Endothelial Gαq/11 is required for VEGF-induced vascular permeability and angiogenesis

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Aims

VEGF A (VEGF-A) is a central regulator of pre- and postnatal vascular development. In vitro studies suggested that heterotrimeric G-proteins of the Gαq/11 family contribute to VEGF receptor 2 (VEGFR2) signalling, but the mechanism and physiological relevance of this finding is unknown. The aim of this study is to understand the role of endothelial Gαq/11 in VEGF-dependent regulation of vascular permeability and angiogenesis.

Methods and results

We show here that VEGF-A-induced signalling events, such as VEGFR2 autophosphorylation, calcium mobilization, or phosphorylation of Src and Cdh5, were reduced in Gαq/11-deficient endothelial cells (ECs), resulting in impaired VEGF-dependent barrier opening, tube formation, and proliferation. Agonists at Gα11-coupled receptors facilitated VEGF-A-induced VEGFR2 autophosphorylation in a Gαq/11-dependent manner, thereby enhancing downstream VEGFR2 signalling. In vivo, EC-specific Gαq/11- and Gαq-deficient mice showed reduced VEGF-induced fluid extravasation, and retinal angiogenesis was significantly impaired. Gαq-deficient ECs showed reduced proliferation, Cdh5 phosphorylation, and fluid extravasation, whereas apoptosis was increased.

Conclusion

Gαq/11 critically contributes to VEGF-A-dependent permeability control and angiogenic behaviour in vitro and in vivo.

Keywords

Heterotrimeric G-proteins • Gαq/11 family • VEGF receptor 2 • Permeability • Angiogenesis

1. Introduction

The growth of new blood vessels from the existing vasculature, a process known as angiogenesis, is crucial during embryonic and postnatal organ development as well as in various remodelling processes of the adult organism.¹ VEGFs are central regulators of pre- and postnatal angiogenesis; their intracellular effects are mediated by three tyrosine kinase receptors, VEGFR1 (FLT-1), VEGFR2 (KDR/FLK-1), and VEGFR3 (FLT-4).² All three receptors are highly expressed in endothelial cells (ECs) during development, and inactivation of any receptor leads to vascular abnormalities and embryonic lethality between embryonic day 8.5 and 10.5 in mice.³ VEGFR2, mainly activated by VEGF-A, plays a key role in the transduction of angiogenic signals and controls EC morphology, chemotaxis, and proliferation.³−⁵ Ligand binding induces VEGFR2 dimerization and activation of the receptor kinase activity, resulting in receptor autophosphorylation. Phosphorylated receptors, in turn, recruit interacting proteins and induce the activation of various intracellular signalling pathways.³⁴ Phosphorylation at Y1175, for example, was shown to result in phospholipase (PLC) γ-dependent calcium mobilization and protein kinase C (PKC) activation, which in turn leads to activation of the mitogen-activated protein kinase pathway and consecutively increased proliferation. In addition, Y1175 phosphorylation induces phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-dependent Akt activation, thereby enhancing cell survival. Phosphorylation at residue Y951 facilitates Src activation, which in turn controls cytoskeletal organization and cell migration.³⁴ Other signalling events induced by VEGFR2 activation are activation of focal adhesion kinase (FAK), paxillin, or small GTPase Cdc42.³

Whether the process of VEGFR2 autophosphorylation can be modulated by factors other than VEGF-A binding is not well understood. In vitro studies suggested that heterotrimeric G-proteins of the Gαq/11 family may contribute to VEGF-induced effects such migration and proliferation.³⁴ and it was suggested that members of this...
G-protein family are required for VEGF-induced VEGFR2 autophosphorylation."6

The Gq/11 family consists of two ubiquitously expressed members, Gq and G12; their α-subunits show a sequence homology of 90% on the amino acid level.7 A large number of G-protein-coupled receptors (GPCRs) mediate their intracellular effects through Gq/11, for example the receptors for permeability-enhancing factors histamine or thrombin.8,9 The major downstream effector pathway of Gq/11 is PLCβ-dependent production of 1,4,5-trisphosphate (IP3) and diacyl glycerol (DAG), giving rise to IP3-dependent intracellular calcium mobilization and induction of various DAG-dependent effects such as PKC activation.9,10 Gq/11-dependent calcium mobilization has been shown to facilitate secretion and cellular contractility; it also plays a role in activation of extracellular signal-regulated kinases 1/2 (ERK1/2) and the FAK family member Pyk2.9,10 Gq/11-dependent PKC activation furthermore facilitates activation of ERK1/2 and consecutive changes in transcriptional activity.9,10 In addition to the canonical PLCβ-dependent signalling cascade, activation of alternative effectors, such as Rho-specific guanine nucleotide exchange factors, PKC ζ, or PI3K, has been described.10 In ECs, Gq/11 is crucial for GPCR agonist-induced phosphorylation of myoin light chain (MLC) kinase, a major determinant of cellular contractility and fluid extravasation.8,11

In addition, mice with tamoxifen-inducible, EC-specific deficiency for Gq/11 are protected from anaphylactic shock induced by agonists at Gq/11-coupled receptors such as histamine or platelet-activating factor.9 However, whether Gq/11 also plays a role in VEGF-induced changes in permeability or VEGF-induced angiogenesis in vivo is unknown. We show in this study that endothelial Gq/11 is crucial for VEGF-induced signalling events and critically contributes to VEGF-dependent permeability control and angiogenic behaviour in vitro and in vivo.

2. Methods

2.1 Experimental animals

The generation and genotyping of mice with tamoxifen-inducible, EC-specific deficiency for Gq/11 has been described previously.5,7 Littermate iEC-Gq/11-Kos (Tie2CreERT2; Gnaqfl/fl;Gna11−/−), iEC-Gaαq-Kos (Tie2CreERT2; Gnaqfl/fl;Gna11−/−), iEC-Gaα11-Kos (Gnaq−/+;Gna11−/−), and control mice (Gnaqfl/fl;Gna11fl/fl) were generated by intercrossing Tie2CreERT2/+;Gnaqfl/fl;Gna11fl/fl mice. Littermate iEC-Gaαq-Kos and control mice were generated by intercrossing Tie2CreERT2+/-;Gnaqfl/fl mice. For induction of Cre-mediated recombination in adult mice, 8- to 12-week-old mice were injected intraperitoneally (i.p.) with 1 mg of tamoxifen dissolved in 50 μL of Miglyol oil on five consecutive days (all genotypes); experiments were performed 1 and 2 weeks after the end of induction.

To induce Cre-mediated recombination in newborn mice, pups received intraperitoneal injections of 50 μg of tamoxifen in 50 μL of Miglyol oil on post-natal days (P) 1, 2, and 3, followed by analysis on Day 6.

To visualize retinal endothelia barrier opening, high mol. wt 2000 kDa of Miglyol oil were injected intraperitoneally into the tail vein and allowed to circulate for 10 min. The dorsal flank was bilaterally shaved, and 20 μl of protease-activated receptor-1 (PAR-1) peptide (an activator of the thrombin receptor subtype protease-activated receptor-1, 10 μg), VEGF (100 ng), and PBS or saline were injected intradermally. Twenty minutes later, mice were sacrificed by cervical dislocation and opening of the thorax, and the dorsal skin was excised. Evans blue was extracted by immersion in formamide for 48 h at 60°C and the amount of blue dye was quantified by spectrometry at 620 nm.

To analyse EC barrier opening in the adult mouse ear, mice were anaesthetized by isoflurane, and 10 μL of PBS, VEGF (100 ng/mL), or PAR-1 peptide (1 mg/mL) were injected into the ear. After 10 min, mice were sacrificed and the ears removed and fixed with 4% PFA for 30 min. The ventral part of the ear skin was removed together with cartilage and muscles, and the dorsal dermis was subjected to immunohistochemical staining.

All animal experiments were performed as approved by local authorities (Regierungspräsidium Darmstadt, Hessen, Germany) and according to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 Antibodies and dyes

Antibodies to Gq/11 (sc-392), -ERK1/2 (sc-7383), and -Src (sc-18) were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies to α-SMA (1A4; 1A4-2D), CRE (γH120.1), F4/80 (BM8), PECAM1 (550274), and αq (612705) were from BD Biosciences (San Jose, CA, USA). Antibodies to Desmin (347580), PECAM1 (550274), -catenin (610153), and Gαq (sc-392), p-ERK1/2 (sc-7383), and c-Src (sc-18) were from Santa Cruz Biotechnology (Dallas, TX, USA). The following antibodies for western blotting and IF staining in human umbilical vein endothelial cells (HUVECs), and VE-cadherin (555289 and 555661) from BD Biosciences (San Jose, CA, USA). Biotinylated IB4 (L2140) was from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Agonist and inhibitors

TNF-α (410-MT), VEGF/AVGVEF-165 (293-VE), and fibroblast growth factor 2 (FGF-2; 233-FB) were from R&D Systems. Thrombin (T4648), histamine (H7125), PAR-1 peptide (SFLRNR-NH2, s1820), FITC dextran (70 kDa: FD70 and 2000 kDa: FD2000), tamoxifen (T5648), and pyrlline maleate salt (PS5144) were from Sigma-Aldrich. PD2 (172889-27-9) was from Merck Millipore; protease inhibitors were from AppliChem (Darmstadt, Germany). The anti-VEGF antibody B20-4.1.1 was a kind gift from Genentech (San Francisco, CA, USA).

2.4 HUVEC culture and siRNA transfection

HUVECs (CC-2517) were cultured in EGM2 complete medium [EGM2 medium (CC3156) and growth factor supplements (CC-4176); Lonza, Basel, Switzerland]. HUVECs (CC-2517) were cultured in EGM2 complete medium [EGM2 medium (CC3156) and growth factor supplements (CC-4176); Lonza, Basel, Switzerland]."
For determination of VEGFR2 phosphorylation under flow conditions, μ-slides 0.4-luer (Cat. 80176, IBIDI, Martinsried) were coated with 70 000 cells per slide and incubated overnight to allow cells to attach. The expression of Gαq and Gα11 was suppressed with siRNAs on two consecutive days according to the manufacturer’s protocol. One day after second transfection, cells were subjected to a shear stress of 12 dyne/cm² for 2 days. After 48 h, the cells were harvested for protein analysis. Key experiments were reproduced with an independent set of siRNAs directed against Gαq and Gα11 (Qiagen SI02780988 and SI04193574; data not shown).

2.5 Tube formation in HUVECs

For tube formation assays, 24-well plates were coated with 200 μL of growth factor reduced GFR Matrigel (BD Biosciences) and incubated at 37°C for 30 min to promote polymerization. Quiescent HUVECs were seeded at a density of 5 × 10⁴ cells per well in the presence or absence of 50 ng/mL VEGF or FGF-2. After 8 h of incubation, a wide field image was taken and pictures were analysed using the ImageJ software (NIH). The term ‘total network’ refers to the number of interconnected ECs per view field.

2.6 Calcium mobilization and inositol monophosphate 1 production in HUVECs

HUVECs were seeded on 96-well plates and siRNA transfection was performed as described above.

For calcium measurements, cells were washed 72 h after transfection with PBS and loaded with 3 μM Fluo-4 AM in Hank’s balanced salt solution (HBSS) containing 10 mM HEPES for 30 min at 37°C. Cells were washed in HBSS and then transferred to an automated fluorescent plate reader (FlexStation-3; Molecular Devices). Cells were stimulated by ligands thrombin (1 U/mL), histamine (10 μM), or VEGF (20 ng/mL). For the determination of the inositol monophosphate 3 (IP₃) metabolite IP₃, cells were stimulated 72 h after transfection with agonists as described above and cumulative IP₃ production was determined 30 min later using the LightCycler 480 Probes Master (Roche, Mannheim, Germany) with primers/probe combination designed by the Roche RT-PCR design centre online tool or Primer-BLAST (NCBI):

GNA12: 5′-GCGGATTCGACCAAGAAGGG/ATCAAACAGAAATTGTTACTGGAGCCATC-3′/#67
GNA13: 5′-TCGGGAAAAACCTGATATGTGAAA/GGTTCAGGTCCAAGAATATGAGCAGATGAAA/CAACCAGCACCTCTC-3′/#3
GNAQ: 5′-GACTACCTTCCAGAATATGTGAAA/GGTTCAGGTCCAAGAATATGAGCAGATGAAA/CAACCAGCACCTCTC-3′/#27
GNA11: 5′-GCATCCAGGAGCACTGACGGGTCAAGGTGGTGGGATGTAGACACCTGCTT-3′/#53
GNA11: 5′-AAAGTCAAACTTGGTGAAGCAGATGAAA/TGGTGTTACTGATGACACCTGCTT-3′/#53
GNA12: 5′-CTCAGGAGCTCAGCTGCTT/TAACAACTTGAGATAGAAA/GGTTCAGGTCCAAGAATATGAGCAGATGAAA/CAACCAGCACCTCTC-3′/#60

2.9 Immunostaining and immunohistochemistry

For immunostainings, HUVECs were grown on collagen-coated coverslip in 24-well plates. Seventy-two hours after siRNA transfection, cells were starved for 2 h in EGM2 medium and then stimulated with thrombin (5 U/mL), histamine (10 μM), VEGF (50 ng/mL), or TNF-α (10 ng/mL) for 10 min. In some cases, cells were pretreated with an Src inhibitor PP2 (1 μM) for 2 h. After stimulation (applicable), cells were washed with PBS and fixed with methanol for 15 min at −20°C, then permeabilized and blocked with Triton X-100 (1%) and 4% BSA for 30 min, and then washed two times with PBS. The cells were immunostained with indicated antibodies in Triton X-100 (1%) and 1% BSA for overnight. Next, cells were washed twice with PBS for 10 min and antibody binding was visualized using species matched, fluorescently labelled secondary antibodies. Nuclei were stained with Hoechst in Triton X-100 (1%) and 1% BSA for 1 h. Images were acquired using an Leica SP5 confocal microscope and analysed using ImageJ (NIH). For statistical evaluation of basal and agonist-induced Cdh5 relocalization, the intensity of Cdh5 staining along 40 representative cell–cell borders was determined per sample.

For retina whole-mount stainings, retinai were dissected and fixed in 4% PFA for 2 h at 4°C or in methanol at −20°C. After fixation, retinae were permeabilized with 0.3% Triton X-100 in PBS for 20 min, then permeabilized and blocked with Triton X-100 (1%) and 1% BSA for 30 min. In some cases, cells were pretreated with an Src inhibitor PP2 (1 μM) for 2 h. After stimulation (applicable), cells were washed with PBS and fixed with methanol for 15 min at −20°C, then permeabilized and blocked with Triton X-100 (1%) and 1% BSA for 30 min, and then washed two times with PBS. The cells were immunostained with indicated antibodies in Triton X-100 (1%) and 1% BSA for overnight. Next, cells were washed twice with PBS for 10 min and antibody binding was visualized using species matched, fluorescently labelled secondary antibodies. Nuclei were stained with Hoechst in Triton X-100 (1%) and 1% BSA for 1 h. Images were acquired using an Leica SP5 confocal microscope and analysed using ImageJ (NIH). For statistical evaluation of basal and agonist-induced Cdh5 relocalization, the intensity of Cdh5 staining along 40 representative cell–cell borders was determined per sample.

2.10 Immunoblotting and immunoprecipitation

For immunoblotting, cells were lysed in radioimmunoprecipitation (RIPA) lysis buffer (150 mM NaCl, 25 mM Tris–HCl, 1 mL of 0.5 M EDTA pH 8.0, 2% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, and 0.25% Triton X-100) containing protease inhibitors (4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (AEBSF) 10 mg/mL, aprotonin 10 mg/mL, leupeptin 10 mg/mL, and pepstatin A 10 mg/mL) and 1 mM of Na₃VO₄. For the detection of tyrosine phosphorylation, cells were treated with 1 mM Na₂VO₃ before they were harvested. Mouse retinai were homogenized with an Ultra Turrax in RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were incubated on a rotating shaker for...
30 min at 4°C, then centrifuged at 18,000 g for 10 min at 4°C. Supernatants were boiled in Laemmli buffer for 5 min and then used for immunoblotting. Proteins were separated in 6 or 8% SDS-PAGE, then transferred onto nitrocellulose membranes, followed by overnight incubation with primary antibodies. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Antibody binding was visualized by enhanced chemiluminescence reagent (Millipore) using Fuji medical X-ray films, and protein bands were quantified using the ImageJ software (NIH).

For protein immunoprecipitation, HUVECs were lysed in ice-cold RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris pH 7.4, 0.1% SDS, 0.25% sodium deoxycholate, 1 μg/mL of each leupeptin, aprotinin, and pepstatin, 1 mM AEBSF, and 1 mM Na3VO4). Protein extracts were purified by centrifugation and incubated with 5 μg of anti-Gαq11 antibody (Santa Cruz Biotechnology, C-19) in the presence of Protein A/G Plus Agarose (Santa Cruz Biotechnology). Precipitated proteins were then centrifuged and washed several times using ice-cold RIPA buffer. Finally, antibody–antigen complexes were eluted by boiling them in Laemmli buffer and samples were then subjected to western blotting. Separated proteins were detected using anti-Gαq (BD Transduction Laboratories, #612705, 1:200) or anti-VEGFR2 (CST, clone 55B11, 1:500, dissolved in 5% BSA) antibodies.

2.11 Ex vivo trypsin sensitivity assay

The analysis of VE-cadherin sensitivity to trypsin was performed as described previously.12,13 In brief, p6 retinae were incubated for 1 h at 37°C in trypsin/EDTA-containing HBSS (0.05% trypsin and 20 mM glucose supplement) or HBSS only (no trypsin and 20 mM glucose supplement). After incubation, the retina was rinsed three times in PBS-containing medium (10% FBS and DMEM) to inactivate trypsin and the retina was lysed in RIPA buffer using microhomogenizer (VWR) for western blot analysis.

Figure 1 Endothelial Gαq11 is required for VEGF-induced effects in vitro. (A) Knockdown efficiency was determined by immunoblotting 72 h after transfection with scrambled control siRNA (siContr) or Gαq11-specific siRNA (siGαq11) (GAPDH as a loading control, representative example of n = 4). (B) VEGF-induced intracellular calcium mobilization was determined in Fluo-4 AM-loaded control and Gαq11-kd HUVEC (representative example of n = 4). (C) VEGF-induced production of IP3, metabolite of IP2, was determined by competitive immunoassay in control and Gαq11-kd HUVEC (n = 8). (D) VEGF-induced phosphorylation of MLC2 in control HUVEC and after knockdown of Gαq11 (n = 5). (E) Effect of Gαq11-kd on VEGF-induced phosphorylation of Src kinases at the activating residue Y416 (p-Src) was determined by immunoblotting (data normalized to total c-Src, n = 5). (F) Effect of Gαq11-kd on VEGF-induced Cdh5 phosphorylation at tyrosine residues 685 and 731 (data normalized to total Cdh5 as a loading control; n = 3). (G) Effect of Src inhibitor PP2 on VEGF-induced Cdh5 phosphorylation (data normalized to total Cdh5, n = 3). (H) The effect of Gαq11-kd on VEGF-induced phosphorylation of Akt (p-Akt) and ERK1/2 (p-ERK1/2) was determined by immunoblotting (phospho-specific signals were normalized to respective total protein signal, n = 3). All data are means ± SEM. Kd, knockdown; n, number of independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed Student’s t-test).
2.12 Statistical analysis

If not indicated otherwise, data are presented as means ± standard errors of the means (SEM). Comparisons between two groups were performed with two-tailed Student’s t-test or ANOVA followed by Bonferroni post hoc test. ‘n’ refers to the number of independent experiments (i.e. transfections) or to the number of mice per group. P-values are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

3. Results

To investigate how G-proteins of the Gq/11 family contribute to VEGF signalling, we performed siRNA-mediated knockdown of Gαq and Gα11 in HUVECs. This resulted in efficient knockdown (Figure 1A), but did not alter the expression of other G-protein α subunits (see Supplementary material online, Figure S1A) or cell morphology and expression of junctional proteins (see Supplementary material online, Figure S1B). Knockdown of Gαq/11 resulted in impaired intracellular calcium mobilization, Src phosphorylation, and VE-cadherin (Cdh5) phosphorylation in response to classical Gq11-coupled receptor agonists such as thrombin or histamine (see Supplementary material online, Figure S1C–G). Interestingly, Gαq/11 deficiency also clearly reduced VEGF-induced calcium mobilization (Figure 1B) and IP3 production (Figure 1C), as well as phosphorylation of MLC2 (Figure 1D), Src (Figure 1E), Cdh5 (Figure 1F), and the Gαq/11 effector PLCβ3 (see Supplementary material online, Figure S1H). Loss of VEGF-induced Src activation seemed to be causal for loss of Cdh5 phosphorylation, since inhibition of Src by PP2 similarly reduced VEGF-induced Cdh5 phosphorylation (Figure 1G). In contrast, VEGF-induced phosphorylation of ERK1/2 and Akt/protein kinase B was not altered (Figure 1H).

We next investigated whether impaired VEGF signalling had consequences for VEGF-dependent endothelial functions such as barrier control, proliferation, or tube formation. We found that VEGF-induced Cdh5 relocalization from junctions was diminished 10 and 30 min after stimulation, whereas TNF-α-induced effects were preserved (Figure 2A). In line with this we found in transwell assays that VEGF-induced increases in permeability for soluble dextran were near abrogated (Figure 2B). VEGF-induced proliferation was strongly reduced in the absence of Gαq/11, whereas proliferation induced by FGF-2, another pro-angiogenic growth factor, was preserved (Figure 2C). Also, VEGF-induced tube formation was impaired, but not FGF-2-induced tube formation (Figure 2D).

In the following step, we addressed the question at what level VEGFR2 and Gαq/11 signalling intersect. One hypothesis is that Gαq/11 may be required to induce full activation of VEGFR2 by facilitating VEGFR2 autophosphorylation.3,4 We found that VEGF-induced VEGFR2 autophosphorylation was impaired in the absence of Gαq/11, especially at residue Y951, and to lesser extent at Y1175 (Figure 3A). Conversely, overexpression of constitutively active Gαq facilitated VEGF-induced VEGFR2 phosphorylation, and this effect was strongly reduced by the Src inhibitor PP2 (Figure 3B). We also found that activation of Gαq/11-coupled receptors by thrombin and histamine facilitated VEGF-induced VEGFR2 phosphorylation in a Gαq/11-dependent manner (Figure 3C).

Figure 2 Gαq/11 is required for VEGF-induced permeability, proliferation, and tube formation in vitro. (A) Redistribution of Cdh5 immunoreactivity from cell junctions 10 and 30 min after stimulation with VEGF or TNF-α in control and Gαq/11-kd HUVEC. For statistical evaluation of basal and agonist-induced Cdh5 relocalization, the intensity of Cdh5 staining along 40 representative cell–cell borders was determined per sample (n = 4). (B) VEGF-induced increases in FITC dextran permeability were determined 90 min after agonist addition in transwell plates containing confluent monolayers of control or Gαq/11-kd HUVEC (n = 8). (Cand D), VEGF- and FGF-2-induced proliferation (C) and tube formation (D) in control and Gαq/11-kd HUVEC (n = 6). All data are means ± SEM. Kd, knockdown; n, number of independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001 (two-tailed Student’s t-test).
Furthermore, we were able to detect a short-lived interaction between VEGFR2 and Gq/11 (see Supplementary material online, Figure 2A). Taken together, these findings suggest that Gq/11-coupled receptors provide a co-stimulatory signal for VEGF-induced VEGFR2 phosphorylation and consecutive intracellular signalling events. To understand the mechanism of Gq/11-dependent facilitation of VEGF signalling, we investigated the possibility that VEGF stimulation induces the release of agonists able to activate Gq/11-coupled GPCRs. To block calcium mobilization induced by VEGF itself, we performed these experiments in the presence of blocking anti-VEGF antibody B20-4.1.1. We found that calcium mobilization induced by supernatant of VEGF-stimulated HUVEC was countermanded by addition of B20-4.1.1 (Figure 3D), indicating that conditioned media did not contain relevant concentrations of agonists at Gq/11-coupled GPCRs.

To test whether Gq/11 was also in vivo required for VEGF effects including opening of the endothelial barrier, we studied cutaneous fluid extravasation in tamoxifen-inducible, endothelial-specific Gq/11-deficient mice (iEC-Gq/11-KO). These mice showed that Evans blue extravasation into the dorsal skin was not only in response to thrombin receptor activation strongly diminished, but also in response to local VEGF application (Figure 4A). Reduced VEGF-induced extravasation in iEC-Gq/11-KOs was also observed in an ear model of FITC dextran extravasation (Figure 4B), and mutant mice showed reduced relocalization of junctional Cdh5 in dermal ear vessels (Figure 4C). Taken together, these findings show that both in vitro and in vivo Gq/11 is required for VEGF-induced opening of the endothelial barrier.

To understand the in vivo consequences of disturbed VEGF signalling in Gq/11-deficient ECs, we studied postnatal angiogenesis in retinae of iEC-Gq/11-KOs. The retina is avascular at birth, and a single superficial layer of blood vessels grows progressively from the centre towards the periphery from P1 until P7. To induce recombination in ECs, newborn mice from intercrosses between Tie2CreERT2; Gnaq<sup>fl/fl</sup>; Gna11<sup>−/−</sup> and Gnaq<sup>fl/fl</sup>;Gna11<sup>−/−</sup> were treated with tamoxifen.
**Figure 4** Gaq11 is required for VEGF-induced barrier opening in vivo. (A) Evans blue extravasation into the skin of the dorsal flank of control and iEC-Gaq11-KOs after intracutaneous injection of 20 μL vehicle, thrombin receptor agonist PAR-1 peptide, or VEGF (n = 8). (B) FITC dextran extravasation into the ear of control and iEC-Gaq11-KO after intracutaneous injection of 20 μL vehicle, PAR-1 peptide, or VEGF (n = 3–4). (C) Endothelial Cdh5 staining in flat mounts of mouse ears fixed 10 min after intracutaneous injection of 20 μL vehicle, PAR-1 peptide, or VEGF (representative example of n = 3–4). All data are means ± SEM. Kd, knockdown; n, number of mice per group; **P < 0.01; ***P < 0.001 (two-tailed Student’s t-test).

**Figure 5** Altered postnatal angiogenesis in iEC-Gaq11-KOs and iEC-Gaq4-KOs. (A–E) Exemplary photomicrographs (A) and statistical evaluation (B–E) of isolectin B4-stained retinae from 6-day-old (p6) control mice and iEC-Gaq4-KOs after tamoxifen treatment on P1–P3 (n = 6). ***P < 0.001 (two-tailed Student’s t-test).
on P1–P3, and retinas were analysed on P6. We found that endothelium-specific inactivation of $G_{aq}$ and $G_{a11}$ (iEC-$G_{aq/11}$-KO) or of $G_{aq}$ only (iEC-$G_{aq}$-KO) resulted in a clear reduction of retinal angiogenesis compared with littermate control mice or $G_{a11}$ single-deficient mice (see Supplementary material online, Figure 3A). Because of the poor odds of obtaining littermate controls and iEC-$G_{aq/11}$-KOs from these matings, all further analyses and quantifications were performed in iEC-$G_{aq}$-KOs and controls generated by intercrossing $Tie2$CreER$^{T2}$; $Gnaq^{-/-}$ mice. Adult iEC-$G_{aq}$-KOs did not differ with respect to blood pressure, heart rate, or body weight from control mice. Retinae of 6-day-old iEC-$G_{aq}$-KOs showed significantly reduced EC coverage, vessel branching, and tip cell numbers, and also the number of filopodia per tip cell was decreased (Figure 5A–E).

In line with an impaired VEGF signalling, ECs of iEC-$G_{aq}$-KOs showed reduced proliferation (Figure 6A) and increased apoptosis (Figure 6B). Retinal vessel pericyte coverage and VEGFR2 expression were not altered (see Supplementary material online, Figure 3B and C), but deep plexus formation was significantly delayed (see Supplementary material online, Figure 3D). To investigate whether retinal vessels of iEC-$G_{aq}$-KO mice showed signs of reduced permeability, we analysed retinal Cdh5 phosphorylation and found it to be diminished in iEC-$G_{aq}$-KO mice (Figure 6C). What is more, Src phosphorylation was significantly reduced in immunoblotting and immunohistochemistry (Figure 6C and D), whereas Akt phosphorylation was not changed (Figure 6C). We also assessed basal fluid extravasation at the angiogenic front after systemic FITC dextran injection and found a reduction of leakage sites in retinae of mutant mice (Figure 6E). Since junction-associated Cdh5 was shown to be more trypsin resistant than non-junctional Cdh5, we analysed the proteolytic sensitivity of Cdh5 by trypsin. We found that protein lysates from iEC-$G_{aq}$-KOs showed reduced trypsin-mediated Cdh5 cleavage (Figure 6F), indicative of reduced availability of disengaged Cdh5.

Taken together, our data reveal an unexpected role of $G_{aq/11}$ in mediating the permeability-increasing and pro-angiogenic effects of VEGF in vitro and in vivo.

4. Discussion

The role of $G_{a11}$ in ECs has been addressed in a number studies, but previous analyses mainly focused on their role in classical GPCR-dependent signalling cascades. We show in this study that endothelial $G_{aq/11}$ does not only mediate the permeability-enhancing

![Figure 6](https://example.com/figure6.png)

**Figure 6** Retinal changes in iEC-$G_{aq/11}$-KOs and iEC-$G_{aq}$-KOs. (A and B) The numbers of proliferating (A) or apoptotic (B) ECs in p6 retinae were determined by immunostaining with antibodies directed against BrdU (A) or activated caspase-3 (B). ECs were identified by their co-staining with isolectin B4 or PECAM1 (n = 3). (C) Immunoblot analysis or phosphorylated Cdh5, Src, and Akt (p-Cdh5, p-Src, and p-Akt) in protein extracts obtained from p6 retinae (phospho-specific signals were normalized to GAPDH as a loading control, n = 3). (D) Retinae from p6 mice were stained with antibodies directed against phosphorylated Src (Y416) and Cdh5 (example of three independent experiments). (E) Retinae from p6 control mice and iEC-$G_{aq}$-KOs were isolated after intracardiac injection of FITC dextran and stained with Cdh5 antibodies. Right side shows magnification of boxed area on the left; arrowheads indicate sites of vascular leakage (example of two independent experiments). (F) Retinae of control and iEC-$G_{aq}$-KOs were treated with 0.05% trypsin for 60 min and total protein extracts were analysed by immunoblotting. VEGFR3 and $b$-catenin were analysed to exclude unspecific degradation of endothelial proteins, GAPDH serves as a loading control (n = 3). All data are presented as means ± SEM. n, number of mice per group; *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed Student’s t-test).
effects of GPCR agonists such as thrombin or histamine, but also plays an important role in the signalling events induced by VEGF. We show that Ga<sub>q/11</sub> is required for VEGF-induced proliferation, barrier opening, and tube formation, which supports and extends findings from previous studies describing a role of Ga<sub>q/11</sub> in VEGF-induced migration and proliferation. With respect to the mechanism by which Ga<sub>q/11</sub> contributes to VEGF-induced effects, we found that activation of Ga<sub>q/11</sub> by GPCR agonists facilitated VEGF-induced autophosphorylation at Y951 and Y1175, two residues crucial for VEGFR2 downstream signalling. This finding is in line with a previous study, demonstrating that bradykinin, another agonist at Ga<sub>q/11</sub>-coupled GPCRs, induces tyrosine phosphorylation of VEGFR2. How Ga<sub>q/11</sub> brings about this facilitation of VEGFR2 phosphorylation is not fully clear. Previous studies and our own analyses indicate that VEGF treatment induces a short-lived interaction between Ga<sub>q/11</sub> and VEGFR2, but whether this interaction is a prerequisite for consecutive phosphorylation events is unclear. Our data also suggest that activation of Src family kinases plays an important role in Ga<sub>q/11</sub>-dependent VEGFR2 phosphorylation. The exact mechanisms of Ga<sub>q/11</sub>-dependent Src activation have not been studied in HUVEC, but data obtained in other cell types point to a role of PLC<sub>B</sub> activation. Studies in rat PC12 cells showed that bradykinin, a typical agonist at Ga<sub>q/11</sub>-coupled receptors, induces c-Src activation through PLC<sub>B</sub>-dependent intracellular calcium mobilization and consecutive autophosphorylation of Pyk2. Studies in rat endothelial cells suggested a role of PLC<sub>B</sub>-dependent PKC activation in Ga<sub>q/11</sub>-dependent Src activation. Bhattacharya et al. demonstrated that PLC<sub>B</sub> is phosphorylated at various residues upon VEGF stimulation, and that VEGF-induced IP<sub>3</sub> production depended on PLC<sub>B</sub>. In line with this, we found that VEGF-induced PLC<sub>B</sub> phosphorylation as well as VEGF-induced IP<sub>3</sub> production and calcium mobilization were reduced after knockdown of Ga<sub>q/11</sub>. How Ga<sub>q/11</sub> is activated in VEGF-stimulated cells is currently unclear. One potential explanation is a VEGF-induced release of Ga<sub>q/11</sub>-coupled receptor agonists, but supernatant transfer experiments did not provide evidence for significant VEGF-induced secretion of factors that activate Ga<sub>q/11</sub>-coupled receptors in an autocrine manner. However, it cannot be excluded that such factors escaped detection because of their localized release or their short half life time. An alternative explanation might be a constant basal tone of Ga<sub>q/11</sub>, that activates G<sub>q/11</sub>-coupled GPCRs, facilitates VEGFR2 autophosphorylation and Plc<sub>B</sub>-dependent intracellular calcium mobilization and consecutive autophosphorylation of Pyk2. In line with this notion, we observed reduced basal IP<sub>1</sub> accumulation in Ga<sub>q/11</sub>-deficient HUVEC and reduced basal VEGFR2 phosphorylation in HUVEC cultured under flow conditions. These data suggest that a tonic activity of Ga<sub>q/11</sub> signalling exists in the absence of exogenous ligands, and that this tonic Ga<sub>q/11</sub> activity facilitates basal VEGFR2 phosphorylation.

Our study shows that VEGF-induced opening of the endothelial barrier is not only impaired in cultured ECs, but also in two in vivo models of VEGF-induced fluid extravasation. That endothelial Ga<sub>q/11</sub> mediates the permeability-enhancing effects of GPCR agonists thrombin, histamine, or platelet-activating factor has been previously shown in EC-Ga<sub>q/11</sub>-KO mice. It is in this context interesting to note that also G-proteins of theGi<sub>13</sub> family, which often couple to the same GPCRs as the Ga<sub>q/11</sub> family, play an important role in mediating VEGF-dependent effects, though through a completely different mechanism: whereas Ga<sub>q/11</sub> facilitates VEGFR2 autophosphorylation and downstream signalling, Ga<sub>q/13</sub> is required for VEGFR2 expression in angiogenic ECs. Taken together, these findings indicate that GPCR signalling in ECs positively regulates VEGF signalling both on the level of transcription and protein phosphorylation, thereby providing important modulatory effects during angiogenesis.

Whether defective angiogenesis in iEC-Ga<sub>q/11</sub>-KO is primarily due to reduced VEGF signalling, disturbed barrier opening, or other factors is currently unclear. Given the central role of VEGF in mediating EC proliferation, survival, and migration, it seems likely that impaired VEGFR2 signalling contributes to observed phenotypes. In line with this notion, we found reduced proliferation and increased apoptosis in retinal ECs of mutant mice. However, the changes in proliferation and apoptosis were not severe enough to compromise endothelial barrier function in general, as shown by the absence of generalized FITC dextran leakage in vitro and in vivo. In addition, loss of permeability-enhancing effects mediated by GPCR agonists and VEGF might contribute to disturbed angiogenesis. Previous studies showed that increased permeability plays an important role in angiogenesis, since blocking antibodies directed against Cdh5 prevented angiogenesis in vitro and in vivo. We found that in the developing retina of iEC-Ga<sub>q/11</sub>-KO mice. It is in this context interesting to note that Ga<sub>q/11</sub>-KOs is primarily due to disturbed angiogenesis. Previous studies showed that increased permeability plays an important role in angiogenesis, since blocking antibodies directed against Cdh5 prevented angiogenesis in vitro and in vivo. We found that in the developing retina of iEC-Ga<sub>q/11</sub>-KO mice. It is in this context interesting to note that Ga<sub>q/11</sub>-KOs is primarily due to disturbed angiogenesis. Previous studies showed that increased permeability plays an important role in angiogenesis, since blocking antibodies directed against Cdh5 prevented angiogenesis in vitro and in vivo. We found that in the developing retina of iEC-Ga<sub>q/11</sub>-KO mice. It is in this context interesting to note that Ga<sub>q/11</sub>-KOs is primarily due to disturbed angiogenesis. Previous studies showed that increased permeability plays an important role in angiogenesis, since blocking antibodies directed against Cdh5 prevented angiogenesis in vitro and in vivo. We found that in the developing retina of iEC-Ga<sub>q/11</sub>-KO mice. It is in this context interesting to note that Ga<sub>q/11</sub>-KOs is primarily due to disturbed angiogenesis. Previous studies showed that increased permeability...


