Endorepellin Affects Angiogenesis by Antagonizing Diverse Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)-evoked Signaling Pathways

TRANSCRIPTIONAL REPRESSION OF HYPOXIA-INDUCIBLE FACTOR 1α AND VEGFA AND CONCURRENT INHIBITION OF NUCLEAR FACTOR OF ACTIVATED T CELL 1 (NFAT1) ACTIVATION

Background: Endorepellin specifically targets endothelial cells via dual-receptor antagonism of α2β1 and VEGF2 to inhibit angiogenesis.

Results: Endorepellin attenuates two major signaling branches of VEGFR2 to transcriptionally repress HIF-1α concurrent with stabilized and cytoplasmically localized NFAT1.

Conclusion: Endorepellin suppresses signaling of VEGFR2 independent of oxygen tension to inhibit angiogenesis.

Significance: Endorepellin via dual-receptor antagonism provides novel mechanisms applicable to similar angiostatic fragments.

Endorepellin, the angiostatic C-terminal domain of the heparan sulfate proteoglycan perlecan, inhibits angiogenesis by simultaneously binding to the α2β1 integrin and the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) on endothelial cells. This interaction triggers the down-regulation of both receptors and the concurrent activation of the tyrosine phosphatase SHP-1, which leads to a signaling cascade resulting in angiostasis. Here, we provide evidence that endorepellin is capable of attenuating both the PI3K/PDK1/Akt/mTOR and the PKC/JNK/AP1 pathways. We show that hypoxia-inducible factor 1α (HIF-1α) transcriptional activity induced by VEGFA was inhibited by endorepellin independent of oxygen concentration and that only a combination of both PI3K and calcineurin inhibitors completely blocked the suppressive activity evoked by endorepellin on HIF1A and VEGFA promoter activity. Moreover, endorepellin inhibited the PKC/JNK/AP1 axis induced by the recruitment of phospholipase γ and attenuated the VEGFA-induced activation of NFAT1, a process dependent on calcineurin activity. Finally, endorepellin inhibited VEGFA-evoked nuclear translocation of NFAT1 and promoted NFAT1 stability. Thus, we provide evidence for a novel downstream signaling axis for an angiostatic fragment and for the key components involved in the dual antagonistic activity of endorepellin, highlighting its potential use as a therapeutic agent.

The complex interplay between tumor cells and their vascularized stroma has profound effects on cancer cell proliferation, migration, metastasis, and angiogenesis. Moreover, the newly formed angiogenic vessels exert an instructive role as a vascular niche by providing a paracrine and “angiocrine” mode of regulation (1). This mechanism involves the secretion and processing of various growth factors and extracellular matrix constituents that influence tumor and endothelial cells in a bidirectional manner, with integrins acting as functional hubs for pathological angiogenesis (2). Heparan sulfate proteoglycans act as depots for pro- and anti-angiogenic factors (3–7), and in concert with members of the fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) family and their receptors, modulate various steps of angiogenesis (8–10). These heparan sulfate-bound factors are dynamically processed by various proteases and heparanase to target their cognate receptor and augment their biological activity during development, tissue remodeling, and cancer growth (9, 11–14).

Perlecan is a key heparan sulfate proteoglycan of basement membranes (15, 16) and cell surfaces (17–19) encoded by a gene encompassing 97 exons (20), with a complex promoter region (21, 22) that drives the expression of a very large protein core of nearly 500 kDa (23). One of the intrinsic characteristics of perlecan is its ability to self-assemble in vitro (24), and this attribute may contribute to the proper formation of basement membranes throughout the body (25, 26). Perlecan is widely distributed in mammalian tissues (27–32) and regulates cell adhesion (33), cardiovascular development (34), epidermal formation (35), and tumor angiogenesis (36–39). Moreover, perlecan is involved in lipid metabolism (40), apoptosis (41), premature rupture of fetal membranes (42), and its expression is often elevated in several types of cancer (43, 44).
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Perlecan shows a clear functional dichotomy. The parent perlecan proteoglycan is pro-angiogenic as shown in gene-targeted studies (45–47), by primarily acting as a co-receptor for FGF2 and VEGFA (48–50). Characterization of the zebrafish perlecan knockdown provides strong genetic evidence linking perlecan to developmental angiogenesis (51). We found that angiogenic blood vessel development of the intersegmental vessels was largely inhibited in the absence of perlecan (51). Notably, knockdown of the α2β1 integrin showed a vascular phenotype similar to that evoked by perlecan knockdown (52). Thus, perlecan functions at multiple levels during the angiogenic cascade influencing endothelial cell migration, proliferation, and lumen formation (53, 54).

In contrast to its parent molecule, the C-terminal domain V of perlecan, named endorepellin to designate its intrinsic anti-angiogenic activity (55), is anti-angiogenic in in vitro and in vivo studies (56–59). Endorepellin can be liberated by cathepsin L (60) whereas its C-terminal module LG3 can be cleaved by bone morphogenetic protein 1 (BMP1)/Tolloid-like proteases (61) releasing a smaller biologically active fragment (41, 56). Specifically, endorepellin triggers a signaling cascade that leads to disruption of the endothelial actin cytoskeleton (56, 62–64). Endorepellin interacts with the α2β1 integrin receptor (56, 63, 65), while simultaneously interacting with the α2β1 integrin and VEGFR2 in endothelial cells (66). Importantly, systemic delivery of endorepellin to tumor xenograft-bearing mice causes a marked suppression of tumor growth and metabolic rate mediated by sustained down-regulation of the tumor angiogenic network (57). Genetic analysis using a siRNA-mediated block of endogenous α2β1 integrin or animals lacking the α2β1 integrin receptor have definitively shown that this is a key receptor for endorepellin and thus for the perlecan protein core (58). Therefore, endorepellin represents a member of the family of cryptic domains residing within larger parent molecules of the extracellular microenvironment that act in a dominant negative manner.

The observations summarized above suggest that perlecan/endorepellin might be directly involved in modulating the VEGFA/VEGFR2 signaling axis. Indeed, we discovered that perlecan binds via endorepellin to both VEGFA/VEGFR2 signaling axis. Indeed, we discovered that endorepellin might be directly involved in modulating the rate mediated by sustained down-regulation of the tumor angiogenic network (57). Genetic analysis using a siRNA-mediated block of endogenous α2β1 integrin or animals lacking the α2β1 integrin receptor have definitively shown that this is a key receptor for endorepellin and thus for the perlecan protein core (58). Therefore, endorepellin represents a member of the family of cryptic domains residing within larger parent molecules of the extracellular microenvironment that act in a dominant negative manner.

EXPERIMENTAL PROCEDURES

Antibodies, Cells, and Reagents—The following rabbit antibodies against human VEGFR2, phospho-Akt1 (Ser-473), total Akt, phospho-3-phosphoinositide-dependent protein kinase 1 (PKD1) (Ser-241), total PKD-1, PI3K p110α, anti-PI3K p85, phospho-p38 MAPK (Thr-180/Tyr-182), phospho-SAPK/JNK (Thr-183/Tyr-185), total SAPK/JNK, phospho-endothelial NOS (eNOS) (Ser-1177), total eNOS, phospho-mTOR (Ser-2448), and total mTOR (7C10) were from Cell Signaling, as well as the mouse anti-rabbit IgG (light chain-specific). Mouse monoclonal antibody (mAb) against human SH2 domain-containing adaptor protein B (Shb) and HDM2, and the rabbit antibodies against HIF-1α and clathrin were from Abcam. mAb against human HIF-1α, HIF-1β, and NFAT-1 and rabbit antibodies against caveolin-1 were from BD Biosciences. Rabbit antibodies against VEGFA and PLCγ were from Santa Cruz Biotechnology. Rabbit anti-endorepellin antibody was described before (56). Affinity-purified goat anti-endorepellin antibody and mouse mAb against human VEGFR2 were from R&D Systems. Rabbit anti-GAPDH was from Advance Immunochemical. Secondary HRP-conjugated goat anti-rabbit and anti-mouse antibodies were from Millipore. Goat anti-mouse and anti-rabbit (Alexa Fluor 488) and goat anti-mouse (Alexa Fluor 594) antibodies were from Invitrogen. Recombinant human VEGFA (VEGF165) was obtained from the National Institutes of Health repository. Human umbilical vein endothelial cells (HUVECs) were purchased from Lifeline Cell Technol-

5 The abbreviations used are: VEGFR2, VEGF receptor 2; eNOS, endothelial NOS3; HIF-1α, hypoxia-inducible factor 1α; HUVEC, human umbilical vein endothelial cell; INCA, inhibitor of calcineurin activity; mTOR, mammalian target of rapamycin; NFAT1, nuclear factor of activated T cell 1; PAE, porcine aortic endothelial; PKD1, 3-phosphoinositide-dependent protein kinase 1; PLCγ, phospholipase Cγ; PMA, phorbol 12-myristate 13-acetate; pVHL, von Hippel-Lindau protein; RACK1, receptor for activated C kinase 1; Shb, SH2 domain-containing adaptor protein B; RTK, receptor tyrosine kinase.
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Endorepellin Blocks Coupling of VEGFR2 to the PI3K Axis and Attenuates VEGFA-evoked PI3K/Akt Signaling in Endothelial Cells—VEGFA-evoked activation of VEGFR2 induces autophosphorylation of VEGFR2 intracellular domain at Tyr-1175 which mediates downstream signaling via two major branches, PI3K and PLCγ (78). Endorepellin initiates a signaling cascade by concurrently binding to VEGFR2 and the α2I domain of the α2β1 integrin (66). This leads to activation of SHP-1 and subsequent dephosphorylation of VEGFR2 at Tyr-1175 (59). Thus, we investigated whether any adaptor/signaling molecules known to bind to Tyr-1175 were disrupted by endorepellin. One of the key adaptor proteins linked to Tyr-1175 is Shb (79). Upon stimulation of PAE-VEGFR2 cells with VEGFA,

and blue channels. Images were acquired in single confocal planes to determine co-localization precisely using the ZEN 2010 software, with filters set at 488/594 nm for dual-channel imaging, and Z-stacks acquired at 0.36-μm intervals. All images were analyzed using ImageJ and Adobe Photoshop CS5.1 (Adobe Systems). To quantify co-localization of VEGFR2 and caveolin or clathrin further we utilized line scanning, a technique that measures the pixels along a single defined axis along the specimen to determine localization of the differentially labeled fluorophores (75, 76). The extent of overlap (defined as two different fluorescent labels displaying independent emission wavelengths that occupy the same pixel) between the two potentially interacting molecules serves as a qualitative assessment of a proximity-dependent localization (77).

 Luciferase and Protein Kinase C (PKC) Assays—After washing cells with PBS, 150 μl of lysis buffer (50 mM potassium phosphate buffer, 2% Triton X-100, 20% glycerol, 4 mM DTT) was added to each well. Cells were lysed at 25 °C for 10 min, centrifuged at 2000 × g for 2 min, and ~100 μl of cleared cell lysate was dispensed into a 96-well ELISA plate, together with 100 μl of luciferase assay buffer (100 mM potassium phosphate, 2 mM DTT, 8 mM MgSO4, 175 μM coenzyme A, 750 μM ATP) and 0.5 mM D-luciferin. A plate luminometer (PerkinElmer Life Sciences) was used for light measurement.

PKC activity was measured with a PepTag nonradioactive PKC assay (Promega) according to the manufacturers’ instructions. Cells were treated with VEGFA (2.6 nm) and endorepellin (100 nm), washed with PBS, and lysed in PKC extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, and leupeptin/aprotinin 1 mg/ml each). The precleared supernatants were assessed for PKC activity using a bright colored fluorescent peptide substrate highly specific for PKC. The enzyme alters the net charge of the peptide substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated forms of the substrate to be separated on an agarose gel. The bands containing phosphorylated peptides were extracted from the gel, heated at 95 °C, solubilized, acidified with glacial acetic acid, and finally evaluated by measuring the absorbance at 570 nm.

Statistical Analysis—All data were expressed as means ± S.E. and statistically analyzed with Student’s t test or paired t test using the Sigma-Stat software 11.0 (SPSS Inc). p < 0.05 was considered statistically significant.

RESULTS

Endorepellin Blocks Coupling of VEGFR2 to the PI3K Axis and Attenuates VEGFA-evoked PI3K/Akt Signaling in Endothelial Cells—VEGFA-evoked activation of VEGFR2 induces autophosphorylation of VEGFR2 intracellular domain at Tyr-1175 which mediates downstream signaling via two major branches, PI3K and PLCγ (78). Endorepellin initiates a signaling cascade by concurrently binding to VEGFR2 and the α2I domain of the α2β1 integrin (66). This leads to activation of SHP-1 and subsequent dephosphorylation of VEGFR2 at Tyr-1175 (59). Thus, we investigated whether any adaptor/signaling molecules known to bind to Tyr-1175 were disrupted by endorepellin. One of the key adaptor proteins linked to Tyr-1175 is Shb (79). Upon stimulation of PAE-VEGFR2 cells with VEGFA,
Shb is phosphorylated and binds directly to Tyr-1175, which then mediates PI3K activity, stress fiber formation, and cell migration (79). We discovered that VEGFA-mediated Shb recruitment was attenuated by endorepellin treatment (Fig. 1, A and G, p < 0.01). Concurrently, endorepellin inhibited the recruitment of PI3K evoked by VEGFA (Fig. 1, A and F, p < 0.01).

Key downstream signaling components of the PI3K pathway include PDK1, a master kinase, which is crucial for the activation of Akt/PKB (80), and subsequent activation of eNOS and mTOR (78, 81). Endorepellin alone evoked no significant changes in PDK1 phosphorylation at Ser-241, but inhibited VEGFA-evoked PDK1 phosphorylation (Fig. 1, B and H, p < 0.01). Moreover, endorepellin significantly suppressed
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VEGFA-evoked phosphorylation of Akt1 at Ser-473 (Fig. 1, C and I, p < 0.01), eNOS at Ser-1177 (Fig. 1, D and J, p < 0.01) and mTOR at Ser-2448 (Fig. 1, E and K, p < 0.01). Collectively, these results indicate that endorepellin, by directly interacting with the VEGFR2 ectodomain, antagonizes a major branch of this RTK signaling axis that controls survival, permeability, and migration (81).

Endorepellin Suppresses HIF-1α Transcriptional Activity Independent of Oxygen Concentration—Next, we investigated the role of downstream targets of mTOR. One of the established targets of mTOR is HIF-1α, known to positively regulate VEGFA transcription (82). Because we did not detect any HIF-1α protein in HUVECs under normoxia (supplemental Fig. 1A) we utilized PAE cells overexpressing the human VEGFR2 (PAE-VEGFR2) (66, 68). In these cells we found abundant HIF-1α under normoxia in contrast to either parental cells or cells expressing the human VEGFR1 (supplemental Fig. 1A). Thus, VEGFR2 overexpression can drive HIF-1α expression and/or enhance HIF-1α stability in these cells. HIF-1α levels were markedly down-regulated by endorepellin in a time-dependent fashion, in contrast to those of the constitutively expressed HIF-1β or von Hippel-Lindau protein (pVHL) (Fig. 2A).

Under normoxia, HIF-1α has a very short half-life (~7 min), and its rapid turnover is mediated by hydroxylation of two proline residues (Pro-402 and Pro-564) by oxygen-dependent prolyl-hydroxylase (82). This leads to ubiquitination by pVHL. E3 ubiquitin ligase and degradation via the 26 S proteasome (83). The lack of changes in pVHL levels, thus, suggests that endorepellin-mediated down-regulation of HIF-1α might be transcriptionally mediated. To address this issue directly, we treated PAE-VEGFR2 with lactacystin, an established proteasome inhibitor (84), with or without endorepellin. We found that HIF-1α levels were increased by lactacystin, an effect counteracted by endorepellin (Fig. 2B). Collectively, these data demonstrate not only a potent transcriptional inhibition of the HIF1A locus evoked by endorepellin, but also implicate post-transcriptional mechanisms to subdue HIF-1α to achieve angioptosis.

Next, we utilized CoCl2 to mimic hypoxia by incubating PAE-VEGFR2 cells with 100 μM CoCl2, a non-oxygen-binding cation that inhibits the oxygen-dependent prolyl-hydroxylase thereby stabilizing HIF-1α (82). Endorepellin suppressed CoCl2-induced HIF-1α levels, without affecting the constitutively expressed HIF-1β (Fig. 2C). Confocal microscopy confirmed the endorepellin-mediated down-regulation of HIF-1α under both normoxic and hypoxic conditions (supplemental Fig. S1, B–E). These data, together with the lack of changes in pVHL levels, suggest that endorepellin causes a transcriptional repression of the HIF-1α/VEGFA axis that is active under normoxic and hypoxic conditions.

To address this issue directly, we generated stable transfectants of PAE-VEGFR2 cells harboring ~1 kb of the human HIF1A promoter cloned upstream of luciferase (71). The cells were stable for several months and showed a dose-dependent inhibition of promoter activity following a 2-h incubation with SU5416 (Fig. 2D), a small molecule inhibitor of VEGFR2 tyro-
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sine kinase (85). Endorepellin caused a dose-dependent (IC_{50} \sim 100 \text{ nM}, Fig. 2E) and time-dependent (t_{1/2} \sim 16 \text{ h}, Fig. 2F) suppression of HIF-1α promoter activity. Notably, endorepellin transcriptional repression of HIF1A required VEGFR2 insofar as only the PAE-VEGFR2_HIF1A-Luc cells, but neither the parental PAE nor the PAE-VEGFR2_HIF1A-Luc cells, responded to endorepellin (supplemental Fig. S2A). Endorepellin-evoked suppression of HIF-1α was also independent of oxygen concentration (supplemental Fig. S2B).

Next, we tested the VEGFR2 inhibitor and endorepellin in stably transfected PAE-VEGFR2 cells harboring a 2.6-kb genomic fragment encompassing the human VEGFA promoter cloned upstream of luciferase (66). In this case, endorepellin and SU5416 equally inhibited transcriptional activity of VEGFA promoter (p < 0.05, and p < 0.01, respectively; Fig. 2G). However, there was no significant additive effect when cells were incubated with both endorepellin and SU5416 (p = 0.7; Fig. 2G). Concurrently, endorepellin inhibited secreted VEGFA levels under hypoxic conditions (p < 0.001, supplemental Fig. S2, C and D) comparable with levels achieved under normoxia (66).

To determine whether the decrease of HIF-1α protein levels detected above and those of VEGFA previously reported (66) were due to a decrease in transcription of HIF1A and VEGFA genes, we performed quantitative real-time PCR. In contrast to VHL, both HIF1A and VEGFA mRNA levels were significantly suppressed by endorepellin (p < 0.001, Fig. 2H). Collectively, these findings corroborate the view that endorepellin is a direct antagonist of VEGFR2 and requires an attenuation of its tyrosine kinase activity to evoke repression of HIF1A and VEGFA expression.

Endorepellin Affects HIF-1α and VEGFA Transcriptional Activity Via Both the PI3K and Calcineurin Pathways—To determine whether endorepellin suppressive activity was solely mediated by PI3K signaling pathway, we utilized LY294002, an established inhibitor of this pathway (80). A 6-h incubation with LY294002 using a concentration (10 \text{ M}) that is specific for PI3K caused a significant (56 ± 3%) inhibition of HIF1A promoter activity even greater than endorepellin (31 ± 8%, Fig. 3A). Notably, combination of endorepellin and LY294002 caused an even further suppression (81 ± 3%, p < 0.001, Fig. 3A). These data suggest that endorepellin might inhibit HIF1A promoter activity via an alternate pathway.

It is well established that the serine/threonine phosphatase calcineurin promotes HIF-1α expression by dephosphorylating receptor for activated C kinase 1 (RACK1) (82). This leads to inhibition of RACK1 dimerization and to a block of RACK1-mediated ubiquitination and degradation of HIF-1α (86). Thus, we utilized INCA-6, a cell-permeable inhibitor of calcineurin activity (87). A 4-h incubation with INCA-6 using an optimal concentration (30 \text{ M}) specific for calcineurin suppression (87) evoked inhibition of HIF-1α promoter activity even greater than endorepellin (p < 0.001, Fig. 3B). Combination of endorepellin and INCA-6 caused an even further suppression down to ~36% of control values (Fig. 3B). Similar effects were obtained with 100 \text{ nM} cyclosporin A, an established inhibitor of calcineurin (88) (data not shown). Notably, INCA-6 also suppressed VEGFA promoter luciferase activity in a time-dependent manner (t_{1/2} \sim 3.5 \text{ h}, Fig. 3C). Using a similar protocol we found that INCA-6 suppressed VEGFA levels in PAE-VEGFR2 cells and that combination of both treatments reduced VEGFA protein levels even further (Fig. 3D). Importantly, endorepellin had no repressive effects in addition to a combination of LY294002 and INCA-6 (Fig. 3E). These findings indicate that endorepellin affects both the PI3K/PDK1/Akt1/mTOR and the calcineurin pathways in down-regulating the transcription of HIF1A and VEGFA genes.

Endorepellin Inhibits VEGFA-evoked Recruitment of PLCγ and Inhibits PKC/JNK/AP1 Pathway in Endothelial Cells—Because Tyr-1175 is the major binding site of PLCγ on the VEGFR2 intracellular domain (78) and this residue is dephosphorylated by endorepellin-evoked activation of SHP-1 (59), we determined whether endorepellin would affect the VEGFA-mediated recruitment of PLCγ in HUVECs. Using co-immunoprecipitation we found that VEGFA-mediated PLCγ recruitment was completely blocked by endorepellin treatment (Fig. 4A). We then investigated whether the activity of PKC, a known downstream effector of PLCγ, was also affected by endorepellin. We utilized a quantitative nonradioactive PKC assay, which measures total kinase ability to convert an artificial peptide substrate from +1 to −1. In other words, upon phosphorylation of the synthetic PKC substrate by PKC, the overall net charge changes from +1 to −1 to allow for a delineation of the phosphorylated and unphosphorylated forms. We found that endorepellin was capable of significantly blocking the VEGFA-evoked PKC activity in HUVECs (p < 0.001, Fig. 4B). In agreement with these findings, endorepellin attenuated VEGFA-induced phosphorylation of c-Jun N-terminal kinase (JNK) at Thr-183/Tyr-185 (Fig. 4C).

To investigate further the signaling events downstream of PKC/JNK, we generated stable transfectants of PAE-VEGFR2 harboring an AP1-luciferase reporter construct (70). This vector contains four in tandem repeated copies of the AP1 elements from the human IL2 gene cloned upstream of the minimal IFNB promoter (essentially a TATA box) (Fig. 4D). Mass cultures (i.e. nonclonal cultures) of PAE-VEGFR2AP1-Luc showed a dose-dependent (IC_{50} \sim 25 \text{ nM}, Fig. 4E) and time-dependent (t_{1/2} \sim 6 \text{ h}, Fig. 4F) transcriptional repression of AP1-luciferase promoter activity by endorepellin. Moreover, endorepellin counteracted VEGFA activity in inducing AP1 promoter activity (supplemental Fig. S3A).

Next, we determined whether AP1-luciferase endothelial cells would respond to phorbol 12-myristate 13-acetate (PMA), an analog of diacylglycerol which is released by PLCγ and activates PKC. We found that AP1-dependent luciferase activity was induced by PMA, and endorepellin was capable of markedly suppressing this effect (p < 0.001, Fig. 4G). Consistent with these findings, treatment of these cells with Rottlerin, a PKC inhibitor, suppressed AP1 promoter activity, albeit there was an additive effect with endorepellin (supplemental Fig. S3B). Collectively, our findings implicate endorepellin in interfering with VEGFA activation of the PLCγ/PKC/JNK/AP1 signaling axis.

Endorepellin Inhibits VEGFA-evoked Nuclear Translocation of NFAT1 and Promotes NFAT1 Stability—One of the key transcription factors regulated by calcineurin is NFAT1 (89). VEGFA-evoked activation of calcineurin evokes dephosphorylation of NFAT1 at specific serine residues which leads to acti-
vation of the protein and its subsequent translocation into the nuclei where NFAT1 induces several genes involved in growth control and angiogenesis (90). Thus, we hypothesized that endorepellin could interfere with this important pathway. Using confocal microscopy, we found that VEGFA evoked rapid and efficient NFAT1 translocation into the endothelial cell nuclei (Fig. 5B), in contrast to either vehicle or endorepellin (Fig. 5, A and C, respectively). However, a preincubation with endorepellin significantly blocked NFAT1 nuclear translocation (Fig. 5D). Quantification of NFAT1 nuclear translocation, based on the NFAT1 nuclear/cytoplasmic ratio, showed a significant (84 ± 3%) inhibition by endorepellin (p < 0.01, Fig. 5E).

Next, we examined the intracellular levels of NFAT1 following treatment with the calcineurin inhibitor cyclosporin A (100 nm), endorepellin (100 nm), or the proteasome inhibitor lactacystin (10 μM). We discovered that endorepellin caused a stabilization of the inactive form of NFAT1 (the upper band in Fig. 5F is the serine-phosphorylated inactive form of NFAT1). Both cyclosporin A and lactacystin stabilized NFAT1 in a similar fashion. In contrast, there was no effect on the levels of HDM2, the human homolog of the murine double-minute gene 2 (91), an E3 ubiquitin ligase that targets NFAT1 for proteasomal degradation (91), suggesting a transcriptional effect of endorepellin. To address this possibility directly, we utilized an NFAT-luciferase reporter construct that contains four tandem-repeated copies of NFAT/AP1 elements from the human IL2 gene cloned upstream of the minimal IFNB promoter (70). In these mass cultures of PAE-VEGFR2 NFAT-Luc cells, there was a marked induction of promoter luciferase activity by VEGFA and a marked suppression by preincubation with endorepellin (p < 0.05, Fig. 5G). Notably, endorepellin by itself significantly suppressed NFAT-luciferase promoter activity (p < 0.01, Fig. 5G).
We conclude that endorepellin, by suppressing VEGFA-evoked activation of calcineurin, interferes with NFAT1 nuclear translocation and evokes stabilization of the inactive NFAT1 species. Moreover, endorepellin-evoked suppression of calcineurin activity also affects the levels of NFAT1 transcriptional activity and downstream events such as VEGFA levels (compare Fig. 3).

Endorepellin Induces Physical Down-regulation of VEGFR2 by Evoking Internalization via Caveosomes—We have previously shown that endorepellin causes a physical down-regulation of both α2β1 integrin and VEGFR2 in HUVECs and PAE-VEGFR2 cells (66). Thus, we hypothesized that a possible mechanism for VEGFR2 down-regulation by endorepellin would be by inducing receptor internalization via caveosomes, a process that leads to degradation of several RTKs in contrast to clathrin-mediated internalization which allows for RTK downstream signaling and recycling to the cell surface (92). Using confocal microscopy, we discovered a progressive colocalization of VEGFR2 and caveolin-1 in HUVECs exposed to endorepellin (Fig. 6, C and E), but not in vehicle-exposed cells (Fig. 6A). By 30 min, large perinuclear vesicles were detectable in the endorepellin-treated cells (Fig. 6E). Co-localization of caveolin and VEGFR2 was further proven by using Z-stacks and line scanning profiles. Comparable results were obtained using PAE-VEGFR2 cells (supplemental Fig. S5).

**FIGURE 4.** Endorepellin inhibits VEGFA-evoked recruitment of PLCγ and inhibits PKC/JNK/AP1 pathway in endothelial cells. A, representative co-immunoprecipitation studies using anti-VEGFR2 antibodies in HUVECs treated for 10 min as indicated, followed by immunoblotting against PLCγ or VEGFR2. B, quantitation of total PKC activity in HUVECs treated for 10 min with VEGFA (2.6 nM), endorepellin (100 nM), or both. The cell lysates were subjected to the PepTag nonradioactive PKC assay as described under “Experimental Procedures.” The phosphorylated peptide bands were excised, and PKC activity was quantified by spectrophotometry. Values are means ± S.E. (error bars) (n = 3). ***, p < 0.001. C, representative immunoblotting of HUVEC lysates treated for 10 min with either VEGFA (2.6 nM), endorepellin (100 nM), or both as indicated. The blots were reacted with anti-phospho-JNK1/2 followed by horseradish peroxidase conjugated to anti-rabbit IgG. The blots were stripped and re-probed with total JNK1/2 antibodies. The data represent at least three separate experiments with similar results. D, schematic of the AP1-reporter construct. E, transcriptional dose-response inhibition of AP1 luciferase activity by endorepellin. Stably transfected PAE-VEGFR2<sup>AP1-Luc</sup> cells were exposed to increasing concentrations of endorepellin for 6 h. Values are means ± S.E. from two experiments run in triplicate. F, transcriptional time course inhibition of AP1 promoter luciferase activity by endorepellin (100 nM). Values are means ± S.E. from three experiments run in triplicate. G, AP1 promoter transcriptional activity enhanced by PMA and counteracted by endorepellin (6-h incubation). Values derive from two independent experiments ± S.E. (n = 10 for each group). ***, p < 0.001.
endorepellin leads to VEGFR2 internalization and degradation predominantly via a caveosome-mediated pathway. We have previously established (66) corroborating evidence that clearly demarcates a time-dependent (0–40 min) decrease in total VEGFR2 protein with endorepellin (100 nM). These data reinforce the confocal co-localization microscopy presented herein.

DISCUSSION

Our central hypothesis is that perlecan and its bioactive parts affect endothelial cell behavior by simultaneously engaging adhesion and angiogenic receptors, such as the α2β1 integrin and VEGFR2, respectively. According to our current working model of dual-receptor antagonism (Fig. 7), endorepellin would act as an allosteric inhibitor of VEGFR2 by binding via LG1-LG2 domains to a region distal to the canonical binding site for the natural ligand VEGFA (i.e. Ig2–3). The terminal LG3 domain would bind to the α2β1 integrin thus bringing together the two receptors, perhaps in a multicomplex signaling apparatus at the cell surface of endothelial cells. This dual-receptor binding leads to rapid internalization and degradation of both receptors, a process that can be inhibited by integrin α2β1-blocking antibodies (59) and mediated by caveolin-positive and clathrin-negative endocytic compartments. Biochemically, the engagement of the two receptors by endorepellin evokes the activity of SHP-1, a powerful tyrosine phosphatase that is recruited to the tail of the α2 subunit of α2β1 integrin and rapidly dephosphorylates several RTKs including VEGFR2 at Tyr-1175 (59). The longstanding implications of the current work expand on dual-receptor antagonism by examining the subsequent consequences on various signaling pathways directly orchestrated by VEGFR2 while discovering novel signaling pathways attenuated by endorepellin. We further discovered that endorepellin attenuates activation of the PI3K/PDK1/Akt/mTOR, calcineurin pathway, and by preventing PLCγ recruitment, precludes PKC/JNK/AP1 activation. These effects are attributable to endorepellin blocking VEGFA-evoked signaling through VEGFR2. Further corroborating these observations is the ability of endorepellin to inhibit VEGFA expression as a direct function of VEGFR2 antagonism leading to receptor internalization via caveosomes coincident with reducing HIF-1α under conditions.

FIGURE 5. Endorepellin inhibits VEGFA-evoked translocation of NFAT1 and promotes NFAT1 stability. A–D, representative confocal images of PAE-VEGFR2 cells following 20-min treatment with vehicle (Control), VEGFA (2.6 nM), endorepellin (100 nM), or endorepellin plus VEGFA. Note that VEGFA induces nuclear (Nu) translocation of NFAT1 (green, B), in contrast to endorepellin (C). However, a 2-h preincubation with endorepellin significantly blocks NFAT1 nuclear translocation (D). E, quantification of NFAT1 nuclear translocation based on the distribution of the NFAT1 nuclear/cytoplasmic ratio. Note that VEGFA-evoked NFAT1 nuclear localization is significantly decreased upon preincubation with endorepellin compared with treatment with VEGFA alone (**, p < 0.01). The values represent the mean nuclear to cytoplasmic ratio of NFAT1 ± S.E. (error bars). Control, n = 31; VEGFA, n = 37; endorepellin, n = 39; combined, n = 50. **, p < 0.01. F, representative immunoblotting of PAE-VEGFR2 cells following an 18-h treatment with cyclosporine A (100 nM), endorepellin (100 nM), or lactacystin (10 μM), as indicated. The membranes were reacted with antibody against NFAT1 or HDM2, the human homolog of the murine double-minute gene 2. G, NFAT promoter luciferase activity following a 6-h treatment with vehicle (Control), VEGFA (2.6 nM), endorepellin (100 nM), or in combination. Values represent the mean ± S.E. of three independent experiments run in quadruplicate. *, p < 0.05; **, p < 0.01.
normoxia via transcriptional and post-transcriptional mechanisms. Moreover, consistent with a blockade of the calcineurin pathway, endorepellin prevents VEGFA-dependent nuclear translocation of NFAT with further stabilization of the inactive species. Therefore, collectively these results detail suppression of two major signaling branches culminating in an attenuation of VEGFA and HIF1A expression while stabilizing inactive NFAT1 (Fig. 7). Thus, endorepellin, by acting at the receptor level, is capable of emanating diverse signaling events that converge onto repression of VEGFA transcriptional activity. We should point out that our current working model has a dynamic multiscale nature, i.e. it is based on experiments performed for short time (10–30 min) to address proximal receptor activity and for several hours for addressing distal transcriptional effects. Interestingly, other anti-angiogenic inhibitors, such as thrombospondin-1 (93) and TIMP-2 (94), can also modulate VEGFR2 activity and other integrin receptors, suggesting that dual-receptor antagonism might be a general biological process shared by other angiostatic proteins.

Notably, mutation of mouse Tyr-1173 to Phe (corresponding to human Tyr-1175 in VEGFR2) results in a loss-of-function phenotype and embryonic lethality, indicating that signaling via Tyr-1175 is essential for VEGFR2 functions during vasculogenesis (95). The adaptor molecule Shb binds to Tyr-1175 and is required for VEGFA-induced activation of PI3K signaling in endothelial cells (78). VEGFA evokes autophosphorylation of VEGFR2 at Tyr-1175 by recruiting Shb to the intracellular domain of the receptor (78). However, endorepellin abrogates this recruitment and attenuates downstream signaling via PI3K. It is well established that both the p110α catalytic and p85 regulatory subunits of PI3K are directly involved in mediating VEGFA/VEGFR2 signaling (78). Hence, we sought to determine whether these two subunits would directly associate with VEGFR2 upon VEGFA treatment. We observed that both the p110α and p85 subunits could be co-precipitated in complex with VEGFR2 in response to VEGFA and this “association” was significantly attenuated by endorepellin, with concurrent suppression of Akt phosphorylation.

Gene-targeting studies using eNos−/− mice have shown eNOS plays a key role in VEGFA-induced angiogenesis and vascular permeability in a PI3K/Akt-dependent manner (96). Indeed, VEGFA induces NO production via eNOS phosphoryl-
ation at Ser-1177 by Akt, a process that is blocked by PI3K inhibitors (96). We found that endorepellin inhibited the VEGFA-induced phosphorylation of eNOS at Ser-1177 and phosphorylation of mTOR at Ser-2448. This activity is notable because mTOR regulates the expression of HIF-1α, a DNA-binding protein that promotes adaptation and survival under hypoxia by inducing the transcription of numerous genes, including VEGFA (82). Here, we discovered that VEGFR2 overexpression was capable of driving HIF-1α expression and/or enhancing HIF-1α stability in PAE-VEGFR2 cells and that endorepellin markedly down-regulated HIF-1α levels under normoxic and hypoxic conditions, including VEGFA (82). Here, we discovered that VEGFR2 overexpression was capable of driving HIF-1α expression and/or enhancing HIF-1α stability in PAE-VEGFR2 cells and that endorepellin markedly down-regulated HIF-1α levels under normoxic and hypoxic conditions, including VEGFA (82). Here, we discovered that VEGFR2 overexpression was capable of driving HIF-1α expression and/or enhancing HIF-1α stability in PAE-VEGFR2 cells and that endorepellin markedly down-regulated HIF-1α levels under normoxic and hypoxic conditions, including VEGFA (82).

We noticed that blocking the PI3K pathway with LY294002 did not completely block the downstream effects of endorepellin. Thus, we investigated whether endorepellin could affect calcineurin, a serine/threonine phosphatase that dephosphorylates NFAT1 and causes its nuclear translocation where NFAT1 activates many genes including HIF1A (89). In this study, we found that VEGFA induced NFAT1 translocation into endothelial cell nuclei, and endorepellin efficiently counteracted this activity. Moreover, endorepellin induced stabilization of the inactive (phosphorylated) species of NFAT1 and blocked VEGFA-evoked transcriptional activity of NFAT1-promoter luciferase.

We would like to propose a unifying hypothesis for endorepellin activity: we favor a scenario where endorepellin would concurrently evoke receptor degradation and trigger a negative signaling emanating from the VEGFR2 itself. First, we discovered that endorepellin evoked VEGFR2 intracellular internalization primarily via caveosome-mediated endocytosis, and further downstream signaling of PLCγ, such as VEGFA-induced PKC activity, phosphorylation of JNK at Thr-183/Tyr-185, and AP1 promoter activity. Thus, endorepellin interferes with VEGFA activation of the PLCγ/PKC/JNK/AP1 signaling axis.

Calcineurin is a serine/threonine calcium-dependent phosphatase that dephosphorylates NFAT1 and causes its nuclear translocation where NFAT1 activates many genes including HIF1A (89). In this study, we found that VEGFA induced NFAT1 translocation into endothelial cell nuclei, and endorepellin efficiently counteracted this activity. Moreover, endorepellin induced stabilization of the inactive (phosphorylated) species of NFAT1 and blocked VEGFA-evoked transcriptional activity of NFAT1-promoter luciferase. We would like to propose a unifying hypothesis for endorepellin activity: we favor a scenario where endorepellin would concurrently evoke receptor degradation and trigger a negative signaling emanating from the VEGFR2 itself. First, we discovered that endorepellin evoked VEGFR2 intracellular internalization primarily via caveosome-mediated endocytosis,

**FIGURE 7. Working model of endorepellin as a dual-receptor antagonist.** Endorepellin could act as an allosteric inhibitor of VEGFR2 by binding via LG1-LG2 domains to a region distal to the canonical binding site of the natural ligand VEGFA (i.e. Ig2–3). The terminal LG3 domain would bind to the α2β1 integrin and could bring together the two receptors. This dual receptor binding leads to rapid internalization and degradation of both receptors, activation of SHP-1, and dephosphorylation of key Tyr residues in the VEGFR2 intracellular domain, importantly Tyr-1175. This biological process causes downstream attenuation of two main signaling axes initiated by engagement of PI3K and PLCγ to the Tyr-1175 of VEGFR2 intracellular domain. This leads to suppression of HIF1A, NFAT1, and AP1 transcriptional activities which negatively affect VEGFA transcription.

INCA-6 also suppressed VEGFA-promoter luciferase activity in a time-dependent manner, and this correlated with a decline in VEGFA protein. Importantly, endorepellin could not inhibit beyond the inhibition observed for the combination of both LY294002 and INCA-6, indicating that endorepellin affects both the PI3K/PDK1/Akt1/mTOR and the calcineurin pathways in down-regulating the transcription of HIF1A and VEGFA genes.

We found that VEGFA-mediated PLCγ recruitment to Tyr-1175 (Fig. 7), the major binding site of PLCγ on the VEGFR2 intracellular domain (78), was completely blocked by endorepellin. Concurrently, endorepellin attenuated downstream signaling of PLCγ, such as VEGFA-induced PKC activity, phosphorylation of JNK at Thr-183/Tyr-185, and AP1 promoter activity. Thus, endorepellin interferes with VEGFA activation of the PLCγ/PKC/JNK/AP1 signaling axis.
in contrast to VEGFA that primarily evokes a clathrin-mediated endocytosis and recycling (96). Physical down-regulation of this RTK, essential for endothelial cell survival and motility/proliferation, provides an attractive explanation for a mechanism to control pathological angiogenesis that could be applicable to other matrix-derived angiogenic inhibitors. Our findings are supported by recent studies that showed a dual receptor antagonism of engineered VEGFA variants that could simultaneously bind VEGFR2 and αvβ3 integrin to elicit a robust anti-angiogenic effect (97). Second, endorepellin by binding distally (Ig1-7) to the canonical VEGFA-binding (Ig2-3) site on the VEGFR2 could act as an allosteric inhibitor, independent of VEGFA concentrations. This binding could prevent dimerization as shown recently for monoclonal antibodies targeting the non-ligand binding site of VEGFR2/3 and preventing homo- and heterodimerization, signal transduction, and microvascular sprouting (98). In conclusion, this would negatively affect endothelial cell biology and lead to a profound anti-angiogenic effect. Therefore, endorepellin and perhaps similar compounds targeting the noncanonical ligand binding site of VEGFR2 could be used together with established therapies, such as antibodies toward the ligand or receptor, as a novel angiogenic effect. Therefore, endorepellin and perhaps similar compounds targeting the noncanonical ligand binding site of VEGFR2 could be used together with established therapies, such as antibodies toward the ligand or receptor, as a novel anti-angiogenic approach in preclinical studies or directly in clinical use.

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