Protein Kinase D activity controls Endothelial Nitric Oxide synthesis

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Summary

Vascular endothelial growth factor (VEGF) regulates key functions of the endothelium such as angiogenesis or vessel repair in processes involving endothelial Nitric Oxide Synthase (eNOS) activation. On the other hand, one of the effector kinases that become activated in endothelial cells upon VEGF treatment is Protein Kinase D (PKD). We show herein that PKD uses eNOS as substrate leading to its activation with the concomitant increased ·NO synthesis. Using mass spectrometry, we show that the purified active kinase specifically phosphorylates recombinant eNOS on Ser1179. Treatment of endothelial cells with VEGF or PDBu activates PKD and increases eNOS Ser1179 phosphorylation. In addition, pharmacological inhibition of PKD and gene silencing of both PKD1 and PKD2 abrogate VEGF signaling, resulting in a clear diminished migration of endothelial cells in a wound-healing assay. Finally, inhibition of PKD in mice results in an almost complete disappearance of the VEGF-induced vasodilatation as monitored through the determination of the diameter of the carotid artery. Hence, our data indicate that PKD is a novel regulatory kinase of eNOS in endothelial cells whose activity orchestrates mammalian vascular tone.

Introduction

Vascular endothelial growth factor (VEFG) is a key physiological regulator of both vascular development during embryogenesis (vasculogenesis) and blood-vessel formation in the adult (angiogenesis). VEGF is mainly secreted from mesenchymal, stromal and epithelial sources to act on endothelial cells in both arteries and veins and its receptors are transmembrane proteins that belong to the receptor tyrosine kinase family (Ferrara et al., 2003; Ho and Kuo, 2007; Olsson et al., 2006). The angiogenic effects of VEGF are primarily mediated by VEGF receptor-2 (VEGFR2) (Ho and Kuo, 2007). Upon VEGF binding to endothelial cells, VEGFR2 undergoes dimerization and tyrosine kinase autophosphorylation, which, in turn, activates various signaling cascades (Byrne et al., 2005). The best characterized action of VEGF on endothelial cells is the ability to promote their growth, stimulation of DNA synthesis and proliferation (Parenti et al., 1998). In addition, VEGF is also a survival factor for endothelial cells *in vitro*, preventing apoptosis induced by serum starvation in a process mediated, at least partially, by the PI3K/Akt pathway (Fujio and Walsh, 1999; Gerber et al., 1998a; Gerber et al., 1998b). Furthermore, a constitutively active Akt was sufficient to promote survival of serum-starved endothelial cells (Gerber et al., 1998b).

At present, two isoforms of the PKD family, PKD1 and PKD2, have emerged as novel crucial molecular players not only in VEGF signaling and angiogenesis but also in other important signaling pathways in the cardiovascular system (Evans and Zachary, 2011; Ha and Jin, 2009). It has also been confirmed that VEGF-mediated PKD activation in endothelial cells promotes both proliferation and migration (Hao et al., 2009; Qin et al., 2006; Wong and Jin, 2005), with PKD activation involving PKC-mediated PKD phosphorylation (Zugaza et al., 1996). Although PKD activity functionally seems to regulate migration, proliferation and apoptosis in endothelial cells, the identification of PKD substrates in endothelial cells has remained elusive (Avkiran et al., 2008).

As early as 1993, Brock and coworkers reported that the vasodilatation induced by VEGF on endothelial cells was dependent on the activity of endothelial Nitric Oxide Synthase (eNOS), since preincubation with eNOS inhibitors such as L-NMMA blocked the relaxation of coronary arteries induced by VEGF (Ku et al., 1993). Subsequently other reports showed that systemic administration of eNOS inhibitors to rabbits bearing a corneal implant blocked the migration of endothelial cells induced by VEGF (Ziche et al., 1997). In addition, pharmacological doses of VEGF are known to stimulate endothelial nitric oxide (·NO) formation and reduce blood pressure in animals and humans (Hood et al., 1998; Janvier et al.,

2005) whereas inhibition of VEGFR2 using a specific antibody rapidly increases blood pressure in mice (Facemire et al., 2009). In humans, VEGF also maintains a normal endothelial control of vasomotor tone since injection of a monoclonal anti-VEGF antibody into the brachial artery inactivates circulating VEGF hence decreasing endothelium-dependent vasodilatation within 15 min (Thijs et al., 2013).

VEGF-dependent phosphorylation of eNOS Ser 1179 and the subsequent boost in ·NO synthesis has been for some time considered to be mediated directly by activated Akt (Protein Kinase B) (Fleming, 2010; Forstermann and Sessa, 2012; Michel and Vanhoutte, 2010). However, it is becoming clear that Akt is not the only activated kinase to target eNOS Ser1179 upon VEGF treatment of endothelial cells. Expression of a dominant negative Akt mutant in bovine endothelial cells only partially inhibits phosphorylation of eNOS Ser1179 induced by VEGF (Boo et al., 2002). Furthermore, a very evident phosphorylation on eNOS Ser1179 could still be observed in lung endothelial cells derived from Akt knock-out mice injected with VEGF (Schleicher et al., 2009).

Therefore, given that VEGF-mediated actions proceed through the activation of various protein kinases, including PKD, it is feasible that PKD could be involved in the phosphorylation-dependent activation of eNOS induced by this growth factor. Supporting this idea, Hao and coworkers showed in a recent report that an antibody recognizing phosphorylated PKD substrates labeled prominently a band of approximately 135 kDa in human endothelial cells treated with VEGF. Importantly, the intensity of this band decreased partially after PKD2 silencing (Hao et al., 2009). Herein, we have analyzed the possible participation of PKD in eNOS phosphorylation and activation. We show that, indeed, eNOS is a PKD substrate both *in vitro* and in transfected cells. In addition, we show that PKD1 and PKD2 can associate to eNOS in cells and also that silencing of these PKD isoforms abrogates eNOS-mediated wound-healing process in VEGF-treated endothelial cells. Finally, we also report that mice injected in the carotid artery with a PKD inhibitor display a clearly diminished response towards VEGF-mediated vasodilatation, a process that requires eNOS activation. In summary, we have identified eNOS as the first PKD substrate specific of endothelial cells and shed light on a novel regulatory mechanism of eNOS activity with importance for the understanding of various circulatory pathologies.

Results

eNOS is a novel substrate of PKD1

Since VEGF activates PKD isoforms in endothelial cells we wondered whether PKD could be using eNOS as a direct substrate in the endothelium and the possible functional outcome. Given that VEGF triggers and array of signaling pathways in cultured endothelial cells, we decided to initially perform *in vitro* kinase assays using recombinant proteins. We incubated recombinant bovine eNOS with purified active catalytic domain of PKD1 fused to GST (PKD1-cat active) in the presence of ATP. In order to identify phosphorylated residues in eNOS, the product of the *in vitro* kinase reaction was digested with trypsin and subjected to HPLC and peptide fragmentation by MALDI TOF/TOF (Fig. 1A). Several hundred eNOS-derived peptides were obtained, but only one significant phosphopeptide was identified. *De novo* sequencing of an eluted tryptic peptide with a mass of 1174,502 Da revealed that it corresponded to sequence TQpSFSLQER (residues T1177-R1185 of bovine eNOS) and the phosphorylated residue was assigned to the Ser residue present at the third position (pS; b_3 in Fig. 1A). Therefore, eNOS Ser1179 located within the C-terminus was the serine phosphorylated by PKD1 *in vitro*. In order to corroborate the mass spectrometry data, we used a commercially available phospho-specific antibody recognizing this phospho-site (eNOS-pSer1179) and performed an *in vitro* kinase assay as above followed by immunoblot analysis. The eNOS-pSer1179 antibody only detected phosphorylated eNOS when pre-incubated with active catalytic domain of PKD1 in the presence of ATP (Fig. 1B).

Expression of eNOS, PKD1 and PKD2 in endothelial cells

To validate our *in vitro* data, we further investigated whether in BAEC (Bovine Aortic Endothelial Cells) endogenous eNOS would become phosphorylated by ectopically expressed PKD. Transfection of GFP-tagged wild-type PKD1 or PKD2 into BAEC led to a robust increase in endogenous eNOS phosphorylation on Ser1179 (Fig. 2A). Detection of a high degree of overexpressed PKD1 and PKD2 autophosphorylation using a phosphospecific antibody (PKD-pSer916), that reflects the activation state of both isoforms (Matthews et al., 2000), indicated that they were active when transfected into BAEC. In addition, the increased appearance of VASP (Vasodilator-stimulated Phosphoprotein) phosphorylated at Ser239, a read-out for ·NO release (Sartoretto et al., 2009), suggested that eNOS phosphorylation on Ser1179 mediated by PKD1 and PKD2 results in enzymatic activation and increased ·NO synthesis. BAEC presented a basal level of eNOS Ser1179 phosphorylation that indicated this particular site is already phosphorylated in endothelial cells by endogenous serine kinases,

including PKD. To check PKD content in BAEC, we performed immunoblot analysis and detected bands corresponding to both PKD1 and PKD2 isoforms (Fig. 2B). Since little is known about endogenous PKD expression in endothelium, we also examined the presence of PKD in frozen tissue samples of mouse carotid arteries and its possible colocalization with eNOS by confocal immunomicroscopy. Figure 2C shows that eNOS was present in the monolayer of endothelial cells whereas PKD signal was detected in tunica media and also in endothelium, where it colocalized with eNOS.

PKD-mediated eNOS Ser1179 phosphorylation results in increased ·NO synthesis

Next, we continued exploring eNOS phosphorylation on Ser1179 by PKD using an eNOS construct where this serine had been mutated. For that purpose, we used HEK293T cells cotransfected with wild-type eNOS or the non-phosphorylatable mutant eNOS-Ser1179Ala ($eNOS^{SA}$) together with GFP-tagged wild-type PKD1 or PKD2 (PKD1wt and PKD2wt) (Fig. 3A). As shown in the figure, although there is a basal phosphorylation of wild-type eNOS on Ser1179, transfection of both PKD1 and PKD2 increases this phosphorylation approximately two-fold. As expected, when the phosphorylatable eNOS Ser1179 was mutated into Ala, no phosphorylation could be detected either in the presence or absence of transfected PKD1 or PKD2. In order to further analyze the contribution of PKD activity to the phosphorylation Ser1179 within eNOS, HEK293T cells were cotransfected with both wild-type eNOS and $eNOS^{SA}$ constructs together with GFP-tagged PKD1 wild-type (PKDwt), or constitutively active (PKD1ca) or kinase-inactive (PKD1ki) mutants (Fig. 3B). The increase on eNOS Ser1179 phosphorylation was more significant when wild-type or constitutively active PKD1 were overexpressed and nonexistent when the kinase-inactive mutant was used (Fig. 3B), indicating that phosphorylation of this site in eNOS is indeed regulated by PKD activity. In these cells, Ser1179 phosphorylation signal was only found after wild-type eNOS transfection, and accordingly, no signal was detected when eNOS-Ser1179Ala mutant was used.

It has been previously reported that, mechanistically, eNOS phosphorylation on Ser1179 enhances the rate of electron flux from the reductase to the oxygenase domain of the protein and reduces the relative calcium requirement for the enzyme, thus increasing ·NO synthesis (McCabe et al., 2000). In order to analyze the effect of PKD phosphorylation on eNOS activity, COS-7 cells were transfected with wild-type eNOS or its non-phosphorylatable Ser1179Ala mutant in the presence or absence of PKD1wt, PKD1ca or PKD2wt and eNOS activity was measured as ·NO release with the fluorescent probe DAF2-

DA (4,5-Diaminofluorescein diacetate) (Fig. 3C) and also as [^{14}C]-L-Citrulline formation when [^{14}C]-L-Arginine was used as a substrate (Fig. 3D). As a positive control, we also transfected COS-7 cells with iNOS, since this isoform releases large amounts of ·NO and its activity is not dependent on the intracellular calcium concentrations. Interestingly, the non-phosphorylatable eNOS mutant (eNOS^{SA}), when transfected alone or together with PKD1wt, was unable to release detectable ·NO levels in the absence of added calcium ionophores. In contrast, the ·NO-releasing activity of transfected wild-type eNOS was low but increased significantly when cotransfected with PKD1wt. This effect was slightly but significantly further enhanced by activating PKD1 with PDBu (phorbol 12, 13-dibutyrate), and was blocked after incubating the cells with the eNOS inhibitor L-NAME (Fig. 3C). Likewise, the conversion of [^{14}C]-L-Arg into [^{14}C]-L-Cit in lysates obtained from COS-7 cells transfected with wild-type eNOS augmented significantly when cotransfected with PKD1wt, PKD2wt or PKD1ca. Finally, even when wild-type eNOS was cotransfected with PKD1ca the amount of [^{14}C]-L-Cit formed was significantly diminished when the eNOS inhibitor L-NAME was present (Fig. 3D). Thus, our data indicate not only that PKD1 phosphorylation of eNOS activates ·NO synthesis but also that phosphorylation displaces the calcium/calmodulin-binding curve towards lower Ca^{2+} concentrations. Hence, PKD1 phosphorylation of eNOS activates the enzyme and further confirms the proposal that phosphorylation of Ser1179 mediates the calcium-independent activation of eNOS (Dimmeler et al., 1999; Fulton et al., 1999; McCabe et al., 2000).

PKD1 and eNOS form a complex in mammalian cells

We next analyzed whether PKD1 or PKD2 were able to associate with eNOS. For that purpose, we transfected HEK293T cells with full-length wild-type eNOS or a mutant that lacked the C-terminus (eNOSΔ27) including the phosphorylation site, together with wild-type GFP-PKD1 and GFP-PKD2 (PKD1 or PKD2). Two days after transfection PKD was immunoprecipitated from total lysates using anti-GFP antibody and the presence of eNOS in the immunocomplexes was assessed by detecting the signal for eNOS by immunoblot (Fig. 4A). Our results showed that wild-type eNOS associates with both PKD isoforms, and that the eNOS phosphorylation site was not involved in the association because an eNOS mutant lacking the C-terminus still associated with PKD1 and PKD2. Next, we also examined which PKD1 domains could be mediating this association and whether PKD activity could affect it using GFP-PKD1wt, the constitutively active mutant (PKD1ca), and two mutants in which the cysteine-rich domain (CRD) or the pleckstrin homology domain (PH) had been deleted

(PKD1$^{\Delta CRD}$ and PKD1$^{\Delta PH}$, respectively) (Fig. 4B). These constructs were cotransfected with wild-type eNOS in the presence or absence of PDBu treatment to activate the kinase. Then, lysates were immunoprecipitated with an anti-GFP antibody. As shown in the figure, GFP by itself could not associate to eNOS (lanes 1 and 2) whereas all the PKD constructs tested were able to associate with full-length eNOS independently of PDBu treatment. Hence, the association between the eNOS and PKD1 is not mediated by the CRD or the PH domains and does not require the activation of the kinase or the presence of the phosphorylatable eNOS Ser1179 residue.

In order to complement our studies we examined which region of eNOS could be mediating this association. HEK293T cells were cotransfected with PKD1wt, or the empty vector, together with different eNOS constructs: full-length wild-type eNOS, the C-terminus truncated mutant (eNOS Δ27), the NADPH binding domain (NADPH, aa 988-1205) or the heme-oxygenase domain (HEME, aa 1-521) (Fig. 4C). Two days after transfection and before lysis, cells were left untreated or treated with PDBu for 15 min to activate PKD1. Then, lysates were immunoprecipitated with an anti-eNOS polyclonal antibody and PKD and eNOS were detected. PKD activation by PDBu, although increased eNOS phosphorylation, did not potentiate the formation of eNOS/PKD1 complexes. PKD coimmunoprecipitation was observed with full-length eNOS and with its C-terminus mutant Δ27 but was hardly detectable with the NADPH-binding domains of eNOS or undetectable with the isolated heme-oxygenase. Thus, our results indicate that eNOS associates to PKD1 through residues comprised between the CaM binding sequence and the FAD/FMN modules.

Stimuli that activate PKD in endothelial cells induce eNOS Ser1179 phosphorylation and redistribute both proteins

The fact that VEGF treatment of BAEC leads to the activation of PKD (Wong and Jin, 2005) led us to explore VEGF-induced eNOS phosphorylation. We examined the activation of endothelial PKD through the addition of PDBu for 15 min (Fig. 5A) or VEGF for 30 min (Fig. 5B) and found a substantial increase in PKD Ser916 phosphorylation of approximately two-fold with both stimuli. Under identical circumstances, eNOS Ser1179 phosphorylation is significantly augmented (Fig. 5A and 5B) hence indicating that endogenous PKD isoforms might be responsible, at least in part, for the observed eNOS phosphorylation at this position. Furthermore, the vasodilator peptide bradykinin also rapidly induced PKD activation with the concomitant eNOS Ser1179 phosphorylation (Fig. S1). Importantly, the time course of VEGF-induced eNOS phosphorylation at serine 1179 showed a fast and increasing effect up

to 30 min assayed (Fig. 5C). Thus, these results indicate a correlation between endogenous PKD activation in endothelial cells and eNOS phosphorylation on Ser1179.

It has been described that in non-stimulated endothelial cells eNOS targets to specific intracellular domains, including the Golgi apparatus and cholesterol/sphingolipid-rich microdomains of the plasma membrane (caveolae) and that upon stimulation its localization changes from membranous compartments to the cytosol, being this translocation important for activation-inactivation of the enzyme (Forstermann and Sessa, 2012; Michel and Vanhoutte, 2010). On the other hand PKD is mainly cytosolic, showing some association to intracellular compartments, including the Golgi apparatus, in unstimulated cells and it can rapidly translocate to different subcellular compartments depending on the cellular context and stimulation conditions (Rozengurt, 2011). Here, we analyzed the subcellular localization of endogenous eNOS in BAEC transfected with GFP-tagged PKD1 by confocal microscopy. Transfected BAEC were left untreated or stimulated with VEGF for 15 min, then fixed and immunostained using an anti-eNOS antibody. As shown in Figure 5D, in resting cells both eNOS and PKD1 were excluded from the cell nucleus and enriched in endomembranes and perinuclear areas of the cell (very likely the Golgi apparatus) where a high degree of colocalization could be observed. After VEGF stimulation both proteins suffered a bulk translocation, with their subcellular distribution becoming more cytoplasmic and less condensed in perinuclear areas. No apparent plasma membrane localization of eNOS could be detected in agreement with the previously reported cytoplasmic immunolocalization of activated Ser1179 phosphorylated eNOS (Fulton et al., 2002). In the case of PKD1, some translocation to certain plasma membrane regions could be observed as well. This subcellular redistribution is in agreement with previous studies in HUVEC that reported that, whereas in the absence of VEGF stimulation PKD1 was almost completely absent from the plasma membrane, a short stimulation of endothelial cells with VEGF induced PKD1 translocation to plasma membrane protrusions, likely lamellipodia (di Blasio et al., 2010). Similar results were obtained when GFP-PKD2 was transfected (data not shown).

PKD inhibition or gene silencing markedly diminishes migration of BAEC

We next examined the role of endogenous PKD in BAEC migration, an eNOS-dependent process that plays key roles in angiogenesis (Borniquel et al., 2010; Lopez-Rivera et al., 2005). For that purpose we performed a wound healing assay by stimulating cells with VEGF or PDBu, in the presence of eNOS, PKD or Akt inhibitors (Fig. 6). Cells were monitored for 24 h up to wound closure. As shown in Figure 6A, treatment with the eNOS inhibitor L-

NAME blocked migration induced by the pro-angiogenic factor VEGF and also by PDBu, two compounds that, as demonstrated in Figures 5A and 5B, activate endogenous PKD1/2 and increase phosphorylation of eNOS on Ser1179. Pretreatment of BAEC with the PKD inhibitor Gö6976 and with Akt inhibitors significantly decreased the sealing of the wound in 24 h (Fig. 6B and Fig. S2). These results indicate that PKD activity is required for the migration of endothelial cells *in vitro*, and also that PKD1/2 and, to a lesser extent Akt are involved in this process.

Next, in order to analyze the role of PKD in eNOS Ser1179 phosphorylation BAEC were incubated with VEGF in the presence or absence of Gö6976 (Fig. 7A and Fig. S3). Inhibition of endogenous PKD1 and PKD2 significantly diminished the amount of eNOS-pSer1179 detected in Western blot, hence underpinning the role of this kinase in eNOS activation in endothelial cells. However, PKD1 and PKD2 may activate eNOS and stimulate angiogenic processes through short and long-term mechanisms. Although it has been reported that PKD1 is involved in angiogenesis (Qin et al., 2006; Wong and Jin, 2005), recent results point toward a prominent role of PKD2 in proliferation and migration of endothelial cells (Hao et al., 2009). In order to determine the role of each individual isoform in eNOS activation in BAEC we performed gene knock-down using lentivirus that produce specific shRNA (short hairpin RNAs) targeted against either PKD1 or PKD2. We previously checked that those validated shRNAs were also effective for gene silencing of bovine PKD1 and PKD2 (Fig. 7B). Importantly, the knock down of PKD1 or PKD2 reduced the VEGF-dependent eNOS phosphorylation on Ser1179 in BAEC (Fig. S4, panel A). When wound healing assays were performed the PKD inhibitor Gö6976 and even to a greater extent the specific lentivirus-mediated silencing of either PKD1 or PKD2 had a severe effect on the BAEC wound healing process (Fig. 7C). In addition, eNOS inhibition with L-NAME in PKD1- or PKD2-slienced BAEC revealed the need for ·NO in the wound healing process (Fig. S4, panel B). Hence, our results indicate both PKD isoforms are involved in the VEGF-dependent eNOS activation and migration of BAEC in a wound-healing assay. This is in contrast with previous reports that suggested that PKD1 silencing had only a minor effect on HUVEC migration whereas PKD2 was the key player in this process (Hao et al., 2009).

PKD regulates vascular activity *in vivo*

It is generally accepted that the eNOS-generated ·NO stimulates cGMP (cyclic Guanosine Monophosphate) synthesis by soluble guanylate cyclase which in turn leads to relaxation of vascular smooth muscle (Gruetter et al., 1979). In order to study the role of PKD in vascular

function, endothelium-dependent vasodilatation was analyzed. For that purpose, changes in vessel diameter in the right carotid artery of mice were measured by high-resolution ultrasound imaging. We have previously shown that PKD and eNOS colocalize in the endothelial monolayer in mouse carotid (Fig. 2C). Hence we tested the response to VEGF, a molecule with well-characterized vasodilatatory properties, in the presence and absence of PKD inhibition. VEGF injection in the mouse tail caused a rapid vasodilatation of the carotid artery with the maximal measured diameter increase (over 1,8-fold) observed after 2 min (Fig. 8A). Conversely, PKD inhibition using Gö6976 for 10 min followed by the subsequent injection of VEGF significantly reduced vasodilatation, reaching the carotid artery a maximum diameter of 0.18 mm 4 min post-injection (Fig. 8B). In addition, injection of the PKD inhibitor Gö6976 *per se* had a clear vasoconstrictor effect on mice (Fig. 8B and 8C). To exclude potential intimal smooth muscle abnormalities, we evaluated endothelium-independent vasorelaxation by infusing 10^{-7} mol/L sodium nitroprusside, showing normal vessel dilatation as expected (data not shown). These results indicate that the ·NO signaling pathway is compromised when PKD is inhibited reflected in the vasoconstrictor effect of PKD inhibition and the partial loss of the VEGF-induced vasodilatatory response. Hence, PKD-mediated phosphorylation of eNOS might be responsible, at least in part, for the vasodilatory action of VEGF.

Discussion

eNOS-derived ·NO, one of the key players in vascular homeostasis, not only modulates blood pressure and vascular tone but also displays multiple antiatherogenic roles including antithrombotic, antiproliferative, anti-inflammatory and antioxidant effects (Forstermann and Sessa, 2010). The enzymatic levels of eNOS in the endothelium are in part regulated through phosphorylation on Ser, Thr and Tyr residues (Fleming, 2010; Forstermann and Sessa, 2012). In particular, eNOS phosphorylation on Ser1179 is without doubt the best characterized posttranslational modification of the enzyme, stimulating the flux of electrons within the reductase domain and leading to enzymatic activation. Although an atomic structure of the full-length reductase domain of eNOS is not available, comparison with the nNOS (neuronal NOS) crystal structure (Garcin et al., 2004) suggests that regulatory eNOS C-terminal tail very likely adopts a helical conformation, which probably fits within a negatively charged groove across the FAD/FMN interface, shielding the flavins from solvent. Hence, the activation observed upon eNOS phosphorylation on Ser1179 very likely reflects the displacement of this α-helix and the concomitant increased electron transfer within the

reductase domain. In addition, eNOS Ser1179 phosphorylation (very frequently accompanied by coordinated dephosphorylation of Thr495) diminishes Ca^{2+} sensitivity of the enzyme (Fulton et al., 1999; McCabe et al., 2000; Schneider et al., 2003).

The use of commercially available anti-pSer1179 antibodies for eNOS has revealed that in unstimulated cultured endothelial cells, Ser1179 is only marginally phosphorylated. Upon the application of fluid shear stress (Dimmeler et al., 1999; Gallis et al., 1999), acute mechanical stretch (Hu et al., 2013) or UV light (Park et al., 2011), eNOS becomes rapidly phosphorylated on Ser1179. Likewise, treatment of endothelial monolayers with VEGF (Fulton et al., 1999; Michell et al., 1999), insulin (Salt et al., 2003), estrogens (Haynes et al., 2000), endothelin-1 (Liu et al., 2003) or bradykinin (Fleming et al., 2001; Schneider et al., 2003) leads to eNOS Ser1179 phosphorylation. To date, at least six protein kinases (Akt, AMPK, PKA, cGK-I/PKG, Chk1 and CaMKII) have been involved in the direct or indirect phosphorylation of eNOS on Ser1179, although the ones involved in each particular physiological response vary with the stimuli applied. For example, whereas shear stress elicits Ser1179 phosphorylation by PKA (Boo et al., 2002), treatment with insulin (Montagnani et al., 2001), estrogens (Hisamoto et al., 2001), and sphingosine-1-phosphate (Morales-Ruiz et al., 2001) mainly phosphorylates eNOS via Akt. On the other hand, eNOS phosphorylation on Ser1179 due to the treatment of endothelial cells with bradykinin, Ca^{2+} ionophore or thapsigargin is considered to be mediated through CaMKII activation (Fleming et al., 2001; Schneider et al., 2003).

It has been historically accepted that VEGF-mediated eNOS Ser1179 phosphorylation and subsequent activation was due selectively to Akt activity. In fact, we and others have reported that activated Akt phosphorylates eNOS on Ser1179 (Dimmeler et al., 1999; Fulton et al., 1999; Michell et al., 1999). Moreover, VEGF treatment of endothelial cells results in an activation of Akt (Fontana et al., 2002; Fulton et al., 1999; Michell et al., 1999). However, expression of a dominant negative Akt mutant in BAEC only partially inhibits phosphorylation of eNOS Ser1179 induced by VEGF (Boo et al., 2002). The presence of another kinase participating in this step is inferred even more convincingly from the fact that isolated endothelial cells from Akt knock-out mice injected with VEGF through the jugular vein still show a very prominent eNOS phosphoSer1179 band in immunoblots (Schleicher et al., 2009). Herein we identify PKD1 and PKD2 as novel kinases able to also phosphorylate eNOS on Ser1179. Since the effects of VEGF on PKD activation are well established (Hao et al., 2009; Qin et al., 2006; Wong and Jin, 2005), it is feasible that PKD could be responsible for VEGF-effects on eNOS activation that are independent of Akt.

eNOS forms a cellular complex with the majority of the protein kinases known to act on eNOS Ser1179, such as Akt (Michell et al., 1999), CaMKII (Ching et al., 2011), Chk1 (Park et al., 2011) or AMPK (Hess et al., 2009). We also see a clear association between PKD1 and PKD2 with eNOS and show that the reductase domain of eNOS is involved in this process. However, eNOS phosphorylation is not required for its association with either of the PKD isoforms since eNOS mutants with a deleted C-terminus are still able to form a complex. From our studies we cannot conclude whether eNOS and PKD interact directly or need additional proteins. In the case of Akt, activation of the kinase by VEGF stimulation and subsequent phosphorylation of eNOS on Ser1179 is required for the formation of eNOS/Hsp90 heterocomplexes (Brouet et al., 2001; Fontana et al., 2002). Although the physiological meaning of the association of eNOS to the kinases that use it as substrate is unclear it has been suggested that, at least in the case of Akt and Chk1, chaperone Hsp90 might perhaps function as a scaffold for the recruitment of eNOS and the kinase leading to eNOS activation (Fontana et al., 2002; Park et al., 2011). Interestingly, this chaperone is also required for VEGFR2 signaling to eNOS (Duval et al., 2007). Given the role of Hsp90 in VEGF and eNOS crosstalk, this chaperone could also be mediating the association between PKD1/PKD2 and eNOS in endothelial cells.

Our results clearly show that eNOS is a novel PKD substrate. The consensus sequence of PKD protein substrates presents a hydrophobic residue such as Leu or Ile at position -5, together with a basic residue such as Lys or Arg at position -3 (Doppler et al., 2005; Hutti et al., 2004; Nishikawa et al., 1997). Comparison of this sequence with that of eNOS (Table S1) shows that eNOS phosphorylation site partially fail to fully meet this requirement since a basic Arg residue is present at the -5 position. Interestingly, the autophosphorylation motif located at the C-terminus of PKD1/PKD2 does not fit within the consensus phosphorylation sequence either since an acidic Glu residue is present at -3 position. Various amino acids present within the kinase recognition site of eNOS are also present in other reported PKD substrates, such as Thr present at -2 position in analogy to PI4KIIIb (Hausser et al., 2005) and HDAC5 (Vega et al., 2004), Gln at -1 position in analogy to HDAC5 (Vega et al., 2004) or Phe at +1 position in analogy to Kidins220 (Iglesias et al., 2000).

Growing evidence indicates that functional activities of PKD family in the circulatory system and in vascular biology are of utter importance, particularly those regulating proliferation, migration and tubulation of endothelial cells that are crucial for angiogenesis (Evans and Zachary, 2011). In this regard, VEGF-dependent PKD activation is known to be required for endothelial cell migration (Hao et al., 2009; Shin et al., 2012), proliferation (Hao

et al., 2009) and tumor angiogenesis (Azoitei et al., 2010). Our study shows that VEGF treatment activates both PKD1 and PKD2 in BAEC, a result that is in accordance to studies performed in other endothelial cells (Evans et al., 2008; Evans and Zachary, 2011; Ha et al., 2008; Ha and Jin, 2009; Qin et al., 2006). We also find that pharmacological inhibition of PKD or specific silencing of PKD1 or PKD2 hampers BAEC migration in wound healing, a process known to be dependent on eNOS.

The role of endothelial PKD isoforms in vascularization of tumours is becoming more evident, since recent data indicate that the abnormal proliferation of HUVECs observed in the presence of dimethylhydrazine is dependent on PKD1. Downregulation of this kinase using siRNA inhibited abnormal proliferation and formation of vascular neoplasms (Nam et al., 2012). PKD is known to regulate cell migration through different mechanisms, being the one associated to its role in the regulation of actin dynamics very well documented (Olayioye et al., 2013). Indeed, many PKD substrates regulate cellular migration through this mechanism, resulting their phosphorylation in inhibition or enhancement of migration (Olayioye et al., 2013).

Here, we discover an additional mechanism by which PKD can control endothelial cell migration in response to VEGF by having a direct action on eNOS phosphorylation and activation. Whether all these mechanisms are interconnected or how important might be the contribution of each of them to endothelial function remains to be determined. In summary, our findings reinforce the pivotal role played by PKD in endothelial cell biology, adding to the list of PKD functions its ability to directly phosphorylate and activate eNOS, a critical enzyme for endothelium physiology.

Materials and Methods

Materials, chemicals and antibodies

VEGF, Phorbol-12, 13-dibutyrate (PDBu), Gö69761, Tricibirine, Akt 1/2 kinase inhibitor, L-N^Gnitroarginine methyl ester (L-NAME), 4,5- Diaminofluorescein diacetate (DAF2-DA), Protein A-Sepharose, 2′,5′-ADP-Sepharose, adenosine 2′(3′)-monophosphate mixed isomers, (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (BH_4) and ATP were from Sigma Co. (St. Louis, MO, USA). Ni-NTA resin was from Qiagen (Chatsworth, CA, USA) and Glutathione Sepharose was from Amersham (Buckimhamshire, UK). L-Arginine was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). [^{14}C]-L-Arg was from GE Healthcare (Uppsala, Sweden). Phosphatase inhibitors PhosSTOP were from Roche. Mouse monoclonal antibodies anti-eNOS and anti-eNOS-phospho-Ser^{1179} (Ser^{1177} in human eNOS and Ser^{1179} in bovine eNOS) were from BD Transduction laboratories. Mouse monoclonal antibodies recognizing total VASP and PKD2, and rabbit polyclonal antibodies anti-VASP-phospho-Ser239, anti-PKCμ (recognizing total PKD1/2) and anti-PKCμ-phospho-Ser^{910} were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We produced antibodies against eNOS and GFP immunizing rabbits with purified bovine eNOS or GFP. Anti-tubulin monoclonal antibody, horseradish peroxidase conjugated anti-rabbit and anti-mouse secondary antibodies and the fluorescent secondary antibodies labeled with Cy2 or Cy3 were from Sigma.

Cloning and expression of eNOS

The molecular cloning, recombinant expression and purification of eNOS has been previously described (Rodriguez-Crespo et al., 1996; Rodriguez-Crespo et al., 1997; Rodriguez-Crespo et al., 1999).

Cloning and expression of recombinant active catalytic domain of PKD1 fused to GST

The C-terminal region of PKD1 (Ser558-Leu918; PKD1cat) containing the full-length catalytic domain was amplified with pBS-PKD1 as template. The PCR product was cloned in pDONR201 by a recombination reaction with BP clonase (GATEWAY system, Invitrogen-Life Technologies; Carlsbad, CA, USA), to generate the construct pENTR-PKD1cat. After automated sequencing, PKD1cat was subcloned in pDEST15 using LR clonase. This vector for prokaryotic expression generates PKD1cat fussed to glutathione S transferase (GST; GST-PKD1cat) of approximate molecular weight of 65 kDa that was purified following standard

methods and stored at -20°C. This protein is constitutively active since it lacks the regulatory autoinhibitory domain.

Cell culture and transfection, immunoprecipitation, immunofluorescence, confocal microscopy, and tissue immunostaining

We followed the procedures previously reported (Navarro-Lerida et al., 2006; Navarro-Lerida et al., 2007).

[^{14}C]-L-Arginine to [^{14}C]-L-Citrulline conversion assay

We followed the procedure previously reported (Navarro-Lerida et al., 2004). Briefly, COS-7 cells were transfected with the desired construct and 48 h after transfection cells were harvested, sonicated for 5 seconds and separation of [^{14}C]-L-Arg and [^{14}C]-L-Cit was performed in a Dowex resin. We used approximately 1 µCi of [^{14}C]-L-Arg per condition.

Micro-ultrasound Imaging

We followed the procedures already published by our group (Herranz et al., 2012). Flow dilatation was measured before and after infusion of VEGF (30 µL of a 10 µg/mL solution in water), the PKD inhibitor Gö6976 (30 µL of a 225 µg/mL solution in DMSO), or both substances. Readings were taken immediately after injection (0 min; t 0) and after 1, 2, 4 and 10 min. To test endothelium-independent vasorelaxation, sodium nitroprusside was infused at 10^{-7} mol/L (30µL) and recordings taken as above. Each condition was tested in at least four animals. Image analyses were performed off-line from recorded loops using the automated system software provided by the manufacturer (Visualsonics). The study was conducted following the guidelines of the Spanish Animal Care and Use Committee, according to the guidelines for ethical care of experimental animals of the European Union (2010/63/EU). This study conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S.National Institute of Health (NIH Publication No. 85–23, revised 1996).

Phosphorylation of eNOS by active catalytic domain of PKD1 (GST-PKD1cat)

Phosphorylation was determined performing *in vitro* kinase assay. Briefly, purified full-length eNOS and GST-PKD1cat were mixed in kinase buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1 mM dithiothreitol), and subjected to an *in vitro* kinase assay for 1 h at RT in the presence or absence of 100 µM final concentration of ATP. Samples were analyzed by mass spectrometry or immunoblot.

Identification of PKD1-phosphorylated residue in eNOS by mass spectrometry or MALDI TOF/TOF

In vitro phosphorylated eNOS was digested with trypsin and analyzed by HPLC followed by MALDI TOF/TOF and peptide fragmentation and *de novo* sequencing in the Proteomic Studies Unit (Unidad de Proteómica; Facultad de Farmacia Parque Científico de Madrid, UCM, Madrid, Spain).

Wound-healing assays

BAEC migration was determined using a scratch wound assay as previously reported (Borniquel et al., 2010; Lopez-Rivera et al., 2005).

Production of lentiviruses

Validated shRNA cloned in pLKO.1 vector, against human PKD1 (two constructs: TRCN0000195251 and TRCN0000002124), named shPKD1-A and B, respectively, and against human PKD2 (TRCN0000001948 and TRCN0000001950) named shPKD2-A´ and B´ were purchased from Sigma (MISSION® shRNA lentiviral plasmids). Lentiviral production was performed in HEK293T following standard procedures.

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Author contributions

T.I. and I.R.-C. designed the study, I.R.-C., C.A.-R., L.S.-R., C.Z., and M.G.-P. performed the experiments. T.I., C.A.-R. and I.R.-C. wrote the paper.

Legends to Figures

Fig. 1. Identification of Ser1179 in bovine eNOS as the site targeted by PKD1 phosphorylation. Purified bovine eNOS (accession number P29473) was phosphorylated by purified active catalytic domain of PKD1 fused to GST (PKD1-cat active) in an *in vitro* kinase assay using ATP and Mg^{2+}. (A) After the phosphorylation reaction, the sample was completely digested with trypsin and the resulting peptides analyzed by HPLC coupled to MALDI-TOF/TOF. The MS/MS spectra of the tryptic eNOS peptide ^{1177}TQpSFSLQER^{1185} (Mass, 1174,502 Da) is shown. Nearly complete b-ion (N-terminal fragmentation) and y-ion (C-terminal fragmentation) series are visible, and fragmentation of the precursor unequivocally reveals that Ser1179 is the phosphorylation site. The typical generation of dehydroalanine from phosphoserine through loss of phosphate during analysis is observed by a -98 Da shift of b- and y- ions. No other phosphopeptides could be detected among the over 200 peptides resolved by HPLC coupled to MALDI-TOF/TOF analysis. (B) Purified eNOS phosphorylation by PKD1 at Ser1179 was detected by immunoblot using a specific antibody recognizing phospho-Ser1179 within eNOS. PKD activity was detected using the phospho-specific antibody recognizing autophosphorylated Ser916. PKD and eNOS immunoblots are also shown.

Fig. 2. PKD1 and PKD2 are expressed in endothelial cells and their overexpression in BAEC enhances eNOS phosphorylation on Ser1179. (A) BAEC were transfected with either GFP or GFP-tagged wild-type PKD1 or PKD2. One day after transfection the medium was replaced with fresh serum-free medium with extra L-Arg (5 mM) and BH_4 (15 μM). Cellular lysates were analyzed by immunoblot to detect eNOS phosphorylation on Ser1179. PKD1 or PKD2 activity was also determined using phospho-Ser916 antibody (Matthews et al., 2000). Detection of phosphorylated VASP (VASP-pSer239) as a doublet of 45 kDa and 50 kDa was used as a measurement of downstream signaling activated by ·NO production. Levels of total endogenous eNOS and VASP, or transfected PKD are also shown. Tubulin immunoblot was used as protein loading control. Data are representative of three independent experiments. (B) Immunoblot analysis of endogenous levels of PKD1 and PKD2 in BAEC, using an antibody that recognizes both isoforms (PKDt) or another antibody detecting specifically PKD2. (C) Endogenous PKD expression in endothelium. Confocal microscopy image of a transverse section 20 μm thick of a mouse carotid artery immunostained for eNOS and PKD. Cells showing positive strong PKD immunoreactivity (green) were identified in the

endothelium and vascular smooth muscle cells. Endothelial cells showed also a high staining for eNOS (red). Nuclei were stained with DAPI (blue). Scale bar: 50 μm.

Fig. 3. PKD activity regulates eNOS Ser1179 phosphorylation and activation of the synthesis of ·NO. (A) HEK293T cells were cotransfected with either GFP vector (-), wild-type GFP-PKD1 (PKD1wt) or wild-type GFP-PKD2 (PKD2wt), and full-length wild-type eNOS or its non phosphorylatable mutant eNOS-Ser1179Ala ($eNOS^{SA}$). (B) HEK293T cells were cotransfected with either GFP vector alone (-), wild-type GFP-PKD1 (PKD1wt), constitutively active GFP-PKD1 (PKD1ca) or kinase inactive mutant of PKD1 (PKD1ki) together with full-length wild-type eNOS or its non phosphorylatable mutant eNOS-Ser1179Ala ($eNOS^{SA}$). In both panels A and B, 24 h after transfection, the medium was replaced with serum-free medium and 24 h later cells were lysed. Total lysates were analyzed by immunoblot. Panels on the right represent the quantification of the immunoblot signals corresponding to the ratio eNOS-pSer1179/eNOS, and expressed relative to the value obtained in cells transfected with eNOS plus GFP (arbitrarily assigned a value of 1). Data are mean ± s.e.m. for three independent determinations, **P*<0.05. (C) COS-7 cells were transfected with empty pcDNA3 plasmid, full-length wild-type eNOS or the non-phosphorylatable mutant eNOS-Ser1179Ala ($eNOS^{SA}$) together or not with PKD1wt. In a different well, iNOS was transfected, which served as a measure of large amounts of cellular released ·NO. One day post-transfection the medium was replaced with fresh serum-free medium with additional L-Arg (5 mM) and BH_4 (15 μM) and where indicated 48 h after transfection cells were treated with PDBu (1 μM) or L-NAME (500 μM) before washing with medium and incubated with the fluorescent ·NO sensor DAF2-DA (25 μM) for 4h. Subsequently, the monolayer was extensively washed with medium and the fluorescence was detected between 505 and 525 nm using an excitation wavelength of 488 nm. A minimum of four large monolayer fields of over 400 cells were captured. Fluorescence was quantified through pixel to pixel intensity determination and the resulting fluorescence corresponding to cells transfected with the empty vector (DAF) was subtracted from each condition (plot). Data are mean ± s.d. for four determinations. **P*<0.05 in relation to cells transfected with the empty vector (DAF). (D) COS-7 cells were cotransfected with either GFP vector alone (-), GFP-tagged wild-type PKD1, PKD2 or constitutively active PKD1 (PKD1ca) together with full-length wild-type eNOS. In a different well, iNOS was transfected, which served as a measure of large amounts of [^{14}C]-L-Cit generation. One day post-transfection the medium was replaced with fresh serum-free medium and when indicated cells were treated with L-NAME (500 μM). 48 h after transfection cells were harvested, sonicated for 5 seconds

and conversion of [^{14}C]-L-Arg to [^{14}C]-L-Cit was analyzed as previously described (Navarro-Lerida et al., 2004) using 1 μCi for each condition. The resulting [^{14}C]-L-Cit measurement obtained for non-transfected cells was subtracted from each condition and the [^{14}C]-L-Arg to [^{14}C]-L-Cit conversion (%) was referred to cells transfected with iNOS to which a 100% conversion was assigned. Data are mean ± s.e.m. for 3 independent experiments. *P<0.05 in relation to cells transfected only with eNOS.

Fig. 4. PKD1 or PKD2 and eNOS form a complex in transfected cells albeit phosphorylation on eNOS Ser1179 is not necessary for this association. (A) HEK293T cells were cotransfected with either GFP vector alone (-), wild-type GFP-PKD1 (PKD1) or wild-type GFP-PKD2 (PKD2), together with full-length wild-type eNOS or with an eNOS construct that lacks the C-terminal tail, eNOSΔ27 (aa 1-1178 hence lacking the phosphorylatable Ser1179). 24 h after transfection medium was replaced with serum-free medium and one day later cells were lysed and immunoprecipitated with anti-GFP antibody (Ip: GFP). The presence of eNOS and PKD in immunocomplexes was analyzed by immunoblot using anti-eNOS and anti-GFP antibodies, respectively. Levels of eNOS-pSer1179, total eNOS, PKDs, GFP alone and tubulin in total lysates are also shown. (B) HEK293T cells were cotransfected with either GFP vector alone (-), wild-type GFP-PKD1 (PKD1wt), constitutively active GFP-PKD1 (PKD1ca), or mutants lacking the PH domain (PKD1$^{\Delta PH}$) or the cysteine-rich domain (PKD1$^{\Delta CRD}$) together with full-length wild-type eNOS. 24 h after transfection the medium was replaced with serum-free medium and one day later cells were treated (+) or not (-) with 1 μM PDBu for 15 min. Cell lysates were immunoprecipitated and analyzed together with total lysates as described for panel A. (C) HEK293T cells were cotransfected with either pcDNA3 vector alone or wild-type PKD1 together with different eNOS constructs: full-length eNOS (F.L. eNOS), the eNOSΔ27 mutant, the isolated eNOS NADPH domain (aa 988-1205), the eNOS heme-oxygenase domain (HEME, aa 1-521), and the empty vector (empty). 24 h after transfection the medium was replaced with serum-free medium and one day later cells were treated (+) or not (-) with 1 μM PDBu for 15 min, followed by lysis and immunoprecipitation with anti-eNOS antibody (Ip: eNOS). Cell lysates and immunocomplexes were analyzed as described for panel A. Please note that the commercial antibody recognizing total PKD has been elicited using the C-terminus of PKD (including the phosphorylatable Ser916) as epitope and hence it recognizes with less efficiency phosphorylated than non-phosphorylated PKD (Matthews et al., 2000). In all cases results are representative of three independent experiments.

Fig. 5. PDBu and VEGF stimulation of BAEC results in PKD activation and eNOS phosphorylation, and the subcellular redistribution of both proteins. (A-C) BAEC were seeded and 24 h later the medium was replaced with serum-free medium. The following day, cells were treated with PDBu (1 μM) for 15 min (panel A), VEGF (10 ng/ml) for 30 min (Panel B) or with VEGF (10 ng/ml) at different times (Panel C). Total lysates were analyzed by immunoblot for eNOS-pSer1179, eNOS, PKD-pSer916, PKD1 and tubulin. Graphs represent the quantification of the immunoblot signals corresponding to the ratio PKD-pSer916/PKD1 or eNOS-pSer1179/eNOS, and expressed relative to the value obtained at time 0 min, that represents basal phosphorylation. Data are mean ± s.e.m. for three independent determinations, **P*<0.05 versus untreated cells. (D) Treatment of BAEC with VEGF causes a fast redistribution of endogenous eNOS and transfected PKD1. BAEC seeded on coverslips were transfected with wild-type GFP-PKD1 (PKD1wt). 24 h later medium was replaced with serum-free medium and 48 h post-transfection cells were treated or not with VEGF (10 ng/ml) for 15 min. The subcellular location of eNOS (red) and PKD (green) was analyzed by confocal microscopy. Merge images are also shown. Nuclei were stained with DAPI. Scale bar: 20 μm.

Fig. 6. Effects of eNOS, PKD and Akt inhibition in BAEC wound-healing. Migration of BAEC was determined using a scratch wound assay. BAEC were seeded and 24 h later the medium of confluent monolayers was replaced with serum-free medium. The following day, medium was again replaced with fresh serum-free medium with extra L-Arg (5 mM) and BH_4 (15 μM) and cells were pre-treated or not with (Panel A) the eNOS inhibitor L-NAME (500 μM) or (Panel B) the PKD inhibitor Gö6976 (20 μM), or two Akt inhibitors: Akt 1/2 inhibitor (20 μM) or Tricibirine (1 μM) for 1 hour before challenging cells with VEGF (10 ng/ml) or PDBu (1 μM) in the presence of the inhibitors. Each cell monolayer was scraped with a 10 μl pipette tip to create a cell-free zone. BAEC migration was quantified by taking pictures each 30 min during 24 h and by assessing the percentage of area recovery as previously described (Reinhart-King, 2008). A minimum of three independent wound-healing experiments were performed for each condition. Graphs on the right represent the area recovery (%). Data are mean ± s.d. for three determinations. **P*<0.05 versus cells not incubated with L-NAME (A) or cells not treated with kinase inhibitors (B).

Fig. 7. Gene silencing of PKD1 or PKD2 severely affect VEGF-induced wound-healing in BAEC. (A) BAEC were seeded and 24 h later the medium was replaced with serum-free medium. The following day, cells were pretreated or not with Gö6976 (10 μM) for 4 h and,

where indicated, stimulated with VEGF (10 ng/ml) for 30 min. Total lysates were analyzed by immunoblot for eNOS-pSer1179, eNOS and tubulin. (B) Lentiviral particles carrying empty pLKO.1 vector or commercially available validated shRNAs against PKD1 (lanes A and B) or PKD2 (lanes A´ and B´) cloned in pLKO.1 vector were used to transduce BAEC. Cells were collected at least 72 h later and total protein extracts were probed by Western blotting with antibodies designated. Western blots show that the shRNAs effectively knocked-down PKD1 (upper panel) or PKD2 (lower panel) expression in BAEC. (C) VEGF-mediated endothelial cell migration was assayed after incubation with the PKD inhibitor Gö6976 (20 μM) or after gene silencing of PKD1 or PKD2 using lentiviruses. Migration of BAEC stimulated with VEGF (10 ng/ml) was determined using a scratch wound assay. Data are mean ± s.d. for three determinations. *P<0.05 versus cells transduced with control lentivirus.

Fig. 8. VEGF-mediated vasodilatation of mouse carotid artery is compromised by inhibition of PKD. High-frequency ultrasound measurement of changes in carotid artery diameter, in response to 30 μl-intravenous tail injection of (A) 10 μg/ml VEGF or (B) 225 μg/ml PKD inhibitor Gö6976 followed by VEGF. Vasodilatation is expressed as the carotid diameter reading (4 mice/group, mean ± s.d.). (C) Real time non-invasive anatomical location of the mouse left common carotid artery by B-mode high frequency ultrasound (left panel, upper figure), and visualization of blood flow by color doppler ultrasound (left panel, lower figure). Real time cine-loop recording to detect internal carotid lumen diameter by M-mode high frequency ultrasound of the same vessel as before. The micrographs represent recording sections corresponding with three full heart cycles. Vertical lines represent internal lumen systolic carotid diameter of arteries corresponding to mice injected with VEGF for 5 min (upper panel) or mice injected with PKD inhibitor for the same time (lower panel).

References

Avkiran, M., Rowland, A. J., Cuello, F. and Haworth, R. S. (2008). Protein kinase d in the cardiovascular system: emerging roles in health and disease. *Circ Res* **102**, 157-63.

Azoitei, N., Pusapati, G. V., Kleger, A., Moller, P., Kufer, R., Genze, F., Wagner, M., van Lint, J., Carmeliet, P., Adler, G. et al. (2010). Protein kinase D2 is a crucial regulator of tumour cell-endothelial cell communication in gastrointestinal tumours. *Gut* **59**, 1316-30.

Boo, Y. C., Sorescu, G., Boyd, N., Shiojima, I., Walsh, K., Du, J. and Jo, H. (2002). Shear stress stimulates phosphorylation of endothelial nitric-oxide synthase at Ser1179 by Akt-independent mechanisms: role of protein kinase A. *J Biol Chem* **277**, 3388-96.

Borniquel, S., Garcia-Quintans, N., Valle, I., Olmos, Y., Wild, B., Martinez-Granero, F., Soria, E., Lamas, S. and Monsalve, M. (2010). Inactivation of Foxo3a and subsequent downregulation of PGC-1 alpha mediate nitric oxide-induced endothelial cell migration. *Mol Cell Biol* **30**, 4035-44.

Brouet, A., Sonveaux, P., Dessy, C., Balligand, J. L. and Feron, O. (2001). Hsp90 ensures the transition from the early Ca2+-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. *J Biol Chem* **276**, 32663-9.

Byrne, A. M., Bouchier-Hayes, D. J. and Harmey, J. H. (2005). Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). *J Cell Mol Med* **9**, 777-94.

Ching, L. C., Kou, Y. R., Shyue, S. K., Su, K. H., Wei, J., Cheng, L. C., Yu, Y. B., Pan, C. C. and Lee, T. S. (2011). Molecular mechanisms of activation of endothelial nitric oxide synthase mediated by transient receptor potential vanilloid type 1. *Cardiovasc Res* **91**, 492-501.

di Blasio, L., Droetto, S., Norman, J., Bussolino, F. and Primo, L. (2010). Protein kinase D1 regulates VEGF-A-induced alphavbeta3 integrin trafficking and endothelial cell migration. *Traffic* **11**, 1107-18.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601-5.

Doppler, H., Storz, P., Li, J., Comb, M. J. and Toker, A. (2005). A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J Biol Chem* **280**, 15013-9.

Duval, M., Le Boeuf, F., Huot, J. and Gratton, J. P. (2007). Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase. *Mol Biol Cell* **18**, 4659-68.

Evans, I. M., Britton, G. and Zachary, I. C. (2008). 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. *Cell Signal* **20**, 1375-84.

Evans, I. M. and Zachary, I. C. (2011). Protein kinase D in vascular biology and angiogenesis. *IUBMB Life* **63**, 258-63.

Facemire, C. S., Nixon, A. B., Griffiths, R., Hurwitz, H. and Coffman, T. M. (2009). Vascular endothelial growth factor receptor 2 controls blood pressure by regulating nitric oxide synthase expression. *Hypertension* **54**, 652-8.

Ferrara, N., Gerber, H. P. and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat Med* **9**, 669-76.

Fleming, I. (2010). Molecular mechanisms underlying the activation of eNOS. *Pflugers Arch* **459**, 793-806.

Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E. and Busse, R. (2001). Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* **88**, E68-75.

Fontana, J., Fulton, D., Chen, Y., Fairchild, T. A., McCabe, T. J., Fujita, N., Tsuruo, T. and Sessa, W. C. (2002). Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* **90**, 866-73.

Forstermann, U. and Sessa, W. C. (2010). Nitric oxide synthases: regulation and function. *Eur Heart J* **33**, 829-37, 837a-837d.

Forstermann, U. and Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *Eur Heart J* **33**, 829-37, 837a-837d.

Fujio, Y. and Walsh, K. (1999). Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem* **274**, 16349-54.

Fulton, D., Fontana, J., Sowa, G., Gratton, J. P., Lin, M., Li, K. X., Michell, B., Kemp, B. E., Rodman, D. and Sessa, W. C. (2002). Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J Biol Chem* **277**, 4277-84.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601.

Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G., Berk, B. C., Aebersold, R. et al. (1999). Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem* **274**, 30101-8.

Garcin, E. D., Bruns, C. M., Lloyd, S. J., Hosfield, D. J., Tiso, M., Gachhui, R., Stuehr, D. J., Tainer, J. A. and Getzoff, E. D. (2004). Structural basis for isozyme-specific regulation of electron transfer in nitric-oxide synthase. *J Biol Chem* **279**, 37918-27.

Gerber, H. P., Dixit, V. and Ferrara, N. (1998a). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* **273**, 13313-6.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998b). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* **273**, 30336-43.

Gruetter, C. A., Barry, B. K., McNamara, D. B., Gruetter, D. Y., Kadowitz, P. J. and Ignarro, L. (1979). Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrosoamine. *J Cyclic Nucleotide Res* **5**, 211-24.

Ha, C. H., Jhun, B. S., Kao, H. Y. and Jin, Z. G. (2008). VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix metalloproteinase expression and angiogenesis. *Arterioscler Thromb Vasc Biol* **28**, 1782-8.

Ha, C. H. and Jin, Z. G. (2009). Protein kinase D1, a new molecular player in VEGF signaling and angiogenesis. *Mol Cells* **28**, 1-5.

Hao, Q., Wang, L., Zhao, Z. J. and Tang, H. (2009). Identification of protein kinase D2 as a pivotal regulator of endothelial cell proliferation, migration, and angiogenesis. *J Biol Chem* **284**, 799-806.

Hausser, A., Storz, P., Martens, S., Link, G., Toker, A. and Pfizenmaier, K. (2005). Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat Cell Biol* **7**, 880-6.

Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C. and Bender, J. R. (2000). Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res* **87**, 677-82.

Herranz, B., Marquez, S., Guijarro, B., Aracil, E., Aicart-Ramos, C., Rodriguez-Crespo, I., Serrano, I., Rodriguez-Puyol, M., Zaragoza, C. and Saura, M. (2012). Integrin-linked kinase regulates vasomotor function by preventing endothelial nitric oxide synthase uncoupling: role in atherosclerosis. *Circ Res* **110**, 439-49.

Hess, C. N., Kou, R., Johnson, R. P., Li, G. K. and Michel, T. (2009). ADP signaling in vascular endothelial cells: ADP-dependent activation of the endothelial isoform of nitric-oxide synthase

requires the expression but not the kinase activity of AMP-activated protein kinase. *J Biol Chem* **284**, 32209-24.

Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N. et al. (2001). Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem* **276**, 3459-67.

Ho, Q. T. and Kuo, C. J. (2007). Vascular endothelial growth factor: biology and therapeutic applications. *Int J Biochem Cell Biol* **39**, 1349-57.

Hood, J. D., Meininger, C. J., Ziche, M. and Granger, H. J. (1998). VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol* **274**, H1054-8.

Hu, Z., Xiong, Y., Han, X., Geng, C., Jiang, B., Huo, Y. and Luo, J. (2013). Acute Mechanical Stretch Promotes eNOS Activation in Venous Endothelial Cells Mainly via PKA and Akt Pathways. *PLoS One* **8**, e71359.

Hutti, J. E., Jarrell, E. T., Chang, J. D., Abbott, D. W., Storz, P., Toker, A., Cantley, L. C. and Turk, B. E. (2004). A rapid method for determining protein kinase phosphorylation specificity. *Nat Methods* **1**, 27-9.

Iglesias, T., Cabrera-Poch, N., Mitchell, M. P., Naven, T. J., Rozengurt, E. and Schiavo, G. (2000). Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J Biol Chem* **275**, 40048-56.

Janvier, A., Nadeau, S., Baribeau, J. and Perreault, T. (2005). Role of vascular endothelial growth factor receptor 1 and vascular endothelial growth factor receptor 2 in the vasodilator response to vascular endothelial growth factor in the neonatal piglet lung. *Crit Care Med* **33**, 860-6.

Ku, D. D., Zaleski, J. K., Liu, S. and Brock, T. A. (1993). Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* **265**, H586-92.

Liu, S., Premont, R. T., Kontos, C. D., Huang, J. and Rockey, D. C. (2003). Endothelin-1 activates endothelial cell nitric-oxide synthase via heterotrimeric G-protein betagamma subunit signaling to protein jinase B/Akt. *J Biol Chem* **278**, 49929-35.

Lopez-Rivera, E., Lizarbe, T. R., Martinez-Moreno, M., Lopez-Novoa, J. M., Rodriguez-Barbero, A., Rodrigo, J., Fernandez, A. P., Alvarez-Barrientos, A., Lamas, S. and Zaragoza, C. (2005). Matrix metalloproteinase 13 mediates nitric oxide activation of endothelial cell migration. *Proc Natl Acad Sci U S A* **102**, 3685-90.

Matthews, S. A., Iglesias, T., Rozengurt, E. and Cantrell, D. (2000). Spatial and temporal regulation of protein kinase D (PKD). *EMBO J* **19**, 2935-45.

McCabe, T. J., Fulton, D., Roman, L. J. and Sessa, W. C. (2000). Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J Biol Chem* **275**, 6123-8.

Michel, T. and Vanhoutte, P. M. (2010). Cellular signaling and NO production. *Pflugers Arch* **459**, 807-16.

Michell, B. J., Griffiths, J. E., Mitchelhill, K. I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., de Montellano, P. R., Kemp, B. E. and Pearson, R. B. (1999). The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr Biol* **9**, 845-8.

Montagnani, M., Chen, H., Barr, V. A. and Quon, M. J. (2001). Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem* **276**, 30392-8.

Morales-Ruiz, M., Lee, M. J., Zollner, S., Gratton, J. P., Scotland, R., Shiojima, I., Walsh, K., Hla, T. and Sessa, W. C. (2001). Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem* **276**, 19672-7.

Nam, S., Choi, S., Lee, J., Kim, J., Song, J. and Bae, Y. (2012). Role of protein kinase Cmicro isoform expression in dimethylhydrazine-induced vascular endothelial proliferation. *Mol Med Rep* **6**, 399-404.

Navarro-Lerida, I., Alvarez-Barrientos, A. and Rodriguez-Crespo, I. (2006). N-terminal palmitoylation within the appropriate amino acid environment conveys on NOS2 the ability to progress along the intracellular sorting pathways. *J Cell Sci* **119**, 1558-69.

Navarro-Lerida, I., Corvi, M. M., Barrientos, A. A., Gavilanes, F., Berthiaume, L. G. and Rodriguez-Crespo, I. (2004). Palmitoylation of inducible nitric-oxide synthase at Cys-3 is required for proper intracellular traffic and nitric oxide synthesis. *J Biol Chem* **279**, 55682-9.

Navarro-Lerida, I., Martinez-Moreno, M., Ventoso, I., Alvarez-Barrientos, A. and Rodriguez-Crespo, I. (2007). Binding of CAP70 to inducible nitric oxide synthase and implications for the vectorial release of nitric oxide in polarized cells. *Mol Biol Cell* **18**, 2768-77.

Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z. and Cantley, L. C. (1997). Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J Biol Chem* **272**, 952-60.

Olayioye, M. A., Barisic, S. and Hausser, A. (2013). Multi-level control of actin dynamics by protein kinase D. *Cell Signal* **25**, 1739-47.

Olsson, A. K., Dimberg, A., Kreuger, J. and Claesson-Welsh, L. (2006). VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* **7**, 359-71.

Parenti, A., Morbidelli, L., Cui, X. L., Douglas, J. G., Hood, J. D., Granger, H. J., Ledda, F. and Ziche, M. (1998). Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem* **273**, 4220-6.

Park, J. H., Kim, W. S., Kim, J. Y., Park, M. H., Nam, J. H., Yun, C. W., Kwon, Y. G. and Jo, I. (2011). Chk1 and Hsp90 cooperatively regulate phosphorylation of endothelial nitric oxide synthase at serine 1179. *Free Radic Biol Med* **51**, 2217-26.

Qin, L., Zeng, H. and Zhao, D. (2006). Requirement of protein kinase D tyrosine phosphorylation for VEGF-A165-induced angiogenesis through its interaction and regulation of phospholipase Cgamma phosphorylation. *J Biol Chem* **281**, 32550-8.

Reinhart-King, C. A. (2008). Endothelial cell adhesion and migration. *Angiogenesis: In Vitro Systems* **443**, 45-64.

Rodriguez-Crespo, I., Gerber, N. C. and Ortiz de Montellano, P. R. (1996). Endothelial nitric-oxide synthase. Expression in Escherichia coli, spectroscopic characterization, and role of tetrahydrobiopterin in dimer formation. *J Biol Chem* **271**, 11462-7.

Rodriguez-Crespo, I., Moenne-Loccoz, P., Loehr, T. M. and Ortiz de Montellano, P. R. (1997). Endothelial nitric oxide synthase: modulations of the distal heme site produced by progressive N-terminal deletions. *Biochemistry* **36**, 8530-8.

Rodriguez-Crespo, I., Nishida, C. R., Knudsen, G. M. and de Montellano, P. R. (1999). Mutation of the five conserved histidines in the endothelial nitric-oxide synthase hemoprotein domain. No evidence for a non-heme metal requirement for catalysis. *J Biol Chem* **274**, 21617-24.

Rozengurt, E. (2011). Protein kinase D signaling: multiple biological functions in health and disease. *Physiology (Bethesda)* **26**, 23-33.

Salt, I. P., Morrow, V. A., Brandie, F. M., Connell, J. M. and Petrie, J. R. (2003). High glucose inhibits insulin-stimulated nitric oxide production without reducing endothelial nitric-oxide synthase Ser1177 phosphorylation in human aortic endothelial cells. *J Biol Chem* **278**, 18791-7.

Sartoretto, J. L., Jin, B. Y., Bauer, M., Gertler, F. B., Liao, R. and Michel, T. (2009). Regulation of VASP phosphorylation in cardiac myocytes: differential regulation by cyclic nucleotides and modulation of protein expression in diabetic and hypertrophic heart. *Am J Physiol Heart Circ Physiol* **297**, H1697-710.

Schleicher, M., Yu, J., Murata, T., Derakhshan, B., Atochin, D., Qian, L., Kashiwagi, S., Di Lorenzo, A., Harrison, K. D., Huang, P. L. et al. (2009). The Akt1-eNOS axis illustrates the specificity of kinase-substrate relationships in vivo. *Sci Signal* **2**, ra41.

Schneider, J. C., El Kebir, D., Chereau, C., Lanone, S., Huang, X. L., De Buys Roessingh, A. S., Mercier, J. C., Dall'Ava-Santucci, J. and Dinh-Xuan, A. T. (2003). Involvement of Ca2+/calmodulin-

dependent protein kinase II in endothelial NO production and endothelium-dependent relaxation. *Am J Physiol Heart Circ Physiol* **284**, H2311-9.

Shin, S., Wolgamott, L. and Yoon, S. O. (2012). Regulation of endothelial cell morphogenesis by the protein kinase D (PKD)/glycogen synthase kinase 3 (GSK3)beta pathway. *Am J Physiol Cell Physiol* **303**, C743-56.

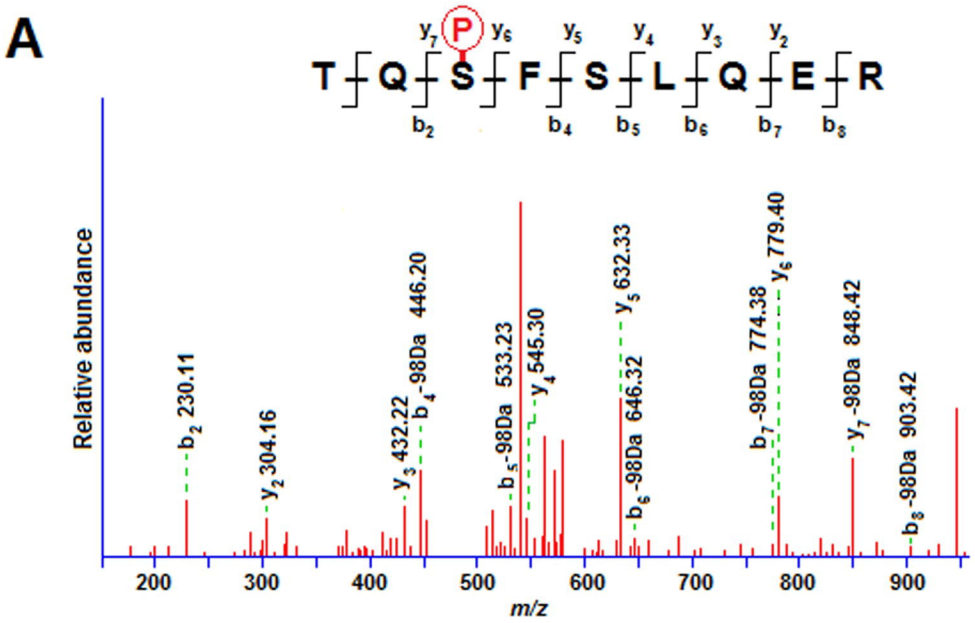
Thijs, A. M., van Herpen, C. M., Sweep, F. C., Geurts-Moespot, A., Smits, P., van der Graaf, W. T. and Rongen, G. A. (2013). Role of endogenous vascular endothelial growth factor in endothelium-dependent vasodilation in humans. *Hypertension* **61**, 1060-5.

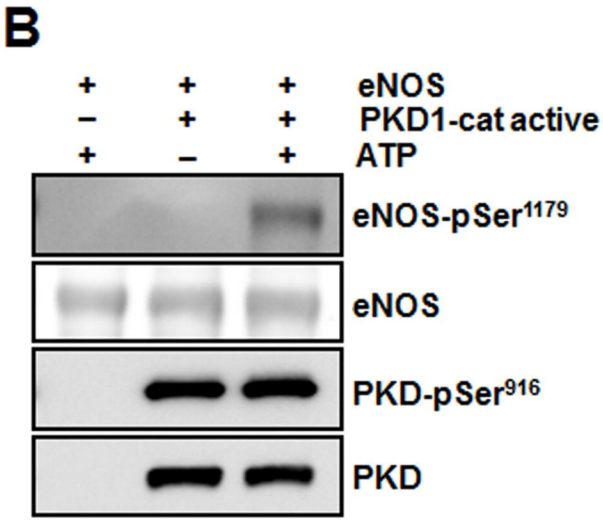
Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N. and McKinsey, T. A. (2004). Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol Cell Biol* **24**, 8374-85.

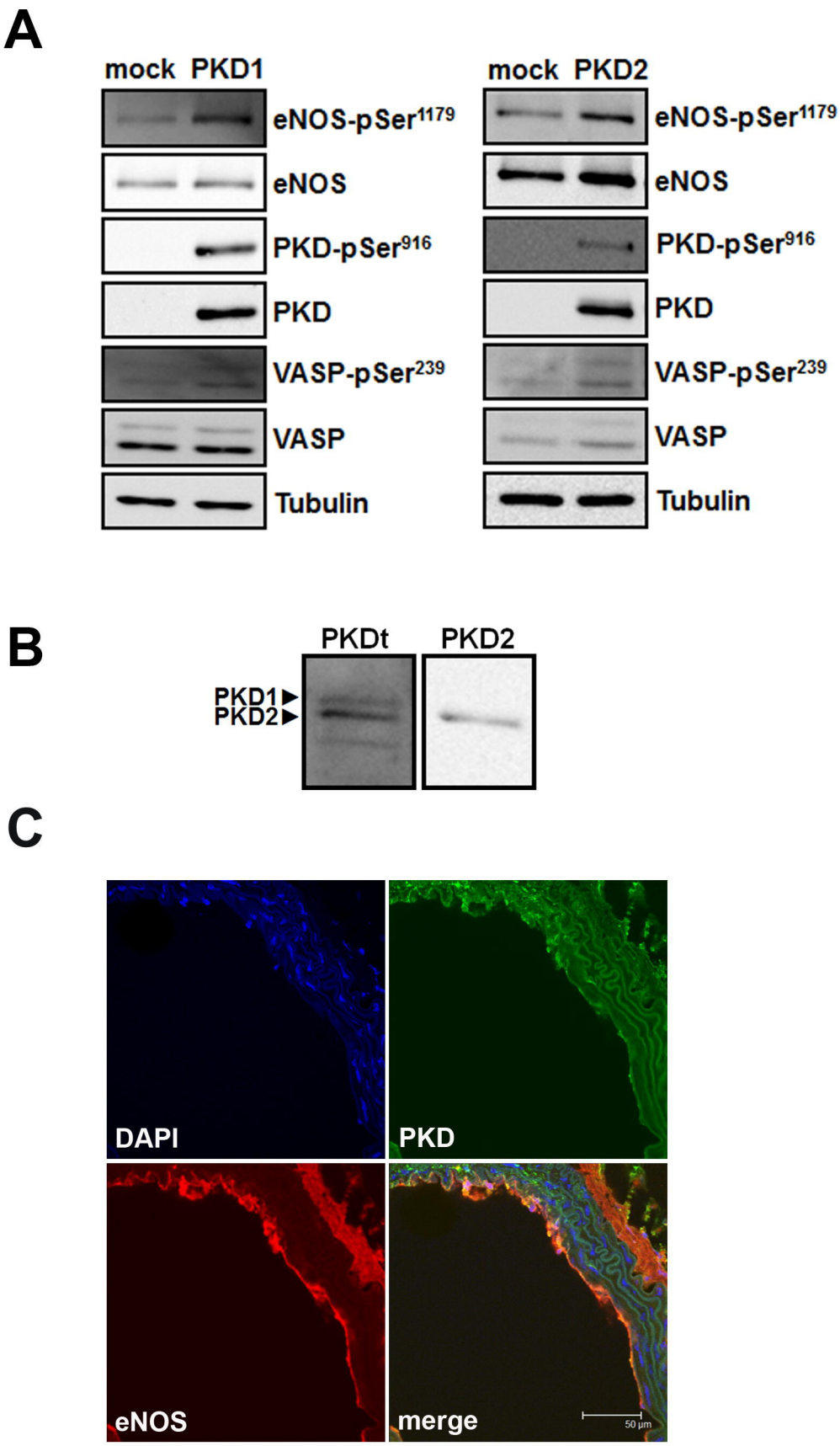
Wong, C. and Jin, Z. G. (2005). Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. *J Biol Chem* **280**, 33262-9.

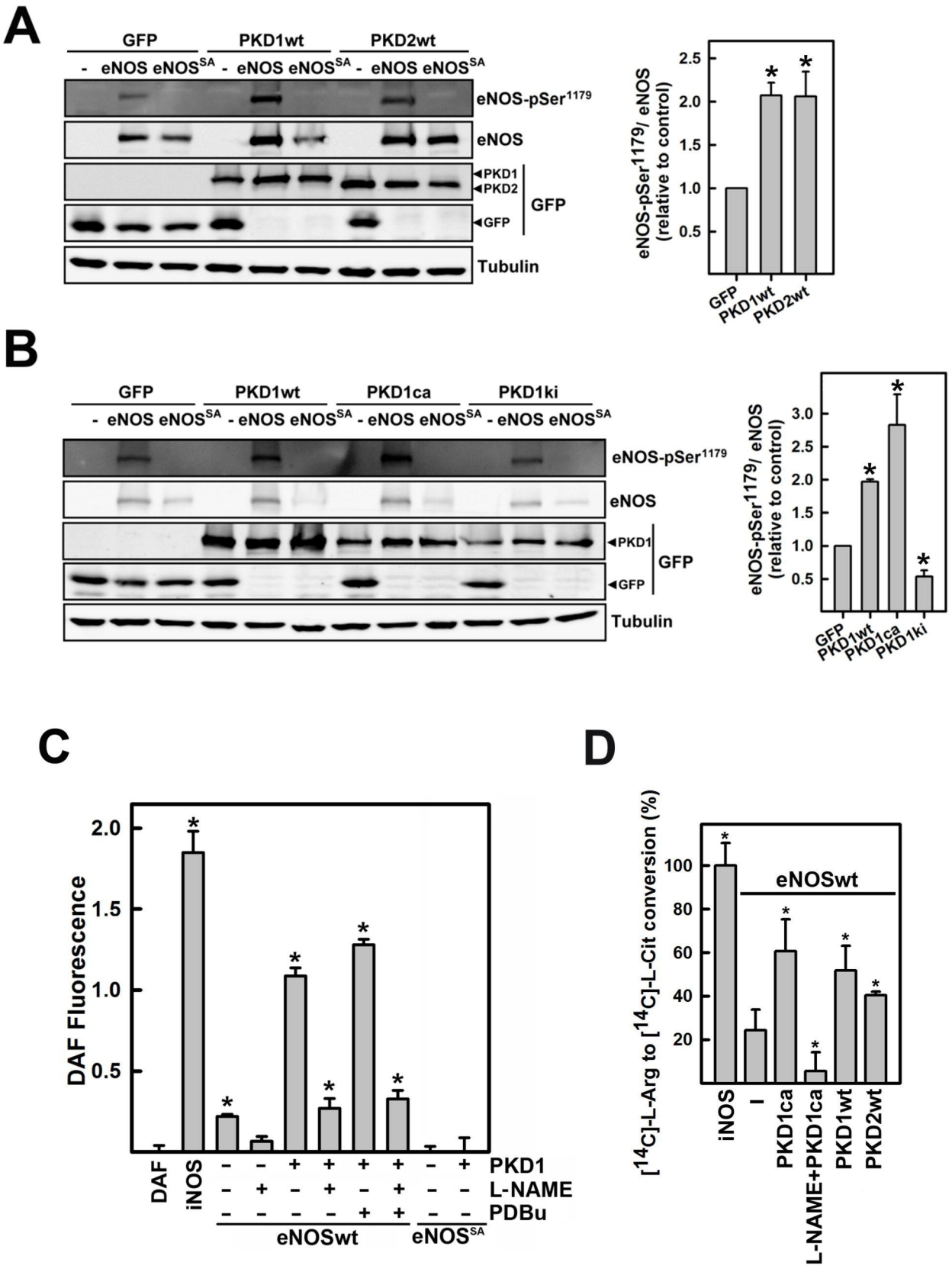
Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H. T., Donnini, S., Granger, H. J. and Bicknell, R. (1997). Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* **99**, 2625-34.

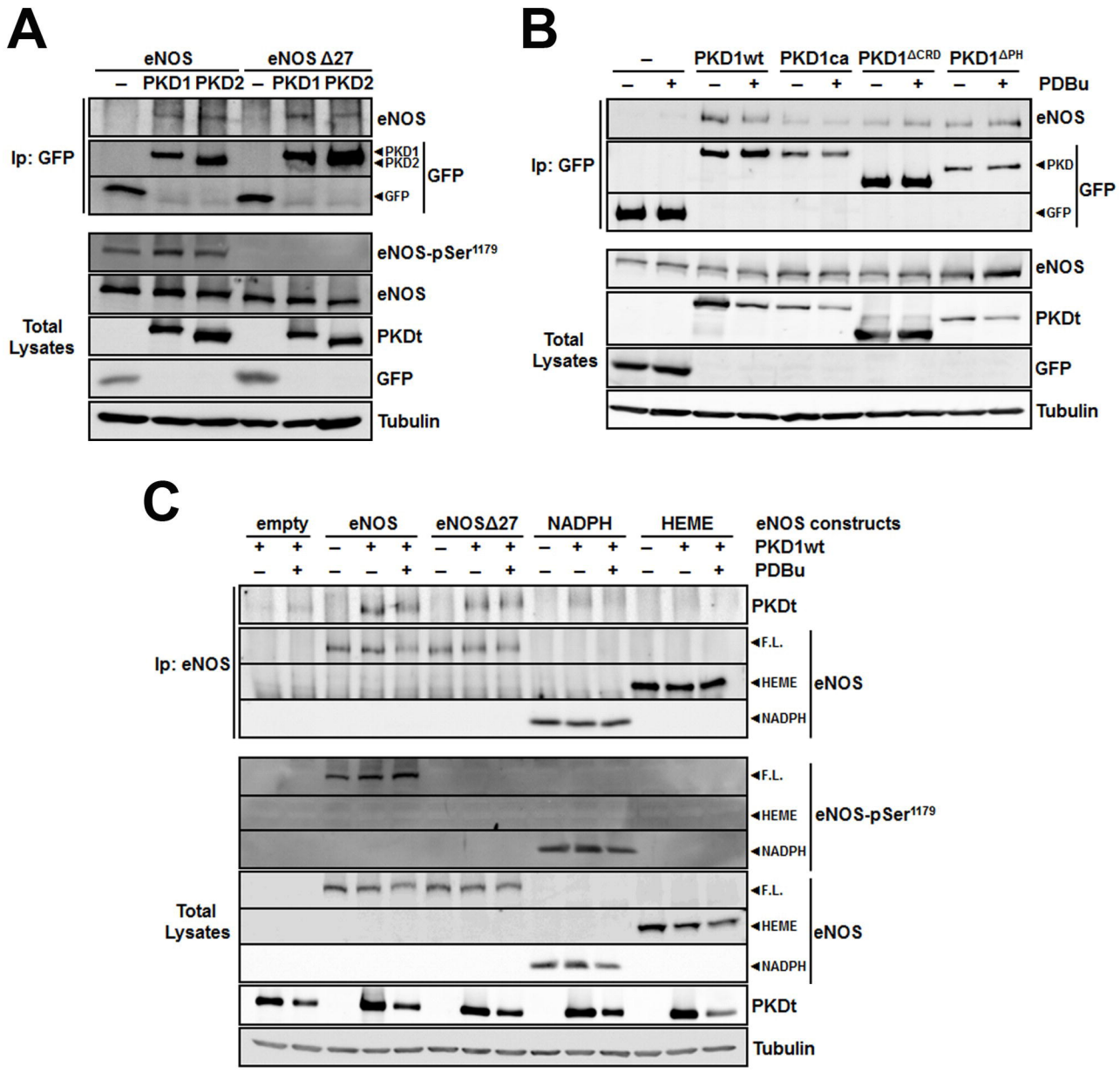
Zugaza, J. L., Sinnett-Smith, J., Van Lint, J. and Rozengurt, E. (1996). Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J* **15**, 6220-30.











Aicart-Ramos et al., Figure 5