Protein Kinase D-dependent Phosphorylation and Nuclear Export of Histone Deacetylase 5 Mediates Vascular Endothelial Growth Factor-induced Gene Expression and Angiogenesis

Chang Hoon Ha1, Weiye Wang1, Bong Sook Jhun1, Chelsea Wong1, Angelika Hausser5, Klaus Pfizenmaier5, Timothy A. McKinsey5, Eric N. Olson1, and Zheng-Gen Jin1,1

From the Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14586, the Institute of Cell Biology and Immunology, University of Stuttgart, 70659 Stuttgart, Germany, Myogen, Inc., Westminster, Colorado 80021, and the Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Vascular endothelial growth factor (VEGF) is essential for normal and pathological angiogenesis. However, the signaling pathways linked to gene regulation in VEGF-induced angiogenesis are not fully understood. Here we demonstrate a critical role of protein kinase D (PKD) and histone deacetylase 5 (HDAC5) in VEGF-induced gene expression and angiogenesis. We found that VEGF stimulated HDAC5 phosphorylation and nuclear export in endothelial cells through a VEGF receptor 2-phospholipase Cγ-protein kinase C-PKD-dependent pathway. We further showed that the PKD-HDAC5 pathway mediated myocyte enhancer factor-2 transcriptional activation and a specific subset of gene expression in response to VEGF, including NR4A1, an orphan nuclear receptor involved in angiogenesis. Specifically, inhibition of PKD by overexpression of the PKD kinase-negative mutant prevents VEGF-induced HDAC5 phosphorylation and nuclear export as well as NR4A1 induction. Moreover, a mutant of HDAC5 specifically deficient in PKD-dependent phosphorylation inhibited VEGF-mediated NR4A1 expression, endothelial cell migration, and in vitro angiogenesis. These findings suggest that the PKD-HDAC5 pathway plays an important role in VEGF regulation of gene transcription and angiogenesis.

Angiogenesis, the formation of new blood capillaries, is an important component of embryonic vascular development, wound healing, and organ regeneration, as well as the pathological processes such as diabetic retinopathies, atherosclerosis, and tumor growth (1–3). Vascular endothelial growth factor (VEGF)2 is essential for many angiogenic processes both in normal and pathological conditions (3). VEGF receptors, VEGFR1 (Flt1) and VEGFR2 (mouse Flk1 or human KDR), are restricted in their tissue distribution primarily to endothelial cells (ECs). The binding of VEGF to its cognate receptors induces dimerization and subsequent phosphorylation of the receptors leading to the activation of several intracellular signaling molecules such as phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), protein kinase C (PKC), nitric-oxide synthase, mitogen-activated protein kinases and focal adhesion kinases (4–7). Gene expression induced by VEGF in ECs plays an important role in controlling angiogenesis. However, the links from VEGF receptors intracellular signaling cascades to gene regulation remain largely elusive.

Protein kinase D (PKD), a serine/threonine protein kinase, has been implicated in the regulation of a variety of cellular functions, including signal transduction (8). Recently we have demonstrated that VEGF rapidly induces activation of PKD via the VEGFR2-PLCγ-PKC pathway, and that PKD is involved in VEGF-induced mitogen-activated protein kinase (ERK1/2) signaling and endothelial cell proliferation (9). Furthermore, it has recently been proposed that PKD regulates gene transcription via the control of class II histone deacetylases (HDACs) subcellular localization in T lymphocytes and in cardiac cells (10–13). However, whether PKD regulates class II HDACs in vascular ECs is unclear.

Histone acetylation/deacetylation has emerged as a fundamental mechanism for the control of gene expression (14, 15). Class II HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) have been shown to mediate interactions with several transcriptional coactivators and confers responsiveness to calcium-dependent signaling (14, 15). For example, HDAC4 controls chondrocyte hypertrophy during skeletogenesis (16), and HDAC7 maintains vascular integrity by repressing cellular signal-regulated kinase; m.o.i., multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PKD, protein kinase D; PKC, protein kinase C; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; BAEC, bovine aortic endothelial cell; siRNA, small interfering RNA; RT, reverse transcriptase; GFP, green fluorescent protein; CaM, calmodulin; WT, wild type.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 15–35 and Table 51.
2 To whom correspondence should be addressed: 211 Bailey Rd., West Henrietta, Rochester, NY 14586. Tel.: 585-276-9783; Fax: 585-276-9829; E-mail: zheng-gen.jin@urmc.rochester.edu.
3 The abbreviations used are: VEGF, vascular endothelial growth factor; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ERK, extracellular signal-regulated kinase; m.o.i., multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PKD, protein kinase D; PKC, protein kinase C; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; BAEC, bovine aortic endothelial cell; siRNA, small interfering RNA; RT, reverse transcriptase; GFP, green fluorescent protein; CaM, calmodulin; WT, wild type.

Received for publication, January 10, 2008, and in revised form, February 20, 2008. Published, JBC Papers in Press, March 10, 2008, DOI 10.1074/jbc.M800264200
matrix metalloproteinase (17). HDAC5 and HDAC9 act as signal-responsive repressors of cardiac hypertrophy (18, 19). However, little is known about the role of class II HDACs in VEGF signaling.

In this report, we show that VEGF stimulates PKD-dependent phosphorylation of HDAC5 at Ser259/498 residues in ECs, which leads to HDAC5 nuclear exclusion and myocyte enhancer factor-2 (MEF2) transcriptional activation. Furthermore, this PKD-HDAC5 pathway mediates a VEGF-induced specific subset of gene expression, cell migration, and tube formation. These findings establish a critical role for PKD and HDAC5 in regulation of gene transcription and angiogenesis by VEGF.

EXPERIMENTAL PROCEDURES

Materials—Plasmids pEGFP-PKD1-WT and pEGFP-PKD1-KN, pCMV-FLAG-HDAC5-WT and pCMV-FLAG-HDAC5-S/A have been described previously (10, 20). Anti-phospho-HDAC5 Ser259/498 (p-HDAC5) antibodies were generated as described previously (10). Anti-PKD and anti-HDAC5 antibodies were from Cell Signaling Technologies. Anti-β-actin antibodies were from Santa Cruz Biotechnology. VEGF and neutralization antibodies against VEGFR1 and VEGFR2 were purchased from R&D System, Inc. All of the pharmacological inhibitors were from Calbiochem.

Cell Culture, Transfection, and Report Assay—Human umbilical vein endothelial cells (HUVECs) were grown in medium 200 with LSGS (Cascade Biologics, Inc.) as described previously (9, 21). BAECs were purchased from Clontech and cultured in medium 199 supplemented with 10% fetal bovine serum as described previously (22). Because the siRNAs and RT-PCR primers used specifically target human proteins or RNA, all experiments related to siRNA and RT-PCR were performed in HUVECs.

Transfections for the reporter assay were performed by electroporation (Bio-Rad). Cells were stimulated 8 h before harvesting, and reporter assays were performed according to the dual luciferase reporter assay using the manufacturer’s recommendations (Promega).

The siRNA duplex targeting PKD1 and transfection have been described previously (9). VEGF stimulation was performed 48 h after siRNA transfection.

Adenovirus Constructs and Infection—Adenovirus constructs encoding human VEGF2 kinase inactive mutant (VEGF2-K868M) were kindly provided by Dr. Masabumi Shibuya (4). Adenoviruses encoding FLAG-HDAC5-WT and FLAG-HDAC5-WT-S/A were generated as described previously (10). Adenovirus expressing GFP-HDAC5-WT, GFP-HDAC5-S/A, and GFP-PKD1-KN were generated from pCMV-FLAG-HDAC5-WT, pCMV-FLAG-HDAC5-S/A (10), and pEGFP-PKD1-KN (20), respectively, using the ViraPower Adenoviral Expression System (Invitrogen). Adenovirus containing β-galactosidase (lacZ) or GFP was used as a control.

ECs were infected with recombinant adenoviruses at the indicated multiplicity of infection (m.o.i.) for 24 h, and then treated with or without inhibitors followed by the application of VEGF (9).

FIGURE 1. VEGF stimulates HDAC5 phosphorylation in endothelial cells. A, BAECs were exposed to VEGF (20 ng/ml) for various times as indicated (A) or to VEGF for 15 min with the indicated concentrations. B, HDAC5 phosphorylation and expression in cell lysates were determined as described under “Experimental Procedures.” Representative immunoblots and quantitative data of HDAC5 phosphorylation normalized with the level of HDAC5 are shown (n = 4). *, p < 0.05 versus control without VEGF treatment.

SDS-PAGE and Western Blotting—SDS-PAGE and Western blot analysis were performed according to standard procedures (9, 22). After incubating with IRDye infrared secondary antibodies (LI-COR Biosciences), immunoreactive proteins were visualized by the Odyssey Infrared Imaging System. Densitometric analyses of immunoblots were performed with Odyssey software (LI-COR Biosciences) (9).
Immunofluorescence—For immunofluorescence experiments, Ad-GFP-HDAC5, Ad-FLAG-HDAC5, and Ad-GFP-PKD1 and their mutants were infected into ECs. GFP-expressing cells were left unstimulated or stimulated with VEGF in the presence or absence of the indicated protein kinase inhibitors. Localization of the fluorescent proteins was assessed by fluorescence microscopy (Olympus BX51; Olympus, Inc.), and the images were captured and analyzed by the Spot software system (RT Color Diagnostic Instruments) (10).

RT-PCR—Total RNA was isolated from cultured ECs using a Total RNA Isolation Kit (Qiagen). First-strand cDNA was synthesized with the SuperScript Preamplification System (Invitrogen). cDNA was amplified by PCR for 30 cycles (Applied Biosystem) (23). Primer sequences are listed in supplemental data Table S1. Human glyceraldehyde-3-phosphate dehydrogenase served as an internal control.

Wound Closure Cell Migration—For the measurement of cell migration during wound closure (24), ECs were seeded on 6-well plates coated with gelatin and grown to confluence. Cells were infected with recombinant adenoviruses where indicated. Monolayers were then disrupted with a cell scraper of ~1.2 mm and photographed at 0 and 12 h after VEGF addition in a light microscope (Olympus CK40) equipped with a digital camera (Olympus DP11). The number of endothelial cells that migrated into the wounded area were counted.

Tube formation assay for in vitro angiogenesis. For the in vitro angiogenesis assay (24), ECs infected with adenoviruses were plated on a thin layer of Matrigel (BD Biosciences) at 5 x 10⁴ cells/well of a 24-well plate in 1% fetal bovine serum EC medium and incubated for 12 h at 37 °C. The capillary-like tube structures were visualized by light microscopy (Olympus CK40) at different time points and imaged with a digital camera (Olympus DP11). The total length of the tube-like structures per field was measured with an ImageJ analyzer as previously described (25). Ten random fields were measured per dish, and the total length per field was calculated.

Statistical Analysis—All values are expressed as mean ± S.E. The significance of the results was assessed by a paired t test between two groups. Differences among 3 or more groups were analyzed by contrast analysis, using the Super analysis of variance. A p value <0.05 was considered significant.

RESULTS

VEGF Stimulates HDAC5 Phosphorylation in Endothelial Cells—To examine the potential role of HDAC5 in VEGF signaling, we first studied HDAC5 phosphorylation in ECs in response to VEGF. BAECs were stimulated with VEGF (20 ng/ml) at different times as indicated, and the phosphorylation of HDAC5 in cell lysates was determined by Western blots with phospho-specific HDAC5 antibodies, which recognize HDAC5 phosphorylated at Ser259 and Ser498 (10). VEGF rapidly induced HDAC5 phosphorylation within 2 min, the activation reached a maximum between 15 and 60 min (Fig. 1A). During the course of VEGF stimulation, the level of total
HDAC5 expression in cells was not changed. Moreover, VEGF induced HDAC5 phosphorylation in a dose-dependent manner at a concentration as low as 1 ng/ml and achieved a maximum of the activation at 10–100 ng/ml (Fig. 1B). The phosphorylation of HDAC5 by VEGF is not limited on BAECs because VEGF also stimulated HDAC5 phosphorylation in similar time- and dose-dependent manners in HUVECs (data not shown).

VEGFR2-mediated PLCγ/PKC Pathway, but Not CaM-CaMK Pathway, Regulates VEGF-induced HDAC5 Phosphorylation—Using neutralizing antibodies against VEGFR1 and VEGFR2 as well as VEGFR2 kinase-inactive mutant, we observed that VEGF-induced HDAC5 phosphorylation is mediated by VEGFR2 but not VEGFR1 (supplemental data Fig. S1).

We further examined signal pathways involved in VEGF-induced HDAC5 phosphorylation. The PLCγ-specific inhibitor U73122 completely blocked HDAC5 phosphorylation (Fig. 2A), whereas the PI3K inhibitor LY294002 did not inhibit VEGF-induced HDAC5 phosphorylation (Fig. 2B). Moreover, the PKC inhibitors GF109203X and Ro-31-8425 inhibited HDAC5 phosphorylation in a dose-dependent manner (Fig. 2, C and D). These results indicate that the PLCγ-PKC pathway mediates VEGF-stimulated HDAC5 phosphorylation in ECs. In contrast, we observed that the calcium-CaM-CaMK pathway was not involved in HDAC5 phosphorylation in ECs by VEGF (supplemental data Fig. S2).

PKD Mediates HDAC5 Phosphorylation by VEGF—We have previously shown that VEGF stimulated PKD phosphorylation and activation in a PLCγ/PKC pathway-dependent manner (9). To examine the potential role of PKD in VEGF-induced HDAC5 phosphorylation, we inhibited the PKD function with three strategies. First a potential PKD inhibitor Go6976 inhibited VEGF-induced HDAC5 phosphorylation in a dose-dependent manner (Fig. 3A). Second, knockdown PKD1 expression in HUVECs by siRNA (9) markedly attenuated VEGF-induced HDAC5 phosphorylation (Fig. 3B). Third, Ad-GFP-PKD1-KN significantly reduced VEGF-induced HDAC5 phosphorylation, whereas the infection of BAECs with Ad-GFP-PKD1-WT enhanced basal HDAC5 phosphorylation (Fig. 3C). Taken together, these data demonstrate an essential role of PKD1 for VEGF-induced HDAC5 phosphorylation in addition, we observed that PKD1 and HDAC5 were phosphorylated by VEGF stimulation in vivo in mouse aortas (supplemental data Fig. S3).

VEGF Stimulates HDAC5 Nuclear Export—To gain insight into the functional significance of HDAC5 phosphorylation in VEGF-mediated signaling events, we studied the effect of VEGF on HDAC5 subcellular localization with HDAC5 fused with GFP. BAECs were infected with adenovirus expressing GFP-tagged HDAC5 WT, and then exposed to 20 ng/ml VEGF. Without treatment of VEGF, GFP-HDAC5 was located primarily in the nuclei of ECs (Fig. 4A). In response to VEGF stimulation, GFP-HDAC5 in the cells underwent a time-dependent nucleocytoplasmic shuttling (Fig. 4A). Nuclear export of HDAC5 was observed at 30 min after application of VEGF, and reached a maximum at 2 h. Exported GFP-HDAC5 remained in the cytoplasm for several hours, and then was gradually imported into the nucleus after a 12-h VEGF stimulation. At 24 h after VEGF treatment, almost all of the GFP-HDAC5 was shuttled back into the nucleus from the cytoplasm (Fig. 4A).

PLCγ-PKC-PKD Pathway Mediates VEGF-induced HDAC5 Nuclear Export—Similar to HDAC5 phosphorylation, the selective pharmacological inhibitors for PLCγ and PKCs abolished VEGF-induced HDAC5 nuclear export (Fig. 4B), but the PI3K inhibitor LY294002, calcium chelator BAPTA/AM, and CaMK II inhibitor KN93 had no effect on HDAC5 nuclear export by VEGF (Fig. 4B).

Consistent with the critical role of PKD in VEGF-induced HDAC5 phosphorylation, the PKD inhibitor Go6976 and siRNA to PKD1 also blocked VEGF-induced HDAC5 nuclear export (Fig. 5, A and B). Moreover, in ECs infected with an adenovirus expressing FLAG-tagged HDAC5 (Ad-FLAG-HDAC5), VEGF also stimulated nuclear export of FLAG-HDAC5 observed by immunocytochemistry with anti-FLAG antibody (Fig. 5C). Co-infection of Ad-FLAG-PKD1-WT induced nuclear export of FLAG-HDAC5 no matter the VEGF treatment or not (Fig. 5C). In contrast, co-infection of Ad-GFP-PKD1-KN blocked nuclear export of HDAC5 in response to VEGF (Fig. 5C). Of note, GFP-PKD1-KN was mainly concentrated in the Golgi and affected cell morphology, which is consistent with a previous report (20).

HDAC5 Phosphorylation on Ser259/498 Sites Is Required for Its Nuclear Export—To determine whether PKD-dependent phosphorylation of HDAC5 at Ser259/498 residues is required...
for HDAC5 nuclear export, we studied subcellular localization of the GFP-HDAC5-S/A mutant, in which serine 259/498 residues were replaced with alanine. ECs were infected with Ad-GFP, Ad-GFP-HDAC5-WT, or Ad-GFP-HDAC5-S/A. After VEGF stimulation for 2 h, GFP-HDAC5-WT was translocated into the cytoplasm (Fig. 5D). In contrast, GFP-HDAC5-S/A remained in the nucleus after VEGF stimulation. Similar results were observed when ECs were infected with Ad-FLAG-HDAC5-WT and Ad-FLAG-HDAC5-S/A (supplemental data Fig. S4). Collectively, the data indicate that PKD-dependent phosphorylation of HDAC5 at Ser259/498 is critical for its nuclear export.

**PKD-HDAC5 Pathway Is Involved in VEGF-induced MEF2 Transcriptional Activation and Gene Expression—**Class II HDACs have been shown to interact with transcription factors of the MEF2 family and play an important role in the repression of MEF2-dependent gene expression (14). Thus, we studied the effect of FLAG-HDAC5-S/A and GFP-PKD1-KN on VEGF-induced MEF2 transcriptional activation in ECs using the 3xMEF2 luciferase reporter assay. In the cells transfected with 3xMEF2 luciferase plasmids and control empty vector, VEGF significantly increased MEF2 transcriptional activity (Fig. 6A).

Co-transfection of FLAG-HDAC5-S/A plasmids and control empty vector, VEGF significantly increased MEF2 transcriptional activity (Fig. 6A). Furthermore, PKD1 was also involved in the process because GFP-PKD1-KN significantly inhibited the VEGF-induced MEF2 transcriptional activation (Fig. 6B).

To determine the role of PKD and HDAC5 in VEGF regulation of genes, we studied the expression of *NR4A1* (also named *Nur77*), a MEF2-dependent immediate-early response gene and orphan nuclear receptor transcription factor previously implicated in tumor cell, lymphocyte, and neuronal growth and apoptosis (23). VEGF strongly stim-
NR4A1 mRNA induction in a time-dependent manner, which peaked at 1 h (Fig. 6C). Both Ad-FLAG-HDAC5-S/A and Ad-GFP-PKD1-KN significantly inhibited the induction of NR4A1 by VEGF (Fig. 6D).

We further explored the potential involvement of PKD1-HDAC5 in regulation of other VEGF responsive genes. HUVECs were infected with Ad-GFP (control), Ad-GFP-HDAC5-S/A, or Ad-GFP-PKD1-KN for 24 h, and then treated with VEGF for 1 h. Using RT-PCR analysis, we found that both HDAC5-S/A and PKD1-KN markedly down-regulated a subset of VEGF-responsive genes, including NR4A1, NR4A2, KLF2 (Kruppel-like factor 2), and KLF4 (Fig. 6E). However, the induction of other VEGF-responsive genes including EGR1, EGR2, FOS, and FOSB were not affected by HDAC5-S/A and PKD1-KN (Fig. 6F).

PKD-HDAC5 Pathway Is Critical for VEGF-induced EC Migration and Tube Formation—Both NR4A1 and KLF2 are involved in VEGF-induced angiogenesis (26) (27). Thus, we...
asked whether the PKD-HDAC5 pathway through regulation of gene expression is implicated in the processes of angiogenesis. In the wound-closure assay (24), VEGF significantly stimulates EC migration (Fig. 7A). Ad-GFP-PKD1-WT increased basal EC migration, which may be due to overexpression of PKD1-WT-increased PKD1 phosphorylation and activation (data not shown) as well as HDAC5 phosphorylation and nuclear export (Figs. 3C and 5C). Ad-GFP-PKD1-WT also enhanced overall the VEGF-induced EC migration. In contrast, Ad-GFP-PKD1-KN markedly decreased VEGF-mediated ECs migration and wound closure (Fig. 7, A and B). VEGF-induced EC migration was also substantially inhibited by Ad-FLAG-HDAC5-S/A infection (Fig. 7, C and D).

In the assay of in vitro angiogenesis, the capability of primary ECs to form capillary-like tube structures was investigated upon cultivation on Matrigel and quantified by measuring the length of the tube-like structure (24, 25). VEGF stimulated capillary-like tube formation in Matrigel (Fig. 8A). Ad-GFP-PKD1-WT infection increased basal and VEGF-induced capillary-like tube formation (Fig. 8, A and B). However, the infection of Ad-GFP-PKD1-KN in ECs significantly attenuated VEGF-induced capillary-like tube formation (Fig. 8, A and B). The infection of Ad-FLAG-HDAC5-S/A had similar inhibitory effects on VEGF-induced capillary-like tube formation (Fig. 8, C and 8D). Taken together, these data indicate a critical role for HDAC5 and PKD1 in regulation of VEGF-induced in vitro angiogenesis.

DISCUSSION

The major findings of this study are that VEGF stimulates PKD-dependent HDAC5 phosphorylation and nuclear export in ECs, and that the PKD1-HDAC5 pathway is involved in VEGF-induced MEF2-dependent gene expression, EC
migration, and tube formation. Because MEF2-dependent transcription and NR4A1 gene expression are implicated in angiogenesis in vitro and in vivo (26), our data suggest that the PKD-HDAC5 pathway is likely to play an important role in VEGF signaling and angiogenesis in physiological and pathological conditions.

Acetylation of chromatin proteins and transcription factors is part of a complex signaling system that is involved in the control of gene expression (14, 15). Recent studies showed that class II HDACs act as signal-responsive repressors of cardiac hypertrophy (18, 28). The present study further revealed an important role of HDAC5 as a VEGF signal-responsive repressor of MEF2 transcriptional activation in ECs. We showed that VEGF promotes phosphorylation of two serine 259/498 residues in HDAC5. Furthermore, we observed that VEGF induced HDAC5 translocated from the nuclei to the cytoplasm after VEGF stimulation of ECs, and increased MEF2 transcriptional activation. Mutation of these serine residues to alanine (HDAC5-S/A mutant) blocked nuclear-cytoplasmic shuttling in response to VEGF. This HDAC5-S/A mutant also inhibited the VEGF-stimulated increase in MEF2 transcriptional activation, NR4A1 expression, and EC migration and tube formation. Our results indicate that the gene repressive action of HDAC5 in ECs is overcome by the PLCγ-PKC-PKD pathway-dependent HDAC5 phosphorylation and nuclear export in response to VEGF.

PKD, as a downstream target of the PLCγ-PKC pathway, has emerged as a key regulator in many cellular functions (29). We recently reported that PKD1 is highly expressed in ECs, and that PKC-dependent PKD1 activation is one of the early signaling events in ECs in response to VEGF (9). In this study, we provide strong evidence

FIGURE 8. PKD-dependent HDAC5 phosphorylation is required for VEGF-induced in vitro angiogenesis. HUVECs infected with (A and B) Ad-GFP (control), Ad-GFP-PKD-WT, or Ad-GFP-PKD1-KN or (C and D) Ad-LacZ (control) or Ad-FLAG-HDAC5-S/A were cultured in Matrigel and measured for in vitro angiogenesis in response to VEGF with tube formation assay. The representative images and the length of tube-like structure were quantified and shown (n = 4). The arrow indicates the tube-like structure. *, p < 0.05 in comparison to value from control; # and &, p < 0.05 versus that from the group treated with VEGF alone.
that PKD1 is a key kinase to mediate HDAC5 phosphorylation and nuclear export in VEGF signaling. We show that HDAC5 phosphorylation and nuclear export is mediated through the VEGF-stimulated VEGFR2-PLCγ-PKC pathway. Our three different approaches including pharmacological inhibitors, siRNA, and the dominant negative mutant of PKD1 further revealed a critical role of PKD1 in mediating VEGF-induced HDAC5 phosphorylation and nuclear export. Of note, overexpression of PKD1-WT alone enhanced HDAC5 phosphorylation and nuclear export as well as cell migration, which may be due to constitutive activation of PKD1 because we observed that overexpression of PKD1 in cells stimulated its phosphorylation and activation (data not shown). Besides PKD, CaMKs has been implicated in phosphorylation and nuclear export of HDAC5 when transfected into COS cells and in H9C2 cardiomyocytes (28, 30, 31). However, in VEGF signaling in ECs, we found that the CaM-CaMK pathway is not involved in these processes.

Signal transduction pathways mediate the biological function of VEGF, including stimulation of cell proliferation, migration, increasing vascular permeability, and angiogenesis (4, 7). Our recent report shows that PKD mediates the VEGFR2-PLCγ-PKC pathway to ERK1/2 activation and EC proliferation (9). In the present study, we further found that PKD has a novel role in VEGF regulation of MEF2 transcriptional activation via targeting on HDAC5. Both overexpression of PKD1-KN and HDAC5-S/A by adenovirus infection significantly inhibited VEGF-stimulated MEF2 transcriptional activation in ECs. MEF2 family transcription factors have been implicated in blood vessel development and vascular integrity (32). Several MEF2-dependent genes, including NR4A1 and KLF2, have been identified (17, 23). In particular, it has recently been shown that NR4A1 regulates VEGF-induced EC proliferation, survival, and tube formation and angiogenesis in vivo (26). In this study, we observed that VEGF stimulated the induction of NR4A1 in ECs in a PKD-HDAC5 pathway-dependent manner, because overexpression of PKD1-KN and HDAC5-S/A blocked VEGF-induced NR4A1 expression. Of note, the peak of NR4A1 induction was 1 h, but the maximal HDAC5 nuclear export appeared at about 2 h of VEGF treatment. One possible explanation for this is that the phosphorylated HDAC5 releases its repressive effects on MEF2 transcription activation before being exported to the cytoplasm, as suggested by previous studies that show that CaM kinase signaling disrupted the MEF2-HDAC5 complex even in the absence of the HDAC5 nuclear export (28). Moreover, VEGF-mediated EC migration and tube formation were inhibited by expression of PKD1-KN and HDAC5-S/A. Although PKD/HDAC5-dependent NR4A1 induction is important for VEGF-induced angiogenesis, it is likely that many other genes in angiogenesis may be targeted by the PKD/HDAC5 pathway. Indeed, we found that the PKD-HDAC5 pathway regulated a subset of gene expressions, including NR4A1, NR4A2, KLF2, and KLF4. In contrast, VEGF-induced early-responsive growth genes, including EGR1, EGR2, FOS, and FOSB, are not affected by PKD-HDAC5 pathway.

In summary, our studies have demonstrated that VEGF stimulates HDAC5 phosphorylation and nuclear export in ECs through the VEGFR2-PLCγ-PKC-PKD pathway. Our experiments also provide evidence supporting a role of PKD and HDAC5 in VEGF-induced MEF2 transcriptional activation, NR4A1 expression, EC migration, and in vitro angiogenesis. Thus, the present findings reveal a critical role of HDAC5 as a PKD substrate in VEGF signaling, and suggest that the PKD-HDAC5 pathway is important for mediating angiogenesis by VEGF. As such, it could help develop new strategies to control physiological and pathological angiogenesis implicated in cardiovascular disease, diabetes, and cancer.

Acknowledgment—We thank Dr. Jane Sottile for critical reading of the manuscript.

REFERENCES

1. Folkman, J. (1995) Nat. Med. 1, 27–31
2. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) Nat. Med. 9, 669–676
3. Carmeliet, P. (2003) Nat. Med. 9, 653–660
4. Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. (2001) EMBO J. 20, 2768–2778
5. Zachary, I. (2003) Biochem. Soc. Trans. 31, 1171–1177
6. Claesson-Welsh, L. (2003) Biochem. Soc. Trans. 31, 20–24
7. Sakurai, Y., Ohgimoto, K., Katoaka, Y., Yoshida, N., and Shibuya, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1076–1081
8. Rozengurt, E., Roy, O., and Waldron, R. T. (2005) J. Biol. Chem. 280, 13205–13208
9. Dong, C., and Jin, Z. G. (2005) J. Biol. Chem. 280, 33262–33269
10. Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N., and McKinsey, T. A. (2004) Mol. Cell. Biol. 24, 8374–8385
11. Dequiedt, F., Van Lint, J., Lecomte, E., Van Duppen, V., Seufferlein, T., Vandenhende, J. R., Wattiez, R., and Kettmann, R. (2005) J. Exp. Med. 201, 793–804
12. Harrison, B. C., Kim, M. S., van Rooij, E., Plato, C. F., Papst, P. J., Vega, R. B., McNally, J. A., Richardson, J. A., Bassel-Duby, R., Olson, E. N., and McKinsey, T. A. (2006) Mol. Cell. Biol. 26, 3875–3888
13. Matthews, S. A., Liu, P., Spitaler, M., Olson, E. N., McKinsey, T. A., Cantrell, D. A., and Scharenberg, A. M. (2006) Mol. Cell. Biol. 26, 1569–1577
14. McKinsey, T. A., and Olson, E. N. (2005) J. Clin. Invest. 115, 538–546
15. Backs, J., and Olson, E. N. (2006) Circ. Res. 98, 15–24
16. Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McNally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A., Karsenty, G., and Olson, E. N. (2004) Cell 119, 555–566
17. Chang, S., Young, B. D., Li, S., Qi, X., Richardson, J. A., and Olson, E. N. (2006) Cell 126, 321–334
18. Zhang, C. L., McKinsey, T. A., Chang, S., Antos, C. L., Hill, J. A., and Olson, E. N. (2002) Cell 110, 479–488
19. Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004) Mol. Cell. Biol. 24, 8467–8476
20. Haussler, A., Link, G., Bamberg, L., Burzlaff, A., Lutz, S., Pfenzermaier, K., and Jahnnes, F. J. (2002) J. Cell Biol. 156, 65–74
21. Jin, Z. G., Wong, C., Wu, J., and Berk, B. C. (2005) J. Biol. Chem. 280, 12305–12309
22. Jin, Z. G., Ueba, H., Tanimoto, T., Lunga, C. O., Griffith, R. M., and Berk, B. C. (2003) Circ. Res. 93, 354–363
23. Youn, H. D., and Liu, J. O. (2000) Immunity 13, 85–94
24. Shen, T. L., Park, A. Y., Alcaraz, A., Peng, X., Jang, I., Koni, P., Flavell, R. A., Gu, H., and Guan, J. L. (2005) J. Cell Biol. 169, 941–952
25. Pi, X., Garin, G., Xie, L., Zheng, Q., Wei, H., Abe, J., Yan, C., and Berk, B. C. (2005) Circ. Res. 96, 1145–1151
26. Zeng, H., Qin, L., Zhao, D., Tan, X., Manseu, E. J., Van Hoang, M., Senger, D. R., Brown, L. F., Nagy, J. A., and Dvorak, H. F. (2006) J. Exp.

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 283 • NUMBER 21 • MAY 23, 2008

14598
Kuo, C. T., Veselits, M. L., Barton, K. P., Lu, M. M., Clendenin, C., and Leiden, J. M. (1997) *Genes Dev.* **11**, 2996–3006

McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 14400–14405

Wang, Q. J. (2006) *Trends Pharmacol. Sci.* **27**, 317–323

McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) *Nature* **408**, 106–111

Sucharov, C. C., Langer, S., Bristow, M., and Leinwand, L. (2006) *Am. J. Physiol.* **291**, C1029–C1037

Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A., and Olson, E. N. (1998) *Development* **125**, 4565–4574