

Angiotensin II Type I and Prostaglandin F₂α Receptors Cooperatively Modulate Signaling in Vascular Smooth Muscle Cells*

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Background: Evidence suggests that FP modulates AT1R physiological responses.

Results: In heterologous and native systems, both receptors allosterically modulated each other's function.

Conclusion: This is likely via the agency of an AT1R/FP heterodimer.

Significance: This may have implications in hypertension management.

The angiotensin II type I (AT1R) and the prostaglandin F₂α (PGF₂α) F prostanoid (FP) receptors are both potent regulators of blood pressure. Physiological interplay between AT1R and FP has been described. Abdominal aortic ring contraction experiments revealed that PGF₂α-dependent activation of FP potentiated angiotensin II-induced contraction, whereas FP antagonists had the opposite effect. Similarly, PGF₂α-mediated vasoconstriction was symmetrically regulated by co-treatment with AT1R agonist and antagonist. The underlying canonical G_{α_q} signaling via production of inositol phosphates mediated by each receptor was also regulated by antagonists for the other receptor. However, binding to their respective agonists, regulation of receptor-mediated MAPK activation and vascular smooth muscle cell growth were differentially or asymmetrically regulated depending on how each of the two receptors were occupied by either agonist or antagonist. Physical interactions between these receptors have never been reported, and here we show that AT1R and FP form heterodimeric complexes in both HEK 293 and vascular smooth muscle cells. These findings imply that formation of the AT1R/FP dimer creates a novel allosteric signaling unit that shows symmetrical and asymmetrical signaling behavior, depending on the outcome measured.

AT1R/FP dimers may thus be important in the regulation of blood pressure.

The receptor for prostaglandin F₂α (PGF₂α,⁶ FP), has been implicated in regulation of complex physiological events, including labor (1, 2), ocular pressure homeostasis (3), smooth muscle contraction, and vascular tone (4). Several groups (including us) have shown that FP activates ERK1/2 in HEK 293 cells via the G_{α_q}-PKC pathway (5–7) and that this response was modulated by both biased allosteric and orthosteric ligands (5, 8). FP has been shown to be involved in blood pressure regulation by the angiotensin II (Ang II) type 1 receptor (AT1R) (9). AT1R plays a critical role in vascular remodeling and is part of the renin-angiotensin system, a key regulator of blood pressure, electrolyte balance, and numerous neuronal and endocrine actions associated with cardiovascular function. Moreover, FP and AT1R are both expressed in vascular smooth muscle cells and regulate signaling events in these cells (10–12), suggesting the possibility of interplay between these receptors and their ensuing cellular responses.

EXPERIMENTAL PROCEDURES

Materials—[³H]PGF₂α, ¹²⁵I-carrier-free radionucleotide, [³H]thymidine, [³H]leucine, and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences. PGF₂α and AL-8810 were from Cayman Chemical (Ann Arbor, MI). Ang II, mouse and rabbit anti-FLAG antibodies, *N*-ethylmaleimide, and poly-L-ornithine hydrobromide were from Sigma. Mouse monoclonal anti-phospho-ERK1/2 (Thr-202/Tyr-204) and rabbit polyclonal anti-total ERK1/2 antibodies were from Cell Signaling (Danvers, MA). Minimum Eagle's medium and

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⁶ The abbreviations used are: PGF₂α, prostaglandin F₂α; AT1R, angiotensin II type 1 receptor; Ang II, angiotensin II; FP, prostanoid F₂α receptor; GPCR, G protein-coupled receptor; VSMC, vascular smooth muscle cell; IP₁, inositol 1-phosphate; BRET, bioluminescence resonance energy transfer; EGFR, EGF receptor; β-Arr2, β-arrestin2; mRFP, monomeric red fluorescent protein.

Allosteric Interactions in AT1R/FP Heterodimers

DMEM were from Hyclone (Logan, UT). Fetal bovine serum (FBS), L-glutamine, Lipofectamine, and gentamicin were from Invitrogen. G418 and puromycin were from Invivogen (San Diego). Phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin were from Bioshop (Burlington, Canada). Bovine serum albumin (BSA) fraction V and AG1478 were from EMD Chemicals Inc. (Gibbstown, NJ). AS604872 (Merck Serono) was synthesized at L'Institut de Recherche en Immunologie et Cancérologie (Université de Montréal, Montréal, Canada) and by Zamboni Chemical Solutions (McGill University, Montreal, Canada). The IP-One homogeneous time-resolved fluorescence assay kit was from CisBio. The UV lamp was from Thermo-Fisher (365 nm, black-ray long wave, model B100AP lamp).

Cell Lines—Primary rat aortic VSMCs were a gift from Dr. Marc Servant (Université de Montréal, Canada) and were grown in DMEM/high glucose supplemented with sodium pyruvate, 10% FBS (v/v), and gentamycin. All experiments were conducted on cells at passages 9–16. Stable HEK 293 cell lines expressing the human FP (FP cells), human AT1R (AT1R cells), or both receptors together (FP/AT1R cells) were generated using pIRES-HA-hFP and/or pcDNA3-FLAG-hAT1R. Stable clones were selected in 0.7 $\mu\text{g/ml}$ puromycin (FP) or 100 $\mu\text{g/ml}$ G418 (AT1R). All cell lines were grown at 37 °C in 5% CO₂. As required, cells were transfected using either standard calcium phosphate co-precipitation (8) or Lipofectamine as per the manufacturer's instructions.

Constructs—pIRES-HA-hFP construct (5) and pcDNA3.1- β -Arr2-mRFP (13) were used as described previously. pcDNA3-FLAG-AT1R construct was made from human AT1R, containing a signal peptide that was amplified by PCR from pRCMV-FLAG-hAT1R using forward primer 5'-ACAAGCTTATGAA-CACGATCATCGCCCTG-3' and reverse primer 5'-GTTCT-AGATCACTCAACCTCAAAACA-3', and was cloned into pcDNA3 using HindIII/XbaI restriction sites in 5' and 3', respectively. The AT1R-YFP construct was made from pcDNA3.1/Zeo(+)-HA-AT1R, amplified by PCR using forward primer 5'-ACCCAGAAGCTTAAAATGGCCCTTAAC-3' and reverse primer 5'-TACCGTCGACTCCACCTCAAAA-CAAGACGCAGG-3'. The PCR fragment was digested with HindIII/SalI and inserted in pEYFP-N1 digested with the same enzymes. GABA-B2-YFP was a generous gift from the laboratory of Dr. Michel Bouvier (Université de Montréal, Canada). To generate the FP-RLuc construct, the coding sequence for human FP was amplified by PCR using pcDNA3.1(+)-hFP (UMR cDNA Resource Center) as a template and the following primers: forward, 5'-GGACCTGTGCGGATCCTCCATGAA-CAATTCCAAACAGC-3' and reverse, 5'-CGCCACCTCCG-GATCCGGTGCTTGCTGATTTCTCTG-3'. Using the In-Fusion cloning system (Clontech), the PCR product obtained was inserted into pIRES-hyg3-cMyc-RLuc vector linearized with BamHI. pcDNA3.1/Zeo(+)-hFP-Venus1 or Venus2 human FP was amplified by PCR from pIRES-HA-FP (5), using forward primer 5'-CAGCACAGTGGCGCCGCCACCATGTCCAT-GAACAATTCCA-3' and reverse primer 5'-GCCACCGCCA-CCATCGATGGTGCTTGCTGATTTCTCTG-3'. The PCR was digested with NotI and ClaI and inserted in the pcDNA3.1/Zeo(+)-hVenus1 or Venus2 vectors, digested with the same

enzymes. pcDNA3.1(+)-AT1R-Venus1 was used as described previously (14).

Peptide Radiolabeling—¹²⁵I-Ang II and [¹²⁵I-Sar¹,Bpa⁸]Ang II were labeled as described previously (15).

Ligand Binding Experiments—HEK 293 cells were plated in poly-L-ornithine-coated 24-well plates at a density of 100,000 cells per well. After 24–48 h, cells were washed once with ice-cold PBS, and binding buffer was added (50 mM Tris, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 0.2% (g/ml) BSA) in the presence of 100,000 cpm of either ¹²⁵I-Ang II or [³H]PGF2 α and increasing concentrations of cold ligand (Ang II or PGF2 α , in half-log increments) in a final volume of 0.5 ml. Nonspecific binding was assessed in the presence of 10 μM cold Ang II (AT₁R) or PGF2 α (FP). To assess allosteric interactions, 1 μM PGF2 α or Ang II or 2 μM L158,809 or AS604872 was used in parallel with both radioligand and cold ligand. Binding was performed overnight at 4 °C. Cells were then washed three times with ice-cold PBS, detached with 0.2 M NaOH for 10 min at room temperature, and solubilized in immunoprecipitation buffer for protein quantification for 10 min at room temperature. Radioactivity was quantified using a γ -counter (¹²⁵I), or lysates were added to 5 ml of scintillation liquid and counted in a β -counter (³H).

Binding Dissociation Kinetics—Transfected HEK 293 cells were washed once with PBS, detached with PBS/EDTA (5 mM) for 5 min at 37 °C, and centrifuged (500 \times g for 5 min). The supernatant was removed, and the cell pellet was homogeneously resuspended in complete binding buffer (50 mM Tris, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 0.2% (g/ml) BSA, with 1 \times protease inhibitor) at a concentration of 50 μg of protein/300 μl . For a single experiment, 24 tubes each containing 50 μg of cells and 100,000 cpm of [³H]PGF2 α or ¹²⁵I-Ang II (20% receptor occupancy) in a final volume of 400 μl were equilibrated for 1.5 h at room temperature. [³H]PGF2 α or ¹²⁵I-Ang II dissociation was initiated by adding 100 μl of 125 μM Ang II or PGF2 α , respectively, resulting in a final concentration of 25 μM cold ligand in 500 μl of complete binding buffer. Binding was quenched at time 0, 1, 2.5, 5, 15, and 30 min (each in triplicate) following the addition of cold ligand by diluting the cells with 3 ml of cold binding buffer and then immediately filtering. Filters were washed twice with 3 ml of cold binding buffer. Total and nonspecific binding (each in triplicate) were assessed by equilibrating the cells (50 μg) with 100,000 cpm of hot ligand in 500 μl for 2 h in the absence or presence of 25 μM cold ligand, respectively. Radioactive signal was counted using either a γ -counter (¹²⁵I) or with scintillation liquid in a β -counter (³H).

Photoaffinity Labeling—AT1R, AT1R/FP, or VSMCs were photolabeled as described previously (16, 17). Briefly, cells were incubated at room temperature for 90 min with 1 nM [¹²⁵I-Sar¹,Bpa⁸]Ang II in 500 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, and 2 mg/ml BSA) with or without cold Ang II (1 μM). After three washes, cells were resuspended in binding buffer and irradiated with UV light for 30 min at 0 °C. Cells were resuspended in lysis buffer (50 mM HEPES, 50 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol) containing 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g/ml}$ leupeptin, 2.5 $\mu\text{g/ml}$ aprotinin, 1 mM pepstatin, and 10 mM N-ethylmaleimide, and after lysis, the clarified cellular lysate was subjected to immu-

noprecipitation with pooled anti-FP (clones 6D12, 7D2, 8E9, 9G3, and 10G10) monoclonal antibody overnight at 4 °C. Protein G-agarose beads were then added for another 4 h. After three washes with lysis buffer, incorporation was quantified using a γ -counter (PerkinElmer Life Sciences Cobra Auto-Gamma). The specific counts/min for photoaffinity labeling was calculated as follow: (cpm from the total photoaffinity labeling with 1 nM [¹²⁵I-Sar¹,Bpa⁸]Ang II) – (cpm from the nonspecific photoaffinity labeling with 1 nM [¹²⁵I-Sar¹,Bpa⁸]Ang II in the presence of 1 μ M Ang II). The net counts/min for immunoprecipitation were calculated as follow: (specific cpm from the protein G-agarose beads in the presence of anti monoclonal antibody) – ((specific cpm from the protein G-agarose beads in the absence of anti monoclonal antibody) + (blank cpm)). In some experiments, immunoprecipitates were solubilized in Laemmli buffer and run on 10% SDS-PAGE. Gels were dried before autoradiography for 5–14 days on a Typhoon FLA 9500 biomolecular imager.

Bioluminescence Resonance Energy Transfer (BRET) Experiments—To detect and analyze interactions between AT1R and FP, BRET was used as described previously (18). For this purpose, HEK 293 cells were seeded in 96-well plates and transfected with a fixed amount of an FP receptor tagged at its C terminus with *Renilla* luciferase (FP-RLuc) and co-transfected with an increasing amount of plasmids encoding AT1R or GABA-B2 (the latter as a negative control), both tagged at their C terminus with YFP (AT1R-YFP, GABAB2-YFP). Cells were assayed 48 h post-transfection. After the addition of the substrate coelenterazine h, emission was measured using an injector-equipped plate reader spectrofluorometer (Synergy 2 from BioTek) at the wavelengths of 485 and 528 nm, corresponding to the maxima of the emission spectra for RLuc and YFP, respectively.

Protein Fragment Complementation Assay Experiments—For confocal microscopy, 48 h after transfection, HEK 293 cells were serum-starved for 30 min, and imaging was performed using a Zeiss LSM-510 Meta laser scanning microscope (Carl Zeiss, Thornwood, NY) equipped with XL-3 temperature chamber with a $\times 63$ glycerol/water immersion lens. Image acquisition was done in single track mode using 514 and 543 nm excitation wavelengths and using BP530–600 and LP560 emission filter sets for Venus/YFP and mRFP, respectively.

Co-localization Analysis—Co-localization between Venus/YFP and mRFP was calculated with Pearson's correlation coefficient, using the JaCoP (Just another Co-localization Plugin) plugin in ImageJ (19). For each image, a region of interest was drawn to calculate the Pearson's correlation coefficient; for all nonstimulated cells, the membrane, where the Venus/YFP signal was most abundant, was chosen. For stimulated cells, the region of interest was chosen where a maximum of endocytic Venus/YFP-labeled vesicles was found.

Cellular Growth Measurements—VSMCs were plated in 24-well plates at a density of 15,000 cells/well in triplicate. After 24 h without serum, cells were treated as indicated in the figure legends for an additional 24 h. The last 8 h of stimulation, 0.5 μ Ci of [³H]thymidine or [³H]leucine was added per well. Cells were then put on ice and washed once with PBS and incubated 15 min in 5% trichloroacetic acid. Cells were lysed in 0.2 M

NaOH, and the incorporated radioactivity was counted using a β -counter.

Vessel Contractility Experiments—Vessel constriction was studied *ex vivo* as described previously (20). Segments of the abdominal aorta (2 mm in length) were isolated from 3-month-old C57BL/6 mice mounted on 20- μ m tungsten wires in small vessel myographs, stretched to optimal isometric tension, and maintained in physiological saline solution (PSS: NaCl, 119 mM; KCl, 4.7 mM; KH₂PO₄, 1.18 mM; MgSO₄, 1.17 mM; NaHCO₃, 25 mM; CaCl₂, 1.6 mM; EDTA, 0.023 mM; glucose, 10 mM; aerated with 12% O₂, 5% CO₂, 83% N₂; pH 7.4) at 37 °C. In these experimental conditions, arterial rings have no resting tone. After a 40-min stabilization period, arterial segments were challenged with 40 mM KCl PSS (KCl was substituted for an equivalent concentration of NaCl). Single cumulative concentration-response curves to Ang II (0.1 nM to 0.3 μ M) and PGF2 α (0.1 nM to 10 μ M) were obtained from independent segments. To determine the impact of activation of the FP on angiotensin-dependent contraction, segments were preincubated with a threshold constricting concentration of PGF2 α (1 μ M) followed 20 min later by a dose-response curve to Ang II. To then assess whether the occupancy alone of the FP could modulate Ang II-induced contraction, arterial segments were preincubated with either one of the antagonists of FP, AS604872 (1 μ M), or AL-8810 (10 μ M), followed 20 min later by a dose-response curve to Ang II. Finally, to assess the effects of the activation and the occupation of AT1R on PGF2 α -induced contraction, arterial segments were preincubated with Ang II (10 nM) and L158,809 (1 μ M), respectively, followed 20 min later by a dose-response curve to PGF2 α . The data were expressed as a percentages of contraction induced by 40 mM KPSS. Student's *t* tests were performed to compare concentration-response curves. Preincubation with the L158,809 had no effects on the resting tension. More importantly, preincubation with Ang II and PGF2 α did not increase tone (0.0 \pm 0.0%, *n* = 4, and 0.06 \pm 0.02%, *n* = 5, respectively).

Western Blotting—VSMCs or stable FP cells (transiently co-transfected with increasing amounts of FLAG-AT1R) were starved for 30 min at 37 °C and stimulated or not with 100 nM PGF2 α or Ang II in the presence or absence of different ligands as described in individual figures. Immunoblotting was then performed with anti-p-ERK1/2 or anti-total ERK1/2. For p-ERK1/2 and total ERK1/2 semi-quantitative analysis by densitometry, the ImageJ program was used. The p-ERK1/2 signal was normalized to total ERK1/2, and fold over basal activation was calculated.

IP₁ Production Assay—This assay was performed as described previously (21). Briefly, VSMCs were grown in 10-cm dishes for 24 h. The cells were then starved without serum. The next day, cells were washed once with PBS and collected in PBS containing 20 mM EDTA. For the assay, 10⁴ cells per well (384-well plate) were used. Cells were first pretreated for 30 min at 37 °C with vehicle, 20 μ M L158,809, or 10 μ M AL-8810 or AS604872. Cells were then treated with increasing concentrations of PGF2 α or Ang II for 60 min at 37 °C. IP₁-d₂ and anti-IP₁-cryptate were added for an additional 2 h at room temperature. Plates were read on a Synergy 2 multimode microplate reader.

Allosteric Interactions in AT1R/FP Heterodimers

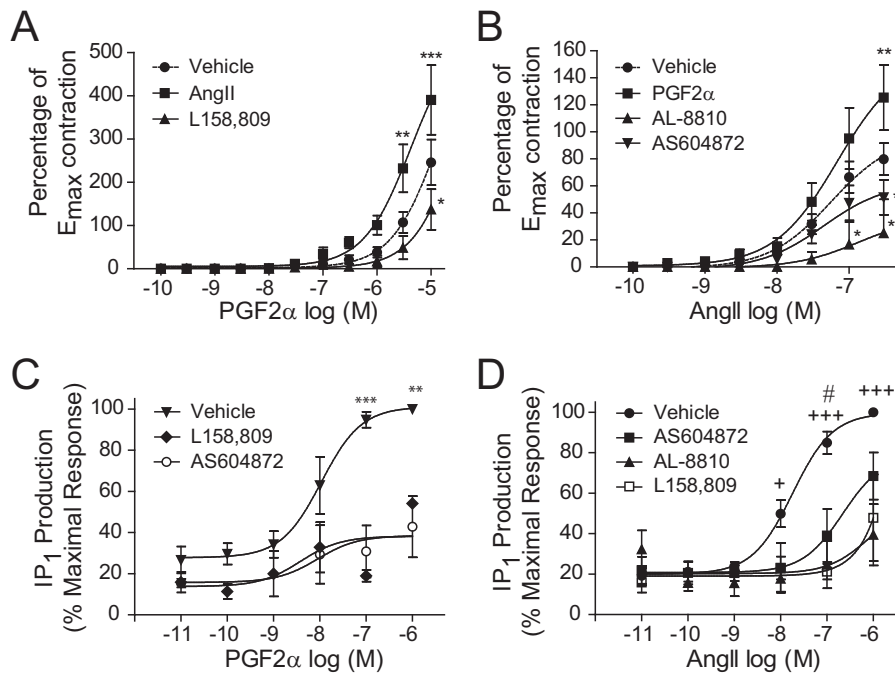


FIGURE 1. Effects of single or dual ligand occupancy on AT1R- or FP-mediated contraction of abdominal aorta. *A* and *B*, effects of 1 μM L158,809 and Ang II (*A*) or 1 μM PGF2 α , AS604872, or 10 μM AL-8810 (*B*) on contraction of abdominal aorta (maximal or E_{max} contraction) as induced by increasing concentrations of PGF2 α (*A*) or Ang II (*B*). *C* and *D*, effects of 2 μM L158,809, 10 μM AS604872, or AL-8810 on PGF2 α - (*C*) or Ang II (*D*)-induced IP₁ production in VSMC. Data are representative of 5 to 8 (*A*) or 5 to 15 (*B*) and 3 (*C*) to 4 (*D*) independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with cells pretreated with vehicle (*A* and *B*) or comparing vehicle with L158,809 (*C*). +, $p < 0.05$; +++, $p < 0.001$ comparing vehicle to AL-8810 (*D*); #, $p < 0.05$, comparing vehicle with AS604872.

Data Analysis—All graphs are represented as the mean value \pm S.E. Intensity of the signals from Western blots and FRET experiments was determined by densitometric analysis with ImageJ. Statistical analysis was performed with GraphPad Prism software with one-way analysis of variance (Table 1 and Figs. 2 and 7) or two-way analysis of variance (Figs. 1, 3, and 6) and, when appropriate, with Bonferroni (comparison between all or selected groups) post hoc tests. A two-tailed p value lower than 0.05 was considered significant.

RESULTS

Abdominal Aorta Contractile Responses to Ang II Are Modulated by FP Occupancy—We first examined the putative interplay between FP and AT1R by assessing the extent to which vascular smooth muscle beds contracted upon occupancy by selective ligands, either singly or for both receptors. Murine abdominal aortic rings were stimulated *ex vivo* with increasing concentrations of PGF2 α , which led to a dose-dependent vasoconstriction (as shown by the % maximal contraction mediated by 40 mM KPSS, Fig. 1A). This response was positively modulated by Ang II pretreatment. A similar potentiation of Ang II-mediated contraction by PGF2 α was also observed (Fig. 1B). Such potentiation could result from convergence on common downstream signaling pathways, such as the G_{α_q} -PLC- Ca^{2+} pathway, which would integrate receptor-mediated VSMC contraction. However, occupation of one receptor with a known antagonist reduced the pressor response mediated by the other receptor. Indeed, either of the selective FP antagonists AS604872 or AL-8810 significantly reduced contraction mediated by Ang II (Fig. 1B), whereas FP-mediated contraction was

reduced in the presence of the selective AT1R antagonist L158,809 (Fig. 1A).

We next determined whether L158,809, AS604872, or AL-8810 modulated PGF2 α - or Ang II-mediated (IP₁) production, a downstream second messenger product of the G_{α_q} -PLC signaling pathway, in VSMCs initially isolated from rat aorta, which endogenously express both FP (22) and AT1R (23). Consistent with the results described above for regulation of aortic contraction, PGF2 α - or Ang II-mediated IP₁ production was blocked by L158,809 (Fig. 1C), or AS604872 and AL-8810 (Fig. 1D), respectively. As controls, both L158,809 (Fig. 1D, *open squares*) and AS604872 (Fig. 1C, *open circles*) inhibited their respective receptor responses. Together, these results suggest regulation at the receptor level between AT1R and FP.

Antagonism of FP Inhibits Ang II-induced Growth in VSMC—Vascular remodeling, which contributes to the development of pathologies such as hypertension, involves many cellular changes, ranging from the rearrangement of extracellular matrix proteins to cell growth (24). In VSMC, many signaling pathways, including those driven by AT1R (25) and FP (22), were found responsible for these changes. To better understand the involvement of the two receptors on VSMC growth, we used [³H]thymidine incorporation as a marker of DNA synthesis and [³H]leucine incorporation as a marker of *de novo* protein synthesis. Only Ang II but not PGF2 α had a significant effect on [³H]thymidine incorporation (Fig. 2A), which was blocked by both AS604872 and L158,809. PGF2 α and Ang II stimulation of VSMC significantly promoted [³H]leucine incorporation in cells (Fig. 2B), although to different extents. These effects were

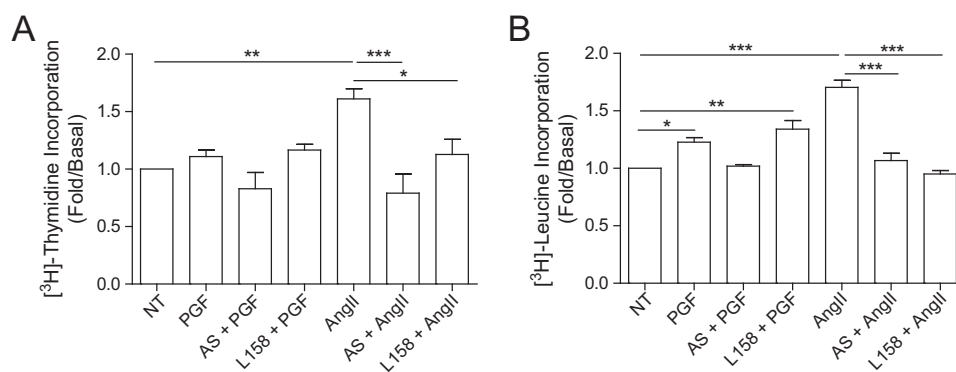


FIGURE 2. **PGF2 α - or Ang II-induced cellular growth is regulated asymmetrically by receptor antagonists.** A and B, [³H]thymidine (A) or [³H]leucine (B) incorporation in VSMC following 30 min of pretreatment with vehicle, 2 μ M L158,809, or 1 μ M AS604872 and 24 h treatment with 10 nM PGF2 α or Ang II. Data are representative of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

selectively blocked by their respective antagonists. However, although L158,809 did not block PGF2 α -mediated [³H]leucine incorporation, AS604872 was as potent as L158,809 in inhibiting responses promoted by Ang II (Fig. 2, A and B), revealing a distinct asymmetry in the regulation of cellular responses modulated via occupation of the two receptors and suggesting possible allosteric interactions.

Modulation of AT1R and FP Signaling via MAPK in VSMCs—We next assessed the extent to which AT1R/FP modulated the ERK1/2 MAPK signaling pathway in VSMCs. Cells were again pretreated with L158,809 and then stimulated for different times with PGF2 α . PGF2 α -induced ERK1/2 activation was potentiated by the AT1R antagonist L158,809 (Fig. 3A, compare *open* and *closed squares* and see representative western blot in Fig. 3B, *middle panel*). In contrast, Ang II-induced ERK1/2 activation was not significantly altered by pretreatment with the FP antagonist AS604872 (Fig. 3C, compare *open* and *closed triangles* and see representative western blot in Fig. 3D, *top panel*), again highlighting an asymmetry in signaling between the two receptors. Control experiments indicated that ERK1/2 activation was completely abrogated in Ang II- or PGF2 α -stimulated VSMC following pretreatment with L158,809 (Fig. 3, A, compare *open* and *closed triangles*, and B, *bottom panel*) or AS604872, respectively (Fig. 3, C, compare *open* and *closed squares*, and D, *bottom panel*). Neither L158,809 nor AS604872 stimulated MAPK activation on their own (Fig. 3B, *top panel*). The potentiation of MAPK activation via the putative AT1R-FP complex promoted by the AT1R antagonist, L158,809, was primarily through EGFR transactivation because this response was blocked by the EGFR antagonist AG-1478 (Fig. 3F). Consistent with this effect, inhibition of the G α_q -PKC pathway, using the PKC inhibitor G α_6983 , did not inhibit L158,809-mediated potentiation of FP-mediated modulation of pERK1/2 (Fig. 3E). Taken together, these data demonstrate that the allosteric asymmetry between FP and AT1R manifests differently depending on the physiological outcome and the relevant downstream signaling pathways measured.

One prediction from these results would be that the effects of AT1R on FP signaling would depend on the relative stoichiometry of the two receptors. We used a stable HA-FP cell line and co-expressed different amounts of FLAG-AT1R. This did not significantly affect the amount of FP expressed in these cells

(see legend to Fig. 4). We examined the effect of increasing AT1R expression on the allostery between the two receptors. First, as we described earlier (8), the coupling of FP to MAPK in HEK 293 cells is distinct from that found in VSMCs. In VSMCs, as we demonstrated above, ERK1/2 MAPK activation by FP depends on EGFR transactivation and was potentiated by L158,809-occupied AT1Rs. In HEK 293 cells, FP, when stimulated by PGF2 α , works through a canonical G α_q - and PKC-dependent pathway (8). That said, in the absence of AT1R, the responses to FP stimulation were identical in the absence or presence of L158,809 (Fig. 4, A and D, *top panel*). At an intermediate level of AT1R co-expression, however, L158,809 pretreatment resulted in an inhibition of FP-stimulated ERK1/2 (Fig. 4, B and D, *middle panel*) phosphorylation especially at early time points suggesting that the consequences of allosteric modulation of FP by stoichiometric amounts of AT1R depend on the cell type studied. Again, the fact that L158,809 did not stimulate either ERK1/2 activation in VSMC or receptor internalization in HEK 293 cells (data not shown) highlights the fact that these effects are likely allosteric and not due to downstream signaling cross-talk. This was also shown when much higher levels of AT1R were co-expressed (Fig. 4, C and D). Indeed, in this latter condition, the allosteric effects were lost as most of the AT1R would not be expected to be associated with FP, despite AT1R signaling being augmented.

AT1R and FP Form Heterodimers—Heterodimerization of GPCRs has been shown to differentially regulate ERK1/2 MAPK signaling through different mechanisms (18, 26–29). To explain this asymmetric signaling regulation between FP and AT1R and to evaluate potential allostery, we next considered heterodimerization as a possible mechanistic basis. We assessed whether the AT1R/FP heterodimer could be detected in a physiological context with endogenous receptors in VSMCs. However, because of the lack of good antibodies to either immunoprecipitate or detect AT1R (30), we used a radio-labeled, photoactivable Ang II analog, [¹²⁵I-Sar¹,*p*-benzoyl-L-phenylalanine⁸]Ang II (or [¹²⁵I-Sar¹,Bpa⁸]Ang II (16, 17)), that can be specifically and covalently linked to AT1R, as a mean to detect both receptors. To validate our system, we first transfected both receptors, alone or together, into HEK 293 cells, which do not express significant levels of either receptor endogenously, and we undertook co-immunoprecipitation experi-

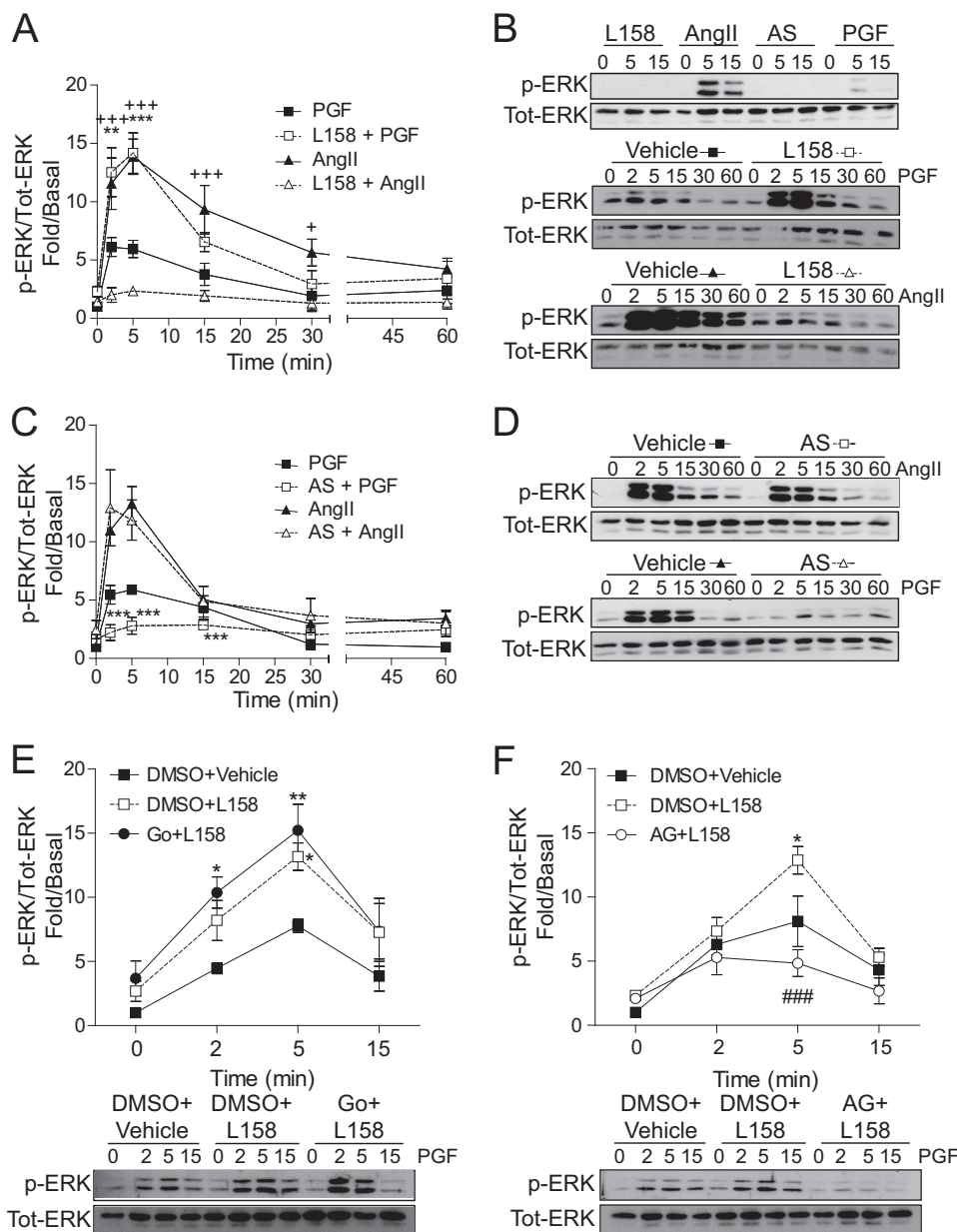


FIGURE 3. L158,809, an AT1R antagonist, potentiates PGF $_{2\alpha}$ -induced ERK1/2 activation. *A*, effect of pretreatment of VSMC with 2 μ M L158,809 on 100 nM PGF $_{2\alpha}$ -induced ERK1/2 activation (100 nM Ang II is used as control). *B*, representative experiment of experiments analyzed in *A*. *Top panel*, L158,809 and AS604872 do not activate ERK1/2 on their own. *C*, effect of VSMC pretreatment with 1 μ M AS604872 on 100 nM Ang II-induced ERK1/2 activation (100 nM PGF $_{2\alpha}$ is used as control). *D*, representative experiment of experiments analyzed in *C*. *E* and *F*, effect of 1 μ M Go6983 (*E*) or 125 nM AG-1478 (*F*) on L158,809 (2 μ M) potentiation of PGF $_{2\alpha}$ (1 μ M)-induced ERK1/2 activation in VSMC. ERK1/2 activation was monitored using anti-p-ERK1/2 antibodies. VSMCs were stimulated with 2 μ M L158,809, 1 μ M Ang II, 1 μ M AS604872, or 1 μ M PGF $_{2\alpha}$ for 5 or 15 min. Cells were lysed in Laemmli buffer and subjected to immunoblotting against phospho- and total (tot) ERK1/2. Total protein loading was monitored using anti-total ERK1/2 antibodies. Data are representative of three (*B*, *top panel*, and *C*), four (*A* and *E*), or five (*F*) independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with PGF $_{2\alpha}$ (closed squares, *A* and *C*) or DMSO + vehicle (*E* and *F*), and +, $p < 0.05$; +++, $p < 0.001$ compared with Ang II (closed triangles, *A*); ###, $p < 0.001$ compared with DMSO + L158,809 and AG + L158,809 (*F*).

ments. HA-FP is immunoprecipitated by the 12CA5 monoclonal anti-HA antibody (Fig. 5*A*, inset). Our results showed that a broad band of around 60 kDa could be labeled and co-immunoprecipitated, which represents glycosylated AT1R (Fig. 5*A*, lanes 1 and 3, respectively). [125 I-Sar 1 ,Bpa 8]Ang II-labeled AT1R was also detected in conditions where FP was immunoprecipitated (Fig. 5*A*, lane 5). [125 I-Sar 1 ,Bpa 8]Ang II incorporation was also quantified using a γ -counter following immunoprecipitation. Co-immunoprecipitation of AT1R and FP pulled out 25 ± 1.0 and $14.8 \pm 0.9\%$ of total [125 I-Sar 1 ,Bpa 8]Ang II-

labeled receptors, respectively, in FLAG-AT1R/FP-transfected HEK 293 cells (Fig. 5*B*). We used a similar approach to detect endogenous complexes in VSMC. However, because of the low amounts of receptor, signals were barely detectable using autoradiography, and association was instead assessed by counting [125 I-Sar 1 ,Bpa 8]Ang II binding in FP immunoprecipitates. Results show that a small amount of endogenous AT1R in VSMC could also be immunoprecipitated ($1.9 \pm 0.2\%$ of total, Fig. 5*B*) using anti-FP mouse monoclonal antibodies that we previously generated and characterized (5). Given the much

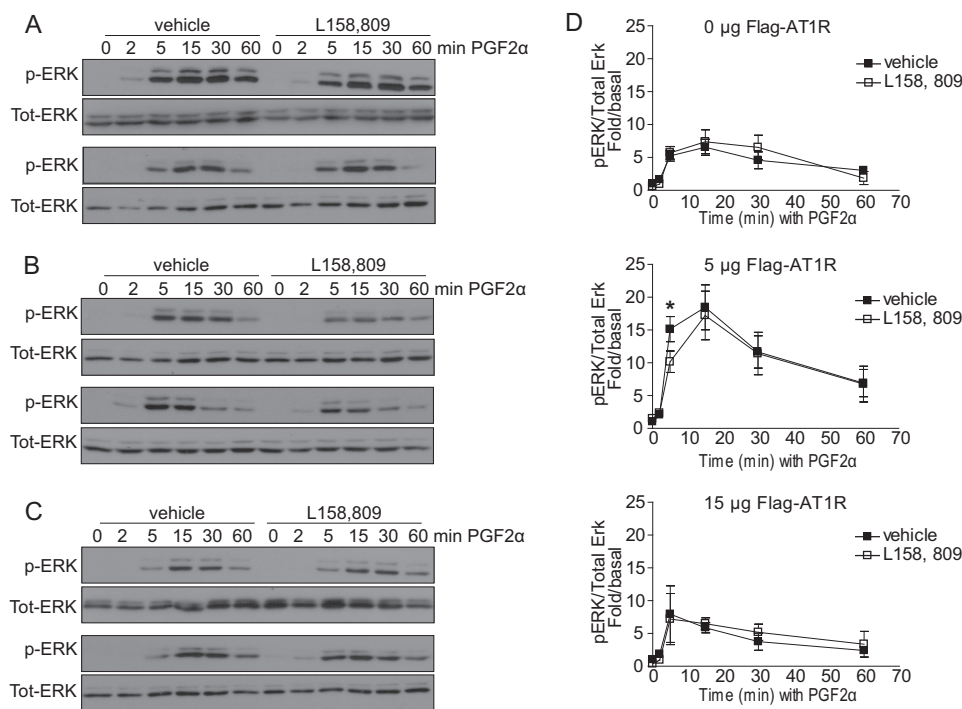


FIGURE 4. Effects of increasing levels of AT1R on FP signaling in HEK 293 cells. Different amounts of FLAG-AT1R (0, 5, and 15 μg of cDNA) were co-transfected into our FP stable cell line. This translated into 2.2 ± 1.0 , 55 ± 6.5 , and 259 ± 69 fmol/mg protein, in three independent experiments. This did not significantly affect the amount of FP expressed in these cells (263 ± 32 , 274 ± 56 , and 226 ± 18 fmol/mg protein, respectively). Time course of FP-mediated activation of ERK1/2 in the presence or absence of 2 μM L158,809 at no (A), intermediate (B), and high (C) levels of co-expressed AT1R is shown. Cells were stimulated with 100 nM PGF2 α for different times, were lysed in Laemmli buffer, and subjected to immunoblotting against phospho- and total (tot) ERK1/2. Total protein loading was monitored using anti-total ERK1/2 antibodies. The two panels represent individual experiments and are representative of at least four independent experiments in each case. D, analysis of Western blotting data using densitometry. Data here are representative of 4–7 independent experiments. *, $p < 0.05$ between vehicle and L158,809.

larger amounts of AT1R (~ 500 fmol/mg protein, data not shown) compared with FP (~ 5 – 10 fmol/mg protein, data not shown) in VSMCs, this small amount of heterodimer is not surprising. Moreover, ^{125}I -Ang II was not detected following FP immunoprecipitation, showing that only [^{125}I -Sar 1 ,Bpa 8]Ang II could be cross-linked to AT1R.⁷

To evaluate the extent to which AT1R and FP could interact in live cells, we next used BRET as an independent means to establish receptor dimers. We used a standard BRET configuration, performing BRET saturation experiments with AT1R-YFP and FP-Rluc. A saturable BRET signal was detected compared with a noninteracting negative control of the GABA-B2-YFP (Fig. 6A). Finally, we also used a fluorescent protein fragment reconstitution assay (31), where the first half of Venus YFP was fused to the AT1R and the second half to FP. These constructs were shown to be functional as they could be stimulated by PGF2 α to activate ERK1/2 (data not shown). When both receptors were co-expressed in HEK 293 cells, reconstituted Venus fluorescence was detected (Fig. 6, B–D) suggesting that the heterodimeric receptor was formed. To test the functionality of this dimer, and to further validate the constructs, we stimulated these cells, which were also transfected with β -arrestin-2 tagged with mRFP (β -Arr2-mRFP) and with either PGF2 α or Ang II. Treatment with Ang II lead to internalization of the receptor heterodimer and an increased co-localization

with β -Arr2-mRFP (Fig. 6, C and D, white bars). PGF2 α had a much less robust effect on β -Arr-2-mRFP recruitment but still showed internalization of the receptor, perhaps by a different endocytic route (Fig. 6, B and D, black bars). FP-Venus1/FP-Venus2 showed no recruitment of β -Arr-2-mRFP following Ang II stimulation (data not shown). Co-localization was shown to be specific as rotating the red signal by 90 $^\circ$ resulted in a significant reduction in the merged signals. Together, using immunoprecipitation, BRET, and fluorescent protein fragment reconstitution, our data suggest that receptor heterodimers provide a mechanistic basis for the interactions between FP and AT1R.

AT1R/FP Dimerization Alters Receptor Binding Properties—Receptor heterodimers have been shown to modulate binding of endogenous ligands between the two receptor protomers (32–35). We thus performed competition binding studies on the putative AT1/FP dimer under various conditions (summarized in Table 1). First, we examined [^3H]PGF2 α binding to FP alone or in AT1R/FP cells. When expressed alone, the K_i value for PGF2 α on FP was of 4.79 ± 1.35 nM (Fig. 7A and Table 1). The presence of AT1R (unoccupied by ligand) was sufficient to increase the affinity of FP for PGF2 α (e.g. lowering the K_i) to 1.85 ± 0.62 nM (Fig. 7A). Despite what seems a modest effect, a 2-fold shift in ligand affinity can have dramatic effects on physiological function *in situ*. Binding of Ang II or L158,809 to AT1R did not further change the K_i value of PGF2 α for FP (1.78 ± 0.92 and 1.63 ± 0.91 nM, respectively, Fig. 7C). In contrast, ^{125}I -Ang II binding to AT1R was only slightly affected by

⁷ E. Goupil, S. Clément, S. A. Laporte, and T. E. Hébert, personal communication.

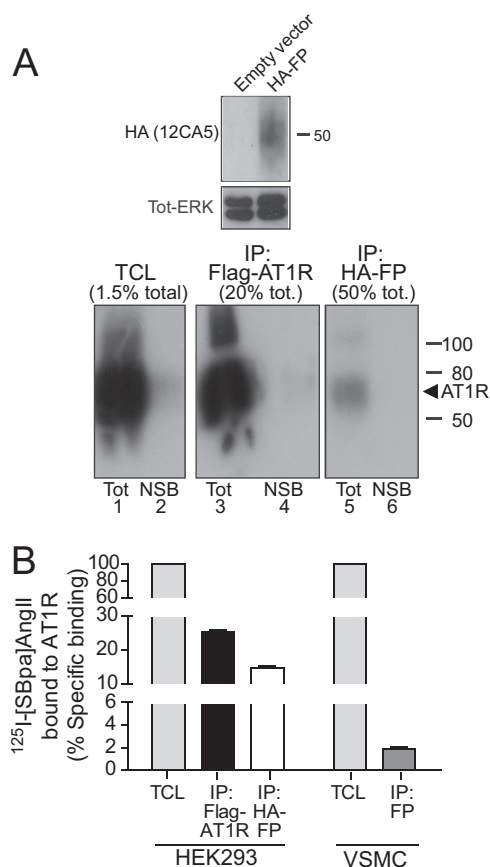


FIGURE 5. AT1R and FP form heterodimers in HEK 293 cells and VSMC. *A*, representative autoradiogram of experiments in HEK 293 cells co-expressing HA-FP/FLAG-AT1R. *TCL*, total cell lysate; *Tot*, total binding; *NSB*, non-specific binding (binding in the presence of 1 μ M Ang II). *Inset*, immunoprecipitation (*IP*) with 12CA5 anti-HA antibodies only pulls down FP when it is transfected into HEK 293 cells and not when empty vector was used. Image is representative of multiple experiments. *B*, co-immunoprecipitation in HEK 293 cells co-expressing FLAG-AT1R and HA-FP or in VSMC. Percent of total binding of [125 I-Sar¹,Bpa⁸]Ang II cross-linked to AT1R and obtained after immunoprecipitation with anti-FLAG (for AT1R) or anti-HA (for FP) in HEK 293 cells (*left*) or anti-FP in VSMC (*right*, see “Experimental Procedures” for details). Data are representative of at least three independent experiments in each case.

simple co-expression of FP (K_i of 1.94 ± 0.63 nM for AT1R alone and 1.20 ± 0.57 nM when FP was present, see Fig. 7*B* and Table 1). However, [125 I]-Ang II binding was altered by occupation of FP with either antagonist (AS604872) or agonist (PGF2 α), increasing AT1R affinity for its agonist by 3- and 2-fold, respectively (1.94 ± 0.63 to 0.48 ± 0.35 and 0.68 ± 0.16 nM; Fig. 7*D*). Finally, in preliminary measures of ligand dissociation kinetics, we noted that the presence of the AT1R resulted in slower dissociation rates (as denoted by the half-life of [3 H]PGF2 α binding to FP or $t_{1/2}$) of [3 H]PGF2 α from FP (no AT1R, $t_{1/2}$ of 7.7 or 7.9 min; 55 ± 6.5 fmol/mg AT1R, 10.4 and 9.7 min; and 259 \pm 69 fmol/mg AT1R, 11.7 and 14.5 min, $n = 2$). These results are in line with our previous study showing the effects of AT1R on FP ligand dissociation (36). Curiously, the effect of [125 I]-Ang II dissociation was the opposite, $t_{1/2}$ of 8.9 and 7.3 min in the absence of FP, and 5.2 and 5.1 min in the presence of FP ($n = 2$). Taken together, our results suggest allosteric regulation within a receptor heterodimer and highlight a functional asymmetry in

how ligand binding and signaling are modulated by occupation of either protomer.

DISCUSSION

This study establishes for the first time mechanisms underlying functional cross-talk between FP and AT1R, which result in differential effects on aspects of AT1R- and FP-mediated cellular signaling and physiological responses. Interestingly, we found both symmetry and asymmetry in these responses with respect to ligand binding, signaling, trafficking, and vascular contractile and growth responses (Fig. 8). Previous studies have demonstrated heterodimerization of AT1R with CB1 cannabinoid receptors (37), the apelin receptor (38), the secretin receptor (39), and the β_2 -adrenergic receptor (40), which in all cases resulted in altered signaling profiles compared with the parent receptors alone. In recent years, much debate has arisen around the question of whether GPCRs serve their physiological functions as monomers (41), dimers (42, 43), or even larger oligomers (44–47). One obvious functional advantage of dimers, more easily understood in the context of heterodimers, is that they can act on each other via allosteric interactions, which may or may not depend on ligand occupation. In this context, GPCR oligomers can function as allosteric machines (48–50). For instance, one ligand-occupied protomer in a heterodimer can act as an allosteric modulator for the other protomer, affecting ligand binding and/or signaling (50). Several groups, including ours, have shown that allosteric communication between the protomers of a dimer could lead to functional selectivity of their downstream cellular signals (18, 27, 33). In fact, a recent study suggested that ghrelin receptor significantly alters D2-dopamine receptor signaling, via heterodimerization in brain regions that never see ghrelin as a neurotransmitter, suggesting a function for the apo-receptor as an allosteric interacting partner, rather than as a signaling receptor in these cells (51). Similar findings were obtained with D1/D2-dopamine receptor dimers (52).

Allosteric regulation and functional selectivity through heterodimerization of AT1R and FP have never been described. Interestingly, the pressor effect of Ang II was potentiated in FP knock-out animals (9) suggesting that basal interactions are important for establishing physiological parameters as for the ghrelin receptor/D2-dopamine receptor pair. We observed asymmetric effects on ligand binding as the presence of AT1R altered the affinity of PGF2 α for FP but not the converse. Furthermore, ligand occupation of FP (either agonist or antagonist) increased the affinity of AT1R for Ang II, but again the converse was not true. Perhaps what is most striking about this asymmetry in ligand binding is that it does not necessarily correlate directly with either signaling or phenotypic outcomes. For example, there was no asymmetry detected when we examined coupling to the canonical G_{α_q} pathway or its downstream physiological outcome of aortic contractility. Occupancy of either receptor with either an agonist or an antagonist had similar positive or negative effects on the other receptor's response whether experiments were performed in VSMCs or in HEK 293 cells. This contrast to the FP knock-out animals suggests that modulation of overall output with respect to contractility depends on both dimer assembly and ligand occupation. How-

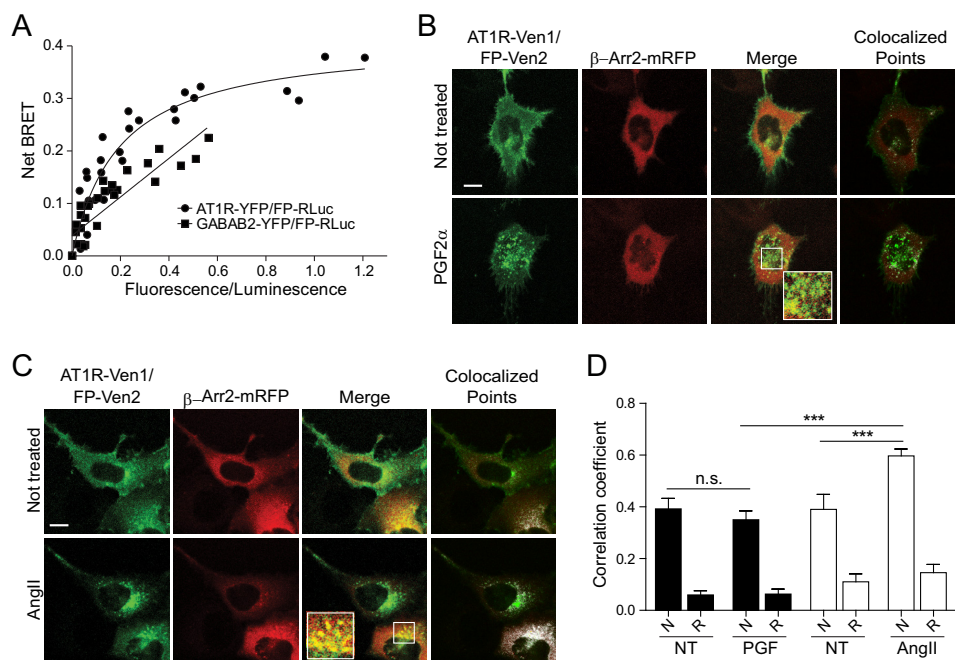


FIGURE 6. Interactions between AT1R and FP detected using BRET or protein fragment complementation assay. *A*, BRET between FP-RLuc (donor) and AT1R-YFP or GABA-B1-YFP (acceptors). BRET saturation curves were produced by transfecting HEK293 cells with a fixed amount of FP-RLuc and co-transfected with an increasing amount of AT1R-YFP (●) or GABAB2-YFP (■) as negative control. *B* and *C*, YFP-Venus was reconstituted using AT1R-Venus1 and FP-Venus2 and co-expressed with β -Arr2-mRFP. Cells were stimulated for 30–45 min with 100 nM PGF 2α (*B*) or Ang II (*C*). Magnified regions (white squares) of the merged images are shown in the corner. Co-localized pixels are shown in white overlaid with the merged signal. Scale bar, 10 μ m. *D*, quantification of co-localization (correlation coefficient) between Venus/YFP and mRFP. Original images (N = normal) were analyzed, then, as a control, the mRFP channel was rotated 90° clockwise (R = rotated). *A*, data are representative of three (GABAB2-YFP) and four (AT1R-YFP) independent experiments (each point representing the mean of experiments conducted in triplicate). *B–D*, quantification is representative of 11–17 cells per condition in three independent experiments. ***, $p < 0.001$, *n.s.*, non-significant; NT, non-treated.

TABLE 1

K_i values for [3 H]PGF 2α or [125 I]-labeled Ang II binding in doubly stable FP/AT1R HEK 293 cells

None represents [3 H]PGF 2α binding to FP expressed alone or [125 I]-labeled Ang II binding to AT1R expressed alone.

Used receptor partner	Radioligand	
	[3 H]PGF 2α on FP	[125 I]-Labeled Ang II on AT1R
None	4.79 ± 1.35	1.94 ± 0.63
AT1R unoccupied	1.85 ± 0.62^a	
L158,809-bound AT1R	1.63 ± 0.91^a	
Ang II-bound AT1R	1.78 ± 0.92^a	
FP unoccupied		1.20 ± 0.57
AS604872-bound FP		0.48 ± 0.35^b
PGF 2α -bound FP		0.68 ± 0.16^c

^a $p < 0.01$ was compared with None for [3 H]PGF 2α binding.

^b $p < 0.01$ was compared with None for [125 I]-labeled Ang II binding.

^c $p < 0.05$.

ever, when ERK1/2 MAPK signaling was examined, we noted a second remarkable asymmetry. FP signaling through ERK1/2 was strongly potentiated by antagonist occupancy of AT1R, although AT1R modulation of ERK1/2 was unaffected by occupancy of FP antagonists, despite increased affinity for Ang II. More remote phenotypic end points dependent on ERK1/2 activation such as DNA or protein synthesis also manifested these asymmetries. Occupancy of AT1R by an antagonist did not modulate FP effects on either outcome, whereas FP antagonists reduced both AT1R-mediated DNA or protein synthesis. Interestingly, canonical $G\alpha_q$ -dependent signaling events mediated by FP in both VSMC and HEK 293 cells were both allosterically antagonized by the L158,809 but only in the presence of AT1R.

Thus, the phenotypic end points downstream of such receptor complexes in a given cell cannot be directly predicted by ligand binding affinity alone, and it may depend on the availability of allosteric modulators such as putative dimer partners and/or effectors associated with each receptor or recruited upon receptor activation. Our findings that the AT1R/FP heterodimer was wired into an EGFR transactivation-dependent ERK1/2 activation mode in VSMC, rather than through the canonical $G\alpha_q$ pathway in HEK 293 cells, which both receptors can engage to signal, support this idea. We have reported changes in signaling pathways when the β_2 -adrenergic receptor formed heterodimers with the oxytocin receptor in a myometrial cell line (18, 27) and highlighted the key role that the cellular context plays into determining what a particular receptor heterodimer does. Moreover, we have previously shown that small molecular allosteric modulators of FP signaling were biased and could act as either positive or negative allosteric modulators depending on the signaling pathway in question (5, 53). The asymmetrical regulation between FP and AT1R for the ERK1/2 pathway and the symmetrical modulation of the $G\alpha_q$ pathway suggest that GPCR allosterism between heterodimers can also lead to pathway selectivity. Furthermore, distinct effects on β -arrestin binding and trafficking of the AT1R/FP heterodimer in response to Ang II or PGF 2α were noted, adding another complexity to their downstream effects.

It is now well established that receptor dimerization can cause changes in conformation within the dimer responsible for altered responses downstream (28). In the context of a

Allosteric Interactions in AT1R/FP Heterodimers

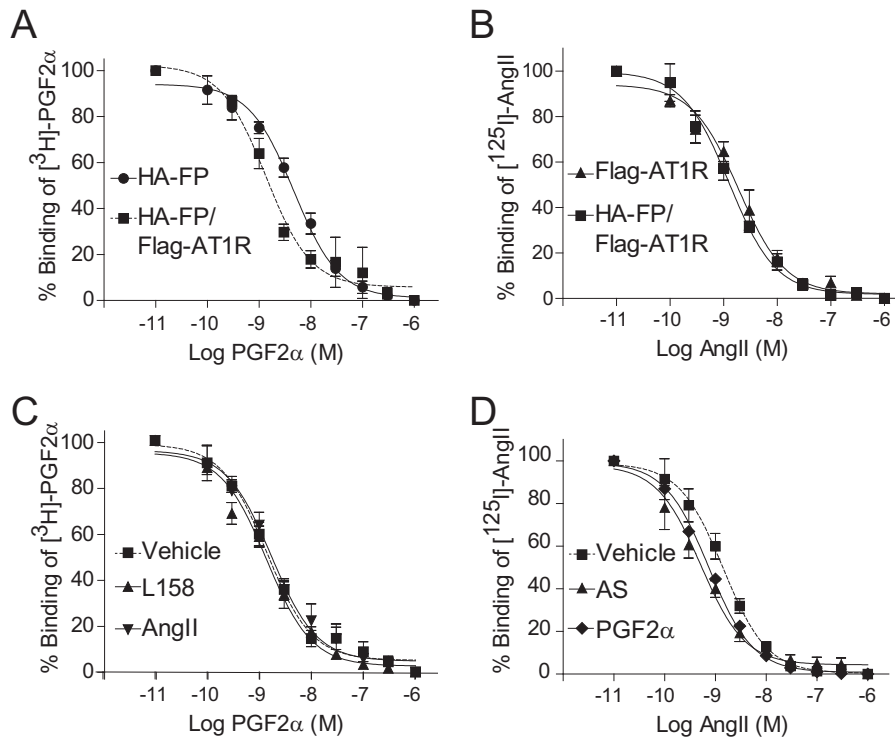


FIGURE 7. **PGF2 α** and **Ang II** binding to the **AT1R/FP** dimer. **A**, [^3H]PGF2 α binding on HA-FP alone or in the presence of unoccupied FLAG-AT1R. **B**, [^{125}I]Ang II binding on FLAG-AT1R alone or in the presence of unoccupied HA-FP. **C**, [^3H]PGF2 α binding on HA-FP in presence of L158,809- or Ang II-occupied AT1R. **D**, [^{125}I]Ang II binding on FLAG-AT1R in the presence of AS604872- or PGF2 α -occupied HA-FP. Data are representative of three to six independent experiments (A–D) performed in duplicate. See Table 1 for the K_i values and statistical analysis.

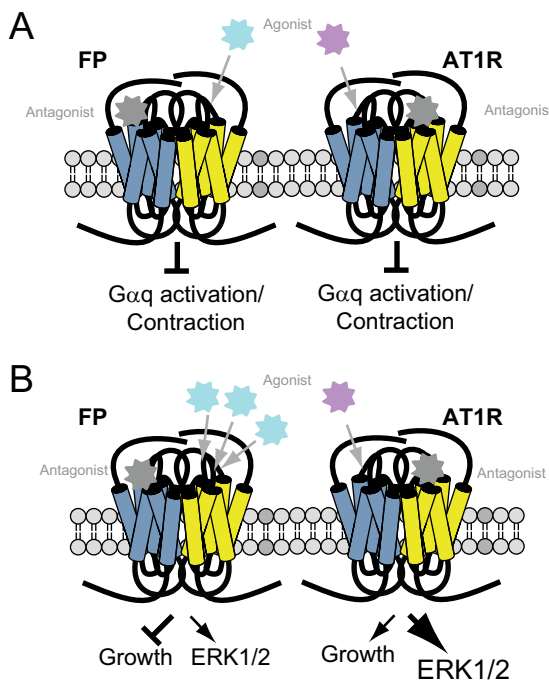


FIGURE 8. **Symmetrical and asymmetrical regulation of AT1R/FP signaling likely through the agency of a heterodimer.** **A**, occupation of either FP (blue) or AT1R (yellow) with their respective antagonist reduces the aortic contractile and G protein-mediated responses promoted by the agonist stimulation of the other protomer. Similarly, occupation of either FP or AT1R with an agonist increased the aortic contractile and G protein-mediated responses promoted by the other receptor. **B**, despite the increased affinity of Ang II for its receptor promoted by the antagonist occupancy of FP, MAPK signaling remained unchanged. However, occupation of FP with antagonist inhibited cell growth induced by Ang II. Finally, occupation of AT1R with an antagonist greatly potentiated FP-dependent MAPK signaling, but it had no effect on PGF2 α -induced cell growth.

D2-dopamine receptor homodimer, one study suggested that protomers are organized asymmetrically with respect to their G protein partners (42), such that occupation of one receptor activates the receptor to facilitate downstream signaling and occupation of the other modulates signaling allosterically without inducing a signal of its own *per se*. This has important implications for the formation and function of receptor heterodimers, in that multiple asymmetrical arrangements become possible depending on the relative orientation of each monomer to the G protein and possibly other effector molecules. This greatly increases the potential organizational complexity of GPCR signaling and suggests that determinants of signaling complex assembly might be of paramount importance in defining signaling specificity in a given tissue, cellular or subcellular compartment (54, 55). Such arrangements might be distinct if complexes are assembled differently, *i.e.* even with the same set of interacting partners, signaling output will be quite distinct. Thus, we must consider both functional and physical asymmetries in the organization of GPCR heterodimers.

Our findings suggest that FP ligands might be useful as a means to allosterically regulate AT1R signaling in hypertension. Recent studies have stressed the importance of developing better AT1R-biased ligands to control its responses by functional selectivity (21, 56). However, the implication of AT1R dimerization with FP, or other GPCRs and the modulation of its signaling by FP ligands, should be taken into consideration as an interesting new therapeutic modality. The use of AT1R blockers to treat hypertension and a parallel therapy with FP ligands to regulate Ang II-induced physiological and pathophysiological

cal responses in the renin-angiotensin-aldosterone system might be considered.

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