Vascular Endothelial Growth Factor (VEGF) Receptor-2 Tyrosine 1175 Signaling Controls VEGF-induced von Willebrand Factor Release from Endothelial Cells via Phospholipase C-γ1- and Protein Kinase A-dependent Pathways

Received for publication, February 9, 2009, and in revised form, June 25, 2009. Published, JBC Papers in Press, July 1, 2009, DOI 10.1074/jbc.M109.019679

Yan Xiong, Yingqing Huo, Chao Chen, Huiyan Zeng, Xiaofan Lu, Chaoliang Wei, Changgeng Ruan, Xiaoyu Zhang, Zhenqian Hu, Masabumi Shibuya, and Jincai Luo

From the Laboratory of Vascular Biology and Laboratory of Calcium Signaling, Institute of Molecular Medicine, Peking University, Beijing 10087, China, the Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, the Jiangsu Institute of Hematology, First Affiliated Hospital of Soochow University, Jiangsu 215006, China, and the Department of Molecular Oncology, Tokyo Medical and Dental University, Tokyo 270-0827, Japan

There is increasing evidence that vascular endothelial growth factor (VEGF) contributes to inflammation independent of its angiogenic functions. Targeting some of the components in endothelial Weibel-Palade bodies (WPBs) effectively inhibits VEGF-induced inflammation, but little is known about how VEGF regulates WPB exocytosis. In this study, we showed that VEGF receptor-2 (VEGFR2), but not VEGFR1, is responsible for VEGF-induced release of von Willebrand factor (vWF), a major marker of WPBs. This is in good contrast to VEGF-stimulated interleukin-6 release from endothelium, which is selectively mediated through VEGFR1. We further demonstrated that VEGFR2-initiated phospholipase C-γ1 (PLCγ1)/calcium signaling is important but insufficient for full vWF release, suggesting the possible participation of another effector pathway. We found that cAMP/protein kinase A (PKA) signaling is required for full vWF release. Importantly, a single mutation of Tyr1175 in the C terminus of VEGFR2, a tyrosine residue crucial for embryonic vasculogenesis, abolished vWF release, concomitant with defective activations of both PLCγ1 and PKA. These data suggest that Tyr1175 mediates both PLCγ1-dependent and PKA-dependent signaling pathways. Taken together, our results not only reveal a novel Tyr1175-mediated signaling pathway but also highlight a potentially new therapeutic target for the management of vascular inflammation.

Vascular endothelial growth factor (VEGF) is a crucial regulator of vasculogenesis, angiogenesis, and vascular permeability (1–5). A number of studies have suggested that VEGF promotes proliferation, migration, and survival of endothelial cells (1, 4). VEGF (also termed VEGF-A) is a member of the growth factor subfamily that includes VEGF-B, -C, -D, and -E and placental growth factor (PIGF). VEGF binds to two high affinity tyrosine kinase receptors, VEGFR1 (also known as Flt-1) and VEGFR2 (also known as KDR/FK-1), whereas VEGF-E binds to VEGFR2 alone, and PIGF binds to VEGFR1 alone. Within the vessel wall, VEGFR2 is selectively expressed in endothelium. In contrast, VEGFR1 is present on both endothelial cells and monocytes (1, 2).

In addition to its role in promoting angiogenesis, there is increasing evidence that VEGF contributes to inflammation independent of its angiogenic functions, although the molecular basis for this effect is incompletely understood (6–8). VEGF is well expressed in the chronic inflammatory skin disease, psoriasis, and in synovial fluid in rheumatoid arthritis (9–12). In addition, previous studies found an association between human severe sepsis/septic shock with elevated circulating levels of VEGF and PIGF (13, 14). Using an in vitro monocyte migration assay and in vivo mouse models of arthritis, several groups, including ours, have suggested that one mechanism by which VEGF causes inflammation is by modulating the infiltration and secretion of monocytes/macrophages via the activation of VEGFR1 (11, 12, 15). On the other hand, emerging evidence suggests that endothelial activation is also important for VEGF-induced inflammation (6, 8, 9). In a mouse model of sepsis, it was demonstrated that the inhibition of VEGFR2, but not VEGFR1, attenuates sepsis mortality, possibly at least in part by suppressing vascular inflammation associated with endothelial activation (9). Consistent with this, ectopic VEGF-A expression in mice enhances leukocyte rolling and adhesion in venules mediated through the P-selectin on the surface of endothelial cells (6). These studies indicate that endothelial activation is another mechanism for VEGF-induced inflammation.

The abbreviations used are: VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CREB, cAMP response element-binding protein; ELISA, enzyme-linked immunosorbent assay; HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; IL, interleukin; PKA, protein kinase A; PLCγ1, phospholipase C-γ1; PIGF, placental growth factor; shRNA, short hairpin RNA; VEGFR1 and VEGFR2, vascular endothelial growth factor receptor 1 and 2, respectively; vWF, von Willebrand factor; WPB, Weibel-Palade body.
VEGFR2/Tyr\textsuperscript{1175} Signaling Controls vWF Release

P-selectin and von Willebrand factor (vWF) are the best characterized constituents of Weibel-Palade bodies (WPBs), endothelial storage granules that also contain various inflammatory mediators (16–18). As a major component in WPBs, vWF is also involved in their biogenesis and thus is used as a marker of WPBs (18, 19). WPB exocytosis, which gives rise to rapid release of vWF and other mediators such as interleukin-8 (IL-8) (17), and translocation of P-selectin from within granules to the endothelial surfaces triggering leukocyte rolling, are critical early events in endothelial activation and vascular inflammation (16). It has been reported that VEGF regulates vWF/ WPB release (20), but the precise roles of VEGF receptors and their downstream effectors in this process have not been defined. In this study, we sought to dissect the signaling pathway by which VEGF induces vWF/ WPB release.

EXPERIMENTAL PROCEDURES

Additional procedures are described in the supplemental Materials and Methods.

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were grown in medium 199 (Invitrogen) containing fibroblast growth factor, heparin, and 20% fetal bovine serum (Hyclone, Ogden, UT). Human aortic endothelial cells (HAECs) were grown in endothelial growth medium 2-MV (Clonetics, San Diego, CA). Cells were used from passages 3 to 6 and were serum-starved in 2% bovine serum albumin for 4 h before stimulation.

RNA Interference—To silence phospholipase C-γ1 (PLC-γ1) and protein kinase A (PKA) (catalytic α and β subunits), we used a lentiviral system (Sigma) for delivering short hairpin RNAs (shRNAs) into HUVECs. The target sequences (see supplemental Materials and Methods) and control scrambled sequences were selected according to an open program on the official website of Massachusetts Institute of Technology. Preparation of lentiviruses and infection of HUVECs were carried out based on a published method (21).

Measurement of vWF and IL-8 by Standard Enzyme-linked Immunosorbent Assays (ELISAs)—HUVECs or HAECs were grown until confluent on 48-well plates and serum-starved for 5 h before stimulation with agonists. The amount of vWF released into the medium was determined from American Diagnostica (Greenwich, CT) or from the Jiangsu Institute of Hematology, Soochow University, Jiangsu, China (22). The amount of IL-8 was measured by an ELISA from R&D Systems (Minneapolis, MN).

Construction of Chimeric Fusion Receptors and Mutants—The chimeric VEGF receptors used in this study comprised the intracellular and transmembrane domains of VEGFR1 (EGLT) or VEGFR2 (EGDR) fused to the extracellular domain of the human EGF receptor (23). Construction of tyrosine-to-phenylalanine mutants of Y1175F and Y1214F was as described before (24). EGDR-Y951F was generated by PCR and cloned into the pMMP retroviral vector. See supplemental Materials and Methods for detailed primer sequences.

Measurement of Intracellular Free Ca\textsuperscript{2+} and cAMP—Intracellular free Ca\textsuperscript{2+} was measured by fluo-4 AM with laser scanning confocal microscopy (Leica, Heidelberg, Germany). Endothelial cells were plated onto collagen-coated coverslips and incubated for 20 min at 37 °C with fluo-4 AM in medium 199 containing 2% bovine serum albumin. For pharmacological studies, cells were pretreated for 30 min with 20 μM 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′,N′-tetracetic acid-acetoxy-methyl ester (BAPTA-AM) before stimulation with 50 ng/ml VEGF, PlGF, or VEGF-E. Similar experiments on EGF stimulation were applied to HUVECs, which were transduced with EGTL, EGDR, and its mutants. The concentration of cAMP in HUVECs was measured according to the manufacturer’s instructions (Amersham Biosciences).

Statistical Analyses—Group differences were analyzed by a standard Student’s t test or by Dunnett’s test (using GraphPad Prism software, otherwise mentioned in the legends of Figs. 3 and 4). All values are expressed as mean ± S.D. Statistical significance was set at a p value less than 0.05 (*) or 0.01 (**).

RESULTS

VEGFR2, but Not VEGFR1, Mediates VEGF-induced vWF Release—A previous study showed that VEGF stimulates WPB exocytosis from HAECs, as evaluated by vWF release as well as P-selectin translocation (20). To extend this finding, we studied the action of VEGF on vWF release from HUVECs by using a standard vWF ELISA. VEGF rapidly induced a time- and dose-dependent vWF release (Fig. 1, A and B), indicating that VEGF promotes vWF secretion from human venous endothelial cells as well. Interestingly, VEGF was somewhat more powerful than thrombin on a molar basis because stimulation with 1 nM VEGF (50 ng/ml) led to 13 milliunits/ml vWF release, whereas stimulation with 38 nM thrombin (1 unit/ml) led to 7.5 milliunits/ml vWF release (Fig. 1B). A recent study has shown that histamine-induced WPBs can undergo a form of exocytosis during which vWF is retained, whereas smaller molecules, such as IL-8, are released (17). Therefore, we also examined IL-8 release from HUVECs in response to VEGF stimulation. VEGF-triggered IL-8 secretion followed a time course similar to that of vWF release (Fig. 1F), suggesting that during VEGF-induced exocytosis, the proportion of WPBs fused with plasma membrane (so-called “lingering kiss”) might be too low to be estimated by our experimental system. This is consistent with the recent study in which the histamine-induced lingering kiss fusion events appeared to be 10–25% (17).

A previous report showed that VEGF induced IL-6 selectively mediated through VEGFR1 but not VEGFR2 (24). To study which receptor mediates the VEGF effect on vWF release, we first examined the expression of VEGF receptors. Both VEGFR1 and VEGFR2 are expressed in HUVECs and HAECs (Fig. 1C). We then used PlGF, a VEGFR1-specific ligand, and VEGF-E, a VEGFR2-specific ligand. Treatment of HUVECs with PlGF resulted in no vWF release, although PlGF was biologically active (supplemental Fig. S1B). In contrast, treatment with VEGF-E rapidly led to vWF release comparable with that of VEGF treatment, indicating that the activation of VEGFR2 alone is sufficient to induce vWF release (Fig. 1, D and E). Similar results were obtained with HAECs (supplemental Fig. S1A). These data suggest that VEGF stimulates vWF release mainly through VEGFR2. VEGF-induced IL-8 release was also mediated via VEGFR2 (Fig. 1F).
To validate further the distinctive roles of VEGFR1 and VEGFR2 in vWF secretion, we employed a chimeric receptor approach in which the extracellular domain of the epidermal growth factor receptor (EGFR) was substituted for that of VEGFR1 (EGFR-Flt-1, designated EGLT) or VEGFR2 (EGFR-KDR, designated EGDR), and both were expressed in HUVECs by using a retrovirus system (23). This system allows independent activation of either receptor by using EGF as an agonist to stimulate the kinase activity of Flt or KDR without activating the endogenous VEGF receptors. As expected, HUVECs transduced with LacZ-expressing viruses did not respond to EGF stimulation. In response to EGF treatment, HUVECs transduced with EGDR rapidly released vWF in a pattern similar to HUVECs responding to VEGF stimulation (Fig. 1, F and G). In contrast, no response was found in HUVECs transduced with EGLT, although it was expressed at a level similar to that of EGDR and became weakly phosphorylated after EGF stimulation (Fig. 1G and supplemental Fig. S2), which is consistent with the nature of VEGFR1 as a weak tyrosine kinase (1, 2). These results confirmed that VEGFR2 plays a predominant role in VEGF-induced vWF secretion.

VEGFR2-triggered PLCγ1/Calcium Signaling Is Important but Insufficient for Full VEGF-induced vWF Release—Intracellular calcium (Ca2+) increase triggers vWF/ WPB exocytosis (16, 17). Previously, several groups, including ours, found that VEGFR2 activates the PLCγ1/ Ca2+ signaling pathway (23, 25). Therefore, we speculated that differential activation of the PLCγ1/ Ca2+ pathway by VEGFR1 and VEGFR2 may account for their different roles in the induction of vWF release.

The abilities of the two VEGF receptors to activate PLCγ1 and Ca2+ mobilization were further compared by approaches using receptor-specific ligands and the chimeric receptors. As shown in Fig. 2, A–D, both approaches confirmed that the activation of VEGFR2, but not VEGFR1, led to PLCγ1 phosphorylation and elevated the level of intracellular Ca2+ in HUVECs. Similar results were obtained with HAECs (supplemental Fig. S3).

A previous study using U73211 (20), a potent PLCγ1 inhibitor, showed that PLCγ1-activated Ca2+ signaling is important for VEGF-triggered vWF/ WPB exocytosis. Because U73122 may affect intracellular Ca2+ levels independent of PLC inhibition (26–28), we employed PLCγ1-specific shRNAs to inactivate its function to avoid nonspecific effects of U73211. PLCγ1-specific shRNAs were constructed and transduced into HUVECs by using a highly efficient lentiviral system (21). shRNA1 was identified as a suitable construct, which reproducibly reduced PLCγ1 expression with an efficiency of ~90% (Fig. 2E and supplemental Fig. S4). The specificity of this construct was confirmed by the observations that it neither reduced VEGFR2 expression (Fig. 2E and supplemental Fig. S4) nor interfered with phorbol 12-myristate 13-acetate-induced vWF release (Fig. 2G), which is known to be independent of Ca2+ signaling (29). Although the expression of a scrambled RNA had no effect on intracellular Ca2+ mobilization, the expression of shRNA1 completely blocked the VEGF-triggered Ca2+ increase at the phases of both initial peak and sustained plateau (Fig. 2F). This result showed that VEGF-induced intracellular Ca2+ mobilization is critically dependent on PLCγ1. We then assessed the effect of PLCγ1 down-regulation on vWF release.
VEGFR2/Tyr1175 Signaling Controls vWF Release

VEGFR2-triggered PKA Activation Is Required for Full VEGF-induced vWF Release—cAMP/PKA signaling is another distinct pathway for the regulation of vWF/Weibel-Palade body exocytosis from endothelial storage granules in response to cAMP-raising agonists (18, 19). So far, however, PKA is known only to negatively regulate VEGF functions, such as the inhibition of VEGF-induced endothelial migration (30, 31). To assess the role of the cAMP/PKA pathway in VEGF-induced vWF release, we first examined the effect of VEGF on the intracellular cAMP level. VEGF stimulation of HUVECs elicited a small, but significant, rise in the intracellular cAMP level as measured by a standard ELISA (Fig. 3A), consistent with a previous report (31). To investigate whether intracellular cAMP increase mediates VEGF-induced vWF release, we incubated HUVECs with VEGF in the presence of cAMP analog Rp-8CPT-cAMPS (Rp). Incubation with Rp significantly reduced vWF release (Fig. 3A), indicating an involvement of cAMP increase in VEGF-induced vWF release. The increase in cAMP levels commonly leads to the activation of PKA, which subsequently results in the phosphorylation of target proteins. The activation of PKA, as estimated by measuring the phosphorylation level of the cAMP-response element-binding protein (CREB), a PKA substrate (32), was increased upon VEGF stimulation (Fig. 3B). To explore the role of PKA in VEGF-induced vWF release, two potent PKA inhibitors (H89 and myristoylated protein kinase inhibitor (14–22)) were used at concentrations that efficiently inhibit the phosphorylation of CREB. Pretreatment of HUVECs slightly, but significantly, inhibited vWF release (20–30% reduction) (Fig. 3C). These data strongly suggest that the cAMP/PKA signaling pathway also plays a role in VEGF-induced vWF release, which is in striking contrast to its negative role in the regulation of endothelial migration (31, 33). To explore the relationship between the PLCγ1 and PKA pathways, PLCγ1 shRNA-expressing HUVECs were pretreated with two PKA inhibitors before VEGF stimulation. PKA inactivation by either inhibitor signifi-

![Figure 2. VEGFR2 mediates PLCγ1 phosphorylation and Ca2+ signaling in HUVECs. A and B, ratio of phosphorylated PLCγ1 (PP-PLCγ1) to total PLCγ1 and Ca2+ mobilization in HUVECs stimulated by VEGF, VEGF-E, or PIGF (50 ng/ml). C and D, ratio of phosphorylated PLCγ1 to total PLCγ1 and Ca2+ mobilization in HUVECs transduced by EGLT, EGDR, or LacZ, with or without EGF (100 ng/ml) stimulation. E, shRNA effect on PLCγ1 expression. Bottom panels, quantitative analysis of Western blot bands of PLCγ1 (left side) and PLCγ2 (right side). **, p < 0.01 versus noninfected cells. F, effects of Ca2+ mobilization on vWF release. **, p < 0.01 versus control. G, effects of PLCγ1 knockdown on VEGF- (left) and phorbol 12-myristate 13-acetate- (PMA; right) induced vWF release (n = 4 ± S.D.). ***, p < 0.01 versus noninfected. Please note that two bands in A and C were detected by the phosphorylated PLCγ1 antibody. The upper band (filled arrowhead) represents phosphorylated PLCγ1 whereas the lower band (open arrowhead) is a nonspecific band (see Footnote 3). Such a detection pattern is also shown in Figs. 3 and 4.

VEGFR2-induced vWF release was decreased significantly (70% reduction) in shRNA1-expressing HUVECs when compared with that in noninfected cells or scrambled RNA-expressing cells (Fig. 2G), indicating that PLCγ1 is essential for VEGF-induced vWF release. The results indicating that inhibition of VEGFR2-triggered Ca2+ signaling by down-regulating PLCγ1 is unable to block VEGF-induced vWF release completely also strongly suggest the possible participation of another effector pathway.
significantly suppressed the residual vWF release (about 25% reduction) (Fig. 3D), indicating that the PKA pathway acts in parallel with the PLCγ1/Ca2+ pathway. To validate further the role of PKA in VEGF-induced vWF release, specific shRNAs targeting the catalytic α and β subunits of PKA were screened, and two efficient shRNAs (shRNA Ca1 and shRNA Cβ1) were identified (Fig. 3E and supplemental Fig. S5). Knockdown of either the α subunit or the β subunit of PKA significantly attenuated VEGF-induced vWF release (Fig. 3F), suggesting that PKA is required for vWF release. To confirm that the VEGF-activated PLCγ1 and PKA pathways are distinct, the effect of PLCγ1 shRNAs on the activation of PKA and the effect of PKA shRNAs on the activation of PLCγ1 were examined. Although PKA shRNAs did not show any inhibitory effect on VEGF-induced PLCγ1 phosphorylation, PLCγ1 shRNAs slightly but insignificantly interfered with VEGF-induced activation of PKA (Fig. 3, H and I). Taken together, the results show that VEGF-induced vWF release requires both PLCγ1/Ca2+-dependent and PKA-dependent signaling pathways. Similarly, PLCγ1 and PKA pathways are also required for VEGF-induced IL-8 release (Fig. 3G).

Identification of Tyr1175 in VEGFR2 as a Molecular Switch to Turn on vWF Release through PLCγ1- and PKA-dependent Signaling Pathways—So far, Tyr951, Tyr1175, and Tyr1214 in VEGFR2, the most prominent noncatalytic auto-phosphorylation sites (4, 34), have been linked to the activation of PLCγ1 (23, 34–36). Therefore, we investigated the roles of these residues in PLCγ1 activation and vWF release by point mutagenesis. We first showed that the various VEGFR2 mutants (Y951F, Y1175F, and Y1214F) were expressed at similar levels in HUVECs (Fig. 4A), and then we compared their capacities to stimulate PLCγ1 phosphorylation. Interestingly, a single mutation Y1175F abolished EGF-induced PLCγ1 phosphorylation (Fig. 4B). Consistently, the Y1175F mutation abolished EGF-induced Ca2+ release and entry (Fig. 4C). In contrast, mutant receptors with Y951F and Y1214F retained their capacities to activate PLCγ1 (Fig. 4B), whereas mutations Y1214F and Y951F did not or only slightly affected Ca2+ mobilization (Fig. 4C). We further examined the abilities of the mutants to stimulate vWF release. Interestingly, the
Y1175F mutant completely lost its ability to induce vWF release, whereas Y951F and 1214F mutants were still able to activate as effectively as the wild type receptor (Fig. 4D), indicating that Tyr1175 is essential for VEGF-induced vWF release. This result also suggests that the Y1175F mutant may fail to activate the cAMP/PKA pathway. Consistent with this, the Y1175F, but not other mutants, had a defective activation of PKA in response to EGF stimulation, which was accompanied by lower cAMP levels than wild type (Fig. 4E).

We show, to our knowledge, for the first time that the cAMP/PKA signaling pathway plays a positive role in the regulation of VEGF function (Fig. 5), in a manner opposite to its inhibitory effect on endothelial migration and tube formation (30, 31). However, this is not unexpected because PKA is a critical effector for vWF/ WPB exocytosis from endothelial storage granules in response to cAMP-raising agonists (18). Interestingly, unlike cAMP-raising agonists, VEGF disrupts endothelial barrier function (38), which may also contribute to inflamma-

DISCUSSION

vWF/ WPB exocytosis is one of the earliest events in endothelial activation and vascular inflammation (16, 19). Targeting some components of WPBs, such as P-selectin, reduced VEGF-induced inflammation (6), suggesting that VEGF stimulation of vWF/WPB exocytosis may be an important mechanism by which VEGF contributes to inflammation. Recently, potent VEGF-capturing reagents such as antibodies against VEGF or inhibitors of VEGF receptor kinase have proven to be very effective in animal inflammation models (8, 9). Because VEGF receptors are critical not only for endothelial cell function but also for cellular processes such as neuronal survival (37), inhibition of specific VEGFR-induced signaling pathways may be preferable in long term treatments, especially for chronic inflammation. In this study, we clearly demonstrate that VEGF is a potent agonist that stimulates vWF/ WPB release from endothelial cells, and this stimulation is mediated by VEGFR2 through both PLCγ1-dependent (in large part) and PKA-dependent (to a lesser extent) dependent signaling pathways (Fig. 5). This is in contrast to the role that VEGFR1 plays in inflammation by promoting the migration and secretion of monocytes/macrophages in which only VEGFR1 is expressed (11, 14). In addition, this is also in good contrast to VEGF-stimulated IL-6 release from endothelium, which is selectively mediated through VEGFR1 (24).
VEGFR2/Tyr1175 Signaling Controls vWF Release

When compared with the vWF release triggered by PLCγ1/Ca2+ signaling, which occurs within 2–5 min after VEGF stimulation, the release induced by cAMP/PKA signaling occurs about 5 min or more after VEGF stimulation and is relatively mild. The use of both signaling pathways by VEGFR2 may ensure rapid and continuous vWF/WPB release in response to VEGF stimulation.

In previous studies, in addition to Tyr1175, the residues Tyr951 and Tyr1214 were also linked to PLCγ1 activation by VEGFR2 (25, 34–36). Our data showed that receptor with the Tyr1175 mutant was completely incapable of activating PLCγ1 phosphorylation and intracellular Ca2+ mobilization, whereas receptors with the Y951F or Y1214F mutation retained these functions (Fig. 4). The discrepancies concerning the residues responsible for PLCγ1 activation found in this study from those in the literature may be due to the different experimental systems. For example, one study on the interaction between Tyr951 and PLCγ1 was performed in a yeast two-hybrid system (35). It has been shown that the data obtained with the yeast system are sometimes inconsistent with protein-protein interaction events in mammalian cells (39, 40). Our work compared the roles of all three of these residues in VEGFR2 in the activation of PLCγ1/Ca2+ signaling in primary human endothelial cells. In a recent report, an adaptor molecule TSAd was suggested to be a more important Tyr951-binding protein than PLCγ1 in the regulation of endothelial cell migration (31), consistent with our finding.

Tyr1175 of VEGFR2 is a critical residue for the regulation of vascular functions. A recent study from the Shibuya group (41) showed that exchange of Tyr1175 (corresponding to Tyr1175 in VEGFR2/KDR) for Phe in mouse VEGFR2/Flk-1 resulted in a loss-of-function phenotype and embryonic lethality, indicating that signaling via Tyr1175 is essential for VEGFR2 functions during mouse vasculogenesis. Our study suggests a new role that Tyr1175 plays in the induction of vWF/WPB exocytosis from endothelial cells within adult vessels. In addition, Tyr1175 mediates the PLCγ1/MAPK (mitogen-activated protein kinase) signaling pathway by which VEGF stimulates endothelial DNA synthesis (25) and also mediates cell migration through phosphatidylinositol 3-kinase mediated by the adaptor proteins Shb and ShcB (1–3). Therefore, Tyr1175 is a unique residue that mediates multiple functions via several signaling pathways in both embryonic development and adult pathophysiological conditions.

The identification of cAMP/ PKA signaling as a novel Tyr1175-mediated pathway raises new questions. For example, how does Tyr1175 regulate the activation of the cAMP/ PKA signaling pathway? Previously, several groups, including ours, found a cross-talk between G protein-coupled receptors and VEGFR2 (42). Therefore, it is possible that Tyr1175 may transmit a signal from VEGFR2 to G protein-coupled receptors through adaptor proteins (such as Shb/ShcB) and in turn trans-activate adenylate cyclase to induce cAMP/ PKA signaling (Fig. 5). It is equally possible that VEGFR2 may activate PKA directly via a CAMP-independent mechanism (43).

In summary, our study demonstrates a key role of Tyr1175 in VEGFR2 in vWF/WPB exocytosis from endothelial cells in response to VEGF stimulation and indicates its usefulness as a potential target in the future development of anti-inflammatory strategies.

Addendum—During the preparation of this manuscript, a report described the role of PKCδ in VEGF-induced vWF secretion (44). Because PKC functions downstream of PLCγ1, it appears that PLCγ1-dependent vWF release from endothelial cells in response to VEGF is mediated through both Ca2+ signaling and PKC signaling pathways.

REFERENCES

1. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) Nat. Med. 9, 669–676
2. Shibuya, M., and Claesson-Welsh, L. (2006) Exp. Cell Res. 312, 549–560
3. Rahimi, N. (2006) Exp. Eye Res. 83, 1005–1016
4. Kowanetz, M., and Ferrara, N. (2006) Clin. Cancer Res. 12, 5018–5022
5. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
6. Detmar, M., Brown, L. F., Schön, M. P., Elicker, B. M., Velasco, P., and Richardson, L., Fukumura, D., Monsky, W., Claflay, K. P., and Jain, R. K. (1998) J. Invest. Dermatol. 111, 1–6
7. Xia, Y. P., Li, B., Hylton, D., Detmar, M., Yancopoulos, G. D., and Rudke, J. S. (2003) Blood 102, 161–168
8. Reinders, M. E., Sho, M., Izawa, A., Wang, P., Mukhopadhyay, D., Koss, K. E., Geethan, C., Luster, A. D., Sayegh, M. H., and Briscoe, D. M. (2003) J. Clin. Invest. 110, 1655–1665
9. Yano, K., Liaw, P. C., Mullington, J. M., Shih, S. C., Okada, H., Bodky, N., Kang, P. M., Toitl, L., Belkoff, B., Buras, J., Simms, B. T., Mizgerd, J. P., Carmeliet, P., Karumanchi, S. A., and Aird, W. C. (2006) J. Exp. Med. 203, 1447–1458
10. Bhushan, M., McLaughlin, B., Weiss, J. B., and Griffiths, C. E. (1999) Br. J. Dermatol. 141, 1054–1060
11. Brown, L. F., Harrist, T. J., Yeo, K. T., Ståhle-Backdahl, M., Jackman, R. W., Berse, B., Tognazzi, K., Dvorak, H. F., and Detmar, M. (1995) J. Invest. Dermatol. 104, 744–749
12. Murakami, M., Iwai, S., Hiratsuka, S., Yamauchi, M., Nakamura, K.,...
Iwakura, Y., and Shibuya, M. (2006) Blood 108, 1849–1856
13. van der Flier, M., van Leeuwen, H. J., van Kessel, K. P., Kimpen, J. L., Hoepelman, A. I., and Geelen, S. P. (2005) Shock 23, 35–38
14. Pickkers, P., Sprong, T., Eijk, L., Hoeven, H., Smits, P., and Deuren, M. (2005) Shock 24, 508–512
15. Sawano, A., Iwai, S., Sakurai, Y., Ito, M., Shitara, K., Nakahata, T., and Shibuya, M. (2001) Blood 97, 785–791
16. Wagner, D. D. (1993) Thromb. Haemost. 70, 105–110
17. Babich, V., Meli, A., Knipe, L., Dempster, J. E., Skehel, P., Hannah, M. J., and Carter, T. (2008) Blood 111, 5282–5290
18. Rondaij, M. G., Bierings, R., Kragt, A., van Mourik, J. A., and Voorberg, J. (2006) Arterioscler. Thromb. Vasc. Biol. 26, 1002–1007
19. Sadler, J. E., Mancuso, D. J., Randi, A. M., Tuley, E. A., and Westfield, L. A. (1991) Ann. N. Y. Acad. Sci. 614, 114–124
20. Matsushita, K., Yamakuchi, M., Morrell, C. N., Ozaki, M., O’Rourke, B., Irani, K., and Lowenstein, C. J. (2005) Blood 105, 207–214
21. Berger, R., Lin, D. I., Nieto, M., Sicinska, E., Garraway, L. A., Adams, H., Signoretti, S., Hahn, W. C., and Loda, M. (2006) Cancer Res. 66, 5723–5728
22. Zhou, Q. S., Zhao, Y. M., Xu, C. S., Yu, Z. Y., Yao, D. Y., Gao, Y. M., and Ruan, C. G. (1992) Thromb. Res. 68, 109–118
23. Zeng, H., Dvorak, H. F., and Mukhopadhyay, D. (2001) J. Biol. Chem. 276, 26969–26979
24. LeCouter, J., Moritz, D. R., Li, B., Phillips, G. L., Liang, X. H., Gerber, H. P., Hillan, K. J., and Ferrara, N. (2003) Science 299, 890–893
25. Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. (2001) EMBO J. 20, 2768–2778
26. Patterson, R. L., van Rossum, D. B., Ford, D. L., Hurt, K. J., Bae, S. S., Suh, P. G., Kurosaki, T., Snyder, S. H., and Gill, D. L. (2002) Cell 111, 529–541
27. Wilscher, N. E., Court, W. J., Ruddle, R., Newbatt, Y. M., Aherne, W., Sheldrake, P. W., Jones, N. P., Katus, M., Eccles, S. A., and Raynald, F. J. (2007) Drug Metab. Dispos. 35, 1017–1022
28. Wong, R., Fabian, L., Forer, A., and Brill, J. A. (2007) BMC Cell Biol. 8, 15–24
29. Carew, M. A., Paleolog, E. M., and Pearson, J. D. (1992) Biochem. J. 286, 631–636
30. Bodnar, R. J., Yates, C. C., and Wells, A. (2006) Circ. Res. 98, 617–625
31. Mizapoiazova, T., Kolosova, I., Usatyn, P. V., Natarajan, V., and Verin, A. D. (2006) Am. J. Physiol. Lung Cell. Mol. Physiol. 291, L718–L724
32. Desloges, N., Rahaus, M., and Wolff, M. H. (2008) Med. Microbiol. Immunol. 197, 353–360
33. Mahadev, K., Wu, X., Donnelly, S., Ouedraogo, R., Eckhart, A. D., and Goldstein, B. J. (2008) Cardiovasc. Res. 78, 376–384
34. Matsumoto, T., Bohman, S., Dixielius, J., Berge, T., Dimberg, A., Magnusson, P., Wang, L., Wikner, C., Qi, J. H., Wernstedt, C., Wu, J., Bruheim, S., Mugishma, H., Mukhopadhyay, D., Spurkland, A., and Claesson-Welsh, L. (2005) EMBO J. 24, 2342–2353
35. Wu, L. W., Mayo, L. D., Dunbar, J. D., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S., and Donner, D. B. (2000) J. Biol. Chem 275, 5096–5103
36. Meyer, R. D., Dayanir, V., Majnoun, F., and Rahimi, N. (2002) J. Biol. Chem. 277, 27081–27087
37. Brockington, A., Lewis, C., Wharton, S., and Shaw, P. J. (2004) Neuropathol. Appl. Neurobiol. 30, 427–446
38. Weis, S., Cui, J., Barnes, L., and Cheresh, D. (2004) J. Cell Biol. 167, 223–229
39. Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K., and Mochizuki, N. (2005) Mol. Cell. Biol. 25, 136–146
40. Legrain, P., and Selig, L. (2000) FES Lett. 480, 32–36
41. Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N., and Shibuya, M. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 1076–1081
42. Zeng, H., Zhao, D., and Mukhopadhyay, D. (2002) J. Biol. Chem. 277, 46791–46798
43. Ma, Y., Pitson, S., Hercus, T., Murphy, J., Lopez, A., and Woodcock, J. (2005) J. Biol. Chem. 280, 26011–26017
44. Lorenzi, O., Frieden, M., Villemin, P., Fournier, M., Foti, M., and Vischer, U. M. (2008) J. Thromb. Haemost. 11, 1962–1969

VEGFR2/Tyr1175 Signaling Controls vWF Release

Iwakura, Y., and Shibuya, M. (2006) Blood 108, 1849–1856