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BRET-based assay to monitor EGFR transactivation by the AT$_1$R reveals G$_{q/11}$ protein-independent activation and AT$_1$R-EGFR complexes

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Abstract

The type 1 angiotensin II (AngII) receptor (AT$_1$R) transactivates the epidermal growth factor receptor (EGFR), which leads to pathological remodeling of heart, blood vessels and kidney. End-point assays are used as surrogates of EGFR activation, however these downstream readouts are not applicable to live cells, in real-time. Herein, we report the use of a bioluminescence resonance energy transfer (BRET)-based assay to assess recruitment of the EGFR adaptor protein, growth factor receptor-bound protein 2 (Grb2), to the EGFR. In a variety of cell lines, both epidermal growth factor (EGF) and AngII stimulated Grb2 recruitment to EGFR. The BRET assay was used to screen a panel of 9 G protein-coupled receptors (GPCRs) and further developed for other EGFR family members (HER2 and HER3); the AT$_1$R was able to transactivate HER2,

but not HER3. Mechanistically, AT_1R-mediated ERK1/2 activation was dependent on $G_{q/11}$ and EGFR tyrosine kinase activity, whereas the recruitment of Grb2 to the EGFR was independent of $G_{q/11}$ and only partially dependent on EGFR tyrosine kinase activity. This $G_{q/11}$ independence of EGFR transactivation was confirmed using AT_1R mutants and in CRISPR cell lines lacking $G_{q/11}$. EGFR transactivation was also apparently independent of β-arrestins. Finally, we used additional BRET-based assays and confocal microscopy to provide evidence that both AngII- and EGF-stimulation promoted AT_1R-EGFR heteromerization. In summary, we report an alternative approach to monitoring AT_1R-EGFR transactivation in live cells, which provides a more direct and proximal view of this process, including the potential for complexes between the AT_1R and EGFR.

Keywords

Angiotensin II; epidermal growth factor receptor; EGFR transactivation; bioluminescence resonance energy transfer; G protein-coupled receptor; receptor heteromerization

1. Introduction

Angiotensin II (AngII) is the major bioactive peptide of the Renin-Angiotensin System (RAS) and influences a broad range of homeostatic and modulatory processes, including cardiovascular and renal physiology. Dysregulation of the RAS is associated with disease via actions on cardiac (1-4), vascular (5,6) and renal (7,8) growth and remodeling, modulation of sympathetic nervous system activity (9-11), endothelial dysfunction (12), angiogenesis (13,14) and inflammation (15-17). AngII acts primarily through the type 1 AngII receptor $(AT_1R[1])$, a G protein-coupled receptor (GPCR), to mediate an array of intracellular signals, including calcium mobilization and generation of reactive oxygen species (ROS), modulation of receptor and non-receptor tyrosine kinases, mitogen-activated protein kinases (MAPK) (including the extracellular-regulated kinase 1/2 (ERK1/2)) and various ion channels (18,19). We and others have demonstrated that the AT_1R can transactivate the epidermal growth factor receptor (EGFR), which in turn modulates cellular growth, tissue remodeling and cellular hypertrophy (20-23).

The EGFR belongs to the ErbB family of receptor tyrosine kinases (RTKs), comprised of four members: EGFR (ErbB1, HER1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (24). HER receptors are activated by a group of epidermal growth factor (EGF) ligands and undergo homo- or hetero-dimerization during their activation, with HER2 being the preferred dimer partner (25). Activated and tyrosine-phosphorylated HER receptors recruit growth factor receptor-bound protein 2 (Grb2) as an initial step in a complex cascade of growth signaling activation. HER receptors can also be 'transactivated' following prior activation of other receptors. Most notably, Ullrich and colleagues first described GPCR-mediated EGFR transactivation, and subsequently proposed a model, whereby activated GPCRs stimulated matrix metalloproteases (MMP), including the ADAMs (A Disintegrin and Metalloprotease), to cleave inactive membrane-bound EGF ligands to activate EGFR (26,27). This model has been broadly accepted, although others have suggested a more

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[1]Nomenclature as per International Union of Basic & Clinical Pharmacology (IUPHAR)

complex mechanism (perhaps cell-specific) involving non-receptor tyrosine kinases (e.g., src and Pyk2) (28-32) and the activation of additional second messengers including calcium (33-35), protein kinase C (32,33,36,37), ROS (23,38) and β-arrestin (36,39). Definitive resolution of the precise mechanism of GPCR-EGFR transactivation requires approaches to monitor this process in living cells, in real-time, as well as the capacity to identify key proteins and interactions involved. While some progress has been made on the latter problem – a siRNA screen was recently used to identify novel mediators of AT$_1$R-EGFR transactivation (40) – to date most readouts of EGFR transactivation have been biochemical, end-point assays that do not capture live cell dynamics or kinetics.

Commonly, GPCR-mediated EGFR transactivation has been defined utilizing the activation of ERK1/2 that is inhibited by the small molecule antagonist of EGFR (i.e., AG1478). However, there are several limitations in using ERK1/2 as a surrogate readout of transactivation. EGFR signal transduction is complex with ERK1/2 phosphorylation representing a signal quite distal from the initial step of EGFR activation. Arguably, a more direct readout would be to examine upstream events, including activation and autophosphorylation of the EGFR. Although a direct readout of phospho-EGFR may mitigate the limitations of the ERK1/2 assay, both phospho-EGFR and phospho-ERK1/2 assays are endpoint approaches and only provide a snapshot of cellular events. More recently, proximity-based assays, such as resonance energy transfer (RET) assays and fluorescein arsenical hairpin (FlAsH) biosensors have evolved to enable dynamic analysis of protein-protein and intra-molecular interactions in real-time (41,42). The development and validation of proximity-based assays has enabled their wide use to characterize receptor interactions. They have become extremely useful tools for studying GPCR biology, and specifically the interaction of GPCRs with G proteins and arrestins (43-46).

In this study, we employed a Bioluminescence-RET (BRET)-based assay to monitor the most proximal event in EGFR activation, namely Grb2 interaction with the activated EGFR. We demonstrate, in live cells and in real-time, that both AngII- and EGF-stimulation can promote Grb2 translocation to the activated EGFR, indicating AT$_1$R transactivation of the EGFR. Having established this approach, we extend it to investigate the molecular processes involved and report evidence for the formation of AT$_1$R and EGFR complexes.

2. Materials and Methods

2.1. Materials

[^{125}I]-Labelled AngII was obtained from ProSearch. HEK293, Chinese hamster ovary (CHO-K1), NIH-3T3, cells were obtained from American Type Culture Collection. HEK293FT were obtained by Thermo Fisher Scientific. HEK-adherent-293 cell line was a generous gift from Dr Dominic Ng, University of Queensland, Australia. AngII was obtained from Auspep or Sigma Aldrich and EGF from R&D Systems or Peprotech. Inhibitors used were YM-254890 (Wako Pure Chemical Industries), Candesartan (AstraZeneca), AG1478 (Merck Millipore) with PP2 and BAPTA obtained from Sigma Aldrich.

2.2. Plasmids

This study used a rat AT1a receptor clone (referred to as AT1R). Preparation of the following cDNA constructs has been previously described: Grb2-Venus (47), β-arrestin2-Venus (48), AT_1R-Vn, β-arrestin2-Rluc8 (49), pcDNA3.1 β-arrestin1-Flag and β-arrestin2-Flag (50), and AT_1R, [TK325]AT_1R and [Y215F]AT_1R (20). EGFR-Vc was supplied from Ichi N. Maruyama (Okinawa Institute of Science and Technology, Okinawa, Japan) (51). The following cDNA constructs were gifts from collaborators: Venus-K-ras from Prof Nevin Lambert (Georgia Regents University) and HA-V_2R from Dr Thierry Durroux (Functional Genomics Institute, Montpellier, France). HA-$β_1$AR was generated by GeneArt (Thermo Fisher Scientific, Regensburg, Germany) and $α_{1b}$AR, $α_{2a}$AR, $β_2$AR, HA-$β_2$R and HA-D_1R cDNAs were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). AT_1R-Rluc8 was generated by inserting AT_1R from AT_1R-Venus into pcDNA3-Rluc8, prepared previously from cDNA kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, CA). EGFR-Rluc8, HER2-Rluc8 and HER3-Rluc8 were generated by inserting EGFR, HER2, and HER3, obtained from Origene (Rockville, MD, USA) into pcDNA3-Rluc8. EGFR-Venus was subsequently generated from EGFR-Rluc8 by replacing the Rluc8 coding region with Venus cDNA from pcDNA3-Venus prepared previously from pcC2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan). Internally EE-tagged $Gα_q$ and $Gα_{12}$ and untagged DN $Gα_q$ Q209L/D277N mutant, $Gα_{11}$ and $Gα_{13}$ were from UMR cDNA resource center (University of Missouri-Rolla (www.cdna.org)). A HEK 293 cell line that lacks both arrestin2 and 3 (β-arrestin 1 and 2) was used as previously described (52).

2.3. Cell Culture & Transfection

NIH-3T3, HEK293, HEK293FT cells and HEK-adherent-293 cell line, which is a derivative of HEK293 with increased adherence, were grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) and antibiotic-antimycotic solution and HEK293FT cells contained 0.3mg/ml glutamine. Stably expressing NHA-AT_1R -HEK293 and HEK293FT cells were maintained as above in DMEM/10% FBS with the addition of 200**μ**g/ml of media of Geneticin (G-148, Gibco). CHO-K1 cells were maintained in α-MEM (Life Technologies) supplemented with 10% FBS and antibiotic-antimycotic solution. All cells were maintained at 37°C in 5% CO2. All cells were used between passages 5 to 30. Cells were transiently transfected using FuGene (Promega) according to manufacturer's instructions or with Lipofectamine 2000 (Invitrogen) as previously described (41). All cells were used 48 hours post-transfection.

2.4. Isolation of VSMC

VSMC isolation and procedures were performed with prior approval from Temple University Institutional Animal Care and Use Committee and in accordance with National Institute of Health Guide for the Care and Use of Laboratory animals. Male Sprague-Dawley rats (350g; Charles River Breeding Laboratory Inc., Wilmington, MA) were used for the preparation of primary VSMC cultures. Rodents were euthanized by CO2 inhalation followed by cervical dislocation. VSMC were prepared from thoracic aorta of rats by

the explant method as previously described (53,54). VSMCs were subcultured in DMEM containing 10% FBS, penicillin and streptomycin. VSMC were used between passages 3 to 12.

2.5. Protein Extraction & BCA Assays

NHA-AT$_1$R-HEK293 were seeded onto 6-well plates at 600,000 cells/well in DMEM/10% FBS. Cells were serum starved for 24 hours and then stimulated with 100nM AngII, 10nM EGF or vehicle for 5 minutes at 37°C. After treatment, cells were washed in ice-cold PBS (Gibco) (pH 7.4) and lysed with 200μL/well of ice-cold RIPA lysis buffer [50mM Tris-HCl pH 7.5, 100mM NaCl, 2mM EDTA, 50mM sodium fluoride, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 10mM sodium pyrophosphate, 10mM sodium orthovanadate] containing Complete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor tablet (Roche). Cells were gently lysed for 1 hour at 4°C and centrifuged at 15,000g for 15 minutes at 4°C. Lysates were scraped from the wells and protein concentration determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For the resolution of all proteins, protein samples were mixed with Laemmli sample buffer (BioRad) containing 8% β-mercaptoethanol and heated at 95°C for 5 minutes.

2.6. SDS-PAGE and Western Blot Analysis

Samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples and a molecular weight standard (Precision Plus Protein™ Dual Color standards, BIO-RAD) were electrophoresed at 100V for approximately 2 hours. Proteins were then transferred electrophoretically onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Merck Millipore). Membranes were incubated with blocking buffer (Odyssey) at room temperature for 1 hour and then with primary rabbit polyclonal anti-p44/42 ERK1/2 MAPK and primary mouse monoclonal anti phospho-p44/42 MAPK P-ERK1/2 (Cell Signaling, Cat #4695 and #9106 respectively) (1:1000 dilution in blocking buffer) at 4°C overnight. Membranes were washed with washing buffer (PBS with 0.1% Tween20) 4 times for 5 minutes each and incubated with a goat anti mouse IRDye 800CW (LI-COR Biosciences, Cat# 926-32210) and donkey anti rabbit IRDye 680RD (LI-COR Biosciences, Cat# 926-32222) for 1 hour at room temperature (1:10,000 dilution in blocking buffer). After further washing, membranes were scanned using an Odyssey Licor Scanner (LI-COR Biosciences). Densitometry analysis of membrane was performed using Image Studio Version 5.2 software.

2.7. Ligand-induced BRET Assays & Receptor-Heteromer Investigation Technology

HEK293 and HEK293FT cells (700,000 cells/well), NIH-3T3 (600,000 cells/well), and CHO-K1 cells (400,000 cells/well), were seeded into 6-well plates and maintained at 37°C and 5% CO$_2$ in complete media supplemented with 10% FBS. 24 hours after seeding, cells were transiently transfected using FuGene (Promega) according to manufacturer's recommendations. 24 hours post-transfection, cells were washed with PBS, and detached using 0.25% trypsin (Gibco), resuspended in FluoroBrite phenol red-free Complete media (Gibco) containing 5% FBS or phenol-red free DMEM containing 25mM HEPES, 0.3mg/ml glutamine, 100IU/ml penicillin and 100μg/ml streptomycin supplemented with 5% FBS and added to poly-D-Lysine-coated (Sigma) 96-well white polystyrene microplate (Nunc

or Gibco) at 100,000 cells/well. Early the following day, cells were serum starved with FluoroBrite phenol red-free media + 10% L-Glutamine or 25mM HEPES with 0.3mg/ml glutamine, 100IU/mL penicillin and 100μg/ml streptomycin for at least 6 hours. 48 hours post-transfection, media was replaced with PBS or Hanks' balanced salt solution (HBSS) containing coelenterazine-h (Promega) at a final concentration of 5μM or furimazine (Promega) at a final concentration of 10μM. BRET[1] measurements were taken at 37°C every 30 seconds using: a M1000 PRO Tecan plate reader (Tecan, Life Sciences) with 430-485 nm and 505-590 nm filters; a CLARIOstar Microplate Reader (BMG Labtech) with 420-480 nm and 520-620 nm filters; or a LUMIstar Omega Microplate Reader (BMG Labtech) with 450-480 nm and 520-550 nm filters. Following baseline readings, cells were treated with vehicle, 10μM AngII or 1μM EGF. The BRET signal observed between interacting proteins was normalized by subtracting the background BRET ratio as follows: the long wavelength emission over the short wavelength emission for a cell sample treated with vehicle was subtracted from the same ratio for a second aliquot of the same cell sample treated with 10μM AngII or 1μM EGF (49). For these BRET kinetic assays, the arrow indicates the first BRET reading following treatment of agonist/vehicle (time of ligand/ vehicle addition). BRET assessment using parental HEK293 and knockout HEK293 has been described previously (41). Ligand- and vehicle-treated wells were assayed in triplicate wells in each independent experiment.

2.8. Bimolecular Fluorescence Complementation (BiFC)

CHO-K1 cells were plated on collagen-coated 35mm glass bottom dishes (MatTek) at a density of approximately 400,000 cells/dish. 24 hours post-seeding, cells were transiently transfected with 0.15μg DNA of Vn-tagged protein and 0.15μg DNA of Vc-tagged protein, to a total of 0.3μg of protein (compensated with empty vector when expressing DNA constructs individually) using FuGene. 24 hours post transfection, cells were serum starved using FluoroBrite phenol red-free media + 10% L-Glutamine for 24 hours. Cells were assayed 48 hours after transfection. Localization of complemented Venus fluorescence was viewed with an Olympus FV1000 inverted confocal (FV1000, Olympus, Tokyo, Japan) 60x oil immersion objective on a heated (37°C) chamber stage.

2.9. Amaxa Nucleofector Transfection

Isolated VSMC (1.0×10^6) were transfected by Amaxa Nucleofector program U-25 in basic Smooth Muscle Cells Nucleofector™ solution (Lonza) containing 1.5μg EGFR-Rluc8, 0.5μg Grb2-Venus with 0.15μg NHA-AT_1R. 24 hours post-transfection, cells were incubated with serum-free DMEM for 24 hours. Cells were assayed 48 hours post-transfection.

2.10. Generation of CRISPR Gene Deletion Cell lines

$G_{q/11}$- and β-arrestin1/2-deleted HEK 293 cell lines were generated using the CRISPR/Cas9 system, as described previously (55,56).

2.11. Data Analysis

All data represent the means ± S.E.M of at least 3 independent experiments. Statistical analysis and curve fitting were carried out using GraphPad Prism version 7.0 (GraphPad

Software Inc., CA, USA). Statistical analysis, indicated in figure legends, was determined using ANOVA with appropriate Dunnett's tests for multiple comparisons or unpaired tests. Differences are considered significant with $p < 0.05$.

3. Results

3.1. BRET readout of AT_1R-EGFR transactivation

To investigate AT_1R-mediated EGFR transactivation, we employed a BRET-based assay to quantitatively monitor, in living HEK293 cells, the recruitment of Grb2 to the EGFR. EGFR-Rluc8 (Renilla luciferase) and Grb2-Venus constructs were co-expressed with the AT_1R, as shown in Fig. 1A. EGF stimulation produced a robust and sustained ligand-dependent BRET signal, indicative of Grb2 translocation and interaction with the EGFR (Fig. 1B). Consistent with the idea that GPCR-mediated transactivation engages only a small percentage of the total EGFR signaling capacity, maximal AngII-mediated Grb2 recruitment and the downstream activation of ERK1/2 represent approximately 20% of that stimulated by the EGF ligand (Fig. 1B). We also investigated whether AngII stimulation recruited Grb2 to other members of the EGFR family, specifically HER2 (preferred dimer partner for EGFR, which is not ligand-activated) and HER3 (which is ligand-activated, but is kinase-deficient). HER2, but not HER3, complexes with Grb2 following AngII-stimulation (inset to Fig. 1B). The BRET-based assay for AT_1R-mediated transactivation extends to additional immortalized cell lines (CHO-K1 and NIH-3T3 cells, Fig. 2, A and B), as well as isolated primary vascular smooth muscle cells (VSMC) (Fig. 2C).

3.2. Screening for EGFR transactivation by multiple GPCRs

A number of GPCRs have been reported to transactivate the EGFR, including the adrenergic, dopamine, bradykinin, vasopressin and adenosine receptors, although the exact mechanisms remain unresolved (18,57). A focused screen of 9 separate GPCRs and their capacity to recruit Grb2 to the EGFR was performed in HEK293FT, as shown in Fig. 3, A-I. Of the subset of GPCRs tested, we only observed an increase in the EGFR-Grb2 BRET ratio for the AT_1R and vasopressin 1b receptor ($V_{1b}R$). For the other GPCRs, their activation led to no change or a slight decrease in the EGFR-Grb2 BRET ratio. We substantiated that these GPCRs were functional in a BRET-based arrestin trafficking assay (insets to Fig. 3 B, D-I), and all assays included EGF activation of the EGFR as an internal control.

3.3. Pharmacological Characterization of GPCR-EGFR transactivation

In order to examine the molecular mechanisms underlying AT_1R-EGFR transactivation, we first examined ERK1/2 activation as an indirect readout. HEK293 cells expressing the AT_1R were stimulated with AngII, leading to the robust and reproducible activation of ERK1/2 (Fig. 4A). To confirm that ERK1/2 activation by AngII was due to EGFR transactivation, cells were pretreated with the EGFR kinase inhibitor AG1478 (Fig. 4, A and B), or the $G_{q/11}$ inhibitor YM-254890, the AT_1R inverse agonist candesartan, the src inhibitor PP2 or calcium chelator BAPTA (Fig. 4, C and D). Consistent with previous studies (34,40), AngII-induced ERK1/2 activation was attenuated by antagonism of the AT_1R, inhibition of the EGFR kinase as well as the inhibition of $G_{q/11}$ (Fig. 4, C and D). Furthermore, AT_1R-EGFR transactivation was blunted by pharmacological inhibition of calcium signaling and

src activity, however to a lesser extent. Taken together, EGFR transactivation (as measured by ERK1/2 activation) by the AT_1R appears to be a $G_{q/11}$-mediated pathway and dependent on EGFR kinase activity.

We next examined whether this pharmacological profile was recapitulated when the BRET based Grb2 recruitment assay was used as a readout for EGFR transactivation. Similar to AngII-mediated ERK1/2 activation, candesartan completely inhibited AngII-stimulated Grb2 recruitment to the EGFR, indicating that transactivation of the EGFR is due to ligand activation of the AT_1R (Fig. 4E). However, AngII-mediated recruitment of Grb2 to the EGFR was not inhibited by pre-treatment with the $G_{q/11}$ inhibitor, YM-254890, which is in stark contrast to the outcome of the ERK1/2-based readout mentioned above. Indeed, YM-254890 treatment led to a sustained BRET signal (Fig. 4E). Additionally, Grb2 recruitment to the EGFR in response to AngII was only partially dependent upon the EGFR kinase activity, as well as src and calcium (Fig. 4, E and F).

3.4. The role of $G_{q/11}$ and β-arrestin in AT_1R-EGFR transactivation

Given, the disparity in the apparent contribution of $G_{q/11}$ to EGFR transactivation, we next sought to interrogate a single point mutant of the AT_1R, $[Y215F]AT_1R$, previously reported to retain high affinity for AngII, but an inability to couple to $G_{q/11}$ mediated signaling (58) (Fig. 5A). Stimulation of the $[Y215F]AT_1R$ promoted Grb2 recruitment to the EGFR approximately equivalent to the wild type AT_1R (Fig. 5B). Similarly, a truncated mutant of the AT_1R, $[TK325]AT_1R$, lacking 34 carboxyl terminal amino acids and deficient in ligand-mediated phosphorylation and recruitment of β-arrestins (59,60) also showed similar levels of Grb2 recruitment as the wild type receptor.

To extend our observations that AT_1R-EGFR transactivation may be independent of $G_{q/11}$ and β-arrestin, we also performed EGFR-Grb2 BRET assays in CRISPR-mediated $G_{q/11}$ and β-arrestin knockout HEK293 cell lines (41). The BRET-based sensors were transfected with the wild type AT_1R into a HEK293 parental (PL) cell line, a $G_{q/11}$ knockout (ΔGq/11) cell line or a β-arrestin1/2 knockout (Δβ-arr1/2) cell line. Comparable AngII-induced EGFR-Grb2 BRET ratios were observed in all three cell lines (Fig. 5C). Rescue experiments (i.e., transfection separately of either G_q, G_{11} or a dominant negative G_q back into the ΔGq/11 cells); or β-arrestin1/2 (transfected separately or together) into the Δβ-arr1/2 cell line were also performed (Fig. 5, D and E). Restoration of $G_{q/11}$ proteins inhibited AngII-mediated transactivation, suggesting, if anything, that $G_{q/11}$ coupling disfavors AngII-mediated transactivation. Reintroduction of β-arrestin1 and β-arrestin2 into the Δβ-arr HEK 293 cells did not affect AngII-mediated EGFR transactivation. Together, these data confirm the independence of $G_{q/11}$ and β-arrestin for AT_1R-EGFR transactivation.

3.5. AT_1R and EGFR exist as complexes

We speculated as to whether the mechanism of GPCR-EGFR transactivation may involve heteromerization of the receptors. In order to study this, we initially used a biomolecular fluorescence complementation (BiFC) assay, whereby complexing between AT_1R and EGFR would result in the complementation of YFP fragments attached separately to the AT_1R (AT_1R-Vn) and the EGFR (EGFR-Vc) (Fig. 6A). AT_1R-Vn and EGFR-Vc were not

fluorescent when expressed separately in cells (Fig. 6, B and C). Co-expression leads to complemented fluorescence with clear membrane localization (Fig. 6, D-F).

To examine whether these complexes are ligand-dependent, we utilized Receptor-Heteromer Investigation Technology (Receptor-HIT), which is most often used on the BRET platform to offer insights into specific ligand-dependent recruitment of proteins to heteromers (61,62). AngII-induced recruitment of β-arrestin2-Venus to unlabeled AT_1R, resulting in BRET with EGFR-Rluc8, is indicative of heteromerization (Fig. 6G). Upon AngII stimulation, an increase in BRET ratio between β-arrestin2-Venus and EGFR-Rluc8 was observed only when the AT_1R was co-expressed (Fig. 6H). We also used a complementary variation of the Receptor-HIT assay, whereby AT_1R-Rluc8, unlabeled EGFR, and Grb2-Venus were co-expressed in living cells, as shown in Fig. 6I. In contrast to the EGFR, the AT_1R does not interact with Grb2 in a ligand-dependent manner. However, if AT_1R and EGFR exist as a heteromer, AngII stimulation will transactivate the EGFR and initiate translocation of Grb2 to the activated EGFR, facilitating BRET between AT_1R-Rluc8 and Grb2-Venus. Even in the absence of exogenous EGFR transfection, some AngII-mediated BRET was observed, indicating the engagement of endogenous EGFR to recruit Grb2-Venus to AT_1R-Rluc8 (Fig. 6J). This effect was enhanced by the ectopic expression of additional unlabeled EGFR (Fig. 6J).

As a final confirmation of ligand-dependent complexing between the AT_1R and the EGFR, we co-transfected cells with AT_1R-Rluc8 and EGFR-Venus, and stimulated with AngII or EGF. Both AngII and EGF appeared to modulate the proximity of the receptors, as shown in Fig. 6, K and L. Together, these experiments indicate that AT_1R and EGFR not only exist in preformed complexes, but that agonist stimulation can modulate their association and/or relative conformations.

AT_1R -EGFR heteromers are unlikely to merely reflect agonist-induced clustering of these proteins in the membrane and/or their concentration and co-trafficking in endosomes, because the association of the EGFR with a membrane marker, K-ras, was decreased following EGF-stimulation, but not AngII-treatment (Fig. 7).

3.6. Pharmacological characterization of AT_1R-EGFR complexes

Ligand-induced changes in proximity between AT_1R-Rluc8 and EGFR-Venus, following AngII- and EGF-stimulation, are shown in Fig. 6, M and N. AngII-mediated increases in BRET between AT_1R and EGFR were inhibited by candesartan, but not AG1478 or YM-254890, indicating that ligand-dependent modulation does not require $G_{q/11}$ coupling or EGFR kinase activity (Fig. 6M). In contrast, EGF modulation of AT_1R-EGFR complexes was abrogated by pretreatment with AG1478 (Fig. 6N). Interestingly, pretreatment with candesartan or YM-254890 further increased the EGF-mediated BRET between AT_1R-Rluc8 and EGFR-Venus (Fig. 6N). These data provide evidence for distinct mechanisms controlling AngII- and EGF-induced modulation of the heteromer.

4. Discussion

GPCR-mediated EGFR transactivation is commonly defined in terms of the activation of ERK1/2, which although informative, is an indirect and distal readout. Herein, we describe a BRET assay, based on the recruitment of Grb2 to the EGFR to quantitatively monitor, in living cells and in real-time, the proximal activation of EGFR. Importantly, we identified that the molecular requirements for EGFR transactivation differed depending on the readout used. For the Grb2-EGFR assay, we showed that EGFR transactivation is independent of $G_{q/11}$ coupling and does not apparently involve the AT_1R carboxy-terminal tail or interaction with arrestin. Using a series of additional BRET-based assays, we provide evidence of preformed complexes between the AT_1R and EGFR that show different attributes, depending on which receptor is activated. In summary, the capacity to interrogate proximal EGFR transactivation provides a platform to better define the complex molecular processes involved.

The development of novel approaches to study GPCR-mediated transactivation in live cells is important, and this study describes a unique BRET-based quantification of Grb2 recruitment to the EGFR as a direct readout of GPCR-mediated transactivation. We have previously used the recruitment of Grb2 to RTKs (EGFR and HER3) as a measure of EGF receptor family activation in HEK293 cells following stimulation with EGF and Heregulin (47). Moreover, others have also identified such BRET approaches as *bona fide* readouts of EGFR activation (63,64). In this study, we initially aimed to determine whether stimulation of the AT_1R could promote Grb2 translocation and interaction with EGFR in a ligand-dependent manner in live cells. AT_1R-mediated EGFR transactivation was readily demonstrable, but was consistently lower in magnitude compared to EGF-stimulation. The selective activation of EGFR by the AT_1R is consistent with many observations that the degree of signaling elicited by GPCRs appears to be only a subset of that obtained following full activation by EGF ligands (27,35,65,66). EGFR transactivation was readily detected in HEK293 cells, which is interesting because a previous study reported that ERK1/2 activation was independent of the EGFR in these cells (65). EGFR-Grb2 recruitment was also recapitulated in other standard cell lines (CHO-K1 and NIH-3T3) and in primary isolated VSMCs, indicating the versatility of this approach to measure EGFR transactivation.

We observed that the AT_1R and $V_{1b}R$ showed consistent recruitment of Grb2 to the EGFR, but we were surprised that other GPCRs (previously reported to cause EGFR transactivation (67-70)) did not. It is important to note that a negative response in our assay does not necessarily mean that the GPCR is not transactivating nor should it call into question the veracity of our assay. There are a number of plausible explanations: firstly, a trivial argument might be that these 'non-transactivating' GPCRs were non-functional – however, we confirmed their activation by using a readout of ligand-dependent β-arrestin trafficking. Secondly, these GPCRs may not be recruiting Grb2 as part of their activation process or, alternatively, there is an association but it is different in nature to that generated by the AT_1R and $V_{1b}R$. Indeed, for a number of the GPCRs that did not show enhanced EGFR-Grb2 association, we actually observed decreased BRET ratios following ligand stimulation. It could be reasonably argued that this reflects dynamic changes in an equilibrium between association, dissociation and reconfiguration of putative EGFR-Grb2 complexes – where

the balance may favor dissociation or reconfiguration that leads to an increased distance between the donor and acceptor moieties. Thirdly, as our assay only reports on the Grb2 recruitment to the EGFR, the possibility exists that receptors other than the EGFR (HER2, HER3 or HER4) and or combinations of dimers between those receptors, are selectively used by these other GPCRs for transactivation. In this regard, it is important to acknowledge that most studies of EGFR transactivation typically use end-point assays, where specificity is inferred via inhibition by the small molecule inhibitor of the EGFR kinase, AG1478. However, high micromolar concentrations of AG1478 (as commonly used) inhibit receptors other than the EGFR (71,72). Importantly, we report the recruitment of Grb2 to HER2, but not HER3, in response to AngII stimulation. Whether a specific GPCR is able to transactivate EGFR, HER2, HER3 or HER4 will require further investigation, using the approaches we have developed. It will be important to consider that HER2 is the preferred dimerization partner for other EGFR family members, and therefore potentially acting in *cis* to modulate other HER receptors. Indeed, HER2 has been shown to be transactivated and/or modulate transactivation (73,74).

Another major observation of our study was the apparent disconnect between pharmacological inhibitors and their relative effect on the readout of transactivation vis-à-vis ERK1/2 *versus* BRET-based Grb2 recruitment assays. We clearly showed that candesartan completely inhibited AngII-induced ERK1/2 phosphorylation and recruitment of Grb2 to the EGFR, indicating that EGFR transactivation is due to ligand activation of the AT_1R in both systems. Interestingly, cells treated with AG1478 showed a complete inhibition of ERK1/2 activation in response to AngII, however only a partial reduction of AngII-mediated transactivation as measured by BRET-based Grb2 recruitment assay, indicating a possible disconnect between proximal recruitment of Grb2 and the eventual activation of ERK1/2. We would argue that Grb2 recruitment is necessary, but not in itself sufficient, to drive ERK1/2 activation following AT_1R activation. Importantly, EGF-mediated recruitment of Grb2 to the EGFR was completely inhibited with pretreatment of AG1478, confirming the efficacy of AG1478.

Even more unambiguous was the relative contribution of $G_{q/11}$ signaling in AngII-mediated transactivation as measured by the ERK1/2 and the BRET-based readout. Although $G_{q/11}$ was absolutely required for ERK1/2 phosphorylation following AngII-stimulation, in contrast, we observed a sustained EGFR-Grb2 interaction when cells were pretreated with YM-254890. One potential confounding issue was that different concentrations of ligands were used to achieve maximal activation in the ERK1/2 and BRET assays (refer to the legend of Figure 4 for detail) and this may have influenced the pharmacological inhibitory profile. However, we observed similar patterns of inhibition of the ERK1/2 readout when cells were stimulated with higher concentration (i.e., 10 μM AngII and 1 μM EGF, data not shown), indicating the differential contribution of $G_{q/11}$ is unlikely to be a mere artefact of ligand concentration.

Previously, it was assumed that transactivation was a consequence of $G_{q/11}$ activation (75), but our data using the proximal assay suggests a re-evaluation of this paradigm might be required. We further adapted the BRET experiments to incorporate mutant versions of the AT_1R, specifically a G protein-uncoupled AT_1R, [Y215F]AT_1R, that does not couple

efficiently to $G_{q/11}$ (20,58). Consistent with previous results, activation of [Y215F]AT_1R resulted in wild type-like Grb2 recruitment to the EGFR. Interestingly, others have reported β-arrestin-mediated ERK1/2 signaling, with an involvement in cardiomyocyte survival and hypertrophy (76-78). Here, we used a carboxy-terminal truncated AT_1R, [TK325]AT_1R, to demonstrate that AT_1R C-tail phosphorylation and β-arrestin interaction is not crucial for Grb2 recruitment. Furthermore, using CRISPR knockout HEK293 cell lines we were also able to show that transactivation (indicated by Grb2 recruitment to the EGFR) appears to be $G_{q/11}$- as well as β-arrestin1/2-independent. This disparity warrants further study and clearly emphasizes the importance of interrogating EGFR transactivation directly at the most proximal point in the process. It is possible that other G proteins might be involved or that the cell background is critical, and this requires further study.

Based on the above observations, a final consideration for the current study was the possibility that GPCR-mediated EGFR transactivation involved receptor heteromerization (79). We initially used a BiFC assay to provide preliminary evidence for heteromers between the AT_1R and EGFR. Although positive, the limitations of this technique in defining ligand-driven heteromer formation meant that we then progressed to Receptor-HIT assays and a BRET-based assay measuring direct association of AT_1R-EGFR. Using all three approaches, we provide evidence that AT_1R and EGFR can exist as preformed complexes that occur constitutively (i.e. in the absence of ligand) and are predominantly localized to the plasma membrane. In addition to preformed complexes, AT_1R-EGFR heteromers appear to be responsive to agonist stimulation and receptor activation. Ligand-induced modulation of the AT_1R-EGFR heteromer was validated using two complementary Receptor-HIT approaches, with both demonstrating that AngII leads to a rapid and robust increase in BRET that required both the AT_1R and EGFR. Lastly, we demonstrate a more 'direct' readout, tagging both receptors and monitoring ligand-induced changes in BRET due to modulation of donor-acceptor proximity. Together, these data strongly indicate that both AngII- and EGF-stimulation can induce the formation of multi-receptor complexes and/or alter the relative conformations of AT_1R and EGFR in pre-existing complexes. An alternative explanation (that these data merely reflect a bystander effect or the co-accumulation of the receptors in endosomes) is not supported by our observation (see Fig. 7) that the trafficking of the EGFR from the membrane occurs with EGF stimulation, but not with AngII.

The concept of GPCRs and EGFRs existing as heteromers is consistent with previous studies. Co-immunoprecipitation studies have shown that agonist stimulation (both AngII and EGF) promotes a multi-protein complex containing the AT_1R and EGFR, however these endpoint assays were not in live cells or in real-time (79). The $β_1$- and $β_2$-adrenergic receptors associate with the EGFR (66,80), and while isoprenaline enhances $β_1$-adrenergic receptor association with EGFR, this is disrupted by EGF-stimulation. Others have also used BRET to provide evidence for the bile acid receptor, TGR5, forming preassembled heterodimers with the EGFR in lipid rafts, however agonist stimulation showed no further enhancement in receptor association (81). Complementary to our studies, Bhattacharya and colleagues showed G protein-coupled receptor 54 interacts with EGFR at the plasma membrane in the absence of agonist. However, only stimulation with its endogenous ligand, kisspeptin, significantly increased their association, while EGF-treatment did not affect the receptor complex (82). Although it appears that many GPCRs can transactivate

EGFRs, heteromerization has only been indicated for a subset. It is still unknown how this interaction affects signaling of either receptor, with the mechanism and/or binding domains that facilitate heteromer formation largely unknown. One possible mechanism that has not been tested directly is the reported contribution of reactive oxygen species (83,84) and their potential oxidation of the EGFR on dimers formation (85).

Whether the complex subserves actual transactivation is difficult to reconcile because specific inhibitors that prevent complex formation are not available. Remarkably, we observed that the modulation of BRET signals associated with AT_1R-EGFR heteromerization differed depending on whether the complex was modulated by activation of the AT_1R or the EGFR. The AngII-induced complex modulation is apparently independent of both $G_{q/11}$ and EGFR kinase activity. G protein-independent transactivation has been reported for β_1-adrenergic receptor and EGFR continued association (66). In contrast, in our hands, EGF-mediated modulation of the AT_1R-EGFR complex was absolutely dependent on the EGFR kinase domain. Moreover, antagonism of the AT_1R or inhibition of $G_{q/11}$ did not inhibit complex modulation, but rather enhanced the EGF-mediated increase in BRET signal between AT_1R-Rluc8 and EGFR-Venus. Whether this enhancement plays any role in receptor activation is unclear, but it is interesting to note that traditional models of monomeric EGFR dimer formation as the active component have been superseded by models that suggest that up to 40% of EGFRs are present as inactive dimers that form higher order active receptor signaling complexes (86-89). This negative modulation of EGFR may be mirrored by the AT_1R, whereby the AT_1R and its associated G protein negatively modulate monomeric/dimeric/oligomeric EGFR.

In summary, the combination of different BRET approaches emphasizes the importance of monitoring the most proximal events of EGFR activation, in live cells and in real-time. Using this approach, we propose a $G_{q/11}$-independent mechanism of EGFR recruitment, instead providing evidence of ligand-modulated AT_1R-EGFR complexes. This work provides a platform to better interrogate the molecular mechanisms underpinning AT_1R-EGFR transactivation, and provides powerful new insights into this underappreciated process.

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Competing Interests

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Abbreviations

AngII	Angiotensin II
AT$_1$R	type 1 angiotensin II receptor
EGFR/HER1/ErbB1	epidermal growth factor receptor
BRET	bioluminescence resonance energy transfer
Grb2	growth factor receptor-bound protein 2
EGF	epidermal growth factor
GPCR	G protein-coupled receptor
HER2/ErbB2	Human epidermal growth factor receptor 2
HER3/ErbB3	Human epidermal growth factor receptor 3
HER4/ErbB4	Human epidermal growth factor receptor 4
RAS	renin angiotensin system
ROS	reactive oxygen species
MAPK	mitogen-activated protein kinases
ERK1/2	extracellular-regulated kinase 1/2
RTK	receptor tyrosine kinase
MMP	matrix metalloproteases
ADAM	a disintegrin and metalloprotease
RET	resonance energy transfer
FlAsH	fluorescein arsenical hairpin
Rluc	Renilla luciferase
VSMC	vascular smooth muscle cells
V$_{1b}$R	vasopressin 1b receptor
PL	parental
BiFC	biomolecular fluorescence complementation
Receptor-HIT	receptor-heteromer investigation technology
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HBSS	Hanks' balanced salt solution

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
PVDF	polyvinylidene fluoride
DN	dominant-negative
AUC	area under the curve

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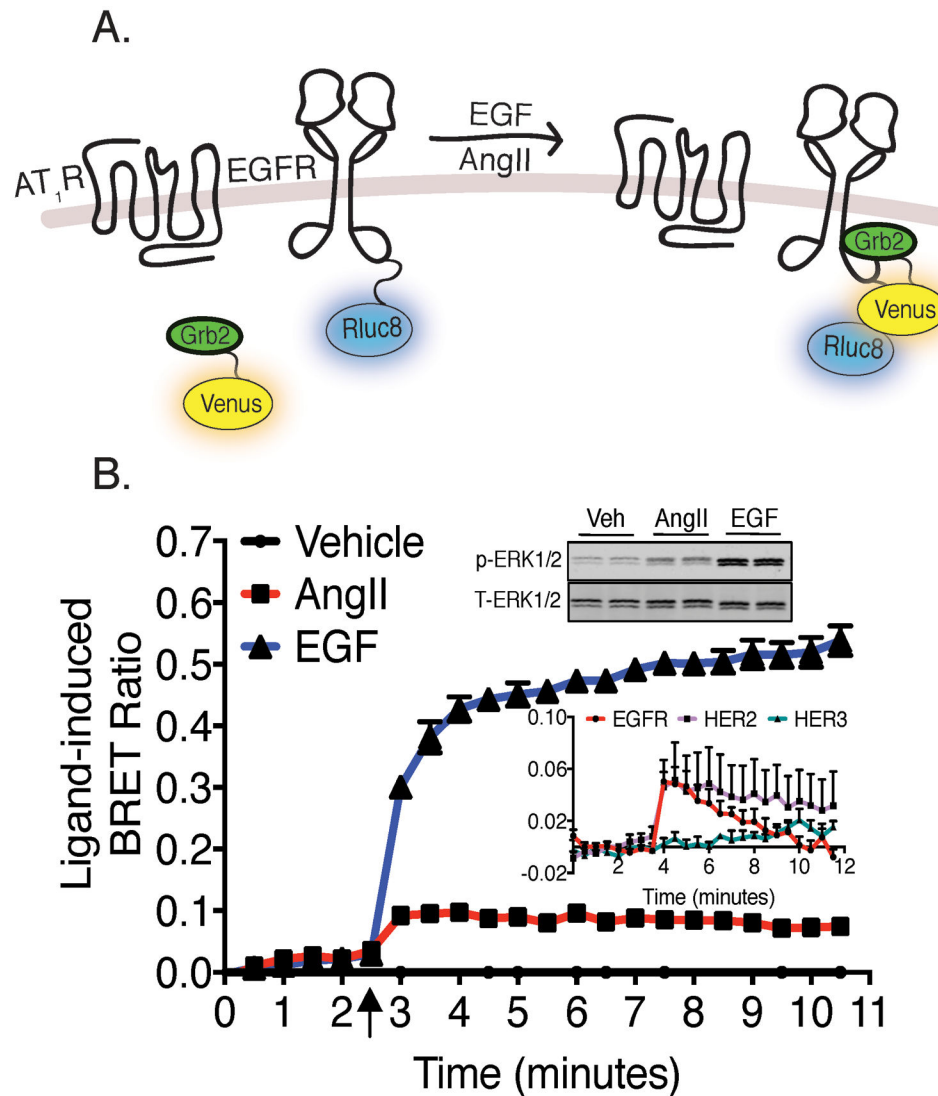
Fig. 1. Development of a BRET assay to study AT$_1$R-mediated EGFR transactivation.
(**A**) Schematic of AT$_1$R-EGFR BRET-based transactivation. EGFR fused to a BRET donor (Rluc8), is co-transfected with Grb2 adaptor protein tagged with a BRET acceptor (Venus) and the AT$_1$R. Stimulation of the AT$_1$R promotes activation of the EGFR and recruitment of Grb2. (**B**) HEK293 cells expressing AT$_1$R, EGFR-Rluc8, and Grb2-Venus were treated with 10μM AngII, 1μM EGF or vehicle. Quantification of ligand-induced BRET ratio (maximum-minimum) between EGFR-Rluc8 and Grb2-Venus following AngII- and EGF-stimulation. Insert is HEK293 cells (stably expressing AT$_1$R) stimulated with 100nM AngII, 10nM EGF or vehicle for 5 minutes before processing for phospho-ERK1/2:total-ERK1/2 (p-ERK:T-ERK) western blots. Blots are representative of 3 independent experiments (**B inset**) Cells expressing AT$_1$R, Grb2-Venus and either EGFR-Rluc8, HER2-Rluc8 or HER3-Rluc8 and stimulated with 10μM AngII. Agonist stimulation is indicated by arrow. Data represent mean ± SEM of 3 independent experiments.

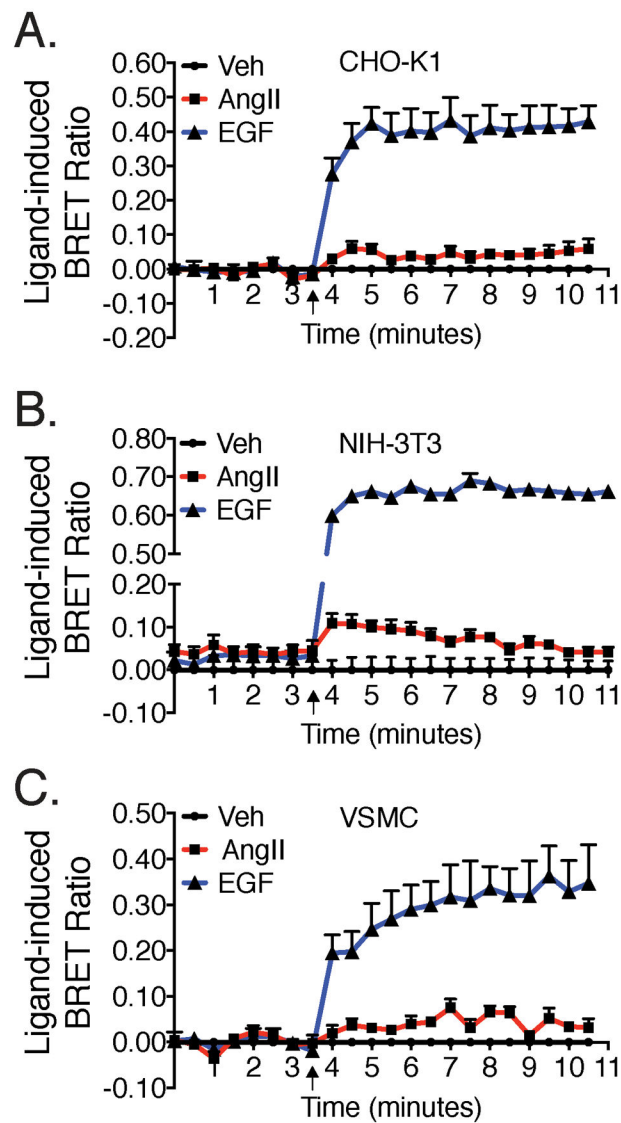**Fig. 2. BRET-based transactivation occurs in a variety of cell lines.**

AT_1R, EGFR-Rluc8 and Grb2-Venus were co-transfected into **(A)** CHO-K1 cells, **(B)** NIH-3T3 cells, and **(C)** primary isolated VSMCs and treated with 10μM AngII, 1μM EGF or vehicle. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3 independent experiments.
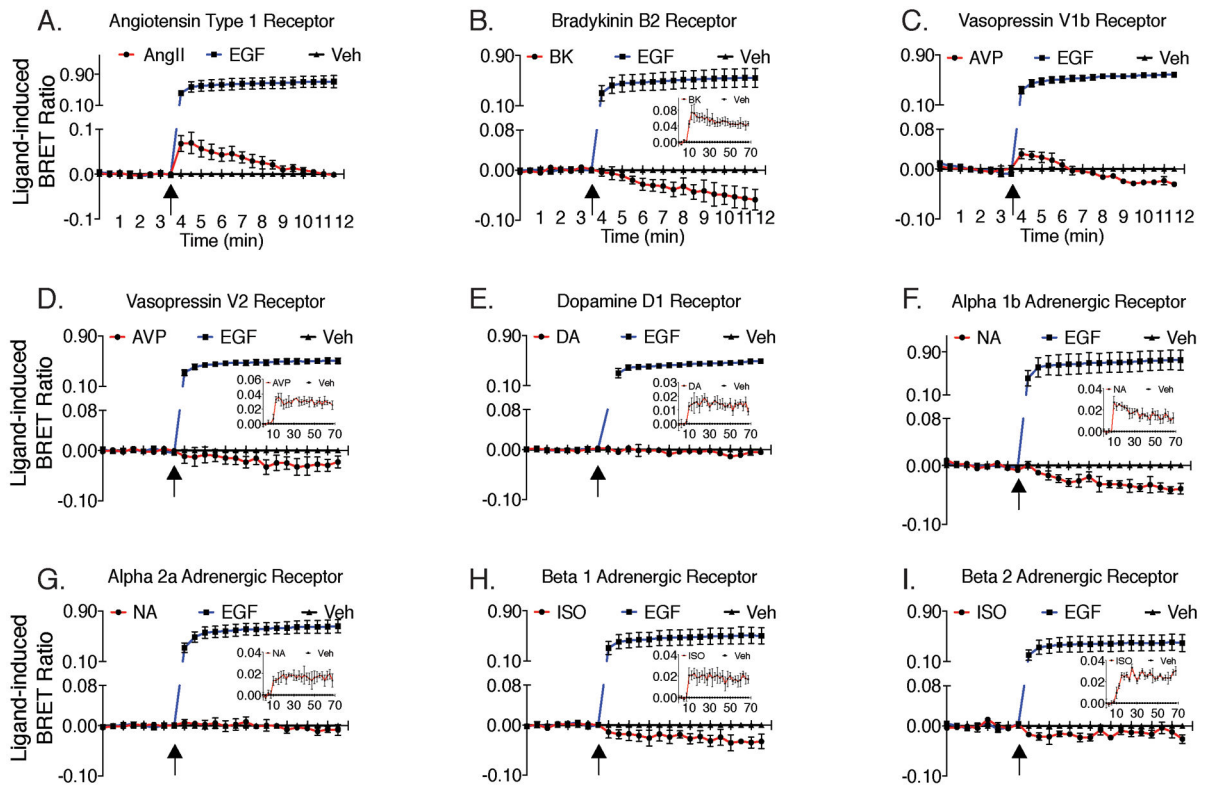
Fig. 3. Screen of multiple GPCRs using the BRET-based transactivation assay.
As per the title of each graph, panels **(A-I)** show 9 different GPCRs expressed in
HEK293FT cells and their ability to induce Grb2-Venus interaction with EGFR-Rluc8
following 1μM EGF or 10μM GPCR agonist stimulation. **(Inset)** shows each GPCR
and their ability to induce β-arrestin2-Rluc8 proximity to plasma membrane marker K-ras-
Venus, following 10μM GPCR agonist-stimulation. Agonist stimulation is indicated by
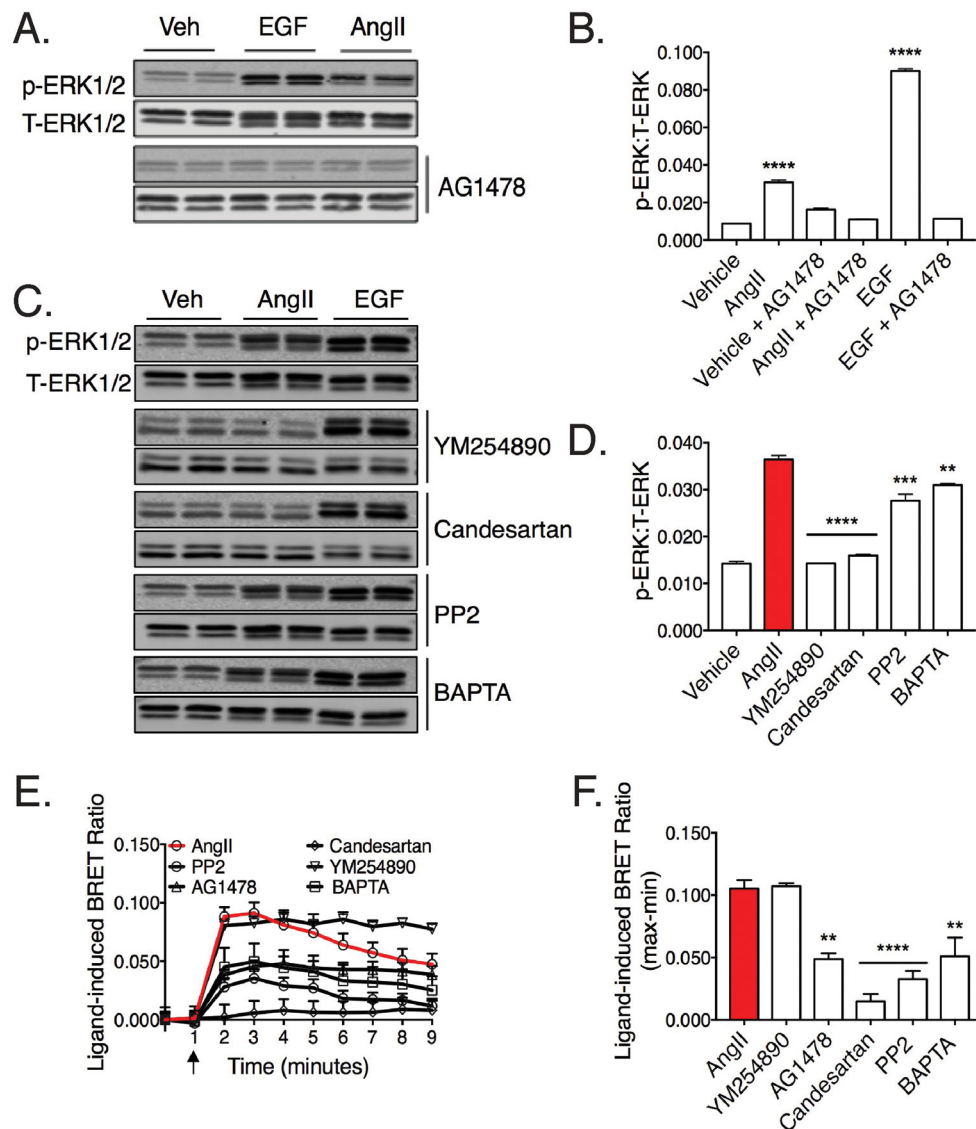arrows. Data represent mean ± SEM of 3-5 independent experiments.

Fig. 4. Analysis of AT$_1$R-EGR transactivation when comparing ERK1/2 phosphorylation and Grb2 recruitment to the EGFR.

(A-B) HEK293 cells stably expressing AT$_1$R were treated with 500nM AG1478 (EGFR inhibitor) for 30 minutes prior to stimulation with 100nM AngII, 10nM EGF or vehicle for 5 minutes before processing for phospho-ERK1/2:total-ERK1/2 (p-ERK:T-ERK) western blots. Blots are representative of 3 independent experiments. (B) Quantification of western blot data using densitometry analysis (ImageJ) illustrates p-ERK1/2 changes. **** $p < 0.001$ vs. vehicle.. (C-D) HEK293 stably expressing AT$_1$R were treated with 1μM YM-254890 (G$_{q/11}$ inhibitor), 1μM Candesartan (AT$_1$R inverse agonist), 10μM BAPTA (Calcium chelator) and 5μM PP2 (src inhibitor) for 30 minutes prior to stimulation with 100nM AngII, 10nM EGF or vehicle for 5 minutes before processing for ERK/p-ERK western blots. Blots are representative of 3 independent experiments. (D) Quantification of western blot data using densitometry analysis (ImageJ) illustrates phospho-ERK1/2 changes. **** $p < 0.001$, *** $p < 0.005$, ** $p < 0.01$ vs. AngII treated cells. (E-F) HEK293 cells transiently expressing

AT$_1$R, EGFR-Rluc8 and Grb2-Venus were treated with 1μM AG1478, 1μM YM-254890, 1μM Candesartan, 10μM BAPTA and 5μM PP2 for 30 minutes prior to stimulation with 10μM AngII, 1μM EGF or vehicle. **(F)** Quantification of ligand-induced BRET ratio (maximum-minimum) between EGFR-Rluc8 and Grb2-Venus following AngII-treatment and in the presence of inhibitors. Agonist stimulation is indicated by arrows. Data represents mean ± SEM of 3 independent experiments. Statistical analysis by a one-way ANOVA with a Dunnett's post-test for multiple comparisons; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$ vs. AngII treated cells.
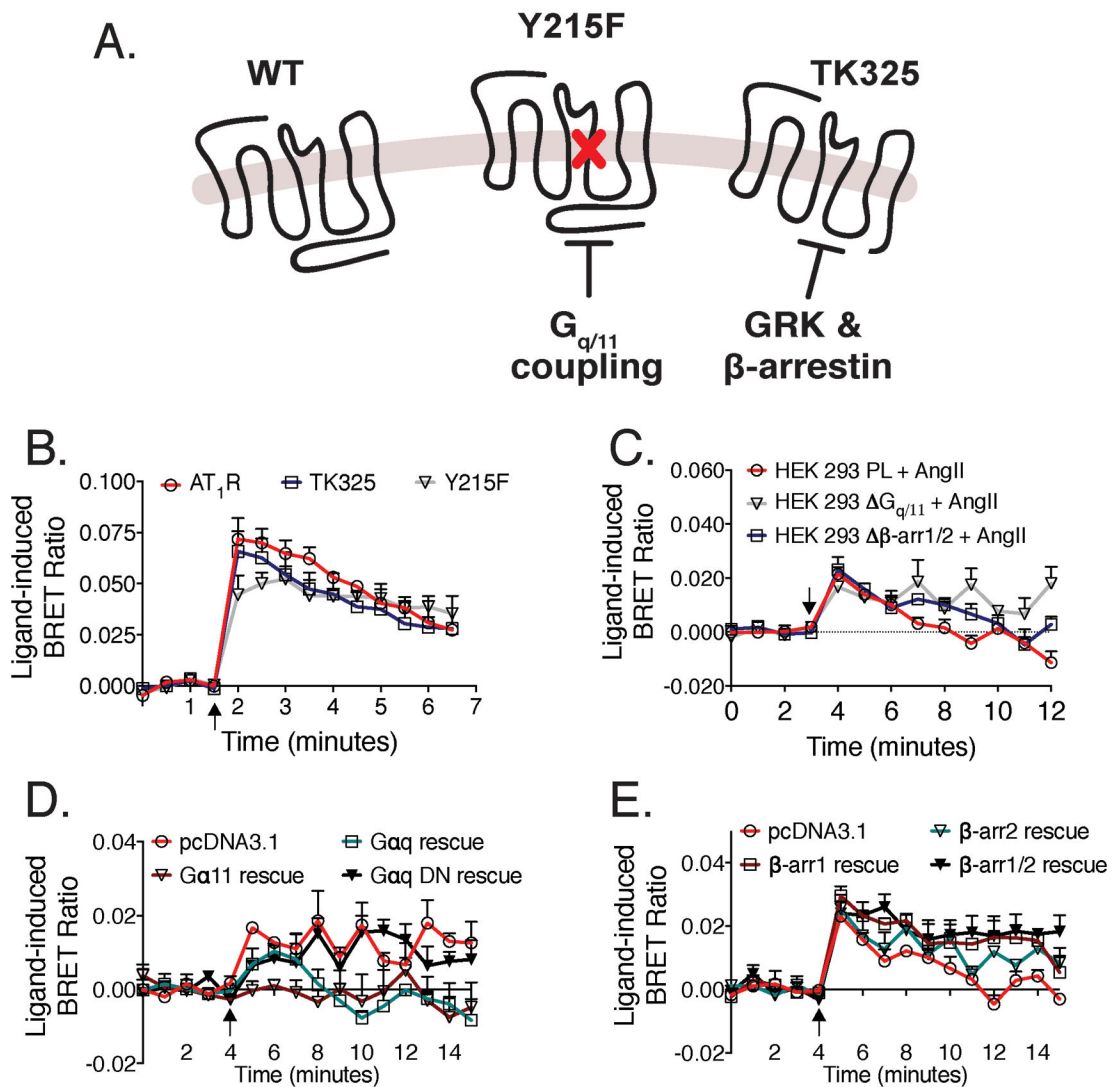
Fig. 5. Dependence on β-arrestin and $G_{q/11}$ in AngII-mediated Grb2 recruitment to the EGFR.
(A) Schematic of wild type AT_1R and mutant AT_1Rs - [TK325]AT_1R (truncated AT_1R deficient in recruiting β-arrestins) and [Y215F]AT_1R (inability to couple to $G_{q/11}$ signaling pathways). **(B)** HEK293 cells expressing EGFR-Rluc8 and Grb2-Venus, alongside wild type AT_1R, [Y215F]AT_1R or [TK325]AT_1R, were treated with 10μM AngII, 1μM EGF or vehicle. **(C)** EGFR-Rluc8, Grb2-Venus and AT_1R were transfected into the HEK293 parental cell line, as well as $G_{q/11}$- and β-arrestin1/2-knockout CRISPR cell lines. **(D)** BRET constructs were expressed in the absence (pcDNA3.1) or presence of either G_{11}, G_q or G_q dominant-negative (DN) and were treated with 10μM AngII. **(E)** BRET constructs were expressed in the absence (pcDNA3.1) or presence of either β-arrestin1, β-arrestin2 or β-arrestin1/2 and were treated with 10μM AngII. All cell lines were treated with 10μM AngII. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3 independent experiments.
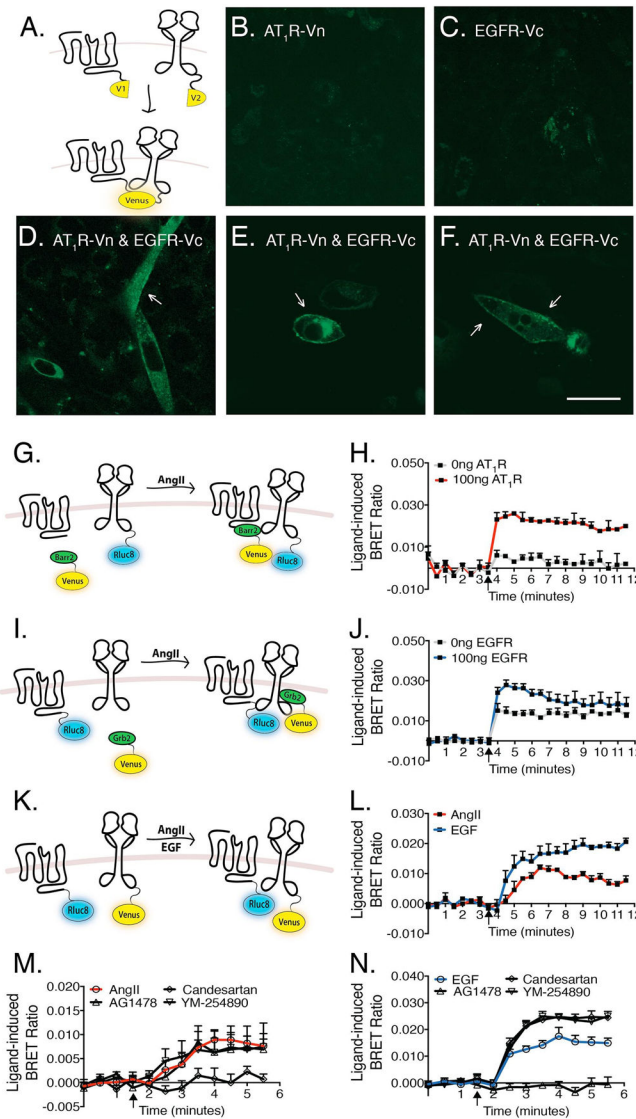
Fig. 6. Detection of AT₁R and EGFR receptor complexes using BiFC and Receptor-HIT.
(A) The Venus fluorophore is split into N-terminal (Vn) and C-terminal (Vc) fragments and genetically fused to AT₁R and EGFR, respectively. Therefore, if AT₁R and EGFR that bear C-terminal Vn and Vc tags form heteromers, fluorescence will be generated via the reconstitution of the Venus fluorophore. **(B, C)** CHO-K1 cells expressing AT1R-Vn or EGFR-Vc constructs separately displayed very low levels of autofluorescence. **(D-F)** Co-expression of AT₁R-Vn and EGFR-Vc in CHO-K1 cells. Scale bar, 30μm. **(G)** If the AT₁R and EGFR exist as a heteromers, AngII-stimulation will activate β-arrestin2 translocation to the AT₁R, enabling BRET to occur between EGFR-Rluc8 and β-arrestin2-Venus. **(H)** HEK293 cells expressing β-arrestin2-Venus and EGFR-Rluc8 in the presence or absence of AT₁R were stimulated with 10μM AngII and the BRET ratio determined. **(I)** In contrast to EGFR, the AT₁R does not interact with Grb2 in a ligand-dependent manner. However, if the AT₁R and EGFR exist as a heteromers, AngII stimulation resulting in transactivation of the EGFR and initiation of Grb2 translocation to the activated EGFR should enable BRET

to occur between AT_1R-Rluc8 and Grb2-Venus. **(J)** HEK293 cells expressing AT_1R-Rluc8 and Grb2-Venus in the presence or absence of transfected EGFR, was stimulated with 10μM AngII. **(K)** A direct readout of ligand-induced changes in AT_1R-Rluc8 proximity to EGFR-Venus. **(L)** Co-expression of AT_1R-Rluc8 and EGFR-Venus in HEK293 cells stimulated with 10μM of AngII or 1μM EGF. **(M-N)** HEK293 cells expressing AT_1R-Rluc8 and EGFR-Venus were treated with 1μM YM-254890, 1μM Candesartan or 1μM AG1478 for 30 minutes prior to stimulation with **(M)** 10μM AngII only or **(N)** 1μM EGF only. Agonist stimulation is indicated by arrows. Data represent mean ± SEM of 3-4 independent experiments.
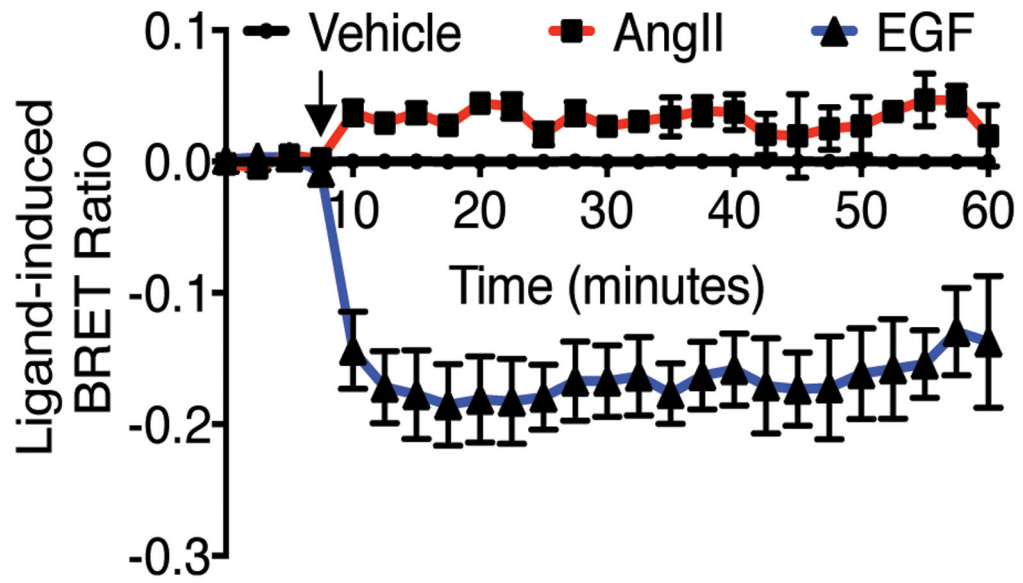
Fig. 7: EGFR localization and trafficking

HEK293 cells expressing AT_1R, EGFR-Rluc8 and K-ras-Venus were treated with 10μM AngII, 1μM EGF and vehicle. Agonist stimulation is indicated by arrows. Data represents mean ± SEM of 5 independent experiments.