Sex differences in the regulation of porcine coronary artery tone by perivascular adipose tissue: a role of adiponectin?

Ahmad: PhD student at University of Nottingham.
Abdulla A Ahmad: PhD student, Faculty of Medicine & Health Sciences
Michael D Randall: PhD, Professor of Pharmacology, Faculty of Medicine & Health Sciences
Richard E Roberts: PhD, Lecturer in Pharmacology, Faculty of Medicine & Health Sciences

Specific Contributions:

- A A Ahmad performed the research
- A A Ahmad, M D Randall, and R E Roberts designed the research study
- A A Ahmad and R E Roberts analysed the data
- A A Ahmad, M D Randall, and R E Roberts wrote the paper.

Running title: Sex differences in PVAT-vascular coupling.

Background and Purpose- As there is sexual dimorphism in the regulation of vascular tone, the aim of this present study was to determine whether there are sex differences in perivascular adipose tissue (PVAT) - mediated regulation of the porcine coronary artery (PCA) tone.

Experimental Approach- Isometric tension recording system was used to record changes in tone in PCAs. Western blot analysis was performed to examine the expression of adiponectin in PVAT and adiponectin receptors (adipo 1 receptor and adipo 2 receptor) and adiponectin binding protein (APPL1) in PCA. The level of adiponectin released from PVAT was measured using ELISA.

Key Results- In the presence of adherent PVAT, contractions to the thromboxane mimetic U46619 and endothelin-1 were significantly reduced in PCAs from females, but not males. In PCAs pre-contracted with U46619, re-addition of PVAT caused relaxation in PCAs from females, but not males. This relaxant response in females was attenuated by combined inhibition of NO synthase (with L-NAME) and cyclooxygenase (with indomethacin). Pre-incubation with an anti-adiponectin antibody abolished the relaxant effects of PVAT. The adiponectin receptor agonist (adipoRon) produced a greater relaxation in PCAs from females compared to males. However, there was no difference in either expression or release of adiponectin from PVAT between sexes. Similarly, there was no difference in expression of adiponectin receptors or the adiponectin receptor adaptor protein APPL1 in PCAs.

Conclusion and Implications- These findings demonstrate a clear sex difference in the regulation of coronary artery tone in response to adiponectin receptor stimulation, which may underlie the anticontractile effects of PVAT in females.

Abbreviations

Adiponectin receptor (adipo receptor), adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1), cyclooxygenase (COX), endothelin-1 (ET-1), NG-nitro-L-arginine methyl ester (L-NAME), myosin light chain (MLC), nitric oxide (NO), nitric oxide synthase (NOS), perivascular adipose tissue (PVAT), porcine coronary artery (PCA), 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U46619).

Introduction

Adipose tissue has traditionally been described as being simply a structural support, with additional lipid storage (white adipose tissue) and thermoregulator (brown adipose tissue) roles (Cannon & Nedergaard, 2004; Mariman & Wang, 2010). However, compelling evidence in recent years has led to the view that adipose tissue is in fact an active secretory organ which releases several bioactive signalling molecules, collectively termed adipokines, which are important in both health and disease (Fortuno *et al.*, 2003; Havel, 2004; Thalmann & Meier, 2007; Yamawaki *et al.*, 2010). Multiple studies have established that there are variations in the function of adipose tissue in different sexes where, for instance, women have higher circulating adiponectin levels compared to men (Arita *et al.*, 2012; Cnop *et al.*, 2003; Nishizawa *et al.*, 2002).

Perivascular adipose tissue (PVAT) is positioned around the blood vessel and is regarded as being distinct from the adventitia. The recent interest in perivascular adipose tissue as an endocrine and paracrine organ has led to a number of studies investigating the characteristics of various fat depots in the body. Soltis and Cassis (1991) firstly described that perivascular adipose tissue significantly attenuated vascular responsiveness of Sprague-Dawley rat aortae to noradrenaline (Soltis & Cassis, 1991). This result was confirmed by Löhn *et al*. (2002) who re-examined the idea of periadventitial vasoregulation. Their study provided additional proof that PVAT-inhibited vasoconstriction induced by a number of additional agonists such as angiotensin II, serotonin and phenylephrine (Lohn *et al.*, 2002). Adiponectin is a major factor secreted by adipocytes (Scherer *et al.*, 1995) and exerts its action by binding to its receptors (adipo 1 receptor and adipo 2 receptor) (Kadowaki & Yamauchi, 2005). Although downstream regulation of signalling of these receptors is yet to be fully identified, overexpression of APPL1 (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1) enhances, and suppression of APPL1 level attenuates, adiponectin signalling and its downstream events (Mao *et al.*, 2006).

Functional studies have found that adiponectin can relax murine mesenteric artery by opening Kv channels (Fesus *et al.*, 2007), and (Greenstein *et al.*, 2009) concluded that adiponectin was the anti-contractile adipokine released from rat mesenteric arterial PVAT. Moreover, a fragment of the adiponectin receptor abolished the PVAT-induced vasorelaxation in human small arteries taken from subcutaneous gluteal fat biopsy samples, which further suggests that adiponectin is a predominant mediator of the relaxation (Greenstein *et al.*, 2009). Noteworthy, there are accumulating data about the potential effect of PVAT in regulating the

vascular tone where PVAT could induce both vasorelaxation and vasoconstriction depending on the anatomical position, species and condition (Nava & Llorens, 2016; Owen *et al.*, 2013; Szasz *et al.*, 2013; Verlohren *et al.*, 2004; Yudkin *et al.*, 2005).

In recent studies we have demonstrated that there are sex differences in the regulation of tone in the porcine coronary artery (Wong *et al.*, 2014). As there are also sex differences in the release of adipokines from adipose tissue in general, in this study we investigated whether there are sex differences in the regulation of vascular tone by PVAT in the porcine coronary artery. Our findings demonstrate that PVAT surrounding coronary arteries from females has an anti-contractile effect, which is not seen in males, indicating that PVAT may have a protective effect on coronary arteries in females, but not males.

Methods

Tissue preparation

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 $CaCl_2$). Experiments were blinded as the experimenter did not know whether the tissues were from male or female animals until after the experiments had been undertaken. 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 and kept overnight in the refrigerator. On the second day, fine dissection was performed to prepare paired PCAs rings from each artery ensuring vessels were not stretched or damaged. PCAs were then cut into rings of approximately 5mm in length and suspended in a multichannel 5 ml organ bath. 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 a Powerlab data acquisition system (ADInstruments) via an amplifier.

Experimental protocol

Organ bath studies

Tissues were initially pre-tensioned to 73.6mN, determined from preliminary studies, and then left to equilibrate for approximately 30-45 minutes. Once a stable baseline was reached, two consecutive responses to 60mM KCl were obtained for standardization. After about 20 min the KCl was washed out with fresh Krebs'-Henseleit solution. Following the return to a stable baseline and, after a further 15-30 minutes, exposure to KCl was repeated. Once again, the tissue was washed out with Krebs'-Henseleit solution, to allow the segment tone to re-stabilize to baseline. The data were calculated and expressed as a percentage of the contraction in response to 60 mM KCl. The KCl response is considered as a reference to the viability and activity of each segment in order to avoid the individual variation in the response of the coronary artery. Thereafter relevant test compounds were added and allowed 60 minutes incubation time. The following test compounds were used: L-NAME (300 µM) to inhibit NO synthase (Wong *et al.*, 2014), and indomethacin (10 µM) (Malinowski *et al.*, 2008) to inhibit cyclooxygenase. Pre-incubation with an anti-adiponectin antibody (1:100; ABIN1857924, antibodies-online Inc., Atlanta, USA) was performed to determine the role of

adiponectin in PVAT-induced vasorelaxation in females. U46619 (9,11-Dideoxy-11α,9α-epoxymethano-prostaglandin F2α) was added cumulatively (1 nM-300 nM) to induce contraction in the arterial rings, which was calculated as a percentage of the contraction in response to 60 mM KCl. In some experiments endothelin-1 (ET-1) (1 nM-300 nM) was used as contractile agent. In other sets of experiments, PVAT (0.3 g) was added to vessels in which the PVAT had previously been removed and pre-contracted with U46619 to a level of 40-60% of the maximum KCl response. In a further set of experiments, cumulative concentration response curves to the adiponectin receptor agonist, adipoRon (1-100 μM) (Okada-Iwabu *et al.*, 2013) were performed in PCAs pre-contracted with U46619.

Western immunoblotting

Polyacrylamide gel electrophoresis followed by Western immunoblotting was performed to determine the expression of adiponectin levels in PVAT, and the adiponectin receptors (adipo 1 receptor and adipo 2 receptor) and APPL1 in PCAs from different pigs from each sex. PVAT or PCA samples were finely dissected and homogenized on ice using Tris-EDTA buffer (pH 7.4) and protease inhibitor cocktail (Calbiochem, VWR International Ltd, Lutterworth, Leicestershire, UK). After estimation of the protein content of each sample using a Lowry protein assay, Laemmli solubilisation buffer was added. Samples were then heated at 100^{o}C for 5 min and centrifuged at 13,000×g for 1 min. PVAT (20 μg) and PCA (20 μg) samples were separated using a 4–20% gradient polyacrylamide gradient gel (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) and then transferred onto nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by Western blotting. Porcine skeletal muscle and rat liver tissues were used as a positive control for adipo 1 receptor and adipo 2 receptor , respectively (Ding *et al.*, 2009). Membranes were then blocked with 5% w/v non-fat milk, or 5% w/v BSA in the case of APPL1, in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h. The blot was then incubated with rabbit anti-adiponectin antibody (1:400 dilution; ABIN1857924, antibodies-online Inc., Atlanta, USA), rabbit anti-adipo 1 receptor antibody (1:500; 250476, Abbiotec, USA), rabbit anti-adipo 2 receptor antibody (1:250 dilution; orb10047, Biorbyt, Cambridge, UK) and rabbit anti-APPL1 antibody (1:2000; mAb 3858, Cell Signaling Technology, UK) in 5% milk, or in 5% BSA in the case of APPL1 antibody, in Tris-buffer overnight at 4°C. Membranes were also incubated with a mouse anti-β-actin antibody (1:50,000 in 5% milk; Sigma-Aldrich, Poole, Dorset, UK) or an anti-mouse myosin light chains (MLC) antibody

(1:1000 in 5% milk; Sigma-Aldrich, Poole, Dorset, UK) as a loading control as well as to quantify porcine proteins. On the second day, the blot was washed in TBST followed by incubation with IRDye 800CW goat anti-rabbit and IRDye 680CW goat anti-mouse secondary antibodies (both 1:10,000 dilution; IRDye, Licor, Cambridge, UK). The immunoblot was then visualized using an Odyssey system from Licor and intensity of the bands measured using Odyssey Image Studio (Licor).

Determination of adiponectin levels released from PVAT

PVAT (0.15g) was incubated in 1ml of Krebs'-Henseleit solution at 37^{o}C. Adiponectin levels in the buffer were then determined using a pig adiponectin ELISA Kit (abx255420; Abbexa, Cambridge, UK) following the manufacturer's protocol. The range of standard curve is 31.25 ng/ml - 1000 ng/ml, sensitivity: < 18.75 ng/ml. The intra-assay coefficient of variation was 10.1.

Data and statistical analysis

Data are expressed as mean ± S.E.M. 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. R_{max} and pEC_{50} values were analysed using a 2-tailed, paired or unpaired Student's t-test to compare differences between 2 groups, as appropriate. Differences between 3 or more groups were assessed using a one-way ANOVA or two-way ANOVA in conjunction with the Sidak's *post-hoc test* to assess possible difference at individual concentrations. $P < 0.05$ was considered statistically significant. Statistical analysis was performed by GraphPad Prism (Version 6). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Materials

All drugs were purchased from Sigma-Aldrich except for endothelin-1 which came from Tocris Bioscience and adiponectin from Antibodies-online Inc. Stock solutions of L-NAME, and adiponectin were dissolved in distilled water. Stock solution of indomethacin was

dissolved in absolute ethanol. Stock solutions of U46619 were made to 10mM in ethanol whilst DMSO was used as a solvent to make 10mM of adipoRon. All further dilutions of the stock solutions were made using distilled water. All stocks were kept frozen at -20^{o}C.

Results

The effects of PVAT on U46619-induced vasoconstriction in PCAs from female and male pigs.

In order to determine the effects of perivascular fat on vascular contraction, concentration-response curves to the thromboxane mimetic U46619 were determined in coronary artery segments from female and male pigs (Figure 1 A and B), with and without adherent PVAT. The presence of fat reduced the maximum contraction to U46619 in coronary arteries from females. There was also a rightward shift in the concentration-response curve. On the other hand, the presence of PVAT had no effect on the U46619 induced-contraction in coronary arteries from male pigs (Figure 1B). Similarly, the endothelin-1-induced vasoconstriction was significantly reduced in coronary arteries from female pigs in the presence of fat with no change in pEC_{50} (Figure 1C). Furthermore, PVAT had no effect on the endothelin-1-mediated vasoconstriction in PCAs from male (Figure 1D). There was no significant difference in the size of the contractile response to 60 mM KCl with PVAT compared to without PVAT (females, control = 8 ± 0.8 g without PVAT compared to 6.4 ± 0.6 g with PVAT; males, control =9.4 ± 1.2 without PVAT compared to 7.7 ± 0.9 g with PVAT)

Acute effects of added PVAT on rings pre-contracted with U46619.

Coronary arterial segments from female pigs with fat removed were pre-contracted submaximally with U46619. Once a stable tone was obtained, 0.3 g PVAT was then added (see Figure 2C). PVAT from female animals caused immediate relaxation (R) (Figure 2 A and C). On the other hand, PVAT from male animals had no significant effect on the U46619-induced vasoconstriction compared to time control (Figure 2B). Furthermore, a crossover study in which PVAT from females was added to PCAs from males showed no vasorelaxant effect (Figure 2D).

The effects of L-NAME and indomethacin on the anti-contractile effect of PVAT in PCAs.

The maximum response of PCAs to U46619 was significantly inhibited in the presence of PVAT, as before (Figure 3). A combination of L-NAME and indomethacin had no effect on the concentration-response curve to U46619 in the absence of PVAT, but this combination prevented the inhibitory effect of PVAT on the U46619 response (Figure 3A and B). On the

other hand, incubation with either L-NAME or indomethacin alone had no effect on the anti-contractile response of PVAT (Figure 3B).

Role of adiponectin in PVAT-mediated vasorelaxation in females

Previous studies have indicated that adiponectin acts through nitric oxide synthase (NOS) and cyclooxygenase-dependent ways. Therefore we determined the role of adiponectin in the relaxation effect seen in the presence of PVAT. Segments of coronary arteries from females were pre-treated with an anti-adiponectin antibody, in order to bind any released adiponectin. In the presence of the anti-adiponectin antibody, the PVAT-induced relaxation was abolished (Figure 4).

Adiponectin expression in PVAT

Our data indicate that PVAT from females, but not males, causes relaxation of coronary arteries through the release of adiponectin. In order to determine whether the differences in the role of PVAT in arteries from male and female animals was due to differences in the amount of adiponectin in PVAT, the expression of adiponectin in PVAT was determined by Western immunoblotting. There was no significant difference in the expression of adiponectin in coronary artery PVAT from female and male animals (Figure 5A,B).

Adiponectin release from PVAT

As there was no difference in the level of expression of adiponectin in PVAT between male and female animals, we determined whether there is a difference in the release of adiponectin from PVAT. Although adiponectin could be detected in the buffer that the PVAT was incubated in, there was no significant difference in the level of released adiponectin between PVAT from male and female animals (Figure 5C).

Responsiveness of porcine coronary arteries to adipoRon

As there was no difference in either the expression of adiponectin or release of adiponectin from PVAT, we determined whether there was a difference in the sensitivity to adiponectin receptor activation between male and female animals. AdipoRon, an adiponectin receptor agonist, produced a concentration-dependent relaxation of coronary arteries from both female and male pigs. However, there was a greater relaxation in arteries from female pigs compared to male (at 100 µM, females: 106±8 % relaxation, n=6; males: 78±6 % relaxation, n=8).

AdipoRon also appeared to be more potent in arteries from females compared to males, based on the observation that adipoRon produced a relaxation at lower concentrations (Figure 6).

Expression of Adiponectin receptors in PCAs

To clarify the difference in the responsiveness of PCAs in different sexes, we measured the level of expression of two different isoforms of adiponectin receptors (adipo 1 receptor and adipo 2 receptor) in PCAs from both sexes, using Western immunoblotting. Numerous bands were detected with the anti-adipo 1 receptor antibody, but none lined up with the band in the rat skeletal muscle positive control at the predicted molecular weight of 40 kDa, suggesting there is no detectable expression of adipo 1 receptor in PCAs from either females or males (Figure 7A). Similarly numerous bands were obtained with the anti-adipo 2 receptor antibody (Figure 7B). A band at the predicted molecular weight of 42 kDa lined up with a band at the same molecular weight in the rat liver positive control was taken to be adipo 2 receptor. This band was expressed in PCAs from both sexes. However, there was no apparent difference in the expression of adipo 2 receptor in PCAs from females compared to males (Figure 7B,C).

Expression of APPL1 in PCAs

As there was no difference in the expression of adiponectin receptors between females and males PCAs, we then investigated the expression of the adiponectin binding protein APPL1 in PCAs by Western immunoblotting. A band for APPL1 was detected at the predicted molecular weight of 82 kDa. However, there was no significant difference in APPL1 expression between PCAs from both sexes (Figure 7D,E).

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 substantial research efforts to establish the mechanistic links between the PVAT and vascular tone regulation. The majority of research in this area has been carried out on blood vessels from male animals and has concentrated mostly on murine aorta and mesenteric vessels. A few studies have determined the role of PVAT in the regulation of coronary artery tone, but this is the first study to compare the effects of PVAT from male and female animals. We have identified clear sex differences in the regulation of coronary artery tone by PVAT, with PVAT from female animals producing an anti-contractile, relaxation response, which was not seen in PVAT from male animals.

In the presence of fat, contractile responses to both the thromboxane receptor agonist U46619 and ET-1 were reduced in coronary arteries from female pigs, but not from male pigs, indicating the release of an anti-contractile compound from the PVAT surrounding the coronary arteries in females. Re-addition of fat removed from the coronary arteries into the tissue baths produced a relaxation response in arteries from females, but not males. In order to confirm this finding, we performed a crossover study in which PVAT from females was added to male arteries. Although PVAT from females induced a relaxation in coronary artery from females, the PVAT from females did not produce a relaxation in coronary arteries from males. Taken together, these data suggest that the PVAT surrounding coronary arteries from female animals may have a protective, anti-contractile role, which may contribute to the reduced incidence of coronary artery disease in females.

The present results are consistent with the studies by (Gao *et al.*, 2007) and (Malinowski *et al.*, 2008) which have shown that PVAT reduces vascular tone in the rat aorta and human internal thoracic arteries, respectively, although sex differences in these responses were not determined. In contrast, previous studies in porcine coronary artery have indicated the release of a contractile agent from PVAT, although again sex differences in the responses were not examined (Owen *et al.*, 2013).

In mouse mesenteric arteries, the anti-contractile effect of PVAT is mediated through either NO or COX-dependent pathways (Lynch *et al.*, 2013). Similarly, in this study, we demonstrated that the anti-contractile effect of PVAT in coronary arteries from female pigs was sensitive to inhibition with a combination of the nitric oxide synthase inhibitor L-NAME and the cyclooxygenase inhibitor indomethacin, whereas inhibition of each enzyme separately had no significant effect. These data indicate the involvement of both NO and

COX metabolites in the PVAT response. This is similar to the findings of (Lynch *et al.*, 2013) who also found that inhibition of both NOS and COX was required to inhibit the anti-contractile response. Dual inhibition is probably necessary because of compensatory activation of the other pathway. In general, this observation is in line with a previous study on aorta from Wistar rats which found that PVAT could exert its anti-contractile effects by secreting a relaxant factor which mediates endothelium-dependent vasorelaxation through NO release (Gao *et al.*, 2007). On the other hand, these findings differ from a previous study by (Malinowski *et al.*, 2008) which was conducted on the human internal thoracic artery where they reported that blocking NOS and COX could not restore the vascular response in intact rings. In fact, these differences may reflect changes in vascular responses in cardiovascular disease as the human vessels used were from patients with severe coronary artery disease and therefore likely to have endothelial dysfunction. In addition, the inconsistency of findings for the role of NO and COX in all previous studies could probably be due to the indefinite sex used.

In order to identify the mechanism underlying the sex difference in the anti-contractile effect of PVAT in the porcine coronary artery, we investigated the role of adiponectin. Adiponectin is the most abundant adipokine in the circulation and has been shown to activate both NO and COX-dependent pathways (Lee *et al.*, 2010; Shibata *et al.*, 2005; Tan *et al.*, 2004). As there is no selective adiponectin receptor antagonist available, we used an anti-adiponectin antibody to bind any adiponectin released from the PVAT and prevent it from activating the receptor on the artery. To this end, we determined the effect of the antibody on the relaxation due to addition of PVAT, rather than using fat-attached arteries. This is because the close proximity of the fat to the blood vessel in fat-attached preparations may not allow the antibody access to bind released adiponectin. Under these conditions, the presence of the anti-adiponectin antibody completely prevented the PVAT-induced relaxation, indicating that adiponectin is likely to be the mediator released from the fat. Therefore, we investigated whether the sex differences in the relaxation were due to differences in adiponectin expression, release or function. Using Western immunoblotting we identified the presence of adiponectin in PVAT from the coronary artery, although there was no difference between male and females. Furthermore, there was no difference in the level of adiponectin released from PVAT, indicating that the difference in the relaxation response is not due to a difference in either expression or release of adiponectin. On the other hand, using an adiponectin receptor agonist, adipoRon, we demonstrated that activation of the adiponectin receptor

produces a greater relaxation and at lower concentrations in coronary arteries from females compared to males. These data indicate that the sex difference in the anti-contractile effect of PVAT is likely to be due to a difference in the responsiveness of the arteries to adiponectin. Noteworthy, responses to adiponectin receptor activation are independent of the endothelium in that denudation of the endothelium did not alter the relaxation to adipoRon in the coronary artery (Supplementary figure 1).

In an attempt to identify the reason for the differences in adiponectin response, we determined the expression of the adiponectin receptors in the coronary artery. Using Western immunoblotting, we identified expression of adipo 2 receptor but not adipo 1 receptor in PCAs from both sexes. However, there was no apparent difference in the expression of adipo 2 receptor between males and females.

Since no sex difference was identified in adiponectin receptors expression, so we investigated the expression of APPL1 which has been identified as an adipo 1 receptor and adipo 2 receptor binding protein (Buechler *et al.*, 2010) and considered as a downstream regulator of adiponectin signalling (Cheng *et al.*, 2007; Mao *et al.*, 2006). Although we have showed an expression of this adaptor protein APPL1 in PCAs, there was no significant difference between sexes. The limitation in our investigation is that the expression of different proteins studied in this research were examined in total homogenate rather than vascular smooth muscle only. In addition, total adiponectin receptors expression were measured rather than the expression of just surface receptors. As adiponectin receptors are distributed in the cytoplasm (Ding *et al.*, 2009) and on the cell membrane (Yamauchi *et al.*, 2003), differences in either cellular or subcellular distribution could underlie the sex differences.

We have previously demonstrated sex differences in intracellular signalling pathways involved in vasorelaxation. In particular, there are sex differences in the relative contribution of the NO and EDH signalling pathways in the porcine coronary artery, as well as differences in the mechanisms underlying the EDH response (Wong *et al.*, 2015a; Wong *et al.*, 2015b). Therefore, differences in signalling may contribute to the sex differences in adiponectin relaxation seen in this study. In addition, malfunction and resistance to adiponectin have been identified in different conditions such as obesity (Bruce *et al.*, 2005; Chen *et al.*, 2005), high fat diet (Mullen *et al.*, 2009) and cardiovascular diseases (Lau *et al.*, 2011). Therefore, we cannot exclude the possibility of adiponectin resistance in the coronary arteries from males.

Although there was reduced relaxation to AdipoRon in porcine coronary arteries from males, there was still a relaxation and not the complete absence of relaxation seen with the addition

of PVAT. The lack of relaxation to PVAT in males could be because the amount of adiponectin released from PVAT is too small to induce a relaxation in males. Alternatively, other studies have indicated that an unknown contractile factor is released from porcine coronary artery PVAT. This may counteract the relaxation response to adiponectin in males.

Clinical relevance of the study

It is becoming increasingly clear that PVAT plays a role in cardiovascular pathophysiology. For instance, an apparent link has been found between an increase in PVAT and coronary artery disease (Verhagen & Visseren, 2011). In addition, several clinical studies have demonstrated vascular protective effects of adiponectin as low plasma levels of adiponectin is associated with hypertension and other cardiovascular diseases (Aprahamian & Sam, 2011; Imatoh *et al.*, 2008; Kim *et al.*, 2013) and high levels of adiponectin are associated with a reduced incidence of acute coronary syndrome and acute myocardial infarction (Mittal *et al.*, 2013; Pischon *et al.*, 2004). The present study demonstrates that only PVAT from females has an anti-contractile effect on the coronary artery tone and thus may contribute to the reduced incidence of cardiovascular disease in pre-menopausal women. However, further studies are required to determine whether similar sex differences in the regulation of tone by PVAT are observed in other blood vessels, and, importantly, whether similar effects are seen in humans.

In summary, the present study illustrates that there are clear sex differences in PVAT function, where PVAT exhibits an anti-contractile responses in PCAs from female pigs, but not male pigs, which appears to be due to increased adiponectin activity. The sex difference in the PVAT response is not explained by a difference in the expression or release of adiponectin, but may be explained by a greater sensitivity of coronary arteries from females to adiponectin receptor activation. This is the first study to demonstrate that PVAT may have a protective effect on coronary arteries in females, which is not present in males. This protective effect could contribute to the cardiovascular protective effects observed in females. Future studies into the role of PVAT on vascular tone should take into account potential sex differences in the responses.

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Conflicts of interest

None.

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Figure 1: Effect of PVAT on U46619 and ET-1 -induced Arterial Contraction. Log concentration-response curves for the vasoconstrictor effects of U46619 (**A & B**) or ET-1 (**C & D**) in the absence (Control) or presence of PVAT (PVAT) in coronary arteries from female (A & C) and male pigs (B & D). Data are expressed as a percentage of the contraction to 60 mM KCl and are mean ± S.E.M. of 12(A), 6(B), 8(C), 7(D) experiments. * indicates $p<0.05$, 2-tailed, paired Student's t-test.

Figure 2: PVAT-induced vasorelaxation in both sexes. Effects of PVAT (0.3 g) in female (**A**) and male (**B**) PCAs. Original trace of recording showing the response of female PCA to addition of PVAT (**C**). Effects of addition of PVAT (0.3 g) from female animals on coronary arteries from male animals **(D)**. Data are expressed as a percentage relaxation from the U46619-induced tone and are expressed as mean ± S.E.M. of 8(A)-8(B)-6(C) experiments, * indicates $p<0.05$, 2-tailed, paired Student's t-test.

Figure 3: Role of NOS and COX in PVAT-induced vasorelaxation. (A) Log concentration-response curves for the vasoconstrictor effects of U46619 in the absence or presence of PVAT in female PCAs with and without pre-incubation with L-NAME (300 µM) and indomethacin (10 µM). Data are expressed as a percentage of the contraction to 60 mM KCl and are mean ± S.E.M. of 11 experiments. (**B)** Maximum response to the vasoconstrictor effects of U46619 in female PCAs with fat attached and either absence or presence of pre-incubation with L-NAME (300 µM) and indomethacin (10 µM). Data are expressed as a percentage of the contraction to 60 mM KCl and are mean ± S.E.M. of 6 experiments. * indicates $p<0.05$, one-way ANOVA v control followed by a Sidak's post hoc test v control.

Figure 4: Role of adiponectin in PVAT-induced vasorelaxtion. Effect of pre-incubation with an anti-adiponectin antibody on PVAT (0.3 g) –induced vasorelaxation in female PCAs. Data are expressed as a percentage change from the U46619-induced tone and are mean ± S.E.M. of 4 experiments. * indicates $p<0.05$, 2-tailed, Student's paired t-test.

Figure 5: Expression and release of adiponectin from PVAT. (A) Expression of adiponectin (30 kDa) and β-actin (42 kDa) levels in 20 μg of PVAT from female (F1-F6) and male (M1-M5) pigs. **(B)** Bar chart showing density of adiponectin bands normalised to b-actin levels, in PVAT from female and male animals. Data are expressed as mean ± SEM. (**C)** Comparison of the levels of adiponectin in the buffer after incubation with PVAT from female or male pigs for 1 hours. Data are expressed as a ratio of adiponectin released to whole weight of PVAT (0.15 g) and are mean ± S.E.M. of 6 female and 6 male PVAT samples.

Figure 6: AdipoRon-induced arterial relaxation. Log concentration-response curves to adipoRon in coronary arteries from female and male pigs. Data are expressed as a percentage change from U46619-induced tone and are mean ± S.E.M. of 6 female and 8 male experiments. * indicates $p<0.05$, (2-way ANOVA followed by a Sidak's post-hoc test).

Figure 7: Expression of adiponectin receptors and adiponectin binding protein. (A) Expression of adipo 1 receptor (40 kDa) and β-actin (42 kDa) levels in 20 μg of PCA from female (F1-F6) and male (M1-M5) pigs. Porcine skeletal muscle is used as a positive control. **(B)** Expression of adipo 2 receptor (42 kDa) and myosin light chain (MLC) (20 kDa) levels in 20 μg of PCA from female (F1-F5) and male (M1-M4) pigs. Rat liver is used as a positive control. **(C)** Bar chart showing density of adipo 2 receptor band normalised to MLC levels, in PCA from female and male animals. Data are expressed as mean ± SEM. **(D)** Expression of APPL1 (82 kDa) and β-actin (42 kDa) levels in 20 μg of PCA from female (F1-F5) and male (M1-M5) pigs. **(E)** Bar chart showing density of APPL1 band normalised to β-actin levels, in PCA from female and male animals. Data are expressed as mean ± SEM.

Figure 1:

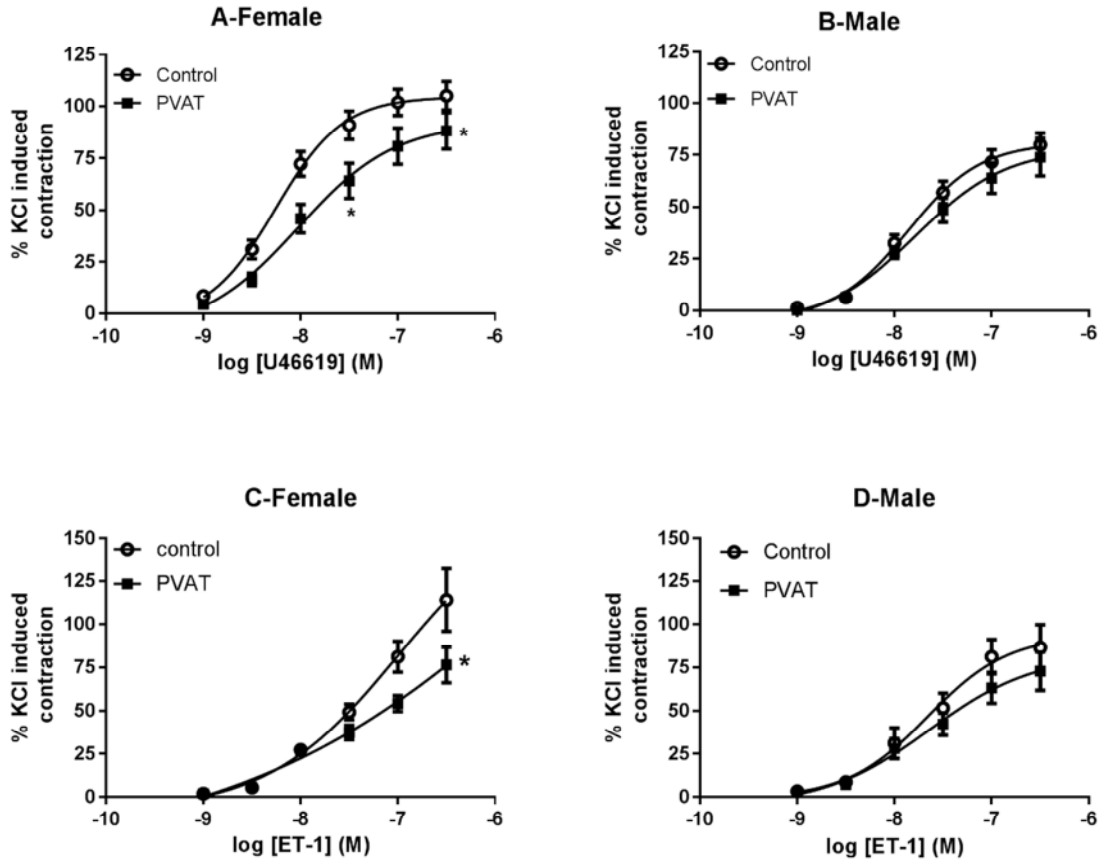

A-Female
Control
PVAT
% KCl induced contraction
log [U46619] (M)
B-Male
Control
PVAT
% KCl induced contraction
log [U46619] (M)
C-Female
control
PVAT
% KCl induced contraction
log [ET-1] (M)
D-Male
Control
PVAT
% KCl induced contraction
log [ET-1] (M)

Figure 2:

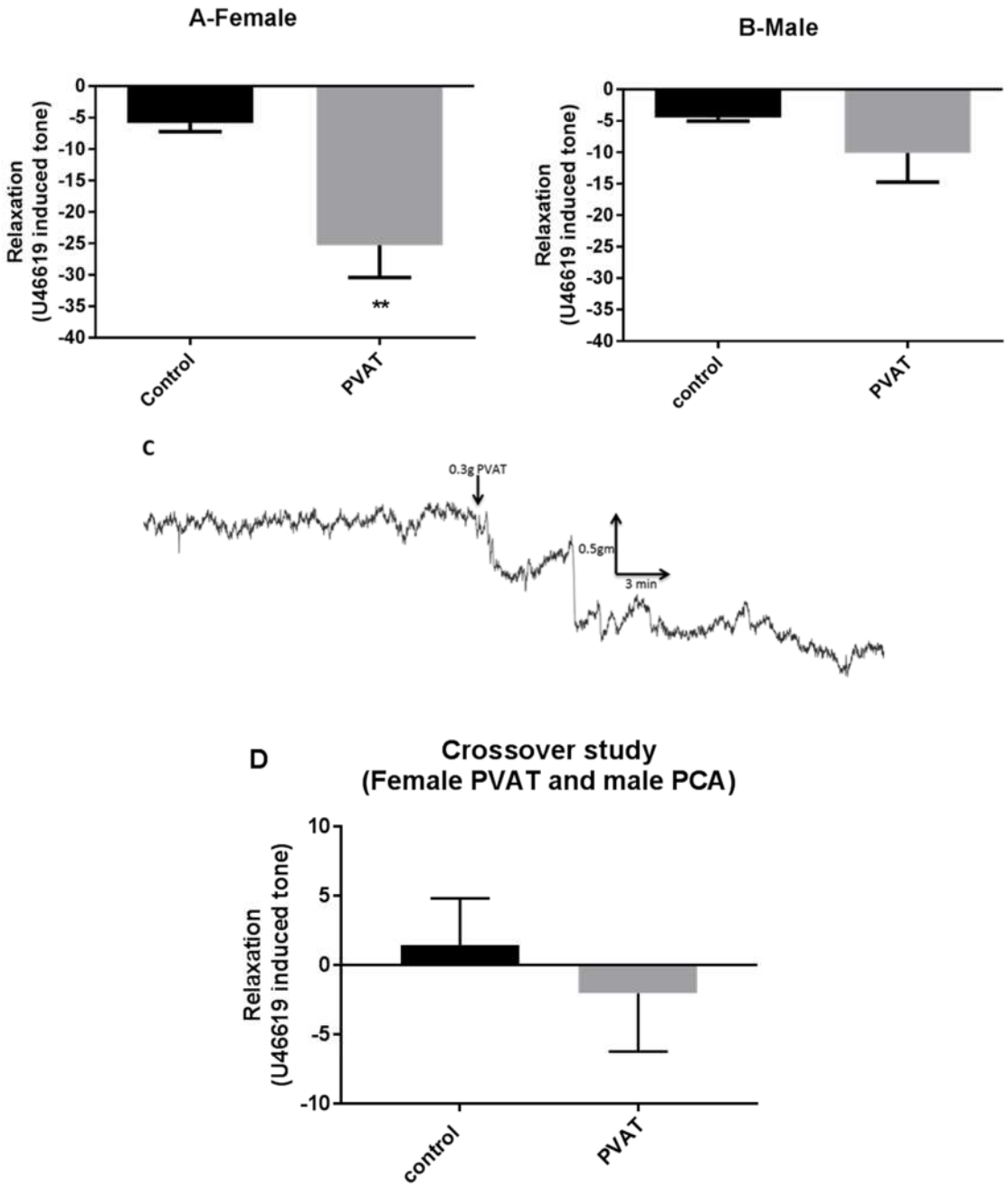
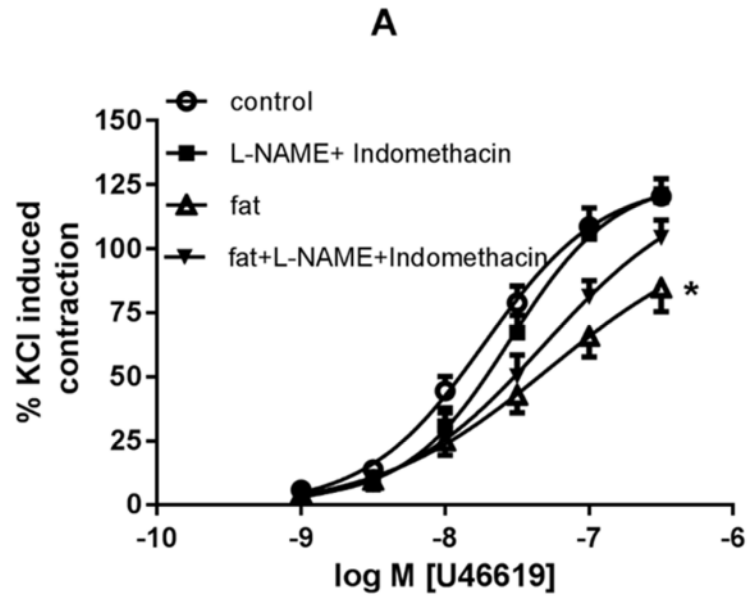

A-Female
Relaxation (U46619 induced tone)
0
-5
-10
-15
-20
-25
-30
-35
-40
**
Control
PVAT
B-Male
Relaxation (U46619 induced tone)
control
PVAT
C
0.3g PVAT
0.5gm
3 min
D
Crossover study
(Female PVAT and male PCA)
Relaxation (U46619 induced tone)
10
5
0
-5
-10
control
PVAT

Figure 3:


A
control
L-NAME+ Indomethacin
fat
fat+L-NAME+Indomethacin
% KCl induced contraction
log M [U46619]
150
125
100
75
50
25
0
-10
-9
-8
-7
-6
*

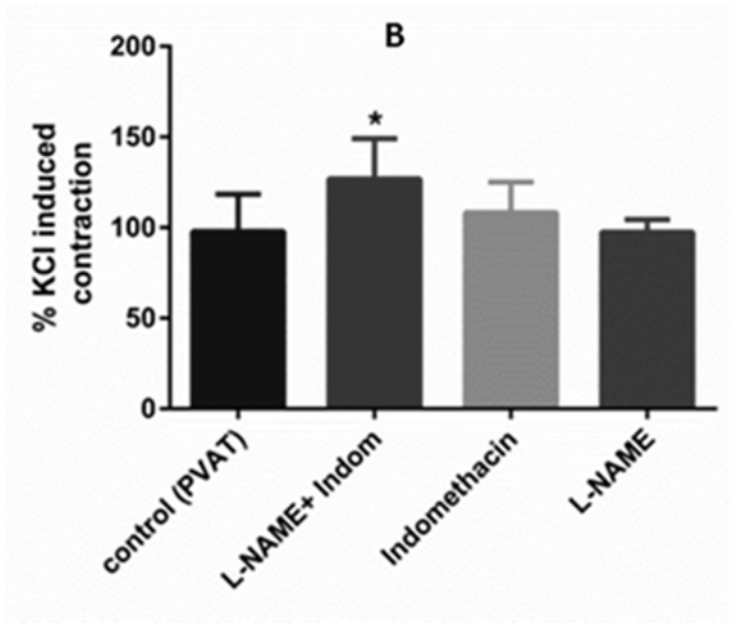

B
% KCl induced contraction
200
150
100
50
0
*
control (PVAT)
L-NAME+ Indom
Indomethacin
L-NAME

Figure 4:

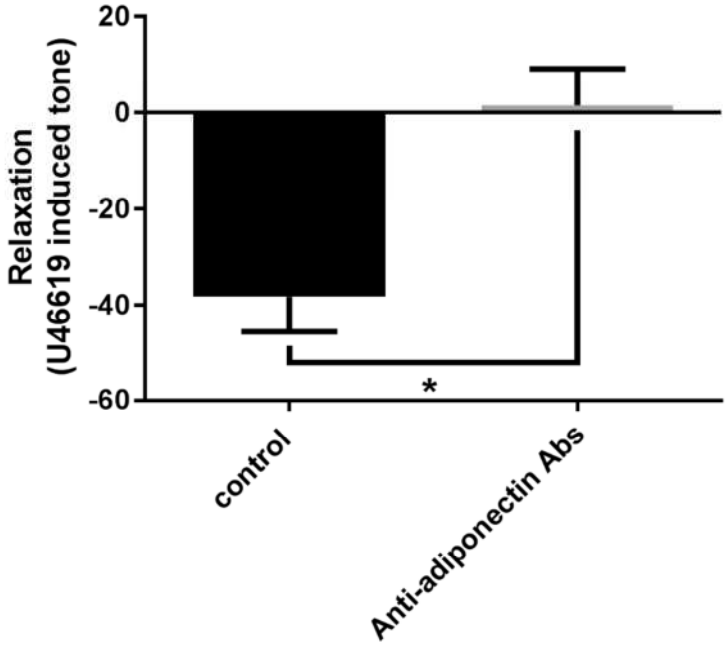
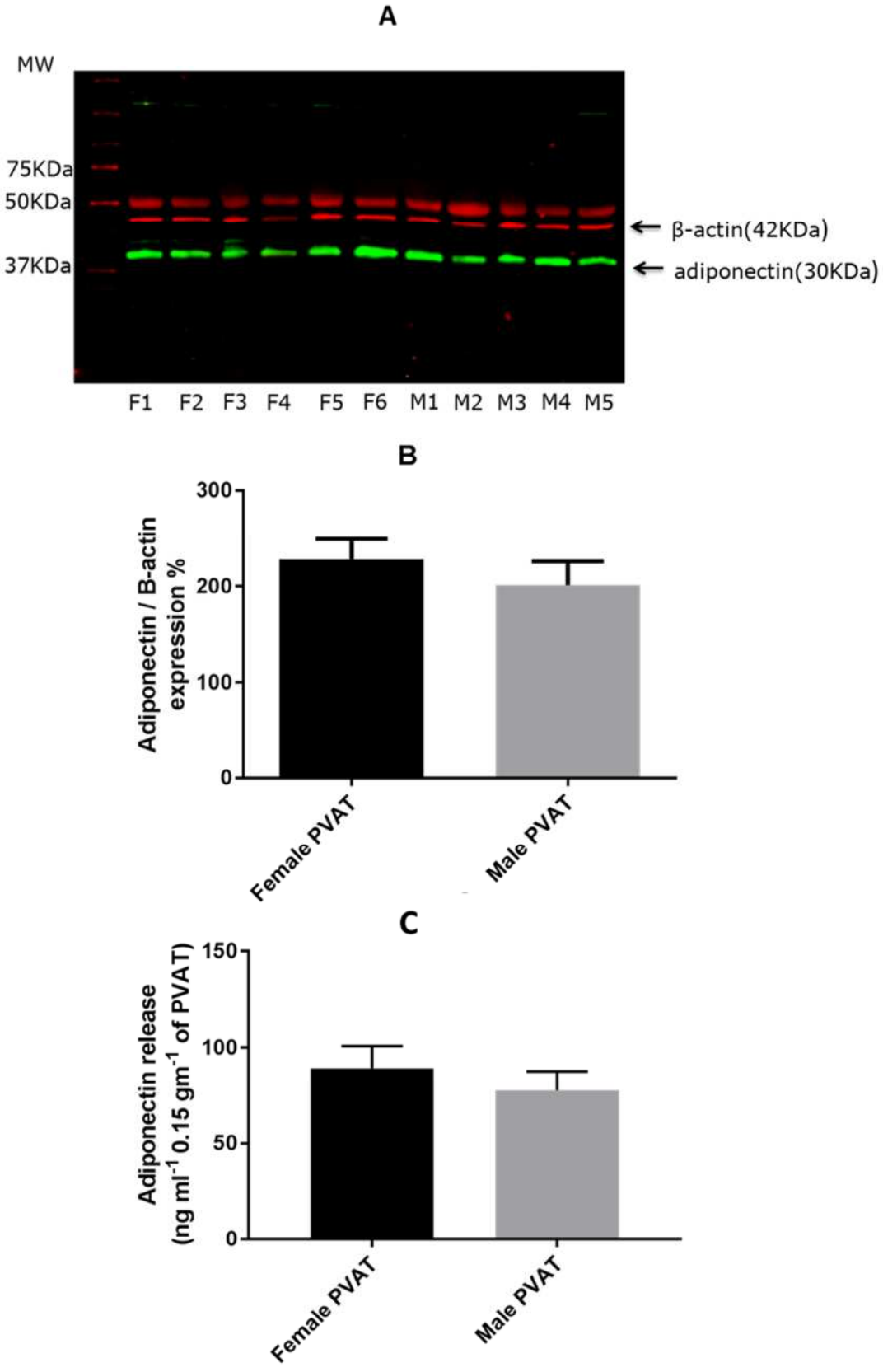

20
0
-20
-40
-60
Relaxation
(U46619 induced tone)
*
control
Anti-adiponectin Abs

Figure 5:


A
MW
75KDa
50KDa
37KDa
β-actin(42KDa)
adiponectin(30KDa)
F1 F2 F3 F4 F5 F6 M1 M2 M3 M4 M5
B
Adiponectin / B-actin expression %
300
200
100
0
Female PVAT
Male PVAT
C
Adiponectin release (ng ml-1 0.15 gm-1 of PVAT)
150
100
50
0
Female PVAT
Male PVAT

1

2

3

Figure 6:

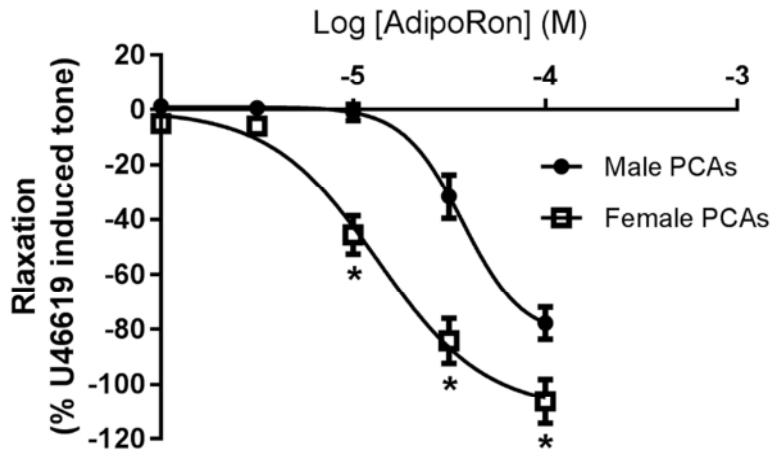
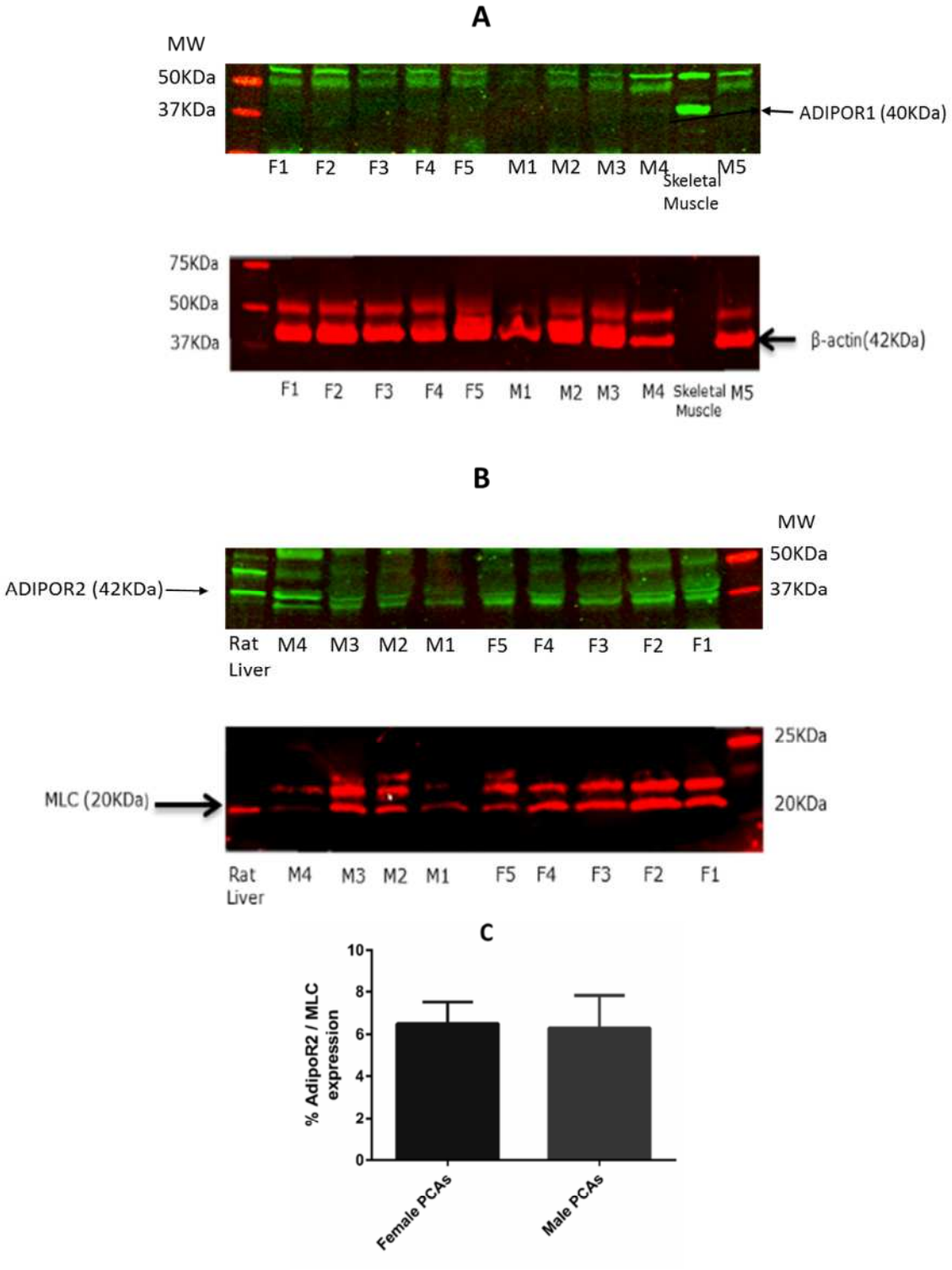
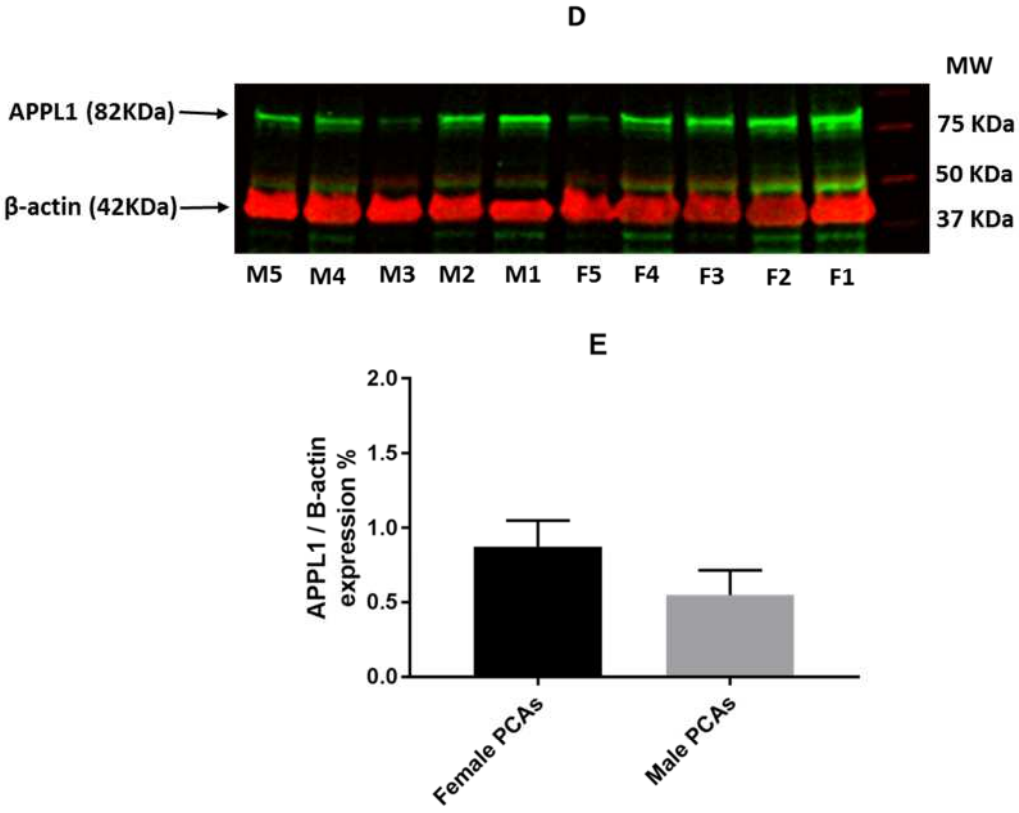

Log [AdipoRon] (M)
20
0
-20
-40
-60
-80
-100
-120
-5
-4
-3
Rlaxation
(% U46619 induced tone)
Male PCAs
Female PCAs
*
*
*

Figure 7:


A
MW
50KDa
37KDa
ADIPOR1 (40KDa)
F1 F2 F3 F4 F5 M1 M2 M3 M4 Skeletal Muscle M5
75KDa
50KDa
37KDa
β-actin(42KDa)
F1 F2 F3 F4 F5 M1 M2 M3 M4 Skeletal Muscle M5
B
MW
50KDa
37KDa
ADIPOR2 (42KDa)
Rat Liver M4 M3 M2 M1 F5 F4 F3 F2 F1
25KDa
20KDa
MLC (20KDa)
Rat Liver M4 M3 M2 M1 F5 F4 F3 F2 F1
C
% AdipoR2 / MLC expression
10
8
6
4
2
0
Female PCAs
Male PCAs


D
MW
APPL1 (82KDa)
β-actin (42KDa)
75 KDa
50 KDa
37 KDa
M5 M4 M3 M2 M1 F5 F4 F3 F2 F1
E
APPL1 / B-actin expression %
2.0
1.5
1.0
0.5
0.0
Female PCAs
Male PCAs

1

2

Supplementary Figure 1:

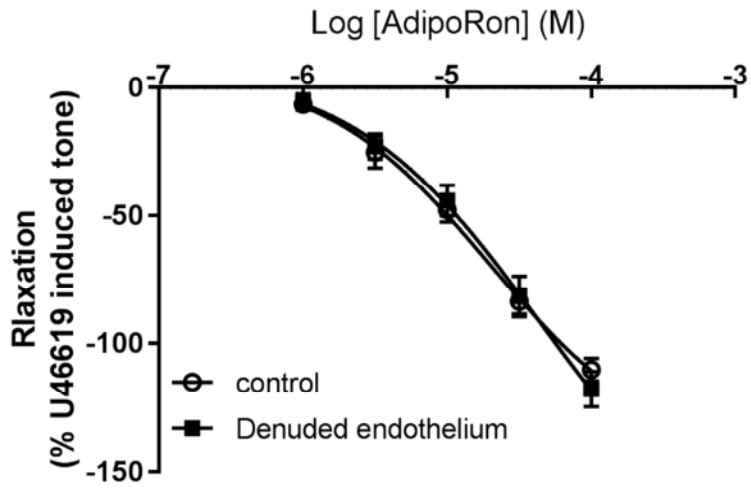


Figure 1: Role of the endothelium in adipoRon-induced arterial relaxation. Log concentration-response curves to adipoRon in intact (control) or endothelium denuded coronary arteries from female and male pigs. Data are expressed as a percentage change from U46619-induced tone and are mean ± S.E.M. of 8 experiments.