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Ang-(1-7) and ET-1 interplay through Mas and ET_B receptor interaction defines a novel vasoprotective mechanism

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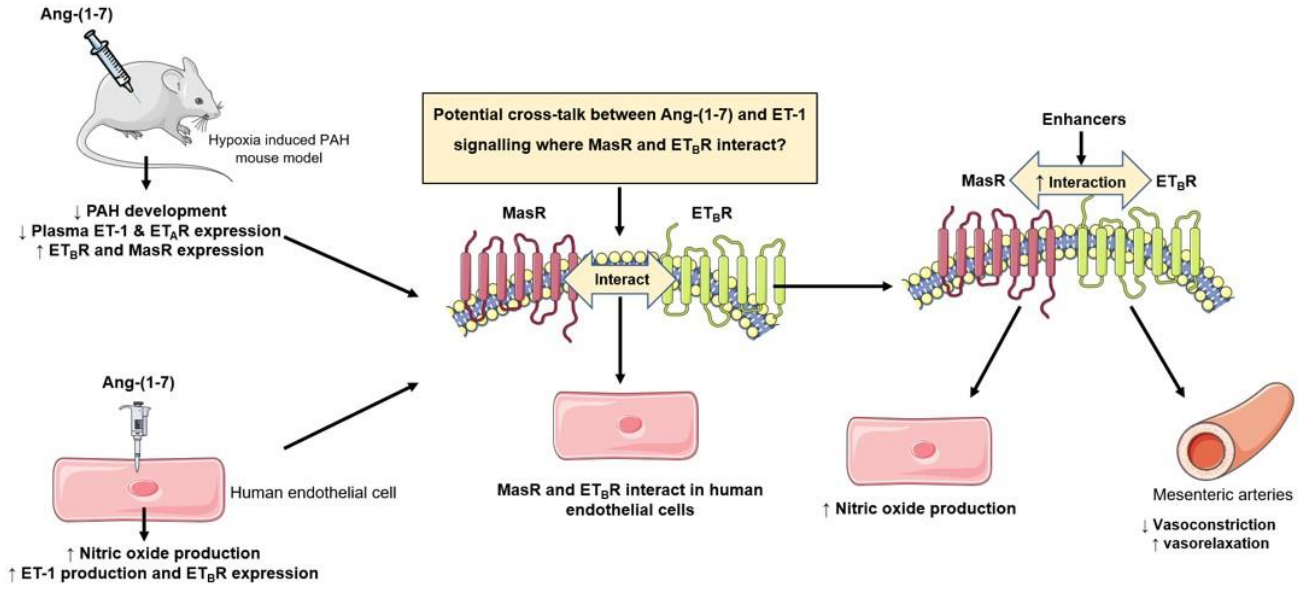
Abstract

Angiotensin (1-7) (Ang-(1-7)) via Mas receptor (MasR) opposes vaso-injurious actions of angiotensin II (Ang II) through ill-defined mechanisms. We hypothesized cross-talk between Ang-(1-7) and the protective arm of the endothelin-1 (ET-1) system involving MasR and endothelin receptor type B (ET_BR). Multiple models were used to address this: *in vivo*, in a mouse model of ET-1-associated vascular injury (hypoxia-induced pulmonary hypertension (PH)); *ex vivo*, in isolated mouse arteries; and *in vitro*, in human endothelial cells. PH mice exhibited pulmonary vascular remodeling, endothelial dysfunction and ET-1-induced hypercontractility. Ang-(1-7) treatment (14 days) ameliorated these effects and increased expression of vascular ET_BR. In human endothelial cells, Ang-(1-7)-induced activation of the eNOS/NO pathway was attenuated by A779 (MasR antagonist) and BQ788 (ET_BR antagonist). A779 inhibited ET-1-induced signaling. Co-immunoprecipitation and peptide-array experiments demonstrated interaction between MasR and ET_BR. Binding sites for ET_BR were mapped to MasR (amino acids 290-314) and binding sites for MasR on ET_BR were identified (amino acids 176-200). Peptides that disrupt MasR:ET_BR association prevented Ang-(1-7) and ET-1 signaling. Using high throughput screening, we identified compounds that enhance MasR:ET_BR interaction, which we termed ‘enhancers’. Enhancers increased Ang-(1-7)-induced eNOS activity, nitric oxide production, and Ang-(1-7)-mediated vasorelaxation, and reduced contractile responses to U46619 (thromboxane A2 analogue). We identify cross-talk between Ang-(1-7) and ET-1 through MasR:ET_BR interaction as a novel signaling network that is vasoprotective. Promoting co-activity between these systems amplifies Ang-(1-7) signaling, increases ET-1/ET_BR-mediated vascular actions and attenuates injurious

effects of ET-1. Enhancing Ang-(1-7)/MasR:ET-1/ET_BR signaling may have therapeutic potential in conditions associated with vascular damage.

Graphical abstract



Nonstandard Abbreviation and Acronyms

Akt	protein kinase B
Ang II	angiotensin II
Ang-(1-7)	angiotensin-(1-7)
eNOS	endothelial nitric oxide synthase
ET_AR	endothelin receptor type A
ET_BR	endothelin receptor type B
ET-1	endothelin-1
FP	fluorescence polarization
GPCR	G protein-coupled receptor
HTS	high-throughput screening
MAP	mean arterial pressure
MasR	Mas receptor
NO	nitric oxide
PI3K	phosphoinositide 3-kinase
PH	pulmonary hypertension
PAH	pulmonary arterial hypertension
RAS	renin-angiotensin-system
ROS	reactive oxygen species
RVH	right ventricle hypertrophy
RVSP	right ventricle systolic pressure

Introduction

Angiotensin-(1-7) (Ang-(1-7)) is a biologically active peptide derived from angiotensin II (Ang II) and angiotensin-(1-9). It is synthesized by angiotensin-converting enzyme 2 (ACE2), primarily in the endothelium, and mediates its effects by binding to Mas receptor (MasR), a G protein-coupled receptor (GPCR) (1,2). The ACE2/Ang-(1-7)/MasR pathway generally opposes Ang II/AT_1R actions and is considered the counter-regulatory axis of the renin angiotensin system (RAS) (3,4). In the vasculature Ang-(1-7) promotes vasodilation and has anti-inflammatory, anti-growth and anti-fibrotic actions (4,5). These effects involve activation of Akt-sensitive endothelial nitric oxide synthase (eNOS) (6), generation of nitric oxide (NO) (7) and production of prostaglandins and endothelium-derived relaxing factor (EDRF) (8,9).

Vasoprotective actions of ACE2 and Ang-(1-7) have been observed in experimental models of cardiac failure, hypertension, kidney disease, cardiac hypertrophy and pulmonary arterial hypertension (PAH) (10-13). Accordingly, strategies to activate or upregulate the ACE2/Ang-(1-7) axis have been proposed as potential new therapeutic approaches in cardiovascular diseases with clinical trials testing the effects of ACE2 and Ang-(1-7) (14,15).

Many of the conditions in which Ang-(1-7) has been shown to be protective are associated with activation of the endothelin-1 (ET-1) system (16,17). Pulmonary hypertension (PH), which is ET-1-sensitive, is ameliorated by the activation of the ACE2/Ang-(1-7) pathway in experimental models (13,18,19). Clinically, patients with PAH are treated with ET receptor blockers (20-22). ET-1 signals through GPCRs, ET_A receptors (ET_AR) and ET_B receptors (ET_BR) that typically induce vasoconstriction and vasorelaxation respectively (23-25). In the vascular system, ET_AR is the main ET receptor subtype in smooth muscle cells, while the ET_BR is the

main receptor type in endothelial cells. In some pathological conditions, ET_BR are expressed in vascular smooth muscle cells (VSMC), where they influence calcium influx, Rho kinase activation and contraction (26). In endothelial cells, ET-1/ET_BR activation leads to eNOS activation, NO production and vasorelaxation and is cardiovascular protective (27) suggesting that the ET_BR is a key regulator of vascular tone. Moreover, in the lungs, kidney, and liver, ET_BR plays a role in ET-1 clearance (28). Downregulation of ET_BR is associated with elevated blood pressure and early onset of renal dysfunction accompanied by reduced sodium excretion and glomerular filtration rate (29).

Cross-talk between Ang-(1-7) and ET-1 has been suggested in obese patients, where Ang-(1-7) decreases ET_AR -induced vasoconstriction measured by forearm blood flow (30). In cultured cardiomyocytes and cardiac fibroblasts, Ang-(1-7)/MasR abrogated ET-1-stimulated proliferation (31). Together these data indicate that Ang-(1-7) may oppose actions of ET-1, possibly via influencing ET_AR signaling (32). The understanding of whether Ang(1-7) affects ET_BR-driven responses is lacking but of interest, as it may lead to vascular protection since ET_BR activation is important in vasodilation and clearance of ET-1.

Molecular processes linking Ang-(1-7) and ET-1 in the vascular system are unclear but networking between signalling pathways and respective receptors may be important. MasR has been shown to dimerize with other GPCR such as AT_1R (33) and AT_2R (34). Accordingly, it is plausible that similar phenomena may occur between MasR and ET-1 receptors. Here we determined mechanisms whereby Ang-(1-7) influences the vascular ET-1 system, focusing on potential interactions between MasR and ET_BR and explored implications of this crosstalk in vascular pathophysiology.

We used a multidisciplinary approach including i) *in vivo* studies in a mouse model of ET-1-associated vascular injury (PH), ii) *ex vivo* studies in isolated intact vessels, iii) *in vitro* experiments in human endothelial cells, iv) high fidelity protein-protein interaction biochemistry and iv) drug discovery strategies. We identified MasR:ET_BR interaction as a node of receptor crosstalk that amplifies Ang-(1-7) signalling and attenuates injurious effects of ET-1. These processes prevent endothelial damage, inflammation and improve vascular function. Through peptide screening we identified compounds that enhance vascular beneficial effects of MasR:ET_BR interaction. Our data suggest that amplifying co-activity between these systems is vasoprotective.

Methods

(See supplementary text for detailed methods)

Data and material availability and sharing

The data and materials used to support the findings of this study are available from the corresponding authors upon reasonable request. Compound structures of the enhancers will be released following patent protection of the intellectual property.

Mouse models

We studied chronically hypoxic mice as a model of PH, as we previously detailed (35). Mice were treated with orally active Ang-(1-7) as described (36). Briefly C57BL/6 mice were divided into 4 groups: normoxia treated with vehicle (V) (β-cyclodextrin) or HPβCD-Ang-(1-7) (Ang-(1-7)) 30 µg/kg/day by oral gavage, hypoxia treated with vehicle (V) (β-cyclodextrin) or HPβCD-Ang-(1-7) (Ang-(1-7)) 30 µg/kg/day by oral gavage. Animals were exposed to normoxia or hypoxia conditions for 14 days and after an additional 14 days of treatment (reversal protocol), *in vivo* assessment of PH effects were performed.

In vivo assessment of hypoxic pulmonary hypertension

In vivo pressure volume loop relation measurements were performed to assess hemodynamic alterations. A closed-chest approach was used (35). Measurement of right ventricular hypertrophy (RVH) was determined using Fulton's index (37). Left ventricular hypertrophy (LVH) was determined as LV weight relative to tibia length.

Vascular reactivity in small muscular pulmonary arteries

Two-millimetre-long segments of intralobar pulmonary arteries were threaded onto 40 µm steel wire and mounted on isometric wire myographs (38).

Lung histology and pulmonary arterial remodelling

Sagittal sections were obtained from the mouse lung. Sections were stained with Elastin Van Gieson (EVG) and microscopically assessed in a blinded fashion. Pulmonary arteries (25 to 100 µm external diameter) associated with an airway distal to the respiratory bronchiole were counted. Pulmonary arteries were considered muscularised if they possessed a double-elastic lamina for at least half the diameter in the vessel cross-section (35). Vessels (48±4) were counted per mouse lung section and the results were averaged and expressed relative to the normoxic vehicle group.

Wire myography to assess mesenteric artery vascular function

Mesenteric resistance arteries from WT mice were dissected as we previously described (39) and arterial segments were mounted on isometric wire myographs.

Cell models

Culture of human endothelial cells. In vitro studies were conducted in human microvascular endothelial cells (HMVECs) (Cascade Biologics, Portland, Oregon, USA) and human aortic endothelial cells (HAEC) (ATCC, Gaithersburg, Maryland, USA). Cells were stimulated with Ang-(1-7), ET-1 or MasR/ET_BR enhancer

compounds and were pre-incubated with pharmacological inhibitors or antagonists: A779 (MasR antagonist; 10μM), BQ788 (ET_BR antagonist; 10μM), BQ123 (ET_AR antagonist; 10μM), L-NAME (NOS inhibitor; 10μM) or LY294002 (PI3K inhibitor; 10μM) for 30 minutes prior to stimulation as indicated in figure legends.

MasR- and ET_BR-expressing MCF-7 cells. Detailed characterisation of putative MasR and ET_BR interactions were performed in cell lines over-expressing the receptors. The MCF-B human breast adenocarcinoma cell line was transfected using Lipofectamine2000™ according to the manufacturer's instructions with pCMV6 vector Human ET_BR variant 1 with a C-terminal Myc-DDK tag or pcDNA3.1 Mas-GFP constructs (kindly provided by Prof. Michael Bader, Max-Delbruck Centre for Molecular Medicine, Berlin).

mRNA expression

Total RNA was extracted from cells or tissues in Trizol reagent. Real time qPCR was carried out on a Taqman PCR apparatus using gene-specific primers to quantify the relative abundance of each gene with SYBR Green I as the fluorescent molecule. The primers used were designed using the online Primer 3 software and listed in Table S1.

Immunoblotting

Total protein from human endothelial cells or mouse lung tissues from the in vivo protocol was extracted in lysis buffer. Total protein lysate was sonicated, cleared by centrifugation and the supernatant collected and protein concentration determined. Equal amounts of protein were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked before the overnight incubation in protein-specific primary antibodies to phospho-eNOS

Ser1177, eNOS VCAM-1 and β-actin, Fluorescence-coupled secondary antibodies were visualized by an infrared laser scanner.

Measurement of endothelial cell NO generation

Cellular NO production was determined using the NO-specific fluorescent 4-Amino-5-methylamino-2′,7′-difluorescein, DAF-FM reagent as previously described (40). Cells were stimulated with either Ang-(1-7) or ET-1 in the presence and absence of A779 and BQ788. Cells were exposed to Ang-(1-7), ET-1 or Mas/ET_BR interaction enhancers 1 to 4.

Measurement of plasma ET-1 levels and endothelial cell-derived ET-1

Plasma was collected from all groups in the *in vivo* protocol. In addition, culture media was collected from cells grown to 80% confluence. Plasma and cell culture media ET-1 levels were measured by ELISA according to manufacturer's instructions.

Investigating MasR:ET_BR interaction in overexpressing cell lines and peptide array analysis

To further explore the potential interaction and crosstalk between MasR and ET_BR, we studied cells overexpressing the two receptor types. MCF-7 human breast adenocarcinoma cells were transfected using Lipofectamine2000™ with pCMV6 vector Human ET_BR variant 1 with a C-terminal Myc-DDK tag or pcDNA3.1 Mas-GFP constructs (kindly provided by Prof. Michael Bader, Max-Delbruck Centre for Molecular Medicine, Berlin). Peptide array studies were carried out 24 hours after transfection.

Peptide array analysis provides a robust approach to gain insights into the basis of specific protein–protein interactions (41).

Co-Immunoprecipitation of flagged MasR and ET_BR in HEK293 cells

An additional strategy was used to further explore the putative association between MasR and ET_BR by studying receptor interaction by co-immunoprecipitation in HEK293 cells transfected with Flag-Mas1 and/or HA-ET_BR (supplementary text for details)

Culture of HEK293 cells and transfection.

HEK293 clonal cell line were used. Cells in 100 mm dish were transiently transfected with 1 μg of Flag-Mas1, HA-ET_BR or both using the polyethylenimine method. Total DNA amount was adjusted to 6 μg with empty vector (pcDNA). As a negative control, cells were transfected with only empty vector (pcDNA). Experiments were conducted 48h after transfection.

Co-immunoprecipitation. HEK293 cells in baseline conditions or pre-treated with Mas:ET_BR disruptor peptide or control peptide and then stimulated with Ang 1-7 and ET-1, alone or in combination. After stimulation, cells were washed in ice-cold PBS, scrapped in RIPA buffer, centrifuged, supernatants were collected and protein concentration determined. Cell lysates were incubated with either anti-FLAG M2 affinity gel or anti-HA affinity matrix. Proteins were eluted from beads for Western blotting. The immunoprecipitated complexes were analysed by immunoblotting.

Identifying compounds that enhance Mas/ET_BR interaction: Screening and characterization of compounds

Having synthesized peptide disruptors of MasR:ET_BR interaction, we next aimed to identify compounds that could enhance the interaction. Screening studies using fluorescence polarization were performed using a purified cytoplasmic region of MasR (GST-Mas cytoplasmic (containing the last 41 amino acids of human MasR)), GST-Mas C-terminal truncated (contains only the last 27 amino acid residues) and pGEX-4T-1 empty vector (contains GST tag only) and peptides corresponding to

ET_BR C-terminal (C^{193}ALSIDRYRA^{202}) or (L^{388}VSKRFKNCF$^{397)}$ with FITC-labelling at the N-terminal. Preparation of cells are detailed in the supplementary text.

Fluorescence polarization high-throughput screening assay to identify compounds that enhance interaction between MasR and ET_BR

High throughput screening against an in-house (University of Glasgow) drug library of 20 064 druggable compounds was carried out to evaluate MasR:ET_BR interaction using fluorescence polarization measurements. Based on peptide array analyses, the optimal interacting sequences with strong binding affinity were selected for the generation of four 10-mer probes: ET_BR (C^{193}ALSIDRYRA^{202}) and ET_BR C-terminal (L^{388}VSKRFKNCF^{397}) with or without FITC-labelling at the N-terminal. The binding of GST-MasR protein to ET_BR peptides was performed by titrating GTS-MasR proteins using a fixed concentration of ET_BR peptide (see supplemental text).

Evaluation of biological and functional effects of Mas/ET_BR enhancers

Of the over 20,000 compounds that were screened using the fluorescence polarization assay for interactions between MasR and ET_BR, 4 were identified as being most potent. We called these compounds ‘enhancers 1-4’. To determine whether candidate enhancer compounds influence vascular cell functional responses, we assessed effects of the 4 candidate compounds on production of NO in human endothelial cells.

Evaluation of effects of Mas/ET_BR enhancers on vascular function

C57BL/6 male mice (12-weeks old) were used for wire myography studies to assess vascular function in isolated intact vessels. Mesenteric resistance arteries from WT mice were dissected and mounted on an isometric wire myograph. Concentration-response curves to U46619 (thromboxane A2 analogue) were generated to evaluate vasoconstriction; while concentration-response curves to acetylcholine (Ach) and to

sodium-nitroprusside (SNP) were performed to evaluate endothelium-dependent and -independent relaxation, respectively. Arteries were preincubated for 30 minutes with 10 µmol/L of each compound (enhancers 1-4) prior to generating dose-responses curves to Ach, SNP or U46619.

Statistical analysis

Results are expressed as mean ± SEM unless otherwise stated. Statistical comparisons of parameters between groups were performed using 2-tailed Student's *t* test, or 1-way and 2-way ANOVA followed by Bonferroni's post hoc tests as appropriate. $P<0.05$ was considered statistically significant. Repeated-measures ANOVA was used for comparison of groups within vascular reactivity studies. Data analysis was conducted using GraphPad Prism 9.0.

Results

Ang-(1-7) prevents pulmonary arterial remodeling, right ventricle dysfunction and pulmonary inflammation in hypoxic mice.

Under normoxic conditions, RVSP and RVH were not different between treatment groups. In hypoxic conditions, RVSP and RVH were increased in vehicle-treated mice (Figure 1A and 1B) and attenuated in mice treated with Ang-(1-7). Mean arterial pressure (MAP) was unchanged across experimental groups (Figure 1C). Body weight was decreased in mice exposed to hypoxia compared to those maintained in normoxic conditions, effects that were unchanged by Ang-(1-7) treatment (Figure S1A).

In hypoxic conditions, mice exhibited significant vascular remodeling. The vascular structure of hypoxic mice treated with Ang-(1-7) was similar to normoxic controls indicating that Ang-(1-7) prevented vascular remodelling (Figure 1D).

Hypoxia significantly increased pulmonary artery mRNA expression of IL-1β (Figure 1E), TNFα (Figure 1F) and MCP-1 (Figure S1B), without influencing IL-6 (Figure S1C) and RANTES (Figure S1D). In Ang-(1-7)-treated hypoxic mice, levels of IL-1β, TNFα and MCP-1 were similar to control groups and significantly lower than in untreated hypoxic mice.

Ang-(1-7) prevents hypoxia-induced elevation of plasma ET-1 concentration, vascular pre-pro-ET-1 transcript levels and ET-1-induced pulmonary artery contraction.

Since ET-1 plays an important role in PH, we assessed whether Ang-(1-7) treatment influences the pulmonary ET-1 system and pulmonary arterial contraction. Hypoxia increased plasma ET-1 levels (Figure 2A) and lung pre-pro-ET-1 mRNA expression (Figure 2B). These effects were significantly reduced by Ang-(1-7) treatment. Additionally, Ang-(1-7) treatment attenuated the hypoxia-induced increase in ET_AR expression (mRNA) (Figure 2C) and caused a significant increase in pulmonary vascular ET_BR gene expression. Vascular expression of MasR was not affected by hepoxia nor by Ang-(1-7) in normoxic and hepoxic mice (Figure S2).

Intralobar pulmonary arteries from normoxic mice (140±10μm ID, n=10) were set up at equivalent pressures of 13.7±0.4 mmHg and vessels from hypoxic mice (n=10) were set up at equivalent pressures of 33.1±0.5 mmHg (42). Contractile responses to ET-1 were significantly increased in vehicle-treated hypoxic mice compared with vehicle-treated normoxic mice (Figure 2E). Ang-(1-7) reversed enhanced contractile responses to ET-1 in vessels from hypoxic mice. Hypoxia increased the sensitivity to ET-1 in vehicle-treated mice compared with normoxic vehicle-treated mice, effects that were reduced in the Ang-(1-7)-treated group.

Crosstalk between Ang-(1-7) and ET-1 leads to NO formation and involves MasR and ET_BR in human endothelial cells.

Having demonstrated *in vivo* that Ang-(1-7) has cardiovascular protective effects and that it modulates the ET-1/ET_BR system in a model of ET-1-sensitive vascular injury, we next dissected the molecular and cellular mechanisms linking these systems in human endothelial cells, where ET_BRs are abundantly expressed. Figure S3A shows that Ang-(1-7) increased mRNA expression of preproET-1 in endothelial cells, an effect blocked by A779, a Mas receptor antagonist. Increases in preproET-1 mRNA levels correlated with an increase in ET-1 production (Figure S3B), which was also inhibited by the MasR antagonist.

Ang-(1-7) and ET-1 are potent stimulators of eNOS leading to NO production. Ang-(1-7) significantly increased eNOS activation, effects that were inhibited by both A779 and BQ788 (Figure 3A). Similar to Ang-(1-7), ET-1 increased phosphorylation of eNOS in a MasR- and ET_BR-dependent manner (Figure 3A). Ang-(1-7) and ET-1 increased NO production, responses that were attenuated by L-NAME, A779 and BQ788 (Figure 3B and 3C).

Ang-(1-7)/MasR/ET_BR attenuates ET-1/ET_AR-induced VCAM-1 expression in human endothelial cells.

An important functional response of endothelial cells to ET-1 is expression of adhesion molecules such as VCAM-1. As shown in Figure S4, ET-1, but not Ang-(1-7), induced a significant increase in expression of VCAM-1. In cells exposed to both peptides, the ET-1-mediated inflammatory response was inhibited, suggesting that Ang-(1-7) negatively regulates endothelial ET-1 signaling. The inhibitory effects of Ang-(1-7) were reversed by A779 and BQ788 (Figure S4), as well as by the NOS inhibitor L-NAME and Akt inhibitor LY294022 (Figure S5A and S5B). ET-1-induced

VCAM-1 expression increase was blocked by the ET_AR antagonist, BQ123, but not by the ET_BR antagonist BQ788 (Figure S4).

Protein-protein interaction between MasR and ET_BR is important for Ang-(1-7)-induced eNOS activation.

Having demonstrated functional evidence in endothelial cells that MasR/ET_BR interactions are important for Ang(1-7) and ET-1-induced NO production and anti-inflammatory effects, we questioned whether the GPCRs, MasR and ET_BR, physically interact in endothelial cells. To investigate this interaction between the two GPCRs and to evaluate the putative binding sites we used peptide array studies in cells overexpressing MasR and ET_BR. The peptide array strategy employs a library of overlapping peptides immobilized on cellulose membranes that encompass the entirety of the human MasR sequence to specifically define the interaction sites of ET_BR interaction by overlaying lysate from cells overexpressing the ET_BR. Figure 4A shows positive binding between peptides in the cytosolic and C-terminal domains of the MasR that confer ET_BR binding.

Using sequence truncation (Figure 4B) and alanine substitution (Figure S6), we were able to further identify a defined region on the MasR responsible for ET_BR binding, which can be mapped to the MasR C-terminal, between amino acids 290-314 as shown diagrammatically in Figure 4C. We also performed reciprocal binding analysis to determine sites of ET_BR that interact with MasR. Using MasR overlays, we identified 10 spots on ET_BR where positive interactions occurred. Of these, 6 were followed up based on the localisation of their sequence within the ET_BR (i.e. transmembrane or intracellular regions), and 4 spots mapping to extracellular domains were excluded. The 6 remaining spots, as shown in Figure S7A, underwent alanine scanning analysis to further identify regions within the ET_BR conferring

specificity for MasR interaction. As shown in Figure S7B we also identified a distinct region (peptide 3 sequence: L-V-P-F-I-Q-K-A-S-V-G-I-T-V-L-S-L-C-A-L-S-I-D-R-Y) on the ET_BR responsible for MasR binding. This region maps to amino acids 176 – 200 of the human ET_BR, mapping to the third transmembrane domain and the second cytosolic loop. These sites of interaction are represented schematically (Figure 4C).

To further explore the association between MasR and ET_BR receptors, we used an additional strategy of co-immunoprecipitation (Co-IP) in HEK293 cells transfected with Flag-Mas1 and/or HA-ET_BR. As shown in figure S8A, upon immunoprecipitation of HA-ET_BR (lanes 1, 2 and 3), co-IP of Flag-Mas1 was observed only in cells co-transfected with both receptors (lane 2). Two major species of Flag-Mas1 appeared in the HA-ET_BR co-IP: one of molecular weight of around 45 kDa and another around 85 KDa (bands 3 and 2, respectively), which likely represents the mature (glycosylated) and unmature (unglycosylated) forms of the receptor (43) respectively. We also detected higher forms above 130 KDa (band 1 and above), which may represents dimers of oligomers. HA-ET_BR in the immunoprecipitates were probed with HA antibody to confirm the receptor's presence in the Co-IP (Figure S8A, lanes 4, 5 and 6). We observed 3 main species of receptors at 37 kDa, 75 kDa and around 125 kDa (bands 3, 2 and 1, respectively). Another form at 45 kDa was also predominantly detected in conditions where only HA-ETBR was expressed (lane 4), potentially representing partially modified receptors. Specificity of detection of antibodies in the Co-IP (e.g. Flag in lane 3, and HA in lane 6) were revealed by the absence of any signals in mock transfected conditions (pcDNA). Similarly, upon immunoprecipitation of Flag-Mas1 (Figure S8B, lanes 1, 2 and 3), Co-IP of HA-ET_BR was detected only in samples from cells

transfected with both receptors (Figure S8B, lane 2). We observed different species of HA-ET_BR in the immunoprecipitates with main ones around 37 kDa (band 3), and 75 kDa (band 2) and higher forms around 125 kDa (band 1). Again, we immunoprecipitated Flag-Mas1 followed by immunoblotting with anti-Flag antibody to confirm the expression of Flag-Mas1 in the different transfection conditions (Figure S8B, lanes 4, 5 and 6) and only detected signals when receptors were transfected (lanes 4 and 5). Here we detected multiple froms of the Flag-Mas1 with major species at around 45 kDa (band 3), 85 kDa (band 2) and higher forms above 130KDa (band 1). We also detected reactive forms of the receptor that were present in the Flag-Mas1's immunoprecipiates at 60-65 kDa and 120 kDa. Together, these findings suggest that Mas1 and ET_BR form complexes under baseline conditions.

We next assessed the effect of agonist stimulation on the ET_BR/MasR complex using Ang-(1-7) and ET-1. HEK293 cells co-transfected with Flag-Mas1 and HA-ET_BR were stimulatd with Ang-(1-7), ET-1 and Ang-(1-7) and ET-1 in combination and performed Flag-Mas1 (Figure 5A) and HA-ET_BR (Figure 5B) immunoprecipitation. As shown in figure 5A-B, after 15 min of stimulation of cells with Ang-(1-7), we observed an increase in the intensity of immunoreactive HA-ETBR (Figure 5A , bands 1 and 2) and Flag-Mas1 (Figure 5B, bands 1 and 2) in the co-immunoprecipitates, suggesting that Ang-(1-7) increases the complex formations between these receptors. ET1 alone or in combination with Ang-(1-7) did not produce the same effects as Ang1-7. Quantification of Co-IPs (Figure 5C-H) revealed a significant increase of higher molecular weight complexes in cells stimulated with Ang1-7 (band 1) as revealed by either the presence of HA-ETBR (Figure 5C) or Flag-MAS-1 (Figure 5F). We also observed a consistant increase in the complex formations of the intermediate molecular weight forms of HA-ETBR and

Flag-MAS following Ang1-7 stimulaion (Figure 5D and G, bands 2), although it did not reach significance. We did not observed any increase in complexes formation of the immature forms of HA-ETBR and Flag-MAS-1 following agonist stimulation (Figure 5E and H, bands 3). Together our findings suggest that Ang1-7 mediates complexe formation of Flag-MAS-1 and HA-ET_BR of only the mature forms of receptors.

Disrupting MasR:ET_BR interaction. **Disruptor peptide attenuates Ang-(1-7)-induced NOS activation in endothelial cells.**

Cell permeable stearated peptides have previously proved successful in disrupting specific protein-protein interactions (44). Based on this approach we used sequences from the ET_BR binding site on MasR (amino acids 290-314) (Figure S9) along with corresponding control peptides, to generate disruptors (and controls) of MasR:ET_BR interaction.

To test the effect of the disruptor peptide on the Mas1/ETbR interaction induced by Ang-(1-7), we performed Co-IPs of Flag-Mas1 in HEK293 cells transfected with both receptors in the presence of the disruptor or control (scrambled) peptide, and asssed the extent to which it affected ET_BR Co-IP. Co-IP experiments and their quantification (figures S9A-C), revealed that Ang-(1-7) increased Mas1/ET_BR interaction (figure S9A, left panel, lane 2 (bands 1 and 2), and figures S9B and C) as compared to vehicle treated cell (figure S9A, lane 1 and figures S9B and C), and that the scramble peptide had no effect on the complexes. However, the disruptor peptide inhibited the complexes formation induced by Ang-(1-7) (figure S9A, left panel (lane 4 vs 5) and figures S9B and C). ET-1 did not alter Flag-MAS-1/HA-ET_BR complexe formation in either the presence of control (figure S9A, left panel, lane 3, and figures S9B and C) or disruptor peptide (figure S9A, left

panel, lane 6, and figures S9B and C). No differences were observed between control and disruptor peptides in vehicle-stimulated conditions, suggesting that the disruptor peptide only inhibits Ang-(1-7)-mediated Flag-MAS-1/HA-ET_BR complexes formation.

To demonstrate that Ang-(1-7)-mediated cellular responses involve MasR:ET_BR interaction in endothelial cells, we investigated the effect of Ang-(1-7) on eNOS activation in the presence of the peptide disruptor and control peptide. As shown in Figure S10, Ang-(1-7) significantly increased eNOS phosphorylation (Ser1177), effects that were inhibited by the MasR:ET_BR disruptor peptide but not by the control peptide.

Enhancing MasR:ET_BR interaction. **Screening, identifying and validating enhancers of MasR/ET_BR interaction using an optimized fluorescence polarization high-throughput screening assay.**

Having synthesized peptide disruptors of MasR:ET_BR interaction, we next aimed to identify compounds that could enhance the interaction. Screening studies were performed using a fluorescence polarization high throughput assay system that was optimized. Details are provided in the supplemental text and Figures S11-S14. Using the optimized system, we performed a primary screen of 20,064 compounds from a bespoke in-house library of compounds built around structures of 116 molecules previously having been shown to influence protein-protein interactions. From this screen we identified 23 potential hits with at least two-fold increase in fluorescence polarisation (Figure S14C). We shortlisted 4 potential candidates that had the greatest affinity for the MasR/ET_BR complex from the initial 23 hits for further validation in the secondary screening through dose response analysis. The IC50 values were calculated upon treatment with unlabelled ET_BR at various

concentrations (Figure S15A and S15B). Furthermore, incubation of FITC-ET_BR and MasR with compounds 1-4 increased the interaction between these receptors, with the effective inhibitory concentration (IC_{50}) average of 5.9 μM (Figure S15C through S15F). We termed the 4 compounds 'enhancers 1-4'.

Enhancer compounds increase endothelial NO production and improve endothelium-dependent vasorelaxation.

To test the biological significance of enhancing MasR/ET_BR interactions, human endothelial cells were exposed to enhancers 1-4 and NO levels was evaluated. Enhancers 3 and 4 increased NO production compared to vehicle-stimulated cells, while no effects were observed for enhancers 1 and 2 (Figure 6A). Finally, we assessed whether enhancing MasR:ET_BR interaction by enhancers 1-4 leads to a functionally relevant response by studying vascular contraction and endothelium-dependent and -independent vasorelaxation in isolated mouse mesenteric arteries. As shown in Figure 6B, contractile responses to the vasoconstrictor U46619 (thromboxane A2 analogue) were reduced by all enhancers, with the most potent anti-contractile effects induced by enhancers 1 and 4. Endothelium-dependent vasorelaxation (acetylcholine-induced responses) was significantly amplified by enhancer 4 but not by teh other compounds (Figure 6C). Endothelium-independent vasorelaxation (SNP-induced responses) were unchanged by any of the enhancers (Figure S16).

To confirm that the effects of enhancer 4 were indeed mediated via MasR/ET_BR interaction, we repeated experiments with the disrupter peptide. Pre-treatment of endothelial cells with a disruptor of MasR/ET_BR interaction blocked enhancer 4-induced NO production (Figure 6D) and eNOS activation (Figure 6E).

Discussion

Vascular injury, often associated with endothelial dysfunction, is a characteristic feature of many cardiovascular diseases. Accordingly identifying processes to ameliorate endothelial damage that will promote vascular health is of clinical importance. Among the many endogenous systems implicated in vasoprotection is the counterregulatory axis of the RAAS, specifically Ang-(1-7), through, as yet ill-defined processes. Here we describe complex interplay between the Ang-(1-7) and ET-1 systems as a novel vasoprotective mechanism, involving MasR:ET_BR heterodimerization leading to eNOS activation and NO production, a key determinant of endothelial function. The MasR:ET_BR cross-talk amplifies Ang-(1-7) signaling and attenuates the detrimental effects of ET-1. Moreover, high throughput screening of a drug library, identified compounds (which we termed 'enhancers') that augment advantageous vascular actions of MasR:ET_BR interaction. Our novel data suggest that amplifying co-activity between these systems is vasoprotective. This paradigm may have therapeutic potential in conditions associated with vascular injury.

Ang-(1-7) is an important player in the RAS and has been shown to oppose adverse effects of Ang II through processes that involve inhibition of PKC, c-Src and, MAP kinases, activation of SHP2 and PLA_2 (4,45-47), stimulation of NO production (6), blunting of Ang II-induced ROS generation (4,48) and inflammation (49). Other studies demonstrated that Ang-(1-7) influences aldosterone and its mineralocorticoid receptor (50). Our data further develop this concept and provide new evidence that Ang-(1-7) networks not only with the RAAS but also with the ET-1 system, by inhibiting ET-1-induced deleterious vascular actions and promoting protective endothelial ET-1/ET_BR-mediated actions (51). Previous studies demonstrated a protective role of Ang-(1-7) in PH (18,19,52), however, a role for Ang-(1-7) through MasR modulating the ET-1 system is new. In our study, mice treated with vehicle

exhibited features of PH when exposed to hypoxic conditions, as evidenced by increased RVSP and RVH, remodelling of pulmonary arteries and increased ET-1 contraction; processes that were attenuated in Ang-(1-7)-treated mice. Clinically, worse clinical outcomes in PAH are associated with elevated circulating levels of cytokines and chemokines including interleukins, IL-1β, IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP1), RANTES, and tumour necrosis factor alpha (TNFα) (53,54). IL-6 influences the proliferation of vascular cells in PAH and, IL-1β and TNFα have been associated with accumulation of extracellular matrix proteins in plexiform lesion observed in PAH (55). In support of this, we observed increased transcript levels of IL-1β, MCP-1 and TNFα in PAH, effects ameliorated by Ang-(1-7), supporting the anti-inflammatory actions of Ang-(1-7). Exact mechanisms whereby Ang-(1-7) modulate ET-1 to have protective vascular effects are yet to be fully elucidated but we show that by interacting with ET_BR, processes involving eNOS and NO lead to vasodilation and anti-inflammatory actions.

The point of crosstalk linking the Ang-(1-7) and ET-1 systems may be through interaction between MasR and ET_BR as demonstrated in our peptide array, co-immunoprecipitation and disrupter studies. To our knowledge such an association has not been previously shown although earlier studies demonstrated that MasR dimerizes with other GPCRs, such as dopamine receptors, bradykinin receptors, and AT_1R and AT_2R (33,34,56,57). Crosstalk between MasR and Ang II receptors has been described in the cardiovascular system, where MasR:AT1R, dimerization anatagonizes Ang II actions (58,59). In CHO-K1 cells with MasR and AT1R co-expression (59), AngII-induced calcium influx was reduced, while AngII/AT_1R-induced vasoconstriction was augmented in MasR knockdown (33). Ang-(1-7) shifts Ang II-responses towards NO production (4,6), as well as for ET-1, as we

demonstrate in the present study. In addition to modulating ET-1 effects through NO-dependent pathways, we found that Ang-(1-7) stimulated an increase in the expression of ET_BR, a phenomenon associated with increased ET-1 production. Although mechanisms for *de novo* ET-1 generation are unclear, it seems that Ang-(1-7) drives the system to a vasoprotective phenotype by promoting ET-1 signaling through upregulation of ET_BR. Farhat et al. (51) showed that activation of ET_BR regulates the abundance of ET-1 mRNA in endothelial cells, a mechanism that might be operational in our system. These events may further attenuate endothelial cell inflammatory processes.

G protein-coupled receptor interaction is a cellular adaptive mechanism that increases cell responsiveness depending on the stimuli (43). The potential of interactions among receptors allows for further or even more effective signaling activation, as this phenomenon may generate an alternative structural response to ligands through allosteric regulation and/or a novel binding pocket. Since Ang(1-7) and ET-1 via MasR/ET_BR interactions lead to improved endothelial function, we focused on identification of compounds that increased MasR/ET_BR interaction. Using robust screening assays, we identified four candidates . These four had the greatest functional affinity for the MasR/ET_BR complex as determined by data from biophysical assays. Of these, two ‘enhancers’ (enhancers 3 and 4) increased generation of NO and improved vascular function. Taken together our *in vitro* and *ex vivo* studies clearly indicate that disrupting MasR:ET_BR interaction attenuates, vasoprotective actions., while enhancing receptor interaction has opposite effects.

In conclusion, we demonstrate a novel signaling network mediated through interaction between MasR and ET_BR. Our findings indicate that Ang-(1-7) negatively modulates injurious actions of ET-1 in PH a models of endothelial dysfunction and

vascular damage, and in human endothelial cells through crosstalk between MasR and ET_BR with involvement of PI3K/Akt/eNOS/NO. For the first time, we identify regions in MasR and ET_BR responsible for receptor interaction. Moreover, using high-throughput screening of a library of small molecules we discovered potential druggable compounds that enhance MasR:ET_BR interaction. While these compounds still await medicinal chemistry characterization and optimization, our observations link the Ang-(1-7) and ET-1 systems and may explain, in part, the beneficial vascular actions of Ang-(1-7) in conditions such as PH, where the ET-1 system is activated. Our findings suggest that enhancing MasR:ET_BR interaction may be a new strategy to promote vascular health and may have important therapeutic potential in cardiovascular disease.

Perspectives

Promoting cross-talk between Ang-(1-7) and ET-1 systems is vasoprotective, where enhancers amplify Ang-(1-7) signaling, upregulate protective endothelial ET-1/ET_BR-mediated actions and attenuate detrimental effects of ET-1. The present work identifies a clinically relevant strategy to induce vasoprotection in experimental models of cardiovascular disease where the ET-1 system plays a major role in injury.

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Author Contributions

Involvement in the conception, hypothesis delineation, and design of the study: ACM, YYS, LLC, YN, SAL, MRM, GSB and RMT. Acquisition of the data or the analysis and interpretation of such information ACM, JK, KYH, YYS, LLC, YN, CHC, YYS, RAL, GT, PP, SB, EG, JEF, SAL, MRM, GSB and RMT. Writing the article or substantial involvement in its revision prior to submission ACM, JK, KYH, , YYS,

LLC, YN, RAS, FRA, MRM, GSB, SAL, RMT. RMT was responsible for overall concept, supervision and funding.

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Figures Legends

Figure 1. Ang-(1-7) treatment ameliorates right ventricle pressure and hypertrophy and, pulmonary arteries injury and inflammation induced by PH. C57BL/6 mice were divided into 4 groups: normoxia treated with vehicle (V) (β-cyclodextrin) or HPβCD-Ang-(1-7) (Ang-(1-7)) 30 µg/kg/day, hypoxia treated with vehicle (V) (β-cyclodextrin) or HPβCD-Ang-(1-7) (Ang-(1-7)) 30 µg/kg/day. Animals were exposed to normoxia or hypoxia conditions for 14 days and after additional 14 days of treatment (reversal protocol), *in vivo* assessment of PH effects were performed: (**A**) Right ventricle systolic pressure (RVSP) (n=5), (**B**) Right ventricle hypertrophy (RVH – Fulton Index) (n=7). (**C**) Mean arterial pressure (MAP) (n=5). (**D**) Pulmonary vascular remodeling was assessed in distal pulmonary arteries after elastin Van Giesen staining in normoxic and hypoxic animals (100 vessels/animal) (n=7). Representative images of pulmonary arteries of each experimental group (scale bar = 50 microns). (**E**) Effect of Ang-(1-7) treatment on IL-1β mRNA levels (n=7) and (**F**) TNFα mRNA levels. (n=7). Data are expressed as mean ±SEM; * $p<0.05$ normoxia V vs. hypoxia V; † $p<0.05$ hypoxia V vs. hypoxia Ang-(1-7) after 2-way ANOVA followed by Bonferroni's post hoc test (A-C, E, F).

Figure 2. **Ang-(1-7) treatment reduces PH-induced ET-1 upregulation.** (**A)** Effect of Ang-(1-7) treatment on ET-1 plasma concentration (pg/mL) (n=5); (**B)** PreProET-1 mRNA levels (n=6); (**C)** ET_AR mRNA levels (n=6); (**D)** ET_BR mRNA levels (n=6). Data are expressed as mean ± SEM; (n=5-10/group); * $p<0.05$ normoxia V vs. hypoxia V; † $p<0.05$ hypoxia V vs. hypoxia Ang-(1-7) after 2-way ANOVA followed by Bonferroni's post hoc test (A-D). (**E**) Vascular reactivity to cumulative concentrations

of ET-1 was evaluated in intralobar pulmonary arteries. * p<0.05 normoxia V vs. hypoxia V; † p<0.05 hypoxia V vs. hypoxia Ang-(1-7) for maximal contraction to ET-1 after non-linear regression (curve fit) and repeated-measures ANOVA analysis (n=6). KCl – potassium chloride.

Figure 3. **Antagonism of MasR and ET_BR blockss eNOS activation and NO production induced by Ang-(1-7) and ET-1 in human microvascular endothelial cells. (A)** HMVECs were exposed to Ang-(1-7) or ET-1 (0.1 μM) for 15 minutes in the presence or absence of A779 (MasR antagonist) (10 μM), BQ788 (ET_BR antagonist) (10 μM) or BQ123 (ET_AR antagonist) (10 μM) and effects on phosphorylation of eNOS (serine 1177) were measured by immunoblotting (n=7). NO production was measured by DAF-FM fluorescence after Ang-(1-7) (**B**) or ET-1 (**C**) for 10 minutes in the presence or absence of L-NAME (NOS inhibitor), A779 or BQ788 (n=6 control/Ang(1-7)/ET-1; n=3 L-NAME/A779/BQ788). Data are expressed as mean ± SEM; * p<0.05 control non-stimulated HMECs (C) vs. Ang-(1-7), ET-1 or ET-1+BQ123 stimulated HMECS; † p<0.05 Ang-(1-7) or ET-1 vs. Ang-(1-7) or ET-1 in the presence of A779 or BQ788 or L-NAME after 1-way ANOVA followed by Bonferroni's post hoc test (A-C). Abbreviations: C – control non-stimulated cells; D – DMSO; E – ET-1; A1-7 – Ang(1-7); A – A779; B – BQ788; B1 – BQ123.

Figure 4. Characterization of ET_BR/MasR interaction by MasR Peptide Array. (**A**) Immobilized peptide "spots" of overlapping 25-mer peptides each shifted along by five amino acids in the entire human MasR sequence probed for interaction with human ET_BR and detection by immunoblotting. Coomassie-stained loading control was used. Sequences in bold were identified as positive interactions. Data are representative of at least 3 separate experiments. (**B**) Truncation of ET_BR:MasR region of interest. MasR region of interest residue truncation analysis. Data is a representative of at least 3 separate experiments. (**C**) Schematic representation of ET_BR/MasR potential biding sites. MasR arrays followed by site-directed mutagenesis identified C-terminal amino acids residues 290-314 as being involved in the association/biding of MasR with ET_BR. Reciprocal binding analysis using ET_BR arrays followed by alanine scanning, indicated that residues 176-200 mapped the site of interaction within the ET_BR.

Figure 5. Ang-(1-7) increases ET_BR and MasR interaction in HEK293 cells co-transfected with Flag-Mas1 and HA-ET_BR. Co-immunoprecipitation (IP) of Flag (**A**) or HA (**B**) was performed in HEK293 cells co-transfected with Flag-Mas1 and HA-ET_BR. Immunoprecipitates were incubated with anti-flag antibody (WB: Flag) or anti-HA antibody (WB: HA). HEK293 cells co-transfected with Flag-Mas1 and HA-ET_BR were stimulated with Ang-(1-7) (100 nM) (lanes 2 and 6), ET-1 (100 nM) (lanes 3 and 7) or Ang-(1-7) (100 nM) and ET-1 (100 nM) in combination (lanes 4 and 8). Quantification of band 1, band 2 and band 3 was performed for Flag IP (**C**-**E**) and HA IP (**F**-**H**). Immunoblots are representative of 3 separate experiments. Quantification data are expressed as mean ± SEM; * $p<0.05$ vs Ctl.

Figure 6. Enhancers of MasR/ET_BR interaction induce NO production and regulate vascular function. (**A**) NO production in human endothelial cells after exposure to ET_BR/MasR interaction enhancers (1-4) (10 µM) for 10 minutes (n=11). Data are expressed as mean ± SEM; * $p<0.05$ vehicle DMSO vs. Enhancer 3 and 4 after 1-way ANOVA followed by Bonferroni's post hoc test. Assessment of vascular functional responses in mesenteric resistance arteries from C57BL/6 mice was performed by wire myography in the presence or absence of ET_BR/MasR interaction enhancers (1-4) (10 µM). (**B**) Maximum contractile response to U46619 (% of KCl response); data are expressed as mean ± SEM; * $p<0.05$ vehicle DMSO vs. Enhancer 3; † $p<0.05$ vehicle DMSO vs. Enhancer 1 and 4 after 1-way ANOVA followed by Bonferroni's post hoc test (n=10). (**C**) Relaxation in response to acetylcholine (Ach); data are expressed as mean ± SEM; * $p<0.05$ vehicle DMSO vs. Enhancer 1; † $p<0.05$ vehicle DMSO vs. Enhancer 4 after 1-way ANOVA followed by Bonferroni's post hoc test (n=5). **(D)** NO production and **(E)** eNOS activation (phosphorylation of Ser1177) in human endothelial cells after exposure to enhancer 4 in the presence or absence of MasR/ETBR receptor interaction disruptor (n=5). Data are expressed as mean ± SEM; * $p<0.05$ vehicle DMSO vs. Enhancer 3 and 4 after 1-way ANOVA followed by Bonferroni's post hoc test.

Novelty and Relevance

What Is New?

Using *in vivo*, *ex vivo* and *in vitro* models we define new pathways linking Ang-(1-7)/MasR and ET-1/ET_BR. Our study demonstrates physical and functional interactions between MasR and ET_BR. To our knowledge, we characterize for the first time sites of interaction between MasR and ET_BR. We provide evidence for targeted druggable compounds, which enhance MasR:ET_BR interaction with beneficial vascular effects.

What Is Relevant

MasR/ET_BR interaction is associated with production of NO leading to vasorelaxation and anti-inflammatory actions. Enhancing co-activity between these systems with drug-like small molecules amplifies these vasoprotective effects through upregulation of endothelial Ang(1-7)/MasR/ET-1/ET_BR signaling and downregulation of of injurious actions of ET-1

Summary

Our findings suggest thatMasR:ET_BR interaction may be a novel approach to promote vascular health. This strategy may have therapeutic potential to improve endothelial dysfunction and vascular damage in cardiovascular diseases

Figure 1

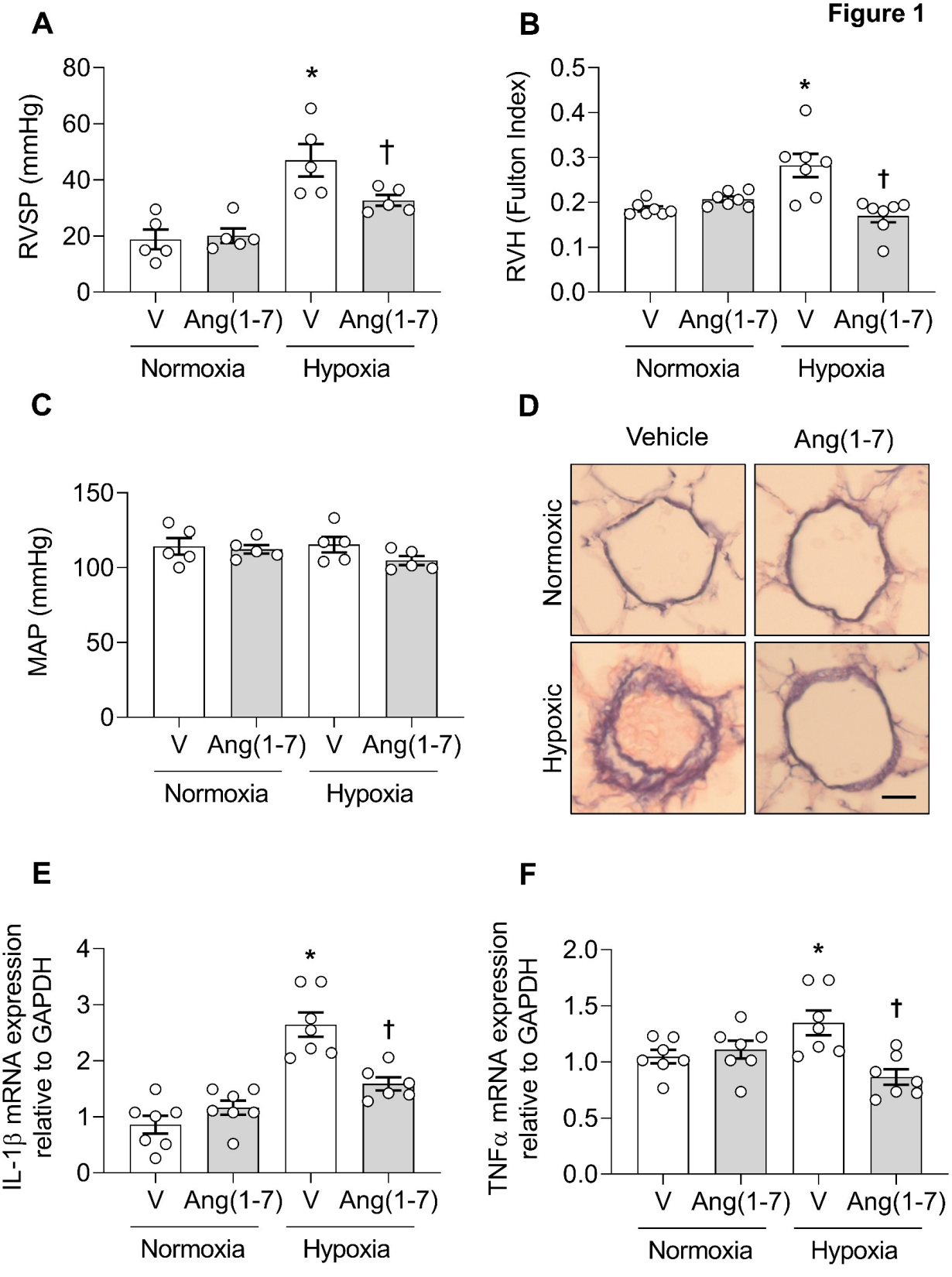

A
RVSP (mmHg)
80
60
40
20
0
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
B
RVH (Fulton Index)
0.5
0.4
0.3
0.2
0.1
0.0
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
C
MAP (mmHg)
150
100
50
0
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
D
Vehicle
Ang(1-7)
Normoxic
Hypoxic
E
IL-1β mRNA expression relative to GAPDH
4
3
2
1
0
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
F
TNFα mRNA expression relative to GAPDH
2.0
1.5
1.0
0.5
0.0
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia

Figure 2

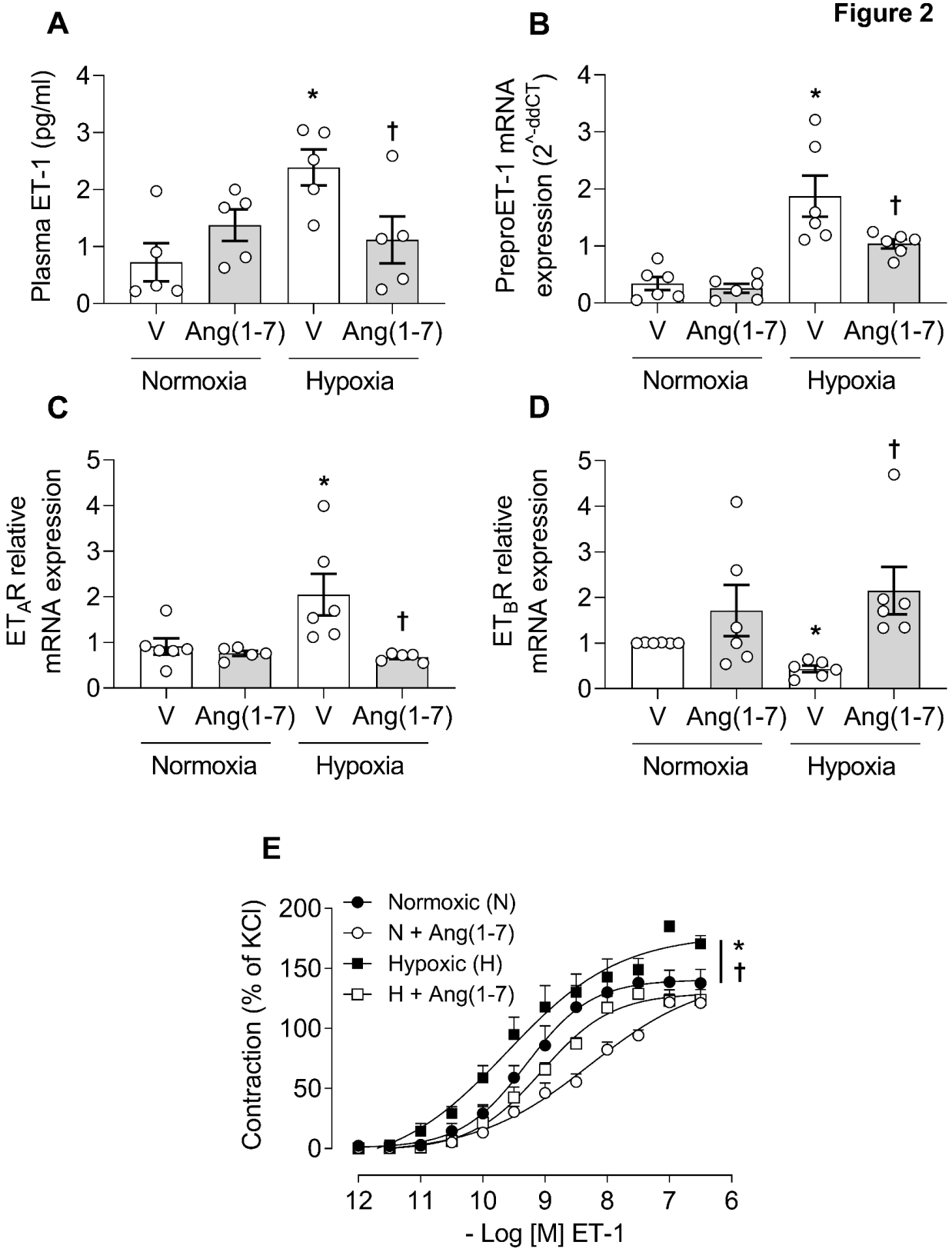

A
Plasma ET-1 (pg/ml)
0
1
2
3
4
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
B
PreproET-1 mRNA expression (2^-ddCT)
0
1
2
3
4
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
C
ETAR relative mRNA expression
0
1
2
3
4
5
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
D
ETBR relative mRNA expression
0
1
2
3
4
5
*
†
V
Ang(1-7)
V
Ang(1-7)
Normoxia
Hypoxia
E
Contraction (% of KCl)
0
50
100
150
200
Normoxic (N)
N + Ang(1-7)
Hypoxic (H)
H + Ang(1-7)
*
†
12
11
10
9
8
7
6
- Log [M] ET-1

Figure 3

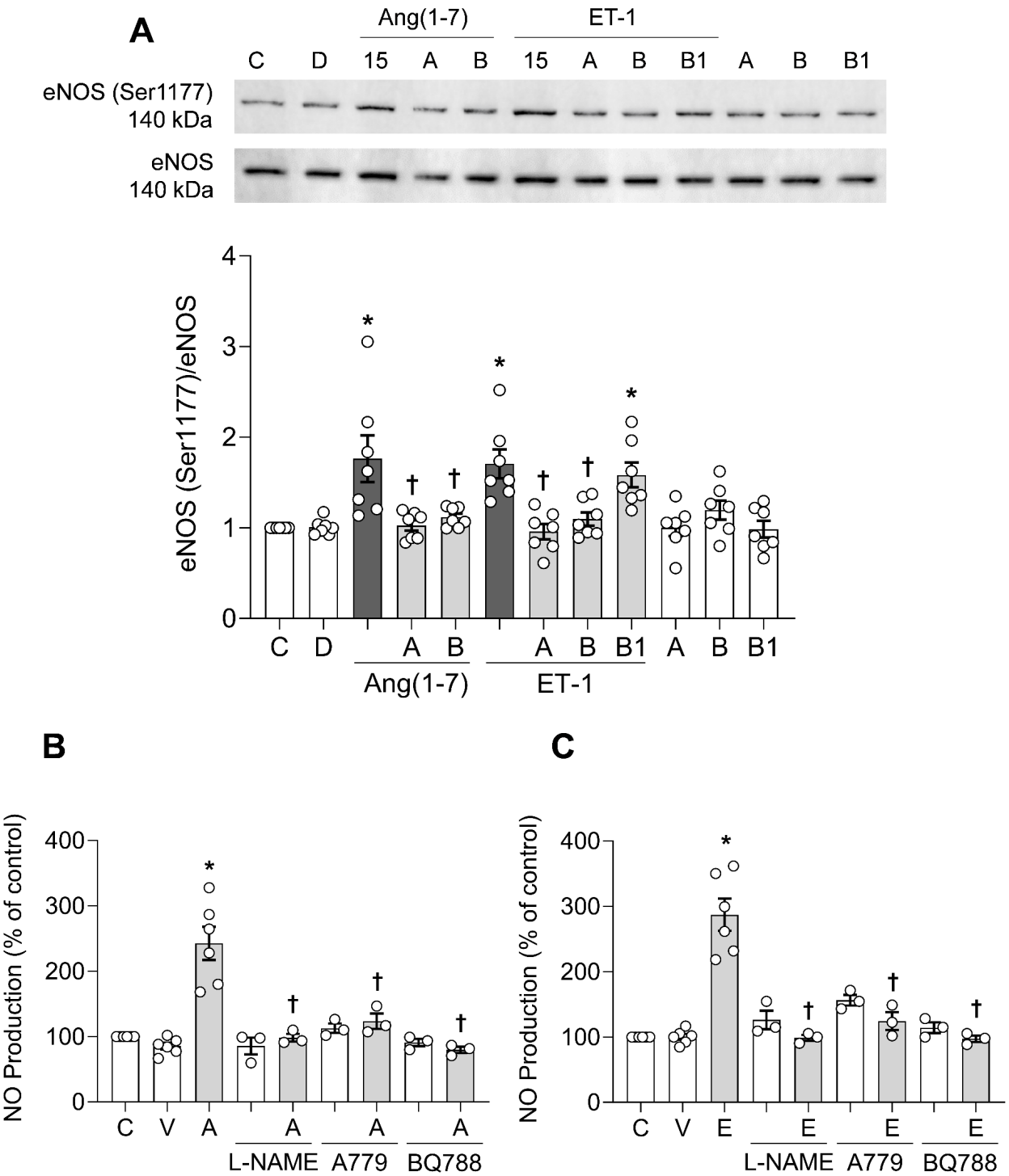
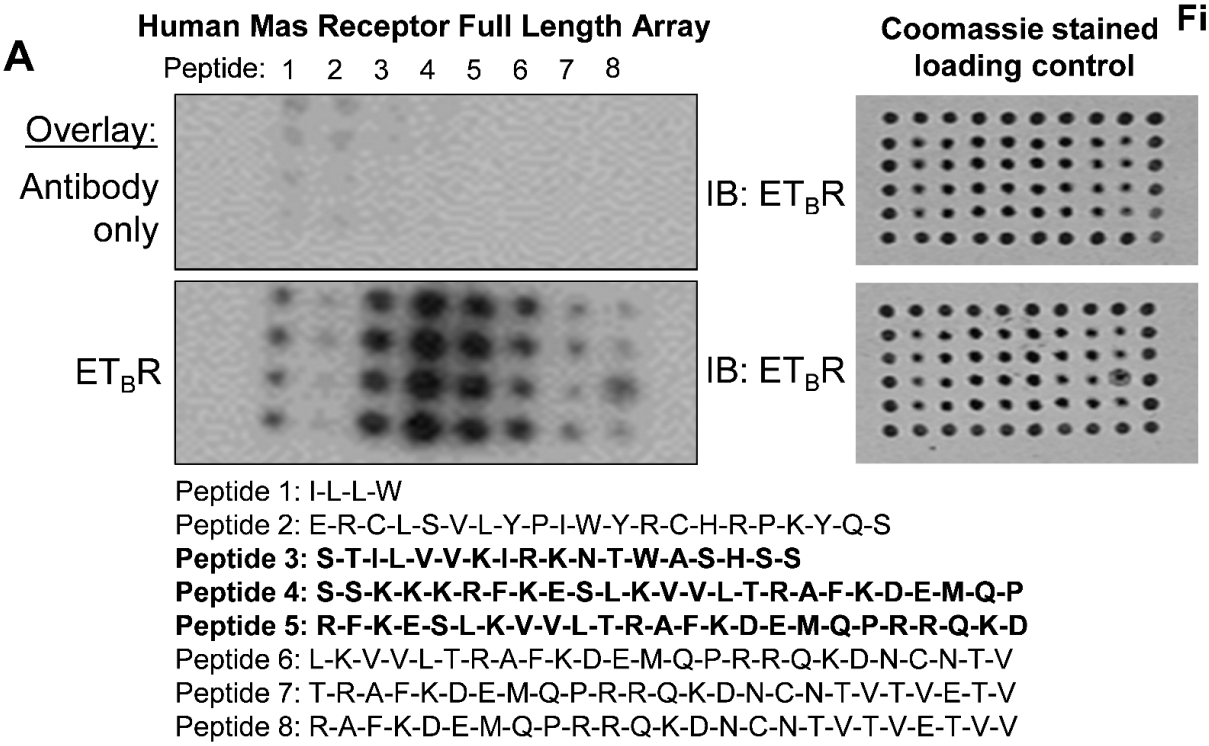

A
Ang(1-7)
ET-1
C D 15 A B 15 A B B1 A B B1
eNOS (Ser1177)
140 kDa
eNOS
140 kDa
eNOS (Ser1177)/eNOS
0 1 2 3 4
C D A B A B B1 A B B1
Ang(1-7)
ET-1
B
NO Production (% of control)
0 100 200 300 400
C V A A A A
L-NAME A779 BQ788
C
NO Production (% of control)
0 100 200 300 400
C V E E E E
L-NAME A779 BQ788

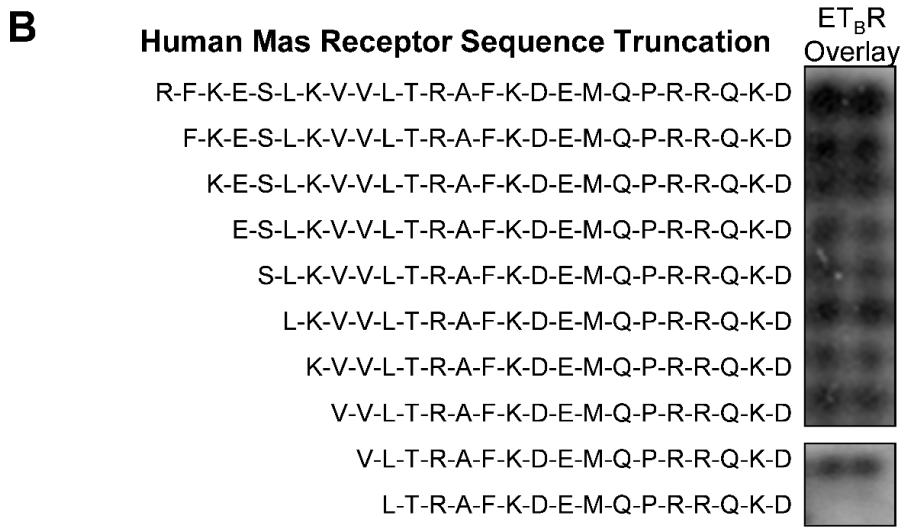
Figure 4

A



Peptide 1: I-L-L-W
Peptide 2: E-R-C-L-S-V-L-Y-P-I-W-Y-R-C-H-R-P-K-Y-Q-S
Peptide 3: S-T-I-L-V-V-K-I-R-K-N-T-W-A-S-H-S-S
Peptide 4: S-S-K-K-K-R-F-K-E-S-L-K-V-V-L-T-R-A-F-K-D-E-M-Q-P
Peptide 5: R-F-K-E-S-L-K-V-V-L-T-R-A-F-K-D-E-M-Q-P-R-R-Q-K-D
Peptide 6: L-K-V-V-L-T-R-A-F-K-D-E-M-Q-P-R-R-Q-K-D-N-C-N-T-V
Peptide 7: T-R-A-F-K-D-E-M-Q-P-R-R-Q-K-D-N-C-N-T-V-T-V-E-T-V
Peptide 8: R-A-F-K-D-E-M-Q-P-R-R-Q-K-D-N-C-N-T-V-T-V-E-T-V-V

B



C

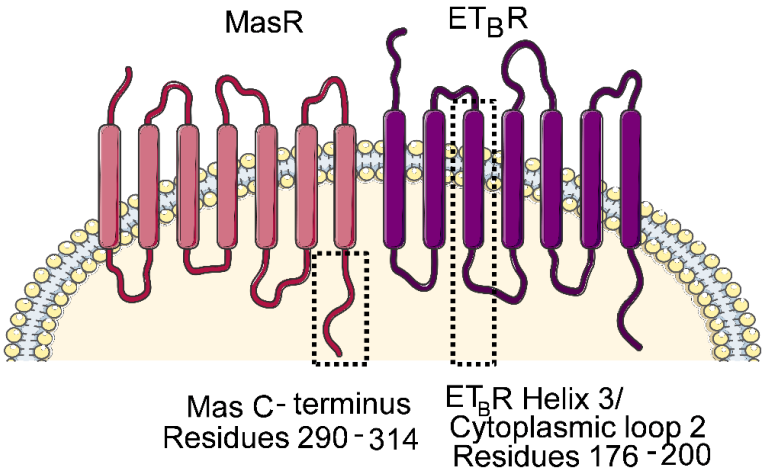
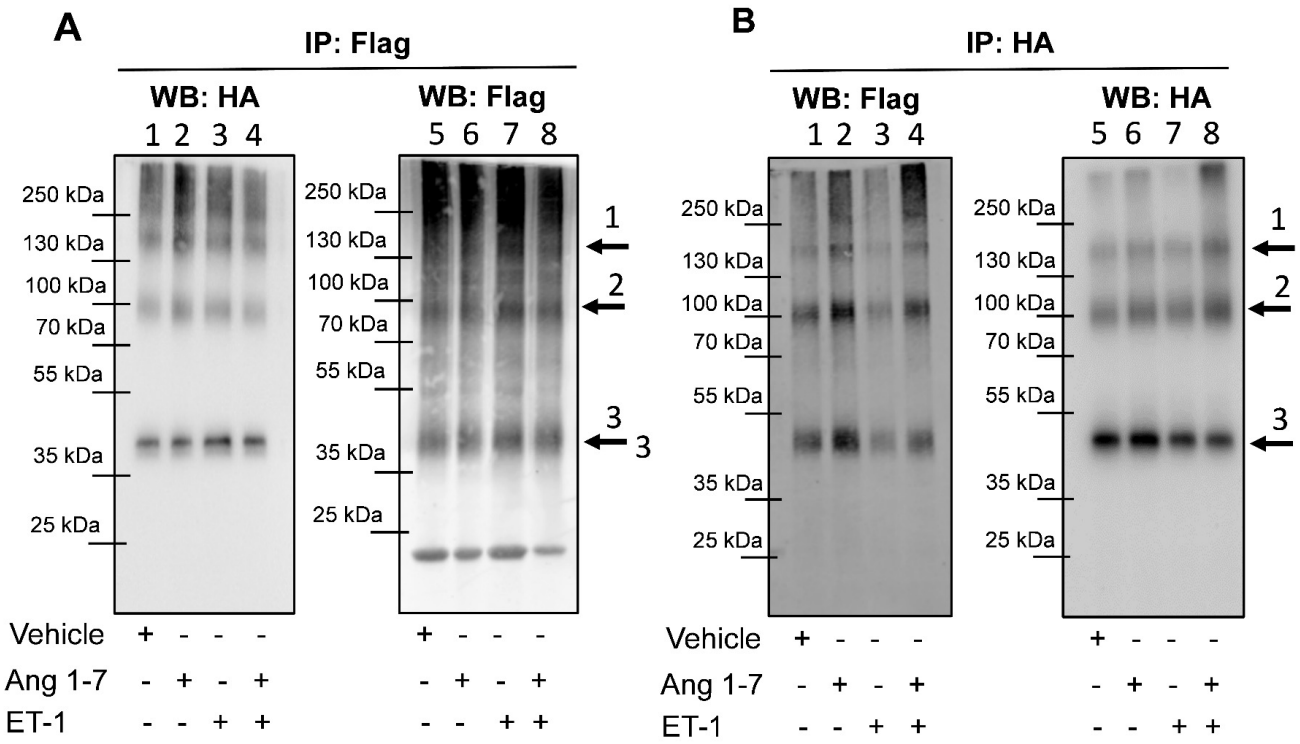
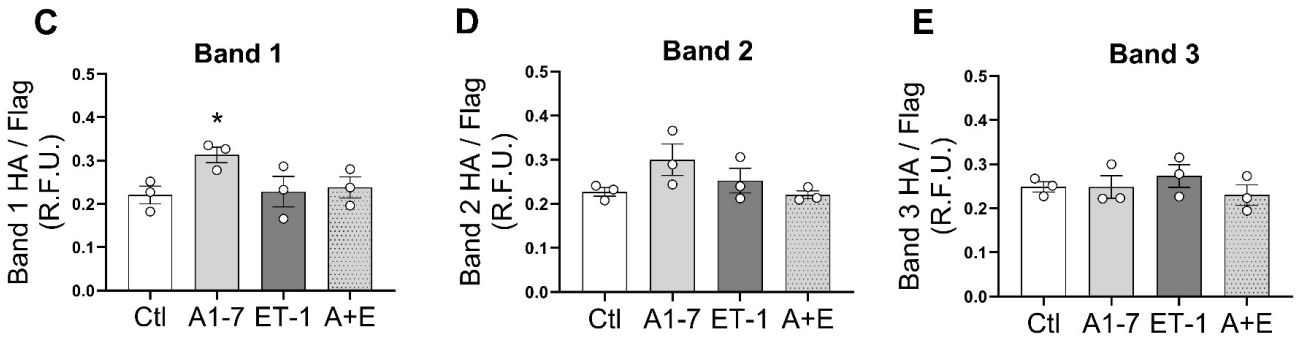


Figure 5



IP: Flag WB: HA



IP: HA WB: Flag

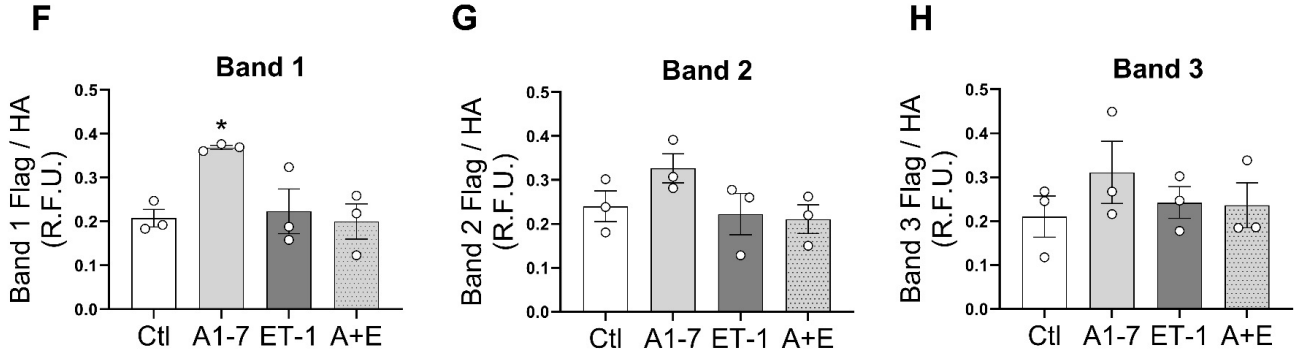
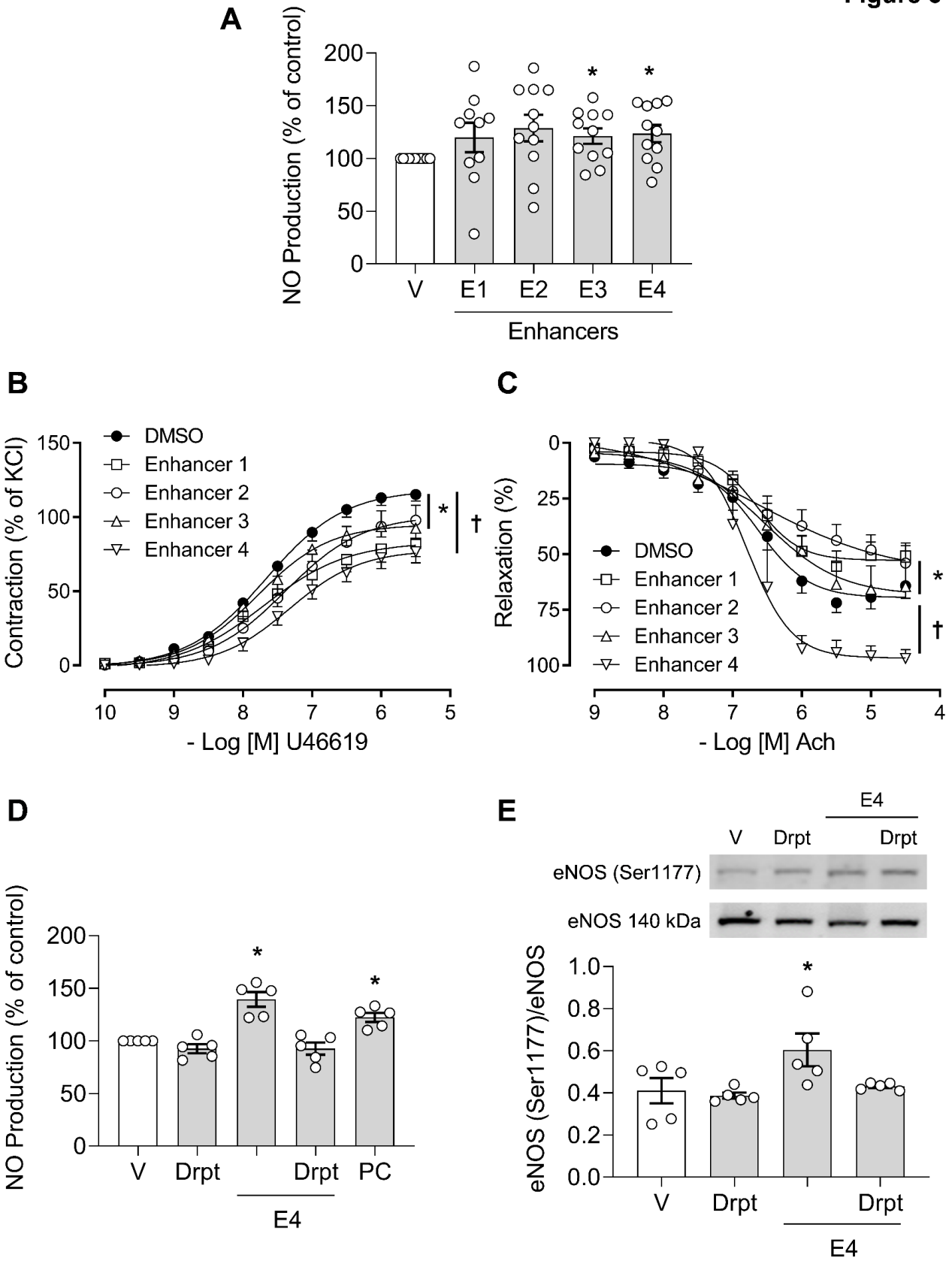


Figure 6


A
NO Production (% of control)
200
150
100
50
0
V
E1
E2
E3
E4
Enhancers
*
*
B
Contraction (% of KCl)
150
100
50
0
DMSO
Enhancer 1
Enhancer 2
Enhancer 3
Enhancer 4
*
†
10
9
8
7
6
5
- Log [M] U46619
C
Relaxation (%)
0
25
50
75
100
DMSO
Enhancer 1
Enhancer 2
Enhancer 3
Enhancer 4
*
†
9
8
7
6
5
4
- Log [M] Ach
D
NO Production (% of control)
200
150
100
50
0
*
*
V
Drpt
Drpt
PC
E4
E
E4
V
Drpt
Drpt
eNOS (Ser1177)
eNOS 140 kDa
eNOS (Ser1177)/eNOS
1.0
0.8
0.6
0.4
0.2
0.0
*
V
Drpt
Drpt
E4