Sex differences in the role of phospholipase A_2-dependent arachidonic acid pathway in the perivascular adipose tissue function in pigs.

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Key Points:

- The fat surrounding blood vessels (perivascular adipose tissue or PVAT) releases vasoactive compounds that regulate vascular smooth muscle tone.

- There are sex differences in the regulation of vascular tone. However, to date, no study has investigated whether there are sex differences in the regulation of blood vessel tone by PVAT.

- This study has identified that the cyclooxygenase products thromboxane and $PGF_{2\alpha}$ are released from coronary artery PVAT from pigs. Thromboxane appears to mediate the PVAT-induced contraction in arteries from females, whereas $PGF_{2\alpha}$ appears to mediate the contraction in arteries from males.

- These sex differences in the role of these prostanoids in the PVAT-induced contraction can be explained by a greater release of thromboxane from PVAT from female animals and greater sensitivity to $PGF_{2\alpha}$ in the porcine coronary artery from males.

Abstract

Previous studies have demonstrated that perivascular adipose tissue (PVAT) causes vasoconstriction. In this present study, we determined the role of cyclooxygenase-derived prostanoids in this contractile response and determined whether there were any sex differences in the regulation of vascular tone by PVAT. Contractions in isolated segments of coronary arteries were determined using isolated tissue baths and isometric tension recording. Segments were initially cleaned of PVAT, which was then re-added to the tissue bath and changes in tone measured over 1 hour. Levels of $PGF_{2\alpha}$ and thromboxane B_2 (TXB_2) were quantified by ELISA and $PGF_{2\alpha}$ (FP) and thromboxane A_2 (TP) receptor expression determined by Western blotting. In arteries from both male and female pigs, re-addition of PVAT caused a contraction, which was partially inhibited by the cyclooxygenase inhibitors indomethacin and flurbiprofen. The FP receptor antagonist AL8810 attenuated the PVAT-induced contraction in arteries from males, whereas the TP receptor antagonist GR32191B inhibited the PVAT-induced contraction in arteries from females. Although there was no difference in $PGF_{2\alpha}$ levels in PVAT between females and males, $PGF_{2\alpha}$ produced a larger contraction in arteries from males, correlating with a higher FP receptor expression. In contrast, release of TXB_2 from PVAT from females was greater than from males, but there was no difference in the contraction by the TXA_2 agonist U46619, or TP receptor expression in arteries from different sexes. These findings demonstrate clear sex differences in PVAT function in which $PGF_{2\alpha}$ and TXA_2 antagonists can inhibit the PVAT-induced vasoconstriction in male and female PCAs, respectively.

Abbreviations

COX, cyclooxygenase; FP, $PGF_{2\alpha}$ receptor; MLC, myosin light chain; PCA, porcine coronary artery; $PGF_{2\text{-}\alpha}$, prostaglandin $F_{2\text{-}\alpha}$; PLA_2, phospholipase A_2; PVAT, perivascular adipose tissue; TP, TXA_2 receptor; TXA_2, thromboxane A_2.

Introduction

Perivascular adipose tissue (PVAT) is positioned around blood vessels and is regarded as being distinct from the adventitia, in spite of an absence of a barrier between them. Compelling evidence in recent years has led to the view that PVAT is in fact an active secretory tissue which releases several bioactive signalling molecules, collectively termed adipokines, which are important in the control of vascular tone in both health and diseases (Fortuno *et al.*, 2003; Havel, 2004; Thalmann & Meier, 2007; Yamawaki *et al.*, 2010). Several studies have suggested a vasorelaxant or, at least, anticontractile effect of various fat depots in the body, proposing a role for TNF-α, H_2S, NO, adiponectin and other adipocyte-derived relaxant factors (ADRFs) (Gao *et al.*, 2007; Fang *et al.*, 2009; Lynch *et al.*, 2013; Virdis *et al.*, 2015). On the other hand, other studies have reported opposing effects of PVAT on vascular tone. Gao *et al*. (2006) proposed that PVAT could augment vasoconstriction induced by perivascular nerve stimulation of the rat superior mesenteric artery. They reported that superoxide production from NAD(P)H oxidase (Gao *et al.*, 2006) and angiotensin II (Lu *et al.*, 2010) are involved in PVAT-mediated potentiation of perivascular nerve stimulation-elicited contraction. Prostanoids are cyclooxygenase metabolites of arachidonic acid (AA) that exert diverse physiological and pathological effects in different systems, including relaxation and contraction of vascular smooth muscle (VSM) (Wright *et al.*, 2001). According to their selectivity for the natural prostanoids, prostaglandins (PG) PGI_2, PGD_2, PGE_2, PGF_2 and thromboxane A_2 (TXA_2), there are five main types of prostanoids receptors IP, DP, EP, FP and TP, respectively. Of these receptors, TP, and FP have the ability to mediate vasocontraction, IP and DP generally mediate vasorelaxation while depending on receptor subtype, EP has the ability to induce both contraction (EP_1 and EP_3) and relaxation (EP_2 and EP_4) (Coleman *et al.*, 1994). Prostanoids released from PVAT have been shown to regulate vascular tone. For example, PGE_2 and PGI_2 released from PVAT surrounding human saphenous vein attenuates noradrenaline-induced contraction (Ozen *et al.*, 2013). Similarly, prostacyclin released from mouse carotid artery PVAT exerts vasorelaxant effects and protects against endothelial dysfunction (Chang *et al.*, 2012). In contrast, another study showed that aortic PVAT from mice is capable of releasing a cyclooxygenase (COX)-derived

adipocyte derived contracting factor (ADCF) that becomes functionally of greater activity in obesity (Meyer *et al.*, 2013), although the identity of this COX metabolite is unknown.

Extensive epidemiological data have revealed the existence of a prominent sexual dimorphism in the incidences of primary vascular diseases that involve excessive vasoconstriction. Thus, among the strongest independent risk factors for coronary artery disease is male sex and a male to female ratio of approximately 2:1 is consistently observed (Nettleship *et al.*, 2009). In contrast, migraine headache (Bartelink *et al.*, 1993) and Raynaud's Disease (Voulgari *et al.*, 2000) all occur in premenopausal women at rates as much as fourfold higher than in men. Furthermore, a previous study has indicated that oestrogen could upregulate the expression of COX-2 and thromboxane synthase in both endothelium and vascular smooth muscle, and upregulate the expression of TP receptors in smooth muscle of female rat aorta, leading to enhanced vasoconstrictor prostanoid function (Li *et al.*, 2008). However, to date, no study has determined whether there are sex differences in the regulation of vascular tone by PVAT.

In the porcine coronary artery, PVAT induces a contraction and enhances agonist and electrical field-stimulated contractile responses (Owen *et al.*, 2013). However, the contractile agents involved in these responses are unknown. PVAT is a potential therapeutic target for controlling coronary artery tone. Therefore, in this present study, we determined whether cyclooxygenase-derived vasoconstrictor prostanoids play a role in PVAT-induced contractile responses and investigated whether there are sex differences in the response of the coronary artery to PVAT.

Methods

Preparing of rings of porcine coronary arteries

Hearts from male and female pigs (large white hybrid pigs, 4-6 months old, weighing ~ 50kg) were obtained from a local abattoir and transported back to the laboratory in ice-cold modified Krebs'-Henseleit solution (118 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 12 mM D-glucose, 1.25 mM $CaCl_2$). The anterior proximal part of the coronary artery was then dissected and placed in Krebs'-Henseleit solution pre-gassed with 5% CO_2 and 95% O_2. PVAT was carefully removed and maintained in Krebs'-Henseleit solution until further use. Thereafter, coronary arteries were cut into rings of approximately 5mm in length and suspended in a multichannel 5 ml organ bath setup. Each bath was filled with 5 ml of Krebs'-Henseleit solution and maintained at 37^{o}C and constantly gassed with carbogen (95% O_2, 5% CO_2). Tension was measured and recorded using isometric force transducers connected to a Powerlab data acquisition system (ADInstruments) via an amplifier.

Characterization of Vascular Responses of Porcine Isolated Coronary arteries

The arteries were initially set to 8 g of tension, determined from preliminary studies, and then left to equilibrate for approximately 30-45 minutes. Once a stable baseline was attained, two consecutive responses to 60 mM KCl were obtained for standardization. Relevant test compounds were then added with 60 minutes incubation time. The following test compounds were used: melittin (10µM) to stimulate PLA_2 activity (Koumanov *et al.*, 2003); indomethacin (10µM) (Malinowski *et al.*, 2008) and flurbiprofen (10µM) (Stanley & O'Sullivan, 2014) to determine the role of cyclooxygenase. To study the role of specific prostanoids, GR32191B (3µM) (He & Yang, 1999) and AL8810 (10µM) (Ramos-Alves *et al.*, 2012) were used as antagonists of TP and FP receptors, respectively. Ethanol or DMSO (0.1% v/v) were used as vehicle controls, as appropriate. Of note, there was no obvious difference in the amount of PVAT per unit length of coronary artery between female and male. After incubation of the coronary artery segments with test compounds, 0.3 g PVAT was added to the organ baths and contractile responses measured.

In a separate series of experiments, cumulative concentration-response curves to the TXA_2 agonist U46619 (1nM-300nM) or $PGF_{2\text{-}\alpha}$ (0.1-100µM) were constructed in coronary artery segments from male and female pigs.

Determination of levels of TXB_2 and $PGF_{2\text{-}\alpha}$ by ELISA.

Levels of TXB_2, a stable metabolite of TXA_2, and $PGF_{2\text{-}\alpha}$ in PVAT were determined by ELISA. PVAT (0.3g) was homogenised in either 0.1M potassium phosphate buffer [pH 7.4] for TXB_2 estimation, or Tris-buffered saline containing 0.1% v/v Tween 20 for $PGF_{2\text{-}\alpha}$ estimation, along with protease inhibitor cocktail (Calbiochem) and indomethacin (10µM). The amount of TXB_2 and $PGF_{2\text{-}\alpha}$ in PVAT samples from male and female animals was analysed by ELISA (ab133022; ab133041), according to the instructions of the manufacturer. TXB_2 and $PGF_{2\text{-}\alpha}$ were detected colourimetrically using an alkaline phosphatase (AP) conjugated- secondary antibody with para-Nitrophenylphosphate (pNpp) as substrate. Colour intensity produced was determined by reading at 405 nm using a SpectraMAX 340 PC microplate reader (Molecular Devices, 50 Wokingham, Berkshire, UK). The results were expressed as pg μg^{-1} of total protein.

Assessment of the PVAT-released TXB_2 by ELISA.

Porcine coronary arterial PVAT from each sex was dissected, weighed and added to an Eppendorf tube containing 1ml pre-gassed Krebs'-Henseleit solution, at 37^oC. For consistency with the functional studies, PVAT was incubated for 2½hr with and without melittin (10µM) in order to investigate the effect of activation of PLA_2 on the level of TXB_2 released from PVAT. The level of TXB_2 in the Krebs'-Henseleit solution was then determined using ELISA (as described above) and the data normalized for weight of PVAT.

Estimation of TP and FP receptor expression in coronary arteries by Western immunoblotting.

Western immunoblotting was performed to determine the expression of TP and FP receptors in segments of coronary arteries from female and male pigs. Arteries were finely dissected from the adherent PVAT and connective tissue and homogenized on ice using Tris buffer [pH 7.4] with protease inhibitor cocktail (Calbiochem, VWR

International Ltd, Lutterworth, Leicestershire, UK). After estimation of the protein content of each sample using the Lowry method, 6x Laemmli sample buffer was added to the samples. Samples were subsequently boiled at 100^{o}C for 5 min and centrifuged at 13000×g for 1 min. Stock solutions (20µg per well) were run on 4–20% gradient polyacrylamide gradient gels (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). Proteins were then transferred onto nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by Western blotting, and then the membrane blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.1% v/v Tween 20 (TBST) at room temp for 1 h. The blot was then incubated with either rabbit anti-TXA_2 receptor antibody (1:250 dilution; ab188897, Abcam, Cambridge, UK) or rabbit anti-$PGF_{2\text{-}\alpha}$ receptor antibody (1:250 dilution; orb185891, Biorbyt, Cambridge, UK) in 5% milk in TBST overnight at 4°C with shaking. An anti-mouse myosin light chain (MLC) antibody was also included as a loading control (1:1000 dilution; Sigma-Aldrich, Poole, Dorset, UK). The following day, the blot was washed in TBST followed by incubation for 1 hour with IRDye 800CW goat anti rabbit (1:10,000) and IRDye 680CW goat anti-mouse (1:10,000) secondary antibodies (IRDye, Licor, Cambridge, UK). Immunoblot was then visualized using an Odyssey system from Licor.

Materials

All drugs were purchased from Sigma-Aldrich except for AL8810 from Santa Cruz Biotechnology and $PGF_{2\text{-}\alpha}$ from Cayman Chemicals. Stock solutions of flurbiprofen, $PGF_{2\text{-}\alpha}$ and GR32191B were dissolved in dimethyl sulfoxide (DMSO). Stock solution of indomethacin and AL8810 were dissolved in absolute ethanol whereas melittin was dissolved in distilled water. Stock solutions of U46619 were made to 10mM in ethanol. All further dilutions of the stock solutions were made using distilled water.

Statistical analysis

Data are expressed as mean ± S.E., where *n* = the number of different animals. The concentration-response curves were fitted to a sigmoidal curve with a variable slope using four parameters logistic equation using GraphPad Prism software. The maximum percentage contraction (R_{max}) and the negative log of concentration required to produce half the maximal contraction of the induced tone (pEC_{50}) were calculated from the fitted

curves. Data were tested for Normality using a Shapiro-Wilk test. Comparisons between two groups of data were analysed using 2-tailed, paired or unpaired Student's t-test, as appropriate. The effects of enzyme inhibitors and receptor antagonists on the PVAT-induced contraction over time were analysed using a two-way ANOVA in conjunction with the Sidak's *post-hoc test* to assess possible difference at individual concentrations. A p value <0.05 was considered to be statistically significant. Statistical analysis was performed by GraphPad Prism (Version 6).

Results

Effects of addition of PVAT on vascular tone of female and male PCAs.

Addition of 0.3 g PVAT caused a time-dependent increase in tone compared to time controls in coronary artery segments from both female and male PCAs (Figure 1). There was inter-individual variation in the size of the contractile response to PVAT therefore adjacent coronary arterial segments from the same animal were always used as controls.

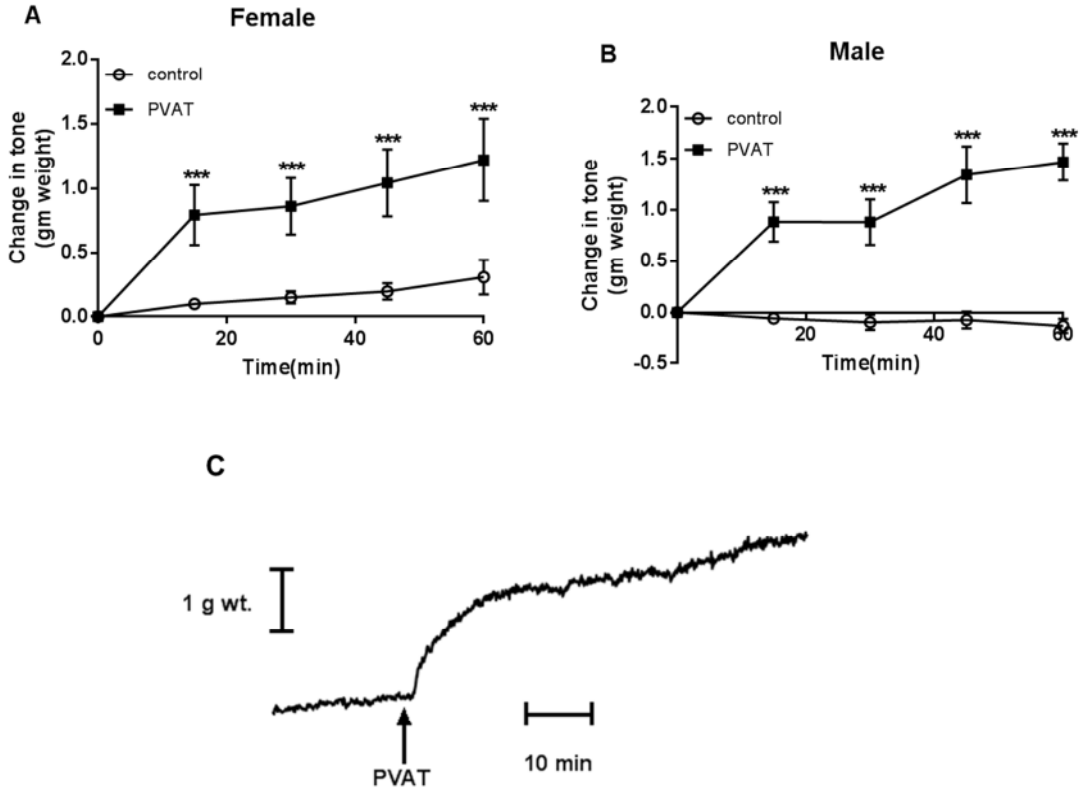

Figure 1: PVAT-induced vasoconstriction. Increase in vascular tone over time following addition of PVAT (0.3g) in female (**A**) and male (**B**) porcine coronary arteries. Data are expressed as change in tone per gram weight and are mean ± SEM of 7(A)- 4(B) experiments. (**C**) Original traces of recording showing the response of female PCA to addition of PVAT. ***$P < 0.001$, (2-way ANOVA followed by a Sidak's post-hoc test) versus control values.

Role of prostanoids in PVAT-induced contraction

Addition of the cyclooxygenase inhibitors indomethacin and flurbiprofen (both 10µM) had no effect on basal tone. However, they caused a significant inhibition in the contraction to PVAT compared to control in both sexes (Figure 2). The thromboxane

receptor antagonist GR32191B (3μM) caused a partial reduction in the PVAT-induced contraction in coronary arteries from female animals (Figure 3A), whereas the responses of coronary arteries from male animals to PVAT were unaffected by GR32191B (Figure 3B). As the TP receptor antagonist had no effect on the PVAT contraction in males, we determined the effect of a FP receptor antagonist. In contrast to the effect of TP receptor antagonism, the FP receptor antagonist AL8810 had no significant effect on the PVAT-induced contraction in coronary arteries from female animals, but reduced the contraction in coronary arteries from male animals (Figure 3 C & D).

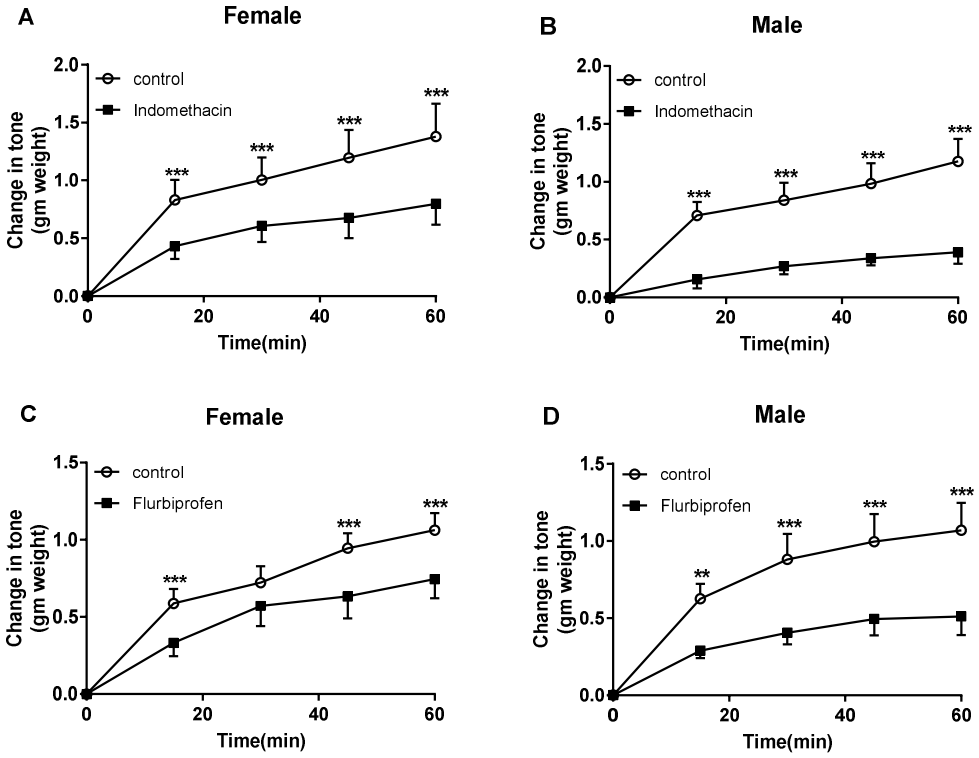

Figure 2: Effect of COX inhibition on PVAT-induced vasoconstriction. Contractile response of the PCAs to PVAT (0.3g) in the absence (control) or presence of 10μM indomethacin (**A & B**) or 10μM flurbiprofen (**C & D**). Data are expressed as change in tone per gram weight and are mean ± SEM from arteries from 15 female (A & C) and 8 male (B & D) animals . * indicates $P < 0.05$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$, (2-way ANOVA followed by a Sidak's post-hoc test) versus control values.

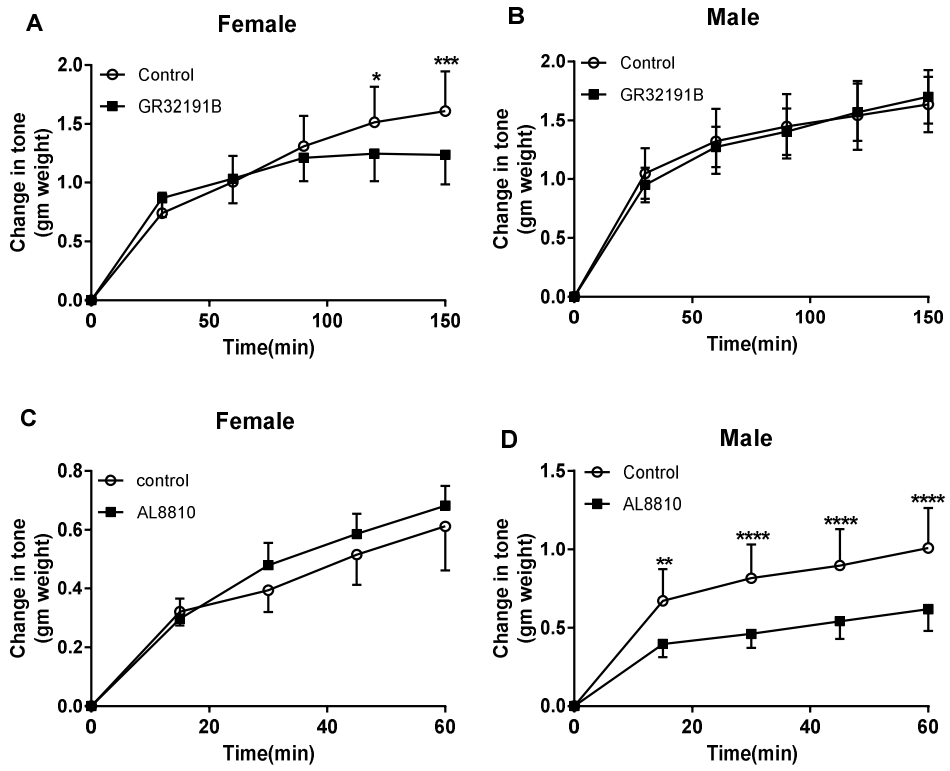

Figure 3: Effect of inhibition of TXA_2 and $PGF_{2\alpha}$ on PVAT-induced vasoconstriction. Effects of pre-incubation with GR32191B (3µM, 1hr) on the response of porcine coronary arteries to PVAT (0.3g) in female (**A**) and male (**B**) pigs. Effects of pre-incubation with AL8810 (10µM, 1hr) on PVAT-induced vasoconstriction in female (**C**) and male (**D**) pigs. Data are expressed as change in tone per gram weight and are mean ± S.E.M. of arteries from 5-6 female (A & C) and 6-7 male (B & D) animals. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$, two-way ANOVA followed by a Sidak's post-hoc test versus control values. NS- not significant.

Determination of the level of TXB_2 and $PGF_{2\alpha}$ in PVAT by ELISA

In order to determine whether the differences in the role of TP and FP receptors in arteries from male and female animals could be due to differences in the content of thromboxane and $PGF_{2\alpha}$ in the PVAT, levels of the stable thromboxane metabolite TXB_2, and $PGF_{2\alpha}$ in PVAT were determined by ELISA. Both TXB_2 and $PGF_{2\alpha}$ were detected in the PVAT from the coronary artery. However, there was no significant difference in the level of TXB_2 or $PGF_{2\alpha}$ in coronary artery PVAT from female and male animals (Figure 4).

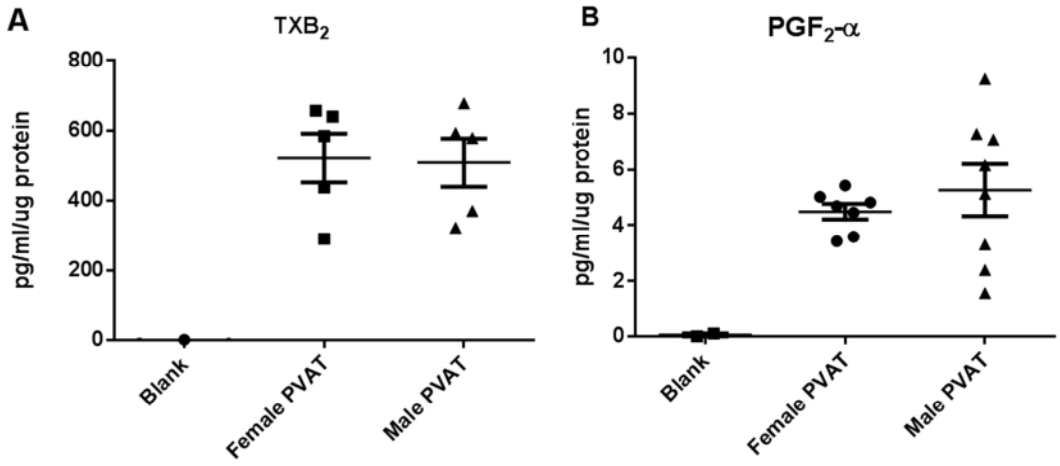

Figure 4: Comparison of the level of TXB_2 and $PGF_{2\alpha}$ in PVAT from both sexes.
Comparison of the levels of the TXB_2 and $PGF_{2\alpha}$ between PVAT from female and male pigs determined by ELISA. Data are expressed as pg per ml per µg protein concentration and are mean ± S.E.M. of PVAT from 5-7 female and 5-8 male animals (**A-B**).

Determination of the level of TP and FP receptor expression in PCAs

As there were no differences in the levels of TXB_2 or $PGF_{2\alpha}$ in PVAT from male and female animals, we determined whether there were differences in the expression of the receptors on the coronary artery, which might explain the differences in the role of these prostanoids. Western immunoblotting for the TP receptor identified a band at the predicted molecular weight of 40kDa. When expressed as a ratio to myosin light chains, used as a loading control, there was no difference in the level of expression of TP receptor between coronary arteries from male and female animals (Figure 5 A & B). Western immunoblotting also detected a band at 50kDa for the FP receptor. In this case, there was a significantly higher level of expression of the FP receptor in coronary arteries from male animals compared to female animals (Figure 5 D & E).

Assessment of the responsiveness of PCAs to TXA_2 and $PGF_{2\alpha}$

As our data indicate differences in the role of TP and FP receptors in the PVAT-induced contractions in coronary arteries from male and female animals, which cannot be explained by differences in the content of thromboxane and $PGF_{2\alpha}$ in the fat, we determined whether there are differences in the contractions in response to TP or FP receptor activation, in the absence of PVAT. There was no difference in the response of

PCAs to the TXA_2 mimetic U46619 between female and male pigs (Figure 5C). On the other hand, the contractile responses to $PGF_{2\alpha}$ were significantly greater in arteries from male pigs (Figure. 5F).

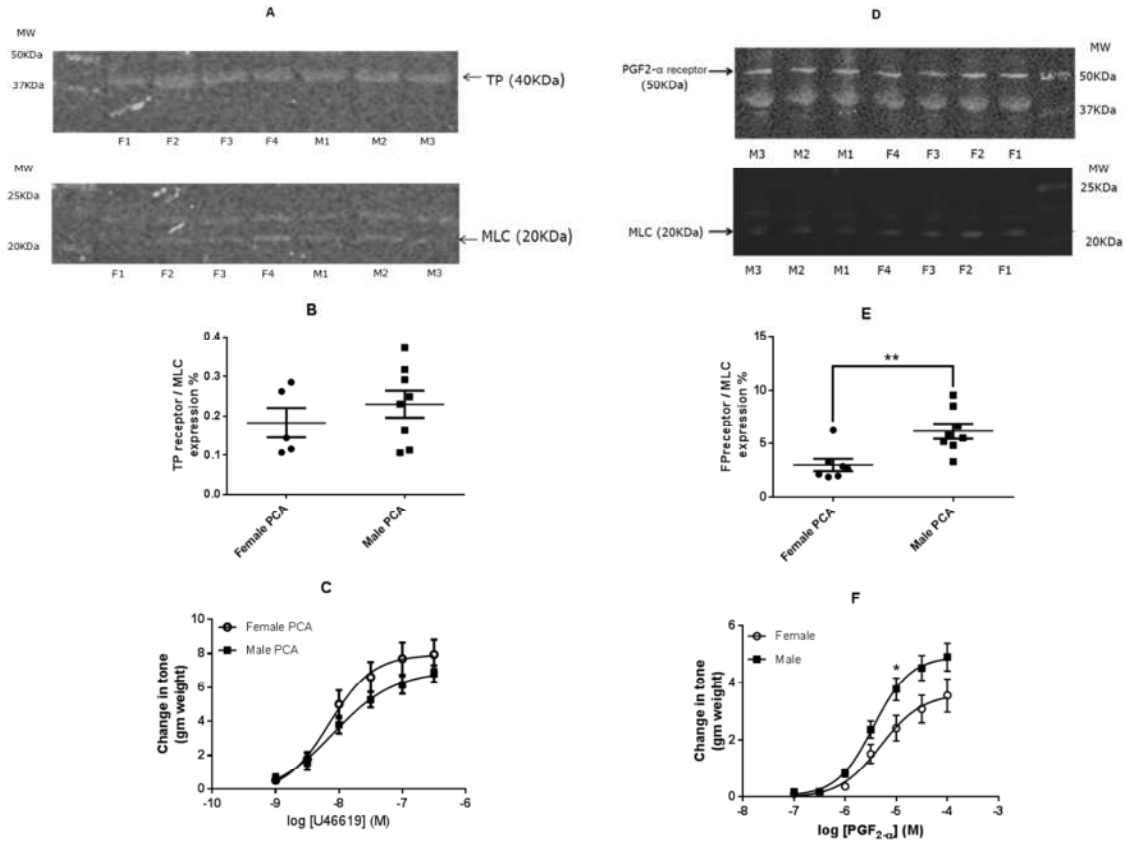

Figure 5: Comparison of the expression of TP and FP receptors, and the responsiveness of PCAs to TXA_2 and $PGF_{2\alpha}$ in both sexes. A. Western immunoblot showing expression of TP receptor (40 kDa) and MLC (20 kDa) levels in 20 µg of coronary arteries from 6 female and 8 male pigs. **B.** Bar chart showing expression levels of TP receptor expression as a percentage of MLC expression in coronary arteries from male and female pigs based on the intensities of their bands. **C.** Log concentration-response curves to the thromboxane receptor agonist U46619 in porcine coronary artery segments from female and male. Data are expressed as change in tone per gram weight and are mean ± S.E.M. of 12 female and 9 male arteries. **D.** Western immunoblot showing expression of FP receptor (50 kDa) and MLC (20 kDa) levels in 20 µg of coronary arteries from 7 female and 8 male pigs. **E.** bar chart showing PGF2-α receptor expression as a percentage of MLC expression in coronary arteries from male and female pigs based on the intensities of their bands. **F.** contractile response to serial concentrations of PGF2α

(0.1-100μM). Data are expressed as change in tone per gram weight and are means ± SEM of 10 Female and 7 male coronary arteries. Data are expressed as mean ± SEM, * indicates $p<0.05$, ** indicates $p<0.01$, (two-tailed, unpaired Student's t-test).

Effect of phospholipase A_2 stimulation on PVAT-induced contractions

Our data indicate that cyclooxygenase produced prostanoids play a role in the PVAT-induced contraction in the porcine coronary artery. In order to support these data further, we investigated the effect of the phospholipase A_2 (PLA_2) stimulator melittin. Addition of melittin (10μM) at baseline tone produced a transient contraction of the arteries, returning to baseline within 15 minutes. Subsequent addition of PVAT led to a significant enhancement of the PVAT-induced contraction in mellitin- treated vessels compared with that of PVAT alone in coronary artery segments from both sexes (Figure 6). The TP antagonist GR32191B caused complete inhibition of the PVAT-induced contraction in the presence of melittin in arteries from female animals and partial inhibition in arteries from male animals (Figure 6 C & D). Surprisingly, the FP receptor antagonist AL8810 had no effect on the PVAT-induced contractions in arteries from male or female animals (Figure 6 E & F). However, in subsequent experiments, we demonstrated that pre-incubation with melittin results in desensitisation of the FP receptor, but not TP receptor-mediated contraction as addition of U46619 and $PGF_{2\text{-}\alpha}$ to the PCA after pre-incubation with melittin resulted in a contractile response to U46619 but not $PGF_{2\text{-}\alpha}$ (Figure 7 A & B).

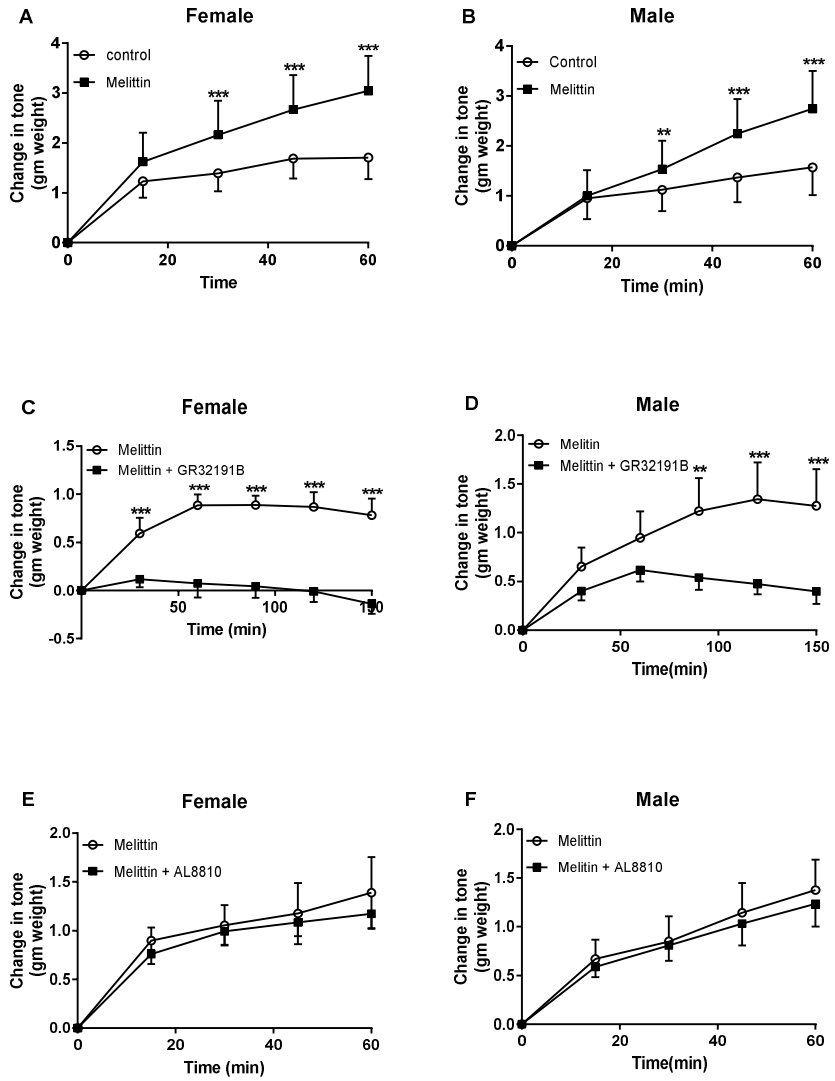

Figure 6 : Effect of phospholipase A2 stimulation on PVAT-induced contractions. A & B. Contractile responses of segments of porcine coronary arteries to fresh PVAT (0.3g) in the absence or presence of melittin (10µM). Data are expressed as change in tone per gram weight and are mean ± SEM from arteries from 5 female (A) and 8 male (B) animals. **C & D**. Effect of pre-incubation with the TP receptor antagonist GR32191B (3µM) on the PVAT-induced contraction, in the absence or presence of melittin (10µM). Data are expressed as change in tone per gram weight and are means ± S.E.M. of 5 animals. **E & F**. Effect of pre-incubation with the FP receptor antagonist AL8810 (10µM) on the PVAT-induced contraction, in the absence or presence of melittin (10µM). Data are expressed as change in tone per gram weight and are

means ± S.E.M. of 4 female (E) and 5 male (F) animals. * indicates $p<0.05$, ** indicates $p<0.01$ and ***indicates $p<0.001$, two-way ANOVA followed by a Sidak's post-hoc test.

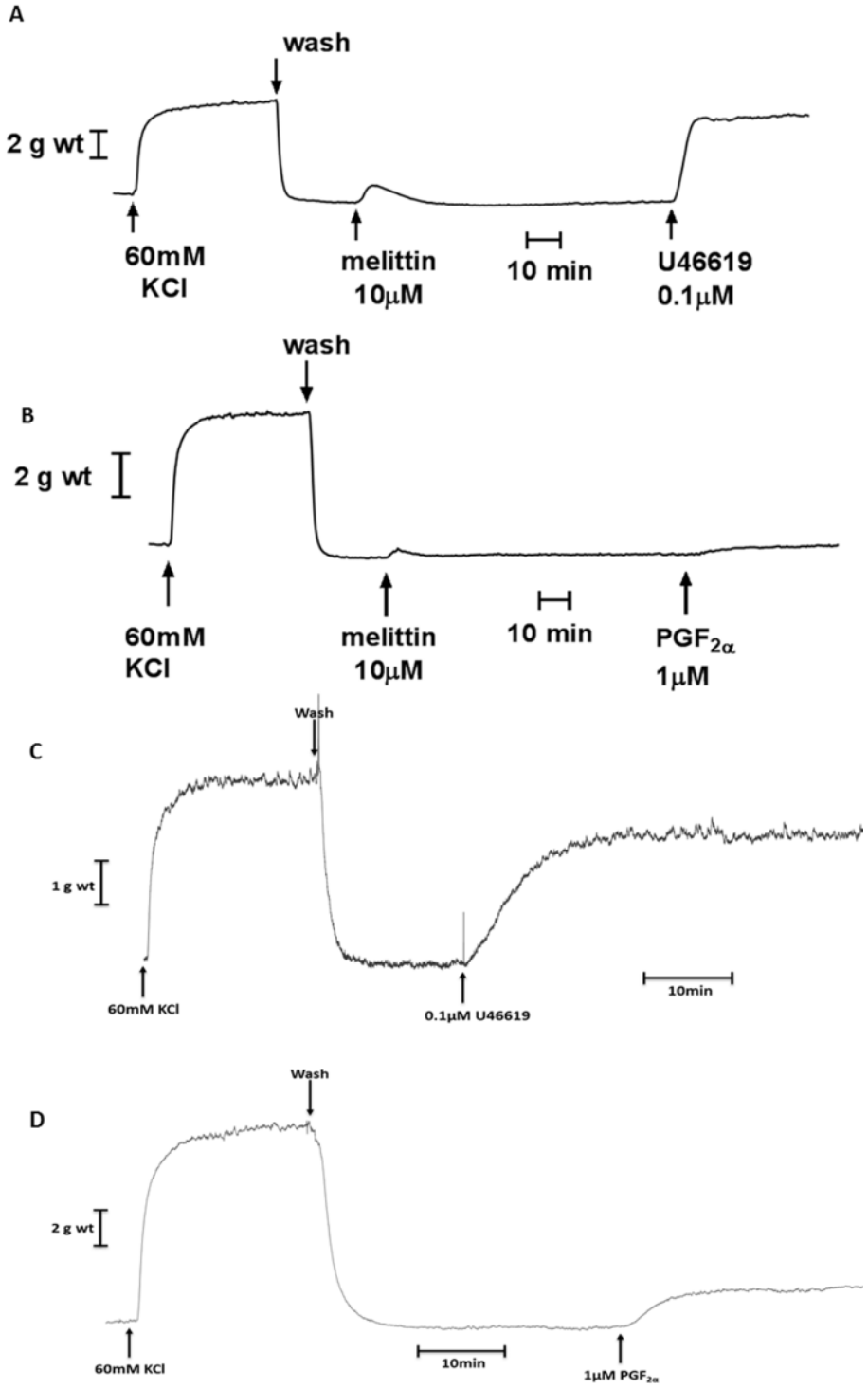

Figure 7: Original traces of recording showing the vasomotor responses of PCA. A & B. Original traces of recording showing the response of melittin-stimulated female PCA to addition of 0.1μM U46619 and 1μM PGF2-α after PVAT pre-treatment (A and B, respectively). **C & D**.

Original traces of recording showing the response of PCA to addition of 0.1μM U46619 and 1μM PGF2-α (C and D, respectively).

Determination of the release of TXB_2 from PVAT

Although there was no difference in the level of TXB_2 in the PVAT from male and female animals, we explored the possibility that there are differences in the release of thromboxane from PVAT. Therefore, we determined the levels of TXB_2 in the buffer after incubation with PVAT. In these experiments it was found that the concentration of TXB_2 was higher in the buffer after incubation with PVAT from female animals compared to PVAT from male animals (Figure 8A). The level of TXB_2 released from PVAT was significantly potentiated after addition of the PLA_2 stimulator (melittin) in both females and males (Figure 8 B & C).

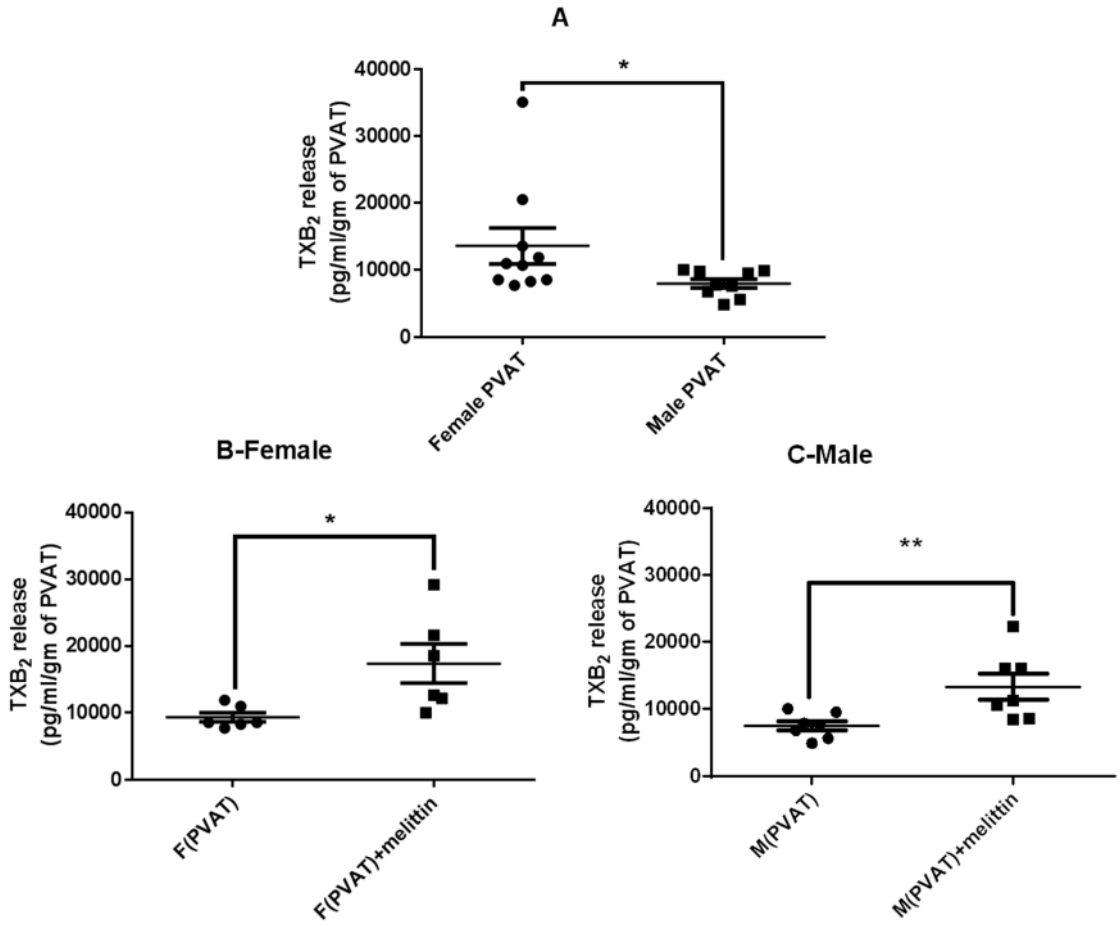

Figure 8 : Comparison of the release of TXB_2 from PVAT in both sexes. A. Comparison of the levels of TXB2 in the buffer after incubation with PVAT from female or male pigs for 2.5 hours. Data are expressed as a pg of TXB2 released per ml per gm of PVAT and are mean ± S.E.M. of 10 female and – 9 male PVAT samples. * indicates $p<0.05$, 2-tailed, unpaired Student's t-test. . **B & C**. Comparison of the levels of TXB2 in the buffer after a 2 ½ hours incubation with PVAT with and without melittin (10µM) from female (B) or male (C) pigs. Data are expressed as pg of TXB2 released per ml per gm of PVAT and are mean ± S.E.M. of 6 female and 7 male PVAT samples. * indicates $p<0.05$, ** indicates $p<0.01$, 2-tailed, paired Student's t-test vs paired controls.

Discussion

The recognition that perivascular adipose tissue is not simply a reservoir for lipid storage but is also a complex endocrine organ, has led to a substantial research effort to establish the mechanistic links between the PVAT and vascular tone regulation. It is becoming increasingly clear that there are differences in the vascular responsiveness in males compared to females (Bubb *et al.*, 2012). For example, we have identified differences in endothelial function between the sexes (Wong *et al.*, 2015). In this present study, we have identified that the PVAT-induced contraction in the porcine coronary artery is mediated, at least in part, through the prostanoids thromboxane and $PGF_{2\alpha}$. Although both prostanoids are present in PVAT, there are sex differences in the vasomotor response of the coronary arteries to PVAT, in that TXA_2 and $PGF_{2\alpha}$ antagonists reduced the PVAT-induced vasoconstriction in females and males, respectively.

Re-addition of PVAT to segments of porcine coronary artery produced a contractile response, which is in line with previous studies, indicating that coronary artery PVAT releases adipose-derived contractile factors (ADCFs) (Owen *et al.*, 2013). However, this previous study did not identify the factor causing the contractile response. In this present study, we have demonstrated that the contraction in response to re-addition of PVAT was partially inhibited by cyclooxygenase inhibitors, indicating a role for contractile prostanoids. Furthermore, the contraction was inhibited by prostanoid receptor antagonists, providing further support for prostanoids in the contractile response. Interestingly, the contractile response to coronary artery PVAT from female animals was inhibited by a TP receptor antagonist, whereas the contractile response to coronary artery PVAT from male arteries was inhibited by a FP receptor antagonist. These data suggest sex differences in the prostanoids mediating the contractile response.

The role of a cyclooxygenase product in the PVAT-induced contraction is similar to that seen in mouse aorta, although in that particular study, only thromboxane levels were measured (Meyer *et al.*, 2013). In this present study we have demonstrated the presence of thromboxane in the PVAT from the porcine coronary artery, although there was no difference in the levels of this prostanoid between male and female

animals. In addition, a concentration-response curve with a TXA_2 agonist (U46619) was performed to assess the responsiveness of PCAs to TXA_2 in both sexes and showed no difference in the response of coronary rings from different sexes to TXA_2. Moreover, Western blot analysis found no difference in TP expression between female and male coronary arteries. These data indicate that the sex differences in the role of thromboxane in the PVAT-induced contraction cannot be attributable to either differences in TP receptor expression or coupling of the receptor to intracellular signalling pathways mediating contraction. We therefore determined whether there are differences in the release of thromboxane from the PVAT by measuring the levels of TXB_2 in the buffer. Interestingly, there was a greater amount of TXB_2 in the buffer containing PVAT from female animals compared to males, suggesting that, although levels of thromboxane are the same in PVAT from both sexes, a greater amount of thromboxane is actually released from the PVAT in females. Although the exact reason for the difference in release of TXB_2 has not been addressed in this study, many reasons could be speculated such as the difference of the expression or function of TXB_2 transporters (for example, multidrug resistance protein 4 or other prostaglandin transporters) which act as a prostanoid efflux pumps (Schuster, 2002; Reid *et al.*, 2003; Rius *et al.*, 2005) or differences in the turnover of released TXB_2. However, the data explain why the PVAT-induced contraction in coronary arteries from female animals is inhibited by the TP receptor antagonist.

The present study also provides evidence that $PGF_{2\alpha}$ is expressed in the PVAT of the coronary artery and is involved in the PVAT-induced contraction in male pigs as FP receptor antagonism by AL8810 significantly reduced the resultant vasocontraction. Therefore, like thromboxane, $PGF_{2\alpha}$ has a role as a PVAT-derived contractile agent. Although there was no difference in the level of $PGF_{2\alpha}$ in the PVAT from male and female pigs, $PGF_{2\alpha}$ itself produced a greater contractile response in coronary arteries from male animals compared to females. This corresponds with a greater expression of the FP receptor in coronary arteries from male animals. Therefore, the sensitivity of the PVAT-induced contraction to FP receptor antagonism in coronary arteries from male animals, but not females, could be explained by differences in FP receptor expression.

In order to confirm the role of prostanoids in the PVAT-induced contraction in the coronary artery, we determined the effect of the PLA_2 activator melittin. Melittin enhanced the PVAT-induced contraction in coronary arteries from both sexes, which correlates with the role of the PLA_2-arachidonic acid- cyclooxygenase pathway. Furthermore, melittin enhanced the production of thromboxane within PVAT and the level of thromboxane released from the PVAT. Antagonism of TP receptors inhibited the contraction to PVAT in coronary arteries from both male and female animals. Interestingly, the FP antagonist no longer inhibited the PVAT-induced contractions in either male or female arteries. However, we found that melittin caused desensitisation of the $PGF_{2\text{-}\alpha}$ contraction but not the TXA_2,-induced contraction, as there was no contraction to $PGF_{2\text{-}\alpha}$ in PCA segments pre-incubated with melittin, whereas the contraction to the thromboxane mimetic U46619 was maintained. This finding which could explain the lack of effect of the FP antagonist under these conditions. We hypothesise that this desensitisation is caused by release of $PGF_{2\text{-}\alpha}$ from the coronary artery in response to melittin.

A limitation of our study is the potential selectivity of GR32191B for TP receptors over FP receptors. However, our data indicate that the larger inhibition of the PVAT response seen with AL8810 in PCAs from males is likely to be due to greater responsiveness and FP receptor expression in males compared to females. Similarly, the greater effect of GR32191B on PVAT responses in females compared to males appears to be due to greater release of thromboxane.

In conclusion, these data demonstrate that PVAT is able to regulate porcine coronary arterial tone through the release of thromboxane and $PGF_{2\alpha}$. To the best of our knowledge, this is the first study that identifies sex differences in PVAT-induced regulation of vascular tone, where thromboxane antagonist could inhibit thePVAT-dependant vasoconstriction in females while $PGF_{2\alpha}$ antagonist is able to inhibit the contraction of PCAs from males. The variation of role TXA_2 and $PGF_{2\alpha}$ in different sexes is further supported by the findings of a greater release of thromboxane from PVAT from female animals and greater expression of FP receptors on the porcine coronary artery from males. It is clear that there are sex differences in the regulation of vascular tone, including the role of endothelium-derived hyperpolarization (EDH)

(Wong *et al.*, 2014). The data presented here enhance our knowledge of the mechanisms underlying these sex differences by demonstrating differences in the adipose-derived contractile factors released from coronary artery PVAT. Adipose tissue, including PVAT, changes under pathological conditions such as obesity. In the porcine coronary artery, obesity enhances the contractile responses of PVAT (Owen *et al.*, 2013). Whether this is due to altered release of prostanoids is unknown. Furthermore, whether the sex difference in the release of prostanoids is maintained in obesity is unknown. Future studies should explore whether these sex differences in PVAT are maintained under pathological conditions.

References

Bartelink ML, van de Lisdonk E, van den Hoogen H, Wollersheim H & van Weel C. (1993). Migraine in family practice: prevalence and influence of sex hormonal status. Fam Med 25, 331-336.

Bubb KJ, Khambata RS & Ahluwalia A. (2012). Sexual dimorphism in rodent models of hypertension and atherosclerosis. British journal of pharmacology 167, 298-312.

Chang L, Villacorta L, Li R, Hamblin M, Xu W, Dou C, Zhang J, Wu J, Zeng R & Chen YE. (2012). Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-gamma deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. Circulation 126, 1067-1078.

Coleman RA, Smith WL & Narumiya S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacological reviews 46, 205-229.

Fang L, Zhao J, Chen Y, Ma T, Xu G, Tang C, Liu X & Geng B. (2009). Hydrogen sulfide derived from periadventitial adipose tissue is a vasodilator. Journal of hypertension 27, 2174-2185.

Fortuno A, Rodriguez A, Gomez-Ambrosi J, Fruhbeck G & Diez J. (2003). Adipose tissue as an endocrine organ: role of leptin and adiponectin in the pathogenesis of cardiovascular diseases. Journal of physiology and biochemistry 59, 51-60.

Gao YJ, Lu C, Su LY, Sharma AM & Lee RM. (2007). Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. British journal of pharmacology 151, 323-331.

Gao YJ, Takemori K, Su LY, An WS, Lu C, Sharma AM & Lee RM. (2006). Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. Cardiovascular research 71, 363-373.

Havel PJ. (2004). Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. Diabetes 53 Suppl 1, S143-151.

He GW & Yang CQ. (1999). Inhibition of vasoconstriction by the thromboxane A2 antagonist GR32191B in the human radial artery. British journal of clinical pharmacology 48, 207-215.

Koumanov K, Momchilova A & Wolf C. (2003). Bimodal regulatory effect of melittin and phospholipase A2-activating protein on human type II secretory phospholipase A2. Cell Biol Int 27, 871-877.

Li M, Kuo L & Stallone JN. (2008). Estrogen potentiates constrictor prostanoid function in female rat aorta by upregulation of cyclooxygenase-2 and thromboxane pathway expression. Am J Physiol Heart Circ Physiol 294, H2444-2455.

Lu C, Su LY, Lee RM & Gao YJ. (2010). Mechanisms for perivascular adipose tissue-mediated potentiation of vascular contraction to perivascular neuronal stimulation: the role of adipocyte-derived angiotensin II. European journal of pharmacology 634, 107-112.

Lynch FM, Withers SB, Yao Z, Werner ME, Edwards G, Weston AH & Heagerty AM. (2013). Perivascular adipose tissue-derived adiponectin activates BK(Ca) channels to induce anticontractile responses. Am J Physiol Heart Circ Physiol 304, H786-795.

Malinowski M, Deja MA, Golba KS, Roleder T, Biernat J & Wos S. (2008). Perivascular tissue of internal thoracic artery releases potent nitric oxide and prostacyclin-independent anticontractile factor. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery 33, 225-231.

Meyer MR, Fredette NC, Barton M & Prossnitz ER. (2013). Regulation of vascular smooth muscle tone by adipose-derived contracting factor. PloS one 8, e79245.

Nettleship JE, Jones RD, Channer KS & Jones TH. (2009). Testosterone and coronary artery disease. Front Horm Res 37, 91-107.

Owen MK, Witzmann FA, McKenney ML, Lai X, Berwick ZC, Moberly SP, Alloosh M, Sturek M & Tune JD. (2013). Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: influence of obesity. Circulation 128, 9-18.

Ozen G, Topal G, Gomez I, Ghorreshi A, Boukais K, Benyahia C, Kanyinda L, Longrois D, Teskin O, Uydes-Dogan BS & Norel X. (2013). Control of human vascular tone by prostanoids derived from perivascular adipose tissue. Prostaglandins & other lipid mediators 107, 13-17.

Ramos-Alves FE, de Queiroz DB, Santos-Rocha J, Duarte GP & Xavier FE. (2012). Increased cyclooxygenase-2-derived prostanoids contributes to the hyperreactivity to noradrenaline in mesenteric resistance arteries from offspring of diabetic rats. PloS one 7, e50593.

Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J & Borst P. (2003). The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 100, 9244-9249.

Rius M, Thon WF, Keppler D & Nies AT. (2005). Prostanoid transport by multidrug resistance protein 4 (MRP4/ABCC4) localized in tissues of the human urogenital tract. J Urol 174, 2409-2414.

Schuster VL. (2002). Prostaglandin transport. Prostaglandins & other lipid mediators 68-69, 633-647.

Stanley CP & O'Sullivan SE. (2014). Cyclooxygenase metabolism mediates vasorelaxation to 2-arachidonoylglycerol (2-AG) in human mesenteric arteries. Pharmacological research : the official journal of the Italian Pharmacological Society 81, 74-82.

Thalmann S & Meier CA. (2007). Local adipose tissue depots as cardiovascular risk factors. Cardiovascular research 75, 690-701.

Virdis A, Duranti E, Rossi C, Dell'Agnello U, Santini E, Anselmino M, Chiarugi M, Taddei S & Solini A. (2015). Tumour necrosis factor-alpha participates on the endothelin-1/nitric oxide imbalance in small arteries from obese patients: role of perivascular adipose tissue. Eur Heart J 36, 784-794.

Voulgari PV, Alamanos Y, Papazisi D, Christou K, Papanikolaou C & Drosos AA. (2000). Prevalence of Raynaud's phenomenon in a healthy Greek population. Ann Rheum Dis 59, 206-210.

Wong PS, Randall MD & Roberts RE. (2015). Sex differences in the role of NADPH oxidases in endothelium-dependent vasorelaxation in porcine isolated coronary arteries. Vascul Pharmacol 72, 83-92.

Wong PS, Roberts RE & Randall MD. (2014). Sex differences in endothelial function in porcine coronary arteries: a role for H2O2 and gap junctions? British journal of pharmacology 171, 2751-2766.

Wright DH, Abran D, Bhattacharya M, Hou X, Bernier SG, Bouayad A, Fouron JC, Vazquez-Tello A, Beauchamp MH, Clyman RI, Peri K, Varma DR & Chemtob S. (2001). Prostanoid receptors: ontogeny and implications in vascular physiology. American journal of physiology Regulatory, integrative and comparative physiology 281, R1343-1360.

Yamawaki H, Tsubaki N, Mukohda M, Okada M & Hara Y. (2010). Omentin, a novel adipokine, induces vasodilation in rat isolated blood vessels. Biochemical and biophysical research communications 393, 668-672.

Additional information

Competing Interest:

None.

Author contributions

All authors contributed to the final version of the manuscript. AAA performed the research; AAA, MDR and RER designed the research study; AAA and RER analysed the data; AAA, MDR, and RER revised the article critically for important intellectual content. All authors approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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