The role of sympathetic nerves and adipocyte catecholamine uptake in the vasorelaxant function of perivascular adipose tissue

Sophie N Saxton¹, Katie E Ryding¹, Robert G Aldous¹, Sarah B Withers¹,², Jacqueline Ohanian¹, Anthony M Heagerty¹.

¹Division of Cardiovascular Sciences, University of Manchester, UK
²School of Environment and Life Sciences, University of Salford, UK

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Address for correspondence
Professor A Heagerty
Division of Cardiovascular Sciences
Manchester Academic Health Science Centre
Core Technology Facility (3rd floor),
46 Grafton Street
Manchester
M13 9NT
United Kingdom
Email: tony.heagerty@manchester.ac.uk
Telephone: +44 161 275 1199

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ABSTRACT

Objective: Healthy perivascular adipose tissue (PVAT) exerts an anti-contractile effect on resistance arteries which is vital in regulating arterial tone. Activation of β<sub>3</sub>-adrenoceptors by sympathetic nerve-derived noradrenaline may be implicated in this effect, and may stimulate the release of the vasodilator adiponectin from adipocytes. Understanding the mechanisms responsible is vital for determining how PVAT may modify vascular resistance in vivo.

Approach and Results: Electrical field stimulation (EFS) profiles of healthy C57BL/6J mouse mesenteric resistance arteries were characterised using wire myography. During EFS PVAT elicits a reproducible anti-contractile effect, which is endothelium-independent. To demonstrate the release of an anti-contractile factor, the solution surrounding stimulated exogenous PVAT was transferred to a PVAT-denuded vessel. Post-transfer contractility was significantly reduced confirming that stimulated PVAT releases a transferable anti-contractile factor. Sympathetic denervation of PVAT using tetrodotoxin or 6-hydroxydopamine completely abolished the anti-contractile effect. β<sub>3</sub>-adrenoceptor antagonist SR59203A reduced the anti-contractile effect, although the PVAT remained overall anti-contractile. When the antagonist was used in combination with an OCT3 inhibitor; corticosterone, the anti-contractile effect was completely abolished. Application of an adiponectin receptor 1 blocking peptide significantly reduced the anti-contractile effect in +PVAT arteries. When used in combination with the β<sub>3</sub>-adrenoceptor antagonist there was no further reduction. In adiponectin knockout mice, the anti-contractile effect is absent.

Conclusions: The roles of PVAT are two-fold. First, sympathetic stimulation in PVAT triggers the release of adiponectin via β<sub>3</sub>-adrenoceptor activation. Second, PVAT acts as a reservoir for NA, preventing it from reaching the vessel and causing contraction.

Abbreviations: DAB - 3,3'-diaminobenzidine; 6-OHDA - 6-hydroxydopamine; ABP - adiponectin blocking peptide; EFS - electrical field stimulation; KPSS – high potassium physiological salt solution; NA - noradrenaline; OCT - organic cation transporter; PBS – phosphate buffer solution; PSS – physiological salt solution; PVAT - perivascular adipose tissue; TTX - tetrodotoxin, WAT - white adipose tissue; VSMC - vascular smooth muscle cell.
INTRODUCTION

Perivascular adipose tissue (PVAT) surrounds the majority of blood vessels, and there is a growing body of evidence that it can release factors which are vital in regulating vascular tone and therefore blood pressure and nutrient delivery\(^1\)\(^-\)\(^3\). The exact mechanisms by which PVAT releases these relaxant factors are still unclear, hindering our understanding of how PVAT modulates vascular resistance.

Sympathetic nerve fibres are abundant within the vasculature and adipose tissue depots, and it is accepted widely that white adipose tissue (WAT) responds to catecholamines secreted from sympathetic nerve fibres\(^4\)\(^-\)\(^6\). Sympathetic denervation of epididymal and inguinal fat pads consisting of WAT in vivo will increase lipid deposits\(^7\)\(^-\)\(^8\), indicating that sympathetic innervation of adipose tissues plays a vital role in lipolysis. The origins of these fibres have been extensively studied using viral transneuronal retrograde tract tracers; namely the pseudorabies virus and the H129 strain of the herpes-simplex virus\(^-\)\(^9\)\(^-\)\(^11\). Injection of these tracers into mesenteric, inguinal, or epididymal fat pads in hamsters, reveals that the sympathetic fibres supplying these fat deposits originate from the central nervous system, starting at the forebrain. However it is unclear if the nerves are innervating the adipocytes directly.

Functional studies of the role of sympathetic nerves in the PVAT anti-contractile effect are conflicting. In conduit arteries, electrical field stimulation (EFS) of PVAT to activate sympathetic nerve fibres has effects dependent upon location\(^12\). In the WAT of the superior mesentery, EFS induced an anti-contractile effect, whereas in the brown adipose tissue of the aorta EFS induced a pro-contractile PVAT effect, which suggests that PVAT function may vary with the phenotype of the adipocytes or size of the vessel. Conversely, previous studies have reported an EFS induced pro-contractile effect in the WAT of the superior mesenteric artery\(^15\), and mesenteric resistance arteries\(^16\). However, the latter study used a much greater voltage than all other studies, and proposed that the pro-contractile effect of PVAT is due to the generation of superoxide anions. The effects of lower voltage stimulations in PVAT-intact mesenteric resistance arteries have not yet been characterised.

Additionally, the mechanisms by which anti-contractile factors are released from PVAT are not fully understood, however evidence suggests that activation of adipocyte \(\beta_3\)-adrenoceptors may play an important role. As previously discussed, lipolysis can be stimulated in WAT by sympathetic stimulation, and this process is mediated via \(\beta_3\)-adrenoceptors present on WAT\(^14\). \(\beta_3\)-adrenoceptors are highly expressed in rodent adipocytes, but not the vasculature\(^15\)\(^-\)\(^16\), and immunohistochemical studies have confirmed that \(\beta_3\)-adrenoceptors are expressed in human PVAT\(^17\). CL-316,243, a \(\beta_3\)-adrenoceptor agonist, is hypotensive in both rodents and dogs\(^18\), and electrophysiological studies demonstrate that the same agonist elicits PVAT-dependent hyperpolarisations of vascular smooth muscle cells (VSMC)\(^19\). The effects of inhibition of adipocyte \(\beta_3\)-adrenoceptors on PVAT function and vessel tension have not yet been characterised.

Adiponectin has been identified as a PVAT-derived relaxing factor, because the application of exogenous adiponectin to mouse mesenteric arteries constricted with noradrenaline (NA) mimics the PVAT anti-contractile effect\(^20\). Furthermore, inhibition of adiponectin receptor-1 abolishes the NA induced PVAT anti-contractile effect\(^20\)\(^-\)\(^21\). In adiponectin deficient mice, the \(\beta_3\)-adrenoceptor agonist CL-316,243 does not induce PVAT-dependent hyperpolarisations observed in control mice\(^19\); therefore it is reasonable to suggest that stimulation of \(\beta_3\)-adrenoceptors within PVAT will trigger the release of adiponectin, but this needs investigating.

It has been suggested that NA transporters may play a role in PVAT’s anti-contractile functions\(^22\)\(^-\)\(^23\). Inhibition of organic cation transporter 3 (OCT3) using corticosterone significantly reduced NA uptake into PVAT, resulting in a higher NA concentration in the
surrounding solution and potentially stimulating a larger VSMC contraction. However it remains to be confirmed if OCT3 plays a functional role in the PVAT anti-contractile effect by allowing PVAT to act as a reservoir for NA, therefore preventing the NA from reaching VSMCs and eliciting contraction.

Therefore, the present studies were designed to examine sympathetic nerves within PVAT, and determine if they play a functional role. We tested the hypothesis that the stimulation of sympathetic nerves within PVAT caused the release of NA. We anticipate that NA will activate adipocyte $\beta_3$-adrenoceptors, triggering the release of adiponectin. In addition, we hypothesise that some NA will be sequestered by OCT3 into adipocytes, thereby preventing the NA from reaching the vessel and eliciting contraction.

MATERIALS AND METHODS

Animal care and ex vivo measurements

All animal procedures complied with the UK Animal (Scientific Procedures) Act 1986 and were performed in accordance with the appropriate Home Office project licence. Male C57BL/6J (Envigo, UK) and Adipoq$^{tm1Chan}$ mice (Jackson Laboratories, USA) were fed a normal chow diet until euthanized at 18-20 weeks old by CO$_2$ asphyxiation. Prior to sacrifice mice were fasted overnight and blood pressure was recorded in conscious, restrained animals using the CODA tail cuff blood pressure monitoring system (Kent Scientific, USA). Immediately after sacrifice mixed blood samples were taken by severing the thoracic aorta. Blood glucose concentration was measured immediately using an automatic blood glucose system. The mesenteric bed and epididymal fat pads were exposed and removed.

Wire myography

Second order mesenteric arterial segments (<250µm) were dissected in ice cold physiological salt solution (PSS) of the following composition (mmol L$^{-1}$): NaCl, 119; KCl, 4.7; MgSO$_4$, 1.17; NaHCO$_3$, 25; KH$_2$PO$_4$, 1.17; EDTA, 0.03; Glucose, 5.5 and CaCl$_2$, 1.6. PVAT was removed from some segments, and left intact on the adjacent segments. A third arterial preparation was used where a clean artery was mounted, and a section of exogenous PVAT was suspended above the vessel (Supplementary figure 1). Arteries were mounted on 40µm diameter wires (Danish MyoTech, Denmark) and left to equilibrate for 30 minutes before normalising in a standardised procedure. Normalisation uses vessel length and wall tension to set vessels at a standardised starting tension. Arteries are stretched in gradual increments using a micrometer, and the passive force is measured using a Powerlab chart recorder (ADInstruments, UK), allowing calculation of wall tension. Using the wall tension, the Powerlab normalisation software (LabChart v7, ADInstruments, UK) can determine the effective pressure following each incremental stretch from the Laplace equation. An exponential curve is fitted using the effective pressure vs the internal circumference of the vessel. Previous studies in the rat mesentery have indicated that the maximal active tension occurs at 90% of the internal circumference, which is stable at a transmural pressure of 100mmHg. Therefore the exponential curve is used to calculate internal circumference at an essential pressure of 100 mmHg, and vessel circumference is set at 90% of this calculated value.

Following normalisation, vessels were left for a further 30 minute equilibration period at 37°C and bubbled at 95% air / 5% CO$_2$ to maintain a pH of 7.4. Following equilibration, vessels were challenged with 60mmol/L high K$^+$ PSS (KPSS, composition in mmol L$^{-1}$: NaCl, 63.7; KCl, 60; MgSO$_4$, 1.17; NaHCO$_3$, 25; KH$_2$PO$_4$, 1.17; K$_2$EDTA, 0.03; Glucose, 5.5 and CaCl$_2$, 1.6) to establish a maximum contractile response and confirm that removal of PVAT does not cause vascular injury. Vessels with a response less than 0.3mN/mm$^2$ were excluded. Responses were continuously recorded using LabChart 7 (ADInstruments, UK).
Electrical field stimulation

Following challenge with KPSS, endothelial function was assessed using 30µM of noradrenaline (NA) followed by a cumulative concentration-response to acetylcholine (Ach) (1 x 10⁻⁷ – 3 x 10⁻⁵M). As consistent with previous studies, vessels with less than 30% relaxation to acetylcholine were discarded. Two platinum plates fixed on either side of the vessel connected to a DMT stimulator (CS4 model, Myopulse software) were used as stimulating electrodes. Vessels were stimulated for 4s at 20V over a frequency range of 0.1-30Hz (0.2ms pulse duration).

To determine the role of the endothelium, EFS responses of vessels with and without endothelium were compared. Endothelium was removed by insertion of equine hair into the vessel lumen to gently remove endothelial cells. When pre-constricted with NA and tested using Ach as described above, vessels with less than 20% relaxation to Ach were considered to be endothelium-denuded, as consistent with previous studies.

Pharmacological assessment

Vessels with and without exogenous PVAT were stimulated with EFS, and then the exogenous PVAT was removed and incubated for 30 minutes with sodium channel inhibitor tetrodotoxin (TTX, 1µM), catecholamine toxin 6-hydroxydopamine (6-OHDA, 2µM), or OCT3 inhibitor corticosterone (100µM), before re-suspending the exogenous PVAT in the bath with its original –PVAT vessel. A combination of SR59230A (1µM) and corticosterone (100µM) was also used in exogenous PVAT experiments.

In protocols using both PVAT-denuded vessels, or PVAT intact vessels, following the first EFS stimulation, β₃-adrenoceptor antagonist SR59230A (1µM), β₃-adrenoceptor agonist CL-316,243 (10µM) or adiponectin receptor-1 blocking peptide (ABP, 3.5µg/ml) was added to the bath and incubated for 30 or 45 minutes, before re-stimulating the vessels. A combination of ABP (3.5µg/ml) and SR59230A (1µM) or CL-316,243 (10µM) was also used in PVAT intact vessel experiments.

The role of superoxide anions was investigated by incubating a pair of ±PVAT vessels with the scavenger superoxide dismutase-polyethylene glycol (SOD-PEG, 200U/ml) for two hours following equilibration and normalisation. As a control, a second pair of ±PVAT vessels were incubated alongside in PSS for two hours. Following the two hour incubation, the EFS protocol was carried out as normal.

The effects of globular adiponectin (5µg/ml) were tested on only –PVAT vessels. Vessels were stimulated once and allowed to recover for 15 minutes before adding adiponectin. The adiponectin was incubated for five minutes before repeating the EFS protocol.

Sample numbers of 8 for each vessel preparation (-PVAT, +PVAT, +exogenous PVAT) were used for all experiments, with the exception of exogenous adiponectin and ABP experiments, for which a n of 4 was used due to the expense of these compounds (see supplementary table 1 for summary of all pharmacological tools).

Solution transfer studies

PVAT-denuded vessels were mounted and stimulated at 10Hz for 4s, followed by a 15 minute recovery period. In separate baths, sections of exogenous PVAT were suspended between the platinum electrodes, and stimulated at 10Hz for 4s. The solution surrounding the stimulated PVAT was transferred to the PVAT-denuded vessels, and the vessels were stimulated for a second time.

A second set of solution transfer experiments were conducted where the exogenous PVAT was first incubated with SR59230A (1µM) for 30 minutes, and were rinsed immediately
before stimulation to prevent transferring the drug to the PVAT vessels during the solution transfer.

**Adiponectin assay**

To quantify the concentration of adiponectin release from PVAT upon EFS, PVAT was harvested from the entire mesenteric bed, and weighed. The PVAT was incubated in a reduced volume myograph bath (3ml) for 30 minutes in PSS bubbled with 95% air / 5% CO₂ to equilibrate, before refreshing the surrounding PSS and stimulating using EFS at 10Hz for 4s (20V, pulse duration 0.2ms). The surrounding supernatant was collected and stored at -80°C until required. To examine the role of β₃-adrenoceptors in adiponectin secretion, in separate PVAT samples the PVAT was pre-incubated with β₃-adrenoceptor antagonist SR59230A (1µM) for 30 minutes, before conducting the protocol as normal.

The concentration of adiponectin in the surrounding supernatant was measured using an ELISA kit (Life Technologies Ltd, UK, cat no. KMP0041). The manufacturer protocol was followed and unmodified. A 1 in 100 dilution of the collected supernatant was used, and adiponectin concentration was determined through measurement of absorbance using a plate reader (BioTek, Northstar Scientific, UK) at 450nm. To account for variation in the amount of PVAT collected from each mesenteric bed, concentration was calculated per 100mg of PVAT.

**Drug preparations**

Noradrenaline (Sigma Aldrich, UK, cat no. A7257), acetylcholine (Sigma Aldrich, UK, cat no. A6625), SODPEG (Sigma Aldrich, UK, cat no. S9549) SR59230A (Bio-techne, UK, cat no. 1151.) and CL-316,243 (Bio-tecne, UK, cat no. 1499) were dissolved in PSS. The blocking peptide for adiponectin receptor 1 was obtained from Enzo Life Sciences (UK, cat no. ALX-151-045-C100), and recombinant mouse globular adiponectin was obtained from Generon (UK, cat no. cyt-432-50ug). TTX (Bio-techne, UK, cat no. 1078) was prepared in a citrate buffer, 6-OHDA (Sigma Aldrich, UK, cat no. H4381) was dissolved in water containing 0.1% of antioxidant sodium metabisulfite (Na₂S₂O₅), and corticosterone (Sigma Aldrich, UK, cat no. 27840) was dissolved in 100% ethanol. Vehicle controls were performed along-side these experiments (supplementary figure 2).

**Immunohistochemistry**

PVAT samples and positive control tissues (kidney or brain) were placed in 4% ice-cold paraformaldehyde for one hour and subsequently washed in 0.1M phosphate buffer solution (PBS). Tissue was then embedded in KP-CryoCompound (Klinipath BV, The Netherlands) and stored at -80°C, prior to serial sectioning at 12µm using a Leica CM 3050 cryostat (Leica Microsystems, Germany). Heat induced antigen retrieval was performed in a citrate buffer, followed by blocking of endogenous peroxidase activity using 3% hydrogen peroxide. Tissues were incubated with 10% goat serum at room temperature for one hour to reduce non-specific antibody binding, in combination with Triton-X 100 (0.1%) to increase permeability. Samples were incubated with antibodies for dopamine β-hydroxylase (10µg/ml, Abcam, UK, cat no. ab96615), β₃-adrenoceptors (10µg/ml, Abcam, Cambridge, UK, cat no. ab59685), or OCT3 (10µg/ml, Sigma Aldrich, UK, cat no. AV44026) overnight at 4°C. Slides were incubated for one hour with a biotinylated goat anti-rabbit secondary antibody (2µg/ml, Abcam, UK, cat no. ab6720) at room temperature. Vectastain ABC complex (Vector Laboratories, UK) followed by the addition of 3,3’-diaminobenzidine (DAB) solution (Vector Laboratories, UK) was used for detection of antibody binding. Negative controls were conducted in PVAT. These were incubated with PBS in place of the primary antibody, and incubation with the secondary antibody and detection methods were conducted as normal. Images were captured using a colour camera (Leica DFC450, Leica Microsystems, Germany) mounted on a microscope (Leica DM5000, Leica Microsystems, Germany).
Statistical analysis

Contractile responses were expressed as a percentage of the maximum contraction elicited with KPSS, as consistent with other studies\textsuperscript{21, 26}. KPSS responses were no different between ±PVAT vessel groups, or before and after drug treatments. Data are shown as the mean±SEM. Normal distribution was confirmed using the Shapiro-Wilk normality test. Differences between the frequency-response curves of ±PVAT vessels were tested using a two-way ANOVA, with a Bonferroni post-hoc test. Before and after treatments within the same vessel types were compared using a repeated-measures ANOVA, again followed by a Bonferroni post-hoc test. The effects of the solution transfer were tested using a paired t-test, and the concentrations of adiponectin provided by ELISA were compared using an unpaired t-test. Statistical tests were conducted using GraphPad Prism (v6, GraphPad Software, USA).

RESULTS

Electrical field stimulation of PVAT triggers the release of an endothelium-independent anti-contractile factor

The effects of EFS were tested on mesenteric vessels with PVAT removed and PVAT left intact. Upon each stimulus there was a frequency-dependent contraction (\(-\)PVAT P<0.0001; 3Hz P<0.05, 10Hz P<0.01, 30Hz P<0.001, n=8), which was significantly reduced with the presence of PVAT (Figure 1A, P<0.001, n=8). Vessels were given 15 minutes recovery before the stimulus was repeated. Following this recovery the anti-contractile effect was reproducible (P<0.001, n=8), therefore electrical activation of nerves within PVAT does elicit a reproducible anti-contractile effect. To demonstrate that this effect is not simply due to PVAT forming a barrier around the tissue, the EFS protocol was repeated in PVAT-denuded vessels with a section of exogenous PVAT suspended above the vessel, and again contractility was significantly reduced in the presence of PVAT (Figure 1B, P<0.0001, n=8).

To confirm that the anti-contractile effect is not due to generation of reactive oxygen species within PVAT upon EFS, ±PVAT vessels were incubated with superoxide scavenger SODPEG for two hours before stimulation. Incubation with the scavenger had no effect on the anti-contractile effect (Figure 1C, P<0.0001, n=8), indicating that our EFS protocol is not damaging the PVAT.

Solution transfer studies were conducted by transferring the solution surrounding a stimulated section of exogenous PVAT to a PVAT-denuded vessel. The vessels were stimulated once before and after the solution transfer. Post-transfer, the contraction was significantly reduced (Figure 1D, P<0.01, n=8), therefore confirming that the exogenous PVAT must have released an anti-contractile factor into its surrounding environment.

To determine the role of the endothelium in the PVAT anti-contractile effect, the effects of EFS on endothelium-denuded vessels were tested. In the absence of endothelium, the PVAT anti-contractile effect persisted (Figure 1E, P<0.0001, n=8), confirming this effect to be endothelium-independent.

The anti-contractile effect of PVAT is dependent on sympathetic nerve firing

The presence of sympathetic nerves was confirmed immunohistochemically in sections of mouse mesenteric PVAT using antibodies for the sympathetic nerve marker dopamine β-hydroxylase (n=5, Figure 2A). To confirm that the anti-contractile response of PVAT elicited by EFS is due to sympathetic nerve activity, TTX and 6-OHDA were used. Exogenous PVAT was incubated with the voltage-gated sodium channel inhibitor TTX, before re-suspending above PVAT-denuded vessels and repeating the EFS protocol. TTX successfully abolished the PVAT anti-contractile effect (Figure 2D, P<0.0001, n=8), confirming the effect to be
neurally mediated. In a similar protocol, catecholamine toxin 6-OHDA was incubated with exogenous PVAT, and again successfully abolished the anti-contractile effect (Figure 2E, P<0.001, n=8), therefore the anti-contractile effect is dependent on sympathetic nerve firing.

**The release of the anti-contractile factor is mediated via β3-adrenoceptors**

The presence of β3-adrenoceptors on mouse mesenteric adipocytes was confirmed using immunohistochemistry (n=5, Figure 3A). To determine if these adrenoceptors modulate vascular tone, specific β3-adrenoceptor agonist and antagonists were used.

Following control responses to EFS ±PVAT vessels were incubated with CL-316,243 (10μM) for 30 minutes (Figure 3D). The agonist significantly enhanced the PVAT anti-contractile effect (P<0.01, n=8), and had no effect on –PVAT vessels (P>0.05, n=8).

The solution transfer experiment shown in Figure 1D was repeated using sections of exogenous PVAT pre-incubated with the β3-adrenoceptor antagonist SR59230A for 30 minutes. Post-transfer there was no longer a significant reduction in contraction (Figure 4A, P>0.05, n=8), therefore confirming that the release of the anti-contractile factor into the surrounding environment must be dependent on β3-adrenoceptor activation. However, when the effects of the β3-adrenoceptor antagonist were tested in vessels with their PVAT left intact, there was only a partial inhibition of the anti-contractile effect (Figure 4B, P<0.01, n=8), and the response of PVAT intact vessels was still significantly lower than that of the PVAT-denuded vessels (P<0.0001). There was no effect of the β3-adrenoceptor antagonist on PVAT-denuded vessels.

**Re-uptake of noradrenaline into PVAT contributes to the anti-contractile effect**

Immunohistochemistry was used to confirm the presence of OCT3 in mouse mesenteric PVAT (n=5, Figure 5A). The functional role of OCT3 was determined by incubating sections of exogenous PVAT with the inhibitor corticosterone, before re-suspending the PVAT above a PVAT-denuded vessel and repeating the stimulus. Similar to the β3-adrenoceptor antagonist, inhibition of OCT3 significantly reduced the anti-contractile effect (Figure 5D, P<0.001, n=8) but again the PVAT remained overall anti-contractile (P<0.001). However, a combination of inhibition of β3-adrenoceptors and OCT3 completely abolished the effect (Figure 5E, P<0.0001, n=8), suggesting that both of these transporters and receptors mediate the anti-contractile functions of PVAT.

**The anti-contractile factor released upon β3-adrenoceptor activation may be adiponectin**

The effects of globular adiponectin were tested on –PVAT vessels. –PVAT vessels were subjected to EFS and allowed 15 minutes to recover. Following the recovery period, globular adiponectin was added and allowed to incubate for five minutes before repeating the stimulus. Globular adiponectin significantly reduced the contractile responses to EFS of PVAT-denuded vessels (Figure 6A, P<0.001, n=4).

Following control responses, ±PVAT vessels were incubated with ABP for 45 minutes. Following incubation, the anti-contractile effect of PVAT was significantly reduced (Figure 6B, P<0.01, n=4), however the PVAT remained overall anti-contractile (P<0.05). ABP had no effect on vessels with PVAT removed (P>0.05).

+PVAT vessels were subjected to a control EFS protocol, before incubating with either SR59230A (1μM) or CL-316,243 (10μM) for 30 minutes. Following incubation, the EFS protocol was repeated for a second time, before performing a second incubation with SR59230A or CL-316,243, in combination with the ABP (5μg/ml), before repeating the EFS protocol for a third time. SR59230A again significantly reduced the anti-contractile effect.
alone (Figure 6C, n=4, P<0.01). When used in combination with the ABP, there was no further reduction in the PVAT anti-contractile effect (n=4, P>0.05). CL-316,243 again significantly enhanced the PVAT anti-contractile effect alone (Figure 6D, n=4, P<0.01), and this enhancement was abolished by ABP (n=4, P<0.01). However, the combination of CL-316,243 and ABP was no different from +PVAT control responses, i.e. there was no reduction to the starting anti-contractile effect, suggesting that ABP may not overcome both basal β₃-adrenoceptor activation, and activation with the agonist.

To quantify the concentration of adiponectin secreted by PVAT upon EFS, PVAT was collected from the entire mesentery and stimulated with EFS in the presence and absence of SR59230A, and the concentration was calculated per 100mg of PVAT (Figure 6E). Upon stimulation at 10Hz, control PVAT released approximately 874.3±65.65ng/ml of adiponectin into the surrounding supernatant (n=8). When pre-incubated with SR59230A, adiponectin secretion is significantly reduced to 378.7±90.4ng/ml (P<0.001, n=8). The results indicate that adiponectin is the anti-contractile factor released from mouse mesenteric PVAT upon β₃-adrenoceptor activation.

To further investigate the importance of adiponectin in PVAT function, the anti-contractile effect was studied in adiponectin knockout (adipo⁻/⁻) mice. As compared to control C57 mice, adipo⁻/⁻ mice had similar body weights (Figure 7A, P>0.05, n=8 for both groups), and similar sized epididymal fat depots (Figure 7B, P>0.05, n=8 for both groups). Blood pressure was recorded in conscious restrained mice using the CODA tail cuff system (Figure 7C). Both systolic and diastolic blood pressure were significantly elevated in adipo⁻/⁻ as compared to C57 controls (P<0.01, n=8 for both groups). Immediately following sacrifice, fasting blood glucose was measured using an automatic monitor, and blood glucose was significantly elevated in adipo⁻/⁻ mice (Figure 7D, P<0.01, n=8). Wire myography was used to assess PVAT function (Figure 7E). ±PVAT vessels from adipo⁻/⁻ mice were subjected to EFS, and there was no difference between ±PVAT vessels (P>0.05, n=8). Following the first stimulus, vessels were incubated with CL-316,243 (10µm) for 30 minutes before repeating the EFS protocol. CL-316,243 had no effect on vessel contractility (P>0.05, n=8). The presence of β₃-adrenoceptors in adipo⁻/⁻ PVAT was confirmed using immunohistochemistry (n=5).
DISCUSSION

This study investigated the potential role for the sympathetic nervous system in controlling the anti-contractile function of PVAT. The main findings of this study were (1) electrical activation of sympathetic nerves in PVAT stimulates the release of a transferable anti-contractile factor, (2) the release of the anti-contractile factor is dependent on β- adrenoceptor activation, (3) the PVAT anti-contractile effect is dependent upon adiponectin (4) adiponectin is essential for normal vascular function, and (5) PVAT acts as a reservoir for sympathetic nerve-derived NA via OCT3, thereby preventing the NA from reaching the blood vessel and causing contraction.

All previous studies have used sympathomimetic drugs to elicit an anti-contractile effect\(^{1, 3, 21, 27, 28}\); therefore the aim of this study was to use EFS to directly activate the nerve fibres, triggering the release of catecholamines. Our EFS protocol successfully elicited an endothelium-independent PVAT anti-contractile effect similar to that reported using sympathomimetics. PVAT-denuded vessels with a section of exogenous PVAT suspended above also exhibited a similar reduction in contractility, confirming that the anti-contractile effect is not simply due to PVAT forming a barrier and insulating the vessel from the EFS. Furthermore, the solution transfer studies illustrate that upon nerve activation, a transferable anti-contractile factor must be released from the PVAT into its surroundings.

Previously it has been shown that a high voltage stimulus induces the release of pro-contractile factors from PVAT\(^{13}\). This study used the superoxide scavenger SODPEG to reduce this pro-contractile effect; thereby confirming the effect to be due to generation of superoxide anions. Generally, superoxide production is considered to be a result of oxidative stress, and leads to vascular dysfunction\(^{29}\). Therefore, production of superoxide in PVAT seems likely to be a result of damage to PVAT caused by the high voltage. In this study, the Gao protocol was replicated, and SODPEG had no effect on contractility, indicating that our protocol is not damaging the PVAT.

This study has used dopamine β-hydroxylase to mark the presence of sympathetic nerves within the mesenteric PVAT. Perhaps a more commonly used marker for sympathetic nerves is tyrosine hydroxylase. However, it has been shown that inflammatory cells also contain tyrosine hydroxylase such as macrophages\(^{30}\), T-cells\(^{31}\), and inflammatory cytokines\(^{32}\). Since there is an abundance of inflammatory cells within PVAT\(^{21, 33, 34}\), it was determined that dopamine β-hydroxylase would be more appropriate.

The most common method for determining if an effect has a neural origin is the use of TTX. Incubation of exogenous PVAT with TTX provided a complete inhibition of the anti-contractile effect; confirming that the anti-contractile effect is indeed neurally mediated. Currently there is no evidence of parasympathetic nerves within PVAT, and morphological studies, found no evidence of cholinergic, vasoactive intestinal peptidergic, or nitrergic nerves, concluding that 97-98% of nerves running within white adipose tissue are noradrenergic\(^{35}\). The rest of the nerves present within PVAT are likely to be sensory nerves\(^{5, 36}\), which may play a role in sensing the products of lipolysis, and feeding back to the central nervous system in order to regulate lipolysis. We were able to confirm the presence of sympathetic nerves functionally using the catecholamine toxin 6-OHDA, which similarly to TTX, completely inhibited the anti-contractile effect of the exogenous PVAT, thereby confirming that the anti-contractile effect is mediated via sympathetic nerve fibres. Furthermore, the anti-contractile factor released upon EFS is endothelium-independent, and in vessels stimulated with exogenous NA\(^{24}\), the PVAT anti-contractile effect is also endothelium-independent. This suggests that the anti-contractile factor released by EFS from sympathetic nerves is NA. One limitation of this study is the lack of information regarding the concentration of NA released by sympathetic nerves within PVAT into the extracellular space adjacent to adrenoceptors. This has not been possible due to the limited sensitivity of commercially available assays.
As consistent with previous studies\textsuperscript{15, 16, 37}, we have confirmed the presence of $\beta_3$-adrenoceptors within rodent mesenteric PVAT. For the first time we have demonstrated a functional role for $\beta_3$-adrenoceptors in the PVAT anti-contractile effect, by significantly reducing the effect using the $\beta_3$-adrenoceptor antagonist. Our solution transfer studies using PVAT incubated with the $\beta_3$-adrenoceptor antagonist, confirm that the release of the anti-contractile factor upon electrical activation of sympathetic nerves is dependent on $\beta_3$-adrenoceptor activation. However, the partial inhibition of the anti-contractile effect by the antagonist in vessels with PVAT intact, reveal that the release of the anti-contractile factor is only one-side of the PVAT story.

Our study has for the first time confirmed the presence of OCT3 in the mouse mesenteric PVAT using immunohistochemistry. This is consistent with previous studies in rat illustrating the presence of OCT3 in aortic and mesenteric PVAT\textsuperscript{23}. OCT3, previously known as the extraneuronal transporter, plays a predominant role in what was originally termed uptake\textsuperscript{2}. It plays a vital role in the transport of solutes such as NA into the peripheral tissues. It has previously been shown that a NA-uptake system exists within PVAT which is mediated via OCT3\textsuperscript{23}, however, our study is the first to demonstrate that inhibition of OCT3 in exogenous PVAT exhibits a significant increase in vessel tension, revealing that the anti-contractile role of PVAT is in part to act as a reservoir for excess NA, preventing the NA from reaching the vessel and eliciting contraction.

This study has indicated that adiponectin is the anti-contractile factor which is released by activation of adipocyte $\beta_3$-adrenoceptors, as secretion of adiponectin from PVAT was significantly reduced in the presence of the $\beta_3$-adrenoceptor antagonist. In addition, application of exogenous adiponectin to PVAT-denuded vessels induced relaxation, and inhibition of adiponectin receptor-1 significantly increased contractile responses in +PVAT vessels. Moreover, the adiponectin blocking peptide (ABP) reduced the enhancement of the anti-contractile effect induced using the $\beta_3$-adrenoceptor agonist, and caused no further reduction in the anti-contractile effect when used in combination with the $\beta_3$-adrenoceptor antagonist. These results are consistent with other studies, which have reported an effect of ABP on responses to NA\textsuperscript{20, 21}. Most importantly, the EFS induced anti-contractile effect is absent in the adiponectin-deficient mouse, which is consistent with previous studies using NA as the vasoconstrictor\textsuperscript{28}. However, there is evidence that the PVAT-derived anti-contractile factor may be agonist-dependent, as when vessels from adiponectin-deficient mice are stimulated with serotonin, the anti-contractile effect is still present\textsuperscript{28}. Furthermore, we have previously reported that addition of ABP to rat vessels challenged with the serotonin, does not have the same antagonistic effect as in vessels constricted with NA\textsuperscript{39}.

This is the first study to report a hypertensive and hyperglycaemic phenotype in adiponectin-deficient mice. Similarly in adiponectin receptor 1 knockout mice, glucose clearance is reduced\textsuperscript{40}. These results indicate that adiponectin is essential for normal blood pressure and glucose clearance; therefore in human obesity where adiponectin bioavailability is reduced\textsuperscript{41} and PVAT function is lost\textsuperscript{42}, these dysfunctions may be contributing to development of metabolic syndrome.

In summary, the sympathetic nervous system in PVAT stimulates an anti-contractile effect which has vital effects on vascular tone and therefore blood pressure and nutrient delivery. The sympathetic nerves release NA, which activates adipocyte $\beta_3$-adrenoceptors, triggering the release of an anti-contractile factor into the surrounding environment. Excess NA is transported into the PVAT via OCT3, preventing the NA from reaching the vessel and increasing contraction. We anticipate that in obesity, where autonomic dysfunction is known to occur, the sympathetic nerves in PVAT may become pathologically overactive, leading to a loss of PVAT anti-contractile function. Loss of the neurally-mediated PVAT anti-contractile effect in obesity may contribute to the development of hypertension and type-II diabetes in obese patients.
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HIGHLIGHTS

- Electrical activation of sympathetic nerves in healthy PVAT stimulates the release of a transferable anti-contractile factor.
- The release of the anti-contractile factor is dependent on β3-adrenoceptor activation.
- Adiponectin may be the anti-contractile factor released upon activation of β3-adrenoceptor activation.
- Adipo−/− mice are hypertensive and hyperglycaemic, and lack an EFS induced anti-contractile effect.
- PVAT acts as a reservoir for sympathetic nerve derived NA via OCT3, thereby preventing the NA from reaching the blood vessel and causing contraction.
Figure 1: EFS triggers the release of a transferable endothelium-independent anti-contractile factor from perivascular adipose tissue. A: Vessels with and without PVAT were subjected to EFS, inducing a frequency dependent contraction (-PVAT: 3Hz P<0.05*, 10Hz P<0.01**, 30Hz P<0.001***#) and given 15 minutes to recover before being stimulated for a second time (-PVAT 1st stim vs +PVAT 1st stim P<0.001***, -PVAT 2nd stim vs +PVAT 2nd stim P<0.001****, -PVAT 1st stim vs -PVAT 2nd stim P>0.05, +PVAT 1st stim vs +PVAT 2nd stim P>0.05, n=8). B: PVAT-denuded vessels were mounted with a section of exogenous PVAT suspended above the vessel, and the EFS protocol was conducted (P<0.0001****, n=8). C: ±PVAT vessels were incubated with SODPEG for two hours before stimulating with EFS (-PVAT vs +PVAT P<0.0001****, -PVAT & SODPEG vs +PVAT & SODPEG P<0.0001***, -PVAT vs -PVAT & SODPEG P>0.05, +PVAT vs +PVAT & SODPEG p>0.05, n=8). D: PVAT-denuded vessels were stimulated at 10Hz, and left to recover for 15 minutes. In a separate bath a section of exogenous PVAT was transferred to the PVAT-denuded vessel, before repeating the 10Hz stimulus (P<0.01**, n=8). E: PVAT vessels with and without endothelium were subjected to EFS (-PVAT +endo vs +PVAT +endo P<0.0001****, -PVAT -endo vs +PVAT -endo P<0.0001++++, -PVAT -endo vs -PVAT +endo P>0.05, +PVAT -endo vs +PVAT +endo P>0.05, n=8). Data shown are mean ± SEM.
Figure 2: The anti-contractile effect is dependent on sympathetic nerves present in PVAT. A: Sections of mouse mesenteric PVAT were stained for dopamine β-hydroxylase (n=5). B: Sections of mouse brain were used as a positive control for dopamine β-hydroxylase staining. C: Adipose tissue sections incubated with TBS in place of primary antibody were used as a negative control. –PVAT vessels with a section of exogenous PVAT suspended above were stimulated with EFS. The exogenous PVAT was removed and incubated for 30 minutes with TTX (D, 1μM, -PVAT vs + exogenous PVAT P<0.0001****, + exogenous PVAT vs TTX treated PVAT P<0.0001++++, -PVAT vs TTX treated PVAT P>0.05, n=8), or 6-OHDA (E, 2μM, -PVAT vs + exogenous PVAT P<0.001***, + exogenous PVAT vs 6-OHDA treated PVAT P<0.001+++ , -PVAT vs 6-OHDA treated PVAT P>0.05, n=8). Data shown are mean ± SEM. Scale bars represent 50μm.
Figure 3: Adipocytes contain functional β3-adrenoceptors. A: Sections of mouse mesenteric PVAT were stained for β3-adrenoceptors (n=5). B: Sections of mouse kidney were used as a positive control for β3-adrenoceptor staining. C: Adipose tissue sections incubated with TBS in place of primary antibody were used as a negative control. D: Following control responses to EFS ±PVAT vessels were incubated with CL-316,243 (10μM) for 30 minutes (-PVAT vs +PVAT P<0.0001****, +PVAT vs +PVAT & CL-316,243 P<0.01++, -PVAT vs -PVAT & CL-316,24 P>0.05, -PVAT vs +PVAT & CL-316.243 P<0.0001), n=8). Data shown are mean ± SEM. Scale bars represent 50μm.
Figure 4: The release of the anti-contractile factor is dependent on adipocyte $\beta_3$-adrenoceptor activation. A: Solution transfer studies using exogenous PVAT pre-incubated with the $\beta_3$-adrenoceptor antagonist SR59230A (P>0.05, n=8). B: Following control responses to EFS ±PVAT vessels were incubated with SR59230A (+PVAT vs +PVAT & SR59230A P<0.01++, -PVAT vs +PVAT & SR59230A P<0.0001****, -PVAT vs +PVAT P<0.0001, -PVAT vs -PVAT vs SR59230A P>0.05, n=8). Data shown are mean ± SEM.
Figure 5: The anti-contractile effect of PVAT is in part due to NA-reuptake. A: Sections of mouse mesenteric PVAT were stained for OCT3 (n=5). B: Sections of mouse kidney were used as a positive control for OCT3 staining. –PVAT vessels with a section of exogenous PVAT suspended above were stimulated with EFS. C: Adipose tissue sections incubated with TBS in place of primary antibody were used as a negative control. The exogenous PVAT was removed and incubated for 30 minutes with corticosterone (D, 100μM, + exogenous PVAT vs + corticosterone treated PVAT P<0.001***, -PVAT vs + corticosterone treated PVAT P<0.001***, -PVAT vs + exogenous PVAT P<0.0001, n=8), or both corticosterone (E, 100μM) and SR59230A (1μM, -PVAT vs + exogenous PVAT P<0.0001****, + exogenous PVAT vs + PVAT treated with corticosterone & SR59230A P<0.0001****, -PVAT vs + PVAT treated with corticosterone & SR59230A P>0.05, n=8). Data shown are mean ± SEM. Scale bars represent 50μm.
Figure 6: Adiponectin is the anti-contractile factor released upon β₃-adrenoceptor activation. A: Following the control EFS protocol, PVAT-denuded vessels were allowed 15 minutes to recover before incubating with recombinant globular mouse adiponectin (5μg/ml) for 15 minutes, and repeating the stimulus (P<0.001***, n=4). B: Following the control EFS protocol, ±PVAT vessels were incubated with ABP (5μg/ml) for 45 minutes (+PVAT vs +PVAT & ABP P<0.0001++++, -PVAT vs +PVAT & ABP P<0.05*, -PVAT vs +PVAT P<0.0001, -PVAT vs -PVAT & ABP P>0.05, n=4). C&D: Following control responses to EFS ±PVAT vessels were incubated for 30 minutes with SR59230A (C, SR 1μM, +PVAT vs +PVAT & SR P<0.0001****, -PVAT vs +PVAT & SR P<0.01++, -PVAT vs +PVAT & SR + ABP P<0.01**, -PVAT vs +PVAT P<0.0001, +PVAT vs +PVAT & SR P<0.01, n=4) or CL-316,243 (D, CL, 10μM, +PVAT vs +PVAT & CL + ABP P<0.01++, -PVAT vs +PVAT & CL + ABP P<0.001***, -PVAT vs +PVAT P<0.0001, +PVAT vs +PVAT & CL P<0.01, n=4) and stimulated for a second time. Next, the vessels were incubated again with SR59230A or CL-316,243, plus the ABP (5μg/ml) for 45 minutes. E: The concentration of adiponectin secreted by 100mg of mesenteric PVAT upon stimulation at 10Hz for 4s, was measured in the presence and absence of SR59230A (P<0.001, n=8). Data shown are mean ± SEM.
**Figure 7: Adipo⁻/⁻ mice lack an EFS induced anti-contractile effect.** Body weights (A) and epididymal fat pad weights (B) of control C57 and adipo⁻/⁻ mice were compared (P>0.05, n=8). C: Blood pressure was recorded in conscious, restrained control C57 mice and adipo⁻/⁻ mice using a CODA tail cuff system (P<0.01**, n=8). D: Following sacrifice mixed blood was collected and fasting glucose concentration was measured using an automatic monitor (P<0.01**, n=8). E: Vessels from adipo⁻/⁻ mice with and without PVAT were subjected to EFS (-PVAT vs +PVAT >0.05, n=5), before incubating with CL-316,243 for 30 minutes and repeating the stimulus (-PVAT vs -PVAT & CL-316,243 P>0.05, +PVAT vs +PVAT & CL-316,243 P>0.05, -PVAT & CL-316,243 vs +PVAT & CL-316,243 P>0.05, n=8). F: Sections of adipo⁻/⁻ mesenteric PVAT were stained for β₃-adrenoceptors (n=4). Data shown are mean±SEM. Scale bar represents 50μm.