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RESEARCH PAPER

Serelaxin-mediated signal transduction in human vascular cells: bell-shaped concentration–response curves reflect differential coupling to G proteins

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BACKGROUND AND PURPOSE
In a recently conducted phase III clinical trial, RELAX-AHF, serelaxin infusion over 48 h improved short- and long-term clinical outcomes in patients with acute heart failure. In this study we used human primary cells from the umbilical vasculature to better understand the signalling mechanisms activated by serelaxin.

EXPERIMENTAL APPROACH
We examined the acute effects of serelaxin on signal transduction mechanisms in primary human umbilical vascular cells and its chronic actions on markers of cardiovascular function and disease.

KEY RESULTS
The RXFP1 receptor, the cognate serelaxin receptor, was expressed at the cell surface in HUVECs and human umbilical vein smooth muscle cells (HUVSMCs), human umbilical artery smooth muscle cells (HUASMCs) and human cardiac fibroblasts (HCFs), but not human umbilical artery endothelial cells. In HUVECs and HUVSMCs, serelaxin increased cAMP, cGMP accumulation and pERK1/2, and the concentration–response curves (CRCs) were bell-shaped. Similar bell-shaped CRCs for cGMP and pERK1/2 were observed in HCFs, whereas in HUASMCs, serelaxin increased cAMP, cGMP and pERK1/2 with sigmoidal CRCs. $G\alpha_{i/o}$ and lipid raft disruption, but not $G\alpha_s$ inhibition, altered the serelaxin CRC for cAMP and cGMP accumulation in HUVSMC but not HUASMC. Longer term serelaxin exposure increased the expression of neuronal NOS, VEGF, ET_β receptors and MMPs (gelatinases) in RXFP1 receptor-expressing cells.

CONCLUSIONS AND IMPLICATIONS
Serelaxin caused acute and chronic changes in human umbilical vascular cells that were cell background dependent. Bell-shaped CRCs that were observed only in venous cells and fibroblasts involved $G\alpha_{i/o}$ located within membrane lipid rafts.

Abbreviations
AHF, acute heart failure; CRC, concentration–response curve; DEA, diethylamine NONOate; eNOS, endothelial NOS; ET_B receptor, endothelin type B receptor; HCF, human cardiac fibroblast; HUAEC, human umbilical artery endothelial cell; HUASMC, human umbilical artery smooth muscle cell; HUVSMC, human umbilical vein smooth muscle cell; iNOS, inducible NOS; nNOS, neuronal NOS; pERK1/2, phosphorylated ERK 1 and 2; PI3K, phosphoinositide 3-kinase; PTX, pertussis toxin; RXFP1 receptor, relaxin family peptide receptor 1

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
β_2-adrenoceptor	eNOS
ET_B receptor	ERK1/2
RXFP1 receptor	Guanylyl cyclase (GC)
RXFP2 receptor	iNOS
Ligand gated ion channels[b]	MMP2
P2X1 receptor	MMP9
	nNOS
	PI3K

LIGANDS	
cAMP	Isoprenaline
cGMP	NF023
EGF	NF449
FGF-2	Nitric oxide (NO)
Forskolin	Relaxin
Hydrocortisone	Suramin
IGF-1	VEGFA
Insulin	Wortmannin

These Tables list key protein targets and ligands in this article 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 are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Acute heart failure (AHF) is a major global health challenge with high morbidity and mortality that represents a great burden on health care (Mosterd and Hoes, 2007). Along with predictions of increasing prevalence, treatment options for AHF have changed little over the last two decades and consequently patients continue to experience high morbidity and mortality. However, in the recent phase III clinical trial (RELAX-AHF), serelaxin (the recombinant form of human relaxin-2) produced a moderate improvement in one of the primary end points, dyspnoea, but also significantly reduced patient mortality at day 180 without any notable side effects (Teerlink *et al.*, 2013). Further analysis of the RELAX-AHF findings showed fewer signs of cardiac, renal and liver damage with early administration of serelaxin, which may contribute to the long-term survival benefit (Metra *et al.*, 2013).

Relaxin is a hormone that mediates cardiovascular adaptations observed during pregnancy and in particular systemic and renal vasodilatation (Conrad, 2010), although these effects are also observed in non-pregnant animals, *ex vivo* studies and in animal models of cardiovascular disease (Masini *et al.*, 1997; 2006; Bani *et al.*, 1998b). There are two distinct actions of serelaxin that have been described in *in vitro* and *in vivo* studies. Rapid serelaxin-mediated responses, observed after stimulation of serelaxin for minutes to hours (<1 h), occur via a $G\alpha_i$/PI3K/cAMP/Akt/eNOS-dependent mechanism in human subcutaneous and rodent renal and mesenteric arteries and also in human coronary artery and aortic endothelial cells (McGuane *et al.*, 2011b). Sustained serelaxin responses, observed after stimulation of serelaxin for days (24–48 h), are seen in rodent small renal and human subcutaneous arteries involving MMPs (gelatinases), endothelin receptor B (ET_B receptor), VEGF and NOS (Jeyabalan *et al.*, 2003; McGuane *et al.*, 2011a).

Although *in vitro* and *in vivo* studies support the potential benefits of serelaxin in humans in cardiovascular disease, there are knowledge gaps in our understanding of the mechanism of action. There is little information on the cells targeted by serelaxin and on signal transduction mechanisms in tissues relevant to the human cardiovascular system that endogenously express the RXFP1 receptor, the cognate serelaxin receptor. However, it is clear that serelaxin affects the tone of blood vessels. In rats, it was recently reported that the RXFP1 receptor is localized to endothelial and smooth muscle cells, although there are marked regional variations in distribution (Jelinic *et al.*, 2014). However, very little is known on the expression of the RXFP1 receptor and the signalling pathways it activates in human arteries and veins. In rats, the effects of serelaxin on arteries have been described in detail (Jeyabalan *et al.*, 2003; Conrad *et al.*, 2004; Conrad and Shroff, 2011) but much less is known of effects in veins. In addition, rat mesenteric arteries and veins both express RXFP1 receptors, yet only the arteries show serelaxin-mediated vascular remodelling (Jelinic *et al.*, 2014).

The effects of serelaxin on arteries and veins could be critical for the understanding of the clinical actions of serelaxin because nitrates, a classical therapy for heart failure, reduce congestion by causing venodilatation. Serelaxin, a known arteriodilator, also reduced congestion without causing hypotension in RELAX-AHF (Teerlink *et al.*, 2013), suggesting that serelaxin could potentially have additional venodilator properties. Therefore, we examined whether serelaxin targets cells in both the arterial and venous vasculature to activate vasodilator signal transduction mechanisms.

In order to address these knowledge gaps, we examined signal transduction mechanisms in primary cells from the human arterial and venous umbilical vasculature and heart, including endothelial cells, smooth muscle cells and cardiac fibroblasts. These were examined for RXFP1 receptor expression and markers of cardiovascular function and disease: short-term effects of serelaxin (<1 h) on cAMP, cGMP and pERK1/2; and the longer term effects of serelaxin (1–48 h) on the expression of VEGF, ET_B receptors, NOS isoforms and MMP activity.

Methods

Cell culture

Primary cultures of human umbilical artery endothelial cells (HUAECs), HUVECs, human umbilical artery smooth muscle cells (HUASMCs), human umbilical vein smooth muscle cells (HUVSMCs) and fetal human cardiac fibroblasts (HCFs: pooled from fetal atria and ventricles) were obtained from ScienCell Research Laboratories (San Diego, CA, USA). Endothelial cells were characterized by their expression of von Willebrand factor (Factor VIII), CD31 and by uptake of acetylated low-density lipoprotein (DiI-Ac-LDL). Smooth muscle cells were characterized by the presence of α-smooth muscle actin and desmin and fetal HCF were characterized by the presence of fibronectin (ScienCell). Cells were maintained in Medium 199 containing 5% FBS, penicillin (100 u·mL^{-1}), streptomycin (100 μg·mL^{-1}) and the relevant growth supplements for optimal growth of each cell type (ScienCell,). Endothelial cells were grown in endothelial cell growth supplement [BSA 10 μg·mL^{-1}, apo-transferrin 10 μg·mL^{-1}, insulin 5 μg·mL^{-1}, EGF 10 ng·mL^{-1}, fibroblast growth factor-2 (FGF-2) 2 ng·mL^{-1}, VEGF 2 ng·mL^{-1}, insulin-like growth factor-1 (IGF-1) 2 ng·mL^{-1}, hydrocortisone 1 μg·mL^{-1}, retinoic acid 10^{-7} M], smooth muscle cells in smooth muscle cell growth supplement (BSA 10 μg·mL^{-1}, apo-transferrin 10 μg·mL^{-1}, insulin 5 μg·mL^{-1}, FGF-2 2 ng·mL^{-1}, IGF-1 2 ng·mL^{-1}, hydrocortisone 1 μg·mL^{-1}) and fibroblasts in fibroblast cell growth supplement-2 (BSA 10 μg·mL^{-1}, apo-transferrin 10 μg·mL^{-1}, insulin 7.5 μg·mL^{-1}, EGF 2 ng·mL^{-1}, FGF-2 2 ng·mL^{-1}, hydrocortisone 1 μg·mL^{-1}) (information provided by ScienCell). Early passage cultures (2–5) were used for all experiments.

Reverse-transcription real-time PCR (RT-qPCR)

RNA was extracted using the TRIzol® reagent (Invitrogen, Mulgrave, Victoria, Australia) and cDNA synthesized using the iScript cDNA synthesis kit (BioRad, Gladesville, NSW, Australia) according to the manufacturer's instructions. RT-PCR was performed using the Taqman Assay for RXFP1 receptors (Invitrogen) according to the manufacturer's instructions.

Radioligand binding

Serelaxin was iodinated with Na^{125}I (Perkin-Elmer, Glen Waverley, Victoria, Australia) using the chloramine-T method as described previously (van der Westhuizen *et al.*, 2010). Whole-cell competition-binding assays were performed as described previously (Halls *et al.*, 2005). Briefly, cells (0.8–1 × 10^6 cells) with [^{125}I]-serelaxin (200 pM) were allowed to compete with unlabelled serelaxin (10 pM–0.1 μM) for 90 min at room temperature. Total binding was determined by radioligand alone whereas non-specific binding was determined by 0.1 μM unlabelled serelaxin.

cAMP and cGMP accumulation assays

cAMP accumulation was determined as previously described (Halls *et al.*, 2006). Briefly, cells were plated into 24-well plates (1 × 10^5 cells per well) and grown overnight to achieve a confluent monolayer. Prior to stimulation, cells were serum starved in M199 medium for 4 h. Where appropriate, the cells were pre-incubated with the PI3K inhibitor wortmannin (100 nM, 30 min), the $G\alpha_{i/o}$ inhibitor PTX (50 ng·mL^{-1}, 18 h), the $G\alpha_s$ inhibitor NF449 (10 μM, 30 min), the $G\alpha_{i/o}$ inhibitor NF023 (10 μM, 30 min), suramin (10 μM, 30 min) or filipin III (1 μg·mL^{-1}, 1 h). Levels of cAMP and cGMP were detected according to the manufacturer's instructions (Perkin-Elmer).

pERK1/2 assay

pERK1/2 was measured using the Surefire ERK kit (TGR BioSciences, Hindmarsh, South Australia, Australia) as described previously (van der Westhuizen *et al.*, 2007). Briefly, cells were plated into 24-well plates (1 × 10^5 cells per well) and grown overnight to achieve a confluent monolayer. Prior to stimulation, cells were serum starved in M199 medium for 4 h. Where appropriate, the cells were pre-incubated with wortmannin (100 nM, 30 min) or PTX (50 ng·mL^{-1}, 18 h). Levels of pERK1/2 were detected according to the manufacturer's instructions (Perkin-Elmer).

Gelatin zymography

Gelatin zymography was performed as described previously to determine changes in levels of MMP2 (gelatinase A) and MMP9 (gelatinase B) in cultured medium samples (Chow *et al.*, 2012).

Western blotting

Western blotting was performed as described previously (Chow *et al.*, 2012). Briefly, 20–30 μg of protein was separated on a polyacrylamide gel, transferred to a PVDF membrane and probed with the following primary antibodies: anti-eNOS antibody (R&D Systems; 1:1000, overnight at 4°C), anti-nNOS antibody (R&D Systems; 1:500, overnight at 4°C), anti-iNOS antibody (Cell Signalling, Beverley, MA, USA; 1:1000, overnight at 4°C), anti-VEGF antibody (Abcam, Cambridge, MA, USA; 1:1000, 2 h at room temperature), anti-ET_B receptor antibody (Pierce, Rockford, IL, USA; 1:1000, overnight at 4°C), anti-β-actin antibody (Cell Signalling; 1:1000, 2 h at room temperature). Membranes were then probed with secondary antibodies (Alexa Fluor 647 anti-rabbit IgG antibody, Alexa Fluor 647 anti-mouse IgG antibody; 2 μg·mL^{-1}; Invitrogen) and scanned using the Typhoon Trio (GE Healthcare, Melbourne, Victoria, Australia), and densitometry was conducted on each band using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data were analysed using GraphPad Prism v6.0 (La Jolla, CA, USA). All data represent the mean ± SEM of '*n*' independent experiments. Signalling assays were conducted in duplicate with each point being taken as the mean of the replicates. Concentration–response curves (CRCs) were fitted using either a sigmoidal or Gaussian model. Binding data were fitted using a one-site competition-binding model to obtain pIC_{50} values that were used to calculate pK_i. Densitometry on bands from gel zymography and Western blotting was conducted using the ImageJ software. Statistical differences among treatment groups were determined by a one-way ANOVA with a Dunnett's *post hoc* test for each cell type studied and statistical significance accepted at $P < 0.05$.

Materials

Serelaxin was kindly provided by Dr D.R. Stewart (Novartis, Basel, Switzerland). Pertussis toxin (PTX), wortmannin, filipin III and suramin were purchased from Sigma (Castle Hill, NSW, Australia). NF023 and NF449 were purchased from Calbiochem (Alexandria, NSW, Australia). TGF-β1 was purchased from R&D Systems (Gymea, NSW, Australia).

Results

Cell surface RXFP1 receptors expression occurs in HUVECs, HUVSMCs, HUASMCs and HCFs, but not HUAECs

RXFP1 receptor mRNA, measured by qPCR, was present in HUAECs, HUVECs, HUVSMCs, HUASMCs, HCFs and human testis (positive control; Figure 1A). Cell surface RXFP1 receptor expression, measured by competition binding of [^{125}I]-serelaxin with unlabelled serelaxin, was detected in HUASMCs, HUVECs, HUVSMCs and HCFs, but not in HUAECs (Figure 1B). Binding affinity correlated well with that observed in HEK cells recombinantly expressing RXFP1 receptors (Supporting Information Table S1). The lack of cell surface expression of RXFP1 receptors in HUAECs was supported by the failure of serelaxin to cause cAMP accumulation (Supporting Information Fig. S1a), cGMP accumulation (Supporting Information Fig. S1b) or pERK1/2 (Supporting Information Fig. S1c) in these cells.

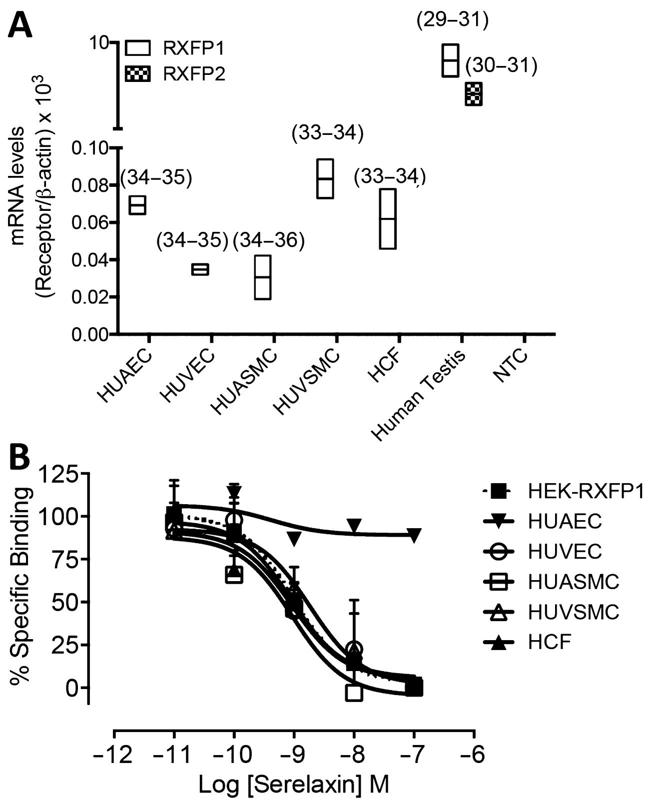

Figure 1

The expression of RXFP1 and RXFP2 receptor mRNA and RXFP1 receptor protein in human primary umbilical vascular cells and human primary cardiac fibroblasts. qPCR (A) was utilized to show expression levels of RXFP1 and RXFP2 receptor mRNA in HUAECs, HUVECs, HUASMCs, HUVSMCs and HCFs relative to β-actin (n = 2). RXFP2 receptor mRNA was only measureable in the positive control. Cell surface RXFP1 receptor protein expression was determined by radioligand binding (B) utilizing [^{125}I]-serelaxin and showed specific serelaxin binding in HEK-RXFP1 cells (n = 6), HUASMCs (n = 4), HUVECs (n = 4), HUVSMCs (n = 3) and HCFs (n = 3), but not in HUAECs (n = 2).

cAMP accumulation in response to acute serelaxin administration

Serelaxin increases cAMP *in vitro* (Halls *et al.*, 2009b) and stimulates inotropy in human atrial myocardium (Dschietzig *et al.*, 2011). In this study, serelaxin (30 nM) increased cAMP accumulation in HUVECs, HUVSMCs and HUASMCs with the response peaking by 30 min (Supporting Information Fig. S2a). In HUVECs (Figure 2A) and HUVSMCs (Figure 2B), serelaxin (30 min) concentration- dependently increased cAMP accumulation to 5% of the forskolin response respectively. CRCs for cAMP accumulation in both cell types were bell-shaped with pEC$_{50}$s of 9.1 ± 0.4 and 9.6 ± 0.4, respectively, for the initial part of the curve. As previous studies have shown that serelaxin-mediated cAMP accumulation in HEK-RXFP1 receptor cells involves Gα_i proteins and PI3K (Halls *et al.*, 2009b), we investigated the effect of PTX (G$\alpha_{i/o}$ inhibitor) and wortmannin (PI3K inhibitor) on the cAMP response. Both PTX (50 ng·mL^{-1}, 18 h) and wortmannin (100 nM, 30 min) significantly inhibited serelaxin-mediated (30 nM) cAMP accumulation in HUVECs (Figure 2A) and HUVSMCs (Figure 2B). In HUASMCs, however, serelaxin (30 min) increased cAMP accumulation but the response was unaffected by PTX (50 ng·mL^{-1}, 18 h) or wortmannin (100 nM, 30 min) pretreatment, suggesting that the response predominantly involved Gα_s (Figure 2C). Most interestingly, and in contrast to HUVECs and HUVSMCs, the CRC to serelaxin in HUASMCs was sigmoidal with a pEC$_{50}$ of 9.0 ± 0.3. In HCFs, serelaxin failed to cause cAMP accumulation (Figure 2D), reflecting previous findings in rat atrial and ventricular fibroblasts (Samuel *et al.*, 2004; Mosterd and Hoes, 2007).

cGMP accumulation in response to acute serelaxin stimulation

Serelaxin-mediated vasodilatation, *in vitro* (Bani *et al.*, 1998a) and *ex vivo* (Jeyabalan *et al.*, 2003; McGuane *et al.*, 2011a,b), occurs via a NOS/NO/cGMP-dependent mechanism. In human vascular cells, serelaxin (30 nM) time dependently increased cGMP accumulation in HUVECs, HUVSMCs, HUASMCs and HCFs with the response peaking by 30 min (Supporting Information Fig. S2b). Serelaxin increased cGMP accumulation concentration-dependently in HUVECs (Figure 3A: pEC$_{50}$ = 8.9 ± 0.4), HUVSMCs (Figure 3B: pEC$_{50}$ = 9.2 ± 0.4), HUASMCs (Figure 3C: pEC$_{50}$ = 9.1 ± 0.3) and HCFs (Figure 3D: pEC$_{50}$ = 9.1 ± 0.3). The maximum serelaxin response was higher in HUVECs and HCFs (75 and 60% of DEA) but lower in HUASMCs (40% of DEA) and HUVSMCs (25% of DEA). PTX (50 ng·mL^{-1}, 18 h) and wortmannin (100 nM, 30 min) pretreatment significantly inhibited serelaxin-mediated (30 nM) cGMP accumulation, confirming the involvement of Gα_i and PI3K, but these responses were qualitatively different in different cell types. In HUVECs, HUVSMCs and HCFs, serelaxin produced bell-shaped CRCs whereas in HUASMCs, as observed for cAMP, it was sigmoidal.

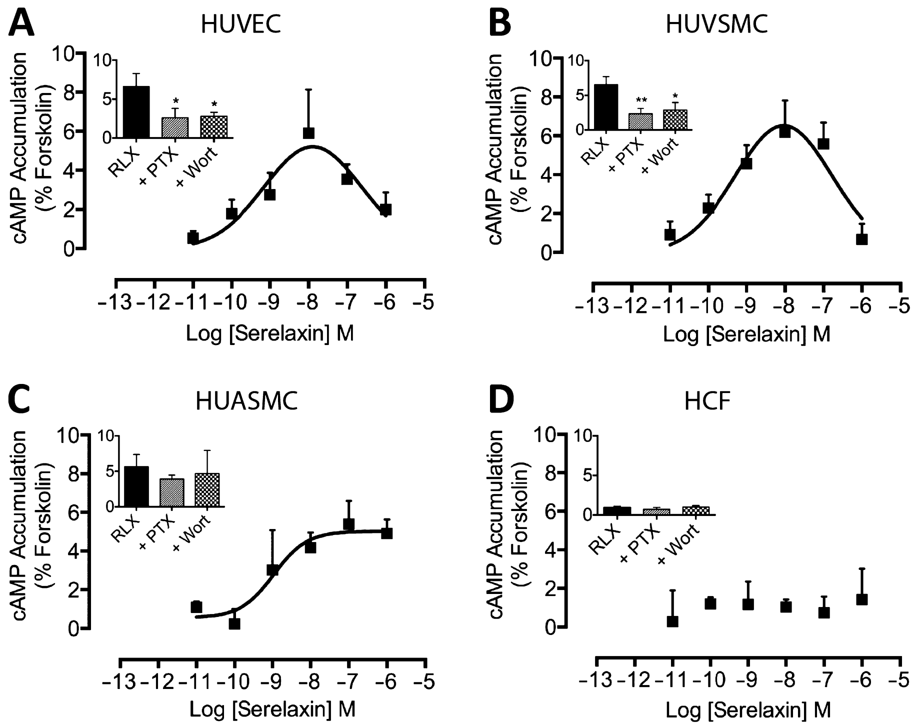

Figure 2

The effect of serelaxin on cAMP accumulation in human primary umbilical vascular cells and cardiac fibroblasts. Serelaxin treatment (30 min) increased cAMP accumulation in (A) HUVECs ($n = 6$), (B) HUVSMCs ($n = 6$) and (C) HUASMCs ($n = 4$), but not in (D) HCFs ($n = 3$). The serelaxin CRC was bell-shaped for HUVECs and HUVSMCs but sigmoidal for HUASMCs. For each cell type, the effect of PTX (50 ng·mL^{-1}, 18 h) and wortmannin (100 nM, 30 min) pretreatment was determined after exposure to serelaxin (30 nM) for 30 min to determine the role of Gα_i and PI3K. Statistical significance was assessed using a one-way ANOVA with a Dunnett's *post hoc* test compared with serelaxin alone: *$P < 0.05$ and **$P < 0.01$.

ERK1/2 phosphorylation in response to acute serelaxin stimulation

ERK1/2, a kinase that promotes cell survival and inhibits apoptosis, is phosphorylated following treatment with serelaxin in HUVECs and vascular smooth muscle cells (Bani *et al.*, 1998b; Zhang *et al.*, 2002; Dschietzig *et al.*, 2003; Masini *et al.*, 2006), but the mechanism involved is not known. Our study is the first to show that serelaxin causes an increase in pERK1/2 in human vascular cells and cardiac fibroblasts in a Gα_i- and PI3K-dependent manner. Serelaxin (30 nM) treatment rapidly increased pERK1/2 in HUVECs (15% of FBS), HUVSMCs (15% of FBS) and HCFs (50% of FBS) peaking at 10 min, and in HUASMCs (25% of FBS) at 5 min with responses that declined to basal after 15 min, except in HUVECs where pERK1/2 levels plateaued after 10 min (Supporting Information Fig. S2c). Responses in all cell types were inhibited by PTX and wortmannin pretreatment suggesting the involvement of Gα_i and PI3K in pERK1/2 responses (Supporting Information Fig. S3a–d). Serelaxin concentration dependently increased pERK1/2 in HUVECs (Supporting Information Fig. S3a, pEC$_{50}$: 9.2 ± 0.3), HUVSMCs (Supporting Information Fig. S3b, pEC$_{50}$: 9.2 ± 0.4), HUASMCs (Supporting Information Fig. S3c, pEC$_{50}$: 9.1 ± 0.4) and HCFs (Supporting Information Fig. S3d, pEC$_{50}$: 9.1 ± 0.3). As in the case of cAMP and cGMP accumulation, CRCs were bell-shaped in HUVECs, HUVSMCs and HCFs, and sigmoidal in HUASMCs.

Role of G proteins in the regulation of serelaxin concentration–response relationships

Previous studies have suggested that differential coupling to G proteins can explain biphasic or bell-shaped concentration–response relationships (Baker and Hill, 2006). As the RXFP1 receptor is known to couple to Gα_s, G$\alpha_{O/B}$ and G$\alpha_{i/o}$ (Halls *et al.*, 2006), we used pharmacological inhibitors to selectively disrupt coupling of Gα_s, G$\alpha_{O/B}$ and G$\alpha_{i/o}$ to RXFP1 receptors. In both HUASMC (Figure 4A) and HUVSMC (Figure 4B), pretreatment with the Gα_s inhibitor NF449 (10 μM, 30 min) reduced the E-max and right shifted the serelaxin CRC for cAMP accumulation. Similar effects were observed on the concentration–response relationships for cGMP accumulation (Figure 5A, B). In HUASMC, pretreatment with the G$\alpha_{i/o}$ inhibitor NF023 (10 μM, 30 min) reduced the E-max of cAMP (Figure 4C) and cGMP (Figure 4C) accumulation without affecting serelaxin potency. In HUVSMC, the G$\alpha_{i/o}$ inhibitor NF023 (10 μM, 30 min) reduced the E-max for cAMP (Figure 4D) and cGMP (Figure 5D) accumulation, but also altered the shape of the CRC from bell-shaped to sigmoidal. These data suggest that Gα_s and G$\alpha_{i/o}$ regulate distinct phases of the serelaxin CRC for cAMP and cGMP

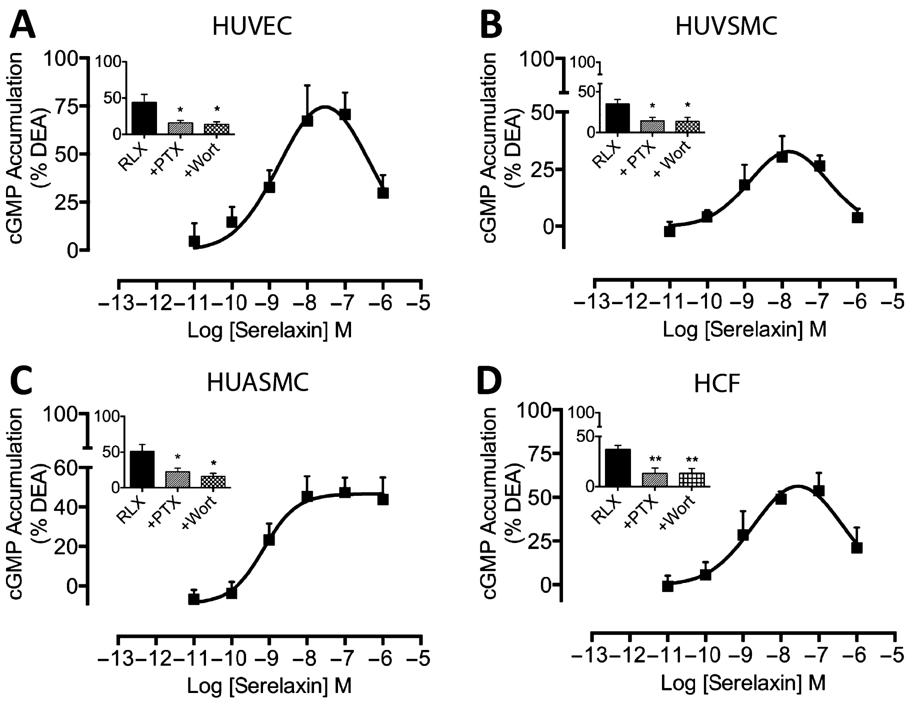

Figure 3

The effect of serelaxin on cGMP accumulation in human primary umbilical vascular cells and cardiac fibroblasts. Serelaxin treatment (30 min) increased cGMP accumulation in (A) HUVECs ($n = 7$), (B) HUVSMCs ($n = 5$), (C) HUASMCs ($n = 6$) and (D) HCFs ($n = 5$). The serelaxin CRC was bell-shaped for HUVECs, HUVSMCs and HCFs but sigmoidal for HUASMCs. PTX (50 ng·mL^{-1}, 18 h) and wortmannin (100 nM, 30 min) pretreatment significantly inhibited serelaxin (30 nM)-mediated cGMP accumulation in each cell type. Statistical significance was assessed using a one-way ANOVA with a Dunnett's *post hoc* test compared with serelaxin alone: *$P < 0.05$ and **$P < 0.01$.

accumulation in primary human vascular cells and that the G-protein coupling is influenced by the cellular background. This was further supported by co-incubation with NF449 and NF023 that totally abolished serelaxin-mediated cAMP (Figure 4E, F) and cGMP (Figure 5E, F) responses in both HUASMC and HUVSMC. As NF449 and NF023 are analogues of suramin, a generic purine receptor antagonist, and as such these peptides are selective antagonists for P2X1 receptors (Soto *et al.*, 1999; Rettinger *et al.*, 2005), we conducted control experiments that showed that pretreatment with suramin (10 μM) had no effect on serelaxin-mediated cAMP (Supporting Information Fig. S4a, b) and cGMP (Supporting Information Fig. S3c, d) accumulation in HUASMC and HUVSMC. Previous studies showed that $G\alpha_i$ can be localized to specific regions of the plasma membrane and RXFP1 receptor coupling to $G\alpha_{i3}$, but not $G\alpha_s$ or $G\alpha_{O/B}$, is dependent on membrane lipid rafts in HEK-RXFP1 receptor cells (Halls *et al.*, 2009a). Therefore, we disrupted lipid rafts in HUASMC by filipin III pretreatment (1 μg·mL^{-1}, 1 h) that caused a drop in E-max for cAMP (Figure 4G) and cGMP (Figure 5G) accumulation without any change in serelaxin potency. Pretreatment of HUVSMC with filipin III (1 μg·mL^{-1}, 1 h) reduced E-max, and converted the bell-shaped CRCs for cAMP (Figure 4H) and cGMP (Figure 5H) accumulation to sigmoidal CRCs. Thus, the effects of filipin III mimicked the effects of the $G\alpha_{i/o}$ inhibitor (NF023), suggesting that both the presence and the location of $G\alpha_{i/o}$ critically shape serelaxin concentration–response relationships.

Changes in expression of VEGF, ET_B and nNOS in human vascular cells following 48 h serelaxin administration

The effects of 48 h serelaxin treatment on protein expression of VEGF, ET_B and NOS are implicated in its vasodilator effects (Dschietzig *et al.*, 2003; Conrad, 2010; McGuane *et al.*, 2011b). Serelaxin (10 ng·mL^{-1}; 1.68 nM) caused a twofold increase in nNOS expression in HUVECs, HUVSMCs and HCFs, and a threefold increase in HUASMCs (Figure 6A–D) that parallels our findings previously observed in rat renal myofibroblasts (Mookerjee *et al.*, 2009). Similarly, and also consistent with previous findings (Alexiou *et al.*, 2013), serelaxin increased the expression of iNOS in HUVECs (~2-fold induction) and had no effect on the expression of eNOS (data not shown). We were unable to detect and measure iNOS and eNOS in HUASMCs, HUVSMCs and HCFs (data not shown). Similar to previous studies in human endometrial cells (Unemori *et al.*, 2000), serelaxin also elevated $VEGF_{165}$ (referred to as VEGF-A) expression by 1.5-fold in HUVECs, HUASMCs and HCFs (Figure 6A, C, D). VEGF-A is the most predominant isoform expressed in the vasculature and it has a crucial role in angiogenesis. There are four different isoforms of VEGF-A ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) of which $VEGF_{165}$ is the most predominant (Ferrara, 2004). Although VEGF expression has been reported to be associated with cAMP accumulation (Unemori *et al.*, 2000), it was unaltered in HUVSMCs (Figure 6B) despite increased cAMP accumulation

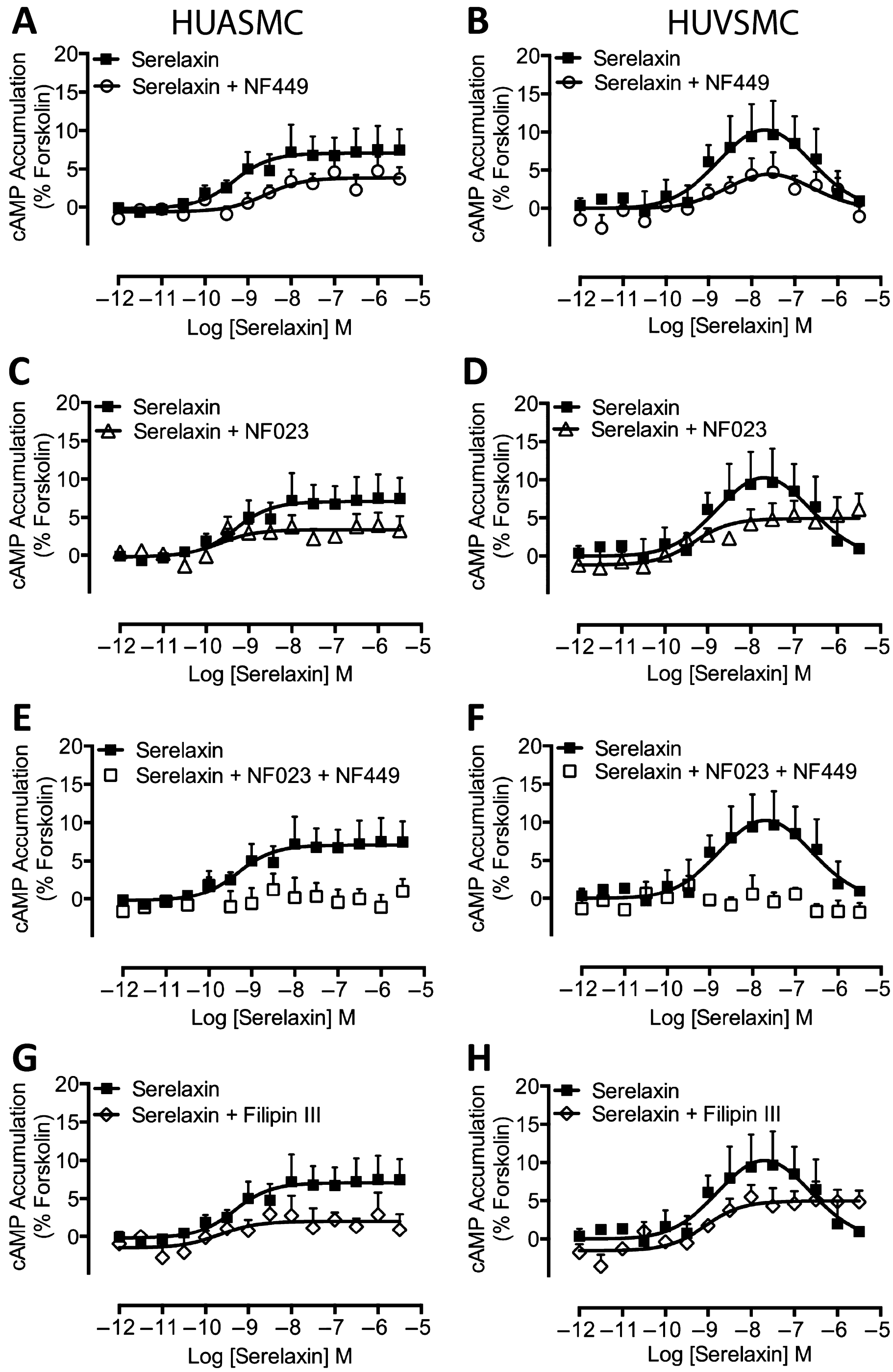

Figure 4

The role of G-protein coupling in serelaxin-mediated cAMP accumulation in human primary umbilical vascular cells. Pretreatment of HUASMC ($n = 6$) in (A) and of HUVSMC ($n = 6$) in (B) with the selective $G\alpha_s$ inhibitor NF449 (10 μM, 30 min) caused a rightward shift and reduced the E-max of the cAMP CRC to serelaxin without modifying the shape of the curve. Pretreatment of HUASMC ($n = 7$) in (C) and of HUVSMC ($n = 6$) in (D) with the selective $G\alpha_{i/o}$ inhibitor NF023 (10 μM, 30 min) reduced the E-max of the cAMP CRC to serelaxin and changed the shape of the curve observed with HUVSMC (D) from bell-shaped to sigmoidal. In both (E) HUASMC ($n = 6$) and (F) HUVSMC ($n = 6$), pretreatment with both NF449 and NF023 completely abolished serelaxin-mediated cAMP responses, showing that the responses result entirely from RXFP1 receptors interaction with G proteins. In (G) HUASMC ($n = 6$) and in (H) HUVSMC ($n = 6$), pretreatment with filipin III (1 $\mu g \cdot mL^{-1}$, 1 h), which disrupts lipid rafts, mimicked the effect of the $G\alpha_{i/o}$ inhibitor NF023 – reducing E-max and converting CRCs from bell-shaped to sigmoidal in HUVSMC.

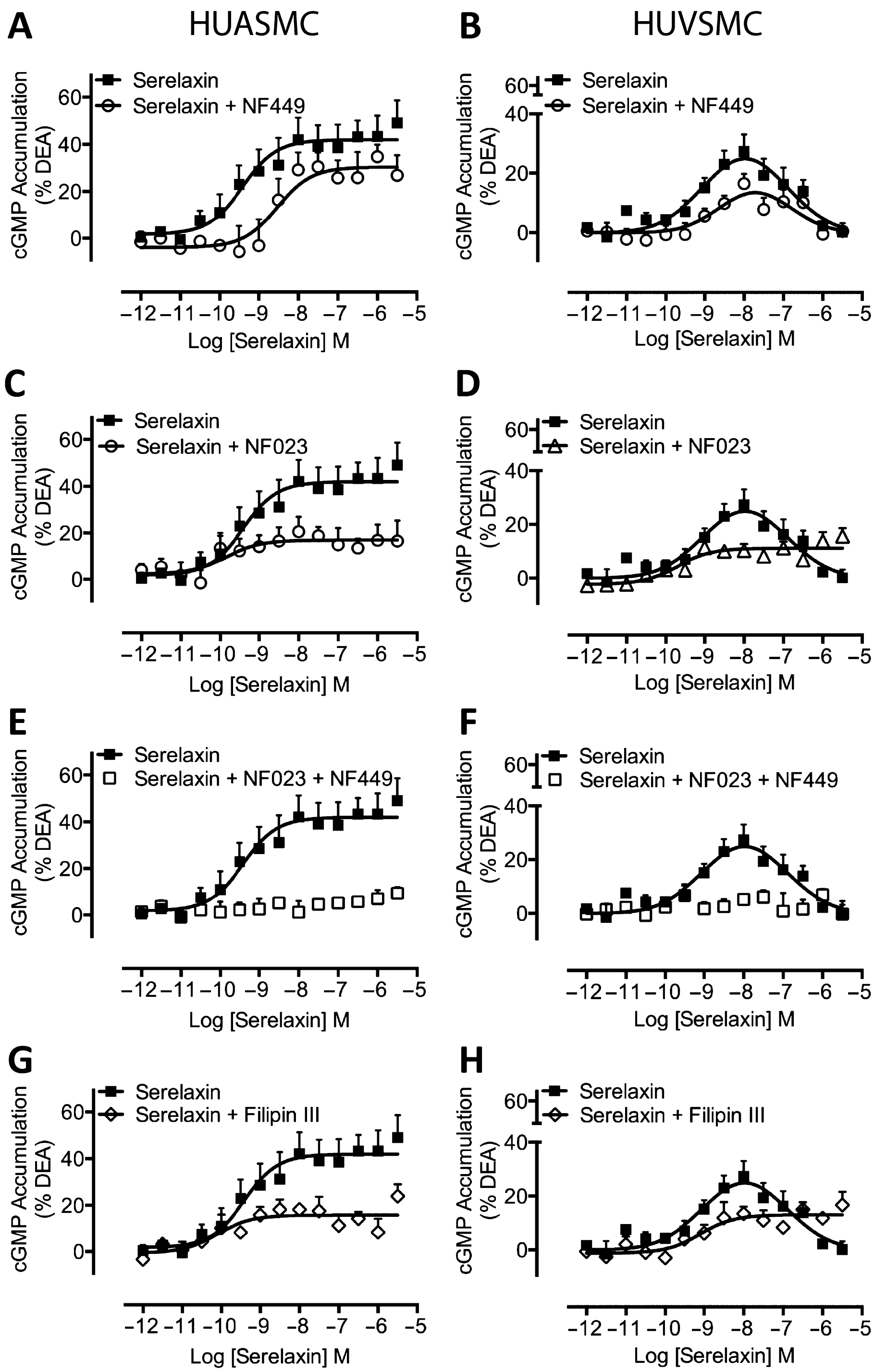

Figure 5

The role of G-protein coupling in serelaxin-mediated cGMP accumulation in human primary umbilical vascular cells. Pretreatment of HUASMC ($n = 6$) in (A) and of HUVSMC ($n = 6$) in (B) with the selective $G\alpha_s$ inhibitor NF449 (10 μM, 30 min) caused a rightward shift and reduced the E-max of the cGMP CRC to serelaxin without modifying the shape of the curve. Pretreatment of HUASMC ($n = 7$) in (C) and of HUVSMC ($n = 6$) in (D) with the selective $G\alpha_{i/o}$ inhibitor NF023 (10 μM, 30 min) reduced the E-max of the cGMP CRC to serelaxin and changed the shape of the curve observed with HUVSMC (D) from bell-shaped to sigmoidal. In both (E) HUASMC ($n = 6$) and (F) HUVSMC ($n = 6$), pretreatment with both NF449 and NF023 completely abolished serelaxin-mediated cGMP responses, showing that the responses result entirely from RXFP1 receptors interaction with G proteins. In (G) HUASMC ($n = 6$) and in (H) HUVSMC ($n = 6$), pretreatment with filipin III (1 μg·mL^{-1}, 1 h), which disrupts lipid rafts, mimicked the effect of the $G\alpha_{i/o}$ inhibitor NF023 – reducing E-max and converting CRCs from bell-shaped to sigmoidal in HUVSMC.

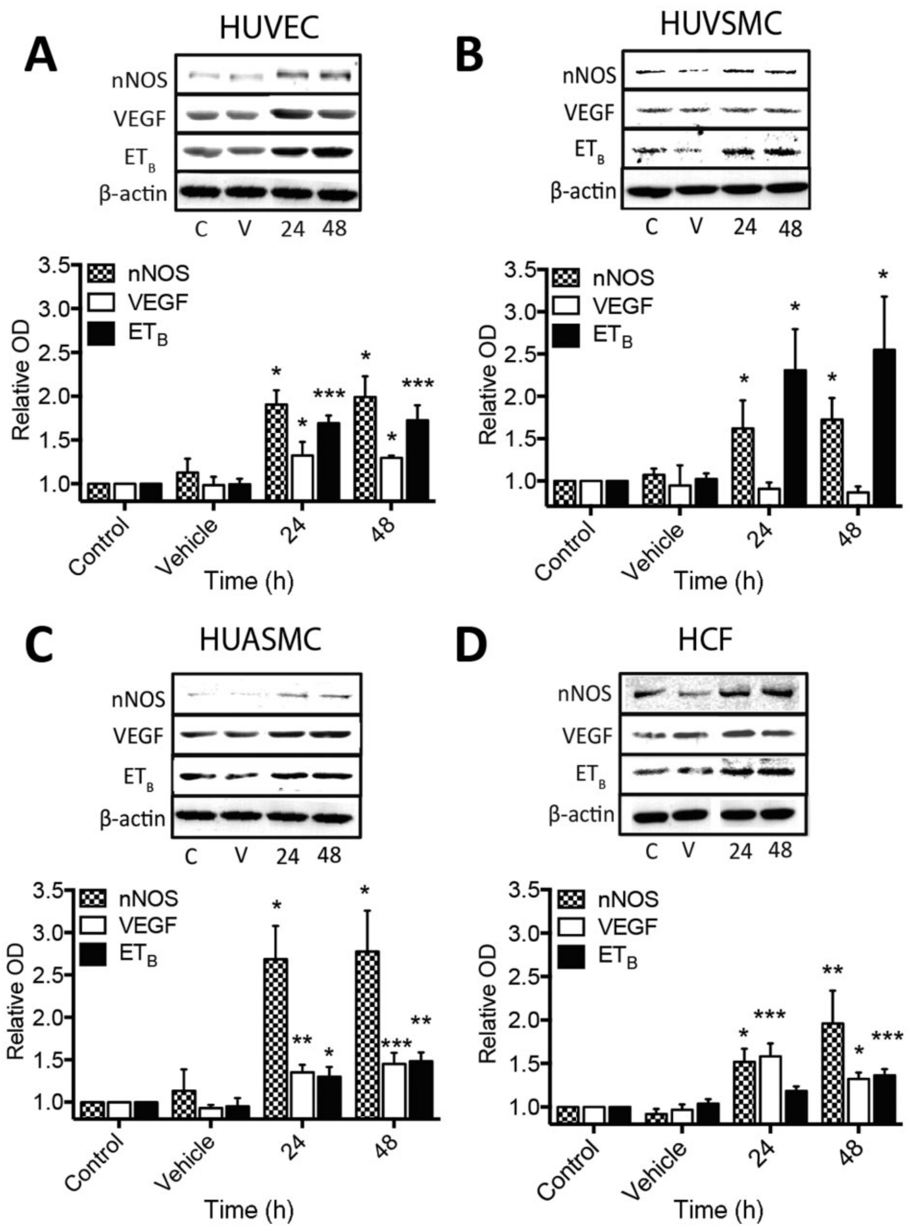

Figure 6

Changes in the expression of nNOS, VEGF and ET_B receptors in human primary umbilical vascular cells and cardiac fibroblasts after serelaxin (1.68 nM) exposure for 24 and 48 h. In HUVECs (A), serelaxin treatment increased the expression of nNOS ($n = 5$), ET_B receptors ($n = 6$) and VEGF ($n = 7$). In HUVSMCs (B), serelaxin treatment increased the expression of nNOS ($n = 5$) and ET_B ($n = 5$) but not VEGF ($n = 4$); however, in HUASMC (C), serelaxin treatment increased the expression of nNOS ($n = 6$), ET_B receptors ($n = 7$) and VEGF ($n = 5$). In HCFs (D), similar to HUVECs and HUASMCs, serelaxin treatment increased the expression of nNOS ($n = 5$), ET_B receptors ($n = 5$) and VEGF ($n = 5$). A representative blot of each protein and β-actin, a loading control, is shown with the densitometry in each figure. Statistical significance was assessed using a one-way ANOVA with a Dunnett's *post hoc* test compared with vehicle alone: $*P < 0.05$ and $**P < 0.01$.

in these cells (Figure 2B). Furthermore, in HCFs, serelaxin increased VEGF expression but had no effect on cAMP accumulation (Figure 2D). The changes may involve the ERK1/2 (Milanini *et al.*, 1998; van der Westhuizen *et al.*, 2007) or NO (Dulak *et al.*, 2000) pathways that are activated in all cell types. Furthermore, and consistent with previous studies (Dschietzig *et al.*, 2003), serelaxin increased ET_B receptor expression in HUVECs and HUVSMCs by two- to threefold, and in HUASMCs and HCFs by 1.5-fold (Figure 6A–D). ET_B receptor expression has been reported to be downstream of MMP and ERK1/2 signalling (Dschietzig *et al.*, 2003; Jeyabalan *et al.*, 2003; Chow *et al.*, 2012), and these pathways were activated in all cell types where ET_B receptor expression was increased (see Figure 7 and Supporting Information Fig. S3).

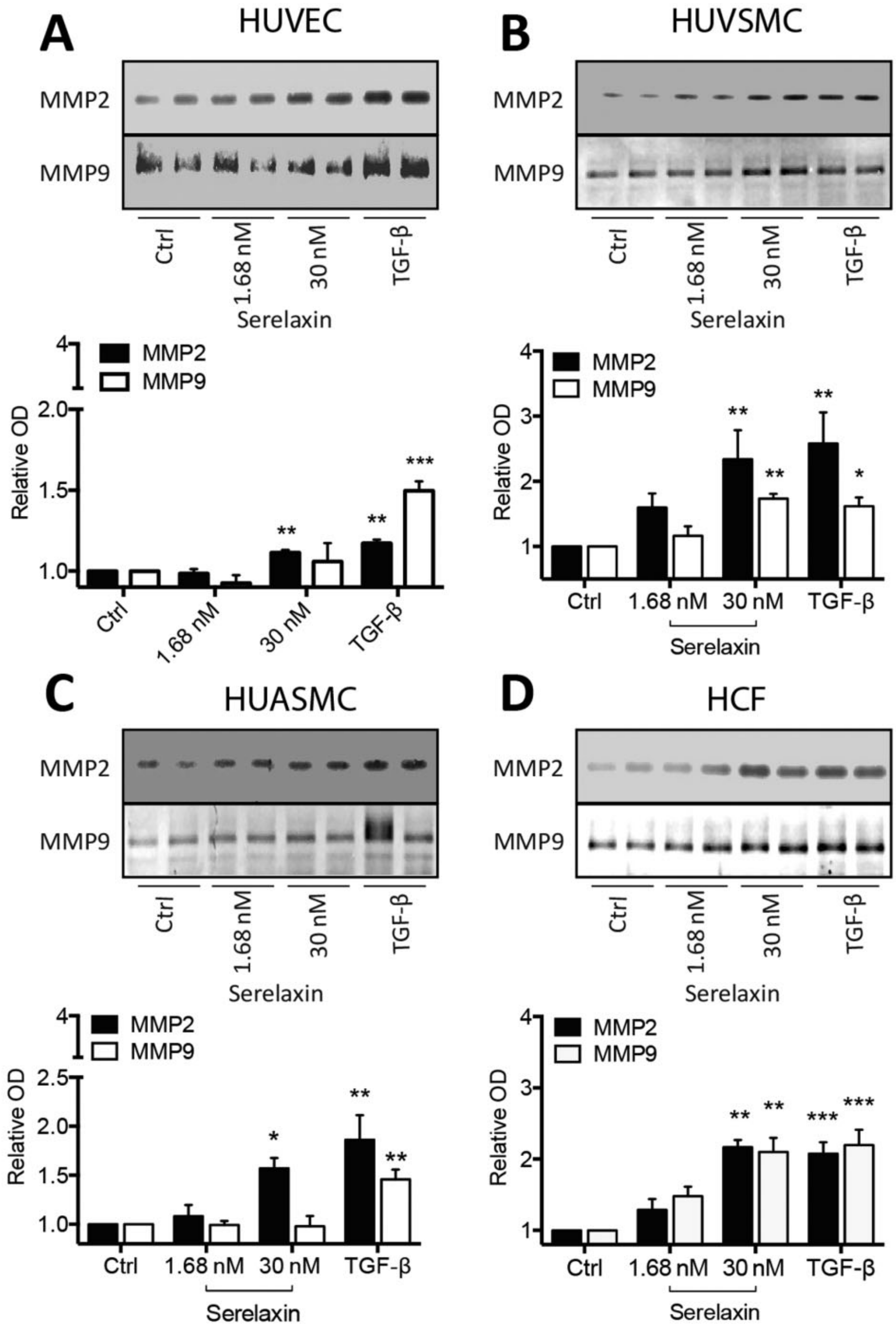

Figure 7

Changes in the activity of MMPs in human primary umbilical vascular cells and cardiac fibroblasts using zymography to assess changes in activity of MMP2 and MMP9 after long-term serelaxin exposure (1.68 and 30 nM) for 48 h. Serelaxin treatment increased the activity of MMP2 in (A) HUVECs ($n = 5$), (B) HUVSMCs ($n = 6$), (C) HUASMCs ($n = 7$) and (D) HCFs ($n = 5$). Serelaxin also significantly increased the activity of MMP9 but only in (B) HUVSMCs ($n = 5$) and (D) HCFs ($n = 5$) and not in (A) HUVECs ($n = 5$) and (C) HUASMCs ($n = 5$). Furthermore, and consistent with the lack of RXFP1 receptor expression (Figure 1), serelaxin had no effect on MMP activity in HUAECs ($n = 2$). A representative scan of each zymography is shown along with the densitometry in each figure. Statistical significance was assessed using a one-way ANOVA with a Dunnett's *post hoc* test compared with control alone: *$P < 0.05$ and **$P < 0.01$.

Changes in MMP activity in human vascular cells following 48 h serelaxin administration

The matrix/collagen-degrading enzymes MMP2 and MMP9 have roles in the remodelling of organs and blood vessels with clear influences on organ function and blood haemodynamics (Jeyabalan *et al.*, 2003). In addition, they convert big ET to ET_{1-32} that activates ET_B receptors to enhance vasodilatation (Fernandez-Patron *et al.*, 1999). *In vivo*, serelaxin increases vascular MMP activity to inhibit myogenic reactivity and promote vasodilatation (Jeyabalan *et al.*, 2006a,b). Our study supports these findings as serelaxin (1.68 and 30 nM for 48 h) increased MMP2 activity in HUVECs, HUVSMCs, HUASMCs and HCFs (Figure 7A–D). Serelaxin (30 nM) caused a three- and twofold increase in MMP2 activity in HUVSMCs and HCFs, but only a 1.2- and 1.5-fold increase in activity in HUVECs and HUASMCs. Serelaxin (30 nM) doubled MMP9 activity in HUVSMCs and HCFs (Figure 7B, D) but had no effect in HUVECs and HUASMCs (Figure 7A, C). In HUAECs, serelaxin had no effect on MMP2 and MMP9 activity (data not shown), consistent with the lack of cell surface RXFP1 receptors expression in HUAECs. Gelatinase activity and/or expression is controlled through a pERK/nNOS/NO/cGMP-dependent pathway (Chow *et al.*, 2012), consistent with our findings as these signalling pathways were activated in all cell types expressing RXFP1 receptors (Figure 3 and Supporting Information Fig. S3).

Discussion and conclusions

In the RELAX-AHF clinical trial, serelaxin infusion over 48 h improved short- and long-term outcomes in patients with acute cardiac failure. Here we have examined the acute (<1 h) and chronic (24–48 h) effects of serelaxin on signalling in human vascular cells in order to better understand the mechanism of serelaxin action in humans experimentally and clinically. This study demonstrates that serelaxin had different effects on human vascular cells dependent on the cellular background. We showed that the bell-shaped CRCs that are a hallmark of serelaxin activity experimentally (Unemori *et al.*, 1996; Danielson and Conrad, 2003; Halls *et al.*, 2006) and clinically (Teerlink *et al.*, 2009) can be demonstrated in human venous cells and cardiac fibroblasts and that they are dependent on the type and location of G proteins present.

Acute serelaxin administration causes rapid dilation of human subcutaneous arteries and rodent renal and mesenteric arteries in a Gα_i/PI3K/Akt/eNOS-dependent manner (McGuane *et al.*, 2011b). The present study demonstrates in human vascular cells that serelaxin increases cAMP, cGMP and pERK1/2 in a Gα_i- and PI3K-dependent manner (Figure 8). Longer term serelaxin effects (up to 48 h) reported include changes in expression of MMPs, ET_B receptors, VEGF and NOS (McGuane *et al.*, 2011a) that were similar to our findings in human vascular cells showing that 48 h serelaxin exposure increased the expression of ET_B receptors, VEGF and nNOS and increased the activity of MMP2, albeit in a cell-dependent manner (Figure 8). Although it is difficult to extrapolate these findings to cells of the systemic vasculature, these changes in human umbilical cells may reflect the haemodynamic changes observed during pregnancy and *in vivo*, particularly the increase in global arterial compliance and decrease in vascular resistance (thereby improving cardiac output) observed in pregnant (Debrah *et al.*, 2006) and non-pregnant animals (Conrad *et al.*, 2004; Xu *et al.*, 2010).

Serelaxin had different effects on human primary cells isolated from the arterial and venous circulations which have implications for our understanding of its clinical actions. Although serelaxin is an arteriodilator (Jeyabalan *et al.*, 2003; Conrad and Shroff, 2011; McGuane *et al.*, 2011b), our study suggests that serelaxin may also have potent venodilating properties in humans. Studies on the rat vasculature suggest that serelaxin has predominantly arteriodilating effects. Rat mesenteric and femoral arteries and veins expressed RXFP1 receptors in endothelial and smooth muscle cells, but serelaxin-mediated remodelling was only observed in the mesenteric arteries (Jelinic *et al.*, 2014) while serelaxin-mediated dilation in rat mesenteric veins has also been observed (Li *et al.*, 2005).

Interestingly, the effects of serelaxin on the expression of NOS isoforms were consistent across the different vascular cells but varied in magnitude. Increased nNOS expression in HUASM and HUVSM cells paralleled previous findings in rat vascular and microvascular smooth muscle cells (Boulanger *et al.*, 1998; Kavdia, 2004), rat renal microvasculature (Ichihara *et al.*, 1998), rabbit cardiomyocytes (Xu *et al.*, 1999) and porcine carotid endothelial cells (Buchwalow *et al.*, 2002). However, serelaxin also increased nNOS expression in umbilical endothelial and smooth muscle cells and cardiac fibroblasts. iNOS was not detected in HUASM and HUVSM cells and was not induced by serelaxin treatment, but in accord with previous findings (Quattrone *et al.*, 2004) serelaxin treatment increased expression of iNOS in HUVECs (data not shown). The findings from HUASM and HUVSM cells contrast with previous studies in vascular smooth muscle cells from rat carotid artery and rat renal cells (Mohaupt *et al.*, 1994; Boulanger *et al.*, 1998).

Interestingly, we demonstrated that serelaxin produced bell-shaped CRCs in HUVECs, HUVSMCs and HCFs, but not in HUASMCs. Our findings suggest that G$\alpha_{i/o}$ associated with lipid rafts has an important role to play in determining the shape of the concentration–response relationship. The pattern of G proteins involved in responses to serelaxin differs with cell type. In HEK cells recombinantly expressing RXFP1 receptors and in human THP-1 cells, serelaxin activation of RXFP1 receptors results in coupling to Gα_s, Gα_{OB} and Gα_{i3}, whereas in rat cardiac fibroblasts there is coupling to Gα_s and Gα_{i3}, in colo16 cells and rat renal fibroblasts to Gα_s and Gα_{OB}, and in T47D cells solely to Gα_s (Halls *et al.*, 2009b; Mookerjee *et al.*, 2009). We have also shown that disruption of membrane rafts in HEK-RXFP1 receptor cells eliminates serelaxin signalling via the Gα_{i3} pathway (Halls *et al.*, 2009a). In HUASMC, cAMP and cGMP concentration–response relationships were sigmoidal, compatible with responses involving Gα_s and Gα_{i3} as inhibition of Gα_s with NF449 or Gα_i with NF023 desensitized the response but did not change the shape of the curves (Figures 4 and 5A, B). Blockade of both Gα_s and Gα_i completely abrogated cAMP and cGMP responses (Figures 4 and 5C). Disruption of lipid rafts with filipin III had a similar effect to Gα_i inhibition (Figures 4 and 5D). In HUVSMC, cAMP and cGMP CRCs were bell-shaped, suggesting that the responses involved not only Gα_s and Gα_{i3}, but Gα_{OB}, and that the latter coupled less efficiently to RXFP1 receptors. Inhibition of Gα_s with NF449 desensitized the responses but did not change the shape of the curves (Figures 4 and 5E). In contrast, inhibition of Gα_i with NF023 both desensitized (blockade of Gα_{i3}) and changed the shape of the curve to sigmoid (Figures 4 and 5F). Again, blockade of Gα_s, Gα_{i3} and Gα_{OB} completely abrogated responses (Figures 4

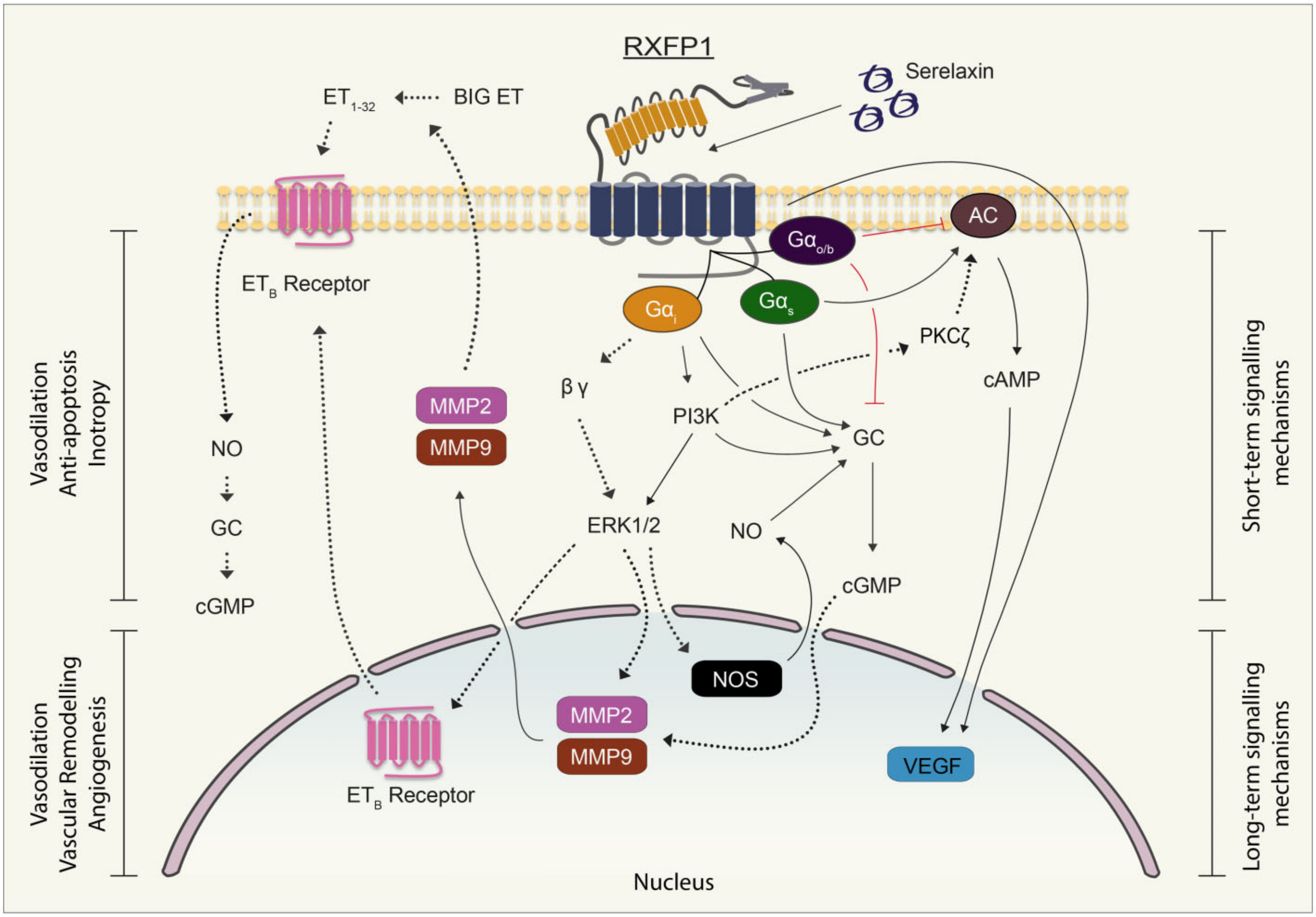

Figure 8

Signal transduction mechanisms employed by serelaxin in human umbilical vascular cells and HCFs and their potential physiological effects. Short-term (<1 h) serelaxin stimulation produces cAMP/cGMP accumulation in vascular cells and pERK1/2 in all cells that is differentially regulated by $G\alpha_s$, $G\alpha_i$, $G\alpha_{OB}$ and PI3K, and these pathways are likely to be involved in the vasodilator and anti-apoptotic effects of serelaxin respectively. In HUASMCs, the serelaxin-mediated cAMP or VEGF response did not involve $G\alpha_{OB}$ and PI3K, whereas in HCFs, the RXFP1 receptor was not coupled to cAMP production. Longer term (24–48 h) serelaxin treatment increased VEGF expression involving both cAMP-dependent and cAMP-independent mechanisms, and these pathways are likely to be involved in angiogenesis. In addition, serelaxin treatment increased the activity of MMP2 and MMP9 to mediate its remodelling actions, but these enzymes are also secreted to convert big ET to ET_{1-32} that activates ET_B receptors to further enhance vasodilatation (Conrad, 2010). Solid lines indicate mechanisms identified in the current study whereas dashed lines indicate previously established mechanisms.

and 5G). Disruption of lipid rafts with filipin III had the same effect as inhibition of inhibitory G proteins (Figures 4 and 5H). The results suggest that both $G\alpha_s$ and $G\alpha_{i3}$ contribute to the increases in cAMP (Halls *et al.*, 2006; 2009a) and cGMP observed in both HUASMC and HUVSMC and that $G\alpha_{OB}$ inhibits these responses but only operates in HUVSMC and interacts with RXFP1 receptors at lower affinity than the other G proteins. Association with lipid rafts seems to be obligatory for the operation of $G_{i/o}$ proteins as disruption with filipin prevented their action. Differential G-protein coupling to RXFP1 receptors has physiological and pathological implications as $G\alpha_i$ expression is increased, whereas $G\alpha_s$ expression is unaffected during heart failure. Additionally, the positive inotropic effects of serelaxin are partially inhibited by $G\alpha_{i/o}$ and PI3K inhibitors in the non-failing human atrial myocardium, whereas in the failing human atrial myocardium, $G\alpha_{i/o}$ and PI3K inhibition completely abolished the inotropic effects (Dschietzig *et al.*, 2011).

Another important observation in our study was that different G proteins mediated distinct phases of the serelaxin CRC with $G\alpha_s$ regulating the initial phase, whereas $G\alpha_{i/o}$ (and possibly $G\alpha_{o/B}$) mediating the latter phase. This bears resemblance to the activation of other family A GPCRs. There is a concentration-dependent switch from G-protein-dependent to G-protein-independent signalling by the β_2-adrenoceptor because low agonist concentrations activated ERK1/2 via $G\alpha_s$, whereas higher agonist concentrations activated ERK1/2 via Src tyrosine kinase (Sun *et al.*, 2007). Similarly, in a human breast cell line MCF-10A, low concentrations of isoprenaline target a β_2-adrenoceptor population localized in membrane rafts and stimulate $G\alpha_s$ to enhance cell adhesion, while higher concentrations activate a β_2-adrenoceptor population outside membrane rafts to inhibit cell proliferation by a $G\alpha_s$ and PKA-dependent signalling pathway (Bruzzone *et al.*, 2014). Likewise, the adenosine A_1 receptor also exists in multiple states that couple to different G proteins. Adenosine A_1 receptor-stimulated cAMP response element-gene transcription produced a biphasic CRC. The inhibitory component was inhibited by PTX whereas the stimulatory component was enhanced, suggesting $G\alpha_i$-coupled inhibition followed by $G\alpha_s$-coupled stimulation (Baker and Hill, 2006).

Despite the exciting findings obtained, there were some limitations to this study. The cells were obtained from human umbilical cords. The umbilical vessels have unique functions in that the umbilical vein transports oxygenated blood to the fetus whereas the artery returns deoxygenated blood to the maternal circulation. The umbilical artery lacks the elastic lamina and adventitia found in other arteries, these being replaced by connective tissue, whereas the umbilical vein contains an unusually thick muscle layer (Spurway *et al.*, 2012), and therefore acts much like an artery. Lack of RXFP1 receptor expression in the umbilical artery endothelial cells appears to be a regional effect as serelaxin causes signal transduction in endothelial cells from human coronary arteries (McGuane *et al.*, 2011b). However, cells from the umbilical cord do provide model systems that allow the study of serelaxin responses in human cells and can provide novel insights into the signal transduction mechanisms activated by serelaxin in the vasculature. Although it is likely that cells from the systemic and renal circulation play a more important role in AHF, the relative lack of availability of such human cells poses a problem. However, we used primary human cells that endogenously express RXFP1 receptors, that are readily available, are relevant to the cardiovascular system and are likely to represent a good model to study physiologically relevant responses. Furthermore, HCFs used in the current study were obtained from human hearts and these cells did not show responses that were markedly different from cells from the umbilical cord. Nonetheless, future studies will extend the number and types of human cardiovascular cells studied. Furthermore, in physiology, cellular crosstalk is likely to impact intra- and extracellular serelaxin signalling. Therefore, future studies investigating serelaxin responses in co-culture systems may establish better models that reflect more closely the pharmacodynamic effects of serelaxin in the treatment of AHF.

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Author contributions

M. S., C. S. S., R. A. B., D. R. S. and R. J. S. participated in research design. M. S. and C. S. S. conducted the experiments. R. A. B. and D. R. S. contributed reagents or tools. M. S. and R. J. S. performed data analysis. M. S., C. S. S., R. A. B., D. R. S. and R. J. S. wrote or contributed to writing of manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Serelaxin stimulation did not cause a functional response in HUAECs. HUAECs do not express RXFP1 receptors at the cell surface (Figure 1), and in accord with this finding, addition of serelaxin (30 nM) did not affect (a) cAMP accumulation (n = 4), (b) cGMP accumulation (n = 4) or (c) pERK1/2 (n = 3). Each point represents the mean ± SEM of four to six independent experiments performed in duplicate.

Figure S2 Time course studies of serelaxin in human primary vascular and cardiac cells. Serelaxin (30 nM) treatment increased (a) cAMP accumulation in HUVEC (n = 7), HUVSMC (n = 3) and HUASMC (n = 4), but not in HCF (n = 3). Serelaxin (30 nM) treatment also increased cGMP accumulation but in all cells including HUVEC (n = 6), HUVSMC (n = 5), HUASMC (n = 3) and HCF (n = 3). CRCs were conducted at 30 min for each cell type. Similarly, serelaxin (30 nM) increased pERK1/2 in HUVEC (n = 9), HUVSMC (n = 4), HUASMC (n = 4) and HCF (n = 3) that peaked between 5 and 10 min after which it returned to baseline. However, in HUVEC, pERK1/2 plateaued after 10 min and remained at that level for up to 60 min. CRCs for pERK1/2 were conducted at 10 min for HUVEC, HUVSMC and HCF but at 5 min for HUASMC. Each point represents the mean ± SEM performed in duplicate.

Figure S3 The effects of serelaxin stimulation on pERK1/2 in human primary vascular cells. Serelaxin stimulation (10 min for HUVECs, HUVSMCs, HCFs and 5 min for HUASMCs) increased pERK1/2 in (a) HUVECs (n = 5), (b) HUVSMCs (n = 6), (c) HUASMCs (n = 6) and (d) HCFs (n = 5). The serelaxin CRCs were bell-shaped for HUVECs, HUVSMCs and HCFs, but sigmoidal for HUASMCs. PTX (50 $ng{\cdot}mL^{-1}$, 18 h) and wortmannin (100 nM, 30 min) pretreatment significantly inhibited serelaxin (30 nM)-mediated pERK1/2 in each cell type. Statistical significance was assessed using a one-way ANOVA with a Dunnett's *post hoc* test compared with serelaxin alone: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Figure S4 The effect of suramin on the dose–response relationship of serelaxin in primary human smooth vascular cells. Pretreatment of suramin (10 μM, 30 min) to HUASMC had no effect on (a) cAMP (n = 3) or (c) cGMP accumulation (n = 3). Similarly, suramin (10 μM, 30 min) pretreatment to HUVSMC had no effect on the dose–response relationship of serelaxin-mediated (b) cAMP (n = 3) or (d) cGMP accumulation (n = 3). Similarly, pretreatment of HUASMC and HUVSMC with 100 μM suramin (30 min) reduced the E-max of (a, b) cAMP (n = 3) and (c, d) cGMP accumulation (n = 3).

Table S1 The binding affinity of serelaxin (pK_i) and the potency (pEC_{50}) of serelaxin for cAMP accumulation, cGMP accumulation and pERK1/2 in human primary vascular cells. Values in parentheses refer to n experiments.