Sexual dimorphism in prostacyclin-mimetic responses of rat mesenteric and coronary arteries.

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Word count: 3646

Author contribution

S.N.B designed and implemented all experiments. S.N.B, E.A.F and L.M preformed experiments, generated and analysed data. S.N.B and I.A.G drafted the manuscript. I.A.G oversaw the project and prepared the submission of the paper. I.A.G provided funding.

Acknowledgements

S.N.B was funded by the British Heart Foundation (Grant #FS/18/41/33762) awarded to I.A.G.

Conflict of interest

The authors declare no conflict of interest.

Data availability

The data generated herein is available upon reasonable request to the corresponding author.

Ethics approval statement

Animals used within the following investigation were handled in strict accordance with the Animal (Scientific Procedures) Act 1986.

Abstract

Background and purpose- Prostacyclin mimetics are widely used clinically. As such it is pertinent to understand the mechanisms underlying the vasoactive response to such agents, yet to date, no study has considered sex as a factor. The aim of this study was to characterise the effect of prostacyclin mimetics, Iloprost and MRE-269, on precontracted arterial tone from male and female Wistar arteries. As a secondary consideration, we investigated *Kcnq*-encoded K_V7 channels as potential downstream targets of prostacyclin-IP-receptor mediated signalling.

Experimental approach- Relative mRNA transcript and protein abundance were determined by RT-qPCR and immunocytochemistry respectively. The effect of Iloprost and MRE-269 was determined on pre-contracted arterial tone in the presence of pharmacological modulators of potassium channels and molecular interreference of $K_V7.1$ within 2nd order mesenteric and left anterior descending arteries from male and female Wistar rats.

Key results- Iloprost evoked a bi-phasic response in male mesenteric arteries, at low concentrations relaxing, then contracting the vessel at high concentration in a process attributed to IP and EP_3 receptors respectively. Secondary contraction was absent in the females, potentially underpinned by a reduction in *Ptger3*. Pharmacological inhibition and molecular interference of $K_V7.1$ significantly attenuated MRE-269 mediated relaxation in male and female Wistar in Diestrus / Metoestrous, but not Pro-oestrus / Oestrus.

Conclusions and implications- Stark sexual dimorphisms in Iloprost mediated vasoactive responses are present within mesenteric arteries. $K_V7.1$ is implicated in IP-receptor mediated vasorelaxation and is impaired by the Oestrus cycle.

What is already known

- Prostacyclin analogue Iloprost evokes enigmatic vasoactive responses through myriad receptors.
- *Kcnq*-encoded K_V7 channels are known targets of endogenous vasoactive signalling cascades, though no functional role for $K_V7.1$ has been identified within the vasculature.

What this study adds

- There are sexual dimorphisms in Iloprost evoked responses. Male mesenteric arteries express a bi-phasic relaxant-contractile response that was absent in females.
- MRE-269 mediated relaxation was impaired Male and Female mesenteric arteries by $K_V7.1$ inhibition, a phenomenon impacted by the oestrus cycle.

Clinical significance

- Sex must be considered as a factor when considering prostacyclin mimetics for as a therapeutic.

Introduction

Prostacyclin (PGI_2, Epoprostenol), a vasoactive derivative of arachidonic acid (Moncada *et al.*, 1976), is an effective regulator of the vasculature due to its anti-thrombotic, anti-inflammatory and potent vasodilatory properties (Barst, Rubin & Long, 1997; Olschewski, 2009). These vasoprotective properties are largely attributed to the binding of PGI_2 to $G_{\alpha s}$ coupled IP prostanoid receptor (Tateson, Moncada & Vane, 1977; Katusic, Santhanam & He, 2012; Vane & Botting, 1995), though the precise mechanisms underlying vasodilatation following IP receptor activation are not defined unequivocally. However, prostacyclin and stable synthetic analogues like Iloprost also contract various arteries via activation of EP_3 receptors (Dusting, Moncada & Vane, 1977; Levy, 1980; Liu *et al.*, 2017). Moreover, a phenotypic switch seen in vascular disease states results in a greater contractile response to prostanoids, contributing to endothelial dysfunction (Levy, 1980; Liu *et al.*, 2017). The diverse response to prostacyclin-mimetics remains largely enigmatic, especially as no studies to our knowledge has yet to investigate prostacyclin-mediated vascular effects within the female animal model. As females have a reduced prevalence for cardiovascular disease, the relative contribution of prostacyclin-mediated relaxations versus contractions may be a contributory factor.

Voltage-gated potassium channels encoded by KCNQ genes (termed K_V7 channels) are key functional components of vasorelaxations generated by several agonists of G_s-linked receptors including β-adrenoceptor (Chadha *et al*., 2012; Stott *et al*., 2016), calcitonin gene related peptide (Chadha *et al*., 2014, Stott *et al*., 2018) and adenosine (Khanamiri *et al*., 2013) in different vascular beds. In addition, K_V7 channels become compromised in arteries from hypertensive animals (Jepps *et al.*, 2011). We therefore speculated whether K_V7 channels were functional components of prostacyclin-mediated responses. As nothing is known about the contribution of K_V7 channels to receptor-mediated relaxations in arteries from female animals, nor the impact of sex on prostanoid mediated vascular responses, we sought to determine whether K_V7 channels were involved with prostacyclin-mediated relaxations in mesenteric and coronary arteries from aged-matched male and female rats. As prostacyclin has a short half-life (Moore, 1982) we characterised the contribution of EP_3 and IP

receptors to responses mediated by the stable analogue Iloprost and defined the contribution of *Kcnq*-encoded K_V7 channels to IP receptor mediated vasorelaxation by pharmacological means consolidated by molecular interference studies. Our data demonstrates a striking sex-dependent difference in response to Iloprost and a novel role for $K_V7.1$ channels in shaping IP-receptor evoked vasorelaxation within rat mesenteric arteries that is Oestrus cycle-sensitive.

Materials and methods

Animal models

Experiments were performed on arteries from male and female Wistar rats (Charles River, Margate, UK) ages 11-14 weeks (200-350 g) from the Biological Research Facility, St George's, London, UK. Animals were housed in cages with free access to water and food (RM1; Dietex Inter-national, UK) with a 12-hour light/dark cycle and constant temperature and humidity (21 ± 1°C; 50% ± 10% humidity) in accordance with the Animal (Scientific Procedures) Act 1986. Animals were kept in LSB Aspen woodchip bedding. Animals were culled by cervical dislocation with secondary confirmation via cessation of the circulation by femoral artery severance in accordance with Schedule 1 of the ASPA 1986.

For the following investigations, 2nd order mesenteric arteries (MAs) and the left anterior descending (LAD) artery were used, identified as the second bifurcation of the superior MA, and the main bifurcation distal to the septal artery respectively. Arteries were dissected, cleaned of fat and adherent tissue and stored on ice in physiological salt solution (PSS) of the following composition (mmol-L^{-1}); 119 NaCl, 4.5 KCl, 1.17 $MgSO_4.7H_20$, 1.18 NaH_2PO_4, 25 $NaHCO_3$, 5 glucose, 1.25 $CaCl_2$.

Oestrus cycle stage determination

Following euthanasia, 50 µL of PSS was inserted into the vaginal canal via a 2-200µL pipette tip and flushed 4-6 times to liberate cells from the surface of the cervix. PSS was removed from the vaginal canal, then placed into an Eppendorf tube on ice. 25 µL of the subsequent cell suspension was mounted on a glass slide and examined under light microscopy (x10 – x20 magnification). Previously described changes in cervical cell histology allowed for the determination of Oestrus cycle stage (Cora, Kooistra & Travlos, 2015) as either (in order of the 4-5 day cycle); Pro-oestrus, Oestrus, Metoestrus or Dioestrus. Cycle stage determination was performed post-experiment during functional investigation as a means of blinding, this was not possible during molecular techniques.

Wire Myography

For functional investigations, ~2 mm arterial segments of LAD or MAs were mounted on 40 μm tungsten steel wire within a myograph chamber (Danish Myo Technology, Arhus, Denmark) containing 5 mL of PSS (composition described above) oxygenated with 95% O_2 and 5% CO_2 at 37°C. Vessels then underwent a passive force normalization process to achieve an internal luminal circumference at a transmural pressure of 100 mmHg (13.3 kPa) to standardize pre-experimental conditions (Mulvany, 1977). Force generated was first amplified by a PowerLab (ADInstruments, Oxford, UK), then recorded via LabChart software (ADInstruments, Oxford, UK). Vessels were then challenged with isotonic high K^+ physiological salt solution (PSS) of the following composition (mmol-L^{-1}): 63.5 NaCl, 60 KCl, 1.17 $MgSO_4.7H_20$, 1.18 NaH_2PO_4, 25 $NaHCO_3$, 5 glucose, 1.25 $CaCl_2$, to determine viability. Vessels were then constricted with 10 μmol-L^{-1} methoxamine, an α-1 adrenoreceptor agonist, and endothelial cell integrity was determined via vasorelaxation in response to 10 μmol-L^{-1} synthetic acetylcholine analogue, carbachol, prior to investigation. Subsequently, vessels were pre-constricted with 300 nmol-L^{-1} thromboxane A2 mimetic U46619. Following which a cumulative concentration effect curve (CEC) was generated in response to either Iloprost (0.001-3 μmol-L^{-1}), IP-receptor agonists Selexipag (0.03-3 μmol-L^{-1}) and MRE-269 (0.01-1/0.01-3 μmol-L^{-1}) or Isoprenaline (0.003-3 μmol-L^{-1}). Vessels were pre-incubated in the presence or absence of a combination of solvent control dimethyl sulphoxide (DMSO) or antagonists of the following: IP prostanoid receptor-CAY-10441 (100 nmol-L^{-1}); EP_3 prostanoid receptor-L-798,106 (300 nmol-L^{-1}); pan-K_V7 channel-Linopirdine (10 μmol-L^{-1}); $K_V7.1$ specific HMR-1556 (10 μmol-L^{-1}); BK_{Ca} iberiotoxin (100 nmol-L^{-1}); K_{ATP}-glibenclamide (1 μmol-L^{-1}) or PKA-KT7520 (1 μmol-L^{-1}) for a period of 10 minutes.

Reverse transcription quantitative polymerase chain reaction

mRNA from whole MAs was extracted using Monarch Total RNA Miniprep Kit (New England BioLabs, Ipswich, Massachusetts, USA) and reverse transcribed via LunaScript RT SuperMix Kit (New England BioLabs, Ipswich, Massachusetts, USA). Quantitative analysis of relative gene

expression was determined via CFX-96 Real-Time PCR Detection System (BioRad, Hertfordshire, UK). Samples were run in BrightWhite qPCR plate (Primer Design, Camberley, UK) in combination with PrecisionPLUS qPCR Master Mix (Primer Design, Camberley, UK) and 300 nmol-L^{-1} of gene specific target primer (Thermofisher scientific, Waltham, Massachusetts, USA) as per manufacturers instruction. Quantification cycle (Cq) was determined via Bio-Rad CFX96 Manager 3.0. Cq values are expressed as normalised values to appropriate housekeeper genes ($2^{-\Delta Cq}$) calnexin (*Canx*) and cytochrome C1 (*Cyc1*). See table 1 for a list of primers used in the following investigation.

Morpholino transfection

Knockdown of $K_V7.1$ in whole MAs was performed by morpholino transfection as described previously (Jepps *et al.*, 2015), briefly; either $K_V7.1$ morpholino nucleotides or mismatch controls (5 µmol-L^{-1}, Genetools, USA) were mixed with Lipofectamine 2000 (ThermoFisher, Paisley, UK) and Opti-MEM (Sigma, UK) and left at room temperature for 2hrs. Morpholino/Lipofectamine/Opti-MEM mixture was added to Dulbecco's modified eagle medium (DMEM) F-12 (Sigma, UK) containing 1%-penicillin/streptomycin. Arteries were added and left for 48hrs at 37°C with 5% CO_2.

Cell culture

As previously (Greenwood & Stott, 2020), Chinese Hamster ovarian (CHO) cells were grown in DMEM/F-12 (Sigma, UK) supplemented in 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37°C. CHO cells were incubated with either a total of 3µg of plasmid containing *Kcnq1* (University of Copenhagen, Denmark) in a Lipofectamine 2000 / Opti-MEM mixture (*Kcnq1* transfected CHO), or lipofectamine / Opti-MEM only for 24 hrs (Non-transfected CHO). Cells were mounted onto glass coverslips, fixed and stained for $K_V7.1$ as below.

Immunocytochemistry

Vascular smooth muscle cells (VSMCs) were isolated from dissected fresh and morpholino transfected MAs via incubation in PSS of the following composition (mmol-L^{-1}): 120 NaCl, 6 KCl, 12

glucose, 10 HEPEs and 1.2 $MgCl_2$ supplemented with 1.75mg/mL Collagenase Type IA, 0.9 mg/mL protease, 1 mg/mL Trypsin inhibitor and 1 mg/mL bovine serum albumin (Sigma, UK) at 37°C for 17 min. Vessels then underwent mechanical trituration by wide bore glass pipette to liberate VSMCs. The subsequent cell suspension was plated onto13mm coverslips in a 24-well plate, supplemented with an equal volume of Ca^{2+} (2.5 mmol-L^{-1}) containing PSS and left to attach for 1hr.

Isolated VSMCs and transfected CHO cells were fixed in 3% paraformaldehyde for 15 min, then stored at 4°C in PBS prior to staining. Cells were then incubated in the following; 100 mmol-L^{-1} glycine in PBS, 5mins; blocking solution (PBS containing 0.1% Triton X-100 and 10% FBS in PBS), 45 mins; primary antibody (K_V7.1, 1:100, Rb, Pineda Antikörper-Service, Germany), overnight at 4°C. The following day, cells were incubated in goat anti-rabbit secondary antibody (1:100, conjugated to Alexa Fluro™ 568), then mounted in Vectasheild (Sigma, P4170). Cells were imaged via Nikon A1R confocal microscope (inverted) on Ti2 chassis (Image Resource Facility, St George's University, London) and total cell fluorescence was analysed using ImageJ software. Anti-body specificity demonstrated by positive staining for K_V7.1 in *Kcnq1* transfected CHO cells (Fig S1.A), but not non-transfected CHO cells (Fig S1.B).

Drugs and reagents

All drugs for the following investigation were procured from Tocris Bioscience (Oxford, UK) unless stated otherwise. CAY-10441 was acquired from Cayman chemical (Michigan, USA).

Data and statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). For functional investigations, contractions are expressed as (%) contraction when normalized to vasoconstriction to 10 μmol-L^{-1} methoxamine and all CECs are expressed as (%) stable contraction in response to 300 nmol-L-1 U46619. For functional experiments involving CECs, a transformed data set was generated using; X=Log(X), to reduce representative skew. Following which, either a four parametric linear regression

analysis was performed using the following equation; (Log(Agonist) vs. response – Variable slope (four parameters Bottom/Hillslope/top/EC_{50})) or a bell-shaped linear regression analysis was performed (only for male MAs in response to Iloprost (0.001-3 μmol-L^{-1}) in the presence of DMSO, due to the nature of low concentration response vs high concentration response, as per- GraphPad Prism's recommendation) using GraphPad Prism (Version 9.0.0) to fit a CEC to the figure. For data comparing multiple groups, a Two way-ANOVA followed by either a *post hoc* Bonferonni test or Šídák's test, was performed for comparison of mean values. Significance values are represented as; P=<0.05 (*). Investigations expressing groups of unequal numbers were gathered due to technical failure or an artefact of cycle stage determination post-experiment during functional investigations. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology in accordance with (Curtis *et al.*, 2018).

Results

Iloprost mediated vasoactive responses

In pre-contracted (300 nmol-L^{-1} U46619) MAs from male rats, Iloprost (0.001-3 μmol-L^{-1}) evoked a bi-phasic response in vessels pre-incubated in DMSO (solvent control), manifest as relaxation at lower concentrations followed by contraction at concentrations >300 nmol-L^{-1} (Fig 1.A,D; black). Iloprost, like other synthetic prostacyclin mimetics, binds to a plethora of prostanoid receptors. In human and rat pulmonary arteries, Iloprost binds to EP_1 / IP with high affinity; $EP_{3/4}$ / FP with moderate affinity and - EP_2 / TP / DP_1 with low affinity (Whittle *et al.*, 2012). We therefore incubated MAs with antagonists of either IP receptor (CAY-10441; 100 nmol-L^{-1}; Clark *et al.*, 2004) or EP_3 receptor (L-798,106, 300 nmol-L^{-1}; Gallant *et al.*, 2002) to discern the underlying mechanisms of the observed phenomena. In the presence of CAY-10441 Iloprost evoked an increase in tension at concentrations above 300 nM (Fig 1.B,D; blue). Conversely, in the presence of L-798,106, Iloprost was an effective and potent relaxant (See representative traces in Fig 1.A,B,C and mean data in Fig 1.D). In LAD arteries from male rats, Iloprost (0.1-10 μmol-L^{-1}) produced no effect on developed tone under control conditions (DMSO; Fig 1.E; black) and a non-significant increase in tone in vessels pre-incubated in CAY-10441 (Fig 1.E; blue). A significant relaxation was produced in response to Iloprost (>3 μmol-L^{-1}; P=<0.05) in the presence of L-798,106 (Fig 1.E; P=<0.05). These data show that Iloprost was a more potent and efficacious relaxant of precontracted MA compared to LAD arteries but in both vessels the relaxation was precluded by activation of pro-contractile EP_3 prostanoid receptors.

In pre-contracted (300 nmol-L^{-1} U46619) MAs from female rats, Iloprost (0.001-3 μmol-L^{-1}) evoked vasorelaxation in vessels pre-incubated with DMSO at all concentrations (Fig 2A; red). These relaxations were ablated by CAY-10441 (Fig 2A; blue; P=<0.05) and significantly enhanced by L-798,106 (Fig 2A; green; P=<0.05). In contrast, LAD arteries from female rats were insensitive to cumulative concentrations of Iloprost (0.1-10 μmol-L^{-1}). Though similarly to MAs, ≥1 μmol-L^{-1} Iloprost evoked significant contractions when vessels were pre-incubated with CAY-10441 (Fig 2B;

blue; P=<0.05) and significant relaxation in vessels pre-incubated with L-798,106 (Fig 2B; green; P=<0.05) when compared to DMSO.

Sex-dependent differences in EP_3 receptor mediated responses in MAs are potentially underpinned by a reduction in *Ptger3*

No difference was observed in the contractile response to Iloprost in LAD arteries from male compared to female rats. In contrast, comparison of MA from male and female Wistars revealed a marked difference in contractile responses produced by Iloprost (Fig 3C,D), both under control conditions and in the presence of the IP receptor antagonist CAY-10441 (Fig 3C,D). Moreover, Iloprost evoked significantly greater contractions of MA's from male rats pre-incubated in CAY-10441 when compared to arteries from females (Fig 3E). Subsequently, we performed Quantitative PCR to determine the relative expression of prostanoid receptors in MAs from both sexes. Figure 3.E shows that expression of *Ptger2/4* respectively was negligible in MA from both sexes whereas *Ptgir* and *Ptger3* were well expressed. There was no sex-dependent difference in *Ptgir* expression, however *Ptger3* was expressed at significantly lower level in MA from female rats compared to MAs from males (Fig 3F). Which potentially underpins the absence of a bi-phasic response to Iloprost in female MAs.

Sex-dependent differences in IP receptor mediated responses in MAs coincide with a reduction in Linopirdine sensitivity

Kcnq-encoded K_V7 channels are known regulators of vascular smooth muscle physiology (see (Barrese, Stott & Greenwood, 2018; Byron & Brueggemann, 2018) for review). As previous reports demonstrate that potassium channels represent downstream targets of prostanoid mediated vasorelaxation (Schubert *et al.*, 1997, 1996; Dumas *et al.*, 1997; Tanaka, Koike & Toro, 2004; Orie, Fry & Clapp, 2006; Lombard *et al.*, 1999) we investigated whether K_V7 channels were functional components of prostanoid receptor mediated vasorelaxation in LAD arteries and MAs.

In the presence of L-798106, precontracted MAs from male rats relaxed more effectively and potently to Iloprost than arteries from females (Fig 4A; P=<0.05). MAs from male (Fig 4B), but not female rats (Fig 4C) were significantly impaired by pre-incubation with 10 μmol-L^{-1} of the pan-Kv7 blocker Linopirdine (Schnee & Brown, 1998; Wang et al., 1998; Yeung & Greenwood, 2005). Thus, the relaxation produced by Iloprost in rat MAs was Linopirdine sensitive in a sex-dependent manner and occurred predominantly via IP receptors, and not $EP_{2/4}$, as their expression was negligible. In LAD arteries, the small relaxation produced by Iloprost in the presence of L-798106 was equipotent in males and females (Fig 4D) and insensitive to Linopirdine irrespective of the sex of the donor animal (Fig 4E,F).

A novel role for K_V7.1 in shaping IP receptor-specific agonists in mesenteric arteries.

Our data suggests Iloprost relaxed precontracted MAs via activation of IP receptors. In order to further investigate IP-receptor mediated signalling, we proceeded to characterise the effects of the selective IP receptor agonist, Selexipag (NS-304; Kuwano *et al.*, 2007; Sitbon *et al.*, 2015). Application of Selexipag produced concentration dependent relaxation of pre-contracted MAs (Fig 5A), however this effect was insensitive to pre-incubation with the IP receptor antagonist CAY-10441 (Fig 5A). Within the body, Selexipag is metabolized into the active compound, MRE-269 (Kuwano *et al.*, 2007). MRE-269 relaxed precontracted MAs more potently than Selexipag and up to threshold of ≥1 μmol-L^{-1} these relaxations were ablated by prior application of CAY-10441 (Fig 5B). Thus, for the following investigations, MRE-269 at concentrations ≤1 μmol-L^{-1} was used as a selective agonist for IP receptors.

The potent relaxation of male MAs produced by MRE-269 was significantly attenuated by pre-incubation with Linopirdine (Fig 6; yellow) when compared to DMSO (Fig 6; black; P=<0.05). In contrast to Iloprost mediated responses, MRE-269 mediated relaxation was also attenuated by pre-incubation with Linopirdine in female MAs (Figure 6.B; P=<0.05). Furthermore, pre-incubation with the K_V7.1 specific inhibitor HMR-1556 (10 μmol-L^{-1}; Gerlach *et al.*, 2001)), also impaired MRE-269-induced relaxations to the same extent as Linopirdine in MAs form male rats (Fig 6.C; green;

P=<0.05) and to a smaller degree, female MAs (Figure 6.D; *P*=<0.05). Subsequently, no apparent difference was observed between male and female MRE-269 mediated relaxation (Fig 6E).

As to date, no functional role for $K_V7.1$ has been identified within the vasculature, additional pharmacological characterisations and molecular interference was used as a secondary technique to corroborate the contribution of $K_V7.1$ to IP-receptor mediated vasorelaxation. Firstly, pre-incubating MA's from male rats with 10 μmol-L^{-1} of the structurally dissimilar $K_V7.1$ antagonist Chromanol 293B (Busch *et al.*, 1996) significantly attenuated MRE-269 (Fig S2). Secondly, 10 μmol-L^{-1} HMR-1556 pre-incubation failed to attenuate Isoprenaline mediated-relaxation of pre-contracted arterial tone in male MAs (Fig S3). Thus, our findings appear not to be a drug-specific phenomenon. Finally, both fresh arteries and those transfected with either mismatch control morpholino or *Kcnq1* targeted morpholino were stained for $K_V7.1$ (as seen in representative images Fig 7.A,B,C). Mean data demonstrates a significant reduction in total cell fluorescence (A.U) in *Kcnq1* morpholino transfected arteries when compared to fresh control (*P*=>0.05; Fig 7.D). Interestingly, vessels transfected with control morpholino also demonstrated a reduction in total cell fluorescence, though this failed to reach significance (Fig 7.D). Functionally, fresh arteries were significantly more sensitive to 300 nmol-L^{-1} $K_V7.1$ activator ML277 (Yu, 2013; Baldwin *et al.*, 2020) mediated relaxation when compared to control and *Kcnq1* morpholino-transfected arteries (*P*=>0.05; Fig 7.E). The relaxation produced by1 μmol-L^{-1} MRE-269 was greater in fresh arteries compared to arteries transfected with control morpholino and significantly superior than *Kcnq1* morpholino transfected arteries (*P*=>0.05; Fig 7.F). Thus, a reduction in $K_V7.1$ protein was observed in conjunction with an attenuated relaxation by MRE-269.

BK_{Ca} and K_{ATP} channels have also been identified as down-stream targets of cAMP-PKA dependent relaxations evoked by Iloprost (Schubert *et al.*, 1996, 1997; Tanaka, Koike & Toro, 2004) in a process attributed to IP receptors but not specified. Here, we demonstrate that BK_{Ca} inhibitor iberiotoxin (100 nmol-L^{-1}; (Candia, Garcia & Latorre, 1992)) but not K_{ATP} inhibitor glibenclamide (1 μmol-L^{-1}; (Ripoll, Jon Lederer & Nichols, 1993)) partially inhibited MRE-269 mediated relaxation in male

MAs (Figure 8.A,B), though this failed to reach statistical significance. However, the PKA inhibitor KT 5720 (1 μmol-L^{-1}; (Kase *et al.*, 1987)) inhibited MRE-269 mediated relaxations significantly (Fig 8.C; *P*=>0.05).

Oestrus cycle-dependent shifts in the sensitivity of MRE-269 mediated vasorelaxation to K_V7 channel modulators

During this investigation, the Oestrus cycle-stage of each female Wistar was catalogued following functional experiments by defined histological changes in cells lifted from the cervix post-euthanasia (as per methods; (Cora, Kooistra & Travlos, 2015)). We observed two-distinct populations of responders to MRE-269 mediated relaxation and its subsequent sensitivity to K_V7 channel modulators, categorized into rats in Diestrus/Metestrus (Di/Met) or Pro-estrus/Oestrus (Pro/Est). As illustrated by figure 9A/C, whereby both Linopirdine and HMR-1556 inhibited MRE-269-mediated relaxation to the same degree in arteries from female in Di/Met (*P*=<0.05). Whereas figure 9.B,D demonstrates no effect of Linopirdine or HMR-1556 pre-incubation on MRE-269 mediated relaxation in MAs from female rats in Pro/Est. Comparably, MRE-269 mediated relaxation in arteries from female Pro/Est rats were significantly less sensitive to the IP-specific agonist than arteries from rats in Di/Met (Fig 9.E), though this failed to reach statistical significance (*P*=<0.06). It is possible therefore, that a shift in K_V7 channel activity throughout the Oestrus cycle partially accounts for negative shifts in sensitivity to IP receptor mediated vasorelaxation.

Discussion

To our knowledge, the present study is the first to highlight sex as a factor in the arterial response to prostacyclin mimetics. The study shows that application of Iloprost to pre-contacted MAs from male rats produced a biphasic response with relaxation at low concentrations, followed by contraction at higher concentrations. Whereas MAs from female arteries presented with mono-phasic relaxation only. In arteries from both sexes the relaxant effect of Iloprost was enhanced by antagonism of EP_3 receptors by L-798106 and was ablated by the IP receptor antagonist CAY-10441. The dominance of Iloprost-mediated contractions in MAs from male rats correlated with a higher level of *Ptger*$_3$ expression. Furthermore, Iloprost mediated relaxations were impaired moderately by the K_V7 channel blocker Linopirdine. Finally, our findings demonstrate that the selective IP receptor agonist MRE-269 was a potent relaxant of pre-contracted MAs from both sexes. This relaxation was inhibited considerably by the pan-K_V7 blocker Linopirdine in arteries from all rats and by the $K_V7.1$ specific blocker HMR-1556 in arteries from males or Di/Met females.

Iloprost evoked vasoconstriction

Whilst PGI_2 is principally regarded as a vasodilator, since it's discovery PGI_2 has been shown to mediated both relaxation and contraction of smooth muscle (Moncada *et al.*, 1976; Dusting, Moncada & Vane, 1977; Liu *et al.*, 2017). PGI_2 has subsequently been identified as an endothelial derived contracting factor produced in response to acetylcholine within rat aorta (Gluais *et al.*, 2005; Rapoport & Williams, 1996), mesenteric (Liu *et al.*, 2017), iliac (Zhang *et al.*, 2021) and renal arteries (Liu *et al.*, 2015; Zhang *et al.*, 2021) in a process attributed to the activation of both EP and TP prostanoid receptors. As described above, synthetic prostanoids activate a plethora of prostanoid receptors. As such, the potential effect of Iloprost includes; 1.) EP_1 receptor activation evoked contraction (Tang *et al.*, 2008); 2.) IP receptor activation mediated relaxation of pre-contracted tone via cAMP-dependent protein kinase A (PKA) activation of large conductance calcium activated potassium channels (BK_{Ca}; (Schubert *et al.*, 1997, 1996; Dumas *et al.*, 1997)), ATP-sensitive potassium channels (K_{ATP}; (Lombard *et al.*, 1999; Dumas *et al.*, 1997; Schubert *et al.*, 1996) and inwardly rectifying K^+ channels

(Orie, Fry & Clapp, 2006). However, it is possible that these phenomena could occur via EP receptor activation; 3.) $G_{i/o}$ coupled EP_3 receptor activation attenuats relaxation via decreasing cAMP / PI3 kinase activation (Orie & Clapp, 2011) and mediates vasoconstriction via PKCδ / Rho-associated protien kinase (Kobayashi *et al.*, 2011); 4.) $G_{\alpha s}$ coupled-EP_4 receptor activation purportedly stimulates nitric oxide/cGMP production (Namkoong *et al.*, 2005).

Concurrent with the work of Liu *et al* (2017), we show that high concentrations of Iloprost evoked contractions in MAs that were ablated by the EP_3 receptor antagonist L-798,106. As Iloprost has a low affinity for TP receptors (Whittle *et al.*, 2012) and all vessels in this study were precontracted with thromboxane A2 mimetic U46619, a TP receptor agonist, TP receptors were not considered for the scope of this investigation. Similar contractile responses were evoked by PGI_2 in MAs in normal and TP receptor knockout mice (Li et al., 2017). Moreover, EP_1 receptor agonists do not elicit contractions in male MAs (Kobayashi *et al.*, 2011) and in agreement with previous findings (Kobayashi *et al.*, 2011), a reduced expression of *Ptger1* was observed when compared to *Ptger3*. Strikingly, Iloprost had negligible contractile effect in MAs from female rats, an observation that has not been reported previously. This was associated with a lower expression level of *Ptger3* in these arteries.

EP_3 is canonically associated with G_i activation and the reduction of intracellular cAMP (Coleman, Smith & Narumiya, 1994; Orie & Clapp, 2011), ultimately resulting in an increase in $[Ca^{2+}]_i$. However, EP_3 receptor activation within MAs from male rats does not influence $[Ca^{2+}]_i$, directly activates PKCδ/ε phosphorylation and activates Rho associated protein kinases (Kobayashi *et al.*, 2011). These conflicting observations are additionally complicated by EP_3 splice variants (EP_3A-D; (Oldfield, Grubb & Donaldson, 2001)), which are purported to differentially bind G_i (EP_3A,D) or G_s (EP_3B,C; (Namba *et al.*, 1993)). We have attributed the sex-dependent differences in the contraction observed in response to Iloprost to the significantly reduced expression of *Ptger3* within females. However, it is possible that differential coupling of secondary signalling messengers to EP_3 receptors could account for said phenomenon. An avenue as of yet, unexplored.

$K_V7.1$ underpins IP receptor mediated relaxation

Our data shows that relaxations of MAs mediated by low concentrations of Iloprost were driven primarily through CAY-10441-sensitive IP receptor activation, which became offset and then swamped by EP_3 receptor activation as [Iloprost] increased. Though slight relaxation was observed during IP receptor inhibition which may be attributed to $EP_{2/4}$ receptors (Orie & Clapp, 2011), but were not determined here. A similar profile was observed in LAD arteries, but the degree of relaxation was considerably less. We were unable to perform Q-PCR to determine if this was due to a difference in receptor expression as the RNA yield from a single LAD artery is small and we would have had to pool arteries from many animals. Additionally, in MAs, but not LAD arteries from male rats the relaxation to Iloprost in the presence of an EP_3 antagonist was attenuated by the pan-K_V7 channel blocker Linopirdine. Similarly, the selective IP receptor agonist MRE-269 produced relaxations of MAs from male rats that were markedly impaired by linopirdine. MRE-269-mediated responses were also PKA-sensitive, but in contrast to previous reports (Lombard *et al.*, 1999; Dumas *et al.*, 1997; Schubert *et al.*, 1996, 1997) not affected by BK_{Ca} nor K_{ATP} blockade.

Kcnq-encoded K_V7 channels are voltage gated potassium channels with a negative threshold for activation that have well identified roles maintaining resting excitability in neurones, cardiac myocytes, epithelia and smooth muscle cells (Barrese et al., 2018). Within the vasculature, of the five subtypes *Kcnq4>Kcqn5>Kcnq1* are the principally expressed transcripts with little to no expression of *Kcnq2/3* (Ohya *et al.*, 2003; Yeung *et al.*, 2007; Jepps *et al.*, 2011; Chadha *et al.*, 2012b). $K_V7.4$/ $K_V7.5$ alone however are implicated in the regulation of the resting membrane potential (Mackie *et al.*, 2008) and basal tone (Joshi, Balan & Gurney, 2006; Mackie *et al.*, 2008; Chadha *et al.,* 2012a; Ng *et al.*, 2011). In addition, pharmacological inhibition or molecular knockdown of $K_V7.4/7.5$ impairs relaxations to many different relaxants including isoprenaline, CGRP, adenosine (G_s linked), atrial natriuretic peptide (cGMP linked) and adipose derived relaxant factors in several arteries (Stott, Barrese & Greenwood, 2016; Chadha *et al.*, 2014; Stott *et al.*, 2015; Chadha *et al.*, 2012b; Gollasch,

2017; Khanamiri *et al.*, 2013; Byron & Brueggemann, 2018; Morales-Cano *et al.*, 2015). Our data suggests that IP receptor activation in male MAs is another GPCR that also relies on K_V7 channels for full functional responses. We currently have no explanation why CAY-10441-sensitive relaxations produced by Iloprost were far less sensitive to Linopirdine than the relaxations produce by a pure IP receptor agonist. This may reflect a contemporaneous inhibitory effect on the K_V7 channels as a consequence of Iloprost activating other receptors. We also observed that Iloprost was a poor relaxant of LADs even when the EP_3 receptors were blocked by L-798106. Previous studies have shown that Rat LAD arteries relax to K_V7 channel activators and contract to K_V7 channel inhibitors (Khanamiri *et al.*, 2013; Hedegaard et al., 2014). The poor relaxant response to Iloprost in LADs therefore does not represent poor K_V7 expression and may reflect either low *Ptgir* expression or ineffective coupling between IP receptor and K_V7 channel.

Surprisingly, MRE-269 evoked CAY-10441-sensitive relaxations in MAs from male rats were also inhibited considerably by both $K_V7.1$ specific inhibitor HMR-1556, Chromanol 293B and molecular knockdown of the channel. In contrast to $K_V7.4$/ $K_V7.5$, the role of $K_V7.1$ within the vasculature remains enigmatic. Though $K_V7.1$ is expressed within VSMCs (Chadha *et al.*, 2012b; Baldwin *et al.*, 2020; Tsvetkov *et al.*, 2017) and $K_V7.1$ specific activators RL-3 and ML277 are potent relaxants of pre-contracted arterial tone (Chadha *et al.*, 2012a; Baldwin *et al.*, 2020), $K_V7.1$ has not been identified as the downstream target of any endogenous vasoactive signalling cascade (Stott *et al.*, 2015; Chadha *et al.*, 2014; Stott, Barrese & Greenwood, 2016). Yet in the present study HMR-1556 produced as full an inhibition as linopirdine, which suggests $K_V7.4/7.5$ do not contribute to the MRE-269 relaxations. Under the same conditions the mixed β-adrenoceptor agonist isoprenaline produced relaxations that were not HMR1556 sensitive. Whilst further work is required to validate these findings, to our knowledge our data is the first to describe an effect on vascular reactivity by $K_V7.1$ inhibition.

Intriguingly when MAs were isolated from female rats the situation was very different than MAs from males. Iloprost responses were only slightly and non-significantly inhibited by Linopirdine in MAs from female rats. In addition, whilst MRE-269 mediated relaxation was attenuated by Linopirdine and

HMR-1556, the effect of the latter was far smaller than in the male. When separated into oestrus cycle stage, arteries from Females in Di/Met expressed sensitivities to HMR-1556 and Linopirdine equivalent to the male, whereas arteries from Females in Pro/Est were entirely insensitive to either, indicating an Oestrus cycle dependent impairment of K_V7 channel activity. Previously, within rat distal-colic crypt cells, 17-β Oestradiol was shown to negatively regulate $K_V7.1$ channel activity (O'Mahony *et al.*, 2007; Alzamora *et al.*, 2011) and promote channel endocytosis (Rapetti-Mauss *et al.*, 2013). Oestradiol levels have previously been demonstrated to be highest within Pro-oestrus rats followed by oestrus, metoestrus and dioestrus (Faccio *et al.*, 2013). We propose that during Pro-oesteus, Oestradiol levels rise, impairing $K_V7.1$ function and or expression during Pro/Est phase, thus reducing the potency of MRE-269 mediated relaxation and thus its HMR-1556/Linopirdine sensitivity which does not recover until Di/Met.

Conclusion

The data of the present study show clearly that there is a remarkable sexual dimorphism in the response of synthetic prostacyclin analogues. In male MAs low concentrations of Iloprost produces a Linopirdine-sensitive relaxation that becomes opposed by an EP_3 receptor-driven contraction as concentrations increase. In contrast, in MAs from female rats only relaxation was observed in response to Iloprost. More strikingly, the potent relaxations to the selective IP receptor agonist MRE-269 were sensitive to the Kv7.1-specific blocker HMR1556 in MAs from males and females in Di/Met but not at all in Pro/Est. The mechanisms underlying these considerable differences in prostacyclin-linked signalling will be the focus of future studies but highlight the importance of considering sex as a determinant in vascular physiology.

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Appendix

Tables

Gene name	(+) Forward primer sequence 3'-5' (-) Reverse primer sequence 5'-3'	Gene accession number	Amplicon
Ptger1	(+) AGTTCGAACGTTGGTCACGA (-) TAAGGTTGCAGCATTGTGCG	NM_001278475.1	112
Ptger2	(+) TATGCTCCCTGCCTTTCACAA (-) GGAGGTCCCACTTTTCCTTT	NM_031088.2	72
Ptger3	(+) GTGCAATTCCTTCCTAATCGCC (-) TCAGGTTGTTCATCATCTGGCA	NM_012704.1	122
Ptger4	(+) ATGAGCATTGAGCGCTACCT (-) AGATGCATAGACGGCGAAGA	NM_032076.3	102
Ptgir	(+) TGACACTTTCGCCTTCGCTA (-) TAGATGGCAGGCAAAGCCAA	NP_001071112.1	156
Tbxa2r	(+) TTGACATTCCCAGGCCCAAA (-) ACGTGATAAGGGGGTCAACA	NM_017054.2	141

Table 1 RT-qPCR primer sequences

Figure legends

Figure 1. Iloprost mediated bi-phasic vasoactive responses within male mesenteric and coronary arteries.

Representative traces of Iloprost mediated (0.001-3µmol-L^{-1}) vasoactive responses within pre-contracted (300 nmol-L^{-1} U46619) mesenteric arteries pre-incubated in; DMSO (A; black) , 100 nmol-L^{-1} CAY-10441 (B; blue) and 300 nmol-L^{-1} L-798,106 (C; green). Mean data for Iloprost mediated vasoactive responses within pre-contracted (300nmol-L^{-1} U46619) mesenteric (D; 0.001-3 µmol- L^{-1}; *n*=7-9) and coronary (E; 0.1-10 µmol- L^{-1}; *n*=5) arteries. All values are expressed as mean ± SEM (D,E). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

Figure 2. Iloprost mediated bi-phasic vasoactive responses within female mesenteric and coronary arteries.

Mean data for Iloprost mediated vasoactive responses (A; 0.001-3 µmol- L^{-1}; B ; 0.1-10µmol- L^{-1}) within female pre-contracted (300 nmol- L^{-1} U46619) mesenteric (A; *n*=7-9) and coronary (B; *n*=7-8) arteries in vessels pre-incubated in DMSO (red), 100 nmol- L^{-1} CAY-10441 (blue) or 300 nmol-L^{-1} L-798,106 (green). All values are expressed as mean ± SEM (A,B). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values *(**=*P*<0.05). (*n*=) number of animals used.

Figure 3. Sex-dependent differences in Iloprost mediated EP receptor evoked vasoconstriction in mesenteric arteries is underpinned by an increased expression in *Ptger3.*

Mean data for Iloprost mediated vasoactive responses (A,B; 0.1-10 µmol-L^{-1}; C,D; 0.001-3 µmol-L^{-1}) within pre-contracted (300 nmol-L^{-1} U46619) male (A-D; black) and female (A-D; red) coronary (A,B) and mesenteric (C,D) arteries in vessels pre-incubated in DMSO (A,C; *n*= 5-9) or 100 nmol-L^{-1} CAY-10441 (B,D; *n*=5-10). Mean data for vasoconstriction in response to 3 µmol-L^{-1} Iloprost in male (black; *n*=15) and female (red; *n*=20) mesenteric arteries in the presence of DMSO or 100nmol-L^{-1} CAY-10441 normalized to peak contraction in response to 10µmol-L^{-1} methoxamine (E). Relative gene expression of prostanoid receptors normalised to stable housekeeper genes expressed as $2^{-\Delta Cq}$ from male (black; *n*=5) and female (red; *n*=6-10) whole mesenteric artery lysates (F). All values are expressed as mean ± SEM (A,B). A two-way statistical ANOVA with a post-hoc Bonferroni or Šídák's test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

Figure 4. Sex-dependent differences in Iloprost mediated IP receptor evoked vasorelaxation in mesenteric arteries is underpinned by a shift in Linopirdine sensitivity.

Mean data for Iloprost mediated vasorelaxation (A,B,C; 0.001-3 µmol-L^{-1}; D,E,F; 0.1-10 µmol-L^{-1}) within pre-contracted (300 nmol-L^{-1}U46619) male (A,B,D,E; black; *n*=5-7) and female (A,C,D,F; red; *n*=8-9*)* mesenteric (A,B,C; 0.001-3 µmol-L^{-1}) and coronary (D,E,F; 0.1-10 µmol-L^{-1}) arteries in vessels pre-incubated in 300 nmol-L^{-1}L-798,106 (A-F) or a combination of 300 µmol-L^{-1} L-798,106 and 10 µmol-L^{-1} Linopirdine (B,C,E,F; yellow). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*= *P*<0.05). (*n*=) number of animals used.

Figure 5. MRE-269, but not selexipag mediated relaxation is inhibited by CAY-10441 in male mesenteric arteries.

Mean data for selexipag (A; 0.03-3 μmol-L^{-1}) and MRE-269 (B; 0.01-10 μmol-L^{-1}) mediated relaxation in vessels pre-incubated in DMSO (A,B; black; *n*=6-7) or 100 nmol-L^{-1} CAY-10441 (A,B; *n*=5-6) in male mesenteric arteries. All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values *(**=$P<0.05$). (*n*=) number of animals used.

Figure 6. Linopirdine and HMR-1556 attenuate MRE-269 mediated vasorelaxation in mesenteric arteries from male and female rats.

Mean data for MRE-269 mediated vasorelaxation (0.001-3 μmol-L^{-1}) within pre-contracted (300 nmol-L^{-1}U46619) male (A,C,E; black; *n*=7-8) and female (C,D,E; red; *n*=11-14) mesenteric arteries in vessels pre-incubated in DMSO (A-E), 10 μmol-L^{-1}linopirdine (A,B; yellow) or 10 μmol-L^{-1} HMR-1556 (C,D,E; green). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=$P<0.05$). (*n*=) number of animals used.

Figure 7. Molecular interference of *Kcnq1* via targeted morpholino knockdown impairs MRE-269 mediated relaxation in male mesenteric arteries.

Representative immunofluorescence showing $K_V7.1$ in isolated vascular smooth muscle cells from fresh (non-transfected; A), Control (Ctrl morph; B) and *Kcnq1 (Kcnq1* morph*;* C) morpholino transfected mesenteric arteries. $K_V7.1$ shown in red, nuclear staining in blue (DAPI [4',6-diamidino-2-phenylindole, dihydrochloride]). Insets show brightfield (BF) images of the cell. Mean data for total cell fluorescence measured in arbitrary units (A.U) for fresh (Black; *n*=3), Ctrl morph (grey, black border; *n*=3) and *Kcnq1* morph (green, grey boarder; *n*=3; D). Mean data for 300nmol-L^{-1} ML277 mediated relaxation (*n*=5-8; E). Mean data for 300 nmol-L^{-1}/ μmol-L^{-1} MRE-269 mediated relaxation (*n*=5-8; F). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Turkey test was used to generate significant values (*=$P<0.05$). (*n*=) number of animals used.

Figure 8. MRE-269 mediated relaxation is attenuated by PKA, but not K_{ATP} and BK_{Ca} inhibition.

Mean data for MRE-269 (A,B,C 0.01-10 μmol-L^{-1}) mediated relaxation in vessels pre-incubated in DMSO (A,B,C; black; *n*=4), 100 nmol-L^{-1} Iberiotoxin (A; blue; *n*=5), 1 μmol-L^{-1} glibenclamide (B; blue; *n*=5) or 1 μmol-L^{-1} KT7520 (C; blue; *n*=5) in male mesenteric arteries. All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values *(**=P<0.05). (*n*=) number of animals used.

Figure 9. K_V7 channel inhibition attenuates MRE-269 mediated vasorelaxation in mesenteric arteries from Diestrus/Metestrus (Di/Met), but not Pro-estrus/Oestrus (Pro/Est) female rats.

Mean data for MRE-269 mediated vasorelaxation (0.001-3 μmol-L^{-1}) within pre-contracted (300 nmol-L^{-1} U46619) Di/Met (A,C,E; black; *n*=5-8) and Pro/Est (C,D,E; red; *n*=5-6) female mesenteric arteries in vessels pre-incubated in DMSO (A-E), 10 μmol-L^{-1} linopirdine (A,B; yellow) or 10 μmol-L^{-1} HMR-1556 (C,D,E; green). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values *(**=P<0.05). (*n*=) number of animals used.

Supplemental figure legends

Figure S1. Representative images of immunocytochemistry of CHO cells demonstrates anti-body specificity.

Chinese hamster ovarian (CHO) cells transfected with *Kcnq1* containing plasmids (A), but not non-transfected CHO cells, present with diffuse labeling of $K_V7.1$, as seen in red (B). Insets contain 4′,6-diamidino-2-phenylindole (DAPI) staining only, blue. Bar = 50μm.

Figure S2. MRE-269 mediated relaxation is attenuated alternate K_V7.1 inhibitor, Chromanol 293B.

Mean data for MRE-269 mediated relaxation in vessels pre-incubated in DMSO (black; *n*=7) or Chromanol 293B 10 μmol-L^{-1} (blue; *n*=7) in male mesenteric arteries. All values are expressed as mean ± SEM. A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values *(*=P<0.05). (*n*=) number of animals used.

Figure S3. Isoprenaline mediated relaxation is unaffected by K_V7.1 inhibition.

Mean data for Isoprenaline mediated relaxation in vessels pre-incubated in DMSO (black; *n*=9) or 10 μmol-L^{-1}HMR-1556 (blue; *n*=9) in male mesenteric arteries. All values are expressed as mean ± SEM. A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=P<0.05). (*n*=) number of animals used.