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Enhanced serelaxin signalling in co-cultures of human primary endothelial and smooth muscle cells

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Running title: Serelaxin signalling in co-cultures of human vascular cells

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Author contributions

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Conducted experiments: Sarwar
Contributed reagents or tools: Bathgate, Stewart
Performed data analysis: Sarwar, Summers
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Summary

Background and purpose – In the phase III clinical trial, RELAX-AHF, serelaxin caused rapid and long-lasting haemodynamic changes. The cellular mechanisms involved are unclear in humans.

Experimental approach – This study examined the effects of serelaxin in co-cultures of human primary endothelial cells (ECs) and smooth muscle cells (SMCs) on cAMP and cGMP signalling.

Key results – Stimulation of human umbilical vein endothelial cells (HUVECs) or human coronary artery endothelial cells (HCAECs) with serelaxin, concentration-dependently increased cGMP accumulation in co-cultured SMCs to a greater extent than in monocultures of either cell type. This was not observed in human umbilical artery endothelial cells (HUAECs) that do not express RXFP1. Treatment of ECs with L-NG-nitro arginine (L-NOARG) (30μM, 30min) inhibited serelaxin-mediated (30nM) cGMP accumulation in HUVECs, HCAECs and co-cultured SMCs. In HCAECs but not HUVECs, pre-incubation with indomethacin (30μM, 30min), also inhibited cGMP accumulation in SMCs. Pre-incubation of SMCs with 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (1μM, 30min) had no effect on serelaxin-mediated (30nM) cGMP accumulation in HUVECs and HCAECs but inhibited cGMP accumulation in SMCs. Serelaxin stimulation of HCAECs but not HUVECs, increased cAMP accumulation concentration-dependently in SMCs. Pre-incubation of HCAECs with indomethacin but not L-NOARG abolished cAMP accumulation in co-cultured SMCs suggesting involvement of prostanoids.

Conclusions and implications – In co-cultures, treatment of ECs with serelaxin caused marked cGMP accumulation in SMCs and with HCAEC also cAMP accumulation. Responses involved EC-derived nitric oxide and with HCAEC prostanoid production. Thus serelaxin differentially modulates vascular tone in different vascular beds.

Key words: serelaxin, co-culture, signal transduction, vasodilation, acute heart failure

List of non-standard abbreviations:

HF, Heart failure; AHF, Acute heart failure; GPCR, G protein coupled receptor; RXFP1, Relaxin family peptide receptor 1; ECs, Endothelial cells; SMCs, Smooth muscle cells; HUAEC, Human umbilical artery endothelial cell; HUVEC, Human umbilical vein endothelial cell; HCAEC, Human coronary artery endothelial cell; HUASMC, Human umbilical artery smooth muscle cell; HUVSMC, Human umbilical vein smooth muscle cell; NO, Nitric oxide; GC, Guanylate cyclase; cAMP, Cyclic adenosine monophosphate; cGMP, Cyclic guanosine monophosphate; DEA, Diethylamine NONOate; PI3K, Phosphoinositide 3-kinase; VEGF, Vascular endothelial growth factor; MMP, Matrix metalloproteinase; NOS, Nitric oxide synthase; ET_B, Endothelin type B

Introduction

Vasodilators are a cornerstone therapy for acute heart failure (AHF). Standard therapies such as loop diuretics, nitrates, β-blockers and ACE inhibitors cause vasodilation and/or prevent vasoconstriction (Hollenberg, 2007). However, most vasodilators exhibit side-effects with hypotension being the most commonly reported example in patients with heart failure (Hollenberg, 2007). Serelaxin, the recombinant form of the human hormone relaxin, presents as a novel treatment option for AHF and in the phase III clinical trial, RELAX-AHF, serelaxin relieved dyspnoea and congestion in patients with AHF but also significantly reduced patient mortality at day-180 without notable side-affects (Teerlink et al., 2013). Serelaxin treatment was also associated with rapid and long-lasting haemodynamic changes including reductions in pulmonary capillary wedge pressure (PCWP), pulmonary artery pressure (PAP: systolic and diastolic), pulmonary vascular resistance (PVR), right atrial pressure (RAP) and systemic vascular resistance (SVR) (Ponikowski et al., 2013). These could be attributed to the vasodilatory effects of relaxin that have been reported *in vitro* (Bani et al., 1998; McGuane et al., 2011b; Sarwar et al., 2014; Boccalini et al., 2015), *in vivo* (Masini et al., 1997; Danielson et al., 1999; Masini et al., 2002; Conrad et al., 2004; Debrah et al., 2005; 2006; Conrad and Shroff, 2011; McGuane et al., 2011a; Segal et al., 2012) and in patients with AHF (Voors et al., 2011; Ponikowski et al., 2013; Voors et al., 2014).

Relaxin acts at RXFP1, the cognate relaxin receptor, that is expressed in endothelial cells (ECs) and smooth muscle cells (SMCs) of arteries and veins, although expression pattern does not always necessarily correlate with function (Jelinic et al., 2013). Studies on human isolated vessels are rare but relaxin does cause vasodilation in human isolated subcutaneous and small systemic resistance arteries (McGuane et al., 2011b). Although the precise cellular mechanisms of the haemodynamic effects of relaxin in humans are poorly understood, two distinct mechanisms have been described. Rapid relaxin-mediated vasodilation occurs via a $G\alpha_i$/PI3K/cAMP/NO-dependent mechanism (McGuane et al., 2011b) whereas sustained relaxin-mediated responses are associated with changes in activity or expression of gelatinases, endothelin receptor B (ET_B), vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) (Dschietzig et al., 2003; Jeyabalan et al., 2003; McGuane et al., 2011a).

We have previously shown that these signalling mechanisms occur in primary ECs, SMCs and fibroblasts from the human vasculature (Sarwar et al., 2014) thereby identifying blood vessels as an important potential target for serelaxin in humans. We also showed that serelaxin had a variety of effects in cells from arteries and veins. However, *in vivo*, the vascular cells are organized as layers in blood vessels, and cross talk between these cells has an important role to play in regulating the function of the vessel. Monocultures *in vitro* fail to integrate this natural physiological organisation of blood vessels and on their own do not reflect the impact of cellular cross talk on signal transduction.

The endothelium is known to release vasoactive substances that act on smooth muscle cells to regulate vessel tone. Acetylcholine and bradykinin cause endothelium-dependent vasorelaxation via their respective GPCRs (Furchgott and Zawadzki, 1980) and the EC/SMC

interactions involve nitric oxide (Palmer et al., 1987), prostacyclin (Radomski et al., 1987) and endothelium-derived hyperpolarizing factor (EDHF) (Bolton et al., 1984). These interactions have been shown to affect cGMP and cAMP signalling, second messengers that are known to regulate cardiovascular function and are altered in disease (Ganz et al., 1986; Majed and Khalil, 2012). Indeed, relaxin mediated relaxation is abolished in human gluteal arteries that are endothelium-denuded (Fisher, 2009) suggesting that relaxin signalling is endothelium-dependent. Since there is a lack of information on the signal transduction mechanisms activated by relaxin in a physiologically relevant environment we have investigated signalling in a cell co-culture model of ECs and SMCs from human arteries and veins in order to better understand serelaxin-mediated signal transduction in human blood vessels.

Methods

Materials

Serelaxin (the recombinant form of human gene 2 relaxin) was kindly provided by Corthera, Inc. (a subsidiary of Novartis AG, Switzerland). 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), L-NG-nitro arginine (L-NOARG) and indomethacin were purchased from Sigma (Australia). Cell co-culture ThinCerts^{TM} were purchased from Greiner Bio-One (Germany).

Human Primary Cells

Primary cultures of human umbilical artery endothelial cells (HUAEC), human umbilical vein endothelial cells (HUVEC), human coronary artery endothelial cells (HCAEC), human umbilical artery smooth muscle cells (HUASMC) and human umbilical vein smooth muscle cells (HUVSMC) were obtained from ScienCell Research Laboratories (San Diego, USA). These cells were characterized as detailed previously (Sarwar et al., 2014). All cells were maintained in Medium 199 containing 5% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and the relevant growth supplements for optimal growth of each cell type. As such, endothelial cells (EC) were grown in EC growth supplement (ECGS), smooth muscle cells in SMGS and fibroblasts in FGS-2 (ScienCell, U.S.A) as detailed previously (Sarwar et al., 2014). Early culture passages (2-5) were used for each cell type.

Cell Culture

For monoculture assays, both ECs and SMCs were plated in standard 24-well CELLSTAR® multiwell plates (Greiner Bio One, Germany) at a density of $2x10^5$ cells per well in a volume of 500µL of growth medium per well. The cells were allowed to adhere and grow overnight. For co-culture assays, ECs were plated on 24-well ThinCerts^{TM} (Greiner Bio One, Germany), comprising translucent membranes with 0.4µm pores, at a density of $1x10^5$ cells per insert in a volume of 400µL of growth medium per insert. Smooth muscle cells were plated in standard 24-well CELLSTAR® multiwell plates (Greiner Bio One, Germany) at a density of $2x10^5$ cells per well in a volume of 500µL of growth medium per well. The cells were allowed to adhere and grow overnight and just prior to the experiment, ThinCerts^{TM} were placed in wells containing smooth muscle cells.

cAMP and cGMP Accumulation

cAMP accumulation was determined as previously described (Sarwar et al., 2014). Briefly, cells grown in mono-cultures, were pre-incubated with stimulation buffer and treated with serelaxin at the given concentrations for 30 min. Forskolin (50µM, 30min) and DEA (1µM, 5min) were used as positive controls for cAMP and cGMP, respectively. Where appropriate, cells were pre-incubated with the NOS inhibitor, L-NOARG; (30µM, 30min), the non-specific cyclooxygenase (COX) inhibitor, indomethacin; (30µM, 30min) or the guanylate cyclase inhibitor ODQ (1µM, 30min). Following stimulation with serelaxin (30min), the cells were rapidly lysed and cAMP and cGMP levels were detected using AlphaScreen cAMP and

cGMP kits (Perkin-Elmer, Australia). For co-culture studies, cells on the ThinCerts™ were stimulated with serelaxin (30min) and/or cells were treated with the relevant inhibitors. Before stimulation with serelaxin, ThinCerts™ were placed directly on top of the wells containing the smooth muscle cells and after completion of the assay, cells were separated and lysed. cAMP and cGMP levels were detected in each cell type using the AlphaScreen cAMP and cGMP kits (Perkin-Elmer, Australia).

Data Analysis

Data was analysed using GraphPad Prism v6.0. Replicates were averaged before entry as a single data point. All data represents the mean ± S.E.M of at least 5 individual experiments unless otherwise indicated in the text. Concentration-response curves were fitted using a sigmoidal or Gaussian distribution function. Statistical significance was determined using a one-way ANOVA with significance accepted at $p<0.05$. If F reached significance, the Dunnett's post hoc test was used to compare groups.

Results

Serelaxin stimulation of HUVEC and HCAEC but not HUAEC enhances cGMP accumulation in co-cultures of HUASMC and HUVSMC

The addition of serelaxin (30min) to HUAEC co-cultured with HUASMC (Figure 1A) or HUVSMC (Figure 1B) failed to produce a cGMP response in HUAEC (Figure 1C, D) or in HUASMC (Figure 1C) or HUVSMC (Figure 1D). This can be explained by the lack of cell surface RXFP1 expression in HUAEC (Sarwar et al., 2014) since direct stimulation of either HUASMC (Figure 1C, dashed line; pEC_{50}: 9.5 ± 0.5) or HUVSMC (Figure 1D, dashed line; pEC_{50}: 9.3 ± 0.3) with serelaxin (30min) produced concentration-dependent increases in cGMP accumulation of 30% and 32% of the DEA response, respectively. The absence of a cGMP response in SMCs co-cultured with HUAEC demonstrates that after addition of serelaxin, although the peptide may penetrate the 0.4μm pores within the insert, it fails to reach a concentration in the SMC chamber sufficient to cause a response.

In contrast, addition of serelaxin (30min) to HUVEC, that do express RXFP1 (Sarwar et al., 2014), when co-cultured with HUASMC, not only increased cGMP accumulation to 27% of the DEA response in HUVEC (Figure 1E; pEC_{50}: 9.8 ± 1.2) but also caused a large, concentration-dependent increase in cGMP accumulation in HUASMC (Figure 1E; pEC_{50}: 9.8 ± 0.5) to 50% of the DEA response, or 1.7 fold higher than the maximal response observed when HUASMC were directly stimulated with serelaxin (Figure 1E, dashed line). Similarly, when HUVEC were co-cultured with HUVSMC (Figure 1F), serelaxin treatment (30min) increased cGMP accumulation to 21% of the DEA response in HUVEC (Figure 1F, pEC_{50}: 9.7 ± 0.6) but also caused a robust increase in cGMP accumulation in the co-cultured HUVSMC reaching 80% of DEA response (Figure 1F, pEC_{50}: 9.5 ± 0.3), or 2.5 times higher than the maximal response obtained with HUVSMC directly stimulated with serelaxin (Figure 1F, dashed line). It was noted that whereas the concentration-response relationship in HUASMC in monocultures was sigmoidal it became bell-shaped in co-cultures with HUVEC.

To examine whether the difference between co-cultures involving HUAEC and HUVEC represented a difference between arterial and venous ECs or a regional difference between ECs, we also utilized co-cultures involving HCAEC. In co-cultures of HCAEC/HUASMC, treatment of HCAEC with serelaxin (30min) produced a modest increase in cGMP accumulation to 27% of the DEA response (Figure 1G, pEC_{50}: 9.8 ± 0.9) but also robustly increased cGMP accumulation in the co-cultured HUASMC reaching 68% of DEA response (Figure 1G, pEC_{50}: 9.7 ± 0.6), or 2.1 times higher than cGMP responses observed in HUASMC directly stimulated with serelaxin (Figure 1G, dashed line). In co-cultures of HCAEC/HUVSMC, treatment of HCAEC with serelaxin (30min) produced a modest increase in cGMP accumulation to about 28% of DEA response (Figure 1H, pEC_{50}: 9.9 ± 0.7) but also increased cGMP accumulation in HUVSMC to 53% of the DEA response (Figure 1H, pEC_{50}: 9.5 ± 0.4), about 1.8 times that of cGMP responses observed in HUVSMC directly stimulated with serelaxin (Figure 1H, dashed line).

Serelaxin-mediated NO generation in HUVEC and HCAEC is responsible for cGMP accumulation in HUASMC and HUVSMC

To determine how serelaxin treatment of HUVEC and HCAEC caused cGMP accumulation in arterial and venous SMCs, we used pharmacological inhibitors to disrupt key signalling pathways. Since in intact blood vessels, NO is known to be generated by ECs to stimulate cGMP in smooth muscle cells (Furchgott and Vanhoutte, 1989), we incubated ECs with the NOS inhibitor L-NOARG and stimulated with serelaxin (30nM, 30min). In monocultures, pre-treatment with the general NOS inhibitor, L-NOARG (30μM, 30min) significantly inhibited serelaxin-mediated cGMP responses (% DEA) in HUVEC (Figure 2A: Serelaxin alone: 24.3±6.4 vs. Serelaxin + L-NOARG: 7.4±3.4, $p<0.05$), HCAEC (Figure 2B: 26.6±4.6 vs. 8.6±2.4, $p<0.05$), HUASMC (Figure 2C: 20.9±2.4 vs. 7.4±3.5, $p<0.05$) and HUVSMC (Figure 2D: 17.6±3.3 vs. 5.7±2.8, $p<0.05$) suggesting that serelaxin-mediated (30nM, 30min) cGMP accumulation in these cells is NO dependent.

We next determined whether NO mediates cross talk between ECs and SMCs by incubating ECs with L-NOARG (30μM, 30min) and serelaxin (30nM, 30min) (Figure 3A). In co-cultures (Figure 3A), pre-treatment of HUVEC with L-NOARG (30μM, 30min) abolished serelaxin-mediated (30nM, 30min) cGMP responses (% DEA) not only in HUVEC (Figure 3B: 36.6±4.2 vs. 9.7±5.7, $p<0.05$, n=6) but also in both HUASMC (Figure 3D: 27.6±3.7 vs. 8.6±2.8, $p<0.05$, n=6) and HUVSMC (Figure 3E: 55.7±7.6 vs. 12.6±7.9, $p<0.05$). In co-cultures with HCAEC, pre-treatment with L-NOARG (30μM, 30min) almost abolished serelaxin-mediated (30nM, 30min) cGMP accumulation not only in HCAEC (Figure 3C: 38.8±5.3 vs. 13.3±6.6, $p<0.05$, n=6), but also in HUASMC (Figure 3F: 70.7±7.7 vs. 21.9±5.5, $p<0.05$) and HUVSMC (Figure 3G: 48.9±8.8 vs. 15.9±3.3, $p<0.05$). These results suggest that endothelial NO production is essential for cGMP responses in co-cultured arterial and venous SMCs.

Serelaxin-mediated prostanoid production in HCAEC but not HUVEC influences cGMP accumulation in HUASMC and HUVSMC

We next determined whether prostanoids had a role in endothelium-dependent responses in co-cultures since previous studies have shown that endothelial prostanoids can act on smooth muscle cells to affect cAMP signalling (Furchgott and Vanhoutte, 1989; Majed and Khalil, 2012). While little is known of the role of prostanoids in vasodilator responses to serelaxin, indomethacin treatment is known to affect responses in some blood vessels (Fisher, 2009).

Indomethacin pre-treatment (30μM, 30min) did not influence serelaxin-mediated (30nM, 30min) cGMP accumulation in monocultures of HUVEC (Figure 2A: serelaxin: 24.3±6.4 vs. serelaxin + indomethacin: 26.6±4.3, NS), HUASMC (Figure 2C: 20.9±2.4 vs. 18.7±3.5, NS) and HUVSMC (Figure 2D: 17.6±3.3 vs. 16.5±3.5, NS) but significantly inhibited serelaxin-mediated cGMP accumulation in HCAEC (Figure 2B: 26.6±4.6 vs. 14.5±1.6, $p<0.05$). In co-cultures, pre-treatment with indomethacin (30μM, 30min) had no effect on serelaxin-mediated cGMP accumulation in HUVEC (Figure 3B: 36.6±4.2 vs. 42.9±13.5, NS, n=6) or on cGMP responses in HUASMC (Figure 3D: 27.6±3.7 vs. 31.2±5.3, NS, n=6) or HUVSMC (Figure 3E: 55.7±7.6 vs. 44.6±10.3, NS), showing that serelaxin does not stimulate prostanoid

production in HUVEC. However, indomethacin pre-treatment did (as in the monocultures) appear to reduce cGMP accumulation in HCAEC (Figure 3C: 38.8±5.3 vs. 28.9±6.2, n=6) and significantly reduced cGMP accumulation in the co-cultures of both HUASMC (Figure 3F: 70.7±7.7 vs. 37.2±10.1, $p<0.05$) and HUVSMC (Figure 3G: 48.9±8.8 vs. 26.7±5.2, $p<0.05$). This suggests that in HCAEC, endothelial prostanoid production has a significant influence on cGMP signalling in arterial and venous smooth muscle cells.

GC activation and cGMP accumulation in HUASMC and HUVSMC is dependent on HUVEC and HCAEC

Since previous studies showed that NO activates guanylate cyclase (GC) in SMCs (Martin et al., 2005), we pre-treated SMCs with the GC inhibitor ODQ and stimulated ECs with serelaxin. In monocultures, pre-treatment with ODQ (1μM, 30min), significantly inhibited serelaxin (30nM, 30min) mediated cGMP accumulation in HUVEC (Figure 2A: Serelaxin: 24.3±6.4 vs. Serelaxin + ODQ: 4.6±4.3, $p<0.05$), HCAEC (Figure 2B: 26.6±4.6 vs. 6.9±4.0, $p<0.05$), HUASMC (Figure 2C: 20.9±2.4 vs. 6.6±3.3, $p<0.05$) and HUVSMC (Figure 2D: 17.6±3.3 vs. 6.6±2.4, $p<0.05$). In co-cultures with HUVEC, pre-treatment of HUASMC or HUVSMC with ODQ (1μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cGMP accumulation in HUVEC (Figure 3I: 36.6±4.2 vs. 34.3±9.1, NS, n=6) but markedly reduced cGMP accumulation in both HUASMC (Figure 3K: 27.6±3.7 vs. 12.3±4.7, $p<0.05$, n=6) and HUVSMC (Figure 3L: 55.7±7.6 vs. 18.9±5.5, $p<0.05$). Likewise in co-cultures with HCAEC, pre-treatment of HUASMC or HUVSMC with ODQ (1μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cGMP accumulation in HCAEC (Figure 3J: 38.8±5.3 vs. 46.4±18.6, NS, n=6), but significantly reduced cGMP accumulation in both HUASMC (Figure 3M: 70.7±7.7 vs. 24.2±8.7, $p<0.05$) and HUVSMC (Figure 3N: 48.9±8.8 vs. 19.1±5.0, $p<0.05$).

Treatment of HCAEC but not HUAEC or HUVEC with serelaxin enhances cAMP accumulation in HUASMC and HUVSMC

In order to examine whether cAMP was another mediator involved in the vasodilator response in SMCs in response to serelaxin treatment, we investigated the effect of the peptide in EC/SMC co-culture (Figure 4A, B) on cAMP accumulation. In co-cultures with HUAECs, serelaxin (30min) treatment failed to produce a cAMP response in HUAECs (Figure 4C, D), HUASMC (Figure 4C) or HUVSMC (Figure 4D). In monocultures, treatment with serelaxin (30min) increased cAMP accumulation in HUASMC (Figure 4C, dashed line, pEC_{50}: 9.6 ± 0.7) and HUVSMC (Figure 4D, dashed line, pEC_{50}: 9.4 ± 0.4), with maximal responses 5% and 6% of the forskolin response, respectively.

In co-cultures of HUVEC (that express RXFP1) with HUASMC, serelaxin treatment (30min) increased cAMP accumulation to 15% of the forskolin response in HUVEC (Figure 4E, pEC_{50}: 9.9 ± 0.6), but there was no increase in cAMP accumulation in HUASMC (Figure 4E) whereas direct stimulation of HUASMC with serelaxin (30min) increased cAMP accumulation concentration-dependently (Figure 4E, dashed line: pEC_{50}: 9.6 ± 0.7). In co-cultures of HUVEC and HUVSMC (Figure 4B), serelaxin treatment (30min) increased cAMP accumulation to 22% of forskolin response in HUVEC (Figure 4F, pEC_{50}: 9.1 ± 0.4), with no

significant effect on cAMP accumulation in HUVSMC (Figure 4F), even though direct stimulation of HUVSMC with serelaxin (30min) increased cAMP accumulation (Figure 4F, dashed line: pEC_{50}: 9.4 ± 0.4).

In co-cultures of HCAEC/HUASMC, treatment of HCAEC with serelaxin increased cAMP accumulation to 16% of the forskolin response (Figure 4G, pEC_{50}: 9.8 ± 0.3). However, treatment of HCAECs with serelaxin (30min) also increased cAMP accumulation in HUASMC to 16% of the forskolin response (Figure 4G, pEC_{50}: 9.30 ± 0.3), or 3.2 times higher than cAMP responses observed in HUASMC directly stimulated with serelaxin (Figure 4G, dashed line). In co-cultures of HCAEC/HUVSMC, treatment of HCAEC with serelaxin (30min) increased cAMP accumulation to 18% of the forskolin response (Figure 4H, pEC_{50}: 9.8 ± 0.4). Stimulation of HCAEC with serelaxin (30min) also increased cAMP accumulation in HUVSMC to 13% of the forskolin response (Figure 4H, pEC_{50}: 9.6 ± 0.3), or 2.2 times higher than cAMP responses observed in HUVSMC directly stimulated with serelaxin (Figure 4H, dashed line; pEC_{50}: 9.4 ± 0.4). Thus in HCAEC not only did serelaxin promote NO release, it also increased the release of another mediator that increased cAMP levels in co-cultured SMCs.

The effects of serelaxin on cAMP signalling in HCAEC co-cultures is dependent on prostanoid secretion from ECs

To provide information on the mediator released from HCAEC by serelaxin treatment to influence cAMP signalling in SMCs, we used pharmacological inhibitors on ECs and SMCs to disrupt key signalling pathways (Figure 5A). In mono-cultures, pre-treatment with indomethacin (30μM, 30min) significantly inhibited serelaxin-mediated (30nM, 30min) cAMP accumulation in HCAEC (Figure 5B: serelaxin: 22.0±3.8 vs. serelaxin + indomethacin: 8.9±1.5, $p<0.05$) but not in HUVEC (Figure 5A: 23.7±5.8 vs. 22.8±5.3, NS), HUASMC (Figure 5C: 16.1±8.0 vs. 15.7±10.1, NS) or HUVSMC (Figure 5D: 10.9±3.0 vs. 9.8±4.2, NS) suggesting that cellular background determines whether serelaxin causes prostanoid production in human primary vascular cells. However, pre-treatment with L-NOARG (30μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cAMP accumulation in HUVEC (Figure 5A: 23.7±5.8 vs. 19.5±1.9, NS), HCAEC (Figure 5B: 22.0±3.8 vs. 19.2±3.1, NS), HUASMC (Figure 5C: 16.1±8.0 vs. 21.3±6.2, NS) or HUVSMC (Figure 5D: 10.9±3.0 vs. 9.3±3.2, NS). Similarly, pre-treatment with ODQ (1μM, 30min) had no effect on serelaxin-mediated (30nM, 30min) cAMP accumulation in HUVEC (Figure 5A: 23.7±5.8 vs. 19.0±3.0, NS), HCAEC (Figure 5B: 22.0±3.8 vs. 22.4±5.3, NS), HUASMC (Figure 5C: 16.1±8.0 vs. 18.8±10.9, NS) or HUVSMC (Figure 5D: 10.9±3.0 vs. 12.2±2.4, NS) suggesting that NOS and GC do not influence cAMP accumulation in human primary vascular cells.

In co-cultures, pre-treatment of HCAEC with L-NOARG had no effect on cAMP accumulation in HUASMC (Figure 6C: 17.8±3.0 vs. 17.3±2.9, NS), HUVSMC (Figure 6D: 27.6±7.1 vs. 26.4±2.9, NS) or HCAEC (Figure 6B: 16.1±2.8 vs. 18.8±4.9, NS) suggesting that endothelial NO had no role in modulating cAMP accumulation. Similarly, pre-treatment of HUASMC or HUVSMC with ODQ (1μM, 30min) had no significant effect on serelaxin-

mediated (30nM, 30min) cAMP accumulation in HCAEC (Figure 6F: 16.1±2.8 vs. 21.9±5.2, NS), HUASMC (Figure 6G: 17.8±3.8 vs. 17.3±3.1, NS) and HUVSMC (Figure 6H: 27.6±7.1 vs. 23.2±3.1, NS), suggesting that GC activation in SMCs had no role in serelaxin-mediated and HCAEC-dependent cAMP accumulation. By contrast, indomethacin pre-treatment of HCAEC (Figure 6B: 16.1±2.8 vs. 8.7±1.7, $p<0.05$) almost abolished the enhanced cAMP response observed in HUASMC (Figure 6C: 17.8±3.0 vs. 6.0±2.0, $p<0.05$) and HUVSMC (Figure 6D: 27.6±7.1 vs. 9.7±3.8, $p<0.05$) suggesting that serelaxin-mediated prostanoid production in HCAEC was regulating cAMP production in both arterial and venous smooth muscle cells.

Discussion and conclusions

Serelaxin caused rapid and long-lasting vasodilatory changes in patients with AHF (Ponikowski et al., 2013) however the cellular and molecular mechanisms involved in humans remain poorly understood. In our previous study utilizing human primary vascular cells, we were able to show that serelaxin targeted cells of the human vasculature to cause short and long-term signalling responses in human ECs, smooth muscle cells and fibroblasts (Sarwar et al., 2014). In this study we demonstrate that the effects of serelaxin on vascular cells are enhanced by cellular crosstalk in an experimental paradigm that allows exchange of mediators between cells.

Vasodilation is a specific effect of relaxin that has been observed in many organs and tissues including the uterus (Bani et al., 1995b; 1999), mammary glands (Bani et al., 1995a), mesocaecum (Bigazzi et al., 1986), kidney (Danielson et al., 1999; Novak et al., 2001; Danielson and Conrad, 2003), liver (Bani et al., 2001), lung (Bani et al., 1997; Alexiou et al., 2013), brain (Chan and Cipolla, 2011; Chan et al., 2013) and heart (Bani Sacchi et al., 1995; Masini et al., 1997). These effects of relaxin can be chiefly ascribed to the stimulation of NO synthesis by cells of the vasculature. *In vitro* studies have shown that relaxin increases NO and/or intracellular cGMP levels in rat and human coronary artery endothelial cells, human umbilical vein endothelial cells, human umbilical artery and vein smooth muscle cells and bovine artery smooth muscle cells (Bani et al., 1998; Failli et al., 2002; Quattrone et al., 2004; Sarwar et al., 2014). This is in accord with our findings in HUVEC, HCAEC, HUASMC and HUVSMC where serelaxin-mediated cGMP accumulation was blocked by the NO-synthase inhibitor L-NOARG and the guanylyl cyclase inhibitor ODQ suggesting that serelaxin activated the NO/GC/cGMP pathway in human ECs and SMCs. To date, most cellular studies of signal transduction of serelaxin in vascular cells have been conducted in monocultures that provide no information on functional coupling between cells.

The vasodilating responses of relaxin have also been observed in a range of different intact blood vessels including rodent aorta, small renal and mesenteric arteries (Dschietzig et al., 2003; McGuane et al., 2011b), human subcutaneous (McGuane et al., 2011b) and human systemic resistance arteries (Fisher, 2009) suggesting that blood vessels are a prime target of relaxin. The different layers of blood vessels play distinct roles in blood vessel function and structure (Lüscher, 1990). Thus the endothelium is in intimate contact with the bloodstream and regulates vascular tone by secretion of vasoactive substances such as NO, prostaglandins and endothelium derived hyperpolarizing factor (EDHF) (Lüscher and Tanner, 1992). However, the effects of relaxin on the secretion of these vasoactive substances and their effects on SMCs have not been reported. Administration of serelaxin to HUVEC or HCAEC produced an enhanced cGMP response in co-cultured SMCs – typically 2-2.5 times that observed in monocultures. cGMP responses in both ECs and SMCs were blocked by addition of L-NOARG to ECs. Similarly, addition of the GC inhibitor, ODQ, to the SMCs blocked the response of serelaxin-stimulated ECs suggesting that serelaxin acted on the ECs to release NO that diffused to SMCs and activated guanylate cyclase to cause cGMP accumulation (Figure 7). This is in accord with previous findings as relaxin-mediated vasodilation was blocked by

nitric oxide synthase and guanylyl cyclase inhibitors in uterine artery rings from mid-pregnant rats (Longo et al., 2003) and human systemic resistance arteries (Fisher, 2009) suggesting a role of NO/cGMP in relaxin-mediated vasodilation in rodents and humans. Interestingly, relaxin has been reported to be more potent than other vasodilators. In isolated and perfused rat and guinea pig heart, relaxin increased coronary flow to an extent that was significantly higher than that obtained with typical vasodilators such as acetylcholine or sodium nitroprusside (Bani Sacchi et al., 1995) suggesting that perhaps relaxin may have additional vasodilatory mechanisms.

In some ECs such as HCAEC, serelaxin was shown, in addition to promoting NO dependent cGMP activation in SMCs, to promote the release of prostanoids to enhance both cGMP and cAMP accumulation (Figure 7). Thus, in HUVEC, indomethacin had no effect on serelaxin-mediated cGMP and cAMP signalling (Figure 2), whereas significant inhibition of both pathways was observed in HCAEC (Figure 2). In HCAEC/SMC co-cultures, indomethacin treatment of HCAEC significantly inhibited cGMP (Figure 3) and cAMP accumulation (Figure 6) in SMCs. Previous studies showed that indomethacin abolished (in patients taking ACE inhibitors) or reduced relaxin-mediated vasodilation in human systemic resistance arteries (Fisher, 2009). Our study is the first to demonstrate this interaction between serelaxin and prostanoids *in vitro* in a system where signalling responses can be studied separately in endothelial and smooth muscle cells, which has important implications for understanding the mechanisms of actions of serelaxin in humans. Serelaxin-mediated local prostanoid production may have paracrine and autocrine actions in particular regions and it is likely that in some tissues serelaxin regulates vascular tone via both prostanoids and NO production. Thus, in rat mesenteric arteries, serelaxin enhanced bradykinin-mediated vasodilation in a NO-dependent manner (Jelinic et al., 2013) whereas serelaxin administration to rats increased the prostacyclin component of chronic bradykinin-mediated vasorelaxation in small mesenteric arteries (Leo et al., 2013).

Cell surface expression of RXFP1 was shown to be essential for cAMP and cGMP responses (Sarwar et al., 2014) not only in ECs but also in co-cultured SMCs since serelaxin treatment of HUAEC (non-RXFP1 expressing cells) had no effect on cAMP and cGMP accumulation in co-cultured SMCs. This further strengthens the notion that serelaxin is predominantly an endothelium-dependent vasodilator that is governed by endothelial RXFP1 expression. This is in agreement with previous findings in human small resistance arteries where relaxin had no effect in endothelium-denuded vessels (Fisher, 2009). Thus serelaxin resembles other vasodilators such as acetylcholine, bradykinin, ATP and substance-P that cause endothelium-dependent vasodilation (Furchgott and Zawadzki, 1980). Another finding in the time course experiments was that serelaxin failed to cause a response in SMCs when added to the inserts containing EC. This suggests that although it is likely that serelaxin penetrates the EC/ThinCert^{TM} barrier, it fails to reach a concentration at the SMCs that can activate a signalling event. We also found that treatment of SMCs by ODQ reduced cGMP responses in these cells following addition of serelaxin to ECs but did not affect cGMP responses in ECs suggesting that ODQ like serelaxin does not pass the ThinCert^{TM} barrier to produce concentrations high enough to be effective. Lastly, responses observed in smooth muscle cells followed the pattern of responses observed in the ECs. We have previously established that

concentration-response relationships in HUASMC are sigmoidal (Sarwar et al., 2014), yet the concentration-response relationship in co-cultures mirrors that found in the ECs (Figure 1E), which for HUVEC and HCAEC were bell-shaped, further strengthening the notion that serelaxin responses in the SMCs were governed by the ECs.

There were some limitations to our study. ECs and SMCs are physically separated by a small gap in our co-culture model however, in normal physiology, ECs and SMCs are in direct contact with each other. There are gap junctions not only between adjacent ECs and SMCs but also between ECs and SMCs that allow the passage of secreted substances. However, these gap junctions play an important role in vasorelaxation involving hyperpolarisation of SMCs that is independent of NO and prostacyclin (Figueroa and Duling, 2009). So although there are clear advantages in working with a system that allows exchange of mediators together with examination of signalling pathways in endothelial and smooth muscle cells, there are other factors in an *in vivo* environment that are not accounted for in the co-culture model including the presence of blood (proteins and cells), blood flow, shear stress, and sympathetic innervation (Rodenwaldt et al., 2007). These important factors that are crucial for tissue function could be incorporated in future studies to determine their roles in serelaxin signalling.

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Figure Legends

Figure 1 cGMP accumulation in co-cultures of human primary vascular smooth muscle cells following addition of serelaxin to endothelium. HUAEC, HUVEC or HCAEC were co-cultured with (A) HUASMC or (B) HUVSMC (all n=5) and the ECs were treated with serelaxin for 30min. Serelaxin addition to HUAEC did not cause cGMP accumulation in HUAEC (▲), (C) HUASMC (□) or (D) HUVSMC (○) co-cultured with HUAEC, whereas direct stimulation of either (C) HUASMC (n=5) or (D) HUVSMC with serelaxin caused a concentration-dependent increase in cGMP accumulation (dashed lines). In contrast, serelaxin addition to HUVEC concentration-dependently increased cGMP accumulation not only in HUVEC (■) but also in (E) HUASMC (□) or (F) HUVSMC (○) co-cultured with HUVEC with the responses in smooth muscle cells being greater or in the case of HUVSMC much greater than cGMP responses to direct stimulation of (E) HUASMC or (F) HUVSMC (dashed lines). A similar pattern of cGMP accumulation was observed with (G, H) HCAEC (●) and (G) HUASMC (□) or (H) HUVSMC (○) co-cultured with HCAEC.

Figure 2 Serelaxin-mediated cGMP accumulation in monocultures of human primary vascular cells (all n=5). Serelaxin (30nM, 30min) increased cGMP accumulation in (A) HUVEC, (B) HCAEC, (C) HUASMC and (D) HUVSMC. Pre-incubation with L-NOARG (30μM, 30min) or ODQ (1μM, 30min) almost abolished serelaxin-mediated (30nM, 30min) cGMP accumulation in all cell types. Pre-treatment with indomethacin (30μM, 30min) significantly inhibited serelaxin-mediated (30nM, 30min) cGMP accumulation in (B) HCAEC but had no effect in (A) HUVEC, (C) HUASMC or (D) HUVSMC. Statistical significance was determined using one-way ANOVA compared with serelaxin using the Dunnett post hoc test with *= $p<0.05$ and **=$p<0.01$.

Figure 3 Serelaxin-mediated cGMP accumulation in human primary vascular smooth muscle cells co-cultured with HUVEC or (A) HCAEC (all n=6 except where otherwise indicated). Stimulation of HUVEC or HCAEC with serelaxin (30nM, 30min) increased cGMP accumulation not only in (B) HUVEC and (C) HCAEC, but also in co-cultures of (D,F) HUASMC or (E,G) HUVSMC. Pre-incubation of HUVEC or HCAEC with L-NOARG (30μM, 30min) before addition of serelaxin (30nM, 30min) significantly inhibited cGMP accumulation in (and (C) HCAEC but also in (D,F) HUASMC and (E,G) HUVSMC. Pre-incubation of HUVEC with indomethacin (30μM, 30min) did not affect serelaxin-mediated (30nM, 30min) cGMP accumulation in (B) HUVEC or in co-incubated (D) HUASMC or (E) HUVSMC (n=5). Pre-incubation of HCAEC with indomethacin (30μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cGMP accumulation in (C) HCAEC but produced marked and significant reductions in cGMP accumulation in co-incubated (F) HUASMC or (G) HUVSMC (n=5). Pre-treatment of HUASMC or HUVSMC with ODQ (1μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cGMP accumulation in (I) HUVEC or (J) HCAEC but reduced or abolished cGMP accumulation in (K,M) HUASMC or (L,N) HUVSMC (n=5). Statistical significance was determined using one-way ANOVA versus serelaxin using the Dunnett post hoc test with *= $p<0.05$, **=$p<0.01$.

Figure 4 cAMP accumulation in co-cultures of human primary vascular smooth muscle cells following addition of serelaxin to endothelium (all n=5). HUAEC, HUVEC or HCAEC were co-cultured with (A) HUASMC or (B) HUVSMC and the endothelial cells were treated with serelaxin for 30 min. Serelaxin added to HUAEC did not cause cAMP accumulation either in (C, D) HUAEC (▲), (C) HUASMC (□) or (D) HUVSMC (○) whereas, direct stimulation of (C) HUASMC or (D) HUVSMC with serelaxin caused a concentration-dependent increase in cAMP accumulation (dashed lines). Although direct addition of serelaxin to HUVEC concentration-dependently increased cAMP accumulation in (E, F) HUVEC (■), there was no significant effect on cAMP accumulation in (E) HUASMC (□) or (F) HUVSMC (○). Direct addition of serelaxin to (E) HUASMC or (F) HUVSMC stimulated cAMP accumulation (dashed lines). Serelaxin concentration-dependently increased cAMP accumulation in (G, H) HCAEC (●) but also caused a robust concentration-dependent increase in cAMP accumulation in both (G) HUASMC (□) and (H) HUVSMC (○).

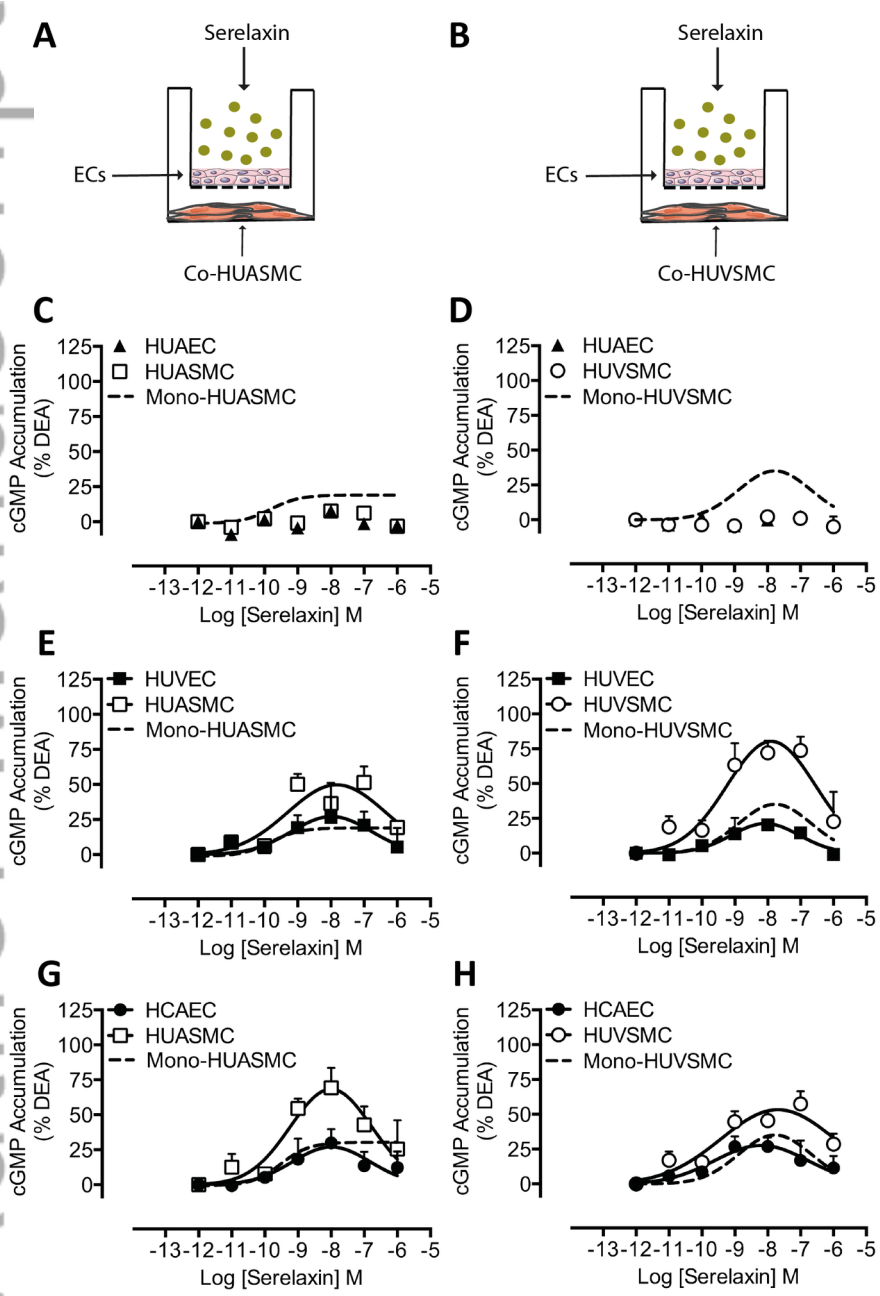
Figure 5 Serelaxin-mediated cAMP accumulation in monocultures of human primary vascular cells (all n=5). Serelaxin (30nM, 30min) increased cAMP accumulation in (A) HUVEC, (B) HCAEC, (C) HUASMC and (D) HUVSMC that was not significantly altered by preincubation with L-NOARG (30μM, 30min) or ODQ (1μM, 30min). Pre-treatment with indomethacin (30μM, 30min) significantly inhibited serelaxin-mediated (30nM, 30min) cAMP accumulation in (B) HCAEC but not in (A) HUVEC, (C) HUASMC or (D) HUVSMC. Statistical significance was determined using one-way ANOVA versus serelaxin using the Dunnett post hoc test with *= $p<0.05$.

Figure 6 Serelaxin-mediated cAMP accumulation in human primary vascular smooth muscle cells co-cultured with HCAEC (A, E, all n=5). Stimulation of HCAEC with serelaxin (30nM, 30min) increased cAMP accumulation not only in (B) HCAEC, but also in co-cultures of (C) HUASMC or (D) HUVSMC. Pre-incubation of HCAEC with L-NOARG (30μM, 30min) before addition of serelaxin (30nM, 30min) had no significant effect on cAMP accumulation in (B) HCAEC, (C) HUASMC or (D) HUVSMC. However, pre-incubation of HCAEC with indomethacin (30μM, 30min) significantly inhibited serelaxin-mediated (30nM, 30min) cAMP accumulation in (B) HCAEC and abolished cAMP accumulation in (C) HUASMC or (D) HUVSMC. Pre-treatment of HUASMC or HUVSMC with ODQ (1μM, 30min) had no significant effect on serelaxin-mediated (30nM, 30min) cAMP accumulation in (F) HCAEC, (G) HUASMC or (H) HUVSMC. Statistical significance was determined using one-way ANOVA versus serelaxin using the Dunnett post hoc test with *= $p<0.05$.

Figure 7 Signal transduction mechanisms activated by serelaxin in co-cultures of human primary vascular cells. Activation of RXFP1 by serelaxin in HUVEC and HCAEC stimulates NO production and activates guanylyl cyclase (GC) and adenylyl cyclase (AC) to produce cGMP and cAMP, respectively. Endothelial NO also diffuses from the endothelial cells across the ThinCert™ membranes and activates GC in both the arterial and venous smooth muscle cells. Additionally in HCAEC (blue lines) but not HUVEC, serelaxin stimulates prostanoid production that produces cAMP accumulation in both arterial and smooth muscle cells.

Statement of conflicts of interest

None



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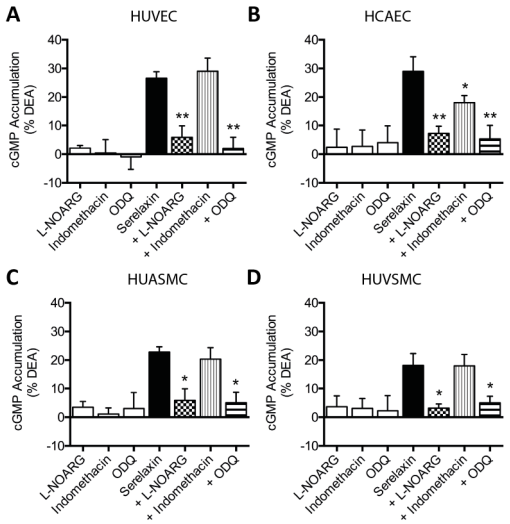

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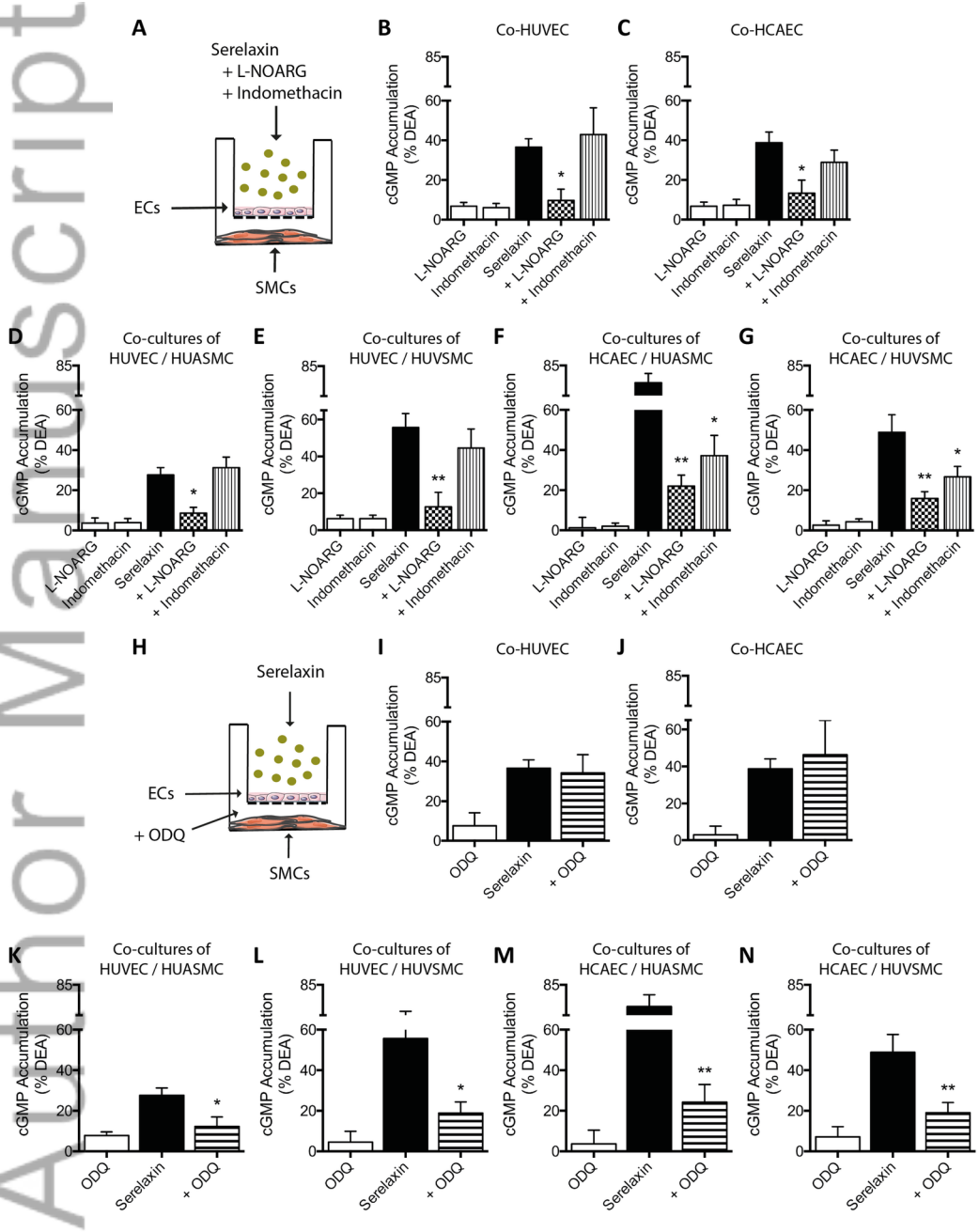

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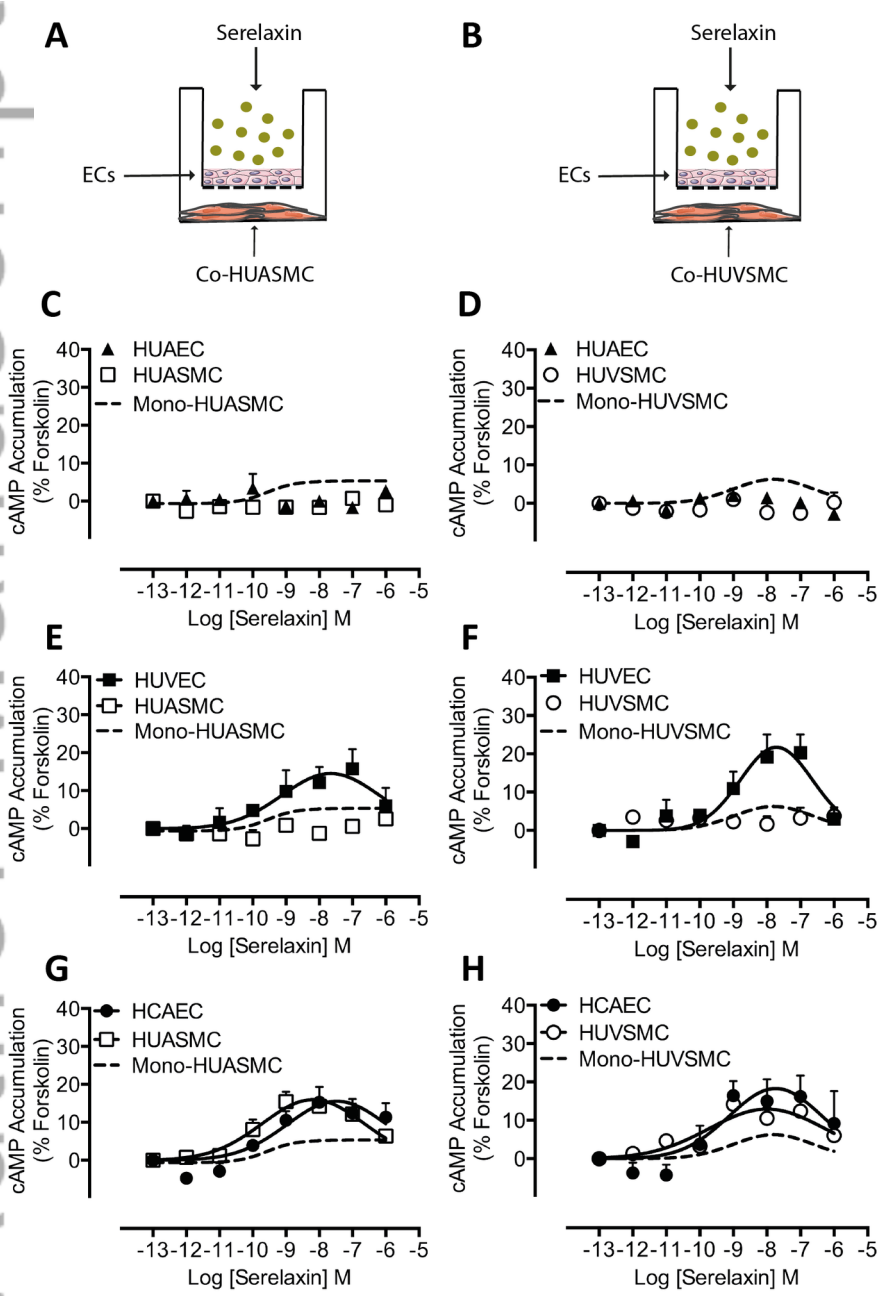

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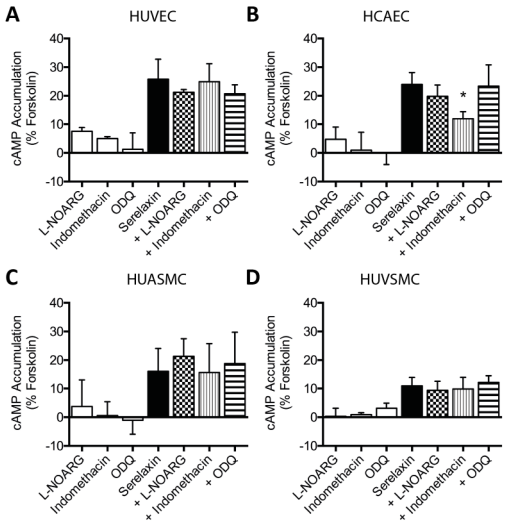

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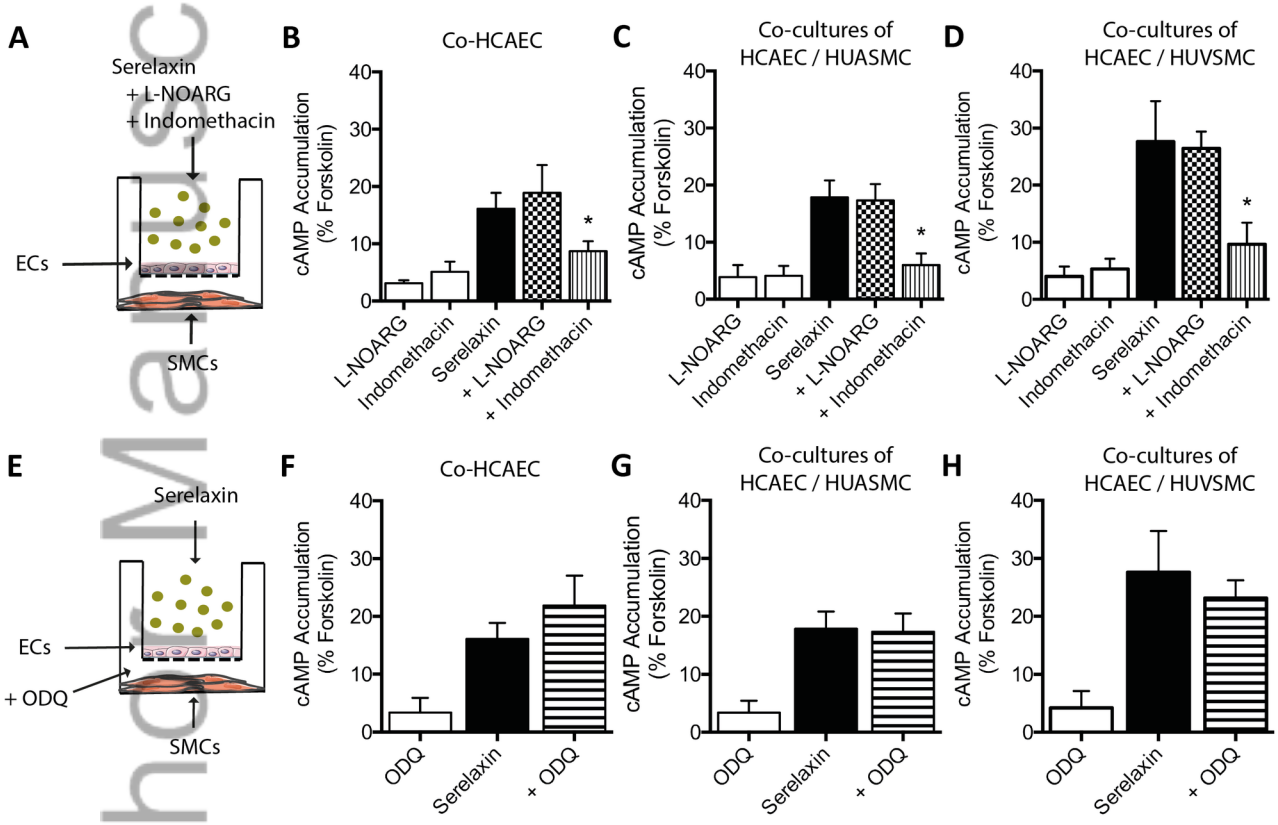
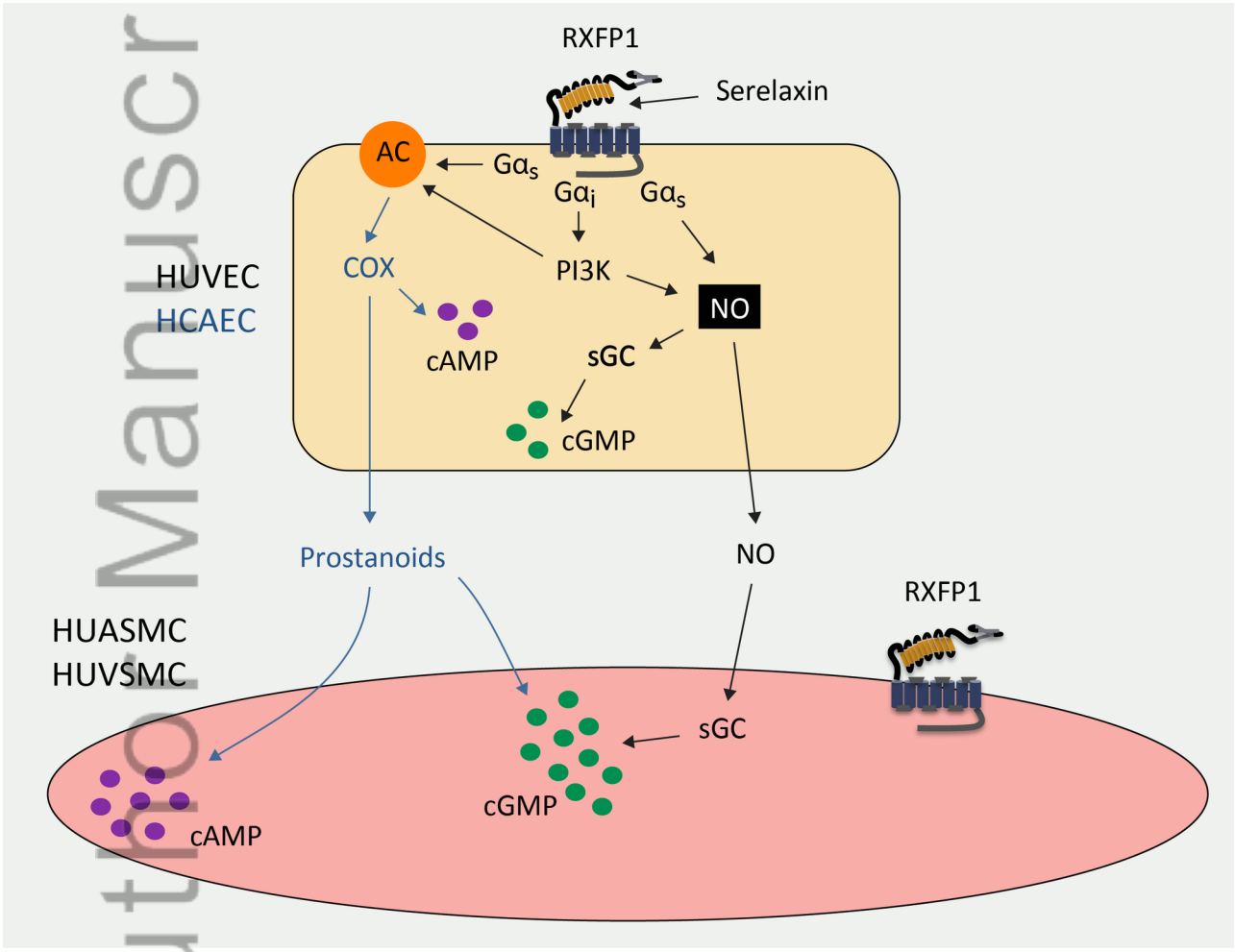


BPH_13371_F6.tif



BPH_13371_F7.tif

2. TABLES OF LINKS - TARGETS

A quick guide to completing the Tables of Links can be found **HERE**. (Please view as a **slideshow** to see the full animation), and a video can be found **here**.

Copy/Paste information into this table as shown in the example in grey shading below

TARGETS			
Nomenclature	**Target Id (insert after the standard URL below, no spaces)**	**Database page citation**	***Concise Guide to PHARMACOLOGY* citation**
RXFP1 receptor	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=351	Roger Summers, Ross Bathgate, Thomas Dschietzig, Michelle Halls, Richard Ivell, Barbara Sanborn, David Sherwood, Steve Sutton. Relaxin family peptide receptors. Accessed on 24/08/2015. IUPHAR/BPS Guide to PHARMACOLOGY, **http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=60**.	Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA and Harmar AJ, CGTP Collaborators. (2013) **The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors.** *Br J Pharmacol.* **170**: 1459–1581.
	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=		
	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=		
	http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=		

This table lists protein targets and ligands which are hyperlinked to corresponding entries in **http://www.guidetopharmacology.org**, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b).

3. TABLES OF LINKS - LIGANDS

A quick guide to completing the Tables of Links can be found **HERE**. (Please view as a **slideshow** to see the full animation), and a video can be found **here**.

Copy/Paste information into this table as shown in the example in grey shading below

LIGANDS			
Ligand name	**Ligand Id (insert after the standard URL below, no spaces)**	**INN only**	**IUPAC Name**
Relaxin (human) H2	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1989	Serelaxin	
	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=		
	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=		
	http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=		

This table lists protein targets and ligands which are hyperlinked to corresponding entries in **http://www.guidetopharmacology.org**, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b).