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In Brief
Kilpatrick et al. have used bioluminescence resonance energy transfer (BRET) and VEGFR2 tagged with NanoLuc luciferase, to demonstrate that oligomeric complexes involving VEGFR2 and $\beta_2$-adrenoceptors can be generated in both cell membranes and intracellular endosomes. These complexes are agonist sensitive and retain their ability to couple to intracellular signaling proteins.

Highlights
- NanoBRET can monitor dimerisation of VEGFR2 and $\beta_2$-adrenoceptors in living cells
- Formation of VEGFR2/$\beta_2$-adrenoceptor complexes was enhanced by agonist stimulation
- $\beta_2$-Adrenoceptor/VEGFR2 complexes were internalized by agonist treatment to endosomes
- Coupling of $\beta_2$-adrenoceptors to $\beta$-arrestin2 was prolonged by VEGFR2 activation

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Complex Formation between VEGFR2 and the $\beta_2$-Adrenoceptor

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INTRODUCTION

Vascular endothelial growth factor (VEGF) is an important mediator of endothelial cell proliferation and angiogenesis via its receptor VEGFR2. A common tumor associated with elevated VEGFR2 signaling is infantile hemangiomata that is caused by a rapid proliferation of vascular endothelial cells. The current first-line treatment for infantile hemangiomata is the $\beta_2$-adrenoceptor antagonist, propranolol, although its mechanism of action is not understood. Here we have used bioluminescence resonance energy transfer (BRET) and VEGFR2 genetically tagged with NanoLuc luciferase to demonstrate that oligomeric complexes involving VEGFR2 and $\beta_2$-adrenoceptor can be generated in both cell membranes and intracellular endosomes. These complexes are induced by agonist treatment and retain their ability to couple to intracellular signaling proteins. Furthermore, coupling of $\beta_2$-adrenoceptor to $\beta$-arrestin2 is prolonged by VEGFR2 activation. These data suggest that protein-protein interactions between VEGFR2, the $\beta_2$-adrenoceptor, and $\beta$-arrestin2 may provide insight into their roles in health and disease.

SUMMARY

Vascular endothelial growth factor (VEGF) is an important mediator of endothelial cell proliferation and angiogenesis (Ferrara, 2009; Shibuya, 2011; Musumeci et al., 2012). VEGF-A mediates its effects on endothelial cells predominantly via the receptor tyrosine kinase (RTK) VEGF receptor 2 (VEGFR2), which also represents an important drug target for cancer angiogenesis (Ferrara, 2009; Claesson-Welsh and Welsh, 2013; Peach et al., 2018a). VEGFR2 signaling is elevated in infantile hemangiomata due to the rapid proliferation of vascular endothelial cells during early infancy (Ou et al., 2014). The current first-line treatment for infantile hemangiomata is the $\beta_2$-adrenoceptor antagonist, propranolol, although its mechanism of action is not fully understood (Léauté-Labrèze et al., 2008; Ozeki et al., 2016; Stiles et al., 2012; Mulcrone et al., 2017). The therapeutic effect of propranolol is mediated by antagonism of the $\beta_2$-adrenoceptor (a G protein-coupled receptor [GPCR]) and appears to result from reduced VEGF-A expression and cell proliferation (Ozeki et al., 2011; Stiles et al., 2012; Mulcrone et al., 2017; Park et al., 2016). $\beta_2$-Adrenoceptor activation has been reported to play a critical role in mediating stress-induced metastasis in breast cancer (Mulcrone et al., 2017; Sloan et al., 2010; Chang et al., 2016) and cancer angiogenesis in prostate cancer (Hulsurkar et al., 2017). For example, skeletal colonization by breast cancer cells is stimulated by a $\beta_2$-adrenoceptor- and VEGF-dependent neo-angiogenic switch (Mulcrone et al., 2017).

In addition to GPCR agonists eliciting changes in the expression of growth factors (such as VEGF-A), there is accumulating evidence for complex interactions between their cognate receptors (Luttrell et al., 1999; George et al., 2013; Pyne and Pyne, 2011; Liebmann, 2011). Three different mechanisms of GPCR-RTK interaction have been identified: (1) GPCR activation of matrix metalloproteases leading to the shedding of heparin-binding growth factors (e.g., heparin-binding epidermal growth factor) and the subsequent activation of their receptors (Daub et al.,
RESULTS

Constitutive and VEGF_{165a}-Induced VEGFR2 Homodimerization

To monitor ligand binding and receptor dimerization of VEGFR2 using BRET, we tagged VEGFR2 on its N terminus with the bright, small (19.1 kDa) NLuc luciferase (Stoddart et al., 2015; Kilpatrick et al., 2017; Peach et al., 2018b) and ligand-induced dimerization of VEGFR2. HEK293 cells stably expressing NLuc-VEGFR2 (Figure 1B), and to demonstrate that the NLuc-tagged VEGFR2 retains its high affinity for VEGF_{165a} (Figure S1A). We were also able to show that constitutive VEGFR2 dimers are formed in HEK293 cells by co-transfecting cells with NLuc-VEGFR2 and an N-terminal HaloTag-labeled VEGFR2 (Figure 1C). In these experiments HEK293 cells were transiently transfected with a fixed concentration of donor NLuc-VEGFR2 cDNA and increasing concentrations of acceptor HaloTag-VEGFR2 cDNA. Cells were treated with either vehicle (open circles) or 1 nM VEGF_{165a} (filled circles) for 60 min at 37°C. Punctate measurements were made for each condition in each individual experiment and values shown are the means ± SEM obtained in six separate experiments. *p < 0.05; **p < 0.001; Student’s t test.

Figure 1. Using NanoBRET to Characterise the Formation of VEGFR2 Homodimers and Ligand Binding at VEGFR2

(A) Schematic representation of the use of NanoBRET to investigate the interaction between NLuc-tagged VEGFR2 (NLuc-VEGFR2) and HaloTag-VEGFR2, or the binding of a fluorescent analog of VEGF_{165a} to NLuc-VEGFR2. (B) NanoBRET saturation binding curves obtained for VEGF_{165a}-TMR binding to NLuc-tagged VEGFR2. HEK293 cells stably transfected with NLuc-VEGFR2 were treated for 60 min with increasing concentrations of VEGF_{165a}-TMR (filled circles). Non-specific binding (open circles) was determined in the presence of 10 nM VEGF_{165a}. Values are means ± SEM from four separate experiments each performed in triplicate. pKD of VEGF_{165a}-TMR was 9.00 ± 0.16 (n = 4). (C) BRET experiments investigated the constitutive and ligand-induced dimerization of VEGFR2. HEK293 cells were transiently transfected with a fixed concentration of donor NLuc-VEGFR2 cDNA (0.05 µg/well) and increasing concentrations of acceptor HaloTag-VEGFR2 cDNA. Cells were treated with either vehicle (open circles) or 1 nM VEGF_{165a} (filled circles) for 60 min at 37°C. Duplicate measurements were made for each condition in each individual experiment and values shown are the means ± SEM obtained in seven separate experiments. *p < 0.05; **p < 0.001; Student’s t test.

Constitutive and VEGF_{165a}-Induced VEGFR2 Homodimerization

To monitor ligand binding and receptor dimerization of VEGFR2 using BRET, we tagged VEGFR2 on its N terminus with the bright, small (19.1 kDa) NLuc luciferase (Stoddart et al., 2015; Kilpatrick et al., 2017; Peach et al., 2018b) and ligand-induced dimerization of VEGFR2. HEK293 cells stably expressing NLuc-VEGFR2 (Figure 1B), and to demonstrate that the NLuc-tagged VEGFR2 retains its high affinity for VEGF_{165a} (Figure S1A). We were also able to show that constitutive VEGFR2 dimers are formed in HEK293 cells by co-transfecting cells with NLuc-VEGFR2 and an N-terminal HaloTag-labeled VEGFR2 (Figure 1C). In these experiments HEK293 cells were transiently transfected with a fixed concentration of donor NLuc-VEGFR2 cDNA and increasing concentrations of acceptor HaloTag-VEGFR2 cDNA. The BRET signal clearly saturated (as would be expected for a specific protein-protein interaction [Mercier et al., 2002]) and then began to decrease at the highest HaloTag VEGFR2 cDNA concentration used, probably due to the concentration of protein not being linearly related to cDNA concentration at high amounts. Importantly, the effects of this concentration were significantly enhanced when cells were treated with 1 nM VEGF_{165a} for 60 min at 37°C (Figure 1C). Analysis of the effect of increasing concentrations of VEGF_{165a} on the homodimerization obtained when 0.05 µg/well HaloTag VEGFR2 cDNA was transiently transfected with 0.025 µg/well of NLuc-VEGFR2 cDNA showed a clear saturable effect with a half maximal effective concentration (pEC_{50} = −log EC_{50}) for VEGF_{165a} of 8.81 ± 0.20 (n = 6; Figure 1D). A similar effect was observed when 0.05 µg/well HaloTag VEGFR2 cDNA was transiently transfected into a stable NLuc-VEGFR2 cell line (Kilpatrick et al., 2017) yielding a pEC_{50} for VEGF_{165a} of 9.40 ± 0.28 (n = 4). These values are very similar to the binding affinity of VEGF_{165a} determined from NanoBRET binding (pK_{i} = 10.17 ± 0.09, n = 7; Figure S1A) and to the pEC_{50} values for VEGF_{165a} of 8.71 ± 0.13 (n = 3; Figure S1A).
obtained using an NFAT reporter assay in cells expressing either NLuc-VEGFR2 or HaloTag-VEGFR2 (9.50 ± 0.06 and 10.00 ± 0.14 for NLuc-VEGFR2 and HaloTag-VEGFR2, respectively; n = 5 in each case; Figure S1B). These values were also similar to those previously reported for the wild-type VEGFR2 (pEC50 = 9.66 ± 0.05 [Carter et al., 2015]) and confirm that the N-terminal NLuc and HaloTag labels do not interfere with intracellular signaling or binding of VEGF165a.

VEGFR2-GPCR Oligomeric Complexes

It is well established that many GPCRs can form homodimers (Ferré et al., 2014; Vischer et al., 2015; Parmar et al., 2016). Here, we have used transient expression of NLuc- and SNAP-tagged GPCR pairs to demonstrate that the two GPCRs (β2-adrenoceptor and adenosine A3 receptors) studied here can form homodimers that are detectable using BRET (Figures 2A and 2B). Transient transfection of donor NLuc-tagged GPCR cDNA and increasing concentrations of acceptor SNAP-tagged GPCR cDNA revealed a clear and statistically significant saturation of the BRET signal that was consistent with a specific protein-protein interaction (Figures 2A and 2B; Table S1). When NLuc-VEGFR2 cDNA was co-transfected with SNAP-tagged GPCR cDNA, evidence for a selective interaction with β2-adrenoceptors was revealed (Figure 2C; Table S1). Clear saturation of the BRET signal was observed that in keeping with close proximity (<10 nm [Mercier et al., 2002]) between VEGFR2 and the β2-adrenoceptor (Figure 2C). Given the propensity for both VEGFR2 and the β2-adrenoceptor to form homodimers, this may represent the transient transfection experiments confirmed that very similar levels of expression of the two GPCRs were achieved in the presence of NLuc-VEGFR2 (Figure S2).

Figure 2. BRET Experiments Investigating GPCR Homo-Dimerization and Complex Formation between GPCRs and VEGFR2

(A and B) GPCR homodimer formation was investigated using transient transfection with NLuc-GPCR cDNA (0.05 µg/well) and increasing concentrations of SNAP-tagged GPCR cDNA for (A) the β2-adrenoceptor (β2-AR) or (B) the adenosine A3-receptor (A3R). Data are means ± SEM from five separate experiments, each performed in duplicate.

(C and D) Complex formation between VEGFR2 and GPCRs. HEK293 cells were transfected with NLuc-VEGFR2 cDNA (0.05 µg/well) and increasing concentrations of SNAP-tagged GPCR cDNA for (C) the β2-adrenoceptor or (D) the adenosine A3-receptor. Data are means ± SEM from five separate experiments, each performed in duplicate.

formation of a larger oligomeric complex. In marked contrast, no evidence for a specific interaction between VEGFR2 and the adenosine A3 receptor was observed (Figure 2D). The BRET signal increased linearly with increasing concentration of acceptor SNAP-tagged adenosine A3-receptor cDNA which is consistent with a non-specific interaction caused by bystander BRET (Figure 2D) (Mercier et al., 2002). A comparison of the expression level of SNAP-tagged β2-adrenoceptors and adenosine A3 receptors in these transient transfection experiments confirmed that very similar levels of expression of the two GPCRs were achieved in the presence of NLuc-VEGFR2 (Figure S2).

A study of the impact of agonist stimulation on the formation of VEGFR2-β2-adrenoceptor complexes (Figure 3) indicated that there was a significant concentration-dependent enhancement of VEGFR2-β2-adrenoceptor complex formation induced by either VEGF165a or isoprenaline (Figures 3C and 3D). Interestingly, in cells transfected with both VEGFR2 and β2-adrenoceptor cDNA, VEGF165a was still able to stimulate VEGFR2 dimerization and both basal and VEGF-stimulated VEGFR2 homodimerization was unaffected by co-stimulation with isoprenaline (Figures 3E and 3G). Similarly, VEGF165a (Figure 3F) and isoprenaline treatment (Figure 3H) did not alter β2-adrenoceptor homodimerization.

To ensure that VEGFR2-β2-adrenoceptor oligomeric complexes were not a result of receptor overexpression, we took advantage of the endogenously expressed β2-adrenoceptors in HEK293 cells. Using CRISPR/Cas9 genome-engineering, we generated HEK293 cells that expressed NLuc-β2-adrenoceptors under the control of the native promoter. These studies showed a significant increase in the BRET ratio for NLuc-β2-adrenoceptors expressed under the endogenous promoter and exogenously transfected with 0.01 µg/well HaloTag-VEGFR2 (fluorophore-labeled HaloTag-VEGFR2 compared with cells where the HaloTag-VEGFR2 was not labeled with fluorophore) (Figures 4A and 4B). This supports the observation of a specific VEGFR2-β2-adrenoceptor oligomeric complex and
also demonstrates that formation and detection of the complexes is independent of tag orientation.

Electroporation of human umbilical vein endothelial cells (HUVECs) with NLuc-VEGFR2 also showed a significant increase in BRET ratio (p < 0.05) when the cells were co-transfected with SNAP-Tag-β2-adrenoceptor cDNA (Figure 4C) suggesting that heterodimers can be formed in endothelial cells. To investigate further the potential for endogenous VEGFR2 and β2-adrenoceptors to interact in HUVECs, we also investigated their ability (alone and in combination) to stimulate cell proliferation. As reported previously (Kilpatrick et al., 2017), 3 nM VEGF<sub>165a</sub> was able to produce a large and significant enhancement of cellular proliferation that could be inhibited by the tyrosine kinase inhibitor, cediranib (Figure S3). A very small but not significant increase in cell number was observed with both high (10 μM) and low (100 nM) concentrations of isoprenaline alone (Figure S3). However, in the presence of 10 μM isoprenaline the response to 3 nM VEGF<sub>165a</sub> was significantly attenuated (Figure S3).

We have previously reported that dimer formation can lead to negative cooperativity between the ligand binding sites of the two partners within an oligomeric complex (Sherbi et al., 2015; May et al., 2011). Therefore, to investigate the potential for negative cooperativity across oligomeric interfaces, we took advantage of our ability to measure ligand binding to NLuc-tagged receptors using BRET and fluorescent ligands (Stoddart et al., 2015; Kilpatrick et al., 2017). Clear saturable and high-affinity specific binding of the fluorescent ligand BODIPY CGP12177-TMR...
to β2-adrenoceptors (Kd = 67.2 ± 3.3 nM, n = 4; Figure S1C) was observed, which was competitively antagonized by non-fluorescent β2-adrenoceptor ligands (Figure S1D). However, no significant alteration in the binding of BODIPY CGP12177-TMR or VEGF165a-TMR to their cognate receptors was observed with the addition of ligands for the corresponding receptor heteromer partner (Figure S4). Thus, VEGF165a did not influence the binding of BODIPY CGP12177-TMR to the β2-adrenoceptor (Figure S4A) and IC118551, CGP12177, propranolol, and isoprenaline did not influence binding of VEGF165a-TMR to VEGFR2 (Figure S4B).

**Functional Impact of VEGFR2-β2-Adrenoceptor Complexes on β2-Adrenoceptor Activation**

To investigate the potential impact of VEGFR2 complexes on β2-adrenoceptor signaling and function, we first studied whether the VEGFR2-β2-adrenoceptor oligomeric complex altered the extent to which an active β2-adrenoceptor could engage with a conformation-sensitive single-domain nanobody (Nb80) that has previously found use in structural studies as a Gs-alpha surrogate protein (Rasmussen et al., 2011; Steyaert and Koblika, 2011). Here we used a GFP-tagged version of Nb80 (Irannejad et al., 2013) in conjunction with a β2-adrenoceptor tagged on its C terminus with NLuc, to establish a NanoBRET assay in living cells to monitor engagement (based on proximity) between an activated β2-adrenoceptor and cytosolic Nb80-GFP. Isoprenaline (10 μM) stimulation of stable Nb80-GFP-expressing HEK293 cells, transiently transfected with β2-adrenoceptor-NLuc, produced a rapid and significant binding of Nb80-GFP to β2-adrenoceptors (Figure S5A; p < 0.001) confirming the ability of Nb80-GFP to detect active conformations of the β2-adrenoceptor. To investigate the impact of VEGFR2 on β2-adrenoceptor signaling, we also transfected cells with either HaloTag-VEGFR2 or an empty control vector. In cells additionally transfected with the control empty vector, isoprenaline stimulated the formation of a complex between the β2-adrenoceptor and Nb80 (log EC50 = –7.91 ± 0.11, n = 6; Figure S5B). This response was competitively antagonized by the high-affinity β2-selective antagonist, ICI 118551 (log Kd = –9.30 ± 0.20, n = 6; Figure S5B). In the presence of VEGFR2, the response to isoprenaline was unaltered, compared with control vector (Figure S5B). The successful expression of HaloTag-VEGFR2 in these cells was confirmed at the end of the experiment by labeling cells with the HaloTag substrate (data not shown).

**Cellular Location of VEGFR2-β2-Adrenoceptor Complexes**

Confocal imaging of SNAP-tagged β2-adrenoceptors and Halo-Tag-transfected VEGFR2 labeled with cell-impermeable dyes showed that, under basal conditions, cell surface β2-adrenoceptors largely remained on the plasma membrane when expressed alone (Figure S5A). However, as reported previously (Kilpatrick et al., 2017), HEK293 cells expressing HaloTag-VEGFR2 showed evidence of constitutive receptor internalization (Figure S5A). Following treatment with 10 nM VEGF165a, VEGFR2 internalization was markedly increased (Figure S5A). β2-adrenoceptor internalization was also stimulated by isoprenaline (10 μM) (Figure S5A). Following co-transfection of HEK293 cells with HaloTag-VEGFR2 and SNAP-Tag β2-adrenoceptor cDNA, the constitutive internalization of VEGFR2 appeared to be accompanied by a low level constitutive internalization of the β2-adrenoceptor (Figure S5A). Isoprenaline (10 μM) stimulated a large internalization of the β2-adrenoceptor that was co-localized with internalized VEGFR2 (Figure S5A). Stimulation of co-transfected cells with 10 nM VEGF165a produced an enhanced internalization of VEGFR2 that was also accompanied by a partial internalization of cell surface β2-adrenoceptors (Figure S5A). Co-localized receptors were readily detected in intracellular Rab5-positive endosomes under both basal or agonist-stimulated (VEGF165a or isoprenaline) conditions (Figure S5B). Control experiments for Rab5 localization are shown in Figure S5B. Structured illumination...
super-resolution microscopy (SIM) confirmed that there was co-localization of HaloTag VEGFR2 and SNAP-Tag β₂-adrenoceptors at intracellular sites, which was elevated after agonist stimulation (Figures 6C and 6D). The Fiji (ImageJ) analysis program CoLoc2 was applied to these SIM images to determine the extent of HaloTag VEGFR2 (green; HaloTag AF488 substrate) and SNAP-Tag β₂-adrenoceptor (red; SNAP-AF647 substrate) fluorescence co-localization (Figure 6D). Circular regions of interest (ROI) were placed on areas of fluorescence at the plasma membrane or intracellular regions (12–15 ROI; Figure 6E). Pearson’s correlation coefficient values were obtained from each ROI, pooled, and expressed as mean ± SEM. A Pearson’s correlation coefficient of +1 is indicative of a perfect co-occurrence between the fluorophores of interest (AF488 and AF647, respectively).

Interaction with β-Arrestin
Receptor internalization and signaling from endosomes are regulated by β-arrestin scaffolding proteins (β-arrestin 1 and 2; also known as arrestin 2 and 3 [Shenoy and Lefkowitz, 2011; Laporte et al., 2000; Luttrell et al., 2001; Eichel and von Zastrow, 2018]). Here we have used the Receptor Heteromer Investigation Technology approach (Jaeger et al., 2014), with β-arrestin2-Venus-YFP in combination with a β₂-adrenoceptor tagged on its C terminus with NLuc, to investigate using NanoBRET the effect of VEGFR2 activation on isoprenaline-induced receptor engagement with β-arrestin2 in cells co-expressing unlabeled HaloTag-VEGFR2 (Figure 7A). Stimulation with 10 μM isoprenaline alone induced β-arrestin2-Venus-YFP engagement with the β₂-adrenoceptor, which reached a peak between 4 and 6 min after addition of the agonist (Figure 7B). Thereafter, the BRET signal declined with time over the next 40 min (Figure 7). However, when 10 nM VEGF₁₆₅α was added at the same time as isoprenaline, the activation of VEGFR2 altered the profile of the β₂-adrenoceptor engagement with β-arrestin2 (Figure 7B). Thus, the peak response was slightly truncated and thereafter a plateau was rapidly achieved (9 min after agonist addition), which was then maintained for the next 30 min (Figure 7B). This plateau remained significantly higher (p < 0.05) than that achieved with isoprenaline alone, where the response continued to decline. This effect of VEGF₁₆₅α was completely prevented by pretreatment with the VEGFR2 RTK inhibitor cediranib (Figure 7B) (Carter et al., 2015). Studies of VEGFR2 phosphorylation using a phospho-specific antibody for the tyrosine residue 1,212 did not provide evidence for enhanced phosphorylation of VEGFR2 following stimulation with isoprenaline in cells co-expressing β₂-adrenoceptors (Figure S6). However, there was a significant degree of constitutive VEGFR2 phosphorylation in vehicle-treated cells (expressing both VEGFR2 and β₂-adrenoceptors) that was inhibited by cediranib.

DISCUSSION
Here, we have demonstrated that VEGFR2 and a GPCR (the β₂-adrenoceptor) functionally interact in highly specific hetero-oligomeric complexes to modulate receptor localization, trafficking, and downstream signaling. This was possible using NanoBRET technology, which sensitively detects interactions between protein-protein pairs (<10 nm apart). We found that the SNAP-tagged-β₂-adrenoceptor was in close proximity to NLuc-VEGFR2, indicative of complex formation. Notably, stimulation of either the GPCR or RTK with a selective agonist (β₂-adrenoceptor with isoprenaline or VEGFR2 receptor with VEGF₁₆₅α) was able to significantly enhance complex formation, as indicated by an increased BRET signal. In contrast, we found no evidence for interaction between the adenosine A₃ receptor and VEGFR2. This indicates that the RTK-GPCR heteromeric interactions observed here are not simply a consequence of non-specific bystander BRET, and that they are consistent with the documented physiological expression of β₂-adrenoceptors and VEGFR2, but not A₃ receptors, on endothelial cells (Claesson-Welsh and Welsh, 2013; Garg et al., 2017; Feoktistov et al.,...
RTK-GPCR hetero-oligomerization was also demonstrated with genome-edited NLuc-β2-adrenoceptor expressed under its endogenous promoter, where the extent of oligomerization was limited to physiological levels by the availability of the β2-adrenoceptor. The level of exogenously expressed HaloTag-VEGFR2 that was available to interact with β2-adrenoceptor in these cells was lower than endogenous levels of VEGFR2 in HUVECs (Peach et al., 2018b), supporting the physiological relevance of the observed hetero-oligomeric interactions.

These findings help to explain a striking feature of previous work that investigated the membrane-diffusional properties of receptor-ligand complexes in living cells using techniques such as fluorescence correlation spectroscopy (FCS) (Briddon et al., 2018). These studies suggested that the receptor species diffusing in the cell membrane were much larger than expected for a single membrane protein (or dimer) diffusing in isolation (Briddon et al., 2018). This suggests that these receptors may be normally present within macromolecular complexes of
much higher molecular mass and/or have restricted lateral diffusion. Since FCS has also identified multiple populations of ligand-receptor complexes for both GPCRs and RTKs, this suggests that multiple signaling complexes may exist in the plasma membrane and elsewhere (Briddon et al., 2018; Thomsen et al., 2016). These observations are consistent with recent work that has identified receptor-G protein interactions at cell surface protein hotspots (Sungkaworn et al., 2017; Calebiro and Sungkaworn, 2018) and also with work that has demonstrated continued intracellular signaling fromGPCRs in endosomes (Irannejad et al., 2013; Eichel and von Zastrow, 2018), and the proposed importance of intracellular location to RTK signaling (Weddell and Imoukhuede, 2017).

Our work has also demonstrated that VEGF_{165a}-induced VEGFR2 homodimerization still occurs in the presence of β-2-adrenoceptor expression, suggesting that the oligomeric complexes detected by NanoBRET may be larger and contain more than just the two specific partner proteins under study. Thus, for example, VEGF_{165a} may be enhancing the formation of oligomeric complexes containing multiple copies of both VEGFR2 and β-2-adrenoceptors since it can enhance the formation of both VEGFR2-VEGFR2 and VEGFR2-β-2-adrenoceptor complexes (as determined by NanoBRET). In addition, β-2-adrenoceptors homodimers appear to be preferred since their formation is not influenced by agonist (isoprenaline or VEGF_{165a}) stimulation.

The studies described here also provide evidence that receptor oligomerization can modulate the localization and trafficking of receptors. Confocal imaging studies in living cells expressing an N-terminal SNAP-tagged β-2-adrenoceptor and an N-terminal-HaloTag variant of VEGFR2 showed that β-2-adrenoceptors largely remained on the plasma membrane under basal conditions, whereas VEGFR2 (as observed by us previously [Kilpatrick et al., 2017]) underwent constitutive internalization. When β-2-adrenoceptors and VEGFR2 were expressed together, there was enhanced constitutive internalization of the β-2-adrenoceptor and some co-localization with VEGFR2 at intracellular sites. This was markedly enhanced following treatment with isoprenaline and co-localized receptors were observed in Rab5-positive intracellular endosomes. A similar situation occurred following VEGF_{165a} stimulation. Collectively these data suggest that, following agonist stimulation of one of the receptor partners, there is enhanced co-localization (Figure 6) and oligomerization (Figure 3) between VEGFR2 and β-2-adrenoceptors within intracellular endosomes.

The findings suggest that receptor hetero-oligomerization may have evolved to expand the scope of downstream signaling that occurs in response to receptor agonism. NanoBRET assays demonstrated that the β-2-adrenoceptor can regularly engage with both a surrogate of the Gs-alpha subunit (Nb80) and with β-arrestin2.

In the case of β-arrestin2, interaction of β-2-adrenoceptors occurred rapidly (within 4–6 min), with a gradual decline in complex formation (indicated by reduced NanoBRET signal), indicating dissociation of the receptor and signaling molecule. While VEGFR2 stimulation with VEGF_{165a} had no effect on the absolute ability of the β-2-adrenoceptor to engage with downstream signaling molecules, it was notable that simultaneous stimulation of co-expressed β-2-adrenoceptors and VEGFR2 altered the temporal characteristics of the interaction between the β-2-adrenoceptor and β-arrestin2. Thus, the maximal level of β-2-adrenoceptor/β-arrestin2 complex formation was truncated, but the rate of complex dissociation was reduced, resulting in sustained complexes for the duration of the experiment (>30 min). The requirement for VEGFR2 activation to alter β-2-adrenoceptor signaling dynamics was confirmed by blocking the effect of VEGF_{165a} by the VEGFR2 RTK inhibitor, cediranib (Carter et al., 2015). Since activation of both VEGFR2 and β-2-adrenoceptor drive internalization of receptor complexes, it is plausible that sustained β-2-adrenoceptor/β-arrestin2 signaling occurs in intracellular endosomes, which may have particular implications for cellular proliferation and adds further complexity to the spatial-temporal control of β-2-adrenoceptor signaling (Irannejad et al., 2013; Eichel and von Zastrow, 2018). These observations may be particularly relevant to the observed attenuation of VEGF-induced cellular proliferation in HUVECs when concentrations of β-2-agonist are used that will stimulate the recruitment of β-arrestin2 to the receptor (Figure 7). Thus, in the presence of 10 μM isoprenaline, the effect of 3 nM VEGF_{165a} on cell proliferation is substantially reduced in human endothelial cells (Figure S2) and this may be related to altered signaling from intracellular endosomes. High concentrations of isoprenaline...
(5–10 μM) have also been shown to enhance VEGF-dependent angiogenic sprouting in HUVECs (Garg et al., 2017). Consistent with this interaction between VEGFR2 and β2-adrenoceptors in physiologically relevant human endothelial cells, we have also obtained evidence for VEGFR2-β2-adrenoceptor oligomerization (detected by BRET) in HUVECs.

The NanoBRET technology used in the current work represents a powerful proximity-based methodology to monitor protein-protein interactions involving different receptors in living cells, as well as ligand binding to cell surface receptors. It reports on the close proximity between two tagged partners that are localized within 10 nm of each other (Stoddart et al., 2018). The NanoBRET methodology also allowed us to investigate if cooperativity occurred across interfaces of the oligomeric complex of VEGFR2 with the β2-adrenoceptor, and demonstrated that no such cooperativity occurs. We have previously shown, using NanoBRET, that agonist-occupied receptor complexes are rapidly internalized to intracellular endosomes (Kilpatrick et al., 2017), and imaging studies in the current study suggest that this is associated with internalization of VEGFR2-β2-adrenoceptor oligomeric complexes. Furthermore, NanoBRET studies demonstrated that these receptor oligomers can associate with, and alter signaling via, β-arrestin2.

In summary, we have demonstrated here that oligomeric complexes involving VEGFR2 and β2-adrenoceptors can be generated in cell membranes and intracellular endosomes that retain their ability to couple to intracellular signaling pathways. Complexes involving the β2-adrenoceptor and VEGFR2 can co-internalize following agonist treatment. Furthermore, VEGF165a or isoprenaline can stimulate further complex formation. VEGF165a treatment can also alter the temporal characteristics of the isoprenaline-stimulated association between the β2-adrenoceptor and β-arrestin2. These data suggest that VEGFR2 and the β2-adrenoceptor may be key components of membrane and endosomal macromolecular complexes, which may have substantial implications for the spatiotemporal control of signaling driven by both of these receptors in physiological or pathophysiological states. Furthermore, the formation of VEGFR2/β2-adrenoceptor complexes may provide insights into their combined effects on endothelial cell proliferation and the therapeutic benefit of the β2-adrenoceptor antagonist propranolol in the treatment of infantile hemangioma. In addition, as both RTK and GPCR family members have been implicated in driving cancer progression and metastasis, the potential formation of distinct RTK/GPCR complexes may represent anti-cancer therapeutic targets for drug discovery efforts to exploit.

**Significance**

The vascular endothelial growth factor receptor 2 (VEGFR2) is a key mediator of angiogenesis and endothelial cell proliferation following binding of its cognate ligand VEGF. Therefore the VEGFR2/VEGF signaling axis is an attractive therapeutic target in the treatment of conditions characterized by aberrant angiogenesis, such as cancer, and the common childhood tumor, infantile hemangioma. Interestingly the β-adrenoceptor antagonist propranolol is the current first-line treatment for infantile hemangioma; however, its mechanism of action is not fully understood, although it is believed to ultimately result in decreased VEGF expression and cell proliferation. Activation of the β2-adrenoceptor has also been suggested to augment VEGFR2 signaling in multiple cancer types. We have used bioluminescence resonance energy transfer (BRET) to demonstrate the existence of oligomeric complexes between VEGFR2 and the β2-adrenoceptor at the plasma membrane and also within intracellular compartments of living HEK293 cells and human umbilical vein endothelial cells (HUVECs). The use of CRISPR/Cas9 gene editing in HEK293 cells illustrated that VEGFR2/β2-adrenoceptor complexes were present, even when the β2-adrenoceptor was expressed at endogenous levels. These complexes can be induced by either VEGFR2 or β2-adrenoceptor selective ligands, and are able to co-internalize to shared intracellular compartments. These oligomeric complexes can couple to intracellular signaling proteins, and the presence of activated VEGFR2 is able to alter the temporal profile of β-arrestin2 coupling to the β2-adrenoceptor in response to ligand stimulation. The existence of VEGFR2/β2-adrenoceptor complexes may explain why the β2-adrenoceptor-selective antagonist, propranolol, is therapeutically beneficial in the treatment of infantile hemangioma. In addition the documented synergy of VEGFR2 and β2-adrenoceptor signaling in many cancer types suggests that the formation of complexes between these two receptors subtypes could represent anti-cancer therapeutic targets.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS


DECLARATIONS OF INTERESTS

R.F.O., M.B.R., K.Z., and K.V.W. are employees of Promega Corporation, which has proprietary rights over the NanoBRET assay and HaloTag technology. K.D.G.P is Chief Scientific Adviser of Dimerix, a spin-out company of The University of Western Australia that has been assigned the rights to the Receptor Heteromer Investigation Technology. K.D.G.P. is an inventor on patents covering the technology and has a shareholding in Dimerix.

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