Characterization of Endocrine Gland-derived Vascular Endothelial Growth Factor Signaling in Adrenal Cortex Capillary Endothelial Cells*

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Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) has been recently identified as a mitogen specific for the endothelium of steroidogenic glands. Here we report a characterization of the signal transduction of EG-VEGF in a responsive cell type, bovine adrenal cortex-derived endothelial (ACE) cells. EG-VEGF led to a time- and dose-dependent phosphorylation of p44/42 MAPK. This effect was blocked by pretreatment with pertussis toxin, suggesting that Go, plays an important role in mediating EG-VEGF-induced activation of MAPK signaling. The inhibitor of p44/42 MAPK phosphorylation PD 98059 resulted in suppression of both proliferation and migration in response to EG-VEGF. EG-VEGF also increased the phosphorylation of Akt in a phosphatidylinositol 3-kinase-dependent manner. Consistent with such an effect, EG-VEGF was a potent survival factor for ACE cells. We also identified endothelial nitric-oxide synthase as one of the downstream targets of Akt activation. Phosphorylation of endothelial nitric-oxide synthase in ACE cells was stimulated by EG-VEGF with a time course correlated to the Akt phosphorylation. Our data demonstrate that EG-VEGF, possibly through binding to a G-protein coupled receptor, results in the activation of MAPK p44/42 and phosphatidylinositol 3-kinase signaling pathways, leading to proliferation, migration, and survival of responsive endothelial cells.

Over the last decade, extensive research has been done on members of the vascular endothelial growth factor (VEGF)1 and angiopoietin families. These proteins are essential for embryonic development and for angiogenesis in a variety of physiological and pathological circumstances (1, 2). VEGF is the most critical inducer of vascular formation and angiogenesis as it is required to initiate the formation of immature vessels by vasculosgenesis or angiogenic sprouting during embryonic development as well as in adults. Members of the angiopoietin family, such as Ang-1 and Ang-2, are required for further remodeling and maturation of the vasculature (3). There are two known VEGF tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) (4). Neuropilin-1 has been shown to be an isoform-specific VEGF receptor able to bind the heparin-binding VEGF isoforms (5). VEGFR-1 is thought to play a negative role in the regulation of angiogenesis acting as a “decoy” receptor at least in some circumstances (6). Compelling evidences indicate that VEGFR-2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF (4). VEGF stimulation leads to a robust autophosphorylation of VEGFR-2 and activation of the MAPK cascade (7, 8), which may directly contribute to endothelial cell proliferation. VEGF also induces tyrosine phosphorylation and activation of PI 3-kinase in endothelial cells (9). Activation of PI 3-kinase, mediated by VEGFR-2, contributes to the chemotactic effects of VEGF (6). Activation of PI 3-kinase is also strongly implicated in VEGF-induced endothelial cell survival via its downstream target serine/threonine kinase Akt (9). Akt promotes cellular survival by suppressing the activity of proapoptotic proteins, such as caspase-9 in monocytes, and by inducing the expression of antiapoptotic proteins, such as Bel-2 and A1, in vascular endothelial cells (10). Effects of VEGF on permeability (11) and vascular tone (12) are believed to be coupled to nitric oxide (NO) production. VEGF has been shown to induce the release of NO from vascular endothelial cells by increasing phosphorylation of endothelial nitric-oxide synthase (eNOS) (13). Phosphorylation of eNOS at Ser-1177 by Akt directly stimulates eNOS activity and NO production (14).

Several lines of evidence suggest the existence of local, tissue-specific regulators of endothelial cell phenotype and growth (15, 16). The morphology and functions of endothelial cells vary extensively among different organs. While endothelial lineage is genetically determined, expression of a set of vascular bed-specific genes is regulated at the transcriptional level following interaction with local signaling pathways (17). Recently we reported the identification of a tissue-specific angiogenic factor, EG-VEGF (18). Expression of human EG-VEGF mRNA is restricted to the steroidogenic glands, ovary, testis, adrenal, and placenta. EG-VEGF promoted proliferation, migration, and fenestration in cultured capillary endothelial cells derived from such endocrine glands. It also induced extensive angiogenesis when delivered in the ovary but not in the skeletal muscle or in the cornea. In contrast, VEGF induced angiogenesis in all the organs tested (18).

EG-VEGF is a member of an emerging new protein family with multiple regulatory functions. The first of such molecules, venom protein A (VPRA) (19), known also as “MIT-1” (20), was purified from the venom of black mamba snake (Dendroaspis polylepis polylepis) as a nontoxic component. EG-VEGF is most likely the human orthologue of VPRA, and the two proteins display 80% homology (18). The other members of this family

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; EG-VEGF, endocrine gland-derived VEGF; ACE, adrenal cortex-derived endothelial; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; GPCR, G protein-coupled receptor; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; VPRA, venom protein A; PTX, pertussis toxin; LPA, lysophosphatidic acid.

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include the secreted protein from the frog Bombina variegata, Bv8 (21) and its mammalian orthologues (22, 23), the digestive enzyme colipase (24), and the Xenopus head-organizer protein dickkopf (25). EG-VEGF/VPRA shows a high degree of homology (70–76%) to the Bv8 protein and its murine and human orthologues. The common structural motif in this family is five pairs of disulfide bonds with conserved span that are predicted to form similar globule foldings. This was demonstrated in the superimposable global folds of the solution structure of VPRA and the crystal structure of colipase despite only 17% sequence identity between these proteins, the least among the five proteins (26).

In the present study we have analyzed the signal transduction events elicited by EG-VEGF in adrenal cortex-derived endothelial (ACE) cells. EG-VEGF was able to activate similar pathways as VEGF, most notably the MAPK and PI 3-kinase/Akt pathways. Both signaling pathways were essential for the activity of EG-VEGF on proliferation, migration, and survival in ACE cells.

EXPERIMENTAL PROCEDURES

Reagents—LY 294002 and wortmannin were from Calbiochem. N'-Nitro-l-arginine methyl ester, PD 98059, anti-eNOS, and pertussis toxin (PTX) were from Biomol, Inc. Anti-phospho-extracellular signal-regulated kinase, anti-Akt, anti-phospho-Akt, and anti-phospho-eNOS were from Cell Signaling. Anti-p44/42 MAPK was from Zymed Laboratories Inc. Annexin-fluorescein isothiocyanate and propidium iodide were from Molecular Probes. The protease inhibitor mixture tablet was from Roche Molecular Biochemicals. Protein A/G beads were from Pierce. Lysoosphosphatic acid (LPA), phosphatase inhibitors, and all other reagents were from Sigma.

Cell Culture and Western Blot—ACE cells were maintained in complete medium (low glucose Dulbecco’s modified Eagle’s medium with 10% calf serum supplemented with 2 mM glutamine and antibiotics). Cells of low passage number (two to six) were used in all studies. For Western blot analysis, cells were cultured in subconfluent conditions for 48 h followed by incubation in serum-free Dulbecco’s modified Eagle’s medium overnight. The medium was changed to assay medium (Dulbecco’s modified Eagle’s medium supplemented with 1 mg/ml bovine serum albumin) for 90 min. At various time points, inhibitors or anti-bodies were added followed by stimulation with growth factors. Cells were lysed in 0.1–1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture, phosphatase inhibitors, 1 mM Na3VO4) by shaking at 4 °C for 20 min. For extracellular signal-regulated kinase, Akt phosphorylation assays, cell lysates were subjected to electrophoresis, transferred, and blotted with anti-phospho-eNOS (Cell Signaling) or eNOS antibody (Biomol). For precipitation of the VEGF receptor, ACE cell lysates from 150-mm plates were incubated with 50 ml of wheat-germ agglutinin beads (Amersham Biosciences, Inc.) for 2 h at 4 °C. The lectin complex was washed with lysis buffer three times and then boiled with SDS-sample buffer followed by electrophoresis, transfer, and blotting with phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc.).

Immunoprecipitation—for the eNOS phosphorylation assay, cell lysates were immunoprecipitated for 4 h with 1 µg of anti-eNOS antibody and 20 µl of protein A/G beads. The immune complex was washed three times with lysis buffer and boiled with SDS-sample buffer. Samples were subjected to electrophoresis, transferred, and blotted with anti-phospho-eNOS (Cell Signaling) or eNOS antibody (Biomol). For precipitation of the VEGF receptor, ACE cell lysates from 150-mm plates were incubated with 50 ml of wheat-germ agglutinin beads (Amersham Biosciences, Inc.) for 2 h at 4 °C. The lectin complex was washed with lysis buffer three times and then boiled with SDS-sample buffer followed by electrophoresis, transfer, and blotting with phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc.).

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RESULTS

EG-VEGF Induces p44/42 MAPK Phosphorylation—We investigated several downstream signaling pathways that might be potentially involved in EG-VEGF’s signaling in ACE cells. Since the MAPK pathway is critical for cellular proliferation, we tested whether EG-VEGF was capable of stimulating p44/42 MAPK phosphorylation. After exposure to various concentrations of EG-VEGF for 10 min, cell lysates were electrophoresed on an SDS-polyacrylamide gel, transferred on a polyvinylidene difluoride membrane, and blotted with phospho-p44/42 MAPK antibody (Fig. 1A). The phospho-p44/42 MAPK antibody only detects p42 and p44 MAPK when catalytically activated by phosphorylation at Thr-202/Tyr-204 (28).

EG-VEGF activates the MAPK pathway in ACE cells. Subconfluent ACE cells were serum-starved overnight and stimulated with the indicated concentrations of growth factors in assay medium. A, dose-dependent activation of p44/42 MAPK phosphorylation. 20 ng/ml VEGF or 2.5, 5, 10, 20, and 40 ng/ml EG-VEGF were added to ACE cells and incubated for 10 min. Cells were lysed, and lysates were subjected to Western blot using an anti-phospho-p44/42 MAPK antibody and reprobed with anti-p44/42 MAPK to normalize the total proteins. B, time course of EG-VEGF stimulation of p44/42 MAPK phosphorylation. 20 ng/ml EG-VEGF was added to ACE cells and incubated for 2, 10, 15, 30, and 60 min. Cells were lysed, subjected to Western blot, and then probed with anti-phospho-p44/42 MAPK antibody as above. C, PD98059 specifically inhibits the phosphorylation of p44/42 MAPK stimulated by EG-VEGF. ACE cells were preincubated with the indicated amount of PD 98059 for 30 min followed by EG-VEGF (20 ng/ml) stimulation for 10 min. Phosphorylated and total p44/42 MAPK proteins were compared. ', minutes.

FIG. 1. EG-VEGF activates the MAPK pathway in ACE cells. Subconfluent ACE cells were serum-starved overnight and stimulated with the indicated concentrations of growth factors in assay medium. A, dose-dependent activation of p44/42 MAPK phosphorylation. 20 ng/ml VEGF or 2.5, 5, 10, 20, and 40 ng/ml EG-VEGF were added to ACE cells and incubated for 10 min. Cells were lysed, and lysates were subjected to Western blot using an anti-phospho-p44/42 MAPK antibody and reprobed with anti-p44/42 MAPK to normalize the total proteins. B, time course of EG-VEGF stimulation of p44/42 MAPK phosphorylation. 20 ng/ml EG-VEGF was added to ACE cells and incubated for 2, 10, 15, 30, and 60 min. Cells were lysed, subjected to Western blot, and then probed with anti-phospho-p44/42 MAPK antibody as above. C, PD98059 specifically inhibits the phosphorylation of p44/42 MAPK stimulated by EG-VEGF. ACE cells were preincubated with the indicated amount of PD 98059 for 30 min followed by EG-VEGF (20 ng/ml) stimulation for 10 min. Phosphorylated and total p44/42 MAPK proteins were compared. ', minutes.

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With this antibody, we observed a dose-dependent phosphorylation of p44/42 MAPK in response to EG-VEGF. A maximal effect was observed in the presence of 20 nM EG-VEGF. We also studied the time course of EG-VEGF-stimulated p44/42 MAPK phosphorylation (Fig. 1B). There was a rapid response starting 2 min after addition of EG-VEGF that peaked at 10 min. After 30 min, the MAPK phosphorylation decreased to a lower, stable level, which was still above the nonstimulated background. To determine the specificity of the MAPK pathway being activated by EG-VEGF, we studied the effect of the MEK1-specific inhibitor PD 98059 on EG-VEGF-induced phosphorylation of p44/42 MAPK. Pretreatment of ACE cells with PD 98059 blocked EG-VEGF-induced phosphorylation of p44/42 MAPK in a dose-dependent manner (Fig. 1C). A complete inhibition of p44/42 MAPK phosphorylation was achieved at a PD 98059 concentration of 10 \mu M.

**EG-VEGF-induced Activation of MAPK Does Not Require VEGF**—Given the similarity in the p44/42 MAPK phosphorylation inductive effects of VEGF and EG-VEGF, we examined the possibility that VEGF release may mediate EG-VEGF downstream signaling. We compared the effect of specific inhibitors on p44/42 MAPK phosphorylation induced by the two growth factors. As expected, a soluble VEGF receptor, mFlt-(1–3) IgG (29), completely inhibited VEGF-induced p44/42 MAPK phosphorylation (Fig. 2A), while a neutralizing monoclonal antibody directed against EG-VEGF blocked the response to EG-VEGF (Fig. 2B). However, mFlt-(1–3) IgG had no effect on EG-VEGF-induced activation. Likewise the anti-EG-VEGF monoclonal antibody did not block VEGF-induced stimulation. Thus, EG-VEGF and VEGF activate the p44/42 MAPK pathway independently.

**EG-VEGF-induced Activation of MAPK Is PTX-sensitive**—We tested the effect of PTX on EG-VEGF-induced MAPK activation (Fig. 3A). PTX specifically modifies the heterotrimeric G protein \(\alpha_i\), blocking signaling pathways of G protein-coupled receptors involving \(\alpha_i\). As a positive control, we tested the effect of PTX on LPA, whose receptor is a \(\alpha_i\)-coupled receptor (30). Pretreatment of ACE cells with 200 ng/ml PTX abolished LPA-induced p44/42 MAPK phosphorylation. In contrast, PTX had essentially no effect on VEGF-stimulated MAPK phosphorylation. This is consistent with the notion that the VEGF receptors are tyrosine kinases. That PTX nearly abolished the EG-VEGF-induced phosphorylation of MAPK indicates that the EG-VEGF receptor is probably a G protein-coupled receptor rather than a tyrosine kinase. To further test this hypothesis, we compared the tyrosine phosphorylation of ACE cell membrane proteins following stimulation with VEGF or EG-VEGF. Phosphotyrosine blots of wheat-germ agglutinin precipitates showed that, while VEGF induced, as expected, tyrosine phosphorylation of a 200-kDa band (consistent with VEGFR-2), there was no evidence of tyrosine phosphorylation in response to EG-VEGF (Fig. 3B). These findings suggest that, despite the similarity of their effects on proliferation and migration, VEGF and EG-VEGF probably utilize different receptor types to activate downstream signaling.

**EG-VEGF Results in Akt Phosphorylation and Promotes Endothelial Cell Survival**—Another important signaling molecule activated by EG-VEGF in ACE cells is Akt. Fig. 4 shows the time course of EG-VEGF-stimulated phosphorylation of Akt at Ser-473. ACE cells were stimulated for the indicated time with or without pretreatment with 300 nM wortmannin. Cell lysates were electrophoresed, transferred, and probed with phospho-Akt (Ser-473) antibodies (Fig. 4, lanes 1 and 2). The activation peaked at 30 min and was sensitive to the PI 3-kinase inhibitor wortmannin (Fig. 4, lane 7). The effect of VEGF on Akt phosphorylation was acute, peaked at 10–30 min, and subsided after 60 min (data not shown). Thus, EG-VEGF leads to PI 3-kinase-dependent activation of Akt in ACE cells.

There is compelling evidence that activation of Akt results in a cell survival signal. This prompted us to examine whether
EG-VEGF is able to promote ACE cell survival in conditions of serum starvation. After 24-h starvation, ACE cells typically show 30% apoptosis as assessed by annexin and propidium iodide staining. Both VEGF and EG-VEGF were able to rescue ACE cells from apoptosis (Fig. 5), indicating that, like VEGF, EG-VEGF is not only a mitogen but also a survival factor for ACE cells. In agreement with earlier reports (9, 31), VEGF had a biphasic effect with high concentrations being progressively less effective.

**EG-VEGF Promotes eNOS Phosphorylation**—Since eNOS is one of the Akt downstream targets and is known to play an important role in homeostasis and angiogenesis of endothelial cells, we examined whether eNOS is regulated by EG-VEGF. Phosphorylation of eNOS on Ser-1177 by Akt is crucial for activation of its enzyme activity. We immunoprecipitated eNOS from ACE cell lysates stimulated with EG-VEGF for 10–90 min and probed for eNOS phosphorylation (Fig. 6). eNOS phosphorylation increased after a 30-min exposure to EG-VEGF. Such increase lasted for the entire incubation period (up to 90 min) and correlated with the degree of Akt phosphorylation. However, the timing of eNOS phosphorylation was slightly delayed compared with Akt phosphorylation.

To confirm that eNOS phosphorylation is downstream to Akt activation, we examined the effect of the PI 3-kinase inhibitor LY 294002 (Fig. 7, lanes 2, 5, and 8). As a positive control, we used 5 mM NG-nitro-L-arginine methyl ester, a competitive eNOS inhibitor, to block the phosphorylation of eNOS (Fig. 7, lanes 3, 6, and 9). We observed inhibition of eNOS phosphorylation induced by either EG-VEGF or VEGF by pretreatment with 10 μM LY 294002, indicating that this event is PI 3-kinase-dependent. Under these conditions, the inhibition ranged between 80 and 90% for EG-VEGF and 60–80% for VEGF.

**Activation of MAPK Is Important for EG-VEGF-induced Cell Proliferation and Migration**—Since EG-VEGF has been recently identified as an endothelial cell mitogen, we examined the contribution of MAPK activation to EG-VEGF-dependent ACE cell proliferation. In a mitogenic assay (Fig. 8A), we found that 10 μM PD 98059 significantly inhibited ACE cell proliferation in response to EG-VEGF (43 ± 6.5%) or VEGF (31 ± 9%). A greater inhibition was achieved using 20 μM PD 98059, but this resulted also in inhibition of basal cell proliferation (data not shown). A similar observation was reported by Parenti et al. (13) in studies of VEGF-stimulated proliferation of coronary endothelial cells. In contrast, 5 mM LY 294002, which effectively blocked Akt phosphorylation induced by EG-VEGF or VEGF, had a much smaller effect (13 ± 0.12% for EG-VEGF and 17 ± 0.8% for VEGF) on proliferation. Thus, these results suggest that activation of the MAPK pathway by EG-VEGF is critical for its function as a mitogen for adrenal gland endothelial cells. Both PD 98059 and wortmannin pretreatment effectively blocked ACE cell migration stimulated by EG-VEGF (Fig. 8B), indicating that activation of both MAPK and PI 3-kinase pathways is required for EG-VEGF to induce this function in ACE cells.

**DISCUSSION**

The present results show that, similar to VEGF, EG-VEGF induces activation of MAPK and PI 3-kinase in ACE cells, leading to proliferation, migration, and survival. The dose-dependent phosphorylation of MAPK p44/p42 was used as a sensitive readout of EG-VEGF activity. The nearly complete inhibition by pretreatment with PD 98059 indicates that the activation of MEK1/2 is necessary for EG-VEGF-induced MAPK p44/42 phosphorylation.

We also observed phosphorylation of Akt on Ser-473 in ACE cells in response to EG-VEGF. Compared with VEGF, Akt phosphorylation induced by EG-VEGF was less intense, yet more prolonged. Similar to that induced by VEGF, EG-VEGF-induced phosphorylation of Akt was wortmannin-sensitive, in-
Indicating that the phosphorylation was downstream of PI 3-kinase activation. The induction of prolonged phosphorylation of Akt contributed significantly to ACE cell migration as reflected in our migration assay and is also likely to be crucial for the antiapoptotic effect of EG-VEGF. However, this pathway appears to contribute less to ACE cell growth relative to that of phosphorylated MAPK p44/42. Akt has been reported to directly phosphorylate eNOS at Ser-1177 (32), which leads to activation of eNOS. Several lines of evidence indicate that eNOS is an important modulator of angiogenesis and vascular tone (33). VEGF has been shown to induce the release of NO from endothelial cells from a variety of species (34). In vitro, VEGF stimulates human endothelial cells to grow in a NO-dependent manner and promotes NO-dependent formation of vessel-like structures in a three-dimensional collagen gel model (35). Inhibition of NO production by eNOS inhibitors significantly inhibited VEGF-induced mitogenic and angiogenic effects (36). Although we found that EG-VEGF-induced phosphorylation of eNOS in ACE cells is dependent on PI 3-kinase and Akt activation, we did not observe significant effects of eNOS inhibitors on ACE cell proliferation or migration (data not shown).

The fact that the effects of EG-VEGF in ACE cells are very similar to those induced by VEGF raised the possibility that EG-VEGF may require VEGF to elicit activation of the MAPK pathway. EG-VEGF and VEGF do not show any sequence or structural homology. Thus, it is very likely that distinct membrane receptors exist for the two molecules. Our data suggest that EG-VEGF receptor might be a G protein-coupled receptor (GPCR) or at least that Goi is required to mediate MAPK activation. EG-VEGF-induced MAPK phosphorylation was PTX-sensitive, whereas PTX failed to inhibit VEGF-induced MAPK phosphorylation in the same experiment. This is consistent with the notion that VEGF activates downstream signaling following binding to receptor tyrosine kinases (4). In contrast, EG-VEGF failed to induce detectable tyrosine phosphorylation in ACE cell membranes. However, given the limited sensitivity of such phosphotyrosine blots, we cannot entirely rule out the possibility that the EG-VEGF receptor may have a weak tyrosine kinase activity. Indeed, it is well established that several receptor tyrosine kinases can cross-talk to GPCR (37). In the case of the IGF-1 receptor, there is evidence not only for an association with multiple G proteins but also for an intrinsic GPCR function (38).

It is now increasingly apparent that, in addition to a well-characterized pathway leading to MAPK activation following ligand-induced tyrosine kinase receptor autophosphorylation, many GPCRs, which lack intrinsic kinase activity, are also able to effectively activate MAPK (39–41). These includes the receptors for such diverse ligands as bombesin, endothelin-1, somatostatin, interleukin-8, oxytocin, LPA, etc. Receptors coupled to heterotrimeric GTP-binding proteins are integral membrane proteins involved in the transmission of signals from the extracellular environment to the cytoplasm (42). Previous studies indicated that MAPK activation by GPCR requires, depending on the cellular context, various nonreceptor tyrosine kinases, including members of the Src family, Csk, Lyn, and Btk.
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(Received for review, see Ref. 42). Accordingly, we found that tyrosine kinase inhibitors such as genistein completely abolish EG-VEGF-dependent MAPK activation (data not shown). Further studies will be required to identify the specific transducers linking EG-VEGF receptor activation to the MAPK pathway.

Several studies indicated the involvement of GPCR in vascular remodeling and angiogenesis. For example, disruption of the Go_{13} gene was found to impair the ability of endothelial cells to develop into an organized vascular system, including sprouting, growth, migration, and remodeling of endothelial cells (43). Receptors for thrombin and angiotensin II are GPCRs involved in the regulation of a number of critical events in the cardiovascular system, including vascular permeability (44), proliferation, and migration of endothelial cells (45, 46) and induction of metalloproteinases production (47). Furthermore, a family of GPCRs (EDG family) serving as receptors for sphingosine 1-phosphate has been shown to play a key role in endothelial cell survival and morphogenesis (48). It will be interesting to examine whether blocking Go_{i} will specifically inhibit the angiogenic effects of EG-VEGF in target tissues.

It is important to emphasize that a different biological activity had been previously attributed to several members of the EG-VEGF protein family. Both VPRA/EG-VEGF (20, 23) and a family of GPCRs (EDG family) serving as receptors for sphingosine 1-phosphate has been shown to play a key role in endothelial cell survival and morphogenesis (48). It will be interesting to examine whether blocking Go_{i} will specifically inhibit the angiogenic effects of EG-VEGF in target tissues.

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