Elabela/Toddler is an endogenous agonist of the apelin APJ receptor in the adult cardiovascular system, and exogenous administration of the peptide compensates for the downregulation of its expression in pulmonary arterial hypertension

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Short title: Down-regulation of Elabela/Toddler in PAH

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Abstract

Background. Elabela/Toddler (ELA) is a critical cardiac developmental peptide that acts through the G protein-coupled apelin receptor, despite lack of sequence similarity to the established ligand apelin. Our aim was to investigate the receptor pharmacology, expression pattern and *in vivo* function of ELA peptides in the adult cardiovascular system, to seek evidence for alteration in pulmonary arterial hypertension (PAH) in which apelin signaling is down-regulated, and to demonstrate attenuation of PAH severity with exogenous administration of ELA in a rat model.

Methods. *In silico* docking analysis, competition binding experiments and down-stream assays were used to characterize ELA receptor binding in human heart and signaling in cells expressing the apelin receptor. ELA expression in human cardiovascular tissues and plasma was determined using RT-qPCR, dual-labelling immunofluorescent staining and immunoassays. Acute cardiac effects of ELA-32 and [Pyr^{1}]apelin-13 were assessed by magnet resonance imaging and cardiac catheterization in anesthetized rats. Cardiopulmonary human and rat tissues from PAH patients and monocrotaline (MCT) and Sugen/hypoxia exposed rats were used to show changes in ELA expression in PAH. The effect of ELA treatment on cardiopulmonary remodeling in PAH was investigated in the MCT rat model.

Results. ELA competed for binding of apelin in human heart with overlap for the two peptides indicated by *in silico* modeling. ELA activated G protein- and β-arrestin-dependent pathways. We detected ELA expression in human vascular endothelium and plasma. Comparable to apelin, ELA increased cardiac contractility, ejection fraction, cardiac output and elicited vasodilatation in rat *in vivo*. ELA expression was reduced in cardiopulmonary tissues from PAH patients and PAH rat models, respectively. ELA treatment significantly attenuated elevation of right ventricular systolic pressure and right ventricular hypertrophy and pulmonary vascular remodeling in MCT exposed rats.

Conclusions. These results show ELA is an endogenous agonist of the human apelin receptor, exhibits a cardiovascular profile comparable to apelin, is down-regulated in human disease and rodent PAH models and exogenous peptide can reduce the severity of cardiopulmonary remodeling and function in PAH in rats. This study provides additional proof of principle that an apelin receptor agonist may be of therapeutic use in PAH in man.

Keywords: Apelin, Elabela/Toddler, Pulmonary Arterial Hypertension, GPCR, Cardiopulmonary

What Is New?

• Elabela/Toddler (ELA) was first identified as an essential peptide in development of the heart in zebrafish, and proposed as a second endogenous ligand at the apelin receptor.

• We have now shown that ELA is widely expressed in the adult human cardiovascular system, localizing to the vascular endothelium and plasma.

• ELA peptides activated G protein- and β-arrestin-dependent pathways with comparable potency to apelin and, crucially, these actions were blocked by apelin receptor antagonists.

• ELA increased cardiac contractility, ejection fraction and cardiac output and elicited vasodilatation in anaesthetized rats *in vivo*.

What are the Clinical Implications?

• Apelin is known to be downregulated in human and animal models of PAH.

• We have demonstrated that ELA did not compensate for the loss of apelin as its expression was also significantly reduced in cardiopulmonary tissues from human PAH patients and in rat models of PAH.

• Treatment with exogenous ELA attenuated right ventricular hypertrophy, systolic pressure and pulmonary vascular remodeling in the monocrotaline rat model of PAH.

• The results suggest that a selective ‘first-in-class’ agonist that mimics the action of the endogenous ligands apelin/ELA is a promising therapeutic strategy in the treatment of PAH distinct from pathways targeted by current clinical treatments.

Introduction

The apelin family of peptides interact with a G protein-coupled receptor named the apelin receptor or APJ. Apelin peptides have an emerging role in the adult cardiovascular system[1] and in embryonic development of the heart[2], and [Pyr[1]]apelin-13 is the most abundant endogenous apelin peptide in human heart[3]. Alteration in the apelin system is thought to contribute to etiology of cardiovascular diseases such as pulmonary arterial hypertension (PAH), a devastating disease with pulmonary vascular remodeling leading to death from right ventricular failure in which a beneficial effect of enhancing apelin receptor signaling has been proposed[1].

Recently, two groups independently identified a well conserved gene encoding a peptide named elabela (ELA)[4] or toddler[5], required for early cardiac development in zebrafish. Intriguingly, the gene (*APELA*) was identified in a region not previously annotated as coding DNA. The *APELA* gene was predicted to express a 54 amino acid protein comprising a 32 amino acid mature peptide (ELA-32). Loss of this gene resulted in a rudimentary or no heart in fish embryos, a phenotype similar to loss of the gene *APLNR* encoding the apelin receptor. Both *APELA* and *APLNR* genes are expressed before gastrulation whereas crucially the established ligand for this receptor, apelin (gene *APLN*), is not present and only expressed later in development. In agreement, deletion of the apelin gene did not produce the same phenotype as deletion of the apelin receptor gene suggesting the presence of a second ligand such as ELA. In support, ELA was demonstrated to internalize the apelin receptor *in vitro*, and activation of the apelin signaling pathway was shown to rescue *APELA* mutants. It is interesting to speculate that ELA might be the first in a series of yet uncharacterized developmental signals[4,5]. From these studies the existence of three peptides was proposed: ELA-32, ELA-21 and ELA-11 (Figure 1A).

Our aim was to investigate the receptor pharmacology, expression pattern and *in vivo* function of ELA peptides in the normal adult cardiovascular system and to seek evidence for alteration in PAH. Using *in silico* molecular modeling and docking we propose a binding mode of ELA to the apelin receptor which is consistent with competition binding experiments, where the binding affinity of ELA was determined in human heart, a clinically relevant target. We have used cell-based pharmacological assays to show that ELA peptides activated the apelin receptor to inhibit cAMP production, induce β-arrestin recruitment and receptor internalization. Results from RT-qPCR experiments indicated the presence of *APELA* mRNA in human blood vessels with immunofluorescence staining confirming presence of mature ELA peptide localized to the vascular endothelium. ELA peptide was also detectable in human plasma. The acute cardiovascular effects of ELA in rat *in vivo* included increased cardiac contractility, ejection fraction and cardiac output and additionally vasodilatation. Immunostaining and RT-qPCR showed reduced expression in cardiopulmonary tissues from human PAH patients and two rat models of PAH, respectively. Crucially, we have demonstrated that ELA can attenuate the severity of changes in cardiopulmonary function/histology in the monocrotaline (MCT) rat model of PAH *in vivo*.

Methods

The Methods section in the online-only Data Supplement provides an expanded description of all experimental protocols. Human tissues samples were obtained with informed consent (Papworth Hospital Research Tissue Bank REC08/H0304/56) and local ethical approval (REC05/Q0104/142). All rodent experiments were performed according to the local ethics committee (University of Cambridge Animal Welfare and Ethical Review Body) and Home Office (UK) guidelines under the 1986 Scientific Procedures Act.

Computational Methods

Docking of ELA-11 and the apelin receptor was conducted using the homology model of apelin and the GOLD docking algorithm as described previously[6].

Competition Binding

Experiments were conducted in homogenate of human ventricle or CHO-K1 cells expressing the human apelin receptor. Affinity (pK_i) and receptor density (B_{MAX} fmol/mg) for ELA-32, ELA-21 and ELA-11 in human left ventricle (LV) were compared by one-way ANOVA, with Tukey's multiple comparison. Data (pK_i and B_{MAX}) for ELA-21 were compared between control LV and PAH LV and between control right ventricle (RV) and PAH RV by 2-tailed Student's *t*-test.

Cell-Based Assays

Inhibition of cAMP accumulation, β-arrestin recruitment and receptor internalization assays were used to determine values of potency (pD_2 ($-\log_{10} EC_{50}$)) and maximum response (E_{MAX}). pD_2 Values were compared using one-way ANOVA with Tukey's multiple comparison

Protein phosphorylation levels for an array of down-stream signalling enzymes and levels of secreted angiogenic factors were determined using pulmonary artery endothelial (PAECs) and smooth muscle (PASMCs) cells treated with 0.1% serum, [Pyr^{1}]apelin-13 or ELA-32 (both 100nmol/L). Data were analysed by one-way ANOVA for matched data with Tukey's multiple comparison.

RT-qPCR

RNA extraction, reverse transcription and quantitative real-time PCR were performed as described in the Supplemental file. For primer sequences see Supplemental Table 1.

Immunostaining

Immunostaining was performed[7] to localize ELA expression in human normal and PAH tissues using ELA antiserum that cross reacted with ELA peptides, but not apelin (Supplemental Figure 1), and to assess the effect of ELA-32 treatment on pulmonary vascular remodeling and cardiomyocyte hypertrophy in the MCT rat model as described in the Supplemental file.

Enzyme Immunoassays

Levels of ELA and apelin in healthy human plasma (n=25) were measured using enzyme immunoassays, compared with Student's t test and the correlation coefficient (Pearson's r) was determined.

MRI and Catheterization

The acute cardiac effects of ELA-32 and [Pyr^{1}]apelin-13 were assessed by magnet resonance imaging (MRI) and cardiac catheterization. MRI was performed in male Sprague Dawley rats (264±2g) anaesthetized with isoflurane (1.5-2.5%, inhaled). Peak effects of ELA-32, [Pyr^{1}]apelin-13 and saline on ejection fraction were expressed as change from baseline and compared by one-way ANOVA with Dunnett's post-test.

In a second study, a pressure volume catheter was inserted to monitor LV hemodynamics in male Sprague Dawley rats (257±7g) anaesthetized with isoflurane (1.5% inhaled). Peak effects of ELA-32, [Pyr^{1}]apelin-13 and saline on LV systolic pressure, cardiac output, stroke volume, contractility (dP/dt_{MAX}) and heart rate were expressed as change from baseline and compared by one-way ANOVA with Dunnett's post-test[8].

Monocrotaline-Induced Rat Model of PAH

Male Sprague-Dawley rats (205±2g) were injected subcutaneously with MCT (60mg/kg, n=18) or 0.9% saline (n=17) on day 0. MCT (n=9) or saline (n=9) exposed animals received daily intraperitoneal injections of ELA-32 (450μg/kg) with the remainder (MCT, n=9; saline, n=8) receiving saline. On day 21, RV hemodynamics, RV hypertrophy and pulmonary vascular remodeling were assessed and group data compared using one-way ANOVA with Tukey's post-test. The effect of chronic ELA administration on systemic blood pressure was investigated by LV catheterization in ELA control and saline control animals (n=5 each group) as described above.

Statistical Analyses

Data are expressed as mean±sem. Data and statistical analyses were conducted in GraphPad Prism 6. $P \leq 0.05$ was considered statistically significant.

Results

ELA Binds to the Apelin Receptor in Human Normal and PAH Heart

Structural alignment of ELA-11 and apelin-13 docked to the apelin receptor indicated they share a significant hydrophobic binding derived from the presence of the C-terminal hydrophobic moiety in a complimentary hydrophobic pocket of the apelin receptor. However, ELA-11 lacks positively charged residues directly corresponding to the important N-terminal "RPRL" motif in apelin-13 required for initial recognition of the peptide, although interestingly there are positively charged amino acids in this region in the longer ELA sequences (Figure 1A). We have recently described a possible pose of apelin-13 interacting with the apelin receptor cavity[6]; given the similarities of the C-terminus we assumed that the pose of this region might be comparable for ELA-11. It has been hypothesized that F10 on ELA-11 may assume a similar pose to F13 on apelin-13[6] and our model indicated that the binding cavity is large enough to accept the additional C-terminal hydrophobic proline (P11) of ELA-11. Following docking analysis the pose that consistently showed the best GOLD docking score is shown in Figure 1B. ELA-11 and apelin-13 showed a high degree of overlap in the binding site (Figure 1C). The ELA-11 pose obtained after docking analysis showed a large number of interactions with the apelin receptor (Figure 1D), mainly involving the hydrophobic residues in the N-terminal and C-terminal section of the peptide (Figure 1E) but also including hydrogen-bonds.

In human LV ELA-32 (pK_i=9.59±0.08) had significantly ($P \leq 0.0001$) higher affinity than ELA-21 (pK_i=8.52±0.11) and [Pyr^1]apelin-13 (pK_i=8.85±0.04) which were comparable. In contrast ELA-11 (pK_i=7.85±0.05) had significantly lower affinity than the other peptides ($P \leq 0.01$) (Figure 2A) suggesting a longer sequence with positively charged residues in the N-terminus is required for optimal binding affinity. This is supported by data for the extended

peptide ELA-14 that competed for binding in CHO-K1 cells with sub-nanomolar affinity (pK_i=9.35±0.02) whereas cyclo[1-6]ELA-11 had lower affinity as expected (pK_i=7.27±0.03).

ELA-21 bound with comparable affinities in RV and LV from human normal and PAH hearts (RV, normal pK_i=8.98±0.04, PAH pK_i=9.30±0.07; LV, normal pK_i=9.31±0.11, PAH pK_i=9.46±0.10) (Figure 2B). In PAH heart, compared to control there was a small (~15%) but significant reduction in apelin receptor density in both LV (PAH 3.42±0.15 fmol/mg, normal 3.96±0.08 fmol/mg; $P \leq 0.05$) and RV (PAH 3.5±0.05 fmol/mg, normal 4.24±0.03 fmol/mg, $P \leq 0.001$).

Receptor Pharmacology of ELA *in vitro*

ELA-32, ELA-21, ELA-11 and [Pyr^1]apelin-13 completely inhibited forskolin-induced cAMP production in a concentration-dependent manner (Figure 3A) with sub-nanomolar potencies (Table 1). The potency of ELA-11 was comparable to the longer ELA peptides indicating that this short sequence retains full biological activity in this G protein-coupled assay.

ELA-32, ELA-21, ELA-11 and [Pyr^1]apelin-13 stimulated β-arrestin recruitment in a concentration-dependent manner (Figure 3B) with similar efficacy. ELA-32 and ELA-21 exhibited comparable pD_2 values and were significantly more potent than ELA-11. A similar rank order of potency was obtained in the internalization assay (Figure 3C), suggesting that a longer sequence is required for optimal activation of the β-arrestin pathway (Table 1). ML221 (Tocris, Bristol, UK) antagonized the concentration-response curves to ELA-32 (Figure 3D) and [Pyr^1]apelin-13 (Figure 3E) in the β-arrestin recruitment assay with comparable pA_2 values of 6.87 and 6.91, respectively. ELA-14 (pD_2=1.09±0.12) and

cyclo[1-6]ELA-11 (pD_2=9.42±0.32) exhibited sub-nanomolar potency in the cAMP assay (Figure 3F) comparable to the other ELA peptides. In β-arrestin ELA-14 (pD_2=9.08±0.04) was equipotent with ELA-32 and cyclo[1-6]ELA-11 (pD_2=7.67±0.02) had comparable potency to ELA-11 (Figure 3G).

In control PAECs and control and PAH PASMCs apelin and ELA-32 increased levels of ERK1/2 phosphorylation and in PAECs there was also a significant increase in phosphorylation of eNOS (Supplemental Figures 2A, 2B, 2C, 2D). Phosphorylation levels of other kinases (Supplemental Figure 3) and levels of secreted angiogenic factors (Supplemental Figures 4 and 5) were unaffected.

ELA is Expressed in Human Cardiovascular Tissues

Expression of *APELA* transcript was observed in all human blood vessels investigated (Figure 4A). Excepting the aorta, there was a trend for *APELA* expression to be higher in arteries compared to veins. Lower levels were detectable in heart and lung (not shown).

Punctate staining of ELA-like immunoreactivity (–LI) and vWF-LI were observed in human PAECs but not co-localized to the same intracellular vesicles (Figure 4B). ELA-LI was found in the intima of coronary (Figure 4C) and mammary artery (Figure 4D), and co-localized with vWF. Endothelial ELA-LI was detectable in blood vessels in heart (Figure 4E) and lung (Figure 4F, Supplemental Figure 6). No ELA-LI was observed in smooth muscle cells or cardiomyocytes.

ELA and apelin were detectable in healthy human plasma at 0.34±0.03nmol/L and 0.26±0.03nmol/L, respectively (Figure 4G) with significantly higher levels of ELA than

apelin (P≤0.05). There was a weak, but significant (P≤0.05), positive correlation between ELA and apelin peptide levels (Pearson's r=0.47) (Figure 4H).

Cardiovascular Effects of ELA in Rodents *in Vivo*

ELA-32 and [Pyr^{1}]apelin-13 directly strengthened cardiac contractions *in vivo*, as illustrated in the representative MRI videos (Supplemental Videos 1 and 2) and snapshots (Figure 5A, 5B). ELA-32 and [Pyr^{1}]apelin-13 dose-dependently increased LV and RV ejection fraction (Figure 5C, 5D). Low dose, ELA-32 (20nmol) and [Pyr^{1}]apelin-13 (50nmol) increased ejection fraction by 10.3±0.7% (P≤0.0001) and 4.9±1.4% (P>0.05) in LV, respectively, compared to saline control (2.0±0.6%) and by 8.0±1.5% (P⩽0.001) and 4.4±0.5% (P>0.05) in RV, respectively, compared to saline control (1.0±0.8%). High dose, ELA-32 (150nmol) and [Pyr^{1}]apelin-13 (650nmol) significantly increased ejection fraction in LV by 13.5±1.7% (P≤0.05) and 15.8±3.6% (P≤0.01), respectively, compared to saline control (1.9±1.0%) and in RV by 9.0±1.8% (P⩽0.05) and 8.7±1.0% (P⩽0.05), respectively, compared to saline control (1.2±1.7%). Effects were short-acting and returned to baseline after 10 minutes for the low dose, but still present at 10 minutes for the high dose. ELA-32 appeared more potent than [Pyr^{1}]apelin-13 as lower doses of ELA-32 were able to achieve equivalent or more pronounced effects as [Pyr^{1}]apelin-13.

In the second rat study ELA-32 (20nmol) and [Pyr^{1}]apelin-13 (50nmol) caused a rapid increase in dP/dt_{MAX} of 1217± 272 mmHg/s (P≤0.01) and 493±144mmHg/s (P>0.05) from baseline, respectively, compared to saline control (49±71mmHg/s). At the higher dose, ELA-32 (150nmol) and [Pyr^{1}]apelin-13 (400nmol) significantly increased dP/dt_{MAX} by 2825±565mmHg/s (P≤0.01) and 3025±680mmHg/s (P≤0.01), respectively, compared to saline control (142±69mmHg/s) (Figure 6A). This effect on contractility coincided with a

significant increase in cardiac output at both doses of ELA-32 (20nmol, 3296±370 relative volume unit/minute (RVU/min), P≤0.0001; 150nmol, 4000±826 RVU/min, P≤0.01) and [Pyr^{1}]apelin-13 (50nmol, 1535±189 RVU/min, P≤0.05; 400nmol, 3989±537 RVU/min, P≤0.01) compared to saline control (352±86 RVU/min; 716±215 RVU/min) (Figure 6B). The increase in cardiac output was mostly due to an increased stroke volume, as end-systolic volume was reduced. Stroke volume was also significantly increased by ELA-32 (20nmol, 8.2±0.9 RVU, P≤0.0001; 150nmol, 9.1±1.9 RVU, P≤0.01) and [Pyr^{1}]apelin-13 (50nmol, 3.6±0.5 RVU, P≤0.05; 400nmol, 9.2±0.9 RVU, P≤0.001) compared to saline control (0.6±3.4 RVU, 1.6±0.5 RVU) (Figure 6C). A trend towards increased heart rate for both peptides did not reach statistical significance (Supplemental Figure 7). This effect was followed by a significant dose-dependent reduction in LV systolic pressure by ELA-32 (20nmol, 14.1±1.6mmHg, P≤0.0001; 150nmol, 17.1±3.2mmHg, P≤0.01) and [Pyr^{1}]apelin-13 (50nmol, 8.2±1.1mmHg, P≤0.01; 400nmol, 8.2±3.2mmHg, P≤0.01), compared to the saline controls (0.6±1.1mmHg; 1.1±0.3mmHg) (Figure 6D). A third, higher dose of [Pyr^{1}]apelin-13 (1300nmol) did not show further increase in any response. In order to confirm contribution of systemic vasodilatation to the reduction in LV systolic pressure, ELA-32 and [Pyr^{1}]apelin-13 were each administered to one animal in which the catheter was placed in the carotid artery and both elicited a comparable reduction in systolic and diastolic blood pressure (not shown). Consistent with the MRI experiment, the cardiovascular effects of ELA-32 and [Pyr^{1}]apelin-13 were short-acting, returning to baseline within 10-20 minutes. Moreover, the response to ELA-32 appeared to be greater than the same dose of [Pyr^{1}]apelin-13.

Down-Regulation of ELA Expression in Human PAH and Rodent Models of PAH

The number of ELA-positive and ELA-negative blood vessels in control human lung sections was 84±3 versus 16±3, compared to 58±5 versus 42±5 in PAH lung (Fisher's exact test,

P≤0.0001, Figure 7A). Compared to controls, the proportion of ELA-positive vessels was significantly reduced while the proportion of ELA-negative vessels was significantly increased in PAH (Supplemental Figure 8).

Expression of *APELA* mRNA in human PAH lung was significantly (P≤0.01) reduced compared to healthy lung (Figure 7B). *Apela* mRNA level in RV was significantly reduced in both Sugen/hypoxia (P≤0.05) and MCT rats (P≤0.01) compared to controls (Figure 7C and 7D). Interestingly, *Aplnr* mRNA in RV was also significantly lower in the MCT rats compared to controls (P≤0.01) (Figure 7E) and there was a trend (not significant, P=0.14) for reduced expression of *Apln* mRNA in these animals (Figure 7F).

Attenuation of MCT-induced PAH by ELA

MCT administration elevated RVSP (81.3±6.0mmHg, P≤0.0001) compared to saline controls (27.4±0.6mmHg). ELA-32 treatment in MCT rats attenuated RVSP (52.5±1.9mmHg, P≤0.0001 vs MCT group) but this was still significantly higher than control RVSP (P≤0.0001 vs saline group) (Figure 8A). Similarly, compared to the saline controls (Fulton index=0.21±0.01), RV hypertrophy was observed in MCT rats (0.46±0.02, P ≤0.0001) that was significantly attenuated by ELA-32 administration (0.32±0.02, P≤0.0001 vs MCT group, P≤0.05 vs saline group) (Figure 8B). MCT increased the percentage of fully muscularized vessels (MCT 28±2% versus saline 8±1%, P≤0.0001) and arteriolar wall thickness (MCT 26±1% versus saline 10±1% P≤0.0001), and this was significantly lessened by ELA-32 (muscularisation, 19±2%, P ≤0.01 versus MCT; 16±1% versus MCT P≤0.0001) (Figure 8C-L). MCT exposure resulted in significant RV hypertrophy indicated by an increase in cardiomyocyte area (WGA staining; MCT 315±9μm^2 versus saline 212±11μm^2,

P≤0.0001), a reduction in cardiomyocyte number/area (MCT 59±2 versus saline 91±1, P≤0.0001) and an increase in GATA4 positive nuclei/area (MCT 44±2 versus saline 30±1, P≤0.0001). There was a significant improvement in these indices following ELA-32 treatment (cardiomyocyte area 228±9μm^2, P≤0.0001 compared to MCT alone; cardiomyocyte number/area 66±2; GATA4 positive nuclei/area 38±1 (both P≤0.05 compared to MCT alone) (Figure 8M, 8N, 8O, Supplemental Figure 9). ELA-32 had no significant effect compared to saline control on any hemodynamic or histological parameter in this study and in particular did not cause systemic hypotension following chronic administration (Supplemental Figure 10). There was some evidence of improved survival in the MCT-ELA group compared to MCT alone as in the MCT group one rat died (day 18) and three died under isoflurane whereas only one MCT-ELA rat died (day 20). Plasma levels of the vasoactive peptides angiotensin-II and BNP-32 were unaltered by ELA-32 treatment (Supplemental Figure 11).

Discussion

This study provides a comprehensive characterization of ELA, originally reported as a regulator of zebrafish cardiac development, in the adult mammalian cardiovascular system. We report for the first time ELA peptides binding to the native human apelin receptor, ELA expression in human blood vessels, attenuation of cardiac dysfunction, cardiac and pulmonary arterial remodeling and peptide and receptor mRNA expression in human PAH and rodent models of PAH in addition to more fully characterizing the cardiovascular profile of ELA compared to apelin.

Receptor Binding and Downstream Signaling

Using cells over-expressing the receptor and ELA conjugated to alkaline phosphatase, Chng *et al*., 2013[4] were the first to suggest that ELA could bind to the human apelin receptor. In this study, we initially employed molecular dynamic simulation based on homology models of the apelin receptor to create a receptor template into which ELA could be reliably docked. Despite lack of obvious sequence similarity, ELA-11 docked within the binding pocket occupied by apelin-13. We subsequently confirmed this in radioligand competition assays using [^{125}I]apelin-13 to demonstrate direct binding of all three ELA peptides to the apelin receptor in human cardiac tissue. Our data from both heart and CHO cells revealed that ELA-32, ELA-21 and ELA-14 bound to the human receptor with sub-nanomolar affinity whereas the linear and cyclo[1-6]ELA-11 displayed a 100-fold drop in affinity, consistent with a recent report using HEK293 cells expressing the apelin receptor[9]. This confirms the importance of positively charged amino acids (which interact via hydrogen bonding with the apelin receptor in our model system) in the longer ELA peptides corresponding to the RPRL motif in apelin-13 that we have previously identified as critical for receptor binding affinity[10]. Similar or lower affinities for ELA conjugated to alkaline phosphatase have been reported in

CHO cells expressing apelin receptor (K_d=0.51nmol/L[11]) and for ELA-32 in isolated rat cardiomyocytes (K_i=38.2nmol/L[12]). Therefore, although a recent report from the co-discoverers of ELA suggested an unidentified cell surface receptor rather than the apelin receptor is responsible for mediating the effects of ELA in human embryonic stem cells[13] our data clearly confirm that in the adult cardiovascular system ELA is a ligand for the apelin receptor.

Next, ELA was tested for its ability to activate G protein-signaling using a cAMP inhibition assay, since the apelin receptor is known to couple via G_i. Ela-32, ELA-21 and ELA-11 were full agonists in this assay with comparable potency to [Pyr[1]]apelin-13 in the sub-nanomolar range, consistent with their high binding affinities. Interestingly, ELA-11 and cyclo[1-6]ELA-11 were not different from the longer forms in terms of potency and efficacy in blocking cAMP accumulation, suggesting that the 11-mer was sufficient for effective G_i protein signaling via the apelin receptor. ELA-14, consistent with our high affinity binding data, has also been reported to activate G protein-dependent and -independent pathways with comparable potency to ELA-32[9] and our data confirm this. Data for ELA-32 have been reported by two other groups with EC_{50} values in the sub-nanomolar[11] to nanomolar[14] range and ELA-mediated inhibition of cAMP production confirmed as pertussis toxin-sensitive[11].

As expected for agonist activation of most GPCRs, apelin binding also triggered recruitment of β-arrestin leading to receptor internalization and β arrestin-dependent signaling[15]. Our data revealed that in contrast to their equivalent potency in the G protein pathway, ELA-32, ELA-21 and ELA-14 were more potent than ELA-11, cyclo[1-6]ELA-11 and [Pyr[1]]apelin-13 in the β-arrestin assays. These data are interesting as they provide additional evidence that extended N-terminal sequences of ELA and apelin peptides are more effective in stimulating

β-arrestin mediated cellular events as previously reported for apelin-17 compared to [Pyr^{1}]apelin-13 in cAMP and internalization assays using the expressed rat receptor[16]. Our data expand on initial studies that demonstrated internalization of eGFP-tagged apelin receptors in zebrafish embryos by exogenous *APELA* mRNA and ELA-21 peptide[5] and ELA-32-induced internalization of GFP-tagged human apelin receptor over-expressed in HEK293 cells[14]. The non-peptide small molecule antagonist ML221 blocked responses to ELA-32 and [Pyr^{1}]apelin-13 in the β-arrestin assay with the same affinity, providing additional evidence that the two ligands bind to the same or overlapping sites on the receptor. Importantly, for drug discovery and therapeutic intervention, these data confirm that apelin receptor antagonists can be designed that will block all apelin signaling irrespective of the endogenous ligand. We conclude from our cell based assays that the truncated form, ELA-11, preferentially activates G protein-signaling and may represent an endogenous G protein-biased apelin receptor ligand. How the different apelin and ELA peptides are integrated in normal apelin receptor physiology and how these may contribute to disease progression remains to be unraveled.

ELA Expression in the Human Cardiovascular System

Having established receptor binding and activation by ELA peptides *in vitro*, we addressed the question of endogenous expression of ELA in relevant human tissues. To date *APELA* mRNA expression has been reported in human embryonic stem cells[4,14] induced pluripotent stem cells[14], kidney[4,14] and prostate[4], and rat kidney[11] with ELA peptide expression only reported in human embryonic stem cells[4]. Our study is the first report of *APELA* transcript and ELA in human blood vessels suggesting that *APELA* is translated into a peptide in the vasculature. Expression was identified in both large and small diameter vessels; for example in heart ELA localized to both epicardial and intramyocardial blood vessels. Specifically,

ELA peptide expression was restricted to the vascular endothelium with no significant localization to vascular smooth muscle or cardiomyocytes. We have previously reported the localization of the apelin receptor to the vascular endothelium, the underlying smooth muscle and to cardiomyocytes[17] raising the possibility that ELA may signal in an autocrine and/or paracrine manner. We have also localized apelin to the vascular and endocardial endothelium[7], and therefore an overlapping distribution of the two peptides is apparent. The significance of two peptides and one receptor will need to be addressed, however in development this is resolved by temporal differences in the expression of each peptide. The expression of *APELA* appeared to be higher in arterial compared to venous tissue, with the exception of the aorta. The implication of this trend is not clear. The presence of ELA peptide in heart was consistent with previous reports on zebrafish, where ELA is critical in cardiac development[4,5] and Perjés *et al.*, 2016[12] recently reported *APELA* mRNA expression in endothelial cells in mouse heart. Staining of cultured primary human endothelial cells verified the presence of ELA peptide in this cell type. Interestingly, ELA did not co-localize with Weibel-Palade bodies[18], suggesting that ELA is produced via a constitutive synthetic pathway rather than via regulated/inducible release. This sub-cellular spatial localization is also observed with apelin[17].

Apelin levels have been measured in human plasma by ELISA[19]. We therefore used a similar ELISA to detect ELA peptides. Both ELA and apelin were detectable in human plasma at sub-nanomolar levels, more indicative of peptides acting as locally released autocrine/paracrine mediators rather than as circulating hormones. Although we observed a correlation between plasma concentrations of ELA and apelin, the relatively narrow age range of our samples did not allow a more detailed correlations between ELA concentration and for example age, sex or BMI.

Cardiovascular Effect of ELA in Rodents *in vivo*

We next tested if ELA modulates cardiovascular functions *in vivo*. In heart, apelin is reportedly the most potent inotrope *in vitro*[3,20] via protein kinase C and extracellular signal-regulated kinases 1/2[21]. Consistent with this are data from *in vivo* studies in rodents and humans. In anaesthetized rats, apelin-16 increased dP/dt_{MAX}[22] and [Pyr^{1}]apelin-13 increased cardiac output and reduced blood pressure[6]. In anaesthetized mice, intraperitoneal injection of [Pyr^{1}]apelin-13 reduced LV end diastolic area and increased heart rate, observed using MRI, whereas hemodynamics measurement by catheterization showed an increased preload recruitable stroke work (a measure of intrinsic contractility) and reduced LV end-systolic pressure[23]. In human volunteers, intracoronary bolus administration of apelin-36 increased dP/dt_{MAX}, and an intravenous infusion of apelin-36 or [Pyr^{1}]apelin-13 increased heart rate and cardiac output[24].

We have used a combination of MRI and invasive catheterization to characterize the cardiac effects of ELA compared to apelin. ELA-32 and [Pyr^{1}]apelin-13 were positive inotropes in the LV, increasing dP/dt_{MAX} consistent with the previous reports for apelin[22,24] and an initial report of increased fractional shortening by ELA-32[9]. In addition, ELA-32 and [Pyr^{1}]apelin-13 increased cardiac output, owing to increased stroke volume and possibly increased heart rate. We also observed for ELA-32 the previously reported apelin-induced reduction in LV end diastolic area[23], resulting in an increased ejection fraction. We could not detect obvious qualitative differences in the cardiac actions of ELA-32 and [Pyr^{1}]apelin-13 however Perjés *et al*., 2016[12] reported that the inotropic effect of ELA-32 *in vitro* in the Langendorff perfused rat heart was dependent on extracellular signal-regulated kinases 1/2 but not on protein kinase C as seen for apelin.

We detected an ELA-32 and [Pyr[1]]apelin-13 induced drop in LV systolic pressure. This is likely a consequence of reduced afterload resulting from peripheral vasodilatation, a known effect of apelin[3,6]. In order to confirm systemic vasodilation, ELA-32 and [Pyr[1]]apelin-13 were administered with the catheter placed in the right carotid artery and both peptides caused a drop in blood pressure in agreement with Murza *et al*., 2016[9]. These *in vivo* data are consistent with recent *in vitro* studies suggesting that ELA has a vasodilatory effect in adult rat coronary arteries[12] and caused relaxation of pre-constricted mouse aortic rings[14] in a reported nitric oxide-independent and partially endothelium-independent manner. Overall, we observed that lower doses of ELA-32 were required to achieve equivalent or more pronounced effects than [Pyr[1]]apelin-13. This is consistent with the ~5-fold higher receptor affinity we determined for ELA-32 compared to [Pyr[1]]apelin-13 in human heart.

Reduced ELA Expression in Human PAH and Rodent Models and Attenuation of MCT-Induced PAH by Exogenous ELA in Rats

Lastly, we addressed the question of the possible alteration in ELA expression in PAH, where apelin expression is known to be reduced contributing to disease pathogenesis. Apelin levels are down-regulated in plasma or serum[19,25] in PAECs[26] and pulmonary microvascular endothelial cells[27] from PAH patients and in RV of MCT-exposed rat[28]. The expression of the apelin receptor was also reduced in RV of MCT rats[28]. We have now shown for the first time that ELA is similarly reduced in pulmonary vessels of PAH patients and in the RV from two rodent models of PAH. In particular, ELA staining was examined in small pulmonary blood vessels which are critical to the pathogenesis of PAH as they undergo vascular remodeling leading to increased vascular resistance and consequently the RV undergoes hypertrophy. We observed a reduction in *Apela* expression in RV of Sugen/hypoxia and MCT-exposed rats and also of *Aplnr* with a trend to reduction in *Apln* in the MCT animals.

This is consistent with a down-regulation of all components of the ELA/apelin/apelin receptor pathway in the RV in PAH. It is important to note that although reduced, *Aplnr* mRNA was still present in the RV, making the receptor amenable for therapeutic manipulation with a goal to replace the down-regulated ELA or apelin peptides. We confirmed this in human heart where the apelin receptor density in PAH compared to normal RV and LV was only reduced by approximately 15%. There has been one study reporting increased *Apela* and *Aplnr* mRNA expression in LV of a mouse model of myocardial infarction[12] but we did not observe this for the receptor in LV from human PAH heart. Finally we have shown that, as reported for apelin[28], administration of ELA-32 attenuated the remodeling of the pulmonary vasculature and hypertrophy of RV cardiomyocytes. This resulted in blunting of the increased RV systolic pressure and hypertrophy induced by MCT in this rat model of PAH.

In conclusion, our study confirms the direct receptor binding of ELA to the apelin receptor in human heart, and provides more details on the docking of this ligand within the receptor using molecular modelling. Using cell-based assays, we compared ELA-32, ELA-21 and ELA-11 with [Pyr^{1}]apelin-13 and found them to be agonists in G protein-dependent and -independent pathways. Our data also demonstrated the widespread presence of *APELA* mRNA and ELA peptide in adult human cardiovascular tissues, and localized the peptide specifically to the endothelium. Furthermore, we demonstrated *in* vivo that ELA increases cardiac contractility, cardiac output and causes vasodilatation. These results show that ELA is an endogenous agonist of the human apelin receptor and exhibits a cardiovascular profile comparable to that of apelin. The relative importance of the two peptides to normal apelin receptor function needs to be explored. However, the down-regulation of ELA expression in PAH and the beneficial effect of ELA administration on cardiac function and

cardiopulmonary remodeling in the MCT rat model of PAH, consistent with that of apelin, supports the potential exploitation of the apelin receptor as a therapeutic target at least in this disease.

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Disclosures

None.

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Figure Legends

Figure 1. ELA peptides bind to the human apelin receptor. (A) Amino acid sequences of ELA-32, ELA-21, ELA-11 and [Pyr^{1}]apelin-13. Disulfide bridges are yellow lines, hydrophobic amino acids shown in green, uncharged polar amino acids in pink, basic amino acids in blue with pyroglutamate in red. (B) Docking of ELA-11 with the apelin receptor. (C) Apelin-13 (atom-colored) and ELA-11 (green) docking showing overlap in the binding site. (D) Ligand interaction diagram showing key close contacts between ELA-11 and the apelin receptor model. (E) Predicted interactions of ELA-11 with the apelin receptor determined by docking analysis. Amino acids in the ELA-11 sequence are color coded to match their predicted sites of interaction on the receptor sequence.

Figure 2. Competition binding curves for ELA peptides in human heart. (A) ELA-32 (■), ELA-21 (▲), ELA-11 (▼) and [Pyr^{1}]apelin-13 (●) in human left ventricle. (B) ELA-21 in normal right ventricle (●), PAH right ventricle (●), normal left ventricle (●) and PAH left ventricle (●). Values are mean±sem, n=3.

Figure 3. Concentration-response curves in cell-based receptor pharmacology assays. (A) Inhibition of forskolin-induced cAMP accumulation. (B) Stimulation of β-arrestin recruitment. (C) Induction of receptor internalization. [Pyr^{1}]apelin-13 (●), ELA-32 (■), ELA-21 (▲) and ELA-11 (▼). Antagonism of (D) [Pyr^{1}]apelin-13 (●, ○) and (E) ELA-32 (■, □) by 30μM ML221 in the β-arrestin assay. Concentration-response curves to ELA-14 (●) and cyclic ELA-11 (●) in (F) cAMP and (G) β-arrestin assays. Values are mean±sem, n=3-5 assays, 2-6 replicates per assay.

Figure 4. Expression of *APELA* transcript and ELA peptide in human cardiovascular tissues. (A) Expression of *APELA* mRNA in human blood vessels determined by RT-qPCR. (B-F) Representative overlay confocal photomicrographs of immunofluorescence staining of (B) human pulmonary artery endothelial cell (PAEC) and (C-F) human cardiovascular tissue. Photomicrographs show ELA-like immunoreactivity in green and the endothelial marker vWF in red. If not indicated, scale bars=75μm. CA, coronary artery (n=11); MA, mammary artery (n=6); RA, radial artery (n=9); PA, pulmonary artery (n=4); UV, umbilical vein (n=6); AO, aorta (n-6); SV, saphenous vein (n=8); LV, left ventricle (n=8), lung (n=6). (G) Levels of ELA and apelin in human plasma (n=25). (H) Correlation between plasma concentrations of ELA and apelin. * P<0.05 compared to ELA.

Figure 5. Cardiac effects of ELA and apelin *in vivo* by MRI in the rat. Representative snapshots of mid-ventricular transverse sections of the heart at end-diastolic and end-systolic points during the 10 minute MRI scan showing the effects of (A) ELA-32 (150nmol) and (B) [Pyr^1]apelin-13 (650nmol). Red bars are drawn to show the approximate diameter of the left ventricle. Dose-dependent increase in (C) left and (D) right ventricular ejection (EF) fraction of the heart in response to ELA-32 (red bars 20 and 150nmol, n=8) and [Pyr^1]apelin-13 (black bars 50 and 650nmol, n=5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to saline control (n=6). ns Not significantly different from saline control.

Figure 6. The *in vivo* effects of ELA-32 and [Pyr1]apelin-13 on the left ventricle of rat heart measured by catheterization. (A) Compared to saline controls (open bars, n=6), ELA-32 (red bars, n=10) and [Pyr^1]apelin-13 (black bars, n=8) caused a significant change in (A) cardiac contractility (dP/dt_{MAX}), (B) cardiac output (CO), (C) stroke volume (SV) and (D)

LV systolic pressure (LVSP). RVU, relative volume unit. ns Not significantly different from saline control. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to saline control.

Figure 7. Altered levels of ELA expression in PAH. (A) Number of blood vessels positive and negative for ELA staining in control (n=4) and PAH (n=4) human lung sections (****P≤0.0001). (B) *APELA* down regulation in human PAH (n=4) compared to normal (n=4) lung (**P≤0.01). *Apela*, mRNA down-regulation in the right ventricle of (C) Sugen/hypoxia (n=6) and (D) MCT exposed (n=5) rats (*P≤0.05, **P≤0.01 respectively, compared to saline (n=7 and 5, respectively). (E) *Aplnr* and (F) *Apln* mRNA expression in right ventricle of saline (n=5) and MCT exposed (n=5) rats (*P≤0.05, compared to saline; ns Not significantly different from saline control).

Figure 8. Attenuation of MCT-induced PAH by ELA-32 in rats. MCT exposure (black bar, n=9) caused an increase in (A) right ventricular systolic pressure (RVSP) and (B) right ventricular hypertrophy measured as RV/(LV+S) (Fulton index) (both ****P≤0.0001) compared to saline control (open bars, n=8). ELA-32 administration red bar (n=9) significantly reduced MCT-induced RVSP and hypertrophy (****P≤0.0001 and ****P≤0.0001 respectively). ELA-32 alone (gray bars, n=9) had no effect and was not different from saline control (open bars, n=8). In tissues from these animals, MCT increased (C) the proportion of fully muscularized vessels in rat lung and (D) wall thickness of larger pulmonary arterioles compared to saline (both ****P≤0.0001) and these changes were attenuated by ELA-32 (**P≤0.01 and ****P≤0.0001, respectively). Immunohistological visualization of remodeling of pulmonary arterioles using α-smooth muscle actin (brown color E-H) and van Giesen's stain (I-L) in sections of lung from (E, I) MCT, (F, J) MCT-ELA, (G, K) ELA alone and (H, L) saline control rats (scale bars=75μm). MCT-induced RV

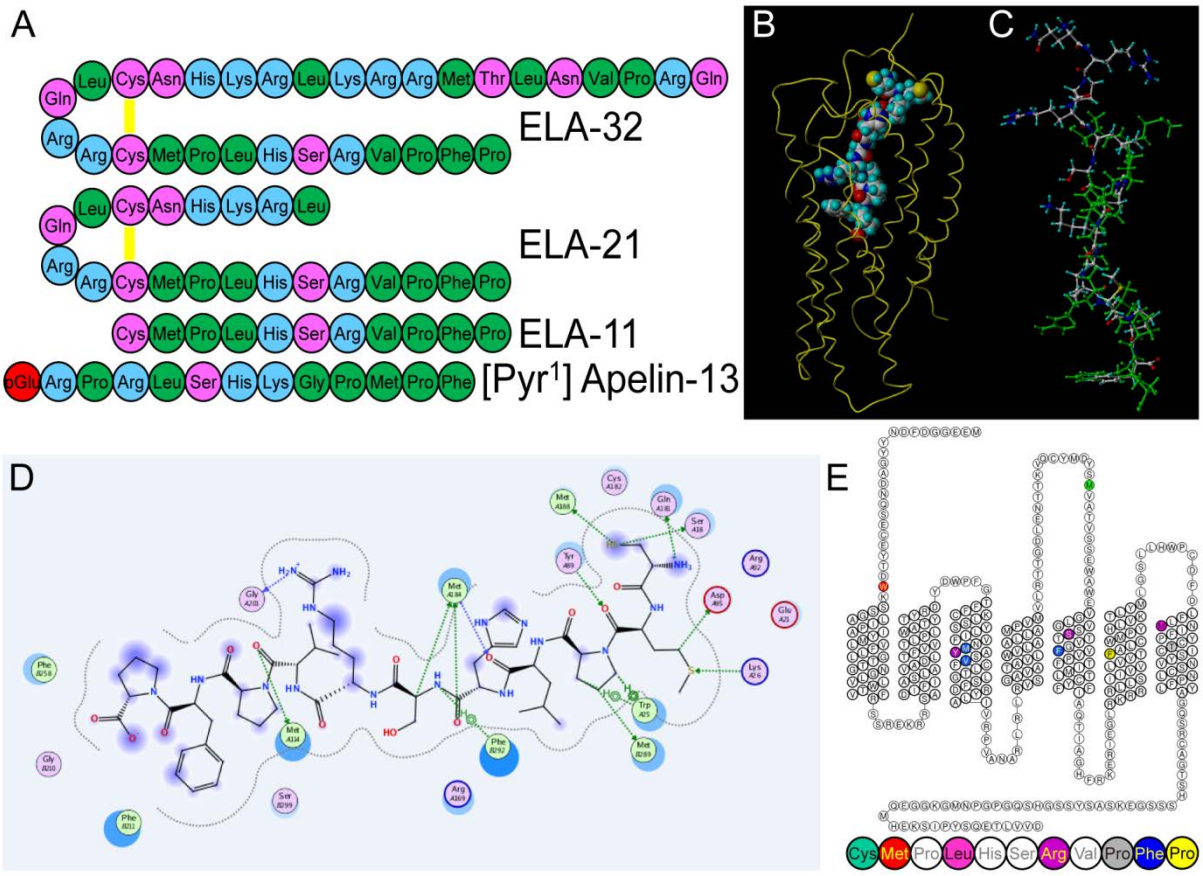
hypertrophy was indicated by (M) an increase in cardiomyocyte area indicated by WGA staining, (N) a reduction in cardiomyocyte number/area and (O) an increase in GATA4 positive nuclei/area compared to saline control (****P≤0.0001) with a significant improvement in these indices following ELA-32 treatment (*P≤0.05, **** P≤0.0001, compared to MCT alone). ELA-32 alone had no effect on any parameter.

Table 1: Potency (pD_2) and efficacy (E_{MAX}) of [Pyr^1]apelin-13, ELA-32, ELA-21 and ELA-11 in cAMP inhibition, β-arrestin recruitment and receptor internalization assays.

Peptide	cAMP		β-Arrestin		Internalization	
	pD_2	E_{MAX}	pD_2	E_{MAX}	pD_2	E_{MAX}
[Pyr^1]apelin-13	9.74±0.11	100±1%	8.10±0.12	100±2%	8.87±0.25	100±2%
ELA-32	9.49±0.07	101±1%	9.05±0.32††	104±4%	9.66±0.36†	102±4%
ELA-21	9.43±0.16	101±1%	9.88±0.29**†††	102±1%	9.55±0.08	96±2%
ELA-11	9.13±0.21	100±1%	7.44±0.18	107±6%	8.13±0.56	105±1%

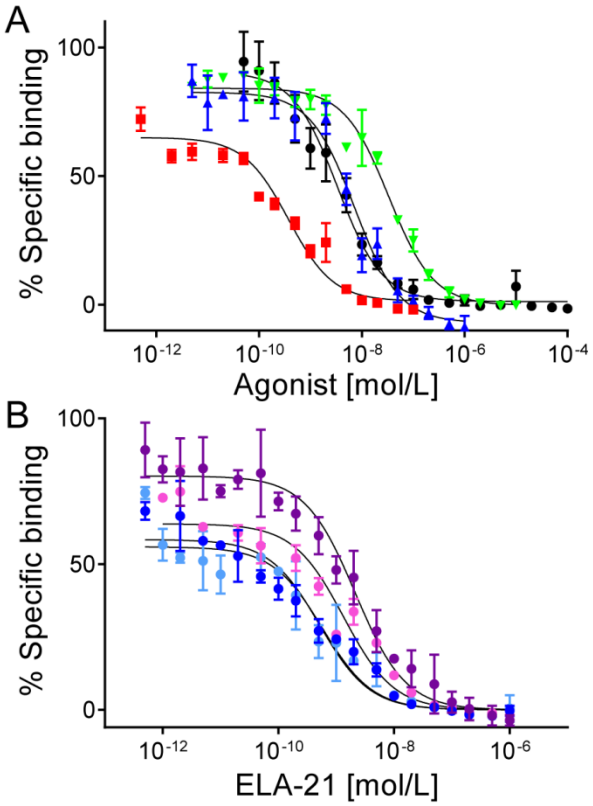
$^{**}P\leq0.01$ Significantly different from [Pyr^1]apelin-13. $^{\dagger}P\leq0.05$, $^{\dagger\dagger}P\leq0.01$, $^{\dagger\dagger\dagger}P\leq0.001$. Significantly different from ELA-11. n=3-4 experiments. Values are mean±sem, n=3-5 assays, 2-6 replicates per assay.

Fig1



1 Fig2

2



3

4 Fig3

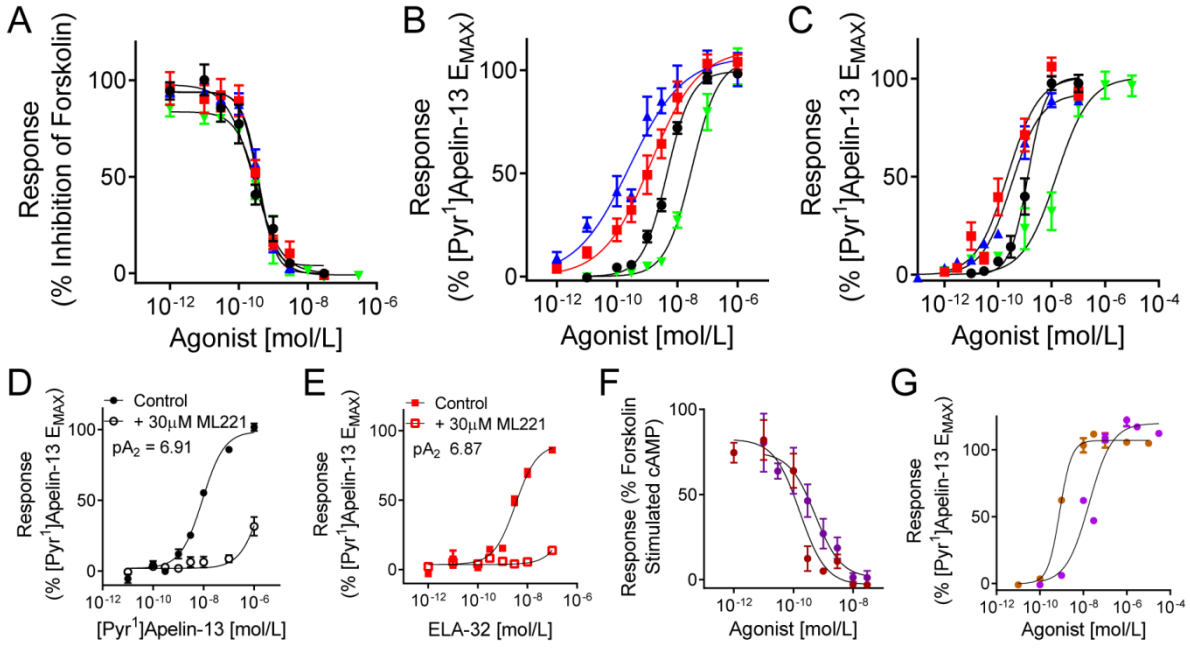


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Fig 4

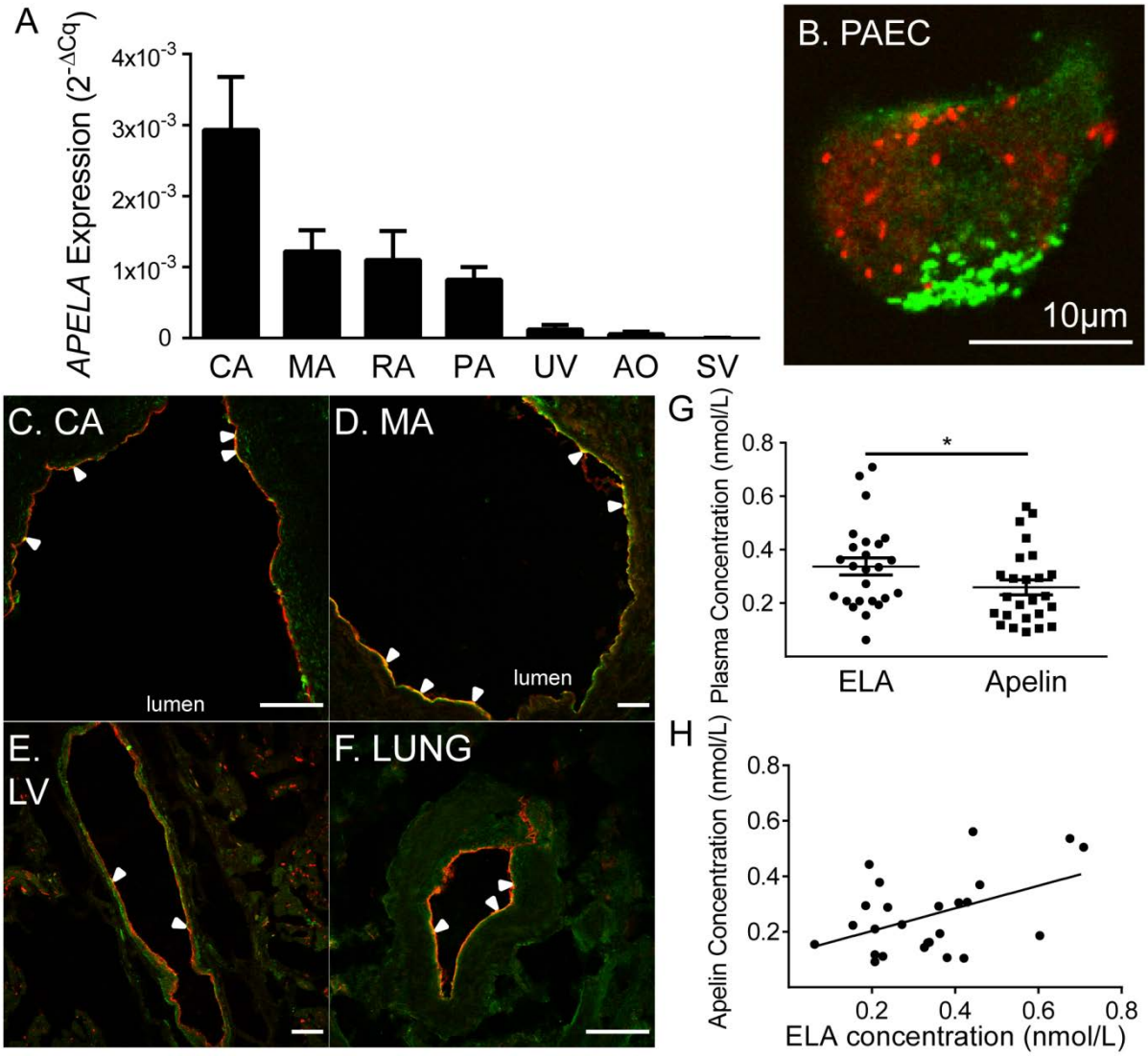
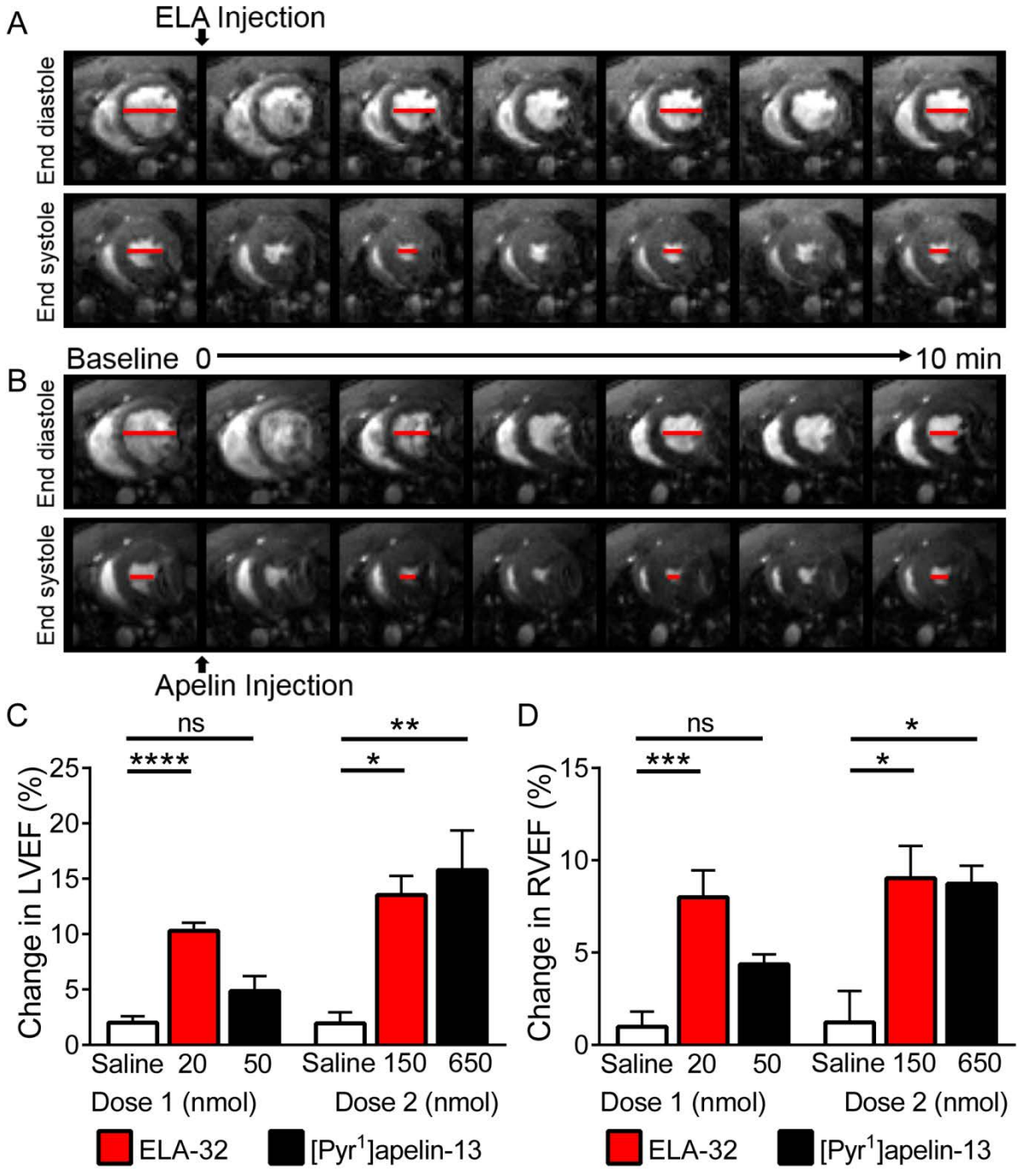

A
APELA Expression (2^-ΔCq)
4x10-3
3x10-3
2x10-3
1x10-3
0
CA
MA
RA
PA
UV
AO
SV
B. PAEC
10μm
C. CA
lumen
D. MA
lumen
E. LV
F. LUNG
G
Plasma Concentration (nmol/L)
0.8
0.6
0.4
0.2
*
ELA
Apelin
H
Apelin Concentration (nmol/L)
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
ELA concentration (nmol/L)

Fig 5

Fig 6


A
ELA Injection
End diastole
End systole
Baseline 0
10 min
B
End diastole
End systole
Apelin Injection
C
ns
**
****
*
Change in LVEF (%)
25
20
15
10
5
0
Saline 20 50
Saline 150 650
Dose 1 (nmol)
Dose 2 (nmol)
ELA-32
[Pyr1]apelin-13
D
ns
*
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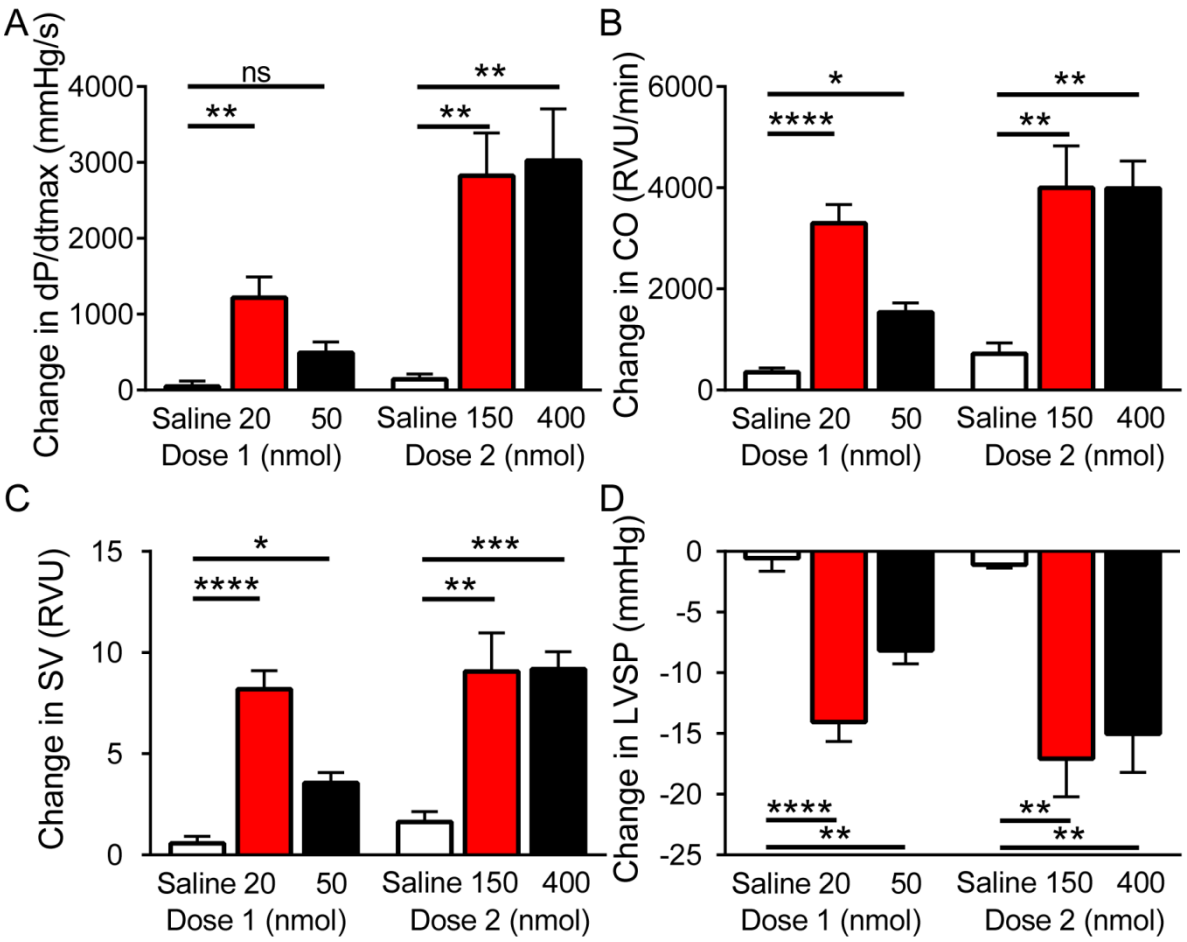
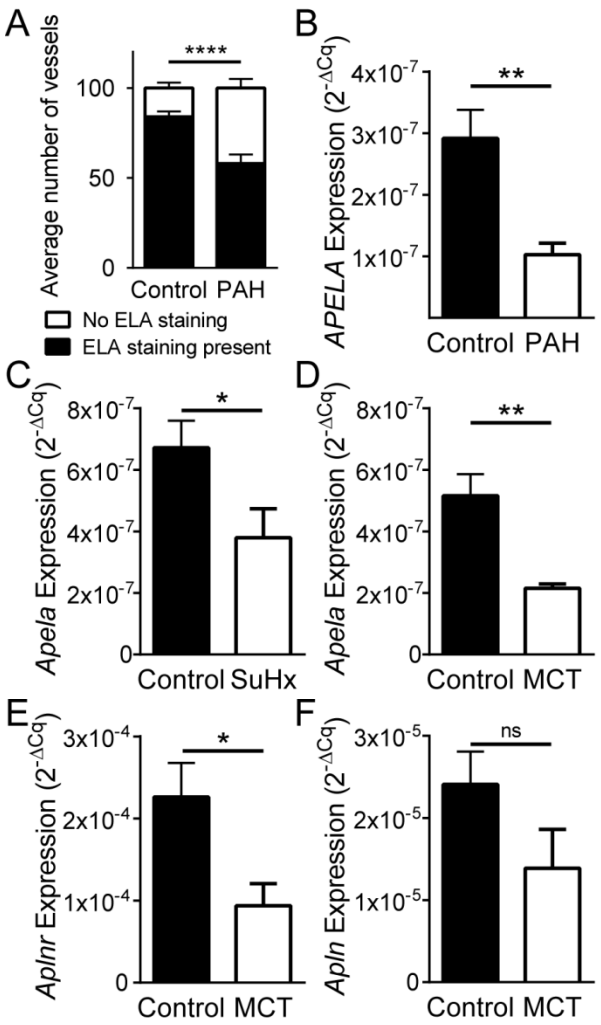
*
Change in RVEF (%)
15
10
5
0
Saline 20 50
Saline 150 650
Dose 1 (nmol)
Dose 2 (nmol)
ELA-32
[Pyr1]apelin-13

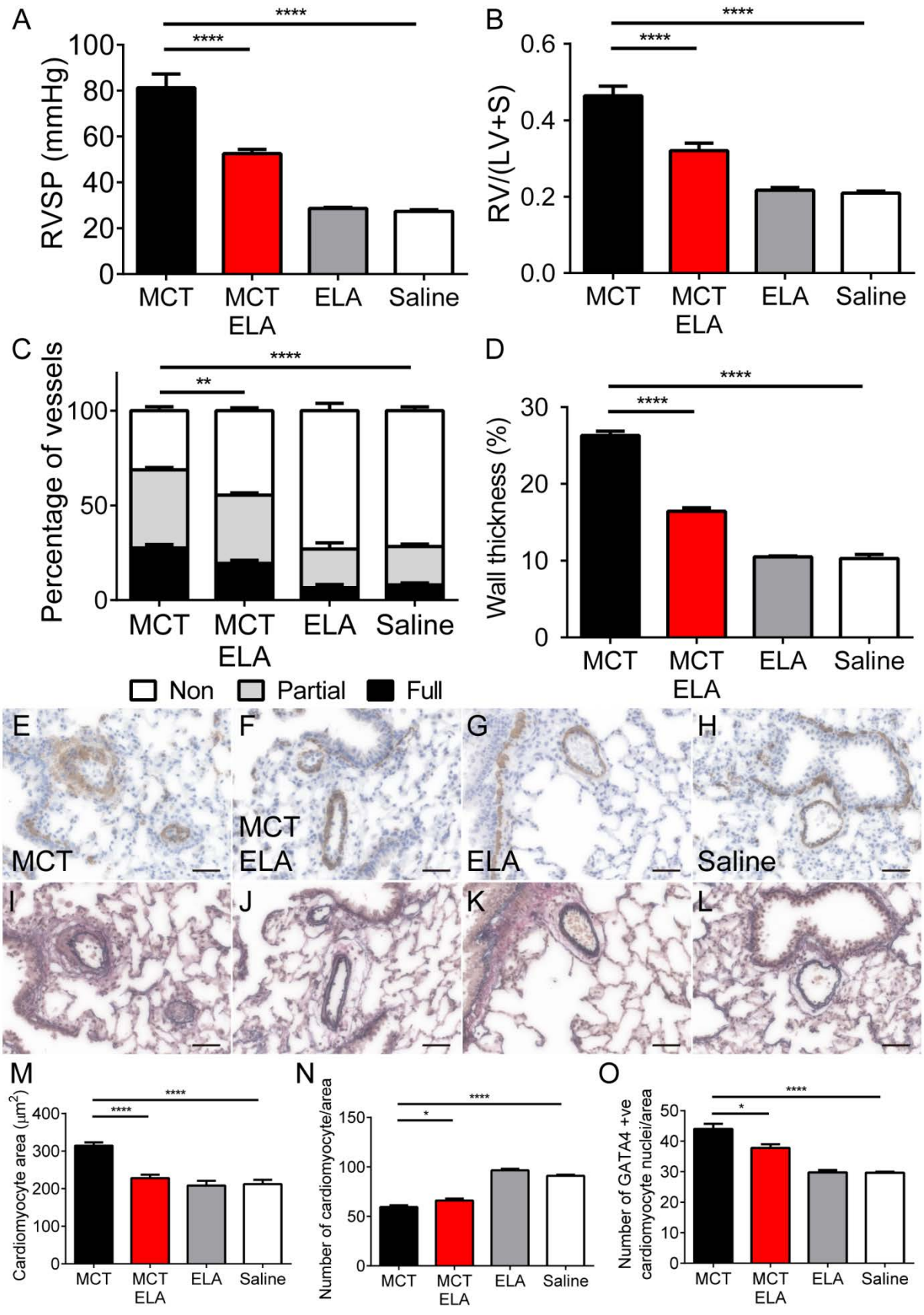
Fig 7


A
Change in dP/dtmax (mmHg/s)
4000
3000
2000
1000
0
ns
**
**
**
Saline 20
50
Saline 150
400
Dose 1 (nmol)
Dose 2 (nmol)
B
Change in CO (RVU/min)
6000
4000
2000
0
*
****
**
**
Saline 20
50
Saline 150
400
Dose 1 (nmol)
Dose 2 (nmol)
C
Change in SV (RVU)
15
10
5
0
*
****
***
**
Saline 20
50
Saline 150
400
Dose 1 (nmol)
Dose 2 (nmol)
D
Change in LVSP (mmHg)
0
-5
-10
-15
-20
-25
****
**
**
**
Saline 20
50
Saline 150
400
Dose 1 (nmol)
Dose 2 (nmol)


A
Average number of vessels
****
100
50
0
Control PAH
No ELA staining
ELA staining present
B
APELA Expression (2-ΔCq)
**
4x10-7
3x10-7
2x10-7
1x10-7
Control PAH
C
Apela Expression (2-ΔCq)
*
8x10-7
6x10-7
4x10-7
2x10-7
0
Control SuHx
D
Apela Expression (2-ΔCq)
**
8x10-7
6x10-7
4x10-7
2x10-7
0
Control MCT
E
Aplnr Expression (2-ΔCq)
*
3x10-4
2x10-4
1x10-4
0
Control MCT
F
Apln Expression (2-ΔCq)
ns
3x10-5
2x10-5
1x10-5
0
Control MCT

1 Fig 9

2


A
RVSP (mmHg)
100
80
60
40
20
0
****
****
MCT
MCT ELA
ELA
Saline
B
RV/(LV+S)
0.6
0.4
0.2
0.0
****
****
C
Percentage of vessels
100
50
0
**
****
Non
Partial
Full
D
Wall thickness (%)
30
20
10
0
****
****
E
MCT
F
MCT ELA
G
ELA
H
Saline
I
J
K
L
M
Cardiomyocyte area (μm²)
400
300
200
100
0
****
****
N
Number of cardiomyocyte/area
150
100
50
0
****
*
O
Number of GATA4 +ve cardiomyocyte nuclei/area
50
40
30
20
10
0
****
*

3

1

2

3

4