β_3-adrenoceptor stimulation of perivascular adipocytes leads to increased fat cell-derived nitric oxide and vascular relaxation in small arteries

Running Title: Vasodilator PVAT function in small arteries

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Abstract

BACKGROUND AND PURPOSE

In response to norepinephrine healthy perivascular adipose tissue (PVAT) exerts an anticontractile effect on adjacent small arterial tissue. Organ bath solution transfer experiments have demonstrated the release of PVAT-derived relaxing factors that mediate this function. The present studies were designed to investigate the mechanism responsible for the norepinephrine-induced PVAT anticontractile effect.

EXPERIMENTAL APPROACH

In vitro rat small arterial contractile function was assessed using wire myography in the presence and absence of PVAT and the effects of sympathomimetic stimulation on the PVAT environment explored using Western blotting and assays of organ bath buffer.

KEY RESULTS

PVAT elicited an anticontractile effect in response to norepinephrine but not phenylephrine stimulation. In arteries surrounded by intact PVAT, the β_3-adrenoceptor agonist, CL-316,243 reduced the vasoconstrictor effect of phenylephrine but not norepinephrine. K_v7 channel inhibition using XE 991 reversed the norepinephrine-induced anticontractile effect in exogenously applied PVAT studies. Adrenergic stimulation of PVAT with norepinephrine and CL-316,243, but not phenylephrine was associated with increased adipocyte-derived nitric oxide production and the contractile response to norepinephrine was augmented following incubation of exogenous PVAT with L-NMMA. PVAT from $eNOS^{-/-}$ mice had no anticontractile effect. Assays of adipocyte cAMP demonstrated an increase with norepinephrine stimulation implicating $G\alpha_s$ signalling in this process.

CONCLUSIONS AND IMPLICATIONS

We have shown that adipocyte-located β_3-adrenoceptor stimulation leads to activation of $G\alpha_s$ signaling pathways with increased cAMP and the release of adipocyte-derived nitric oxide. This process is dependent upon K_v7 channel function. We conclude that adipocyte-derived nitric oxide plays a central role in anticontractile activity when rodent PVAT is stimulated by norepinephrine.

Abbreviations

CO_2, *carbon dioxide*; eNOS, *endothelial nitric oxide synthase*; KPSS, *high potassium physiological salt solution*; L-NMMA, N^G*-monomethyl-L-arginine*; MA, mesenteric artery; NE, *norepinephrine*; nNOS, *neuronal nitric oxide synthase;* PDRF, *PVAT-derived relaxing factor*; Phe, *phenylephrine*; PKA, *protein kinase A*; PSS, *physiological salt solution*; PVAT, *perivascular adipose tissue*; VEGF, *vascular endothelial growth factor*; XE 991, *10,10-bis(4-Pyridinylmethyl)- 9(10H)-anthracenone dihydrochloride*.

Additional Information

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Conflicts of interests

None

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Introduction

Perivascular adipose tissue (PVAT) surrounds virtually all blood vessels and exerts an anticontractile effect in response to various vasoconstrictor agonists (Gao *et al.*, 2007; Greenstein *et al.*, 2009). The mechanisms by which PVAT exerts this effect are the subject of intense investigations and what is clear already from organ bath studies is that adipocytes release transferable relaxing factor(s) (Lohn *et al.*, 2002; Greenstein *et al.*, 2009). However, the precise identity of these is unclear.

Recent evidence has indicated that β_3-adrenoceptors may be involved in the release of PVAT-derived relaxing factors (PDRFs) provoked by sympathomimetic hormones: Weston *et al.* (2013) demonstrated that the specific β_3-agonist, CL-316,243, induced endothelium-independent but PVAT-dependent myocyte hyperpolarization in rat mesenteric artery via the opening of BK_{Ca} channels.

Vascular smooth muscle cell potassium channels are key regulators of membrane potential, with increased or decreased activity resulting in hyperpolarization or depolarization, respectively, meaning that they act as 'off switches' in excitable cells and occupy a central position in the regulation of vascular tone (Ghatta *et al.*, 2006). Myocyte membrane potential is hyperpolarized in the presence of PVAT and substantial evidence points towards a role of potassium channels in the anticontractile mechanism. Pharmacological investigations have led to the identification of several potassium channel candidates including K_v7 (Fésüs *et al.*, 2007; Schleifenbaum *et al.*, 2010) and BK_{Ca} (Gao *et al.*, 2005; Lynch *et al.*, 2013), which appear to undergo differential activation depending on the vascular bed and species investigated. Collectively these studies indicate the emerging importance of PVAT, which appears to exert its anticontractile effect through a variety of complex pathways that may be activated in parallel in response to different agonists.

Therefore, the present studies were designed to investigate the mechanisms responsible for the noradrenaline-induced PVAT anticontractile effect. We report that β_3-adrenoceptor stimulation of adipocytes leads to $G\alpha_s$ signalling, a rise in cAMP and K_v7 channel activation. The PVAT-derived relaxing factor is nitric oxide.

Methods

Group sizes

For all experiments, the group size is provided within the figure legends. For all experiments the number of animals is reported. Assays for cAMP and nitric oxide were performed in duplicate and an average for each animal used for calculation of mean data.

Randomisation

All rats used in this study were healthy adult male Sprague Dawley rats bred at Charles River, UK. Rats were randomly assigned to a numbered cage upon delivery from Charles River and the animals were used when they reached an appropriate size (250-300 g). The pharmacological protocol to be used in each myograph bath was determined prior to mounting the vessels.

Blinding

Experimental blinding was not used for this study as there was one core experimenter responsible for dissection, experimental protocols and analysis. In order to limit experimental bias, data analysis was not performed until experimental data sets were complete. Duplicate data from a minimum of five separate animals was required for all experimental protocols described.

Normalisation

Contractile responses are expressed as mean percentages of KPSS constriction ± standard error of the mean (S.E.M) consistent with previously published data (Greenstein *et al.*, 2009; Withers *et al.*, 2011). This allowed for comparison in contractile changes independent of vessel size.

Statistical Comparison

The data and statistical analysis comply with British Journal of Pharmacology guidelines (Curtis *et al.*, 2015). GraphPad Prism (v6, GraphPad Software, USA) was used for all statistical analyses and P values < 0.05 were considered statistically significant. Contractile responses are expressed as mean percentages of KPSS constriction ± standard error of the mean (S.E.M) consistent with previously published data (Greenstein *et al.*, 2009; Withers *et al.*, 2011) and curves fitted using non-linear regression analysis. $LogEC_{50}$ values were calculated from individual concentration-response curves fitted

using non-linear regression analysis with a sigmoidal function. Data were analysed using two-way ANOVA following by Bonferroni *post hoc* test or by unpaired or paired t-test as appropriate.

Validity of animal model

Male Sprague Dawley rats are a well-established model for the investigation of PVAT function and vascular contractility (Greenstein et al., 2009; Aghamohammadzadeh et al., 2013). The eNOS deficient ($eNOS^{-/-}$) mouse model is well published and commercially available. This model was chosen to confirm the role of eNOS in our proposed mechanism. We have previously shown comparable response of mouse and rat mesenteric arteries to adrenergic stimulation (Withers *et al.*, 2011). Myography protocols detailed below have been validated within our laboratory for rat, mouse and human tissues (Greenstein *et al.*, 2009). Antibodies which were selective to rat antigens were chosen for immunohistochemistry and western blot studies.

Ethical statement

Experiments involving the use of animals were performed in accord with the UK Animals (Scientific Procedures) Act 1986 under the authority of a valid project license and were conducted in accordance with the ARRIVE guidelines. The experiments were approved by The University of Manchester Ethical Review Process. All work was carried out in accordance with the Declaration of Helsinki.

Animals

Healthy male Sprague Dawley rats (250 – 300 g, Charles River, UK) were euthanized by CO_2 inhalation with death confirmed by permanent cessation of the circulation by severing the diaphragm. The mesenteric bed was immediately removed and placed in ice-cold physiological salt solution (PSS in mmol/L: 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.17 KH_2PO_4, 0.03 K_2 EDTA, 5.5 glucose and 1.6 $CaCl_2$).

Male eNOS deficient ($eNOS^{-/-}$) mice were kindly provided by Dr Elizabeth Cottrell (Manchester, UK). $eNOS^{-/-}$ mice were bred in-house at University of Manchester and used at 12-weeks old. As a control for eNOS-/- mice, age matched male C57BL6/j mice

were purchased from Envigo, UK. Mice were euthanized as described above. Mesenteric artery samples obtained and studied as described below.

Wire myography

Small mesenteric arteries (internal diameter: 200 – 300 µm) both with and without PVAT were dissected and mounted on two 40 µm wires in a wire myograph (Danish MyoTech, Denmark). The vessels were gassed (95% air/ 5% CO_2) and maintained in PSS at 37°C for 30 minutes before wall tension and diameter were normalised using a standardised procedure (Mulvany & Halpern, 1977). Isometric tension was continuously recorded (Chart 5, v5.5, AD Instruments, UK). Vessels were challenged with 60 mmol/L high potassium PSS (KPSS in mmol/L: 63.7 NaCl, 60 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.17 KH_2PO_4, 0.03 K_2 EDTA, 5.5 glucose and 1.6 $CaCl_2$) to establish viability and functional endothelial integrity was assessed by relaxation to 10 µmol/L carbachol following stable constriction with 10 µmol/L norepinephrine.

When investigating the role of the endothelium, endothelium was removed by inserting equine hair into the lumen. Success in removing endothelium was tested using 10 µmol/L carbachol as above.

Pharmacological assessment

Pharmacological protocols were used to study the mechanisms involved in mediating the PVAT anticontractile effect. In each case, segments from the same artery were prepared with and without PVAT and incubated either with PSS as a vehicle control or PSS containing the pharmacological tool for 30 minutes. The role of β_3-adrenergic signalling was evaluated by incubation of vessels with the β_3-adrenoceptor agonist, CL-316,243 or the β_3-adrenoceptor antagonist, SR 59230A. The contribution of nitric oxide to PVAT function was assessed by incubation with the non-specific NOS inhibitor, L-NMMA (Aghamohammadzadeh *et al.*, 2013), and Vinyl-L-NIO, which has been shown to be selective for neuronal NOS (nNOS) at a concentration of 100 nmol/L (Babu & Griffith, 1998). Protocols investigated the contribution of K^+ channels through incubation with the the K_v7 channel inhibitor- XE 991 (Li *et al.*, 2013).

Following incubation with pharmacological tools, concentration-responses were constructed through the cumulative addition of norepinephrine or phenylephrine to the myograph bath ($1 \times 10^{-7} - 3.5 \times 10^{-5}$ mol/L). The use of these two vasoconstrictor agonists allowed the role of β-adrenergic signaling to be investigated as norepinephrine has equal affinity for α and β-adrenoceptors, whereas phenylephrine has higher affinity for α-adrenoceptors (Minneman *et al.*, 1994).

Solution transfer experiments

Bioassay experiments were used to study the release of relaxing factors from PVAT (Lohn *et al.*, 2002; Gao *et al.*, 2007; Greenstein *et al.*, 2009). PVAT with and without its adjacent artery was used as a donor, whereas arteries stripped of PVAT were used as recipients. Both the PVAT and recipient arteries were stimulated with 30 µmol/L norepinephrine and once a stable constriction had developed solutions were removed from both baths and transferred from the bath containing the donor PVAT to the recipient artery without PVAT. The change in tension in the recipient artery was recorded. In order to investigate the contribution of external calcium to the anticontractile mechanism, donor PVAT with and without arteries was stimulated in PSS with (1.6 mmol/L) and without calcium. In experiments were PVAT was incubated in calcium-free PSS, the calcium concentration of the solution was restored prior to addition to the recipient artery.

Exogenous PVAT experiments

Concentration-responses to norepinephrine were also constructed in vessels devoid of PVAT but with PVAT suspended on a wire within the myograph bath (exogenous PVAT) to enable examination of the release of release of relaxing factors from PVAT in response to different vasoconstrictor agonists. In some experiments, exogenous PVAT was incubated with various pharmacological tools for 30 minutes before the return of PVAT to the myograph bath to enable further investigation of PVAT mechanisms. Exogenous PVAT was incubated with forskolin to examine the effects of Gαs activation, db-cAMP to assess the role of cAMP, L-NMMA and Vinyl-L-NIO to explore the role of PVAT-derived nitric oxide and XE 991 to investigate $K_v7$activation within PVAT.

Stimulation of PVAT

PVAT samples (~200 mg) were dissected from small mesenteric arteries and incubated in HEPES buffer (pH 7.4, in mmol/L: 127 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 10 HEPES, 2.4 $CaCl_2$ and 11.8 glucose) in the absence (control) or presence of norepinephrine (10 µmol/L) or CL-316,243 (10 µmol/L) for 30 minutes at 37°C. The solution surrounding the PVAT (supernatant) was removed and both tissue and supernatant were snap frozen in liquid nitrogen and stored separately at -80°C until use.

Western blot analysis

In order to investigate the effects of norepinephrine-stimulation on downstream signalling within PVAT, western blot analysis was performed on unstimulated and stimulated PVAT lysate. PVAT samples were homogenised in RIPA buffer containing protease and phosphatase inhibitors using a FastPrep-24 5G instrument with lysing matrix 5 tubes. Protein concentrations were determined using a Bradford assay. Samples (40 µg of protein) were separated using SDS-PAGE electrophoresis on a 12% stain-free SDS-PAGE gel (Bio-Rad Laboratories, UK). Following electrophoresis, samples were transferred to a low-fluorescence PVDF membrane using a Trans-Blot Turbo system and transfer was verified using stain-free imaging. Non-specific binding sites were blocked with 5% BSA in TBS-Tween (0.1% Tween), followed by overnight incubation with primary antibody at 4°C. Specific primary antibodies against AMPKα (0.27 µg/ml), phosphorylated-AMPKα (0.26 µg/ml), β_3-adrenoceptor (5 µg/ml), eNOS (1 µg/ml) were used. This was followed by three 10 minute washes in TBS-Tween at room temperature and exposure to secondary antibody (Donkey anti-rabbit HRP, 0.8 µg/ml) for 1 hour at room temperature. Protein reactivity was detected using Clarity Western ECL substrate chemiluminescent detection reagent and captured digitally using the ChemiDoc MP imaging system. Stain-free technology was used as a normalisation tool as it allowed normalisation to total protein levels through quantification of signal intensity within the whole sample (Ferguson *et al.*, 2005; Gurtler *et al.*, 2013). The chemiluminescent signal intensity of a given protein was normalised to the relative quantification of the corresponding intensity of the total protein and changes in expression were expressed as the fold increase over unstimulated controls, assigning a value of 1 to the control. Data from each replicate were normalised and then averaged across the replicates and data are plotted as mean ± S.E.M.

Immunohistochemistry

PVAT samples were placed in 4% paraformaldehyde for 18 hours and subsequently processed to paraffin wax blocks prior to serial sectioning at 5 µm. Samples were de-waxed using xylene and rehydrated with ethanol prior to immunostaining for $K_v7.4$ expression (4 µg/ml). Heat-induced antigen retrieval was performed followed by blocking of endogenous peroxidase using 3% H_2O_2 and block of non-specific binding sites using goat serum. Sections were incubated with anti-$K_v7.4$ antibody overnight, followed by one hour incubation with Biotin-SP conjugated anti-rabbit secondary antibody (1.5 µg/ml) and detection using Vectastain ABC complex followed by the addition of 3,3'-diaminobenzidine (DAB) solution to allow visualisation of antibody binding. Images were captured using a colour camera (Leica DFC450) mounted on a microscope (Leica DM5000).

cAMP assay

Following stimulation, PVAT samples were homogenised using a FastPrep-24 5G instrument with lysing matrix 5 tubes in ice-cold 0.1 mol/L hydrochloric acid at a 1:5 ratio (*w/v*) to inactivate any phosphodiesterases within the sample. Samples were then centrifuged at 1000 x *g* for 10 minutes to remove insoluble cellular debris and neutralised with 1 mol/L sodium hydroxide. Samples were diluted 100-fold in calibrator diluent before intracellular cAMP levels were determined using the competitive enzyme immunoassay cAMP parameter assay kit.

Nitric oxide levels within PVAT supernatant

Nitric oxide within PVAT supernatant was converted to detectable nitrite by three hour incubation with nitrate reductase (0.17 $U.ml^{-1}$), glucose-6-phosphate dehydrogenase (4.17 U/ml), glucose-6-phosphate (500 µmol/L) and NADPH (1.67 µmol/L) as described previously (Verdon *et al.*, 1995). A Griess reagent kit was then used to determine total nitrite concentration.

Total adiponectin ELISA of PVAT supernatant

Total adiponectin levels in stimulated and non-stimulated PVAT supernatant samples were measured using a commercially available ELISA kit. Stimulated and unstimulated supernatant samples were diluted 1:750 in calibrator diluent before the assay was performed according to the manufacturer's instructions.

Materials

Myography: All salts for physiological salt solutions, norepinephrine, phenylephrine and carbachol were purchased from Sigma-Aldrich, UK. All other pharmacological tools were purchased from R&D Systems, UK.

Western blot analysis: General consumables were purchased from Sigma-Aldrich, UK. Reagents for RIPA buffer were purchased from Roche Diagnostics, UK. FastPrep-24 5G instrument was purchased from MP Biomedicals Inc. Antibodies were purchased as following AMPKα (Cell Signalling Technology, Cat # 2603 RRID: AB_490795) and phosphorylated-AMPKα (Thr172) (Cell Signalling Technology, Cat # 2535, RRID: AB_331250), β3-adrenoceptor (Novus Biologicals, NBP1-00716, RRID:AB_1502640), eNOS (Santa Cruz Biotechnology, Cat # sc-654 RRID: AB_631423) were used. Donkey anti-rabbit HRP, (Jackson ImmunoResearch Laboratories, Cat# 711-035-152, RRID:AB_10015282). All other western blot materials were purchased from Bio-Rad Laboratories, UK.

Immunohistochemistry: General consumables were purchased from Sigma-Aldrich, UK. Antibodies purchased as follows, Kv7.4 (Santa Cruz Biotechnology, Cat # sc-50417, RRID: AB_2131729), Biotin-SP conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, USA, Cat# 711-065-152, RRID: AB_2340593), Vectastain ABC complex and 3,3'-diaminobenzidine (DAB) solution were purchased from Vector Laboratories, UK (ABC: Cat# PK-6100, RRID:AB_2336819 DAB: SK-4100, RRID:AB_2336382).

Assays: All general consumables were purchased from Sigma-Aldrich, UK. The Griess reagent kit was purchased from Promega, USA. The cAMP, adiponectin and adipokine assay kits were from R&D systems, USA.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY.

Results

PVAT produces a transferable anticontractile factor

In endothelium-intact segments of rat mesenteric small arteries, the vasoconstrictor response to norepinephrine (Figure 1A) but not phenylephrine was reduced in the presence of PVAT (Figure 1B). The vasoconstrictor response to norepinephrine was reduced in vessels lacking PVAT with exogenous PVAT suspended in the bath (Figure 1A) suggesting that the anticontractile effect was due to secretion of a soluble factor.

Transfer of solutions from donor PVAT with and without an artery stimulated with norepinephrine to a recipient artery lacking PVAT pre-contracted with norepinephrine produced a significant reduction in tension (Figure 1C) confirming that the anticontractile effect was due to secretion of a soluble factor. Also, relaxation occurred following incubation of donor PVAT with and without an artery in calcium-free external PSS solution and transfer to a recipient artery, indicating that the anti-contractile effect is calcium-independent (Figure 1D).

In endothelium-denuded arteries, the vasoconstrictor response to norepinephrine was not reduced by the presence of PVAT (Figure 1E), confirming that the anticontractile factor is endothelium-independent.

Adipocyte located β_3-adrenergic activation produces a PVAT-derived anticontractile effect via $G\alpha_s$ signaling

Western blot analysis indicated the presence of β_3-adrenoceptors within the PVAT of rat mesenteric arteries (Figure 2A), therefore we investigated the contribution of β_3-adrenergic signaling to the anticontractile effect. The presence of CL-316,243 had no effect on vasoconstrictor response to norepinephrine (Figure 2B) but reduced the effect of phenylephrine (Figure 2C), indicating a role for β_3-adrenoceptor activation in mediating the anticontractile effect of norepinephrine on PVAT. Incubation of vessels with intact endothelium but lacking PVAT with CL-316,243 had no effect on the vasoconstrictor response to either of the agonists tested (norepinephrine: *P* = NS, data not shown; phenylephrine: *P* = NS, Figure 2D) confirming that the changes observed are a consequence of β_3-adrenoceptor activation within PVAT. Moreover, the β_3-adrenoceptor antagonist, SR 59230A, increased the contractile response to norepinephrine in vessels with PVAT (Figure 2E) but had no effect on the response to phenylephrine (Figure 2F).

Incubation of exogenous PVAT with forskolin had no effect on the vasoconstrictor response to norepinephrine (Figure 3A) but reduced the effect of phenylephrine (Figure 3B). Incubation with db-cAMP had no effect on the vasoconstrictor response to norepinephrine (Figure 3C) but reduced the vasoconstrictor response to phenylephrine (Figure 3D). Intracellular cAMP levels were increased from 250 ± 33 pmol/mL to 467.4 ± 54 pmol/mL within PVAT following stimulation with CL-316,243 indicating that β_3-adrenoceptor stimulation involves $G\alpha_s$ signaling (Figure 3E).

K_v7 channel activation contributes to norepinephrine-mediated anticontractile capacity

Incubation with the K_v7 inhibitor, XE 991, did not alter the vasoconstrictor response to norepinephrine in vessels lacking PVAT (Figure 4A) but potentiated the response to norepinephrine in vessels with PVAT (Figure 4B). Incubation of exogenous PVAT with XE 991 prior to placement within the myograph bath reversed the anticontractile effect of PVAT (Figure 4C) indicating that K_v7 channel activation **within** PVAT contributes to the norepinephrine-induced anticontractile capacity. Immunostaining for $K_v7.4$shows

K_v7.4 expression within PVAT, the endothelial layer and the adventital layer of the mesenteric artery (Figure 4D).

Nitric oxide produced by adipocytes plays a key role in the anticontractile effect.

Western blot analysis demonstrated higher eNOS expression within PVAT than the mesenteric artery (Figure 5A). Incubation with the NOS inhibitor, L-NMMA, did not alter the vasoconstrictor response to norepinephrine in vessels with intact endothelium but lacking PVAT (Figure 5B) but the vasoconstrictor response was increased in the presence of PVAT (Figure 5C). Incubation of exogenous PVAT with L-NMMA prior to placement within the myograph bath increased the vasoconstrictor response to norepinephrine (Figure 5D) indicating that PVAT-derived nitric oxide may contribute to the norepinephrine-induced anticontractile capacity. Incubation with D-NMMA, an inactive enantiomer of L-NMMA, had no effect on the contractile response of vessels with PVAT (Figure 5E). Incubation of exogenous PVAT with nNOS inhibitor Vinyl-L-NIO had no effect on the contractile response (Figure 5F), indicating that eNOS may be the NOS isoform responsible for nitric oxide release from PVAT. Moreover, the anticontractile effect of PVAT on the vasoconstrictor response to norepinephrine present in C57BL6/j mice was absent in $eNOS^{-/-}$, mice indicating that eNOS activation contributes to the release of nitric oxide from PVAT (Figure 6).

β_3-adrenoceptor activation within PVAT does not alter adiponectin release

Abundant amounts of adiponectin were found to be basally secreted from PVAT; however this was unaltered following stimulation with either norepinephrine or CL-316,243 (Figure 7).

Discussion

The present study investigated the mechanisms of the norepinephrine-induced PVAT anticontractile effect. The main findings were that (1) β_3-adrenergic signaling contributes to the PVAT anticontractile effect; (2) K_v7 channel activation ***within*** PVAT contributes

to release of a PVAT-derived relaxing factor and (3) PVAT-derived nitric oxide plays a key role in the norepinephrine-induced PVAT anticontractile effect.

Consistent with previously published data (Briones *et al.*, 2005; Garland *et al.*, 2011; Flacco *et al.*, 2013), we demonstrated no functional role of β_3-adrenergic signaling in the contractile response to norepinephrine in vessels lacking PVAT. This suggests that the effects observed in the present study were a consequence of β_3-adrenoceptor activation within the PVAT. This is supported by our previous observations of β_3-adrenoceptor expression within mouse mesenteric PVAT, and the effects of adipocyte β_3-adrenoceptor inhibition on vessel tension (Saxton *et al.*, 2018). In this study, we report a similar β_3-adrenoceptor mediated mechanism in rat PVAT, and have for the first time identified PVAT-derived nitric oxide as a relaxing factor released upon β_3-adrenoceptor activation.

Adipocyte β_3-adrenoceptors are likely activated by sympathetically-derived NA. The role of β_3-adrenoceptors in catecholamine induced lipolysis has been well characterized (Rebuffe-Scrive, 1991; Robidoux *et al.*, 2006; Bartness *et al.*, 2014), and the origin of sympathetic fibers in epididymal and inguinal fat depots have been extensively studied using viral transneuronal tract tracers (Bamshad *et al.*, 1998; Song *et al.*, 2009; Nguyen *et al.*, 2014). Denervation of these fat depots will lead to a decrease in lipolysis (Rooks *et al.*, 2005; Foster & Bartness, 2006), however until recently the importance of sympathetic nerves in PVAT was unknown. Previously, using immunohistochemistry we have confirmed the presence of sympathetic nerves in mouse mesenteric PVAT (Saxton *et al.*, 2018). In addition, we confirmed a functional role for sympathetic nerves in PVAT, as pharmacological sympathetic denervation of PVAT abolished the anticontractile effect. Therefore, it is sympathetically -derived NA which activates β_3-adrenoceptors, triggering the release of a relaxing factor.

Dual coupling of β_3-adrenoceptors to both $G\alpha_s$ and $G\alpha_i$ proteins has been reported in 3T3-F440A adipocytes (Soeder *et al.*, 1999). We highlight an important role of the β_3-adrenoceptor/$G\alpha_s$/cAMP signaling pathway in the PVAT anticontractile capacity as β_3-adrenoceptor stimulation resulted in cAMP production and incubation with forskolin or db-cAMP was able to mimic the PVAT anticontractile effect. This is supported by

previous studies showing β_3-adrenoceptors within cultured adipocytes have a tendency towards $G\alpha_s$ protein coupling following stimulation with physiologically relevant catecholamine concentrations (Soeder *et al.*, 1999; Robidoux *et al.*, 2006).

Our finding that K_v7 channel inhibition does not alter the contractile response to norepinephrine in vessels lacking PVAT was surprising given the key role of K_v7 channels in the regulation of vascular tone (Ng *et al.*, 2011; Chadha *et al.*, 2012). The majority of studies investigating the effect of K_v7 channel inhibition on vascular tone has reported increased myogenicity in the presence of XE 991 rather than investigating its effect on the arterial response to vasoconstrictor agonists or have demonstrated activation/inhibition of currents using single-cell electrophysiology in the absence of spasmogens (Ng *et al.*, 2011; Chadha *et al.*, 2012). Moreover, we found very little $K_v7.4$ expression within the arterial medial layer consistent with the absence of a functional role and our data are supported by an earlier myograph study using rat mesenteric artery (Li *et al.*, 2013).

Substantial evidence suggests a role for voltage-gated K^+ channels, especially K_v7 in mediating the PVAT anticontractile effect in resistance arteries (Verlohren *et al.*, 2004; Schleifenbaum *et al.*, 2010; Zavaritskaya *et al.*, 2013). For the first time, we have shown $K_v7.4$ expression within PVAT and that specific inhibition of K_v7 within PVAT reversed the norepinephrine-induced anticontractile effect in health. This suggests a crucial role for activation of K_v7 located ***within*** PVAT in mediating the release of a relaxing factor in health. These findings contradict previous studies that suggested a role for vascular smooth muscle K_v7 channels in mediating the anticontractile effect (Fang *et al.*, 2009; Schleifenbaum *et al.*, 2010; Zavaritskaya *et al.*, 2013). However, our experimental design allowed specific inhibition of K_v7 within PVAT without affecting smooth muscle channels enabling identification of the tissue location of the channels involved in the PVAT anticontractile effect. Moreover, previous studies did not investigate K_v7 expression within PVAT and on the basis of their results a role for PVAT K_v7 activation could not be excluded.

Our data showing a crucial role for K_v7 within the adipocytes is supported by observations of voltage gated K^+ channels within rat white adipocytes (Ramirez-Ponce *et al.*, 1996) that can be modulated by norepinephrine and cAMP (Ramirez-Ponce *et al.*, 1998). Moreover, evidence has emerged that K_v7 channel activation contributes to the vasodilator response induced by β-adrenoceptor agonists through a mechanism involving cAMP and protein kinase A, because K_v7 channel inhibitor linopirdine was able to inhibit relaxations induced by isoprenaline and forskolin (Chadha *et al.*, 2012) and phosphorylation of $K_v7.4$ by protein kinase A has been reported to shift the activation curve leading to enhanced $K_v7.4$ activity (Chambard & Ashmore, 2005). Taken together with the observed increased cAMP levels, we suggest that β_3-adrenoceptor stimulation within PVAT and subsequent cAMP production leads to K_v7 activation. This leads to the movement of potassium out of the adipocyte down its electrochemical gradient and hyperpolarization that drives calcium entry with subsequent activation of downstream signaling pathways leading to the release of nitric oxide.

Consistent with earlier work, we were able to show that PVAT releases a transferable factor (Lohn *et al.*, 2002; Greenstein *et al.*, 2009; Lynch *et al.*, 2013) and we demonstrate that this factor is adipocyte-derived nitric oxide as the anticontractile effect reversed in the presence of specific PVAT NOS inhibition and stimulation with CL-316,243, but not phenylephrine increased nitric oxide release from PVAT. Consistent with previous studies, the effects of this factor are endothelium-independent (Greenstein *et al.*, 2009; Saxton *et al.*, 2018).

Several studies have demonstrated a link between β_3-adrenergic signaling and nitric oxide production by adipocytes (Canova *et al.*, 2006; Hodis *et al.*, 2011) and recent electrophysiological studies in non-contracted vessels demonstrated that β_3-adrenergic stimulation of PVAT produced myocyte hyperpolarization that could be partially reversed by the presence of L-NMMA (Weston *et al.*, 2013). Moreover, our data support previous findings in human subcutaneous arteries (Greenstein *et al.*, 2009) and mouse mesenteric arteries (Lynch *et al.*, 2013) where NOS inhibition was reported to attenuate PVAT function and we have recently shown a crucial role for activation of protein kinase G, a

downstream signaling component in the nitric oxide pathway, in the norepinephrine-induced anticontractile effect in mice (Withers *et al.*, 2014).

L-NMMA inhibits all three isoforms of NOS (eNOS, nNOS and iNOS) but may also have non-specific effects. However, the lack of effects of the inactive enantiomer, D-NMMA, and the specific nNOS inhibitor Vinyl-L-NIO, strongly suggested that the observed effects of L-NMMA in rat PVAT were a consequence of eNOS inhibition. Endothelial NOS (eNOS) plays a key role in white adipose tissue metabolism (Sansbury *et al.*, 2012) and is activated by increased intracellular calcium (Busse & Mulsch, 1990). Moreover, the PVAT anticontractile effect was absent in $eNOS^{-/-}$ mice, and we were able to detect greater eNOS expression in PVAT than mesenteric artery suggesting that it is the key NOS isoform catalyzing nitric oxide production within PVAT.

Adiponectin promotes nitric oxide production within the endothelium (Cheng *et al.*, 2007) and previous studies within our laboratory have suggested a key role for adiponectin as a relaxing factor mediating the anticontractile effect in human subcutaneous (Greenstein *et al.*, 2009) and mouse mesenteric (Lynch *et al.*, 2013) arteries and in response to β_3-adrenergic stimulation of non-contracted rat mesenteric arteries (Weston *et al.*, 2013). Also, serum levels of adiponectin were increased in rats following seven-day infusion of CL-316,243 (Zhang *et al.*, 2002), and adiponectin secretion from mouse mesenteric PVAT is significantly reduced when incubated with SR 59230A (Saxton *et al.*, 2018). Previously, adiponectin has been shown to induce vasorelaxation in rat (Greenstein *et al.*, 2009) and mouse (Lynch *et al.*, 2013) mesenteric arteries and myocyte hyperpolarization in non-contracted vessels (Weston *et al.*, 2013). We observed high basal adiponectin secretion from PVAT, however no changes in total adiponectin secretion were observed following stimulation with norepinephrine or CL-316,243. This may be because biologically relevant changes in the ratio of high molecular weight: low molecular weight isoforms could not be detected using commercially available ELISA kits (Brochu-Gaudreau *et al.*, 2010). AMPK is a major downstream target of adiponectin (Yamauchi *et al.*, 2007) and we show AMPK phosphorylation in unstimulated PVAT was not altered by β-adrenergic stimulation suggesting that adiponectin may be constitutively

released from PVAT and could facilitate nitric oxide production by the adipocytes in response to β-adrenergic stimulation.

The results presented show that adipocyte β_3-adrenoceptor stimulation leads to increased intracellular cAMP production and KCNQ activation via increased intracellular calcium leading to release of relaxing factors, one of which is nitric oxide (Figure 7). For the first time we demonstrate norepinephrine stimulates release of adipocyte-derived nitric oxide. This nitric oxide is a key PVAT-derived relaxing factor and modulates arterial contraction. These findings support the targeting of NOS for the treatment of PVAT dysfunction associated with obesity and the metabolic syndrome.

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