Sensory innervation of perivascular adipose tissue: a crucial role in artery vasodilatation and leptin release

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Short title: PVAT is crucial for artery sensory nerve function

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Abstract

Aims: Electrical field stimulation (EFS) elicits robust sensory neurogenic relaxation responses in the rat isolated mesenteric arterial bed but these responses are absent or difficult to demonstrate in isolated arteries. We believe that this mismatch is due to the absence of perivascular adipose tissue (PVAT) as it is conventionally removed in studies on isolated vessels. We aimed to determine whether sensory nerves are expressed in PVAT, their physiological roles and their possible interactions with PVAT-derived adipokines.

Methods and Results: Using confocal imaging, enzyme immunoassay (EIA), myography, vascular perfusion and multiplex analysis of rat mesenteric arteries, we show that PVAT is crucial for the roles of sensory nerves in control of vasomotor tone and adipokine release. Immunofluorescence double staining showed co-expression of calcitonin gene-related peptide (CGRP; sensory neurotransmitter) and PGP9.5 (neuronal marker) in PVAT of mesenteric arteries. CGRP release from dissected PVAT, measured using EIA, was increased by capsaicin which activates sensory nerves. EFS in both mesenteric arteries and perfused mesenteric arterial beds, with and without PVAT, demonstrated neurogenic relaxation in the presence of PVAT, which was greatly attenuated in preparations without PVAT. Neurogenic relaxation due to EFS was associated with release of leptin in PVAT-intact mesenteric arterial beds, which was abolished in preparations without PVAT. Exposure to low oxygen was associated with an attenuated leptin and adiponectin release, but an increase in IL-6 release, from mesenteric arterial beds. Exogenous leptin augmented relaxation to CGRP in mesenteric arteries.

Conclusions: These data show, for the first time, expression of sensory nerves within PVAT and that PVAT is crucial for sensory neurogenic vasorelaxation and crosstalk with adipocytes leading to leptin release, which may augment CGRP-mediated relaxation; leptin release is abolished after exposure to conditions of reduced oxygenation.

Non-standard Abbreviations and Acronyms

BAT	brown adipose tissue
CGRP	calcitonin gene-related peptide
EFS	electrical field stimulation
EIA	enzyme immunoassay
IL-6	interleukin-6
NANC	non adrenergic non cholinergic
PVAT	perivascular adipose tissue
PVAT+	perivascular adipose tissue intact
PVAT-	perivascular adipose tissue denuded
SMA	superior mesenteric artery
TRPV1	transient receptor potential vanilloid 1
TTX	tetrodotoxin
WAT	white adipose tissue

1. Introduction

Sensory nerves containing calcitonin gene-related peptide (CGRP) and other neurotransmitters are expressed throughout the cardiovascular system and can regulate vascular tone.[1-5] They are assumed to reside exclusively/predominantly in the adventitia, the outermost layer of blood vessels, which includes elastic and collagen fibres. The rat isolated mesenteric arterial bed preparation has been widely used to define our understanding of sensory neurotransmission in blood vessels;[6-12] in this preparation electrical field stimulation (EFS) elicits pronounced neurogenic vasorelaxation due to activation of sensory nerves and release of CGRP; it is noteworthy that the mesenteric arterial bed is extensively covered in perivascular adipose tissue (PVAT). In contrast, attempts to demonstrate neurogenic relaxation to EFS in rat isolated mesenteric arteries have been unsuccessful; whilst rat isolated mesenteric arteries do relax to EFS, this is not thought to be a sensory nerve generated response and rather is myogenic or dependent on an intact endothelium.[13-15] Moreover, there are surprisingly few reports of sensory neurogenic relaxation in other isolated arteries. Here we present evidence that this is due to the normal process of removing PVAT from artery segments before experimentation, a process that is not normally applied to the whole mesenteric arterial bed preparation.

In this study we tested the hypothesis that sensory nerves are expressed in PVAT. PVAT surrounds most blood vessels except for the cerebral vasculature and microvasculature[16] and is attracting increasing interest as a regulator of vascular function and because of evidence for its involvement in dysfunctional arterial contractility in pathophysiological conditions.[17-21] Sensory nerves are present in white adipose tissue (WAT)[22-25] and brown adipose tissue,[26,27] but their expression and roles in PVAT have not previously been reported. PVAT is a source of adipokines and other vasoactive compounds, but the relationship between these compounds and sensory nerves is also poorly understood. Evidence for interactions between leptin and sensory nerves was shown in WAT, where leptin secreted from the adipocytes increased the activity of sensory nerves innervating the tissue.[25] In the present study we tested the hypothesis that sensory nerves in PVAT have a fundamental role in artery vasodilatation and adipokine release, and that cross-talk may be disrupted under conditions of reduced oxygenation, such as might arise due to adipose tissue expansion in obesity. We provide evidence that the expression and role of sensory nerves in the control of blood vessel contractility has been underestimated, with implications for our understanding of cardiovascular function under physiological conditions and in disease.

2. Methods

2.1 Rat tissue preparation

All experimental procedures were approved by the Animal Care and Use Committee of University of Nottingham, United Kingdom. Male Wistar rats weighing 180-220 g were killed by stunning and exsanguination. The mesenteric arterial beds were isolated and dissected as described previously.[6,10-12] Mesenteric arteries and mesenteric arterial beds were prepared in two conditions, either as PVAT intact (PVAT+) or as PVAT denuded (PVAT-). PVAT removal from mesenteric arterial beds was carried out as described previously.[28] PVAT was carefully removed from mesenteric arteries/arterial beds with fine scissors under a dissecting microscope paying attention not to damage the adventitial layer.

2.2 Mesenteric arterial bed perfusion

The mesenteric arterial beds were perfused as described previously.[6,10-12] Briefly, the superior mesenteric artery was cannulated and the mesenteric arterial bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused while other branches of the superior mesenteric artery were tied off. The preparation was contained within a humid chamber and perfused with Krebs-Henseleit solution at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30; Cole-Parmer Instrument Co., Chicago, U.S.A). The Krebs solution was gassed with 95 % O_2 and 5 % CO_2 and maintained at 37°C. A vasodilatation response was elicited by EFS (0.5-12 Hz, 60 V, 0.1 ms, 30 s) in the presence or absence of PVAT. Exogenous CGRP (1.5×10^{-13}-1.5×10^{-10} moles) was added as bolus doses.

2.3 Wire myography

Fine dissection was used to isolate superior mesenteric and second-order mesenteric arteries of the mesenteric arterial bed using a dissecting microscope. Arteries were cut into ring segments, mounted on fine wires, and placed between the plastic jaws of a dual and multi-channel wire myograph (Danish Myo Technology, Arhus, Denmark). The isometric tension recordings were carried out as described previously.[29] Segments were equilibrated in Krebs solution at 37°C, gassed with 95% O_2 and 5% CO_2. Tensions of 1 g and 0.5 g were applied to superior mesenteric and second-order mesenteric arteries, respectively, in the presence of guanethidine (5μM) and methoxamine (1-10μM) (to block sympathetic neurotransmission and pre-constrict the preparations, respectively). EFS at 0.5-12 Hz, 40 and 60 V, 0.05 and 0.1 ms, 30 s was carried out to investigate nerve mediated vasorelaxant responses. Finally tetrodotoxin (1 μM), a neurotoxin which acts by blocking sodium channels, was added after a stable tone was achieved and then responses to EFS were repeated. In separate experiments leptin (0.1 pM) was added after stable tone to methoxamine had been achieved and, after 5 min, cumulative relaxation responses to CGRP were constructed.

2.4 Immunofluorescence staining

Superior mesenteric arteries, first-order mesenteric arteries, second-order mesenteric arteries and third-order mesenteric arteries were fixed in 4% (w/v) paraformaldehyde for 3 hours. Briefly, samples were sectioned on a cryostat at 40 μm (Leica model CM1900, Heidelberg, Germany). In some of PVAT+ (double staining) preparations, fat from half of the vessel was removed and immunofluorescence staining procedures were conducted as described below with omission of the cutting step; this procedure was conducted with the aim of obtaining more nerve profiles. Immunolabelling was performed using a calcitonin gene related peptide (CGRP) antibody (anti-CGRP, rabbit polyclonal, Merck Millipore, UK) diluted 1:500, and/or antibody to a general neuronal marker, protein gene product 9.5 (anti-PGP 9.5, mouse monoclonal, Abcam, UK) diluted 1:100. The secondary antibody for CGRP was goat anti-rabbit IgG tetramethylrhodamine (TRITC; Thermo Fisher Scientific, Waltham, MA U.S.A), and for PGP9.5 the secondary antibody was goat anti-mouse IgG fluorescein isothiocyanate (FITC; Thermo Fisher Scientific, Waltham, MA U.S.A), 1:500 and 1:200, respectively; these were diluted in PBS + 0.1% (w/v) bovine serum albumin (BSA), and were incubated with the samples at 37^0C for 1 hour followed by further washes with PBS + 0.1% (w/v) BSA. Mesenteric artery segments and isolated PVAT were observed under laser confocal scanning microscopy (Leica SP2, Heidelberg, Germany) and Volocity image software (Perkin Elmer, Massachusetts, U.S.A). Excitation: green laser 488 nm (Argon 100 mW); red laser 561 nm (LASOS, Taser Technik GMH). Emission: FITC 500-550 nm, TRITC 570-620 nm. Lens 63x /1.4 Oil (HCX PL APO CS 63 x 1.4 NA, Oil).

2.5 Enzyme immunoassay (EIA)

CGRP release was quantified using EIA as described previously.[12] PVAT dissected from segments of mesenteric arteries (superior mesenteric and second-order mesenteric arteries) was incubated in Krebs solution with and without capsaicin (10 μM) at 37°C, for 15 min. The solutions were then assayed for CGRP content using rat CGRP EIA kit (SPI-BIO, Bertin Pharma, Montigny-le-Brentonneux, France). CGRP in each sample was extracted using Oasis® HLB Extraction cartridges (Waters, Massachusetts, USA) in acidic conditions. Extracts were incubated for 18 hours in 96-CGRP monoclonal antibody anticholinesterase enzyme (AChE) coated wells. The concentration of rat CGRP was determined by measuring the enzymatic activity of the AChE using Ellman's reagent. The intensity of the colour changes, determined by spectrophotometry, is proportional to the concentration of rat CGRP.

2.6 Multiplex immunoassay

Mesenteric arterial beds with and without PVAT were perfused at 3 ml/min with Krebs-Henseleit solution containing guanethidine (5 μM) and methoxamine (1-5 μM), to inhibit sympathetic neurotransmission and pre-contract the preparations, respectively.[6-12] Fractions of perfusate were collected at raised tone 5 min before EFS (control), and during EFS-evoked sensory nerve stimulation (8 Hz, 60 V, 0.1 ms, 30 s), for 1 min. Mesenteric arterial beds were further perfused for another 1.5 hours with Krebs solution gassed with 95% N_2 and 5% CO_2 to induce low oxygen conditions. Fractions of low oxygen perfusate were then collected before and during EFS as described previously (above).

Fractions were lyophilised and then reconstituted in 150 μl of distilled water per fraction, in order to concentrate the samples. Levels of leptin, adiponectin and interleukin-6 (IL-6) were measured using a rat multiplex cytokine immunoassay kit (RADPCMAG-82K, Milliplex, Merck Millipore, Darmstadt, Germany). The specific antibody-coated magnetic beads were sonicated, mixed with assay buffer, and then added together with the samples onto a plate which was incubated with agitation overnight at 4° C in the dark. After several washes with assay buffer, 50 μl of detection antibodies were added into each well, and the plate was then incubated with agitation for 1 hour at room temperature. 50 μl of Streptavidin-phycoerythrin was then added to each well and the plate was re-incubated with agitation for 30 min. After washing, 100 μl of Bio-Plex™ sheath fluid (Bio-Rad, California, USA) was added to all wells. The plate was read using a Bio-Plex® 200 instrument (Bio-Rad, California, USA), with samples measured in duplicate.

2.7 Materials

Methoxamine hydrochloride and capsaicin were purchased from Sigma-Aldrich (Dorset, United Kingdom). Guanethidine monosulphate (Ismelin®) was purchased from Amdipharm Mercury (London, United Kingdom). Tetrodotoxin and CGRP were purchased from Tocris Bioscience (Bristol, United Kingdom). Leptin was from Source Bioscience UK Ltd. Capsaicin was dissolved in ethanol (stock of 10 mM) while other drugs were dissolved in distilled water.

2.8 Statistical Analyses

Relaxant responses of the mesenteric arterial beds and mesenteric arteries were expressed as a percentage of the decrease in pressure (mmHg) or tension (g) respectively, against the methoxamine-induced raised tone. Data are reported as means ± SEM. Two-way ANOVA with Bonferroni or Sidak's post hoc test was conducted for EFS data, EIA assay of CGRP release, multiplex assay of adipokine release, and CGRP relaxations in mesenteric arteries. Locally Weighted Scatter-plot Smoother (LOWESS) was carried out to determine interpolated X values from unpaired Y values in EIA experiments. All analyses were carried out using GraphPad Prism (Version 6, GraphPad Software, California, USA). P-values of less than 0.05 were considered statistically significant. n indicates number of rats.

3. Results

3.1 Removal of PVAT abolishes sensory vasodilatation responses in the perfused mesenteric arterial bed

The rat mesenteric arterial bed is a preparation with extensive PVAT and a pronounced sensory neurogenic vasorelaxant response.[6-12] EFS (0.5-12 Hz, 60 V, 0.1 ms and 30 s) of perfused mesenteric arterial beds in the presence of guanethidine and methoxamine (to block sympathetic neurotransmission and pre-constrict the preparations respectively) elicited frequency dependent vasodilatation in preparations with intact PVAT (PVAT+) (Figure 1A). We and others have shown that these responses are abolished by capsaicin and tetrodotoxin treatment identifying an involvement of capsaicin-sensitive sensory nerves.[6,7,30] We investigated the effect of removing the PVAT on the sensory neurogenic vasorelaxant response. Neurogenic vasodilatation was virtually abolished in preparations in which PVAT had been removed by dissection (eg. at 12 Hz: PVAT+, 85 ± 3 % vs PVAT-, 8 ± 2 %; n=4) (Figure 1B).

To examine the integrity of vessels in both PVAT+ and PVAT- preparations, bolus injection of exogenous CGRP (1.5×10^{-13}-1.5×10^{-10} moles) was carried out. There was no difference in vasorelaxant responses to CGRP between the preparations (Figure 1C) showing that the ability of the smooth muscle to relax was similar between the preparations with and without PVAT.

3.2 Electrically-evoked neurogenic vasodilatation in isolated mesenteric arteries under isometric tension reveals the role of PVAT and influence of vessel size

To further investigate the possible contribution of sensory nerves in PVAT to the control of vasomotor tone, isometric tension recording was conducted in rat isolated mesenteric arteries with and without PVAT. Responses to EFS were investigated in methoxamine-preconstricted artery segments in the

presence of guanethidine (5 μM) to block sympathetic neurotransmission. In second order mesenteric artery segments with PVAT, EFS elicited frequency-dependent relaxation which was greatly reduced in the presence of tetrodotoxin (eg. at 12 Hz: PVAT+, 67 ± 14 % vs PVAT+ with tetrodotoxin, 12 ±7 %) (P<0.001, n=6) (Figure 2A,B,C). In superior mesenteric arteries with PVAT, relaxation responses to EFS were small (eg. at 12 Hz: PVAT+, 5 ± 3 %) and were abolished in the presence of tetrodotoxin (n=6) (Figure 2D). Thus, in the presence of PVAT, neurogenic relaxation responses to EFS were observed, which were greater in second order mesenteric arteries compared to superior mesenteric arteries (compare Figures 2C and D).

In second order mesenteric arteries without PVAT, the effect of EFS (0.5-12 Hz, 60 V, 0.1 ms, 30 s) was investigated in artery segments from 9 rats: in 2 of the preparations we observed minimal vasodilatation (<5% relaxation at 8 and 12 Hz); in 7 other preparations a vasodilator response was observed, but these responses were tetrodotoxin-resistant, as reported by others.[13-15] The stimulation parameters used were reduced, to 0.5-12 Hz, 40 V, 0.05 ms, 30 s, in an attempt to identify a tetrodotoxin-sensitive neurogenic response; under these conditions only small relaxation responses were observed in the second order mesenteric arteries without PVAT, and these were blocked by tetrodotoxin (eg. at 12 Hz, PVAT-, 5 ± 4 %, PVAT- with tetrodotoxin, 0 ± 0 %, n=4). In superior mesenteric arteries without PVAT, no relaxation responses to EFS (0.5-12 Hz, 60 V, 0.1 ms, 30 s) were observed (eg. at 12 Hz: PVAT-, 0 ± 0 %; n=6). These data identify the essential requirement of sensory nerves in PVAT to vasorelaxant control of rat small mesenteric arteries. The nerves are not parasympathetic vasodilator nerves, since neurogenic relaxations in the rat mesenteric arterial bed are unaffected by atropine (muscarinic cholinergic antagonist).[6]

3.3 CGRP-containing nerve fibres are expressed in PVAT of mesenteric arteries

Immunoreactivity for CGRP was investigated in mesenteric arterial segments (superior mesenteric and second-order mesenteric arteries) both with PVAT removed (PVAT-) (Figure 3A-C) and with PVAT intact (PVAT+) (Figure 3D-F). Immunoreactivity for CGRP was observed both on the surface of the vessels (Figure 3A,B) and within the PVAT (Figure 3D,E). Fibre-like structures were observed and some of them appeared to have neuronal somata (Figure 3A,B). To confirm the expression of CGRP-containing nerves within PVAT, a co-localization protocol was employed on PVAT of the mesenteric arteries. Superior mesenteric and second-order mesenteric artery segments with PVAT intact were co-treated with anti-CGRP (red staining) and anti-PGP9.5 (green staining). Intense double immunoreactivity was observed as yellow staining, and showed the presence of nerves and neuronal somata as shown in Figure 4. In the absence of anti-CGRP nerve antibody (negative control), no immunoreactivity was visualized in the artery segments as shown in Figure 3 (C, F) and Figure 4 (E and J).

3.4 EIA shows CGRP expression in PVAT of mesenteric arteries

To further examine whether the CGRP is associated with sensory nerves in PVAT, dissected PVAT (isolated from superior mesenteric and second-order mesenteric arteries) was incubated in Krebs solution with and without capsaicin (10 μM, 15 min), and then EIA was carried out on samples of the supernatant to measure the amount of CGRP released. In dissected fat isolated from the second-order mesenteric artery, the CGRP level was more than 100% greater after exposure to capsaicin (8.4 ± 1 pg/ml/cm; n=4) than in the absence of capsaicin (3.6 ± 0.4; n=4) (Figure 5). CGRP levels in the supernatant of dissected fat isolated from superior mesenteric arteries were similar with (2.6 ± 0.5 pg/ml/cm; n=4) and without (2.6 ± 0.9 pg/ml/cm; n=4) capsaicin treatment (Figure 5).

3.5 Interactions between sensory nerves and PVAT-derived mediators

Given the above evidence for expression of sensory nerves within PVAT, we investigated whether their activation could affect adipokine release. In mesenteric arterial beds pre-contracted with methoxamine (1-5 μM) and in the presence of guanethidine (5 μM), levels of leptin collected in the perfusate were 800% greater in PVAT+ preparations than in PVAT- preparations (15.6 ± 2.6 pg/ml; n=10 vs 1.9 ± 0.8 pg/ml; n=6) (P<0.05, ANOVA) (Figure 6A). Moreover, in the presence of PVAT, leptin levels measured in the perfusate collected during EFS of sensory nerves were significantly enhanced (28.1 ± 4.4 pg/ml, n=10) (P<0.05, ANOVA) (Figure 6A). Mesenteric arterial beds with intact PVAT released

a significantly greater amount of adiponectin into the perfusate than PVAT- preparations (160 ± 33 ng/ml; n=11 vs 2 ± 0.8 ng/ml; n=5, respectively) (P<0.01, ANOVA) (Figure 6B). However, adiponectin release in PVAT+ preparations was similar during EFS (8 Hz, 60 V, 0.1 ms, 30 s) induced sensory neurogenic activation (129 ± 25 ng/ml; n=11) as under control conditions in the absence of EFS (Figure 6B).

3.6 Effects of reduced oxygen level on the interactions between sensory nerves and PVAT-derived mediators

The effects of oxygen level on perfusate leptin and adiponectin levels were investigated in mesenteric arterial beds with intact PVAT pre-contracted with methoxamine (1-5 μM), in the presence of guanethidine (5 μM) to block sympathetic neurotransmission; we hypothesised that reduced oxygen, such as can occur during adipocyte expansion, would alter adipokine release and consequently the interactions between sensory nerves and adipocytes. The raised tone of the preparations gassed with standard oxygen was decreased with a low oxygen supply (48±9 mmHg vs 14±7 mmHg respectively; n=8), so responses to EFS could not be meaningfully quantified. EFS had no significant effect on leptin release under conditions of reduced oxygenation (standard oxygen, 28.1±4.4 pg/ml; n=10 vs low oxygen, 1.8±0.4 pg/ml; n=8) (Figure 7A). In both standard oxygen and low oxygen, adiponectin was detected in the perfusate of PVAT+ mesenteric arterial beds (Figure 7B). Low oxygen significantly decreased the level of adiponectin collected in the perfusate at basal conditions (standard oxygen, 160.3±32.9 ng/ml; n=11 vs low oxygen, 57.1±15.2 ng/ml; n=7) and during EFS (standard oxygen, 129±24.5 ng/ml; n=11 vs low oxygen, 33.3±8.8 ng/ml; n=9) (P<0.05, ANOVA) (Figure 7B). Under standard oxygen conditions IL-6 was not detected in the perfusate of mesenteric arterial bed preparations, however, levels of IL-6 were significantly enhanced in low oxygen conditions (Figure 7C).

3.7 Leptin augments relaxation responses to CGRP in rat mesenteric arteries

The effect of leptin on CGRP responses was investigated to determine whether reciprocal interactions exist between sensory nerves and PVAT. CGRP elicited concentration-dependent relaxations in the rat second-order mesenteric artery segments and these were augmented in the presence of leptin (0.1 pM) (n=6) (P<0.05, ANOVA). LogEC_{50} values were: control -8.55 ± 0.25 (n=6) and in the presence of leptin -8.87 ± 0.16 (n=6) (Supplemental Figure 1). At this concentration (0.1 pM) leptin had no effect on the tone of the rat second-order mesenteric artery.

4. Discussion

The present study provides the first evidence for the distribution of sensory nerves in PVAT in any species, and shows that sensory nerves in PVAT play a direct role in vasoregulation of rat mesenteric arteries. This study is also the first that examines the direct effect of sensory nerve stimulation on levels of PVAT-derived mediator release. Main novel findings in this study are: (1) the immunofluorescence and EIA studies revealed that sensory nerves are expressed in PVAT; (2) the isometric tension experiments of isolated superior mesenteric and second-order mesenteric arteries indicated that PVAT enhances neurogenic vasorelaxation, and this was greater in smaller arteries (second-order mesenteric arteries) compared to conduit arteries (superior mesenteric artery); (3) the perfusion experiments showed that the absence of PVAT greatly attenuates EFS-evoked neurogenic vasorelaxation in the rat mesenteric arterial bed; (4) sensory nerve activation stimulates leptin release in the presence of PVAT and this interaction was lost with PVAT removal and under conditions of reduced oxygenation; and (5) exogenous leptin augments CGRP-mediated vasorelaxation. A diagrammatic summary of some of the key findings is shown in Figure 8.

4.1 Responses to EFS in isolated mesenteric arteries and perfused mesenteric arterial beds in the presence and absence of PVAT

The rat perfused mesenteric arterial bed preparation has been widely used to investigate sensory neurotransmission since EFS of the sensory nerves elicits robust vasorelaxant responses; it is a vascular preparation that is extensively covered in PVAT, although this is never specifically mentioned by users

of the preparation.[6-12] For the first time, we investigated the effect of PVAT removal on the sensory neurogenic vasorelaxant response in the mesenteric arterial bed. EFS responses in rat perfused mesenteric arterial beds were greatly attenuated in preparations in which PVAT had been removed, consistent with the concept that the presence of PVAT enhances neurogenic vasodilatation. There are a number of possibilities that may contribute to this observation. The simplest explanation is that sensory nerves are expressed within PVAT and directly contribute to neurogenic vasodilatation of the mesenteric arterial bed, in line with the immunostaining and EIA evidence for the expression of CGRP-containing nerves in PVAT. However, we also present evidence for a selective release of leptin from PVAT concomitant with electrical stimulation of sensory nerves (discussed below), thus CGRP and/or other neurotransmitters released from sensory nerves may also act at adipocytes to release adipokines, which then act to enhance CGRP function and/or release.

In isometric tension experiments in methoxamine-preconstricted second order mesenteric artery rings with PVAT intact, in the presence of guanethidine to block sympathetic neurotransmission, EFS elicited frequency-dependent vasodilatation. Tetrodotoxin almost abolished the EFS responses, which indicates that the responses were neurogenic. In contrast, responses to EFS in second order mesenteric artery rings without PVAT were either very small or were not blocked by tetrodotoxin, as reported by others in rat isolated mesenteric arteries and in other arteries from different species.[13-15] These data identify fundamental differences in the vasorelaxant responsiveness of mesenteric arteries to EFS depending on the absence or presence of PVAT, where the presence of PVAT enhances neurogenic vasorelaxation. Classically, artery ring preparations are prepared for pharmacological investigation with PVAT removed, thus the present study may explain why there are so few reports of sensory neurogenic relaxations in isolated vessel preparations. In vessels with PVAT, neurogenic relaxation in second-order mesenteric arteries was much larger than in the superior mesenteric artery, suggesting that neurogenic vasorelaxation is dominant in smaller or resistance arteries compared to conduit arteries, in line with evidence that smaller arteries are more densely innervated than conduit arteries.[31] Given the crucial role of small arteries, these data indicate for the first time an important contribution of sensory nerves in PVAT to neurogenic control of blood vessel tone which could modulate vascular resistance and blood pressure.

The absence of PVAT had a more pronounced effect on sensory neurogenic relaxation than expected given that the immunofluorescence staining showed that sensory nerves densely innervate blood vessels in all orders (superior mesenteric, first-, second- and third-order mesenteric arteries) of PVAT-denuded preparations. Confocal microscopy also showed that despite the removal of PVAT, there was an abundance of sensory nerves at the adventitia, consistent with previous evidence for a high density of CGRP-containing nerves on the surface of clean mesenteric arteries.[6,8,9,30,31] Exogenous CGRP produced similar responses in PVAT+ and PVAT- preparations, indicating that the ability of the preparations to relax was unchanged. These observations highlight the importance of PVAT intactness in regulating vasodilatation, and support the possibility of interactions between sensory nerves at the adventitia with adipocytes, and/or continuity between adventitial sensory nerves and sensory nerves in PVAT as suggested for sympathetic nerves.[32] The contribution of PVAT to neurogenic vasorelaxation described here appears to be distinct from its known anticontractile effect, based on the different sensitivities of CGRP-mediated vasorelaxation and the anticontractile effect to blockade with potassium channel inhibitors.[33,34]

4.2 The presence of sensory nerves within PVAT of mesenteric arterial beds

Immunofluorescence staining of PVAT-denuded segments of rat superior mesenteric artery, and first-, second-, and third-order mesenteric arteries, showed the presence of extensive innervation with perivascular nerve fibres immunoreactive to anti-CGRP. Similar observations in mesenteric arterial preparations without PVAT have been reported previously.[6,8,9,30,31] CGRP-immunoreactivity was also visualized within PVAT in PVAT intact mesenteric arteries. A double staining protocol using anti-CGRP and anti-PGP 9.5 antibodies was applied, and co-localization of the staining was evident, which indicates the presence of sensory nerves within PVAT. Interestingly, neuronal somata were visualized in segments of both PVAT intact and PVAT denuded preparations. Traditionally, cell bodies of CGRP-containing nerves were believed to be located only in the dorsal root ganglia. However, there is evidence

that adventitial neuronal somata containing mRNA encoding CGRP are present in rat small mesenteric arteries,[35] which is in line with the current observation. A more recent study revealed that the adventitial neuronal somata express a number of genes including paladin and CaSR which participate in response to injury/stress and vasodilatation.[36] Collectively these studies demonstrate that sensory neuronal somata may reside in the adventitia and PVAT of rat mesenteric arteries.

CGRP release from dissected PVAT in the presence and absence of capsaicin, an agonist at vanilloid receptor subtype 1 (TRPV1), which releases CGRP from sensory nerves, was investigated using EIA. We found that the CGRP level in the supernatant of dissected PVAT, isolated from second-order mesenteric arteries, was significantly greater with capsaicin treatment than in the absence of capsaicin. CGRP levels in the physiological solution bathing dissected PVAT from superior mesenteric arteries were similar with and without capsaicin treatment. These observations suggest that sensory nerves are distributed in PVAT, and that the density of sensory nerves in PVAT is greater in resistance mesenteric arteries than in conduit mesenteric arteries, in line with our pharmacological studies. There is evidence that adipocytes from human abdominal fat express mRNA for CGRP, but CGRP protein expression was absent under control conditions and was only induced after treatment with LPS.[37] Thus, a source of CGRP within PVAT in cell types other than sensory nerves seems unlikely.

4.3 Release of endogenous leptin during EFS of perfused mesenteric arterial beds with intact PVAT, and effect of exogenous leptin on CGRP-mediated vasorelaxation

EFS-induced sensory neurogenic vasorelaxation corresponded with an enhanced leptin release in PVAT+ preparations, which suggests that there is a link between endogenous leptin and sensory nerves. Both basal levels of leptin and the EFS-mediated release of leptin were virtually abolished by PVAT removal, consistent with PVAT as the source of the leptin. Presumably, CGRP and/or other neurotransmitter(s) released from activated sensory nerves during EFS act at receptors on the adipocytes in PVAT to cause leptin release. The release of leptin appeared to be selective since EFS did not alter adiponectin release in either PVAT+ or PVAT- preparations. Adiponectin levels detected in the perfusate of mesenteric arterial beds with intact PVAT were virtually abolished in preparations without PVAT, consistent with PVAT as the source of adiponectin. Exogenous leptin augmented CGRP-mediated relaxations in mesenteric arteries, identifying a mechanism which may contribute to the augmentation by PVAT of neurogenic vasodilatation, although it should be recognised that PVAT is a source of numerous vasoactive factors in addition to leptin[17-21] which may modulate sensory neurotransmission. The mechanism by which leptin augments CGRP-induced relaxation may involve an augmented reduction in intracellular Ca^{2+} concentration[38] or sensitivity, or may involve the endothelium.[39] Leptin may also act pre- as well as postjunctionally. Leptin secreted from WAT was shown to act as a paracrine factor to increase the activity of spinal sensory nerves in WAT in Siberian hamsters.[25] More recently, leptin receptors were shown to be expressed on sensory neurons, and the deletion of leptin signalling in vagal afferent neurons resulted in hyperphagia and obesity.[40] PVAT-derived angiotensin II augmented sympathetic neurotransmission in rat mesenteric arteries,[41] but is unlikely to be involved here since it is a vasoconstrictor which inhibits CGRP release from sensory nerves in the rat mesenteric arterial bed.[7] Superoxide ions have been shown to promote sensory neurogenic relaxation in the rat isolated mesenteric arterial bed[42] and, since superoxide ions can be generated by rat mesenteric arterial PVAT (where they have been shown to enhance sympathetic neurotransmission),[43] this provides another mechanism by which sensory neurogenic relaxation may be augmented by PVAT. The present data add new insight into the crosstalk between leptin and sensory nerves.

We investigated whether reduced tissue oxygenation, which can arise due to adipose tissue expansion,[44-46] can alter adipokine release, either with or without activation of sensory nerves. Under conditions of reduced oxygenation, levels of leptin and adiponectin in the mesenteric arterial bed perfusate were lower compared to control conditions, significantly so for adiponectin, and EFS failed to evoke a release of leptin. IL-6 levels were increased. Our data are consistent with pharmacological evidence for the generation of IL-6 in the PVAT of hypoxic (95% N_2, 5% CO_2 for 2.5 hours) rat isolated mesenteric arteries.[47] In our mesenteric arterial beds, methoxamine-induced tone was greatly attenuated in low oxygen conditions, likely through myogenic mechanisms as shown in rat hypoxic pre-constricted

mesenteric arteries.[48] These data suggest that myogenic mechanisms may predominate over sensory neurogenic control to cause vasorelaxation and an increase in blood flow under conditions of reduced oxygenation. The data are consistent with the fact that vascular tone regulation is achieved by an interplay between factors including myogenic, neurogenic and PVAT influences, and that their relative impact and cross talk is influenced by the levels of tissue oxygenation. Communication between perivascular sympathetic nerves and the endothelium has also been demonstrated.[49,50]

4.4. Limitations of the study

The mesenteric artery of rodents is surrounded by WAT, while human PVAT has characteristics of both WAT and BAT (beige adipose tissue).[51] With regard to leptin, plasma levels correlate positively with adipose tissue mass in both humans and rodents; leptin appears to operate mainly in the long-term control of feeding behaviour and energy balance, but may also function in short term control in rodents.[52] Various levels and durations of oxygenation have been used by others to investigate the effects of low oxygen on vascular function and in the present study the effects of gassing with atmospheric oxygen could additionally have been considered. Acute hypoxia, as used in the present study and by others,[47] can trigger an acute inflammatory reaction, as evidenced by the release of IL-6, and it should be noted that this is different to low-grade inflammation associated with adipose tissue hypoxia in obesity.[53,54] Nonetheless, the *in vitro* effects reported here may be qualitatively similar to those observed *in vivo*.

5. Conclusions

This study has provided evidence that sensory nerves are expressed within the PVAT of rat mesenteric arteries and enhance neurogenic vasodilatation in rat isolated mesenteric arterial beds and mesenteric arteries. Interactions between sensory nerves within PVAT and adipocytes have also been revealed, since leptin release was enhanced by EFS of sensory nerves only in mesenteric arterial bed preparations with intact PVAT, and exogenous leptin augmented CGRP-induced relaxations. The present study also indicates that a low oxygen level reduces adiponectin and leptin levels measured in mesenteric arterial beds with PVAT, but enhances levels of IL-6. Collectively, these data indicate the presence of sensory nerves within PVAT, which play an integral role in vasocontractility and regulate PVAT-derived leptin release. The expression and role of sensory nerves in the cardiovascular system is potentially far greater than previously recognised since traditionally neurogenic control of isolated blood vessels has been investigated in preparations from which the PVAT has been removed, and this could be very relevant to the pathophysiology of diseases of the cardiovascular system including hypertension and obesity.

Funding

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Conflict of Interest

None

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Figure legends

Figure 1: Removal of PVAT attenuates sensory neurogenic vasorelaxation in the rat isolated mesenteric arterial bed. A, Representative traces show that electrical field stimulation (EFS; 0.5-12 Hz, 60 V, 0.1 ms, 30 s) of rat mesenteric arterial beds perfused with Krebs solution containing guanethidine (5 μM) and methoxamine (1-5 μM), at raised tone, produces frequency-dependent decreases in perfusion pressure due to vasodilatation in preparations with intact perivascular adipose tissue (PVAT+), while in preparations without PVAT (PVAT-) the neurogenic vasorelaxant responses are abolished. **B,** In PVAT+, EFS at 0.5-12 Hz, 60 V, 0.1 ms and 30 s elicited frequency-dependent vasodilatation (n=4). In PVAT-, the EFS vasodilator response was attenuated (n=4). **C,** There was no difference between preparations with and without PVAT with regard to relaxation responses to bolus doses of calcitonin gene-related peptide (CGRP) (P>0.05, n=5). ****P<0.001, PVAT+ vs PVAT-.

Figure 2: Neurogenic relaxations in rat isolated mesenteric artery segments with intact PVAT. Responses shown are to electrical field stimulation (EFS; 1-12 Hz, 60 V, 0.1 ms, 30 s) in methoxamine (1-10 μM) pre-constricted rat mesenteric arteries in the presence of guanethidine (5 μM) to block sympathetic neurotransmission. **A,B** Representative traces showing EFS-induced relaxation responses of second order mesenteric arteries with intact perivascular adipose tissue (PVAT+) in the absence and presence of tetrodotoxin (TTX, 1 μM). Comparison of EFS-induced neurogenic vasorelaxation in the presence of PVAT (PVAT+) in second order (n=6) (**C**) and superior mesenteric arteries (n=6) (**D**). EFS-evoked vasorelaxation in second order mesenteric arteries was attenuated with the addition of tetrodotoxin (TTX, 1 μM) (n=6). ***P<0.001, PVAT+ vs PVAT+ and TTX.

Figure 3: CGRP-immunoreactivity in PVAT, and on the surface of PVAT-intact and PVAT-denuded rat mesenteric arteries. Representative images of calcitonin gene-related peptide (CGRP) immunoreactivity (red) on the surface of perivascular adipose tissue (PVAT)-denuded segments of rat superior mesenteric artery (**A**), second order mesenteric artery (**B**), and PVAT+ segments of superior mesenteric artery (**D**), and second order mesenteric artery (**E**). Specificity of the antibody was examined by removal of the primary antibody (Control); PVAT- preparation (**C**) and PVAT+ preparations (**F**). DNA was counterstained with DAPI (blue). Arrows indicate immunoreactive fibre-like structures (**a**), and adventitial neuronal somata (**b**). Scale bar = 50 μm, (n=6).

Figure 4: Co-localization of CGRP and PGP 9.5 (neuronal marker) within PVAT of rat mesenteric arteries. Double staining for calcitonin gene-related peptide (CGRP) and PGP 9.5 within perivascular adipose tissue (PVAT) of rat mesenteric arteries: Anti-CGRP staining in PVAT of superior

mesenteric artery (red) (**A**), anti-PGP 9.5 staining in PVAT of superior mesenteric artery (green) (**B**), merged image shows some neurons co-expressing CGRP and PGP 9.5 in PVAT of superior mesenteric artery (yellow) (**C**). 3-dimensional merged image showing some neurons co-expressing CGRP and PGP 9.5 marker in PVAT region of superior mesenteric artery (**D**). In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control) in PVAT of superior mesenteric artery, no staining was observed (**E**). Anti-CGRP staining in PVAT of second order mesenteric artery (red) (**F**), anti-PGP 9.5 staining in PVAT of second order mesenteric artery (green) (**G**), merged image shows some neurons co-expressing CGRP and PGP 9.5 in PVAT of second order mesenteric artery (yellow) (**H**). 3-dimensional image of some neurons co-expressing CGRP and PGP 9.5 in PVAT of second order mesenteric artery (**I**). In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control) in PVAT of second order mesenteric artery, no staining was observed (**J**). Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate CGRP and PGP 9.5 double labelled: a = nerve, b =cell body/neuronal somata. Scale bars for A-D, F, G and H = 23 μm, I = 16.46 μm and E and J= 50 μm (n=6 preparations from 2 rats).

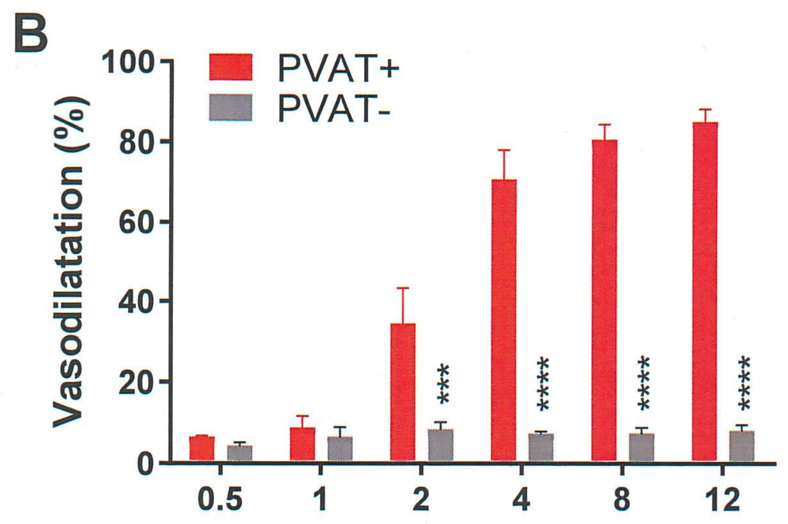
Figure 5: Capsaicin-evoked CGRP release in rat mesenteric PVAT. Concentration of calcitonin gene-related peptide (CGRP) in the supernatant of dissected perivascular adipose tissue (PVAT) isolated from segments of rat superior mesenteric artery (SMA) and second order mesenteric artery in the presence and absence of capsaicin (10 μM, 15 min); capsaicin enhanced CGRP release from PVAT isolated from the second order mesenteric artery (n=4-5). **P<0.01.

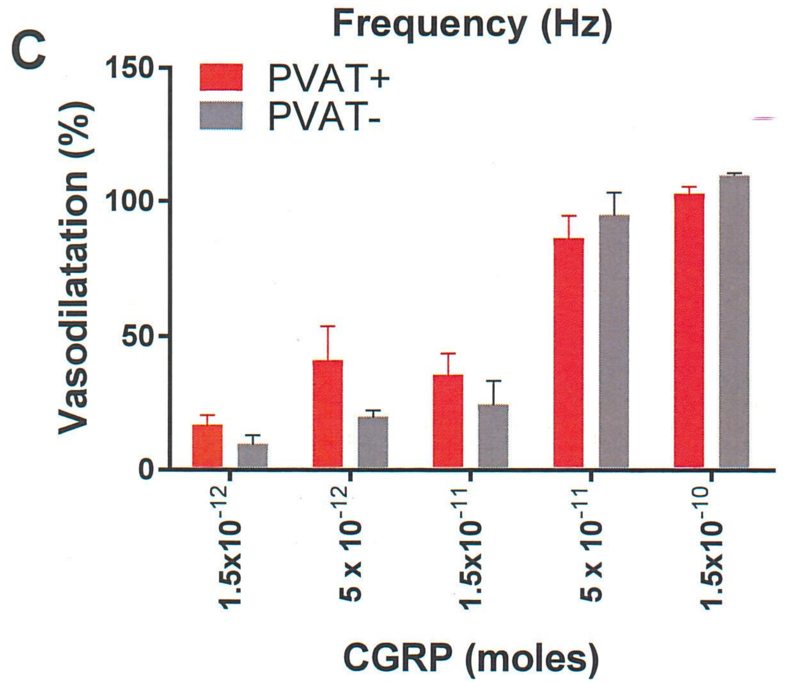
Figure 6: **Electrical field stimulation evokes leptin release in mesenteric arterial bed preparations with intact PVAT, but not in PVAT-denuded preparations.** Tone of the mesenteric arterial bed preparations was raised with methoxamine, guanethidine (5 μM) was present to block sympathetic constriction, and electrical field stimulation (EFS; 8Hz, 60 V, 0.1 ms, 30 s) was applied. **A,** In the presence of perivascular adipose tissue (PVAT+), leptin release was greater both under basal (control) conditions and during EFS than in preparations without PVAT (PVAT-). EFS enhanced leptin release in PVAT+ preparations (n=6-10). **B,** The absence of PVAT attenuated the adiponectin level collected in the perfusate in both basal (control) conditions and during EFS (n=5-11). *P<0.05, **P<0.01, ***P<0.001.

Figure 7: The effect of oxygen level on mediators release during electrically-evoked neurogenic vasorelaxation in rat mesenteric arterial bed preparations with perivascular adipose tissue (PVAT+). Preparations were submaximally pre-contracted with methoxamine, guanethidine (5 μM) was present to block sympathetic neurotransmission, and electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) was applied. **A,** The leptin level was reduced in conditions of low oxygen level (gassing with 95% N_2 5% CO_2) compared to standard oxygen levels (gassing with 95% O_2 5% CO_2) (n=7-10). **B,** The level of adiponectin collected in the perfusate was also greater in standard oxygenation (n=7-11). **C,** The IL-6 level was increased in a reduced oxygen level compared to a standard oxygen level in both basal conditions and during EFS (n=3-7). *P<0.05, **P<0.01, ****P<0.0001; ANOVA with Bonferroni post test.

Figure 8: Expression and roles of sensory nerves in perivascular adipose tissue. Sensory nerves containing calcitonin gene-related peptide (CGRP) and other sensory neurotransmitters are present in perivascular adipose tissue as well as in the adventitia. Following neurogenic activation, CGRP and other sensory neurotransmitters are released from these sensory nerves and cause vasorelaxation, predominantly involving activation of smooth muscle CGRP receptors (CGRP-R). The sensory neurotransmitters also act to promote the release of leptin from the adipocytes, which augments CGRP-induced vasorelaxation through pre- and/or postjunctional mechanisms. NKA, neurokinin A; Lep-R, leptin receptor; SP, substance P.

Figure 1





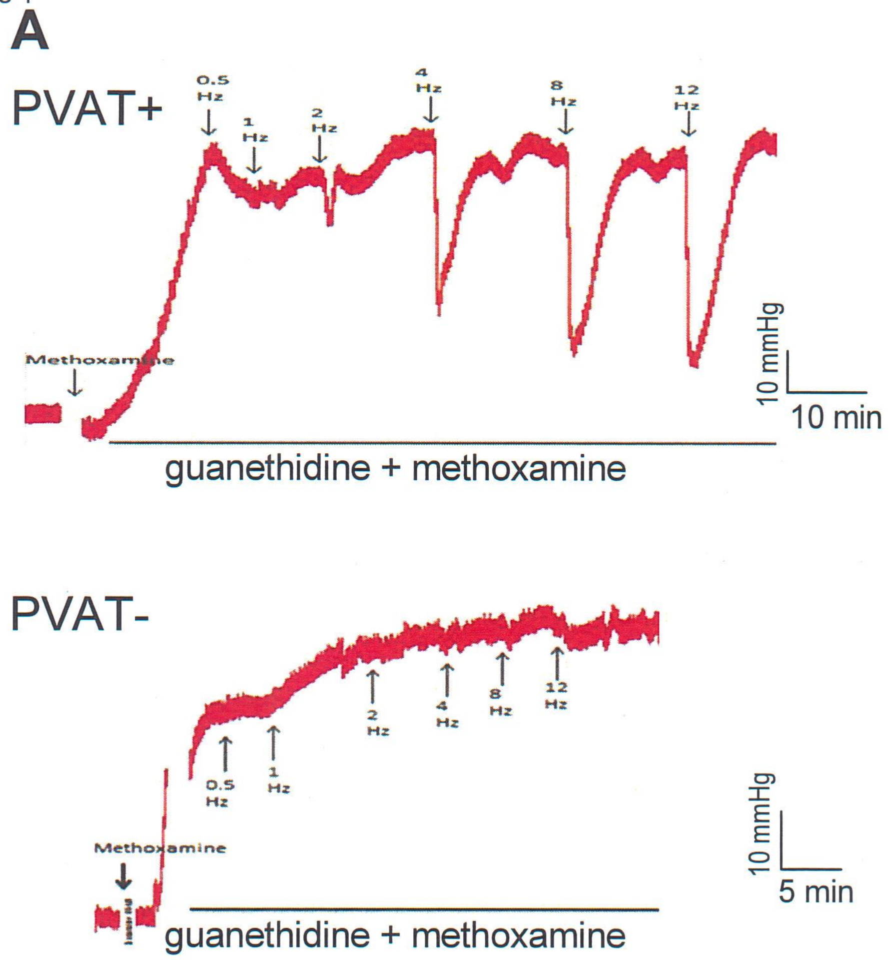


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Figure 2

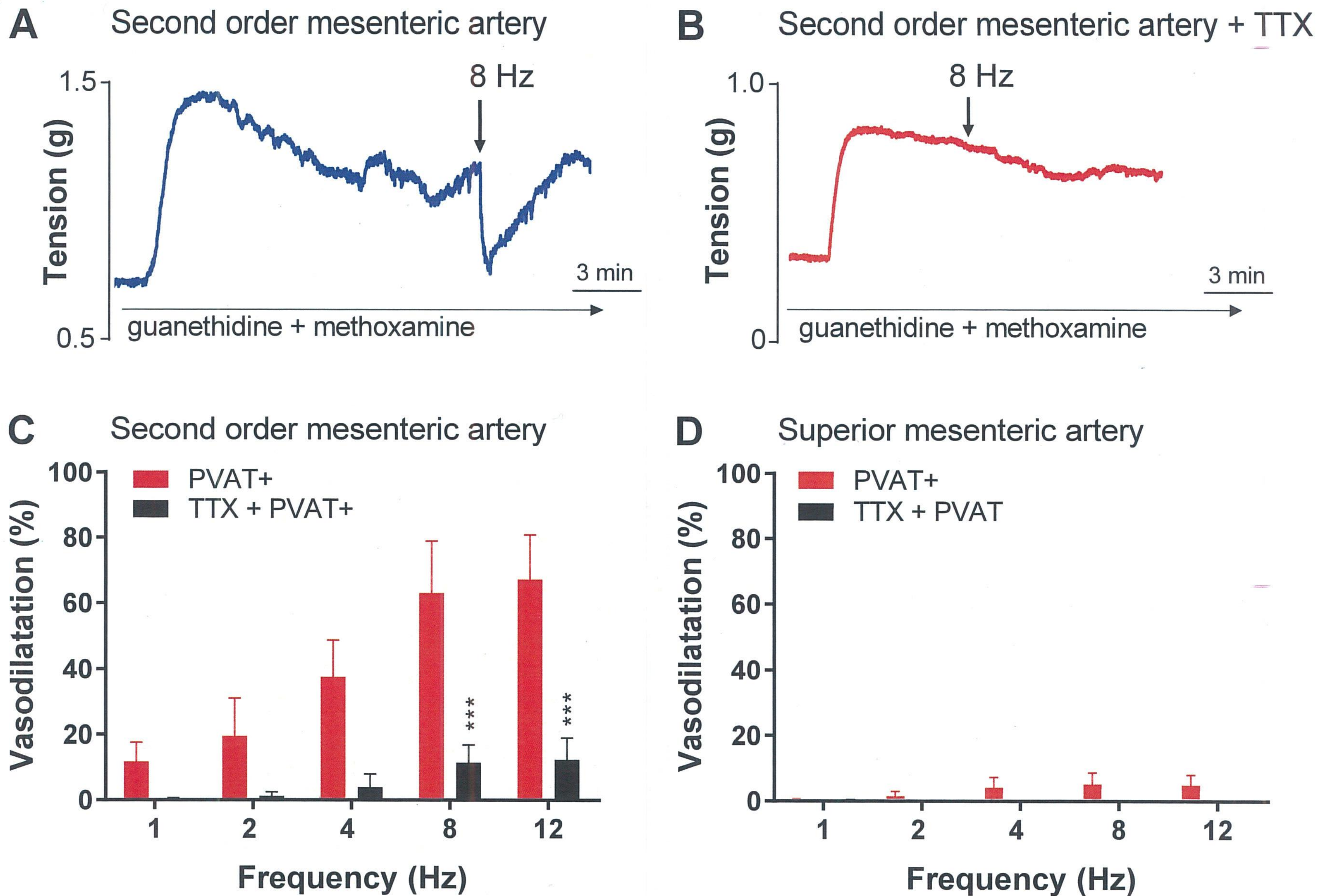

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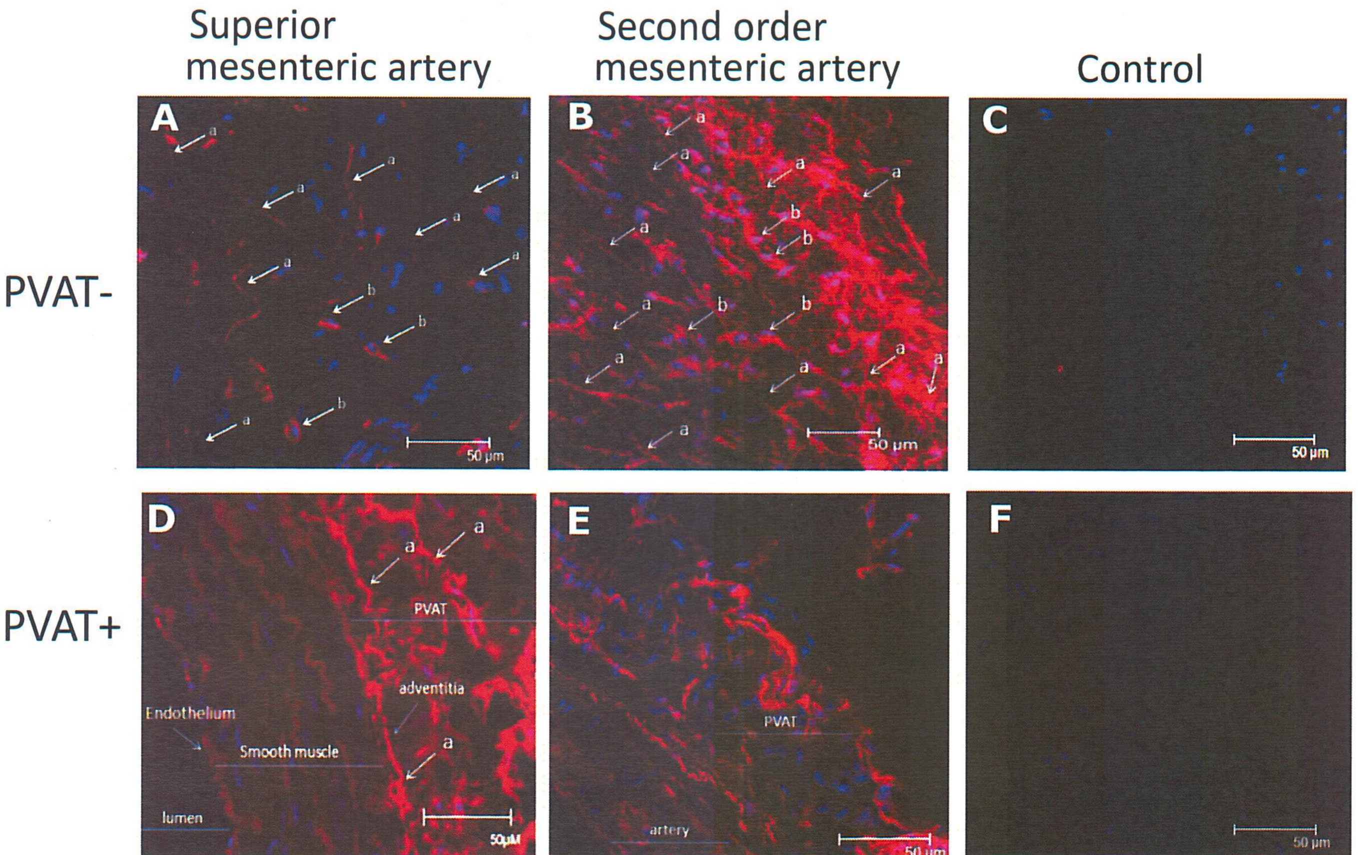
Figure 3



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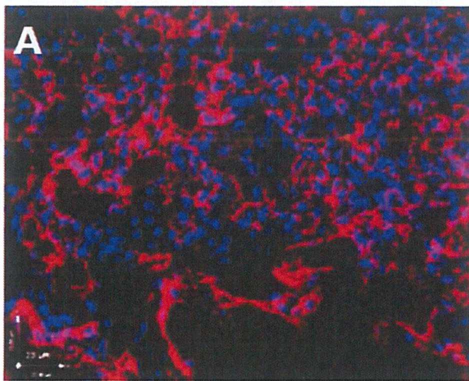
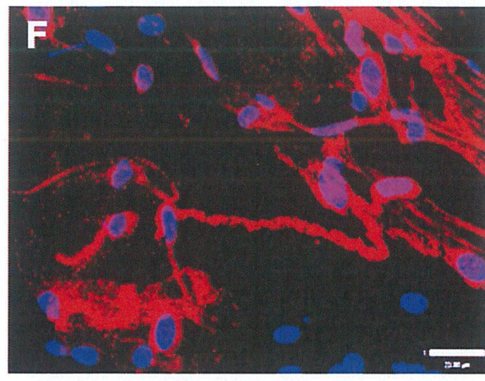
Superior mesenteric artery

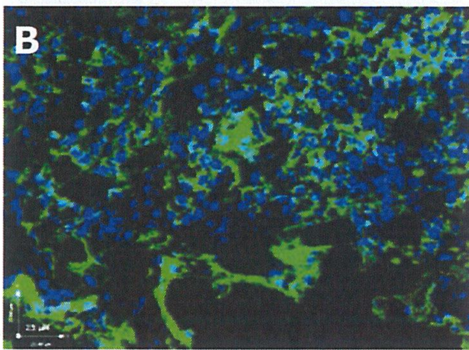
2nd order mesenteric artery

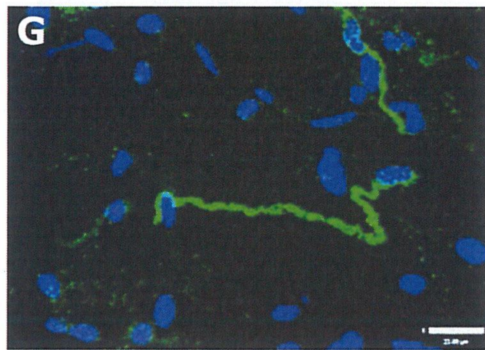
CGRP



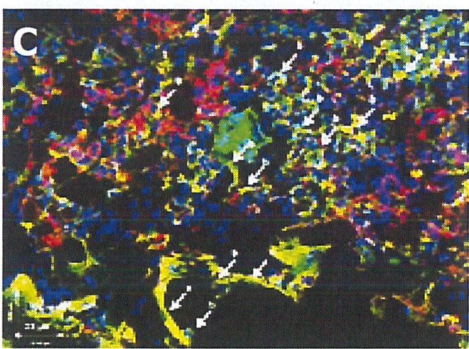


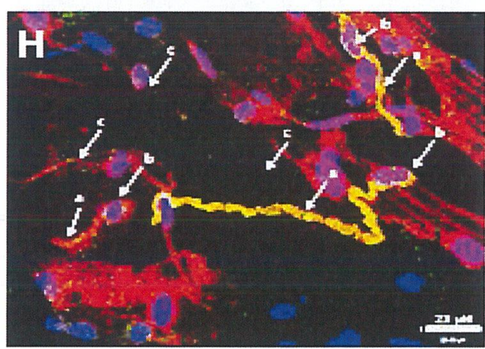
PGP9.5



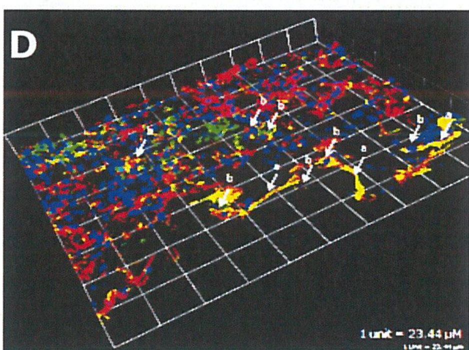


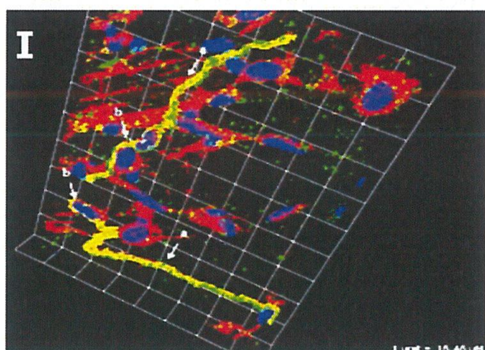
CGRP+
PGP9.5



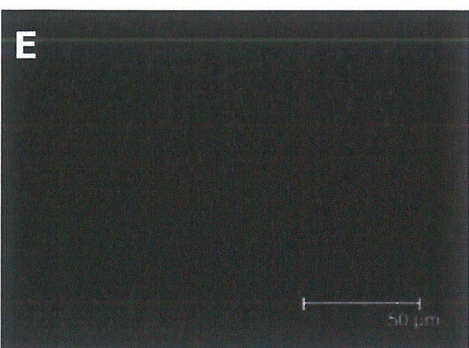


CGRP+
PGP9.5





DAPI



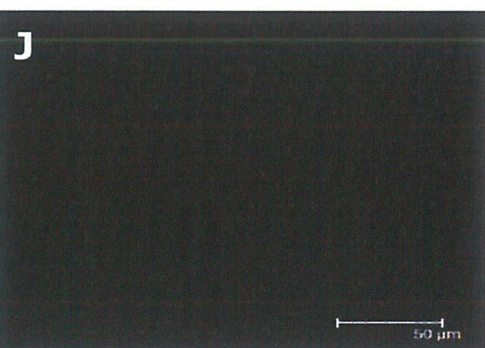


Figure 4: Co-localization of CGRP and PGP 9.5 (neuronal marker) within PVAT of rat mesenteric arteries. Double staining for calcitonin gene-related peptide (CGRP) and PGP 9.5 within perivascular adipose tissue (PVAT) of rat mesenteric arteries: Anti-CGRP staining in PVAT of superior mesenteric artery (red) (**A**), anti-PGP 9.5 staining in PVAT of superior mesenteric artery (green) (**B**), merged image shows some neurons co-expressing CGRP and PGP 9.5 in PVAT of superior mesenteric artery (yellow) (**C**). 3-dimensional merged image showing some neurons co-expressing CGRP and PGP 9.5 marker in PVAT region of superior mesenteric artery (**D**). In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control) in PVAT of superior mesenteric artery, no staining was observed (**E**). Anti-CGRP staining in PVAT of second order mesenteric artery (red) (**F**), anti-PGP 9.5 staining in PVAT of second order mesenteric artery (green) (**G**), merged image shows some neurons co-expressing CGRP and PGP 9.5 in PVAT of second order mesenteric artery (yellow) (**H**). 3-dimensional image of some neurons co-expressing CGRP and PGP 9.5 in PVAT of second order mesenteric artery (**I**). In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control) in PVAT of second order mesenteric artery, no staining was observed (**J**). Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate CGRP and PGP 9.5 double labelled: a = nerve, b =cell body/neuronal somata. Scale bars for A-D, F, G and H = 23 µm, I = 16.46 µm and E and J= 50 µm (n=6 preparations from 2 rats).

Figure 5

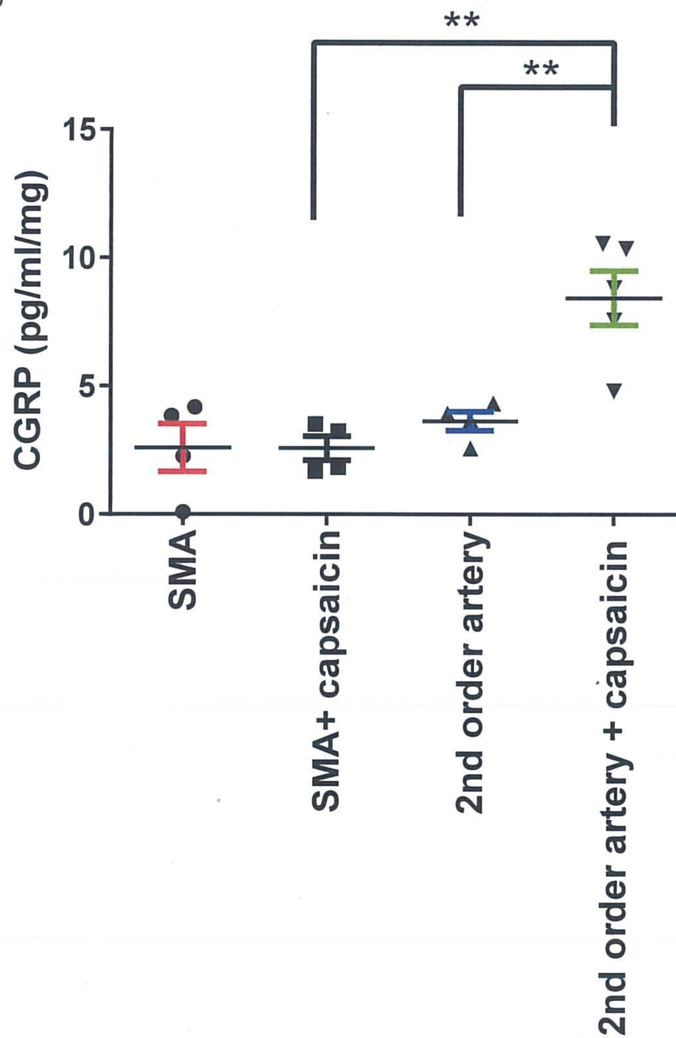


Figure 5: Capsaicin-evoked CGRP release in rat mesenteric PVAT. Concentration of calcitonin gene-related peptide (CGRP) in the supernatant of dissected perivascular adipose tissue (PVAT) isolated from segments of rat superior mesenteric artery (SMA) and second order mesenteric artery in the presence and absence of capsaicin (10 μM, 15 min); capsaicin enhanced CGRP release from PVAT isolated from the second order mesenteric artery (n=4-5). **$P<0.01$.

Figure 6

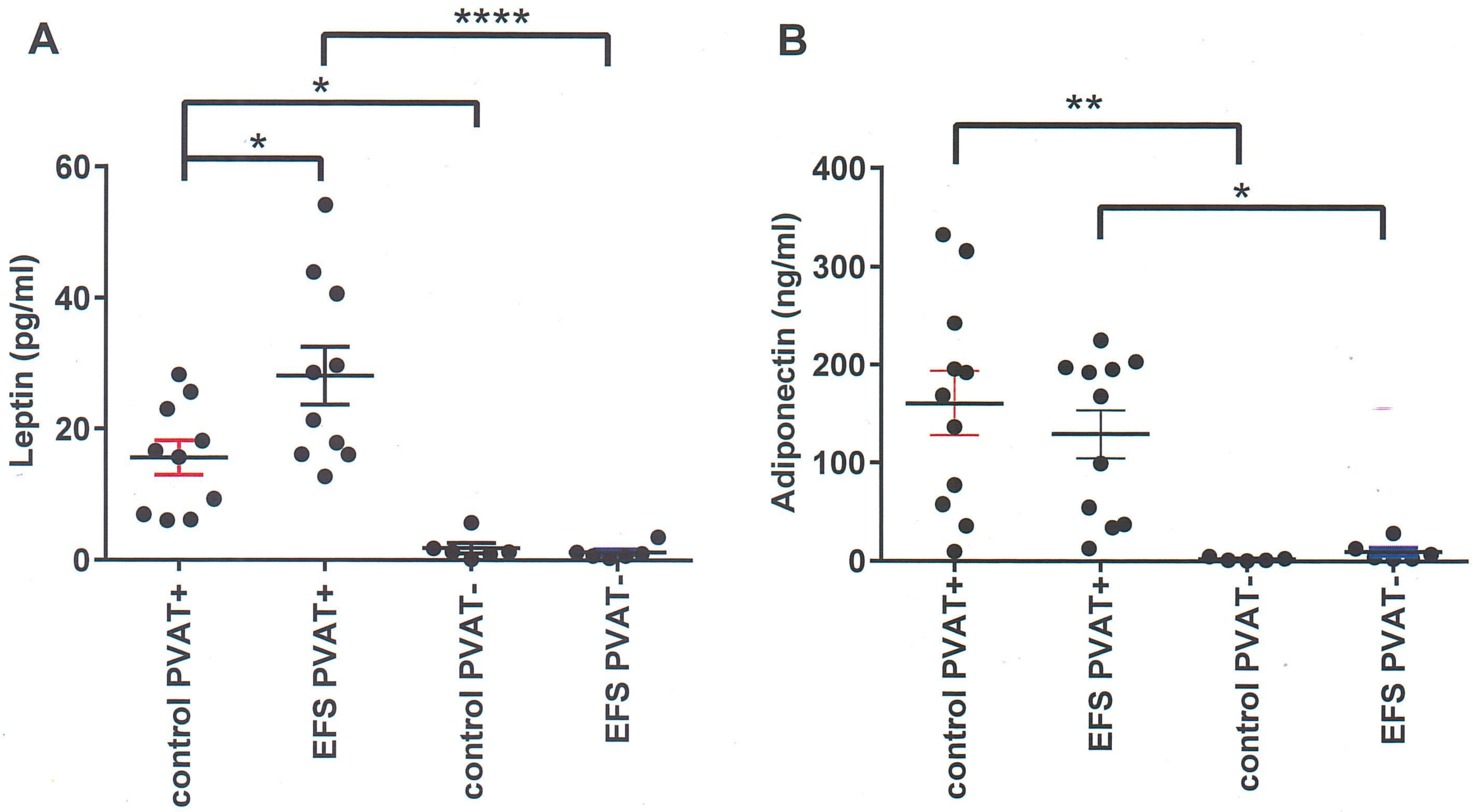


Figure 6: Electrical field stimulation evokes leptin release in mesenteric arterial bed preparations with intact PVAT, but not in PVAT-denuded preparations. Tone of the mesenteric arterial bed preparations was raised with methoxamine, guanethidine (5 μM) was present to block sympathetic constriction, and electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) was applied. **A**, In the presence of perivascular adipose tissue (PVAT+), leptin release was greater both under basal (control) conditions and during EFS than in preparations without PVAT (PVAT-). EFS enhanced leptin release in PVAT+ preparations (n=6-10). **B**, The absence of PVAT attenuated the adiponectin level collected in the perfusate in both basal (control) conditions and during EFS (n=5-11). *P<0.05, **P<0.01, ***P<0.001.

Figure 7

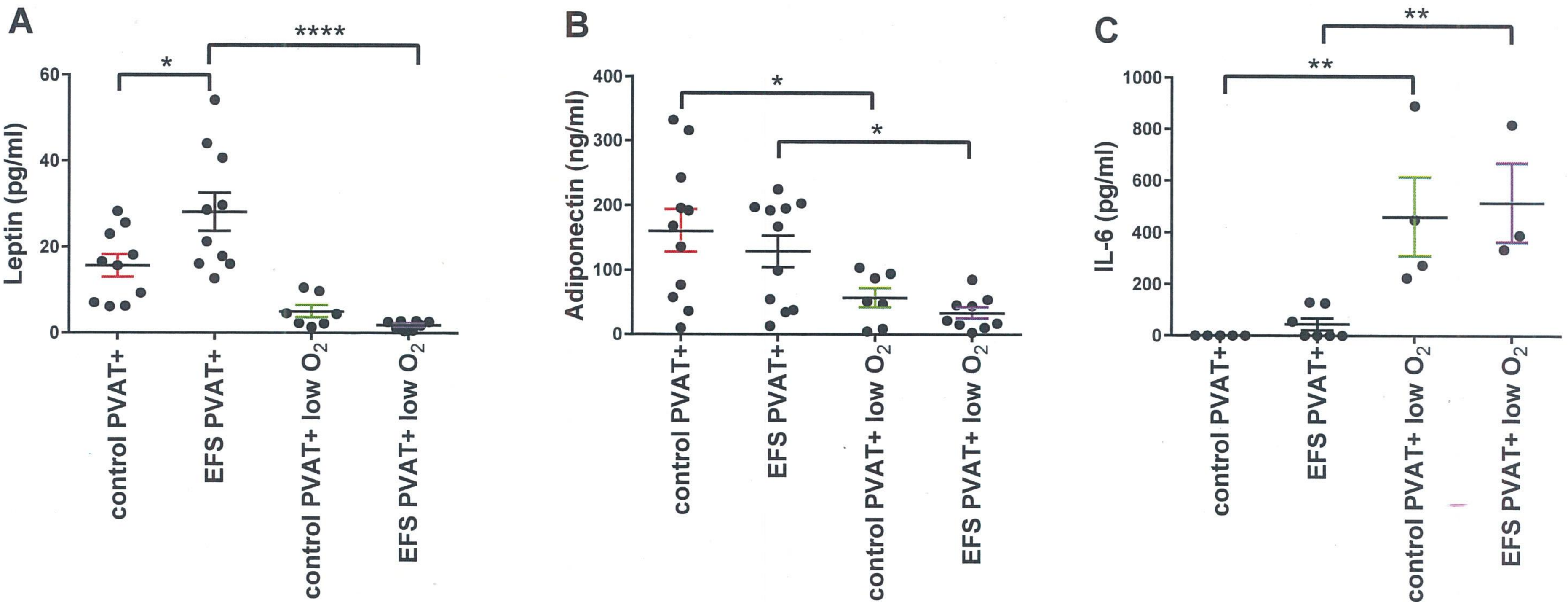


Figure 7: The effect of oxygen level on mediators release during electrically-evoked neurogenic stimulation of rat mesenteric arterial bed preparations with perivascular adipose tissue (PVAT+). Preparations were submaximally pre-contracted with methoxamine, guanethidine (5 μM) was present to block sympathetic neurotransmission, and electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) was applied. **A**, The leptin level was reduced in conditions of low oxygen level (gassing with 95% N_2 5% CO_2) compared to standard oxygen levels (gassing with 95% O_2 5% CO_2) (n=7-10). **B**, The level of adiponectin collected in the perfusate was also greater in standard oxygenation (n=7-11). **C**, The IL-6 level was increased in a reduced oxygen level compared to a standard oxygen level in both basal conditions and during EFS (n=3-7). *P<0.05, **P<0.01, ****P<0.0001; ANOVA with Bonferroni post test.

Figure 8

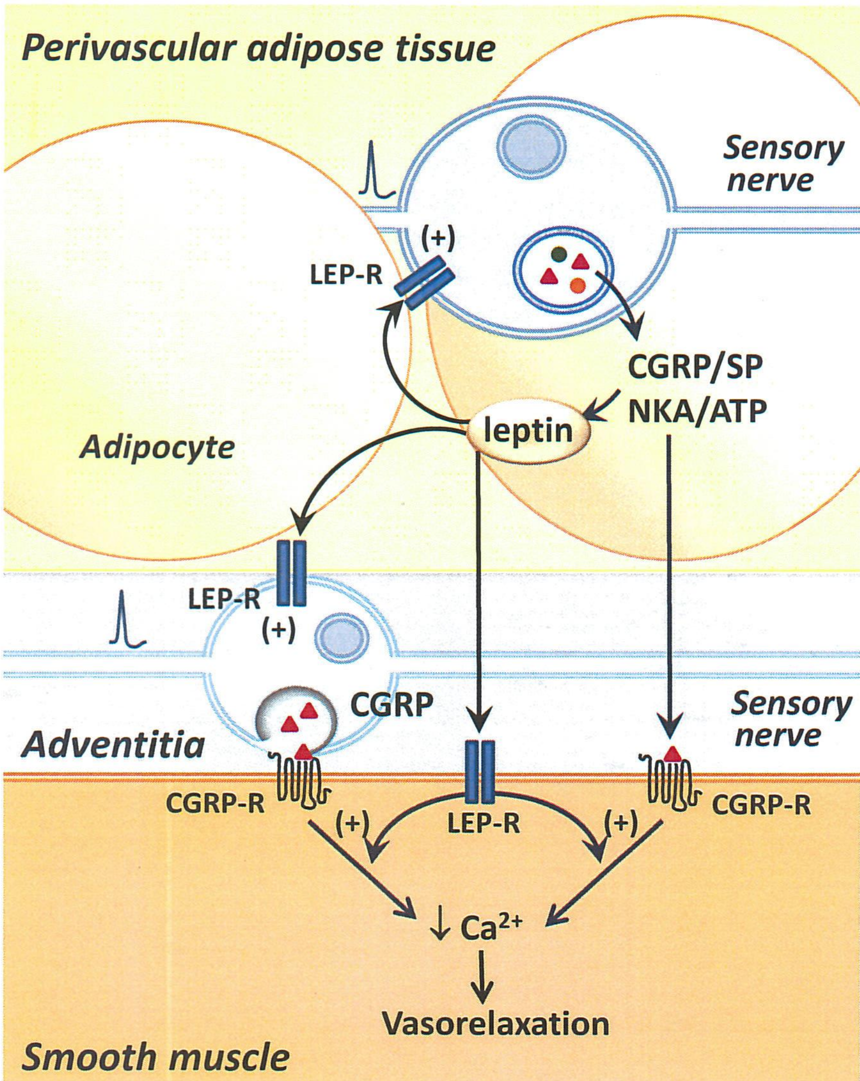


Figure 8: Expression and roles of sensory nerves in perivascular adipose tissue. Sensory nerves containing calcitonin gene-related peptide (CGRP) and other sensory neurotransmitters are present in perivascular adipose tissue as well as in the adventitia. Following neurogenic activation, CGRP and other sensory neurotransmitters are released from these sensory nerves and cause vasorelaxation, predominantly involving activation of smooth muscle CGRP receptors (CGRP-R). The sensory neurotransmitters also act to promote the release of leptin from the adipocytes, which augments CGRP-induced vasorelaxation through pre- and/or postjunctional mechanisms. NKA, neurokinin A; Lep-R, leptin receptor; SP, substance P.