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Vascular endothelial growth factor induces heat shock protein (HSP) 27 serine 82 phosphorylation and endothelial tubulogenesis via protein kinase D and independent of p38 kinase

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ABSTRACT

Proteomic analysis identified HSP27 phosphorylation as a major change in protein phosphorylation stimulated by Vascular Endothelial Growth Factor (VEGF) in Human Umbilical Vein Endothelial Cells (HUVEC). VEGF-induced HSP27 phosphorylation at serines 15, 78 and 82, but whereas HSP27 phosphorylation induced by H_2O_2 and TNFα was completely blocked by the p38 kinase inhibitor, SB203580, VEGF-stimulated serine 82 phosphorylation was resistant to SB203580 and small interfering(si)RNA-mediated knockdown of p38 kinase and MAPKAPK2. The PKC inhibitor, GF109203X, partially reduced VEGF-induced HSP27 serine 82 phosphorylation, and SB203580 plus GF109203X abolished phosphorylation. VEGF activated Protein Kinase D (PKD) via PKC, and siRNAs targeted to PKD1 and PKD2 inhibited VEGF-induced HSP27 serine 82 phosphorylation. Furthermore recombinant PKD selectively phosphorylated HSP27 at serine 82 in vitro, and PKD2 activated by VEGF in HUVECs also phosphorylated HSP27 selectively at this site. Knockdown of HSP27 and PKDs markedly inhibited VEGF-induced HUVEC migration and tubulogenesis, whereas inhibition of the p38 kinase pathway using either SB203580 or siRNAs against p38α or MAPKAPK2, had no significant effect on the chemotactic response to VEGF. These findings identify a novel pathway for VEGF-induced HSP27 serine 82 phosphorylation via PKC-mediated PKD activation and direct phosphorylation of HSP27 by PKD, and show that PKDs and HSP27 play major roles in the angiogenic response to VEGF.

1. Introduction

Vascular Endothelial Growth Factor (VEGF or VEGF-A) is essential for angiogenesis during development and in the pathogenesis of human pathologies including cancer and eye diseases [1,2]. VEGF exerts its diverse biological effects in endothelial cells through high affinity binding to two tyrosine-kinase receptors, Flt-1 (VEGFR1) and KDR (VEGFR2) [3,4]. KDR is activated through ligand-stimulated receptor dimerisation and trans(auto)phosphorylation of multiple tyrosine residues in the cytoplasmic domain [5,6], triggering an array of early signaling events followed by short- and long-term cellular biological effects including production of prostacyclin and nitric oxide, increased cell survival, migration, proliferation and angiogenesis [4,6–14]. The function of Flt-1 in the endothelium is unclear, but it is thought to regulate the activity of VEGF partly by acting as a decoy receptor, and in part though direct regulatory effects on KDR [1]. VEGF has been reported to induce Heat Shock Protein (HSP) 27 phosphorylation via a p38 kinase-dependent pathway [15], but the mechanisms involved in VEGF regulation of HSP27 phosphorylation and the role of this protein in VEGF function remain poorly understood.

HSP27 is a ubiquitous and abundantly expressed member of the small heat shock protein family, phosphorylated in response to diverse stress stimuli, including osmotic stress, reactive oxygen species, and inflammatory cytokines. HSP27 is thought to play major roles in the regulation of apoptosis, organisation of the actin cytoskeleton, and cell migration [16,17], and also protects cardiomyocytes against ischaemic injury [18]. The major pathway through which HSP27 phosphorylation is regulated is the p38 kinase cascade [19,20]. HSP27 is directly phosphorylated at serines 15, 78 and 82 by the p38 kinase substrate, MAPK-activated protein kinase-2 (MAPKAPK-2). Large HSP27 multimers are formed by unphosphorylated HSP27, and phosphorylation at serine residue 82 in particular, promotes dissociation of multimers, whereas phosphorylation at serine 15 is implicated in dimer interaction with actin [21].

Abbreviations: ERK1,2, extracellular signal-regulated kinases 1 and 2; HUVEC, human umbilical vein endothelial cells; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase-2; p38 kinase, p38 mitogen-activated protein kinase; PKC, protein kinase C; PKD, protein kinase D/PKCμ; TNFα, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; VEGFR1/Flt-1, VEGF receptor 1/fms-like tyrosine kinase 1; VEGFR2/KDR, VEGF receptor 2/kinase-insert domain-containing receptor.

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Despite the potential importance of HSP27 for migration and survival, key functions involved in VEGF-dependent angiogenesis, previous studies have not directly examined the role of HSP27 in these or other functions of VEGF. In the present study, we investigated both the signal transduction mechanisms mediating VEGF-induced HSP27 phosphorylation and the role of HSP27 in endothelial biological responses to VEGF. Our findings identify a novel pathway mediated via Protein Kinase D (PKD) and independent of p38 kinase that plays the major role in VEGF regulation of phosphorylation at serine 82, whereas phosphorylation at serines 15 and 78 occurs predominantly via p38 kinase. We further demonstrate that while inhibition of p38 kinase has little effect on the migratory response to VEGF, knockdown of PKDs and HSP27 inhibits VEGF-mediated cell migration and tubulogenesis.

2. Materials and methods

2.1. Materials

VEGF-A_{165} and PlGF-1 were from R&D Systems Ltd. (Abingdon, UK). TNF-α and H_2O_2 were from Sigma Inc (Poole, UK). All inhibitors were from Calbiochem Inc (Nottingham, UK). Antibodies to total ERKs 1 and 2, PKCα, PKDs, phospho-PKD (serines 738/742, equivalent to serines 744/748 in mouse), phospho-PKD (serine 916), total HSP27, phospho-HSP27 (serine 82), and phospho-Threonine 202/Tyrosine 204 ERKs1 and 2, and total MAPKAPK2 were from Cell Signalling Technology Inc (Herts, UK). Antibodies to p38 kinase, phospho-Threonine 180/Tyrosine 182 p38 kinase, were from BD Biosciences (Oxford, UK). The antibody to phospho-HSP27 (serine 78) was from Upstate Biotechnology Inc (Hampshire, UK) and the antibody to phospho-HSP27 (serine 15) was from Cambridge Bioscience (Cambridge, UK). The antibody to PKD2 was obtained from Universal Biologicals Ltd (Cambridge, UK). All other reagents were of the highest grade available.

2.2. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from TCS CellWorks (Buckingham, UK) and cultured on gelatin-coated plates in endothelial basal medium (EBM; Cambrex BioScience Ltd, Nottingham, UK) supplemented with gentamycin–ampicillin, epidermal growth factor and bovine brain extract (Singlequots; Cambrex) and 10% (v/v) foetal bovine serum (FBS) (complete EBM). For experimental purposes, fully confluent HUVECs at passages 2–4 were pre-incubated overnight with 1% FBS in EBM prior to addition of factors and other treatments. Where appropriate, inhibitors were pre-incubated with the cells in serum-free EBM for 30 min before treatment.

2.3. Two-dimensional electrophoresis and mass spectrometry

Confluent HUVECs were incubated overnight in EBM media containing 1% FBS. Media was then replaced with serum-free EBM containing 25 ng/ml VEGF-A_{165} for 10 min. Cells were washed with phosphate-buffered saline (PBS) and lysed in 2D buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS). Lysates were clarified by centrifugation and 50 μg protein were subjected to isoelectric focussing on a 24 cm acrylamide strip, pH gradient 3–10 using an IPGphor (GE Healthcare, Little Chalfont, UK). Equilibration of the strips to the SDS buffer system (6 M urea, 2% w/v SDS, 30% v/v glycerol and 0.002% w/v bromophenol blue in 50 mM Tris–HCl buffer; pH 8.8) was performed in two 15 min incubations, the first in SDS buffer containing 1% w/v dithiothreitol, the second in SDS buffer containing 2.5% w/v iodoacetamide. Electrophoresis in the second dimension was performed after applying the strips to 10% acrylamide gels at 2.5 W/gel for 30 min, then 12.5 W/gel. Gels were fixed by overnight incubation in 40% v/v ethanol/10% v/v acetic acid and silver stained using a mass spectrometric compatible protocol [22]. Spots of interest were excised from the gel and underwent reduction by DTT, followed by alkylation with iodoacetamide and dehydration in acetonitrile. The dehydrated gel plugs were rehydrated with 20 ng/μl trypsin (Trypsin Gold; Promega, Southampton, UK) and incubated overnight at 37 °C in a humid chamber. Trypsin digests were spotted onto a stainless steel target together with matrix (a saturated solution (~10 mg/ml) of α-cyano-4-hydroxycinnamic acid diluted 1:2 in 33% v/v acetonitrile and 0.1% v/v trifluoroacetic acid) and peptide masses were determined by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a MALDI-TOF Mass Spectrometer (Bruker Autoflex, Bruker Daltonics, Coventry, UK). Proteins were identified by submitting the peptide masses to the Mascot online database (http://www.matrixscience.com). Silver stained gels were scanned on an ImageScanner (Amersham) and individual protein spots quantitated with ImageJ software (US National Institutes of Health; http://rsb.info.nih.gov/nih-image/Default.html).

2.4. Immunoblotting

After treatments, cells were washed with PBS and lysed in buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM disodium EDTA, 1% v/v Igepal CA-630, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, supplemented with Complete™ protease inhibitor cocktail (Roche Diagnostics, Welwyn Garden City, UK). Whole cell lysates were clarified by centrifugation at 16,000 *g* for 30 min, adjusted to SDS-PAGE sample buffer (62.5 mM Tris–HCl pH 6.8, 100 mM dithiothreitol, 2% w/v SDS, 10% v/v glycerol, 0.002% w/v bromophenol blue), and heated to 95 °C–100 °C for 3 min. Equivalent amounts of protein were separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Watford, UK). Membranes were blocked with 5% w/v non-fat dry milk and 0.1% v/v Tween-20 in PBS, for 1–2 h at room temperature, before being probed with the primary antibody by overnight incubation at 4 °C. Detection was via a horseradish peroxidase-linked secondary antibody (Dako, Ely, UK) and ECL plus reagents (GE Healthcare), following the manufacturer's protocol. Autoradiograms of immunoblots were digitised on an ImageScanner (Amersham) and individual protein bands quantitated with ImageJ. Any differences in HSP27 phosphorylation due to variations in protein loading were routinely corrected by normalizing to levels of total ERKs1/2 immunoblotted on the same membranes as were used for phosho-HSP27 immunoblot, thus avoiding artefacts arising from comparisons with other membranes or from stripping and reprobing membranes with total HSP27 antibody.

2.5. In vitro kinase assay

Different amounts (0–200 ng) of recombinant PKD-1 (Calbiochem) were added to kinase buffer (4 mM MOPS, 4 mM $MgCl_2$, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 50 μM DTT, pH 7.2) containing 0.1 μg recombinant HSP27 (Upstate Biotechnology Inc). The kinase reaction was started with the addition of 100 μM ATP. The reaction was allowed to proceed at room temperature for 15 min before termination by the addition of SDS-PAGE sample buffer and heating to 95–100 °C for 3 min. Samples were analysed by immunoblotting as described above and probing with antibodies to total HSP27 or HSP27 phosphorylated at serines 15, 78 or 82.

2.6. Immunoprecipitation

Cells were washed in serum-free medium and treated with VEGF or vehicle for 10 min, then washed twice in ice-cold PBS and lysed by scraping the cells in lysis buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 4 mM EDTA, 1% v/v Triton X100, protease inhibitor cocktail, phosphatase inhibitor cocktail: 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM sodium orthvanadate). Lysis was allowed to continue on ice for 30 min, after which the lysate was clarified by centrifugation (30 min 16,000 *g* 4 °C) and the insoluble material removed. The lysate was then pre-cleared by incubating with protein A/G plus agarose beads (Autogen Bioclear Ltd, Calne, UK) for at least 1 h followed by incubation with PKD2 antibody overnight at 4 °C, and further incubation with protein A/G agarose beads for 1 h Immunoprecipitates were washed three times with lysis buffer and used in an in vitro kinase assay as detailed above. Briefly, immune complexes were washed and resuspended in 90 μl kinase buffer containing 3 μg recombinant HSP27 and the mixture was allowed to equilibrate to room temperature for 5 min. The kinase reaction was started with the addition of 10 μl of 100 μM ATP, and an aliquot was immediately removed, mixed with an equal volume of 2× SDS-PAGE buffer and heated to 95 °C–100 °C for 3 min to terminate the reaction (reaction time=0 min). The reaction was allowed to proceed at room temperature, and aliquots were removed at the time points indicated. Samples were stored at −70 °C prior to analysis by immunoblotting.

2.7. Transfection with small interfering RNA (siRNA)

Specific siRNAs (Ambion Ltd Huntingdon, UK, or Dharmacon RNA Technologies Inc) were resuspended in nuclease-free water to yield a stock concentration of 50 μM. HUVECs in 6-well plates were allowed to reach ~70% confluence before transfection. siRNAs diluted to 1 μM with Opti-MEM media (Invitrogen, Paisley, UK), containing no serum or antibiotics, were incubated for 25 min with 10 μl Oligofectamine™ Reagent (Invitrogen) in a total volume of 200 μl. Thereafter, HUVEC monolayers were washed with Opti-MEM media and the complexes were added onto the cells at a final siRNA concentration of 200 nM. In parallel wells, Silencer™ control siRNA (Ambion or Dharmacon Inc), which is a non-targeting scrambled siRNA, was used at the same concentration. After transfection for 4 h, the medium was adjusted to 10% FBS (v/v) and cells were incubated overnight. Media was then replaced with complete EBM and the cells incubated for a further 24 h. The knockdown effects of siRNAs were then confirmed by immunoblotting.

2.8. Cell migration

Transwell cell culture inserts made of transparent, low pore density polyethylene terephthalate (PET) with 8 μm pore size (Falcon; BD Biosciences, Oxford, UK), were inserted into a 24-well plate. Serum-free media supplemented with or without 25 ng/ml VEGF-A_{165} or vehicle was placed in the bottom chamber and HUVECs in suspension (1.5×10^5 cell/well in serum-free EBM) were added to the top chamber and incubated at 37 °C for 4 h. HUVECs that had not migrated or had only adhered to the upper side of the membrane were removed before membranes were fixed and stained with a Reastain Quik-Diff kit (IBG Immucor Ltd, West Sussex, UK) using the manufacturer's protocol and mounted on glass slides. HUVECs that had migrated to the lower side of the membrane were counted in four random fields per well at 20× magnification using an eye piece indexed graticule.