Relaxin reduces endothelium-derived vasoconstriction in hypertension: revealing new therapeutic insights.

Short running title: Relaxin-prostanoid interaction in hypertension

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Authorship contribution statement:

CHL, HHN, SM, MJ & CQ performed the research and analysed the data

CHL, TR, UR, RR, MT & LJP designed the study;

CHL & LJP wrote the paper;

All authors revised the paper critically for important intellectual content and approved the final submission of paper.

Abstract

Background and Purpose: Endothelium-derived vasoconstriction is a hallmark of vascular dysfunction in hypertension. In some cases, an overproduction of endothelium-derived prostacyclin (PGI_2) can cause contraction rather than relaxation. Relaxin is well-known for its vasoprotective actions, but the possibility that this peptide could also reverse endothelium-derived vasoconstriction has never been investigated. We tested the hypothesis that short-term relaxin treatment mitigates endothelium-derived vasoconstriction in spontaneously hypertensive rats (SHR).

Experimental Approach: Male Wistar Kyoto rats (WKY) and SHRs were subcutaneously infused with either vehicle (20 mM sodium acetate) or relaxin (13.3 μg/kg/h) using osmotic minipumps for 3 days. Vascular reactivity to the endothelium-dependent agonist acetylcholine, was assessed *in vitro* by wire-myography. Quantitative PCR and liquid chromatography mass spectrometry were used to identify changes in gene expression of prostanoid pathways, and prostaglandin production, respectively.

Key Results: Relaxin treatment ameliorated hypertension-induced endothelial dysfunction by increasing nitric oxide-dependent relaxation and reducing endothelium-dependent contraction. Notably, short-term relaxin treatment upregulated mesenteric PGI_2 receptor (IP) expression, permitting PGI_2-IP mediated vasorelaxation. In the aorta, reversal of contraction was accompanied by suppression of the hypertension-induced increase in prostanoid-producing enzymes and reduction in PGI_2-evoked contractions.

Conclusion and Implications: Relaxin has region-dependent vasoprotective actions in hypertension. Specifically, relaxin has distinct effects on endothelium-derived contracting factors and their associated vasoconstrictor pathways in mesenteric arteries and the aorta. Taken together, these observations reveal the potential of relaxin as a new therapeutic for vascular disorders that are associated with endothelium-derived vasoconstriction including hypertension.

Keywords: vascular endothelium, prostanoids, hypertension, relaxin

Bullet point summary:

What is already known?

- The peptide hormone relaxin elicits vasoprotective actions in the macro- and microvasculature.

What does the study address?

- Short-term relaxin treatment attenuates endothelium-derived vasoconstriction in arteries isolated from hypertensive rats.

Clinical significance

- Short-term relaxin infusion reverses hypertension-induced endothelial dysfunction by limiting endothelium-derived vasoconstriction in a region-dependent manner.
- Relaxin is a potential therapeutic drug for vascular diseases associated with exaggerated endothelium-derived vasoconstriction.

Non-standard Abbreviations and Acronyms:

EDH	endothelium-derived hyperpolarization
EDCF	endothelium-derived contracting factors
EDC	endothelium-derived contraction
Indo	indomethacin
IK_{Ca}	intermediate-conductance calcium-activated potassium channel
IP/*Ptgir*	prostacyclin receptor
KPSS	high potassium physiological saline solution
LCMS	Liquid Chromatography Mass Spectrometry
L-NAME	Nω-nitro-L-arginine methyl ester
PSS	physiological saline solution
SK_{Ca}	small-conductance calcium-activated potassium channel
SNP	sodium nitroprusside
TP	Thromboxane receptor
TRAM34	1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole
R_{max}	maximum relaxation
Rn18S	ribosomal 18S
RXFP1/*Rxfp1*	relaxin/insulin-like family peptide receptor 1

Introduction

Essential hypertension is associated with vascular endothelial dysfunction (Bernardo, Weeks et al., 2010; Vanhoutte, Zhao et al., 2016). Endothelium-derived nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarization (EDH) are major contributors to vasorelaxation in healthy blood vessels. In hypertension, the production and vasodilator capacity of NO and EDH are diminished and contribute to endothelial dysfunction (Leung & Vanhoutte, 2015; Tang & Vanhoutte, 2010; Vanhoutte, Zhao et al., 2016). In addition, the endothelium favours the production of endothelium-derived contracting factors (EDCF) that enhance the contraction of blood vessels in hypertensive subjects. Specifically, in the aorta of spontaneously hypertensive rats (SHR), the production of cyclooxygenase (COX)-derived PGI_2 is paradoxically increased, with an associated reduction in prostacyclin receptor (IP) expression (Félétou, Verbeuren et al., 2009; Gluais, Lonchampt et al., 2005). As a consequence, the excessive endothelium-derived PGI_2 acts on the TP and/or other prostanoid receptors to cause contraction rather than vasorelaxation, indicating that PGI_2 shifts from being a vasodilator to an EDCF (Liu, Zhan et al., 2017). Targeting vascular prostanoid pathways may reveal new effective treatments for endothelial dysfunction in hypertension.

It is well-established that the peptide hormone relaxin has vasoprotective actions in the cardiovascular system (Jelinic, Marshall et al., 2019; Jelinic, Marshall et al., 2018; Leo, Jelinic et al., 2017; Leo, Jelinic et al., 2016a). These actions occur through activation of its major receptor, relaxin/insulin-like family peptide receptor 1 (RXFP1), which is localized to endothelial and vascular smooth muscle cells of blood vessels (Jelinic, Leo et al., 2014; Ng, Jelinic et al., 2015). Short-term relaxin infusion (2-3 days) enhances endothelial vasodilator function by upregulating endothelial NO synthase (eNOS), COX2 and IP expression (Jelinic, Leo et al., 2017), thereby increasing NO-mediated relaxation and PGI_2 production in healthy mesenteric arteries (Leo, Jelinic et al., 2016b). In the aorta, relaxin treatment also reverses endothelial dysfunction by stimulating NO and/or PGI_2 production in the aorta (Dschietzig, Brecht et al., 2012; Ng, Leo et al., 2016; Pini, Boccalini et al., 2016). Previous studies have reported inconsistent findings regarding the potential of relaxin to reverse vascular dysfunction in SHR. For example, relaxin treatment has minimal effects on myogenic tone and flow-mediated vasodilation in mesenteric arteries of SHR (van Drongelen, van Koppen et al., 2013). However, in SHR cerebral parenchymal arterioles, relaxin treatment decreases myogenic tone and increases EDH-type relaxation (Chan, Sweet et al., 2013). In SHR aorta, relaxin infusion reverses adverse vascular remodeling and increases arterial compliance (Xu,

Chakravorty et al., 2010). However, none of these studies investigated the potential for relaxin to mitigate the detrimental effects of EDCFs in blood vessels from hypertensive individuals. Given the importance of EDCFs in the pathogenesis of vascular dysfunction in hypertension, it is essential to resolve whether or not relaxin mitigates EDCF-mediated contraction in the setting of hypertension.

Therefore, the key objective of the present study was to investigate the effects of short-term relaxin treatment on vascular function, focusing on EDCF pathways, in both small mesenteric arteries and aorta of SHR. We also examined whether or not short-term relaxin treatment elicits benefits within the heart, specifically indications of left ventricle (LV) remodeling as reported with longer durations (14 days) of relaxin infusion (Lekgabe, Kiriazis et al., 2005) in SHR. Here, our results reveal that short-term relaxin treatment mitigates endothelium-dependent contraction (EDC) in both the mesenteric artery and aorta of SHR. Although the overall therapeutic endpoint is a reduction in EDC, relaxin has region-specific actions on the vasculature through novel influences on EDCF generation/bioavailability and their associated vasoconstrictor pathways. To our knowledge, these findings are the first demonstration of an interaction between relaxin and EDCFs in the context of hypertension-induced vascular dysfunction *in vivo*. They also reveal compelling new insights for development of relaxin-based peptides to treat vascular disorders underpinned by endothelium-derived vasoconstriction.

Methods

All procedures were approved by the Faculty of Science, The University of Melbourne Animal Experimentation Ethics Committee (The University of Melbourne, AEC 1413186.1) and conform to ARRIVE (Animal Research: Reporting In Vivo Experiments) (Kilkenny, Browne et al., 2010), BJP's Ethical Policies, and the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes. All rats were housed in individually ventilated large cages with paper bedding (2 rats per cage) at the School of BioSciences SPF1 Animal House Facility. They were maintained on an automated time cycle of 12h light/dark at 20°C, with standard food pellets (Barastock, VIC, Australia) and water available *ad libitum*.

Animals

The SHR is a well-established animal model of essential (or primary) hypertension, which is one of the most studied animal models for vascular disease in the context of hypertension (Chan, Sweet et al., 2013; Debrah, Conrad et al., 2005b; Parikh, Patel et al., 2013; Xu, Chakravorty et al., 2010). In this study, male SHR (body weight: 330-410g) and normotensive Wistar-Kyoto rats (WKY) (body weight: 350-420g) aged 24-26 weeks old, (Animal Resource Centre, WA, Australia) were housed and maintained under standard conditions, as described above. Equal group size of n=15 rats was designed for each group of this study. It is estimated that approximately 10% of SHR may not develop hypertension (systolic blood pressure > 150 mmHg), thus, 2 extra SHR were included in each group (n=17 per group for SHR). Systolic blood pressure of rats was measured using non-invasive tail cuff plethysmography (Kent Scientific Corporation, Connecticut, USA), as previously described (O'Sullivan, Marshall et al., 2016). The systolic blood pressure of SHR (mean: 179 ± 6 mmHg, n = 34) was significantly higher than the WKY (mean: 121 ± 7 mmHg, n = 15). In this study, all the SHR (n=34) developed hypertension and were included. Blood pressure was not measured during relaxin infusion in this study as it has previously been shown that relaxin treatment has no effect on blood pressure in SHR (Chan, Sweet et al., 2013; Debrah, Conrad et al., 2005; Parikh, Patel et al., 2013; Xu, Chakravorty et al., 2010), and also in another SHR study from our laboratory (unpublished data). The SHR were randomly divided into two groups to receive either vehicle (20 mmol/l sodium acetate, n=17) or recombinant human relaxin-2 (13.3 µg/kg/h, n=17) for a period of 3 days. The WKY received an equivalent volume of the vehicle alone for 3 days (n=15). Considering the principles of animal ethics (Replacement, Reduction, and Refinement), we did not add 2 more WKY to this study to have equal group sizes (n=17 per group) as these additional animals would not have changed the outcomes of the statistical analysis. Similarly, given that we and many others have investigated the vascular effects of relaxin in normotensive rats (e.g. WKY+placebo vs WKY + relaxin) (Jelinic, Leo et al., 2017; Jelinic, Leo et al., 2014; Leo, Jelinic et al., 2014b; van Drongelen, van Koppen et al., 2013), we concluded that repeating this experiment with an extra cohort of relaxin-treated WKY would not provide new knowledge of relevance to this study.

Subcutaneous infusion of relaxin

On the day of surgery, all surgical instruments were sterilised using a glass bead steriliser (Germinator 500, Medtex, Mt Waverly, VIC, Australia) and gauze was sterilised by autoclaving. Aseptic conditions were maintained throughout surgery. For subcutaneous

infusion of relaxin or vehicle, all animals were anaesthetized with 2% isoflurane (Univentor 400, Agnthos, AB, Sweden) in oxygen via inhalation. Rats were determined to be within the surgical plane of anaesthesia by the absence of pedal withdrawal, toe pinch and corneal reflexes (both left and right). Respiration rate and colour of the mucous membranes were also monitored throughout the surgery to prevent isoflurane overdose. Rats were placed on a heating pad set to 37°C during surgery to prevent hypothermia. Ocular lubricant was applied to the eyes to prevent irritation and the surgical site was shaved and swabbed with Microshield 5 disinfectant (Clorohexidine gluconate 5% w/v; Johnson & Johnson Medical Pty Ltd., North Ryde, NSW, Australia). Once anaesthetized, a 2 cm incision was cut between the shoulder blades and the subcutaneous tissue was separated from the cutaneous tissue via blunt dissection to make a pocket large enough for the minipumps. The rats were implanted with a 3-day Alzet osmotic minipump (Model 1003D, Bioscientific, Gymea, NSW, Australia) under the skin to infuse either relaxin or vehicle. Bupivacaine (2 – 4 drops of 0.5%, Pfizer Australia Pty Ltd., WesRyde, NSW, Australia) was applied to the incision site prior to closure for analgesia with 3 to 4 simple interrupted stitches tied with a surgeon's knot using sterile 4-0 polyglactin-90 coated vicryl sutures with a 19 mm 3/8 circular reverse cutting needle (Ethicon, Johnson & Johnson Medical Pty Ltd., North Ryde, NSW, Australia). After surgery, the rats were transferred to a heated clean cage to monitor for signs of pain and distress. Once the rats regained consciousness, they were transferred back to the animal facility where regular welfare-related assessments (monitoring for any signs of pain, infection or distress) were carried out daily. All rats recovered fully after surgery, without any indication of pain or other ailments that required intervention.

Three days post-onset of relaxin infusion, blood samples were obtained from the LV via cardiac puncture under 2% isoflurane anaesthesia. Plasma concentrations of relaxin were measured in duplicate using the Human Relaxin-2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. Human recombinant relaxin was detected in the plasma of relaxin-treated SHR after 3 days of infusion (mean: 101 ± 9 ng/ml), but was below the level of detection in the plasma of placebo-treated WKY and SHR. The limit of detection of detection was 15.6 pg/ml and the intra- and inter coefficients of variation were 2.3 - 4.7% and 5.5 - 10.2%, respectively.

After blood collection, the animals were killed via cervical dislocation under anaesthesia. The mesenteric arcade was isolated and immediately placed in ice cold Krebs bicarbonate solution

(mmol/l: 120 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 D-glucose, and 2.5 $CaCl_2$, bubbled with carbogen (95% O_2 and 5% CO_2). Small mesenteric arteries (third-order branch of the superior mesenteric artery, internal diameter ~300 μm) and abdominal aortae were isolated, cleared of fat and loose connective tissue, cut into rings 2 mm in length and mounted on a Mulvany-Halpern wire-myograph (model 610M, Danish Myo Technology, Aarhus, Denmark). For myography experiments, we were unable to achieve equal group sizes between or within animal group comparisons in some cases because we tested combinations of multiple inhibitors in vessels from WKY or SHR-treated with placebo or relaxin. For example, control (absence of any inhibitors) curves will have the highest experimental number as these need to be done in every single experiment before the effects of inhibitors can be compared. The remaining arteries were snap frozen in liquid nitrogen and stored at -80˚C for further analysis. The heart was isolated and immediately placed in ice-cold Krebs bicarbonate solution. The whole heart was then blotted dry and weighed before isolating the LV. After weighing the LV, a portion of the LV was snap frozen in liquid nitrogen and stored at -80˚C for further analysis.

Assessment of vascular reactivity *ex vivo*

After the mesenteric arteries and aortae were mounted on the myograph, the vessels were allowed to stabilize at zero tension for 15 min before normalisation, as described previously (Leo, Jelinic et al., 2016b). All experiments were performed at 37˚C, and the organ baths were bubbled with carbogen. Vascular reactivity was assessed, as previously described, with the following modifications (Leo, Hart et al., 2011). Briefly, mesenteric arteries were maximally contracted with KPSS ((mmol/l: 25 NaCl, 100 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11.1 D-glucose, and 2.5 $CaCl_2$) and the integrity of the endothelium was subsequently determined, as described previously (Leo, Jelinic et al., 2014b). To evaluate vascular smooth muscle reactivity to vasoconstrictors, cumulative concentration-response curves to the thromboxane A_2 mimetic, U46619 (0.1 nmol/l-1 μmol/l), were constructed. Similarly, to assess endothelial and vascular smooth muscle function, mesenteric arteries were submaximally precontracted to a similar level (70-80% KPSS) using phenylephrine (0.1-3 μmol/l) and cumulative concentration-response curves to the endothelium-dependent agonist, ACh (0.1 nmol/l-10 μmol/l), and the endothelium-independent vasodilators, sodium nitroprusside (SNP, 0.01 nmol/l-10 μmol/l) and iloprost (1 pmol/l-0.1 μmol/l), were determined. In addition, responses to ACh were examined after 20 mins incubation with

different combinations of pharmacological blockers including the NO synthase inhibitor, L-NAME (200 μmol/l), the cyclooxygenase (COX) inhibitor indomethacin (Indo, 1 μmol/l), the selective COX1 inhibitor SC560 (1 μmol/l), the selective COX2 inhibitor NS398 (1 μmol/l), the intermediate conductance calcium-activated potassium channel IK_{Ca} inhibitor TRAM34 (1 μmol/l), the small conductance calcium-activated potassium channel (SK_{Ca}) inhibitor apamin (1 μmol/l), the thromboxane receptor antagonist SQ29548 (10 μmol/l) and the prostacyclin synthase (PGI_2S) inhibitor U51605 (1 μmol/l).

In another separate set of experiments, the effects of relaxin treatment on endothelium-dependent contraction was determined in endothelium-intact mesenteric arteries and aortae. Mesenteric arteries were incubated with the combination of L-NAME, TRAM34 and apamin and the aorta was incubated with L-NAME alone for 45 mins. After 45 mins of incubation, endothelium-dependent contraction was evoked by cumulative addition of ACh (10 nmol/l-100 μmol/l). In some cases, the role of the endothelium was investigated in endothelium-denuded arteries. The endothelium was denuded by rubbing the lumen of the vessel with a hair and the endothelium was deemed denuded if ACh-induced relaxation was abolished. To investigate the mechanisms of endothelium-dependent contraction, responses to ACh were also examined in the presence of Indo, SC560, NS398 and SQ29548.

Assessment of basal NOS activity

The effects of relaxin treatment on basal levels of NO release were also examined through the addition of L-NAME (200 μmol/l) in endothelium-intact rings submaximally contracted with phenylephrine (10-100 nmol/l) to approximately ~20% of KPSS-mediated contraction. Under these conditions, contraction to L-NAME is considered to reflect the level of NO synthase (NOS) activity (Kahlberg, Qin et al., 2016).

Measurement of prostaglandin metabolites

Segments of mesenteric arteries (~5 branches) and a single aortic ring (2-3 mm) were placed in 0.5 ml or 1 ml of Krebs-HEPES buffer at 37°C, respectively. The equilibration time was 1 h during which the buffer was changed every 15 min. At the end of the incubation period, ACh (10 μmol/l) was applied for 10 min. The mesenteric segments and aortic ring were removed and the buffer collected and snap frozen in liquid nitrogen and stored at −80°C for further analysis. The arteries were dried overnight and dry tissue weight was measured. Briefly, 500 μl of buffer was diluted with 1 mL of hexane:ethyl acetate (1:1 v/v) containing

20 µl of 1 M citric acid and 20 µl of 1% BHT. After the separation of the organic and the water layer, the organic layer was collected. The organic layer was dried down and reconstituted in 100 µl of methanol. The prostaglandins metabolites in the plasma and bathing solution of ACh (10 µmol/l)-stimulated mesenteric arteries were measured by liquid chromatography mass spectrometry. A total of 24 prostaglandin standards from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) were used in the liquid chromatography mass spectrometry (Supplemental Table 1).

Eicosanoids were detected and quantified using Agilent 1290 LC system connected to an Agilent triple quad (QQQ 6490) mass spectrometer. Reverse-phase separation was performed on an Agilent XDB C18 (2.7µm) 2.1 x 150 mm column. Eicosanoids were eluted using a mobile phase consisting of (A) 0.1% acetic acid and (B) acetonitrile /isopropanol (90/10, v/v). Gradient elution was carried out for 10 min at a flow rate of 0.6 mL/min. Gradient conditions were as follows: 0–1.0 min, 25% B; 1.0–8.0 min, 25–95% B; 8–8.5 min, 95% B, 8.51 – 10.0 min, 25% B. A 10 µL aliquot of each sample was injected onto the column in which temperature was kept at 40˚C. Throughout the analysis, samples were kept at 5˚C, and mass spectrometry detection was performed using multiple reaction monitoring (MRM). Mass spectrometer parameters were optimised for each analyte and detected in negative mode. Nitrogen gas was employed as the collision gas and capillary voltage, fragmentor voltage and collision energy were set to 4000V, 380V and 60 V, respectively. The sheath gas temperature was maintained at 350˚C and the collision gas flow was 12 L/min. Data acquisition was performed using MassHunter acquisition software and all data analysis was performed using MassHunter quantitative software (Agilent).

Similarly, 6-keto prostaglandin $F_{1\alpha}$, TxB_2 and 8-isoprostane were measured in ACh (10 µM)-stimulated aortic rings using EIA kits from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. For ELISA experiments, we did not manage to achieve equal group sizes as some of the vessels from the WKY group were used for other experiments, resulting in n=6 for WKY and n=8 for SHR+placebo or SHR+relaxin. The buffer was diluted 1:50 and used for the measurement of 6-keto prostaglandin $F_{1\alpha}$. Undiluted samples were used for the measurement of TxB_2 and 8-isoprostane. The absorbance counts were normalised to dry tissue weight.

RNA extraction and quantitative PCR

Frozen blood vessels and left ventricles were pulverized and resuspended in 1 ml TriReagent (Ambion Inc., Scoresby, VIC, Australia) and total RNA was then extracted according to the manufacturer's instructions. Extraction of RNA from very small amounts of vascular tissue can result in poor quality and/or quantity of RNA (A_{260}:A_{280} ratios < 1.8). In these instances, we exclude these samples from further analysis (mesenteric arteries: 1 WKY+placebo, 1 SHR+relaxin; aorta: 1 SHR+placebo). RNA pellets were resuspended in 15-20 µl RNA Secure™ (Ambion). Quality and quantity of RNA was analysed using the NanoDrop ND1000 Spectrophotometer (Thermo Fischer Scientific Australia Pty Ltd, Scoresby, VIC, Australia) with A_{260}:A_{280} ratios > 1.8 indicating sufficient quality for qPCR analysis. First strand cDNA synthesis used 1 µg of total RNA in a 20 µl reaction containing random hexamers (50 ng/µl) and 200 units of Superscript™ III (Invitrogen, Mulgrave, VIC, Australia).

The comparative cycle threshold ($2^{-\Delta Ct}$) method of quantitative real-time polymerase chain reaction (qPCR) was used to analyse cyclooxygenase 1 (*Ptgs1*), and 2 (*Ptgs2*), Prostacyclin synthase (*Ptgis*), and receptor (*Ptgir*), Thromboxane synthase (*TXAS*), and receptor (*Tp*), GTP cyclohydrolase I (*Gch-1*), *Rxfp1* and endothelial NOS (*Nos3*) gene expression in mesenteric and aorta of rats. Rat-specific forward/reverse primers and 6-carboxyl fluorescein–labelled (FAM) Taqman probes were designed and purchased from Biosearch Technologies (Novato, CA, USA). Primers were designed to span intron/exon boundaries. qPCR was performed in triplicate on the Applied Biosystems ViiA7 PCR machine (Life Technologies, Mulgrave, VIC, Australia) using 96-well plates with 10 µl volume reactions in triplicate containing SensiMix (Bioline) and 10 µmol/l of primers and FAM-labelled probe. Ribosomal 18S (*Rn18s*) was used as the reference gene. Negative template controls substituting cDNA with water or RT negative controls substituting the reverse transcriptase in the cDNA synthesis, were included on each plate. Similarly, qPCR was used to assess potential actions of relaxin on the LV: expression of brain natriuretic peptide (*Nppb*), connective tissue growth factor (*Ctgf*), transforming growth factor-β1 (*Tgfb1*), tumour necrosis factor-α (*Tnf*) and NADPH oxidase 2 (*Cybb*) in rat left ventricles. Rat-specific forward/reverse primers (GeneWorks, Thebarton, SA, Australia) were generated from GenBank. Real-time PCR reaction was determined by SYBR green chemistry using the Applied Biosystems 7500 fast real-time PCR system with triplicate samples of 12.5 µl containing SYBR Green PCR Master Mix (Applied Biosystems, Scoresby, VIC, Australia) and 10 µmol/l (gene of interest) of primers. Ribosomal 18S (*Rn18s*) was used as the reference

gene. Details of the rat specific primers are in Supplemental Table 2. The mean C_T value of WKY was used as the internal calibrator and subtracted from the mean C_T value of gene of interest, and then analysed using $2^{-\Delta Ct}$ method to reduce unwanted sources of variation, as described previously (Marshall, Leo et al., 2016; Marshall, Ng et al., 2016). These normalised data (ΔC_T) were expressed as fold change relative to means of controls (WKY) and presented as mean ± SEM.

Cardiomyocyte morphology

A portion of the LV was embedded in paraffin wax, cut into sections of 5 µm, and mounted on SuperFrost PLUS slides (Menzel-Gläser, Braunschweig, Germany). After overnight drying at 37˚C, the sections were stained with hematoxylin and eosin (H&E) (Australian Biostain Pty Ltd, Traralgon East, VIC, Australia) for the determination of cardiomyocyte cross-sectional area and width. Cardiomyocyte cross-sectional area and width were determined from the same image of 100 individual cardiomyocytes per rat, calculated from cell outlines using Image J (RRID:SCR_003070) (Ng, Leo et al., 2017). The counting and quantification of cardiomyocyte cross-sectional area and width was performed by another operator who was blinded to the experimental groups.

Reagents

All drugs were purchased from Sigma-Aldrich (St Louis, MO, USA), except for U46619, U51605, SC560 and NS398 (Cayman Chemical Company, Ann Arbor, MI, USA). All drugs were dissolved in distilled water, with the exception of indomethacin, which was dissolved in 0.1 mol/l sodium carbonate and U46619, which was dissolved in 100% ethanol (final concentration less than 0.1% ethanol) as 1 mmol/l stock solution and subsequent dilutions were in distilled water. Furthermore, SC560 and NS398 were dissolved in 100% DMSO, and U51605 was dissolved in 100% EtOH.

Statistical analyses

The data and statistical analysis in this study comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis, Alexander et al., 2018). For all experimental protocols, based on 15% SD, we will be 80% powered to detect 20% changes at $P<0.05$ with n=9 per group. Concentration-response curves for rat mesenteric arteries were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 5.0, GraphPad Software (RRID:SCR_002798), San Diego, CA,

USA) to calculate the sensitivity of each agonist (pEC_{50}). Relaxation to vasodilators was measured as a percentage of precontraction to phenylephrine, with maximal relaxation, R_{max}. Group pEC_{50} and R_{max} values were compared using one-way ANOVA with post-hoc analysis using Tukey's test or Student's unpaired t-test, as appropriate. Concentration-response curves were also analysed with repeated measures two-way ANOVA (treatment vs. concentration). *Post-hoc* analysis was only performed when the F value was greater than F critical value, indicating that there was no variance in homogeneity. $P < 0.05$ was considered statistically significant. Certain experiments were undertaken in duplicate (ELISA) and triplicate (qPCR) to ensure the reliability of single values. Data analysis and data presentation from these experiments used the single values obtained from the mean of the technical replicates. Statistical analysis was only performed using these independent values (not technical replicates) with $n>5$, and any data with sample size of $n<5$ are indicated as exploratory observations. n refers to number of animals or independent experiments. Outliers were excluded in data analysis and presentation, where indicated. An outlier is predefined when an individual data point is 2 standard deviations from the mean. The details of which experiment(s) and how many outliers were excluded are indicated within the respective figure legends.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding, Sharman et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

Results

Relaxin treatment reverses endothelial dysfunction in the mesenteric arteries of SHR.

Endothelial dysfunction was evident in the mesenteric arteries of SHR as a significant reduction in the sensitivity, but not maximum relaxation in response to ACh compared with WKY (Figure 1A). Relaxin treatment for 3 days significantly reversed this endothelial dysfunction. Relaxation to the endothelium-independent vasodilator, SNP, was comparable between the 3 groups (Figure 1B), indicating that vascular smooth muscle function was not affected. The endothelial dysfunction evident in SHR was partially explained by a reduction

in basal NOS activity, which was reversed by relaxin treatment (Figure 1C). Despite changes to basal NOS activity, *Nos3* was not altered by hypertension or relaxin treatment (Supplemental Figure I, A). Moreover, relaxin had no effect on the expression of the rate limiting enzyme for tetrahydrobiopterin synthesis, GTP cyclohydrolase I (*Gch-1*), the levels of which were significantly reduced in SHR (Supplemental Figure I, B). Importantly, the beneficial effects of relaxin were independent of any changes in *Rxfp1* expression in mesenteric arteries (Supplemental Figure I, C).

To explore the mechanism(s) by which relaxin reverses endothelial dysfunction in mesenteric arteries of SHR, we evaluated vascular reactivity to ACh in the presence of various pharmacological inhibitors. In the presence of Indo+L-NAME, relaxin treatment for 3 days did not improve the impaired classical EDH-type relaxation observed in SHR (Figure 1D). In WKY, a significant component of relaxation remained in the presence of Indo+L-NAME+apamin+TRAM34. However, it was abolished in both placebo- and relaxin-treated SHR, suggesting that hypertension *per se* impairs the contribution of non-classical EDH responses (Edwards, Feletou et al., 2010) (Figure 1E). In contrast, the impaired NO-mediated relaxation evident in SHR (Figure 1F) was reversed by treatment with relaxin.

A key finding was the significant contribution of COX-derived vasoconstrictor prostanoids in SHR mesenteric arteries, demonstrated by an increase in sensitivity to ACh in the presence of a non-selective COX inhibitor, Indo (Figure 2B). This increased ACh sensitivity was not observed in WKY rats (Figure 2A) or relaxin-treated SHR (Figure 2C), indicative that relaxin is correcting the vasoconstrictor prostanoid imbalance. To further explore the involvement of NO and prostanoids, vascular reactivity to ACh was evaluated in the presence of TRAM34 + apamin to abolish the actions of the 'classical' EDH (Supplemental Figure II). Interestingly, we observed EDC in response to the higher ACh concentrations in SHR. The EDC was almost completely abolished by Indo, the PGI_2 synthase inhibitor, U51605 and TP antagonist, SQ29548 (Supplemental Figure II, B-C), suggesting these contractions are mediated by COX-derived PGI_2 targeting the TP. Furthermore, the magnitude of this ACh-evoked contraction was significantly reduced in relaxin-treated SHR (Supplemental Figure II, A), indicating that this constrictor pathway was downregulated by relaxin treatment.

Relaxin treatment inhibited endothelium-dependent contraction in SHR.

The nature of EDC in mesenteric arteries of SHR was further investigated by assessing the response to ACh in the presence of L-NAME+TRAM34+apamin (Figure 3). ACh-evoked concentration-dependent contraction that was significantly increased in SHR compared to WKY; it was endothelium-dependent as contraction was abolished in endothelium-denuded arteries (data not shown). Consistent with data in Supplemental Figure II, this EDC was significantly reduced in relaxin-treated SHR (Figure 3A). Furthermore, EDC was also significantly increased in the aorta of SHR, and this was similarly reduced by short-term relaxin treatment (Figure 3B-C).

Relaxin-dependent activation of prostanoid pathways

We investigated the underlying mechanisms associated with the reduction in ACh-induced contraction in SHR after relaxin treatment. Blockade of COX1 and TP abolished ACh-mediated contraction, indicating their involvement in EDC in the SHR (placebo- and relaxin-treated) mesenteric arteries (Figure 3D-E). Furthermore, inhibition of COX2 (with NS398) had minimal effects on the contraction to ACh (Figure 3D-E). Conversely, COX1-derived ACh-evoked contraction was significantly reduced with relaxin treatment (Figure 3F). The involvement of COX-derived prostanoids was also demonstrated in the aorta as EDC was completely abolished by Indo (Figure 3C).

We further tested our hypothesis that relaxin treatment in SHR could directly modulate prostanoid synthesis by analysing gene expression of key enzymes in the prostanoid synthesis pathway. In the mesenteric arteries, neither hypertension nor relaxin had any significant effects on expression of prostanoid-generating enzymes; *Ptgs1*, *Ptgs2*, *Ptgis* or *Tbxas1* (Figure 4A-D). In contrast, expression of *Ptgs1* (but not *Ptgs2*) was significantly increased in the aorta of SHR and reduced by relaxin treatment (Figure 5A-B). Similarly, *Ptgis* and *Tbxas1* in the aorta were significantly upregulated by hypertension; their expression was also reduced in relaxin-treated SHR (Figure 5C-D).

Our previous findings demonstrated that relaxin treatment for 3 days stimulates PGI_2 production in mesenteric arteries from normotensive Wistar rats (Leo, Jelinic et al., 2016b). On this basis, we used liquid chromatography mass spectrometry to screen for the potential involvement of 24 different prostaglandin metabolites (Supplemental Table 2) to further explore if prostanoid production was altered by relaxin treatment in the mesenteric arteries. Eight out of 24 metabolites were detected, whereas the remaining 16 were below the

detection limit. ACh-stimulated production of arachidonic acid (Figure 6A), $PGF_{2\alpha}$, docosahexaenoic acid (DHA), PGA_2/PGB_2 and LTB_4 in the mesenteric arteries was not affected by hypertension or relaxin treatment (Supplemental Figure III, A-D). In contrast, ACh-induced release of 6-keto $PGF_{1\alpha}$ (stable metabolite of PGI_2), 8-iso-$PGF_{2\alpha}$ (non-enzymatic peroxidation of arachidonic acid) and PGE_2/PGD_2 was significantly increased in SHR; the increase in the latter two metabolites was not significant in relaxin-treated SHR compared with WKY (Figure 6B-D). In the aorta, release of 6-keto $PGF_{1\alpha}$ was significantly increased but marginal for TXB_2 after ACh-stimulation in SHR, but relaxin treatment elicited no further effects (Figure 6E-F).

Relaxin-mediated activation of prostanoid receptors in the vascular smooth muscle

Contractions to the TP agonist, U46619 were increased in mesenteric arteries of SHR compared with WKY; this hyper-responsiveness to U46619 was significantly reduced in relaxin-treated SHR (Figure 7A). In addition, relaxin reversed the impaired vasodilation to the IP agonist, iloprost (Figure 7B), suggesting that this may be underpinned by changes in receptor numbers, affinity of the ligand to the receptor or activity of the signalling pathway. We conducted a different experiment to determine if activation of IP was directly contributing to the contraction in the SHR aorta. In WKY, iloprost elicited ~20% contraction (Figure 7C) but this was significantly increased to ~60% in SHR. Relaxin treatment reduced this iloprost-evoked contraction to below 20% (Figure 7C). Similarly, the sensitivity to U46619-induced contraction was increased in aorta of SHR compared with WKY (SHR pEC_{50}: 7.41 ± 0.05 vs WKY pEC_{50}: 7.07 ± 0.03). However, in contrast to the mesenteric arteries, this hyper-sensitivity to U46619 was not significantly reduced (SHR+relaxin pEC_{50}: 7.29 ± 0.06) in the aorta of relaxin-treated SHR (Supplemental Figure IV). In support of our pharmacological data, *Ptgir* expression was significantly reduced in the mesenteric arteries of SHR and was upregulated after relaxin treatment (Figure 8A). However, there were minimal effects on *Tbxa2r* expression (Figure 8B). In contrast, there were significant increases in *Ptgir* and *Tbxa2r* expression in the aorta of SHR, and a clear downregulation of both receptors after relaxin treatment (Figure 8C-D). Collectively, these data illustrate that relaxin can directly mediate vascular smooth muscle responses to prostanoids in both arteries, but by distinct mechanisms.

Relaxin had no effect on cardiomyocyte size or biomarkers of fibrosis in the LV of SHR.

The effects of relaxin on the LV have previously been shown in the SHR myocardium but only after 14 days of infusion (Lekgabe, Kiriazis et al., 2005). Therefore, in the current study we decided to evaluate the additional beneficial effects of relaxin on the LV after a much shorter duration of treatment. Body weights and tibial length were comparable in all groups regardless of hypertension or relaxin treatment (Figure 9, A-B). Whole heart and LV weights were significantly increased in SHR compared to WKY and this did not change after relaxin treatment (Figure 9, C-D). Exploratory histological assessment of the LV demonstrated that cardiomyocyte cross-sectional area and width (Figure 10, A-C, n=4) were increased in SHR. This was accompanied by a significant increase in expression of B-type natriuretic peptide (*Nppb*) in the LV (Figure 10, D). Short-term relaxin treatment had no effect on cardiomyocyte cross-sectional area or width Figure 10, A-C) but *Nppb* expression was increased (Figure 10, D).

We also analysed expression of biomarkers of early onset of fibrosis, connective tissue growth factor (CTGF) and transforming growth factor-β (TGFβ). Both *Ctgf* and *Tgfb* expression were significantly increased in the LV of SHR compared to WKY and remained increased after relaxin treatment (Figure 10, E-F). Similarly, *Cybb* was increased in the LV of SHR but was not reduced after relaxin treatment (Supplemental Figure V, A). Neither hypertension nor relaxin treatment affected tumour necrosis factor-α (T*nf*) (Supplemental Figure V, B).

Discussion

This study demonstrates that endothelial dysfunction in SHR is attributed to the reduction in the vasodilator effects of NO and EDH, and enhanced production of EDCFs. Endothelium-dependent contraction in mesenteric arteries is, at least in part, mediated by increased production of COX1-derived PGI_2. As a result of decreased expression of IP in the mesenteric arteries of SHR, the excessive production of PGI_2 acts on TP to cause contraction. Short-term relaxin infusion reverses hypertension-induced endothelial dysfunction by increasing NO and reducing EDC, without any impact on EDH-type relaxation. More importantly, relaxin treatment increases the expression of IP, thus allowing PGI_2 to act on IP to elicit vasorelaxation. Endothelium-dependent contraction is also enhanced in the aorta of SHR and reversed by relaxin treatment. However, the mechanisms of relaxin action on vasorelaxation and EDC in the two arteries are different. Specifically, relaxin suppresses the hypertension-associated increase in prostanoid-producing enzymes

(COX1, PGIS and TXAS) and diminishes the PGI_2-evoked contraction in the aorta of SHR. In summary, we have shown that relaxin diminishes EDC in the vasculature in addition to stimulating vasodilator pathways (Supplementary Figure VI). This important finding opens up new possibilities for relaxin as a potential therapeutic for vascular diseases that are underpinned by enhanced prostaglandin-mediated EDC.

Many studies demonstrate that short-term relaxin treatment augments endothelial vasodilator function in resistance-size vessels under normotensive conditions; however, the vascular effects of relaxin in hypertension are less consistent (Chan, Sweet et al., 2013; Xu, Chakravorty et al., 2010). Discrepancies between studies on SHR are attributed to the severity of the disease, age of SHR, duration of treatment and/or vessel-specific effects of relaxin. Furthermore, the short-term (2-3 days) effects of relaxin on EDC have not been considered. In our study that used SHR (aged 24-26 weeks old), we demonstrated that subcutaneous infusion of relaxin for 3 days improved endothelial vasodilator function in the mesenteric arteries. This is likely to be independent of a reduction in blood pressure because relaxin has no effect on systolic blood pressure in SHR (Chan, Sweet et al., 2013; Debrah, Conrad et al., 2005b; Parikh, Patel et al., 2013; Xu, Chakravorty et al., 2010). Consistent with previous studies (Gluais, Lonchampt et al., 2005; Michel, Man et al., 2008; Tang, Jensen et al., 2008; Tang & Vanhoutte, 2009), one of the important contributors to endothelial dysfunction in the SHR is the overproduction of COX-derived vasoconstrictor prostanoids (Félétou, Verbeuren et al., 2009; Tang & Vanhoutte, 2009) and enhanced EDC. Of significance, relaxin treatment completely counteracted this EDC in both mesenteric arteries and aorta of SHR. In all cases, EDC was abolished by indo, confirming that a COX-derived product was responsible for causing contraction in these arteries.

Cyclooxygenases are the first enzymes involved in the biosynthetic pathway that lead to prostanoid formation from arachidonic acid. There are 2 isoforms of COX, the constitutive isoform, COX1 and the inducible isoform, COX2 (Félétou, Huang et al., 2011). In this study, we demonstrated that EDC to ACh were blocked by selective COX-1 (but not selective COX-2) inhibition. Furthermore, enhanced COX-mediated contraction in SHR occurs without changes to COX1 and COX2 mRNA expression in the mesenteric arteries. In contrast, in SHR aorta relaxin treatment reversed the increase in COX1, but not COX2 expression. Consistent with our data, EDC are abolished in aortae of COX1 knockout mice but not in COX2 knockout mice (Tang, Ku et al., 2005; Tang & Vanhoutte, 2008). Our

previous work demonstrated that short term relaxin treatment increased COX2 expression in normotensive mesenteric arteries, suggesting the role of COX2 cannot be completely excluded (Leo, Jelinic et al., 2016b). Indeed, our data indicate a small contribution of COX2-derived EDC in the mesenteric arteries as also reported in previous studies (Martelli, Testai et al., 2013; Wong, Leung et al., 2009). Given the greater contribution of COX1-derived EDC in SHR, it is more likely that relaxin mitigates COX1-derived vasoconstrictor prostanoids. Collectively, relaxin treatment reduces EDC in SHR arteries regardless of its action on COX1 or COX2 gene expression. However, it is important to note that no alteration in COX gene expression does not rule out any potential changes in protein levels or enzymatic activities.

Cyclooxygenase-derived prostanoids consist of a variety of biologically active eicosanoids that are formed from the short lasting but biologically active PGH_2, through the action of a set of synthases namely PGD, PGE, PGF, PGI and TXA synthases. These different synthases convert PGH_2 to their respective eicosanoids such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. These prostaglandins will preferentially interact with their specific G protein-coupled receptors, which are classified in five subtypes DP, EP, FP, IP and TP on the vascular smooth muscles cells (Félétou, Huang et al., 2011). Prostanoid production in aorta and renal arteries is altered by hypertension (Gluais, Lonchampt et al., 2005; Michel, Man et al., 2008). For example, PGI_2S is upregulated, and accompanied by increased PGI_2, PGE_2 and 8-iso $PGF_{2\alpha}$, the product of oxidative modification of free fatty acids. In parallel, IPs are downregulated causing these prostanoids to converge and bind to the overexpressed TP and/or EP, promoting vasoconstriction (Félétou, Verbeuren et al., 2009; Gluais, Lonchampt et al., 2005; Liu, Zhan et al., 2017; Michel, Man et al., 2008; Tang & Vanhoutte, 2009). To characterize which eicosanoids were altered by relaxin treatment, we measured prostanoid production in mesenteric arteries and aorta using LCMS and ELISA, respectively. Hypertension selectively increased PGI_2 and 8-iso $PGF_{2\alpha}$ production. In the aorta, increased PGI_2 production was associated with increased PGI_2S gene expression. PGE_2/PGD_2 were also elevated in the SHR mesenteric arteries. In our previous study using normotensive rats, we demonstrated that relaxin infusion for 3 days enhanced bradykinin-mediated PGI_2 production in mesenteric arteries. It is important to note that there was a compensatory increase in PGI_2 in SHR, so it was not clear if relaxin treatment would further exacerbate or mitigate PGI_2 production. Surprisingly, relaxin had minimal effects on prostanoid production in SHR aorta and mesenteric arteries. One possible explanation is that PGI_2 is

already significantly elevated in SHR, preventing relaxin from causing any further increase. In rat mesenteric arteries, PGE_2 and 8-iso $PGF_{2\alpha}$ evoke vasoconstriction and some of this is mediated via interaction with TP receptors ((Kobayashi, Murata et al., 2011; Kondo, Okuno et al., 1980)). Enhanced production of these constrictor prostanoids may contribute to the TP-mediated contraction in SHR mesenteric arteries. Our data indicate that relaxin may downregulate the production of some vasoconstrictor prostanoids (PGE_2/PGD_2 and 8-iso $PGF_{2\alpha}$) in SHR mesenteric arteries but it failed to reach statistical significance.

Previous studies demonstrate that short term relaxin infusion upregulates IP expression in mesenteric arteries of normotensive rats (Jelinic, Leo et al., 2017). Conversely, IP expression is decreased in the aorta of male relaxin gene-deficient mice; this is accompanied by impaired functional responses to the PGI_2 analog, iloprost (Ng, Jelinic et al., 2015). Similarly, endothelial dysfunction is associated with upregulation of vasoconstrictor prostanoids in the mesenteric arteries of relaxin-deficient mice (Leo, Jelinic et al., 2014a). These findings suggest that relaxin is an important regulator of PGI_2-IP pathway in the vasculature of normotensive animals. In this study, IP and TP are both upregulated in the aorta of SHR. Moreover, we also showed a decrease in IP, but not TP in the mesenteric arteries. Similarly, there were differential effects of relaxin on the expression of these receptors. Specifically, relaxin treatment reduced the expression of TP and IP in the SHR aorta, whereas it increased IP in the mesenteric arteries. Taken together, our findings support the hypothesis that relaxin regulates IP expression/activity in the mesenteric arteries, which restores the ability of PGI_2 to cause vasorelaxation in SHR vessels. In addition, we have uncovered a completely different mechanism of relaxin action in the aorta. This involves a relaxin-mediated decrease in TP and IP expression, thereby suppressing the vasoconstrictor action of PGI_2 and other prostanoids.

In this study, relaxin treatment reversed EDC in the vasculature by altering IP expression and/or activity. There is limited information in the literature to explain regulation of IP receptor expression in vascular cells. Given that several key transcriptional regulators such as estrogen response element, sterol response element binding protein or enhancer binding proteins are involved in the complex regulation of IP expression (Reid & Kinsella, 2015), it is possible that relaxin may interact with any of these to alter IP expression. However, additional experiments beyond the scope of this study will be required to prove this hypothesis. Other than IP expression, the activity of IP can also be influenced by GPCR

interacting proteins (Reid & Kinsella, 2015). One of such GPCR interacting proteins is known as postsynaptic density 95/disc large/zonula occludens-1 domains (PDZK1) (Reid & Kinsella, 2015). Unphosphorylated PDZK1 is constitutively associated in a complex with the IP. PDZK1 is thought to increase IP expression at the plasma membrane and enhance agonist binding. Upon receptor activation, IP undergoes an agonist-induced conformational change leading to dissociation of PDZK1. The free PDZK1 is dependent on cAMP-dependent PKA-mediated phosphorylation to trigger its re-association with the IP (Reid & Kinsella, 2015). In SHR, production of PGI_2 is enhanced, suggesting that there may be an increase in the proportion of PDZK1 that is not associated with IP. Relaxin increases cAMP accumulation and triggers PKA-medicated phosphorylation in cells (Singh, Simpson et al., 2015). Therefore, it is possible that relaxin treatment may cause the re-association of free PDZK1 with the IP, leading to an increase in the availability of 'activatable' IP receptors, and therefore IP activity in the SHR arteries. This possibility requires exploration in future studies.

In addition to EDCFs, impaired NO bioavailability and EDH also contribute to endothelial dysfunction in hypertension. Vascular oxidative stress and uncoupling of eNOS occur in SHR arteries, leading to decreases in NOS activity and NO bioavailability (Macarthur, Westfall et al., 2008; Mason, Kubant et al., 2006). In the current study, relaxin reversed hypertension-induced endothelial dysfunction in the mesenteric arteries by increasing basal NOS activity and NO-mediated relaxation, without any changes in eNOS and GTPCH1. This suggests that relaxin may regulate eNOS function at a post-translational level to increase NO bioavailability. We have previously shown that relaxin enhances NOS activity via reduction of vascular oxidative stress (Leo, Fernando et al., 2017), activation of eNOS phosphorylation at ser1177 (Leo, Jelinic et al., 2016b; Leo, Jelinic et al., 2014b; McGuane, Debrah et al., 2011) and upregulation of eNOS dimerization (Jelinic, Leo et al., 2017). Furthermore, the ability of relaxin to increase NO bioavailability and prevent endothelial dysfunction are observed in a number of disease conditions, including TNF-α incubation (Dschietzig, Brecht et al., 2012), cigarette smoke (Pini, Boccalini et al., 2016), arteriosclerosis (Tiyerili, Beiert et al., 2016) and diabetes (Ng, Leo et al., 2016; Ng, Leo et al., 2017). Previous studies also reported that acute intravenous injection of relaxin for 3 hours increases the contribution of EDH to endothelium-dependent relaxation in the resistance-size arteries, but it primarily involves the activation of IK_{Ca} (Chan, Sweet et al., 2013; Leo, Jelinic et al., 2014b; Marshall, O'Sullivan et al., 2017). The impaired EDH-type

responses in the SHR mesenteric arteries were not reversed by 3 days of relaxin treatment. In contrast, 14 days of relaxin treatment decreases myogenic tone and increases EDH-type relaxation in the cerebral parenchymal arterioles (Chan, Sweet et al., 2013). Therefore, a longer duration of relaxin treatment may be required to reverse the impaired EDH-type relaxation in SHR (Chan, Sweet et al., 2013).

Although relaxin produces vasoprotective effects in mesenteric arteries and aorta, this has not translated into a direct effect on blood pressure in the SHRs. However, this does not preclude marked changes in the circulation, such as systemic arterial resistance, global arterial compliance or cardiac output. Indeed, many other published studies also show that relaxin's vasoprotective actions are independent of any normalisation of blood pressure (Chan, Sweet et al., 2013; van Drongelen, van Koppen et al., 2013; Xu, Chakravorty et al., 2010). Of note, relaxin treatment reduces arterial load (by decreasing systemic vascular resistance and increasing arterial compliance) while also increasing cardiac output, heart rate and stroke volume in hypertensive rats, without any effect on mean arterial pressure (Debrah, Conrad et al., 2005a).

Although the primary focus of this study was the vasculature, it provided an opportunity to assess any additional beneficial effects on the myocardium after 3 days of relaxin infusion. This is because anti-fibrotic and anti-hypertrophic actions of relaxin were reported in the myocardium of 9- to 10-month-old SHR after 14 days of treatment (Samuel, Royce et al., 2017; Samuel, Summers et al., 2016). It is important to note that the SHR used in our study were relatively young (6-7 months). Nevertheless, there was evidence of cardiomyocyte hypertrophy and increased expression of biomarkers of early onset cardiac fibrosis in the SHR at this age. In our study, we showed that a 3-day infusion of relaxin had no significant effect on LV cardiomyocyte morphometry or expression of four biomarkers of fibrosis and hypertrophy we selected to analyse. On this basis, we decided not to investigate fibrosis in the LV any further. However, as we did not directly measure collagen deposition or conduct a more comprehensive analysis of fibrosis, we cannot conclude that 3 days of relaxin treatment had no anti-fibrotic effects on the myocardium. Importantly, our data clearly demonstrate that a 3-day relaxin infusion is sufficient to modify many aspects of vascular dysfunction associated with hypertension, but the short time course of infusion was not sufficient to have significant cardiac effects.

In conclusion, short-term relaxin treatment reverses hypertension-induced vascular dysfunction through novel interactions with EDCFs and their associated vasoconstrictor pathways. Relaxin clearly has region-dependent effects on the vasculature, although the overall therapeutic endpoint is a reduction in EDC by restoring endothelium-derived PGI_2 vasodilator pathways. We propose that relaxin or its newer generation mimetics such as B7-33 or ML290 could be progressed as novel therapeutics for the treatment of the vascular complications of hypertension and/or other vascular diseases that are associated with increased endothelium-derived vasoconstriction (Leo, Jelinic et al., 2019; Ng, Leo et al., 2018).

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure 1

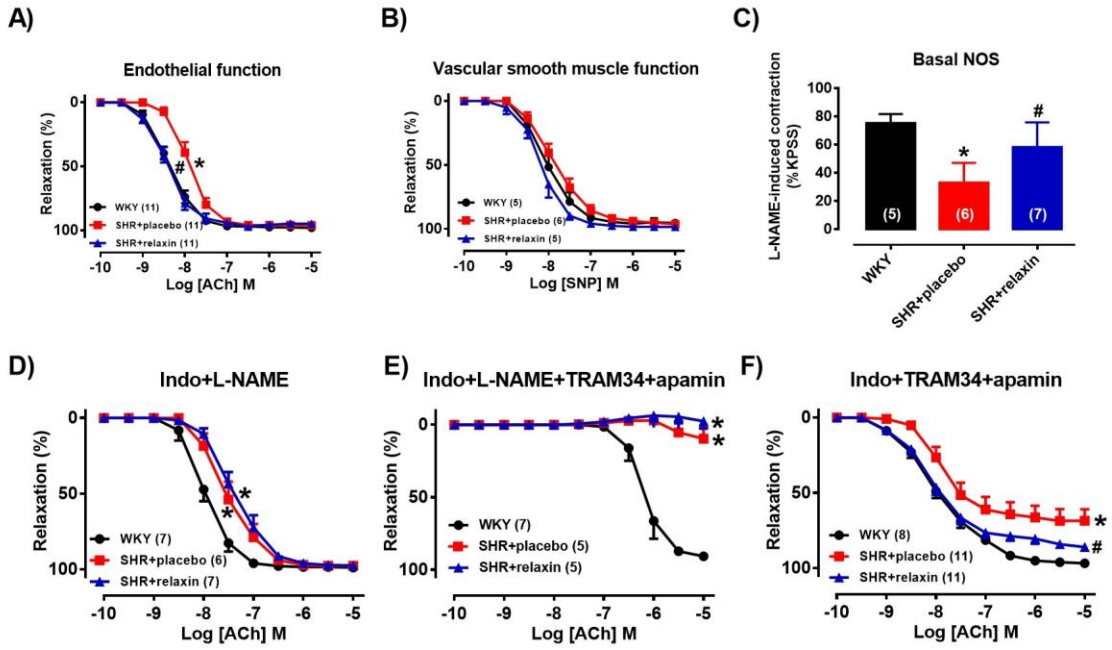

Figure 1: Short term relaxin treatment reverses endothelial dysfunction by increasing NO-mediated relaxation but not EDH-type relaxation. Concentration–response curves to ACh (A) and SNP (B), and basal NOS activity (C) of mesenteric arteries from WKY+placebo (●), SHR+placebo (■) or SHR+relaxin (▲) for 3 days. Concentration–response curves to ACh in the presence of Indo+L-NAME (D, inhibitors of COX and NOS), Indo+L-NAME+TRAM34+apamin (E, inhibitors of COX, NOS, IK_{Ca} and SK_{Ca}, respectively) and Indo+TRAM34+apamin (F, inhibitors of COX, IK_{Ca} and SK_{Ca}, respectively) in endothelium-intact mesenteric arteries of WKY+placebo (●), SHR+placebo (■) or SHR+relaxin (▲) for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, $P < 0.05$, (1-way ANOVA, Tukey's post-hoc test).

Figure 2

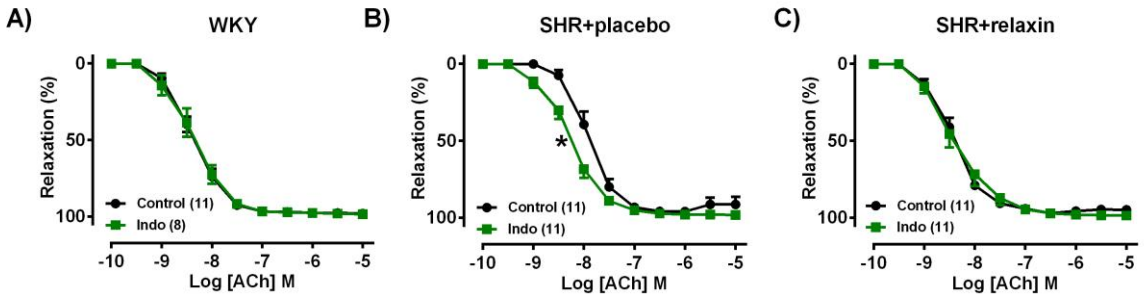

Figure 2. Short term relaxin treatment reverses the contribution of COX-derived vasoconstrictor prostanoids. Concentration–response curves to ACh in endothelium-intact mesenteric arteries of WKY+placebo (A), SHR+placebo (B) or SHR+relaxin (C) in the absence (control) or in the presence of Indo (1 μmol/l, non-selective COX inhibitor). Number of rats used per experimental group are shown in parentheses. * Significantly different to control (unpaired student's t-test).

Figure 3

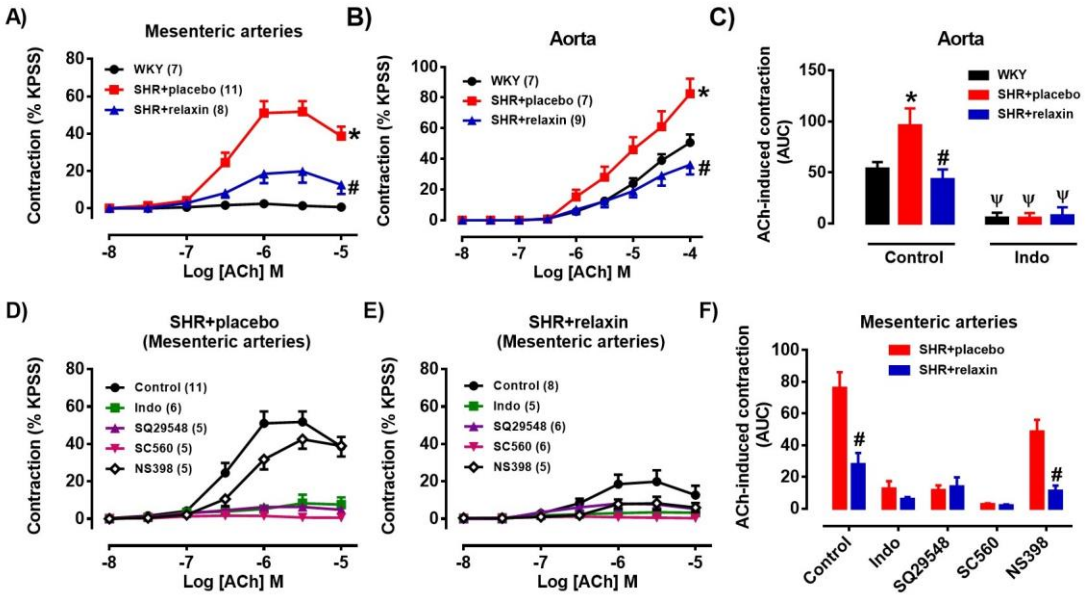

Figure 3. Short term relaxin treatment reverses endothelium-dependent contraction in mesenteric arteries and aorta. Concentration–response curves to ACh in endothelium-intact, resting mesenteric arteries (A, in the presence of L-NAME+TRAM34+apamin) or aorta (B-C, in the presence of L-NAME or Indo+L-NAME, respectively) of WKY+placebo (●), SHR+placebo (■) or SHR+relaxin (▲) for 3 days. Concentration–response curves to ACh in endothelium-intact mesenteric arteries of SHR+placebo (D), SHR+relaxin (E) and AUC (F) in L-NAME+TRAM34+apamin (control) or in the presence of Indo (1 μmol/l, non-selective COX inhibitor), SC560 (1 μmol/l, COX1 inhibitor), NS398 (1 μmol/l, COX2 inhibitor) and SQ29548 (10 μmol/l, TP antagonist). Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, P <0.05, (1-way ANOVA, Tukey's post-hoc test).

Figure 4

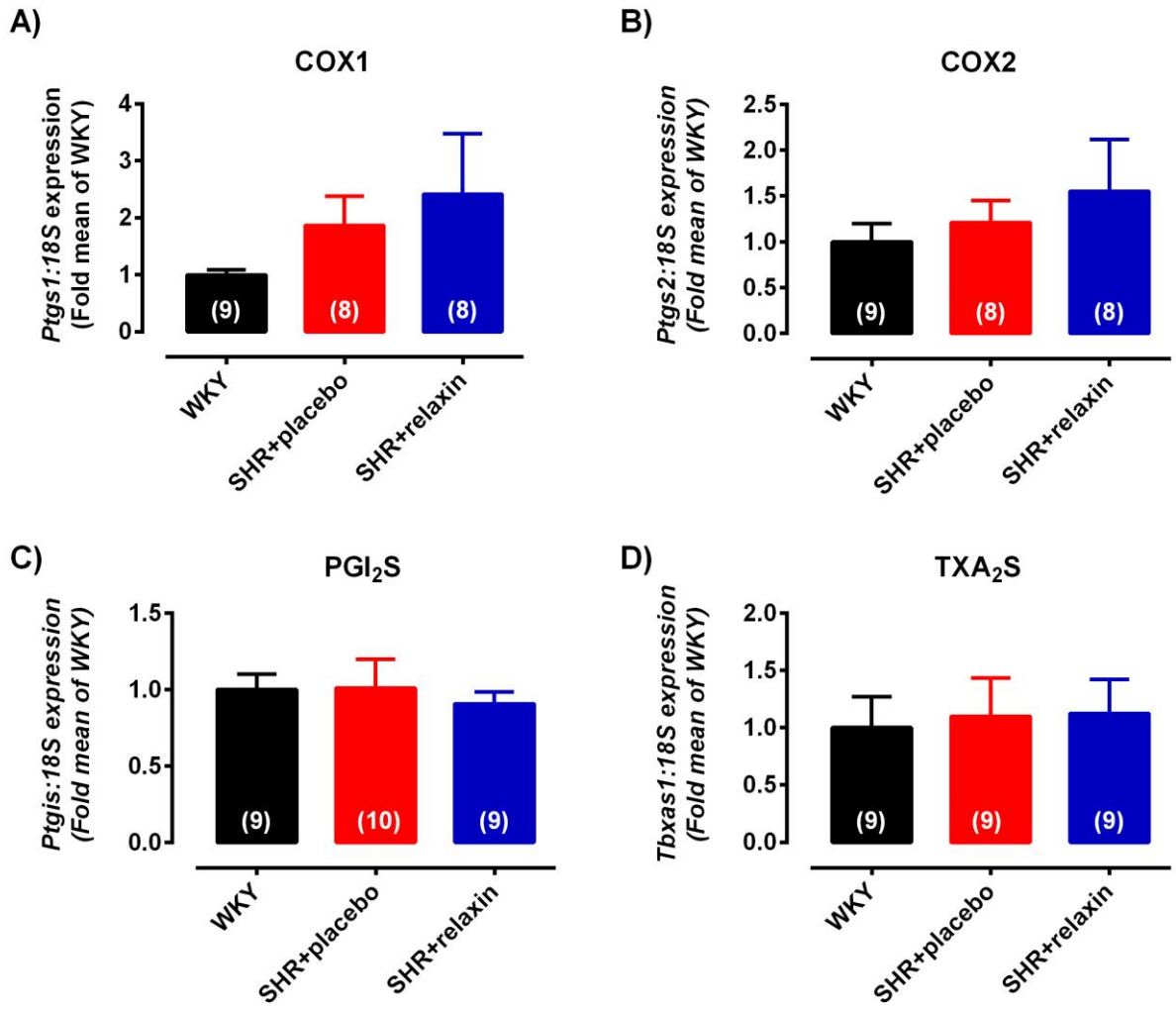

Figure 4. Gene expression of prostanoid-synthesising enzymes in the mesenteric arteries. Quantitative analysis of *Ptgs1* (A), *Ptgs2* (B), *Ptgis* (C), *Tbxas1* (D) expression in mesenteric arteries of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Number of rats used per experimental group are shown in parentheses. Outlier exclusion: 2 SHR+placebo and 1 SHR+relaxin (A-B), 1 SHR+placebo (D).

Figure 5

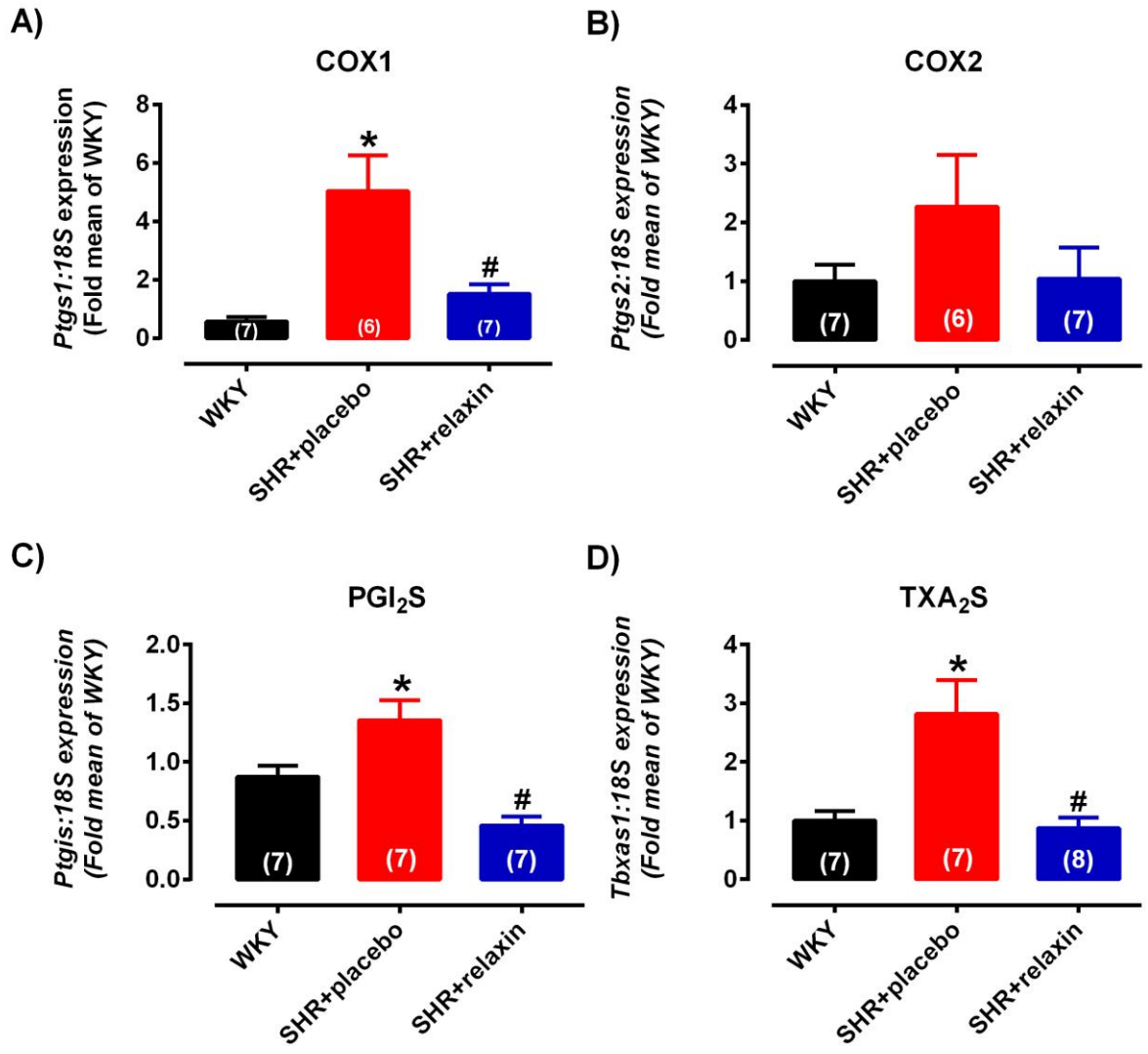

Figure 5. Gene expression of prostanoid-synthesising enzymes in the aorta. Quantitative analysis of *Ptgs1* (A), *Ptgs2* (B), *Ptgis* (C), *Tbxas1* (D) expression in aorta of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, P <0.05, (1-way ANOVA, Tukey's post-hoc test). Outlier exclusion: 1 WKY, 1 SHR+placebo and 1 SHR+relaxin (A-B), 1 WKY and 1 SHR+placebo (C), and 1 WKY (D).

Figure 6

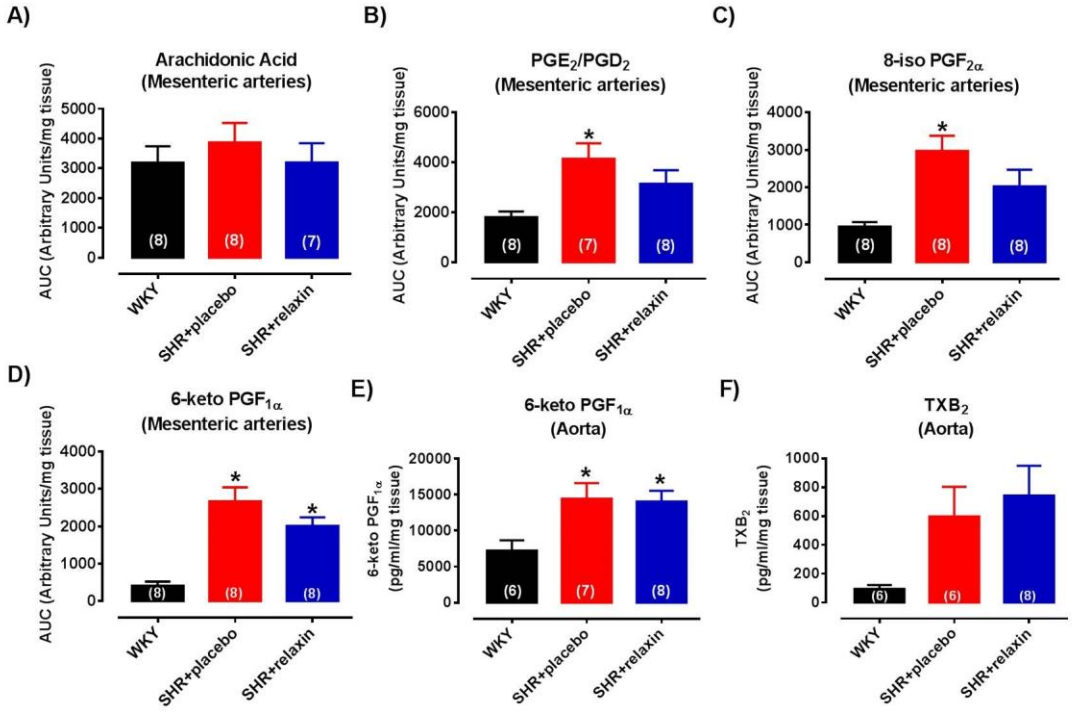

Figure 6. Metabolite profiling of prostaglandin production in mesenteric arteries (A-D) and aorta (E-F). Measurement of arachidonic acid (A), PGE_2/PGD_2 (B), 8-iso $PGF_{2\alpha}$ (C) and 6-keto $PGF_{1\alpha}$ (D-E) and TXB_2 (F) production in ACh (10 µmol/l)-stimulated mesenteric arteries (A-D) and aorta (E-F) from WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. PGE_2/PGD_2 are grouped together as the retention time cannot be sufficiently distinguished. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, $P < 0.05$, (1-way ANOVA, Tukey's post-hoc test). Outlier exclusion: 1 SHR+relaxin (A), 1 SHR+placebo (B), 1 SHR+placebo (C) and 2 SHR+placebo (D).

Figure 7

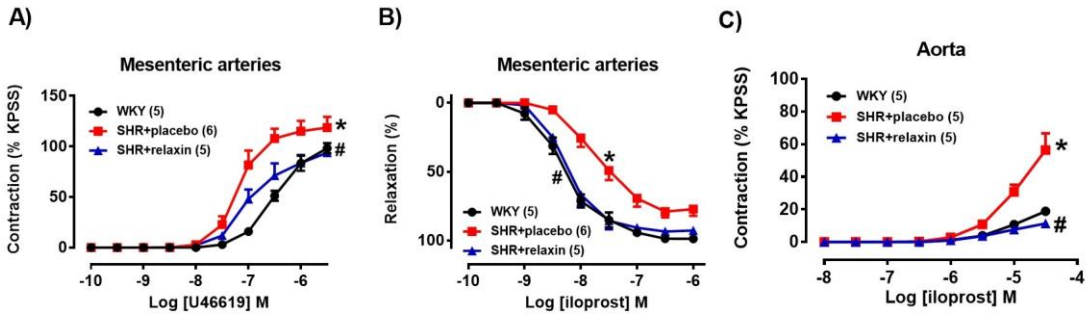

Figure 7. Short-term relaxin treatment altered vascular reactivity to prostanoid receptor activation in the mesenteric arteries and aorta. Concentration–response curves to U46619 (A) and iloprost (B) in endothelium-intact mesenteric arteries and contraction to iloprost (C) in the presence of L-NAME (NOS inhibitor) of endothelium-intact, resting aorta from WKY+placebo (●), SHR+placebo (■) or SHR+relaxin (▲) for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, P <0.05, (1-way ANOVA, Tukey's post-hoc test).

Figure 8

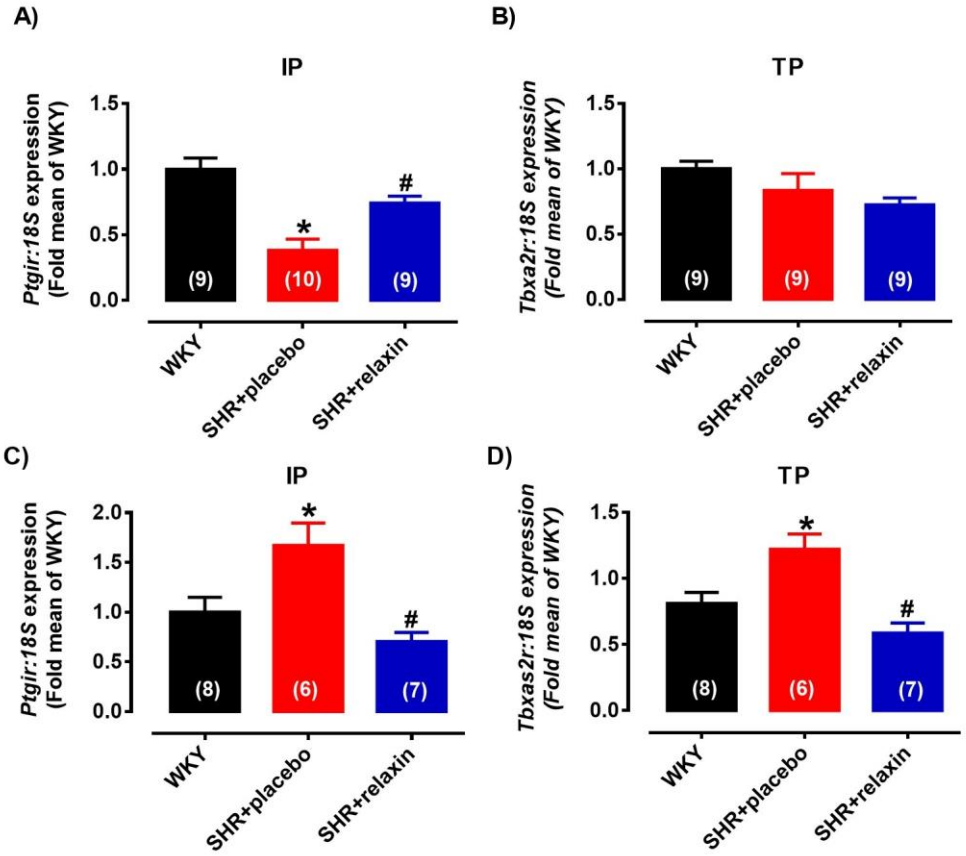

Figure 8. Gene expression of TP and IP in mesenteric arteries (A-B) and aorta (C-D). Quantitative analysis of *Ptgir* (A,C) and *Tbxa2r* (B,D) expression in the mesenteric arteries (A-B) and aorta (C-D) of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY, # significantly different to SHR+placebo, P <0.05, (1-way ANOVA, Tukey's post-hoc test). Outlier exclusion: 1 SHR+placebo (B), 1 SHR+placebo and 1 SHR+placebo (C-D).

Figure 9

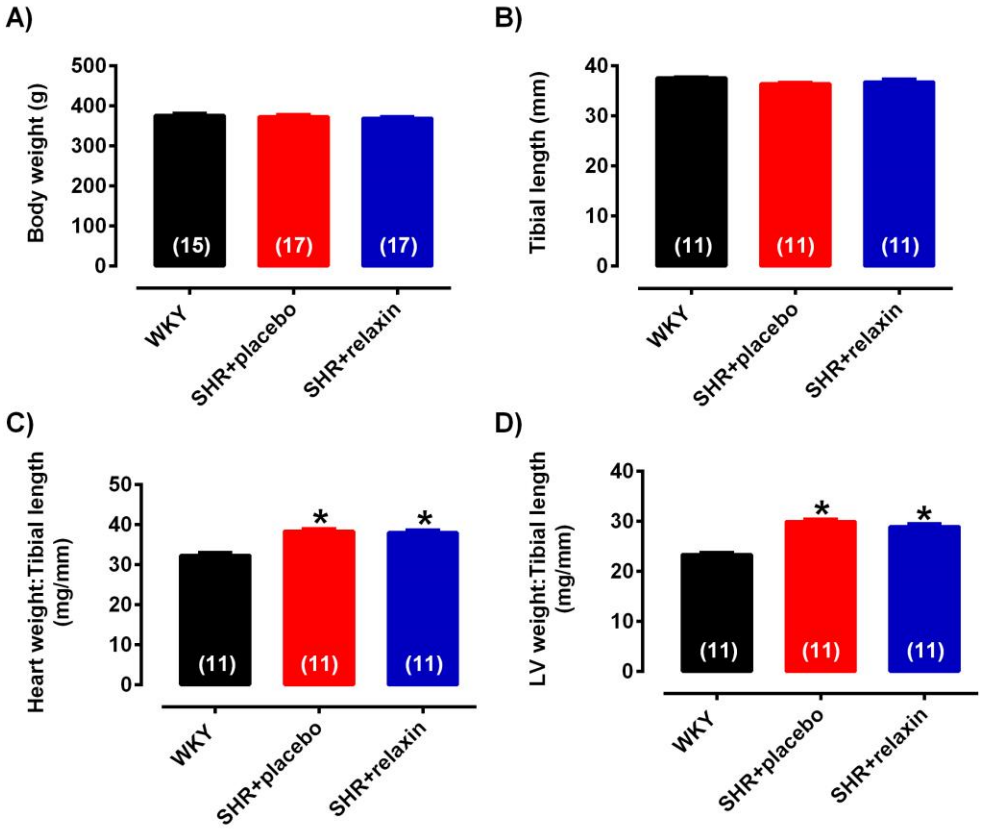

Figure 9. Short term relaxin treatment had no effect on heart and left ventricle weights. Body weights (A), tibial length (B), heart weight (C) and left ventricle weight (D) of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY+placebo, $P < 0.05$, (1-way ANOVA, Tukey's post-hoc test).

Figure 10

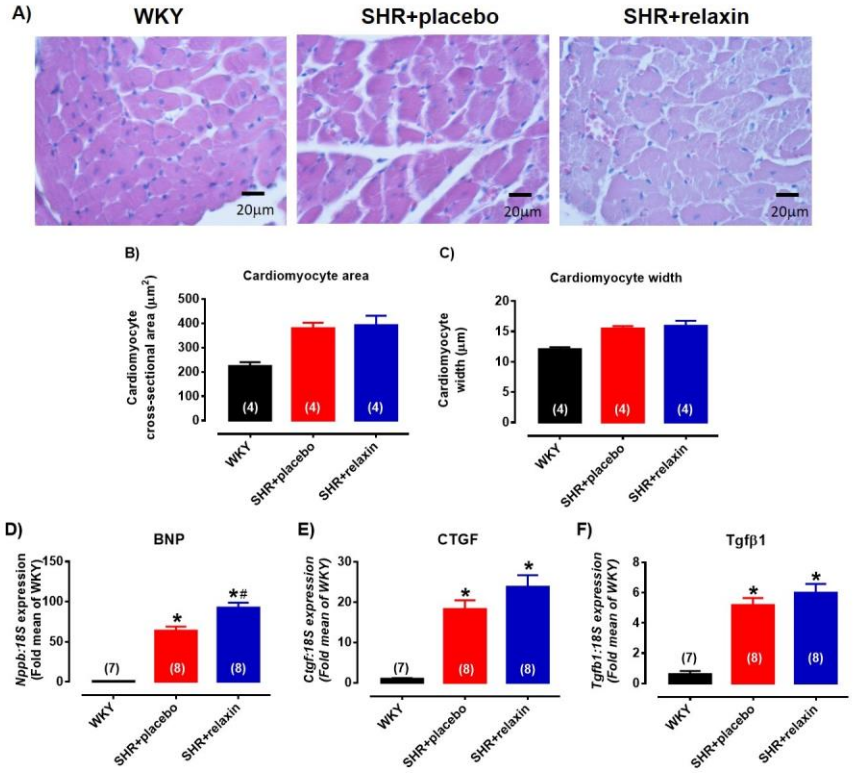

Figure 10. Short-term relaxin had no effect on cardiac hypertrophy and biomarkers of early onset fibrosis. Representative images (A) and quantification of cardiomyocyte cross-sectional area (B) and width (C) of H&E-stained cardiomyocytes in the left ventricle of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Quantitative analysis of *nppb* (D), *ctgf* (E) and *tgfb* (F) mRNA expression in the left ventricle of WKY+placebo, SHR+placebo or SHR+relaxin for 3 days. Number of rats used per experimental group are shown in parentheses. * Significantly different to WKY+placebo, # significantly different to SHR+placebo, P <0.05, (1-way ANOVA, Tukey's post-hoc test). Outlier exclusion: 1 WKY (D-F).