 % with an IC_{50} of approx. 30 nM (results not shown).

The role of p85 in Ang II stimulated PAI-1 expression was evaluated in VSMCs transfected with a dominant-negative Δp85 PI3K regulatory subunit. At 2 days after incubation of VSMCs with adenoviral vectors that contained expression constructs for either Δp85 or β-galactosidase, cells were stimulated with Ang II (3 h, 100 nM), and PAI-1 mRNA expression was examined. The levels of Ang II-stimulated PAI-1 expression in untransfected and β-galactosidase-transfected cells were similar (Figure 1B), and show that the transfection protocol did not induce PAI-1 expression or block Ang II-induced PAI-1 expression. By contrast, Ang II-stimulated PAI-1 expression in cells transfected with Δp85 was decreased by 48.2 % ± 14.4 % ($P < 0.05$), compared with β-galactosidase-transfected control cells (Figure 1B). These findings show that PI3K activity, via a p85-sensitive mechanism, contributes to Ang II-stimulated PAI-1 expression in VSMCs.

In order to determine whether PI3K also contributed to Ang II-stimulated PAI-1 protein production, VSMCs were incubated with Ang II for 18 h in the presence or absence of LY294002, and PAI-1 levels in the conditioned medium were quantified by Western blot analysis using an anti-PAI-1 antibody. Treatment of cells with 20 μM and 50 μM LY294002 decreased Ang II-stimulated PAI-1 synthesis by 70 % and to basal level respectively (Figure 2A). Since Src kinase has been implicated in AT$_1$-mediated transactivation of IGF-1, EGF-R (epidermal growth factor-receptor), and the activation of p85-coupled PI3K [8,20], the role of tyrosine phosphorylation in Ang II-stimulated PAI-1 synthesis was examined. Treatment of cells with 1 μM and 10 μM PP2, a Src-related kinase inhibitor, decreased Ang II-stimulated PAI-1 levels in the conditioned medium by 43 % and 89 % respectively (Figure 2B). These results confirm that tyrosine phosphorylation, possibly via an Src related kinase, contributes to Ang II-stimulated PAI-1 expression.

To further characterize the mechanism which mediates Ang II stimulation of PI3K, lysates from unstimulated cells and cells treated with Ang II (100 nM, 5 mins) were precipitated with

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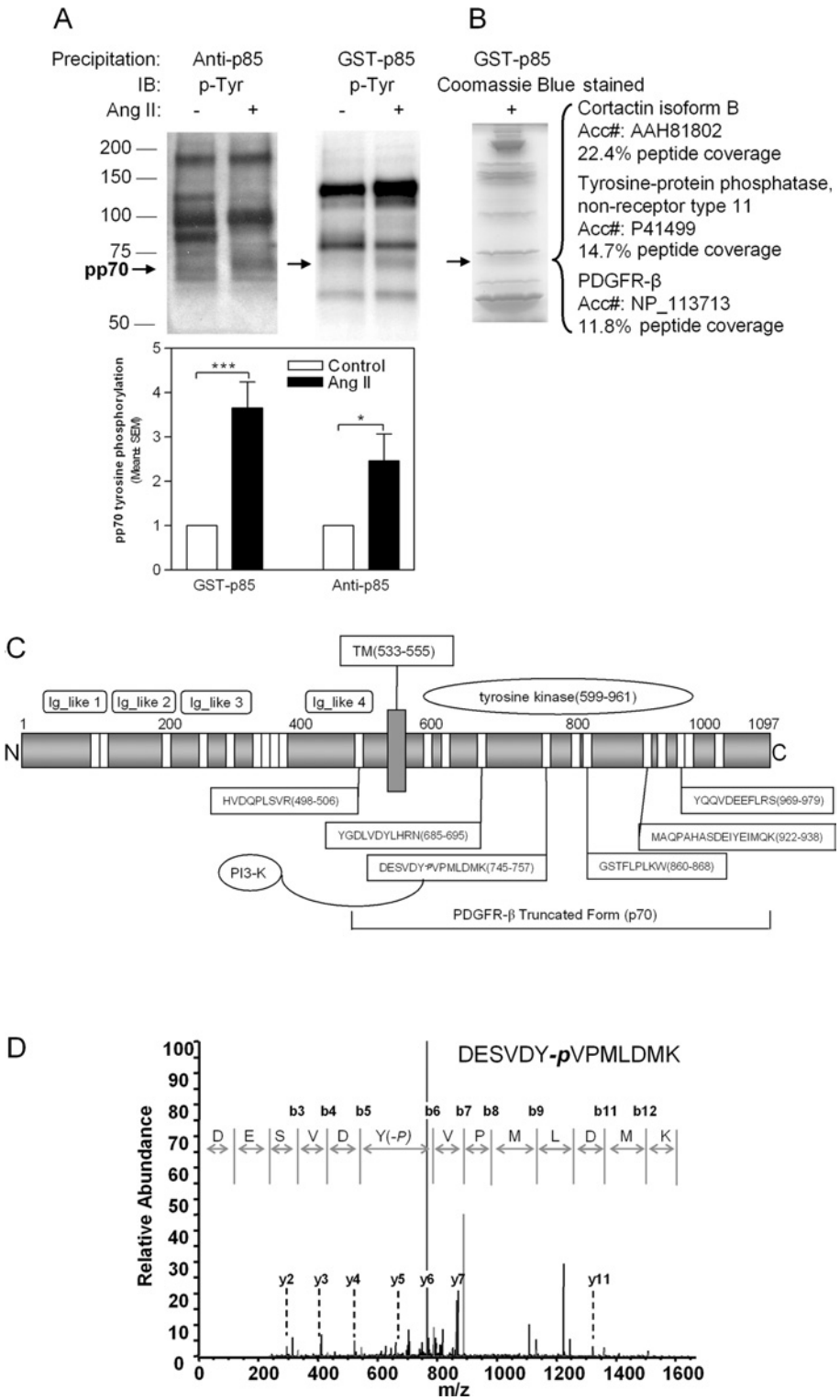

Figure 3 Identification of Ang II-stimulated p85 binding proteins

(**A**) VSMCs were stimulated with 100 nM Ang II for 5 min. Cell lysates were precipitated with an anti-(PI3K p85) antibody or the GST fusion protein containing the N-terminal SH2 domain of p85 and were subsequently Western blotted with an anti-p-Tyr antibody. Representative immunoblots (IB) and a bar graph show quantification of the tyrosine phosphorylation of a 70 kDa band are shown. The results are expressed as the fold change in stimulation over the basal level. Data shown are the means ± S.E.M. for at least 3 independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$. (**B**) PI3K-binding proteins were isolated from cell lysates of Ang II-simulated VSMCs and were stained with Coomassie Blue. The segment of the gel at 70 kDa was analysed by LC-MS/MS and the proteins identified are shown. (**C**) Schematic diagram shows the peptide coverage of the PDGFR-β identified by MS in the p70 kDa band. The locations of the peptide sequences identified in the p70 kDa band are indicated. The locations of peptides identified for the PDGFR-β isolated from the 190 kDa band are indicated as white bars. The phosphorylated tyrosine on p70 PDGFR-β, identified by LC-MS/MS, is indicated by ($^{-p}$). (**D**) MS/MS spectra of the Tyr^{751}-phosphorylated peptide (DESVDY-***p***VPMLDMK) of PDGFRβ, isolated from the 70 kDa band shown in Figure 3(B). Fragmentation y^{+} ions and b^{+} ions are labelled.

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GST–p85, or immunoprecipitated with an anti-p85 antibody. Proteins that bound to p85 were separated by SDS/PAGE, tyrosine-phosphorylated proteins were visualized by anti-phosphotyrosine antibody immunoblotting. Comparison of Western blots of these p85-associated proteins revealed that the amount of the tyrosine-phosphorylated 70 kDa protein present in both GST–p85 and anti-p85 antibody precipitates was increased after Ang II stimulation by 3.5- and 2.5-fold respectively (Figures 3A and 3B). In addition, we observed two tyrosine-phosphorylated bands, located at 85 and 120 kDa, which were associated with p85 immunoprecipitates from unstimulated cells, but not from Ang II-stimulated cells. These results suggest that Ang II produces multiple changes in the proteins associated with PI3K and subsequently an increase in the association of p85 with a protein of approx. 70 kDa.

In order to identify this 70 kDa p85-binding protein, VSMCs were stimulated with Ang II (100 nM, 5 mins) and p85-binding proteins were isolated using GST–p85 affinity beads. The isolated protein fraction was separated by SDS/PAGE and visualized by Coomassie Blue staining. The protein band at 70 kDa was excised, trypsinized and characterized by LC-MS/MS analysis. Three proteins were identified in this band, including cortactin isoform B (accession number AAH81802), tyrosine-protein phosphatase non-receptor type 11 (accession number P41499; PTP2), and PDGFR-β (accession number NP_113713). The identification of PDGFR-β in this band was unexpected, since the full-length PDGFR-β migrates at 190 kDa. Mapping of the tryptic peptides identified in the pp70 (phosphorylated p70) band revealed six tryptic peptides of the PDGFR-β, including five in the cytosolic domain and only one peptide (HVDQPLSVR) located approx. 25 amino acids N-terminal to the membrane-spanning sequence (Figure 3C). In addition, further analysis of MS/MS spectra identified phosphorylation on Tyr^{751} of the tryptic peptide, DESVDY^{-p}VPMLDMK (amino acids 745–757) (Figure 3D), which has been shown to mediate SH2 binding to p85 [21]. MS analysis of a trypsin digest of the band at 190 kDa identified additional PDGFR-β tryptic peptides located in both the extracellular and cytosolic domains (Figure 3C). These results suggest that the phosphorylated PDGFR-β in the 70 kDa band contains the cytosolic and membrane-spanning domains of the PDGFR-β and is truncated in the juxtamembrane region of the ectodomain.

The effect of Ang II on cortactin, PTP2 and p70 PDGFR-β binding to GST–p85 was examined by immunoblot analysis. This study confirmed that all of these three proteins bound to GST–p85 in the absence of stimulation with Ang II (Figure 4A). Stimulation with Ang II did not affect the levels of cortactin and PTP2 binding to GST–p85. By contrast, Ang II increased p70 PDGFR-β binding to GST–p85 by approx. 2-fold ($P < 0.05$). In order to determine whether Ang II could also increase endogenous p85 binding to phosphorylated p70 PDGFR-β, cell lysates from control and Ang II-stimulated cells were immunoprecipitated with an anti-p85 antibody and immunoblotted with an anti-PDGFR-β (p-Tyr^{751}) phospho-specific antibody. This experiment showed that Ang II stimulated a 2-fold increase in the amount of phosphorylated p70 PDGFR-β associated with p85 (Figure 4B).

The effect of Ang II on the phosphorylation of the truncated receptor in total cell lysates was also examined. Immunoblotting of the cell lysate with a C-terminal antibody detected both full-length (190 kDa) and truncated (70 kDa) forms of the PDGFR-β (Figure 4). The protein level of p190 PDGFR-β was 5.3-fold greater than that of p70 PDGFR-β. Stimulation with Ang II did not affect the relative amounts of these two forms of the PDGF receptor. Immunoblotting with anti-phosphospecific PDGFR-β antibodies revealed that Ang II induced phosphorylation of both Tyr^{1021} and Tyr^{751} residues of the p70 PDGFR-β by 2-fold, but did not significantly affect phosphorylation of the full-length

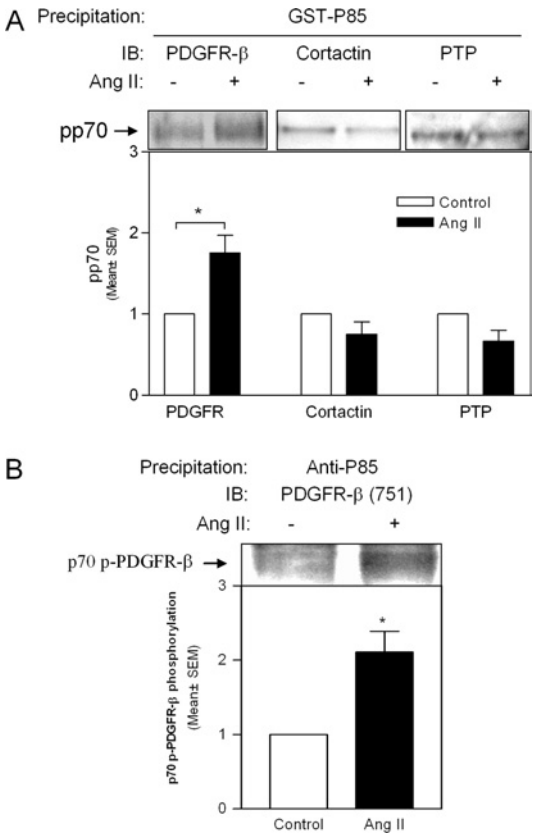

Figure 4 Ang II-stimulated p85-binding and tyrosine phosphorylation in a truncated PDGFR-β mutant

VSMCs were stimulated with Ang II. The resulting lysates were precipitated with GST–p85 fusion protein (**A**) or an anti-(PI3K p85) antibody (**B**) and immunoblotted (IB) with antibodies against the PDGFR-β C-terminal domain, cortactin, PTP or p-PDGFR-β (p-Tyr^{751}), as indicated. Representative immunoblots and a bar graph show quantification of p70 PDGFR-β phosphorylation (values are expressed as the fold change compared with unstimulated or treated controls; means $\pm$ S.E.M.) for at least three independent experiments. Significant differences are indicated as $^*P < 0.05$.

PDGFR-β (Figure 5). Time-course studies demonstrated that Ang II-stimulated phosphorylation of the p70 PDGFR-β at Tyr^{1021} occurred rapidly after 2 min, followed by a gradual return towards basal levels after 1 h (Figure 6A). Pre-treatment of cells with the AT_1 receptor antagonist candesartan (CV-11974, 1 μM) completely blocked Ang II-stimulated phosphorylation of the p70 PDGFR-β (Figure 6B).

Stimulation with PDGF (25 ng/ml, 5 min) increased Tyr^{1021} phosphorylation of both the full-length and p70 PDGFR-β (Figure 7A). Pre-treatment of cells with the PDGFβ-R kinase inhibitor, AG1296 (5 μM), decreased PDGF-stimulated Tyr^{1021}-phosphorylation of 190 kDa PDGFR-β by 70 % and completely blocked phosphorylation of the 70 kDa receptor. Ang II did not affect phosphorylation of the full-length receptor. However, Ang II stimulated Tyr^{1021}-phosphorylation on the p70 PDGFR-β by 3.6 ± 1.4-fold, and this response was not affected by AG1296. By contrast, treatment of cells with PP2 decreased Ang II-stimulated phosphorylation of Tyr^{1021} by 62 % ($P < 0.05$) (Figure 7B).

DISCUSSION

The present study identified a 70 kDa fragment of the PDGFR-β as a signalling adapter that could mediate AT_1-receptor-stimulated

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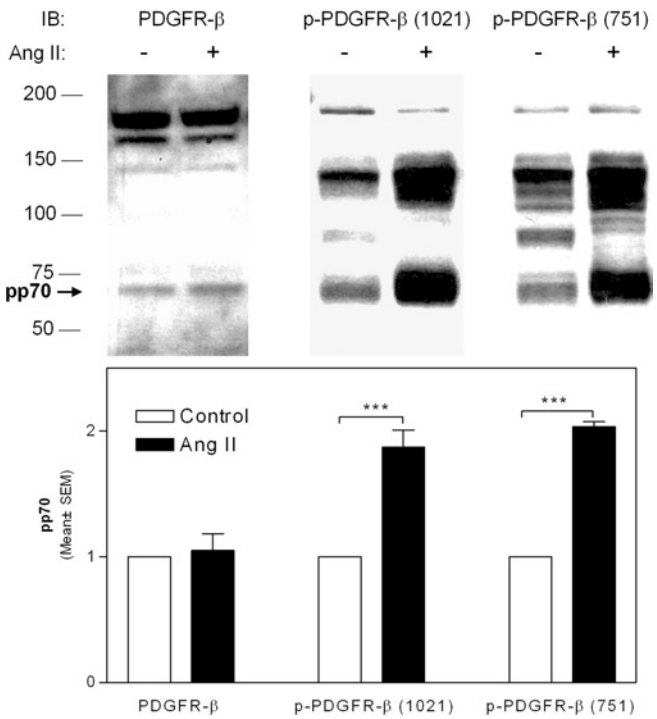

Figure 5 Effect of Ang II on PDGFR-β phosphorylation

VSMCs were stimulated with 100 nM Ang II for 5 min and cell lysates were immunoblotted (IB) with an anti-PDGFR-β antibody, and anti-PDGFR-β phosphospecific antibodies against p-Tyr^{1021} and p-Tyr^{751}. Results are expressed as the fold change in stimulation (means $\pm$ S.E.M) compared with unstimulated cells. Bar graphs show the results for at least three independent experiments. ***$P < 0.001$; stimulated versus unstimulated cells.

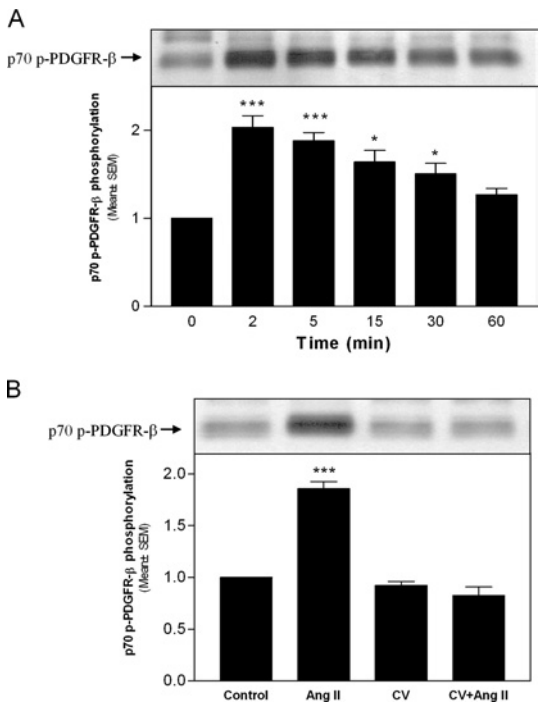

Figure 6 Time course and effect of AT_1 receptor antagonism on Ang II-stimulated phosphorylation of p70 PDGFR-β

VSMCs were stimulated with Ang II for the indicated times (**A**). Cells were pre-treated with candesartan (CV 11974) for 30 min followed by stimulation with Ang II (100 nM) for 5 min (**B**). Immunoblotting was performed with anti-phospho-PDGFR-β (p-Tyr^{1021}) antibodies. Results are expressed as the fold change in stimulation (means $\pm$ S.E.M) compared with unstimulated cells. Bar graphs and immunoblots show the results for at least three independent experiments. Significant differences are indicated as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ for stimulated versus unstimulated cells.

activation of PI3K in VSMCs, suggesting a novel role for truncated growth factor receptors in G-protein-coupled-receptor signalling. Although there is growing evidence that PI3K contributes to Ang II's hypertrophic effects in VSMCs [1,3,4,20], the mechanisms that couple the AT_1 receptor to the PI3K pathway are not fully understood. In the present study, we report that inhibition of PI3K by overexpression of Δp85 decreased Ang II-stimulated PAI-1 mRNA expression by 50–60 %. A similar response was also observed after treatment of VSMCs with the PI3K inhibitors LY295002 or wortmannin and by the Src family tyrosine kinase inhibitor PP2. Since the Δp85 construct contains its SH2 domains but does not bind the p110 catalytic subunit, these results suggest that Ang II-stimulated tyrosine phosphorylation of adapter proteins is required for its activation of PI3K.

To identify the tyrosine-phosphorylated proteins that bind to p85 after stimulation with Ang II, we used a GST fusion protein containing the p85 N-terminal SH2 domain as 'bait', and characterized affinity-purified proteins by Western blotting of tyrosine-phosphorylated proteins and MS/MS. As expected, a number of tyrosine-phosphorylated proteins were isolated by affinity purification using the anti-p85 antibody, both in the basal state and after stimulation with Ang II. Anti-phosphotyrosine Western blotting of proteins bound to p85 revealed a 70 kDa band from both anti-p85 antibody and GST–p85 precipitates that was increased after stimulation with Ang II. The identification of PDGFR-β in the 70 kDa band was surprising, since, to our knowledge, this fragment had not previously been shown to accumulate in VSMCs.

Peptide coverage obtained by MS indicated that this PDGFR-β fragment contains the intracellular domain and short segment of the fourth extracellular Ig-like domain, including at least 35 residues N-terminal to the membrane-spanning sequence (residues 533–555). The p70 PDGFR-β lacked most of the extracellular sequence, including the second and third Ig-like domains that mediate PDGF ligand binding [22]. Stimulation of VSMCs with Ang II increased tyrosine-phosphorylation of the p70 PDGFR-β at Tyr^{751} and Tyr^{1021} and its binding to both GST–p85 and endogenous p85.

Although Ang II-stimulated transactivation of the PDGF receptors has been previously reported [23,24], the present study is the first to identify a truncated growth factor receptor as a potential signalling adapter for the AT_1 receptor. In addition, our results show that the AT_1 receptor preferentially phosphorylates the truncated p70 PDGFR-β compared with its effect on the full-length receptor at 190 kDa. Ang II-stimulated phosphorylation of the p70 PDGFR-β was blocked by the AT_1 receptor antagonist CV 11974. PDGF also induced phosphorylation of the p70 PDGFR-β, a response inhibited by the PDGF tyrosine kinase selective inhibitor AG1296. By contrast, Ang II-induced phosphorylation of the 70 kDa receptor was not affected by AG1296, suggesting that phosphorylation of this receptor fragment does not require the PDGFR-β tyrosine kinase. Ang II-stimulated phosphorylation of p70 PDGFR-β was partially inhibited by PP2, a Src family kinase inhibitor, but not by the Jak2 kinase inhibitor, AG490 (results not shown). A previous study has shown that AG1296 did not inhibit Ang II-stimulated tyrosine-phosphorylation of p190 PDGFR-β and 66 kDa Shc (Src homology and collagen homology), which was immunoprecipitated using an anti-Shc antibody [23]. In the present study, we did not detect Shc peptides in the proteomic analysis of the p70

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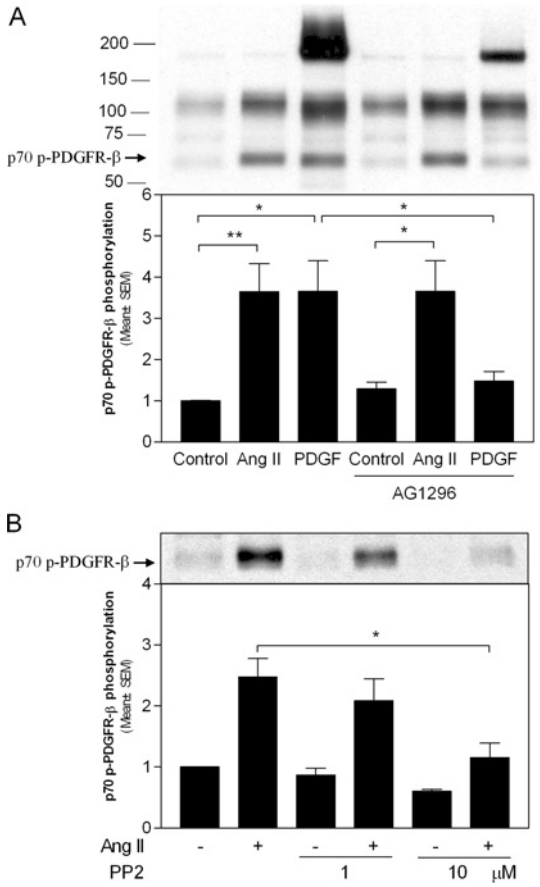

Figure 7 Inhibition of p70 PDGFR-β phosphorylation

VSMCs were pre-treated with either AG1296 (**A**) or PP2 (**B**) for 15 min, followed by a 5 min stimulation with Ang II or PDGF, as indicated. Immunoblotting was performed with phospho-PDGFR-β (p-Tyr^{1021}) antibodies. Results are expressed as fold change in stimulation (means $\pm$ S.E.M.) compared with unstimulated cells. Bar graphs show the results for at least three independent experiments. Significant differences are indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

band isolated by p85 immunoblotting, and demonstrated using two independent methods, including MS and immunoblotting using phosphospecific anti-PDGFR-β antibodies, that the Ang II-stimulated phosphorylation detected in the p70 protein band is associated with a PDGFR-β sequence.

Previous studies have shown that both α and β isoforms of the PDGFR can be proteolytically cleaved. The soluble 90 kDa fragment of the extracellular domain of the PDGFR-α, which retains its ability to bind PDGF-BB, has been isolated from the conditioned medium of cultured human osteosarcoma cells [25], and the report also described detection of a 70 kDa cellular fragment of the receptor and suggested that these fragments were generated via proteolytic cleavage. Okuyama et al. [26] have reported cathepsin B-mediated cleavage of the PDGFR-β in hepatic stellate cells. Proteolysis of the extracellular domain of the PDGFR has been implicated as a mechanism of receptor down-regulation, and the release of a soluble extracellular fragment that retains ligand-binding activity could act as a competitive autocrine/paracrine inhibitor. Our results suggest that the intracellular fragment of the PDGFR-β accumulates in VSMCs and functions as an adapter that contributes to Ang II-stimulated recruitment of PI3K to the membrane.

While the AT_1 receptor activates both p85-coupled (class Ia) and G$\beta\gamma$-coupled (class Ib) PI3K isoforms [1,6], the contribution from these two PI3K activation pathways to specific AT_1 receptor actions in VSMCs is not fully understood. We show that the dominant-negative Δp85-mutant decreased Ang II-stimulated PAI-1 mRNA levels (Figure 1), suggesting that p85-coupled PI3K contributes to AT_1-induced PAI-1 expression. By contrast, G-protein-coupled PI3K γ deficiency in mice has been shown to block Ang II-induced hypertension and the generation of reactive oxygen species [27]. Indeed, PI3K γ is required for AT_1 activation of L-type calcium channels and protein kinase B/Akt phosphorylation [27]. Thus the contribution of the truncated form of the PDGFR-β to the total pool of Ang II-stimulated PI3K activity and its downstream effects will require further studies.

Since Δp85 inhibits Ang II-induced PAI-1 expression, we propose that Ang II-signalling in VSMCs is mediated by a heterodimeric class Ia PI3K. Using a proteomic method we have identified an ectodomain-truncated form of the PDGFR-β as a signalling adapter that contributes to AT_1-receptor-stimulated recruitment of PI3K to the membrane. These findings reveal a novel pathway for AT_1-receptor-stimulated recruitment of PI3K in VSMCs.

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