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DISSERTATION

Voltage-gated K^+ (K_v) channels and control visceral arteries tone by perivascular adipose tissue

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Abstract

Background: Perivascular adipose tissue (PVAT), is now recognized as dynamic paracrine organ and important metabolic sensor. PVAT regulates vascular function by releasing perivascular adipose relaxing factors (PVRFs), particularly a transferable adipocyte-derived relaxing factor (ADRF). Although, the exact nature of ADRF is unknown, adiponectin, Ang 1-7, H_2S and palmitic acid methyl ester (PAME) have been proposed as ADRF candidates. Recent evidence has implicated VSMC K_v7 channels to play a key role for vasodilation by ADRF. Consistently, the anti-contractile effects of PVAT are abolished by the K_v7 channel blocker XE991 in rat and mouse visceral arteries. There is also a suggestion that diphenyl phosphine oxide-1 (DPO-1) sensitive $K_v1.5$ channels may play a role in the control of arterial tone by ADRF.

Methods: Wire myography was used to measure vasocontractions of the arteries in mice, rats and human. PVAT was either left intact or removed from the arterial rings. PAME and H_2S were used as vasodilators. K_v channels were blocked by XE991 and DPO-1. PAME and H_2S were detected by GC-MS or fluorescence microscopy. Total RNA was extracted from snap-frozen tissues in mouse model using an RNeasy RNA isolation kit. Quantitative analysis of target mRNA expression was performed with quantitative real-time PCR (RT-PCR).

Results: We found that PVAT displayed anti-contractile effects in both human and mouse mesenteric arteries and rat aortas. The anti-contractile effects were inhibited by XE991, but not DPO-1. PAME (EC_{50} ~1.4 μM) was capable to produce relaxations of PVAT-removed rat aortas but not human arteries. These effects were abolished by XE991. Our results also show that presence of PVAT [(+) fat] diminished the contractile effects of 5-HT; however, lack of CTH did not influence this anti-contractile effects in mouse mesenteric arteries.

Conclusions: Our data suggest that K_v7 channels are involved in the anti-contractile effects of PVAT on arterial tone in human mesenteric arteries, similarly to the rat aorta and mouse mesenteric arteries. $K_v1.5$ channels in VSMCs are not the downstream mediators of the XE991 effects on PVAT-dependent arterial vasorelaxation. PAME contributes to PVRF-dependent vasorelaxation of rat aorta but not human visceral arteries. We also show that CTH-produced H_2S is not introduced in ADRF (or PVAT) effects.

Zusammenfassung

Hintergrund: Das perivaskuläre Fettgewebe (PVAT) wird heute als dynamisches, parakrines Organ und wichtiger metabolischer Sensor angesehen. PVAT reguliert die vaskuläre Funktion durch Freisetzung perivaskulärer adipöser relaxierender Faktoren (PVRFs), insbesondere eines übertragbaren Adipozyten-abgeleiteten relaxierenden Faktors (ADRF). Obwohl die genaue Natur von ADRF unbekannt ist, wurden Adiponektin, Angiotensin, H_2S und Palmitinsäuremethylester (PAME) als ADRF-Kandidaten vorgeschlagen. Jüngste Beweise haben impliziert, dass VSMC-K_v7-Kanäle eine Schlüsselrolle für die Vasodilatation durch ADRF spielen. Konsequenterweise werden die anti-kontraktilen Effekte von PVAT durch den K_v7-Kanalblocker XE991 in den viszeralen Arterien von Ratten und Mäusen beseitigt. Es gibt auch einen Vorschlag, dass Diphenylphosphinoxid-1 (DPO-1) -empfindliche K_v1.5-Kanäle eine Rolle bei der Kontrolle des arteriellen Tonus durch ADRF spielen können.

Methoden: Um Vasokontraktionen von Mäusen, Ratten und menschlichen Arterien zu messen, wurde die Drahtmyographie verwendet. PVAT wurde entweder intakt gelassen oder aus den Arterienringen entfernt. Als Vasodilatatoren kamen PAME sowie H2S zum Einsatz. K_v-Kanäle wurde von XE991 und DPO-1 blockiert. Der Nachweis von PAME und H_2S erfolgte mittels GC-MS bzw. Fluoreszenzmikroskopie. Das Mausmodell wurde mittels RT-PCR bewertet.

Ergebnisse: Wir fanden, dass PVAT anti-kontraktile Effekte sowohl in menschlichen Mesenterialarterien als auch in Mausarterien und Rattenaorten zeigte. Die kontraktilen Wirkungen wurden durch XE991, nicht jedoch durch DPO-1 gehemmt. PAME (EC_{50} ~ 1,4 µM) war in der Lage, Relaxationen von PVAT-entfernten Rattenaorten, aber nicht von menschlichen Arterien zu erzeugen. Diese Effekte wurden von XE991 unterbunden. Unsere Ergebnisse zeigten weiterhin, dass das Vorhandensein von PVAT [(+) Fett] die kontraktilen Wirkungen von 5-HT verringerte; das Fehlen von CTH beeinflusste diese kontraktilen Effekte in den Mesenterialarterien der Maus jedoch nicht.

Diskussion: Unsere Daten legen nahe, dass K_v7-Kanäle an anti-contractile effects von PVAT auf den Arterien Tonus in menschlichen Mesenterialarterien beteiligt sind, ähnlich wie die Aorta der Ratte und die Maus-Mesenterialarterien. K_v1.5-Kanäle in VSMCs sind nicht die nachgeschalteten Mediatoren der XE991-Effekte auf die PVAT-abhängige arterielle Vasorelaxation. PAME trägt zur PVRF-abhängigen Vasorelaxation der Rattenaorta bei, jedoch nicht der menschlichen Viszeralarterien. Wir zeigen auch, dass CTH-produziertes H_2S nicht in ADRF- (oder PVAT-) Effekte eingeführt wird.

1. Introduction

Perivascular adipose tissue (PVAT), which surrounds the aorta and its vascular branches, as well as many other arteries, is now recognized as dynamic paracrine organ and important metabolic sensor [1]. PVAT does not only provide mechanical protection to vessels but also regulates vascular function by releasing perivascular adipose relaxing factors (PVRFs), particularly a transferable adipocyte-derived relaxing factor (ADRF), which diminishes the contractile actions of vasoconstrictors such as phenylephrine (PE), serotonin (5-HT), angiotensin II and U46619 [2]. The anti-contractile effect of PVAT has been observed in both large and small arteries of rats, mice, pigs and human. The anti-contractile effects of PVAT rely on the opening of K^+ channels in vascular smooth muscle cells (VSMCs) [3]. This action occurs without involvement of NO, prostaglandin I_2 (PGI_2) or endothelium-derived hyperpolarizing factor (EDHF) [4].

VSMC K_v7 channels are considered to play a key role in vasodilation by ADRF released from PVAT [2]. Consistently, the anti-contractile effects of PVAT are abolished by the K_v7 channel blocker, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991) in rat and mouse visceral arteries [5]. Although the exact nature of ADRF is unknown, adiponectin, Ang 1-7, H_2S and palmitic acid methyl ester (PAME) have been proposed as ADRF candidates [6; 7]. XE991 is a widely used K_v7 channel blocker, which inhibits $K_v7.1$ homomeric or $K_v7.1$/KCNE channels (IC_{50} of ~0.8μM and 11.1 μM, respectively), KCNQ2/3 channels (EC_{50}~1 μM), KCNQ4 (EC_{50} ~5.5μM), and KCNQ5 (EC_{50} ~65 μM). Noteworthy, XE991 can also inhibit other K_v channels, such as ERG (KV11; EC_{50} ~110μM) and $K_v1.2/1.5$, $K_v2.1/K_v9.3$ channels (~30% inhibition at 10 μM) in heterologous expression systems. A recent study showed that K_v channels in VSMCs of mouse mesenteric arteries are very sensitive to XE991 (EC_{50} ~60 nM), suggesting that these channels may contribute to PVAT control of arterial tone [8]. However, a very recent study suggested that diphenyl phosphine oxide- 1 (DPO-1) sensitive $K_v1.5$ channels could contribute to the K_v current in VSMC [9].

PAME is one of the most abundant fatty acids in mammalian cells, and represents an endogenous naturally occurring fatty acid methyl ester [10]. This compound has been reported to have the ability to inhibit Kupffer cells which are resident macrophages in the liver regulating inflammatory processes by secretion of TNF-alpha and NO. PAME is also known to exhibit anti-fibrotic effects and to act as a potent vasodilator released in retina and myometrium [11; 12]. A recent report identified PAME as a novel potent vasodilator released from PVAT in rat aorta, which exhibits vascular relaxation by opening K_v channels in smooth muscle cells [7]. Although these findings suggest that PAME could represent a potential mediator in control of vasotonus and blood pressure in rats, the role of K_v7 channels in PVAT regulation of human arterial tone and vasodilatory PAME effects remains to be established.

Hydrogen sulfide (H_2S) is produced in small amount by some cells of the mammalian body and has a number of biological signaling functions. H_2S is endogenously generated by cystathionine β-synthase (Cbs)

and cystathionine γ-lyase (Cth) [13]. It acts as a relaxant of smooth muscle and as a vasodilator. We have previously shown that CTH inhibitors, including propargylglycine or β-cyanoalanine, inhibit ADRF effects in aortas of rats but not mice. Although these findings suggest that H_2S could represent a potential mediator in control of vasorelaxation, the role of CTH-produced H_2S in PVAT remains to be characterized.

Therefore, we tested whether K_v7 and $K_v1.5$ channels are involved in the control of arterial tone and its regulation by PVAT or not. Our study also aimed at extending our current knowledge on ADRF candidates.

2. Aims and Hypotheses

The main objective of the experimental work for my thesis was to study the roles of voltage-gated K^+ (K_v) channels in PVAT regulation of arterial tone in mice, rats and human arteries. Specifically, the following hypotheses were tested:

2.1 Hypothesis #1

We tested the hypothesis that XE991-sensitive K_v (K_v7) channels and PAME are involved in the anti-contractile effects of PVAT on both rat aorta and human mesenteric arteries.

2.2 Hypothesis #2

We tested whether $K_v1.5$ channels are involved in the control of arterial tone and its regulation by PVAT or not.

2.3 Hypothesis #3

This study investigated whether CTH-produced H_2S is an ADRF or not.

3. Methods

3.1 Animal preparation.

Cth$^{-/-}$ mice that were backcrossed for >10 generations to achieve >99.2% genetic homogeneity on C57BL/6J background were used. Gene expression was evaluated by RT-qPCR in the mouse model. Either litter- or age-matched (10–14 weeks old) male wildtype (129S6 background) mice were used as controls. Male Sprague Dawley rats (250–300 g, 10-15 weeks old) were obtained from Charles River, Germany, Berlin. Mesenteric tissue was taken from patients undergoing surgical treatment. All patients provided informed consent for participation in this study. All experimental procedures were performed in accordance with the German legislation on protection of animals. Animal care followed American Physiological Society guidelines, and local authorities (Landesamt für Gesundheit und Soziales Berlin, LAGeSo) approved all protocols.

3.2 Isometric contractions of murine mesenteric arteries.

Mesenteric arteries were removed, quickly transferred to cold (4°C) oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS: 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, and 1.6 $CaCl_2$ in mmol/L), and dissected into 2-mm rings. Perivascular fat and connective tissues were either removed (fat (–)) or left intact (fat (+)). Each ring was positioned between two stainless steel wires (0.0394-mm in diameter) in a 5-ml PSS-filled organ bath of Small Vessel Myograph (DMT 610M, Danish Myo Technology, Aarhus, Denmark). The PSS was continuously oxygenated and kept at 37°C (pH 7.4). Mesenteric rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mm Hg. This normalization procedure was performed to obtain the passive diameter of the vessel at 100 mm Hg. The software LabChart 5 was used for data acquisition and display. The rings were pre-contracted with the KCl buffer (119 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, and 1.6 $CaCl_2$ in mmol/L) and equilibrated until a stable resting tension was acquired. To test the endothelial function, vessels were pre-contracted with 1 µmol/L PE. After all vessel segments had reached a stable contraction plateau, increasing doses of acetylcholine (Ach) were administered to the organ baths. The periadventitial regulation of the vascular tone was studied in vessels contracted with serotonin (3 to 1000 nmol/L). Tension is expressed as a percentage of the steady-state tension (100%) obtained with PE or isotonic external 60 mmol/L KCl, respectively. In some arterial rings the endothelial layer was mechanically disrupted by gently rubbing the luminal surface forth several times with scissors. Endothelium integrity or functional removal was confirmed by the presence or absence of the relaxant response to 1 µmol/L Ach. The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display [14].

3.3 Isometric contractions of rat aortas.

Male Sprague-Dawley rats were killed, and the thoracic aortas were removed, and quickly transferred to cold (4°C) oxygenated (95% O_2/5% CO_2) PSS, and dissected into 2 mm rings, respectively. Perivascular fat and connective tissue were either removed [(-) fat] or left [(+) fat] intact as previously described. The rings were placed under force of 20 mN. The bath solution volume was 20 mL of a vessel myograph (Schuler tissue bath system, Hugo Sachs Elektronik, Freiburg, Germany). After 1h equilibration, contractile force was measured isometrically using standard bath procedures and solutions as described. Cumulative concentration response curves were obtained in the presence and absence of the K^+ channel or an enzyme inhibitor, XE991. Tension was expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60 mM KCl. To test for the presence of functional endothelium, rings were contracted with 1 µM PE and once the vessels reached a stable maximum tension, the vessels were stimulated with 10 µM ACh and relaxation was confirmed (greater than 80%). In some rings, the endothelium was removed by gently abrading the luminal surface of the vessel with a stainless steel pin to determine the contribution of the endothelium to PAME relaxation. Functional endothelium was considered absent if 10 µM ACh did not produce relaxation [15].

3.4 Isometric contractions of human arteries.

Procedures were performed in accordance with the ethics guidelines of the National Health and Medical Research Council of Germany. All patients provided informed consent for participation in this study. Mesenteric tissues were taken from 12 patients (1 female and 11 males) undergoing surgical treatment of bowel carcinoma or inflammatory bowel disorders [colon cancer (n=3), sigmoid cancer (n=4), colon adenoma (n=1), and sigmoid diverticulitis (n=1)]. Immediately after lower intestinal surgery, mesenteric arteries were excised from resected mesenteric tissue, and quickly transferred to cold (4°C) oxygenated (95% O_2/5% CO_2) PSS, and dissected into 1 mm rings. PVAT was either removed [(-) fat] or left [(+) fat] intact as previously described. Each ring was positioned between two stainless steel wires in a 5-mL organ bath of a Small Vessel Myograph (DMT 610M; Danish Myo Technology, Denmark). The organ bath was filled with PSS. The bath solution was continuously oxygenated with a gas mixture of 95% O_2 and 5% CO_2, and kept at 37°C (pH 7.4). The rings were placed under force of 3 mN. The software Chart5 was used for data acquisition and display. The rings were pre-contracted with 60 mM KCl and equilibrated until a stable resting tension was acquired. Chemicals were added to the bath solution if not indicated otherwise. Vessels were pre-contracted with either 5-HT or PE. All chemicals were added to the bath solution (PSS) [16].

3.5 Bioassay experiments of rat aortas.

We transferred aliquots of bath solution from rat aorta with PVAT incubated in a donor bath chamber to vessel preparations without PVAT in an acceptor bath chamber of the Schuler tissue bath system (Hugo Sachs Elektronik, Freiburg, Germany). Cumulative response curves were obtained in the presence and absence of 5-HT (total incubation time, 5 min). The volume of the solutions in the bath was 20 mL. In most experiments, transfer interval of aliquots was 15 to 20 min; the volume of the aliquots was 3 or 5 mL. Transfer of bath solution aliquots from aortic vessels without PVAT or fresh PSS did not affect contraction of vessel preparations without PVAT in the acceptor bath chamber.

3.6 Measurements of PAME by GC/MS

GC/MS analysis was performed using an Agilent ChemStation. For determination of endogenous PAME concentrations in bath solutions, rat peri-aortic and human visceral adipose tissue (3 g each) were incubated in 15 mL-Eppendorf tubes with 10 mL PSS solutions, with or without 5-HT 5 µM (30 min, in 37^{o}C water bath). PSS was oxygenated (95% O_2/5% CO_2) for 30 min before use. After removal of adipose tissue, the PSS solution was dissolved in hexane (1:3 volume ratio), extracted and vortexed. Next, 1 mL of water was added to the solution. In order to ensure that the concentration of PAME between the aqueous and the lipophilic phase was in equilibrium, the samples were shaken by hand for 4 min. Thereafter, the phases were separated by centrifugation and the lipophilic hexane phase containing fatty acid methyl esters was removed and dried under nitrogen. The fatty acid methyl ester residues were re-dissolved in 50 µL hexane and transferred into an autosampler vial. Samples were analyzed by using a fully automated Agilent 7890A-5977B system equipped with a flame ionization detector. Peaks of re-dissolved PAME were identified by comparison with PAME standard and their nominal concentrations were determined [30; 31]. (+) Fat masses were measured in rat aortic (2 mm) and human mesenteric artery (1 mm) rings (n=6 each) to calculate magnitudes of effective [PAME] in the 20 mL and 5 mL myograph bath chambers, respectively [17].

3.7 Quantitative polymerase chain reaction (PCR).

Total RNA was isolated from snap-frozen tissues using an RNeasy RNA isolation kit (Qiagen). RNA concentration and quality were tested by NanoDrop-1000 spectrophotometer (PeqLab). For cDNA synthesis, 0.2–2 µg of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative analysis of target mRNA expression was performed with quantitative real-time PCR using the relative standard curve method. TaqMan analysis was conducted using an Applied Biosystems 7500 Sequence Detector (Life Technologies). The expression levels of the target genes were normalized by 18S ribosomal RNA levels.

3.8 H_2S measurements and Fluorescence microscopy.

To detect H_2S production, isolated organs were incubated in PBS containing 50 µmol/L WSP-1 (Washington State Probe-1, Cayman Chemical), a recently developed fluorescent sensor for H_2S (22). After 45-min incubation, the samples were frozen. Tissue samples were homogenized and centrifuged, and the supernatants were analyzed for the fluorescence intensity at ex 465 nm/em 525 nm. In parallel, the PBS where the tissue was incubated was also analyzed for the fluorescence.

Briefly, mice were anesthetized with isoflurane and rapidly isolated resistance arteries were cut into 1.0–1.5-mm long ring segments with care taken not to injure the endothelium. Each mesenteric segment was placed on an individual well of cell culture dishes containing 1.0 mL of Krebs-HEPES buffer supplemented with 2 mmol/L l-arginine in a light-protected humidified 37°C chamber for 30 min (the initial equilibration) and the basal fluorescence was recorded for 20 min. The mesenteric resistance arteries were then incubated for 45 min with 5 µmol/L DAF-FM.

For detection of H_2S by fluorescence sensors, isolated mesenteric artery rings (1.5 mm tick) were incubated for 45 min at 37°C with WSP-1 (Cayman, distributed by Alexis, Grünberg, Germany) for H_2S [18]. Hoechst was added to stain nuclei and incubated for additional 15 min. Rings were washed and recorded. The green fluorescence was visualized with the FITC (fluorescein-isothiocyanate) filter setting and the blue fluorescence with the DAPI filter setting, using a Zeiss Axioplan-2 imaging microscope with the computer program AxioVision 4.8.

3.9 Statistical analysis.

Data were analyzed by Prism version 5.0 (GraphPad Software, La Jolla, California, USA) and were shown as mean ± SD. Paired and unpaired Student‘s *t*-tests and ANOVA were used for appropriate analyses. A value of *p* less than 0.05 was considered statistically significant and *n* represents the number of arteries.

For a complete description of the methods see:

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

Dmitry Tsvetkov, Jean-Yves Tano, Mario Kassmann, **Ning Wang**, Rudolf Schubert, and Maik Gollasch. The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone. Front Physiol. 2016 Aug; 4;7:335. doi: 10.3389/fphys.2016.00335.

István András Szijártó, Lajos Markó, Milos R. Filipovic, Jan Lj Miljkovic, Christoph Tabeling, Dmitry Tsvetkov, **Ning Wang**, Luiza A. Rabelo, Martin Witzenrath, André Diedrich, Jens Tank, Noriyuki Akahoshi, Shotaro Kamata, Isao Ishii, Maik Gollasch. Cystathionine γ-Lyase–Produced Hydrogen Sulfide Controls Endothelial NO Bioavailability and Blood Pressure. Hypertension. 2018 Jun; 71(6):1210-1217. doi: 10.1161/HYPERTENSIONAHA.117.10562.

Copies of these publications are attached in the **Appendix**.

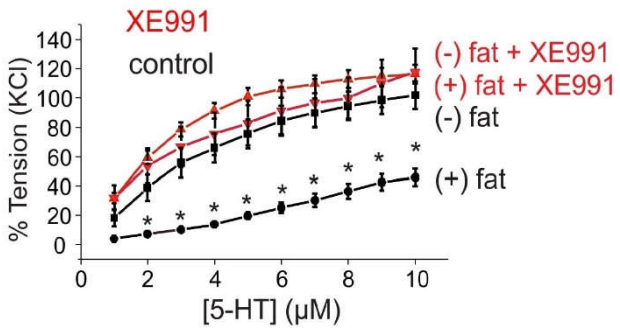

Figure 1

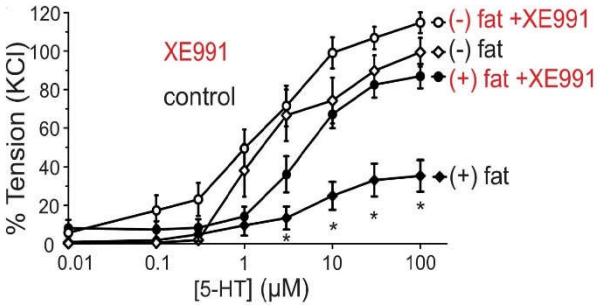

Figure 2

***FIGURE 1** | Presence of anti-contractile effects of perivascular adipose tissue (PVAT) in rat aorta and their inhibition by XE991.*

***FIGURE 2** | Presence of anti-contractile effects of perivascular adipose tissue (PVAT) in human mesenteric arteries and their inhibition by XE991.*

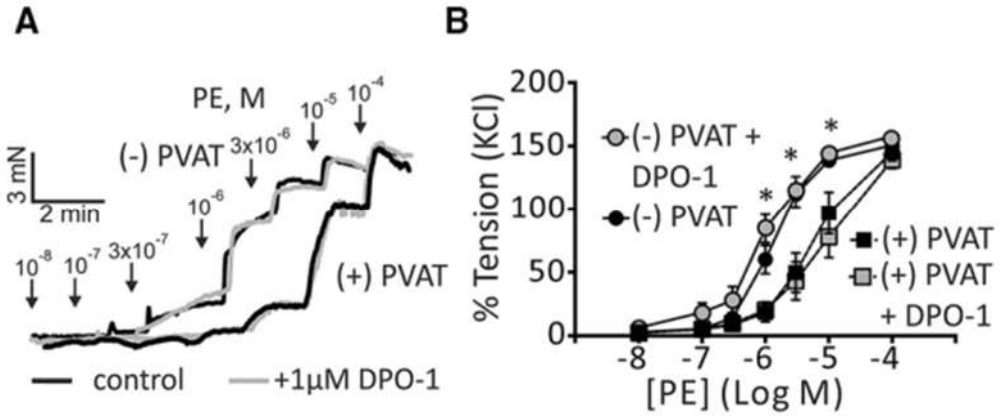

Figure 3

***FIGURE 3** | Presence of anti-contractile effects of perivascular adipose tissue (PVAT) in mouse mesenteric arteries and their inhibition by DPO-1. **(A)** Original traces showing the effects of 1 μM DPO-1 on PE-induced contractions in (−) PVAT and (+) PVAT mesenteric artery rings compared with control rings without DPO-1. **(B)** Concentration-response relationships for PE-induced contractions in Kcna5$^{+/+}$ (+) PVAT (n = 8)*

*or (−) PVAT (n = 9) mesenteric arteries in the absence of DPO-1 or in Kcna5$^{+/+}$ (+) PVAT (n = 8) and (−) PVAT (n = 9) arteries after 30 min of pre-incubation with 1 μM DPO-1. *p < 0.05, for (−) PVAT vs. (+) PVAT or (−) PVAT + DPO-1 vs. (+) PVAT + DPO-1; repeated-measures two-way ANOVA, followed by Bonferroni post hoc test.*

4. Results

4.1 K_v7 channels play an important role in the control of arterial tone by PVAT

We first investigated the role of Kv7 channels in the anti-contractile effects of PVAT in rat aortas. Rat aortic rings without PVAT ((-) fat) showed stronger contractions to 5-HT (relative increase, 60-100% between 2-10 μM 5-HT) than vessels with PVAT ((+) fat) (Figure 1). Incubation of the vessels with the Kv7 channel inhibitor XE991 ((30 μM, 30 min) inhibited the anti-contractile effects of PVAT mediated by transferable ADRF in rat aortic rings. XE991 slightly (less than 20%) increased contractions of (-) fat rat aortic rings in response to 5-HT, but there was no difference between contractions of (-) fat and (+) fat rings in response to 5-HT. Contractions of (-) fat and (+) fat aortic rings obtained by 60 mM KCl containing PSS were not significantly different (13.07 + 1.29 mN, n=16 vs. 10.81 + 1.19 mN, n=14, P>0.05, respectively).

Similar results were observed in human visceral arteries. Human mesenteric arteries without PVAT ((-) fat) showed significantly stronger contractions in response to 5-HT than vessels with (+) fat (Figure 2). Incubation of the vessel rings with XE991 (30 μM, 30 min) inhibited the anti-contractile effects of PVAT. XE991 did not affect 5-HT-induced contractions in (-) fat human mesenteric artery rings.

PAME (EC_{50} ~1.4 μM; maximal relaxation E_{max} ~25%) was capable of producing relaxations of (-) fat rat aortas. PAME relaxations were not affected by removal of the endothelium. Pretreatment of aortic rings with XE991 (30 μM, 30 min) prevented the relaxant effects of PAME. PAME up to 10 μM failed to induce relaxations of PVAT-removed human mesenteric arteries. Pretreatment of the vessels with XE991 (30 μM, 30 min) did not affect the lack of PAME effects. Taken together, the results indicate that PAME at relatively high concentrations can induce slight relaxations in pre-contracted rat aortic rings, which are mediated by opening of K_v7 channels. In contrast, PAME is not a potent vasodilator (up to 10 μM) of human mesenteric arteries.

For a complete description of the results see:

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

4.2 K_v1.5 channels do not play a role in the control of arterial tone by PVAT

In this study, we found that K_v1.5 channels are not involved in the regulation of arterial tone of systemic visceral arteries of mice and rats by PVAT. In this set of experiments, we used the K_v1.5 channel blocker DPO-1 at concentrations assumed to be specific and potent for K_v1.5 channel inhibition (Figure 3). In the presence of 1 μM DPO-1, mesenteric artery rings (−) fat displayed similar contractions in

response to PE compared to non-treated (−) fat control rings. The 95% CI for EC_{50} of control and DPO-1 treated rings were 1.21–1.79μM and 0.78–1.40μM, respectively. The anti-contractile effects of PVAT were also unchanged by 1 μM DPO-1: the 95% CI for EC_{50} of control (+) fat and 1 μM DPO-1 (+) fat treated rings were 3.99–8.14μM and 4.99–10.05μM, respectively. Experiments on rat mesenteric arteries showed similar results: Cumulative dose-response curves in response to 5-HT were similar in vessel rings in the absence or presence of DPO-1. Altogether, the results suggest that $K_v1.5$ channels do not play a functionally relevant role in the control of arterial tone by PVAT, in both mouse and rat arteries.

For a complete description of the results see:

Dmitry Tsvetkov, Jean-Yves Tano, Mario Kassmann, **Ning Wang**, Rudolf Schubert, and Maik Gollasch. The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone. Front Physiol. 2016 Aug; 4;7:335. doi: 10.3389/fphys.2016.00335.

4.3 CTH-produced H_2S is not an ADRF

Cth mRNA was detectable in all tested tissues of *Cth*$^{+/+}$ but not in *Cth*$^{-/-}$ mice. CTH protein levels were detected as ≈44 kDa bands. CTH protein was not detected in mesenteric arteries of *Cth*$^{-/-}$ mice. We assessed basal endogenous levels of H_2S in mesenteric artery rings by fluorescence microscopy using specific fluorescent probes, Washington State Probe-1. Lower H_2S was detected in *Cth*$^{-/-}$ arterial rings, compared with *Cth*$^{+/+}$ arteries.

In mesenteric arteries, presence of (+) fat diminished the contractile effects of 5-HT (3–1000 nmol/L); however, lack of CTH did not influence the anticontractile effects. ACh concentration-dependent relaxation was observed in *Cth*$^{+/+}$ and *Cth*$^{-/-}$ mesenteric artery rings. The rings were constricted with PE (1 μmol/L) and then stimulated with increasing concentrations (3–3,000 nmol/L) of ACh in the absence or presence of glibenclamide (a CTH inhibitor). Pretreatment of the vessels with glibenclamide (1 μmol/L) did not modify the magnitude of ACh-induced relaxation.

For a complete description of the results see:

István András Szijártó, Lajos Markó, Milos R. Filipovic, Jan Lj Miljkovic, Christoph Tabeling, Dmitry Tsvetkov, **Ning Wang**, Luiza A. Rabelo, Martin Witzenrath, André Diedrich, Jens Tank, Noriyuki Akahoshi, Shotaro Kamata, Isao Ishii, Maik Gollasch. Cystathionine γ-Lyase–Produced Hydrogen Sulfide Controls Endothelial NO Bioavailability and Blood Pressure. Hypertension. 2018 Jun; 71(6):1210-1217. doi: 10.1161/HYPERTENSIONAHA.117.10562.

5. Discussion

PVAT is a local deposit of adipose tissue surrounding the vasculature. It is present throughout the body and has local effects on blood vessel contractility [19]. PVAT plays a potent anti-contractile role in the control of arterial tone along arterial segments of different vascular beds and species. This vascular regulation depends on the anatomical integrity of the vessels and is mediated by a transferable "adipocyte-derived relaxing factor" (ADRF) which opens smooth muscle K+ channels [20]. In the present study, we investigated the roles of K_v7 channels and ADRF candidates in PVAT regulation of arterial tone in rat and human vessels.

The major findings include that XE991-sensitive K_v (K_v7) channels are involved in the anti-contractile effects of PVAT on mouse mesenteric arteries, rat aortas and human mesenteric arteries. KCNQ-type K_v channels mediated by XE991- inhibit the effects of the PVAT. Simultaneously, we exclude $K_v1.5$ channels as potential downstream candidates in this process. Furthermore, PAME displays properties of a PVRF in rat aorta, where it may contribute to paracrine PVAT regulation of arterial tone, but not in human mesenteric arteries. Our data indicate that PAME is not an ADRF. We also show that CTH-produced H_2S is not involved in ADRF effects (or PVAT effects).

5.1 K_v7 channels and ADRF

We found that PAME was capable to produce relaxations of rat aortas. These effects were inhibited by XE991. In contrast, PAME at similar concentrations did not relax human mesenteric arteries. Together, these data suggest that PAME could contribute to PVAT relaxations by activating K_v7 channels in rat aorta, but not in human mesenteric arteries. The results are in line with the idea that 4-AP is not inhibiting K_v7 channels in rat aorta. We next explored the role of PAME and K_v7 channels in the anti-contractile effects of PVAT in rat aorta using a bioassay approach [21; 22]. In these experiments, we confirmed earlier findings that 5-HT induces vessel relaxation by releasing a transferable vasoactive substance (ADRF) from PVAT into the bath solution [23; 24]. We found that these aliquots produced less potent relaxations in rat aortas without PVAT suggesting that 5-HT is able to stimulate the release of ADRF from PVAT. Moreover, inhibition of K_v7 channels in (-) fat aortic rings by XE991 disrupted these effects in our bioassay experiments. We previously demonstrated that ADRF effects occur without involvement of the endothelium [25]. Therefore, the present data demonstrate that 5-HT induces ADRF release from PVAT, which displays anti-contractile properties through activation of XE991-sensitive (K_v7) K_v channels in vascular smooth muscle cells. Then we focused on PAME bioactivity and release under these conditions. This part of our study is important for understanding the role of PAME as a putative ADRF and/or paracrine PVRF. We found that PAME (EC_{50} ~1.4 μM) only slightly relaxed rat aortas (E_{max}, about 25%), whereas similar concentrations of PAME had no effects on human mesenteric arteries.

Most vessels possess some amount and type of PVAT, varying from mostly brown fat (thoracic aorta) to mixed brown and white fat (mesenteric vessels) [26]. We were able to detect endogenously released PAME in bathing solutions of both rat peri-aortic and human visceral adipose tissues. Furthermore, 5-HT was capable of inducing PAME release from rat peri-aortic adipose tissue, indicating a humoral active release process. However, the measured nominal concentrations of PAME were too low to explain transferable ADRF properties in both vascular preparations under study. Taken together, it is suggested that PAME is actively released from PVAT and displays properties of relaxing factor in rat aorta, but not in human mesenteric arteries, where it may contribute as paracrine PVRF to PVAT regulation of arterial tone, independently of metabolism of endogenous lipid epoxides.

5.2 K_v1.5 channels and ADRF

In recent studies, K_v1.5 channels in the heart are essential in coupling myocardial blood flow to cardiac metabolism [27; 28]. Moreover, hypertension is associated with altered expression of vascular K_v1.5 channels. Therefore, K_v1.5 channels may represent interesting putative targets of PVAT, which raised the question of their potential involvement in the regulation of arterial tone by PE in mouse mesenteric arteries. Our results suggest that the K_v1.5 channel inhibitor DPO-1 at 1 µM displayed no effect on vasocontractions in the absence or presence of PVAT.

In addition, our results demonstrate that K_v1.5 channels are not involved in the control of mesenteric arterial tone and its regulation by PVAT in mouse and rat mesenteric arteries. The nature of the 10 µM DPO-1 sensitive component is unclear, but it is most likely related to non-specificity of this drug, for example, in targeting vascular K_v1.3 and/or KCNQ channels. Importantly, the inhibitory effects of XE991 on PVAT vasorelaxation are rather related to inhibition of KCNQ-type K_v channels. These data unequivocally substantiate the hypothesis of different targets of perivascular relaxing factor(s), which employ distinct mechanisms to mediate an anti-contractile effect. Further studies should focus on the enhancement of these relaxing factors, as these will be beneficial for the patients with cardiovascular diseases.

5.3 H_2S and ADRF

The physiological and biomedical importance of hydrogen sulfide (H_2S) has been recognized in the cardiovascular system as well as in the rest of the body [29]. In blood vessels, CTH is one of H_2S-producing enzymes expressed in both smooth muscle and endothelium as well as periadventitial adipose tissues [30]. Regulation of H_2S production from CTH is controlled by a complex integration of transcriptional, post-transcriptional, and post-translational mechanisms in blood vessels. Our results provide evidence that CTH-produced H_2S is an important endogenous gasotransmitter in the cardiovascular system, which interacts with the NO signaling pathway to increase peripheral arterial resistance and blood pressure in the systemic circulation. CTH/H_2S is a candidate of adipocyte-derived relaxing factor. CTH inhibitor, such

as propargylglycine (PPG), cannot inhibit adipocyte-derived relaxing factor effects in mice [13]. It is possible that PPG is a weak CTH inhibitor in the murine vasculature. Our data using *Cth*$^{-/-}$ arteries provide firm evidence that CTH/H_2S is not involved in PVAT regulation of arterial tone in mice [31].

In summary, XE991-sensitive K_v (K_v7) channels are involved in the anti-contractile effects of PVAT on mouse mesenteric arteries, rat aortas and human mesenteric arteries. We conclude that DPO-1 sensitive $K_v1.5$ channels in VSMCs are not the downstream mediators of the XE991 effects on PVAT-dependent arterial vasorelaxation. PAME contributes to PVRF-dependent vasorelaxation of rat aorta but not human visceral arteries. We also show that CTH-produced H_2S is not an ADRF(s).

For a complete description of the discussion see:

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

Dmitry Tsvetkov, Jean-Yves Tano, Mario Kassmann, **Ning Wang**, Rudolf Schubert, and Maik Gollasch. The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone. Front Physiol. 2016 Aug; 4;7:335. doi: 10.3389/fphys.2016.00335.

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7. Affidavit

I, Ning Wang, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic Voltage-gated K^+ (K_v) channels and control visceral arteries tone by perivascular adipose tissue. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspondence to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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7.1 Declaration of any eventual publications

Ning Wang had the following share in the following publications:

Publication I: 70 % Contribution

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

Impact Factor (2016/2017): 4.134

Contribution in detail: Design of experimental protocols, preparation of vessels, isometric contraction force measurements in rat and human arteries, analysis and interpretation of data, writing the first draft of the manuscript, interpretation of results, and literature searches.

Publication II: 20 % Contribution

Dmitry Tsvetkov, Jean-Yves Tano, Mario Kassmann, **Ning Wang**, Rudolf Schubert, and Maik Gollasch. The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone. Front Physiol. 2016 Aug; 4;7:335. doi: 10.3389/fphys.2016.00335.

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Publication III: 15 % Contribution

István András Szijártó, Lajos Markó, Milos R. Filipovic, Jan Lj Miljkovic, Christoph Tabeling, Dmitry Tsvetkov, **Ning Wang**, Luiza A. Rabelo, Martin Witzenrath, André Diedrich, Jens Tank, Noriyuki Akahoshi, Shotaro Kamata, Isao Ishii, Maik Gollasch. Cystathionine γ-Lyase–Produced Hydrogen Sulfide Controls Endothelial NO Bioavailability and Blood Pressure. Hypertension. 2018 Jun; 71(6):1210-1217. doi: 10.1161/HYPERTENSIONAHA.117.10562.

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Contribution in detail: Design of experimental protocols, consulting literature materials, preparation of vessels, isometric contraction force measurements in mouse mesentery arteries, preparation of mice and human fat solution samples, analysis and interpretation of data.

Signature, date and stamp of the supervising university teacher

Prof. Dr. med. Dr. rer. nat. Maik Gollasch

Signature of doctoral student

Ning Wang

8. Selected Publications

Publication #1: Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue.

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

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Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue

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Background: Perivascular adipose tissue (PVAT) exerts anti-contractile effects on visceral arteries by release of various perivascular relaxing factors (PVRFs) and opening voltage-gated K^+ (K_V) channels in vascular smooth muscle cells (VSMCs). Palmitic acid methyl ester (PAME) has been proposed as transferable PVRF in rat aorta. Here, we studied PVAT regulation of arterial tone of human mesenteric arteries and clarified the contribution of K_V channels and PAME in the effects.

Methods: Wire myography was used to measure vasocontractions of mesenteric artery rings from patients undergoing abdominal surgery. Isolated aortic rings from Sprague-Dawley rats were studied for comparison. PVAT was either left intact or removed from the arterial rings. Vasocontractions were induced by external high K^+ (60 mM), serotonin (5-HT) or phenylephrine. PAME (10 nM–3 μM) was used as vasodilator. K_V channels were blocked by XE991, a K_V7 (KCNQ) channel inhibitor, or by 4-aminopyridine, a non-specific K_V channel inhibitor. PAME was measured in bathing solutions incubated with rat peri-aortic or human visceral adipose tissue.

Results: We found that PVAT displayed anti-contractile effects in both human mesenteric arteries and rat aortas. The anti-contractile effects were inhibited by XE991 (30 μM). PAME (EC_{50} ~1.4 μM) was capable to produce relaxations of PVAT-removed rat aortas. These effects were abolished by XE991 (30 μM), but not 4-aminopyridine (2 mM) or NDGA (10 μM), a lipoxygenases inhibitor. The cytochrome P450 epoxygenase inhibitor 17-octadecynoic acid (ODYA 10 μM) and the soluble epoxide hydrolase inhibitor 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA 10 μM) slightly decreased PAME relaxations. PAME up to 10 μM failed to induce relaxations of PVAT-removed human mesenteric arteries. 5-HT induced endogenous PAME release from rat peri-aortic adipose tissue, but not from human visceral adipose tissue.

Publication I

Conclusions: Our data also suggest that K_v7 channels are involved in the anti-contractile effects of PVAT on arterial tone in both rat aorta and human mesenteric arteries. PAME could contribute to PVAT relaxations by activating K_v7 channels in rat aorta, but not in human mesenteric arteries.

Keywords: XE991, adipocyte-derived relaxing factor (ADRF), perivascular adipose tissue (PVAT), KCNQ channels, K_V channels

INTRODUCTION

Perivascular adipose tissue (PVAT), which surrounds the aorta, its vascular branches and many other arteries, is now recognized as dynamic paracrine organ and important metabolic sensor (Szasz et al., 2013; Gil-Ortega et al., 2015; Gollasch, 2017). PVAT does not only provide mechanical protection to vessels but also regulates vascular function by releasing perivascular adipose relaxing factors (PVRFs), particularly a transferable adipocyte-derived relaxing factor (ADRF), which diminishes the contractile actions of vasoconstrictors such as phenylephrine (PE), serotonin (5-HT), angiotensin II and U46619 (Löhn et al., 2002; Yiannikouris et al., 2010). The anti-contractile effect of PVAT has been observed in both large and small arteries of rats, mice, pigs and humans (Bunker and Laughlin, 1985; Szasz and Webb, 2012; Gollasch, 2017). The anti-contractile effects of PVAT rely on the opening of K^+ channels in vascular smooth muscle cells (VSMCs) (Tano et al., 2014). This action occurs without involvement of NO, prostaglandin I_2 (prostacyclin) or endothelium-derived hyperpolarizing factor (EDHF) (Löhn et al., 2002; Li et al., 2013).

VSMC K_v7 channels are considered to play a key role for vasodilation by ADRF released from PVAT (Gollasch, 2017). Consistently, the anti-contractile effects of PVAT are abolished by the K_v7 channel blocker XE991 in rat and mouse visceral arteries (Löhn et al., 2002; Schleifenbaum et al., 2010; Tsvetkov et al., 2016b). Although, the exact nature of ADRF is unknown, adiponectin, Ang 1–7, H_2S and palmitic acid methyl ester (PAME) have been proposed as ADRF candidates (Fang et al., 2009; Lee et al., 2011; Gu and Xu, 2013; Lynch et al., 2013). The effects of adiponectin on vascular tone are mediated by activation of calcium-activated K^+ (BK_{Ca}) channels on VSMCs and adipocytes and by endothelial mechanisms (Lynch et al., 2013; Baylie et al., 2017), or K_v channel-dependent mechanisms (Fésüs et al., 2007). PAME is one of the most abundant fatty acids in mammalian cells (Lau et al., 2017), and represents an endogenous naturally occurring fatty acid methyl ester (Fukuda et al., 1967). This compound has been reported to have the ability to inhibit Kupffer cells which are resident macrophages in the liver regulating inflammatory processes by secretion of TNF-alpha and NO (Cai et al., 2005). PAME is also known to exhibit anti-fibrotic effects (Fukunishi et al., 2011) and to act as potent vasodilator released in retina and myometrium (Lee et al., 2010, 2011; Crankshaw et al., 2014). A recent report identified PAME as novel, potent vasodilator released from PVAT in rat aorta, which exhibits vascular relaxation by opening K_v channels in smooth muscle cells (Lee et al., 2011). Although these findings suggest that PAME could represent a potential mediator in control of vasotonus and blood pressure in rats, the role of K_v7 channels in PVAT regulation of human arterial tone and vasodilatory PAME effects remains to be established. Therefore, we tested the hypothesis that XE991-sensitive K_v (K_v7) channels are involved in the anti-contractile effects of PVAT on human mesenteric arteries. Furthermore, we investigated the contribution of endogenous PAME to PVAT regulation of arterial tone in human mesenteric arteries and the role of K_v7 channels in vasodilatory PAME effects. Isolated aortic rings from Sprague-Dawley rats were studied for comparison. Finally, we tested whether PAME might contribute to PVAT regulation of arterial tone by involving metabolism of endogenous lipid epoxides.

MATERIALS AND METHODS

Isometric Contractions of Rat Vessels

The local animal review board of Berlin (LAGESO) approved all studies, according to American Physiological Society criteria. Male Sprague-Dawley rats (200–300 g, 8–10 weeks; Charles River, Sulzfeld/Berlin Germany) were killed, and the thoracic aortas were removed, and quickly transferred to cold (4°C) oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS), and dissected into 2 mm rings, respectively. Perivascular fat and connective tissue were either removed [(−) fat] or left [(+) fat] intact as previously described. The rings were placed under force of 20 mN. The bath solution volume was 20 mL of a vessel myograph (Schuler tissue bath system, Hugo Sachs Elektronik, Freiburg, Germany). After 1 h equilibration, contractile force was measured isometrically using standard bath procedures and solutions as described (Dubrovska et al., 2004; Kohn et al., 2012; Brennan et al., 2016).

Cumulative concentration response curves were obtained for PAME (Löhn et al., 2002) in the presence and absence of the K^+ channel or enzyme inhibitors, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991); 4-aminopyridine (4-AP); nordihydroguaiaretic acid (NDGA); 17-octadecadiynoic acid (ODYA); or 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA). Tension was expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60 mM KCl. To test for the presence of functional endothelium, rings were contracted with 1 μM PE and once the vessels reached a stable maximum tension, the vessels were stimulated with 10 μM acetylcholine (ACh) and relaxation was confirmed (>80%) (Löhn et al., 2002). In some rings, the endothelium was removed by gently abrading the luminal surface of the vessel with a stainless steel

pin to determine the contribution of the endothelium to PAME relaxation. Functional endothelium was considered absent if 10 μM ACh did not produce relaxation (Löhn et al., 2002).

In bioassay experiments, we transferred aliquots of bath solution from aorta with PVAT incubated in a donor bath chamber to vessel preparations without PVAT in an acceptor bath chamber of the Schuler tissue bath system (Hugo Sachs Elektronik, Freiburg, Germany). Cumulative response curves were obtained in the presence and absence of 5-HT (total incubation time, 5 min). The volume of the solutions in the bath was 20 mL. In most experiments, transfer interval of aliquots was 15–20 min; the volume of the aliquots was 3 or 5 mL. Transfer of bath solution aliquots from aortic vessels without PVAT or fresh PSS did not affect contraction of vessel preparations without PVAT in the acceptor bath chamber (Löhn et al., 2002).

Isometric Contractions of Human Vessels

Procedures were performed in accordance with the ethics guidelines of the National Health and Medical Research Council of Germany. All patients provided informed consent for participation in this study. Mesenteric tissue was taken from 12 patients (1 female, 11 males) undergoing surgical treatment of bowel carcinoma or inflammatory bowel disorders [colon cancer ($n = 3$), sigma cancer ($n = 4$), rectal cancer ($n = 1$), colon adenoma ($n = 1$), Crohn's disease ($n = 1$), and sigmoid diverticulitis ($n = 1$)]. The mean age of the patients was 69 years (range: 46–80), the mean BMI of the patients was 25 kg/m^2 (range: 20–30 kg/m^2), which is expected for the general population, since adopting the WHO classification is that ~50% or more of the general adult population will always be in the overweight range (now pre-obese, BMI 25–30 kg/m^2), at least in the US and Western Europe (Nuttall, 2015). Few patients were taking drugs, including β-blockers ($n = 4$), angiotensin-converting-enzyme inhibitors ($n = 2$), metformin ($n = 2$), calcium channel blocker ($n = 1$), diuretic ($n = 1$), or fibrates ($n = 1$). Immediately after lower intestinal surgery, mesenteric arteries were excised from resected mesenteric tissue, and quickly transferred to cold (4°C) oxygenated (95% O_2/5% CO_2) PSS, and dissected into 1 mm rings. PVAT was either removed [(−) fat] or left [(+) fat] intact as previously described (Schleifenbaum et al., 2014). Each ring was positioned between two stainless steel wires in a 5-mL organ bath of a Small Vessel Myograph (DMT 610M; Danish Myo Technology, Denmark) (Tsvetkov et al., 2016a). The organ bath was filled with PSS. The bath solution was continuously oxygenated with a gas mixture of 95% O_2 and 5% CO_2, and kept at 37°C (pH 7.4). The rings were placed under force of 3 mN. The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display. The rings were pre-contracted with 60 mM KCl and equilibrated until a stable resting tension was acquired. Chemicals were added to the bath solution if not indicated otherwise. Vessels were pre-contracted with either 5-HT or phenylephrine. All chemicals were added to the bath solution (PSS).

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

PAME measurements were performed by Shanghai Ingeer Certification Assessment Co, Ltd (ICAS, Shanghai, China). GC/MS analysis was performed using an Agilent ChemStation. For determination of endogenous PAME concentrations in bath solutions, rat peri-aortic and human visceral adipose tissue (3 g each) were incubated in 15 mL-Eppendorf tubes with 10 mL PSS solutions, with or without 5-HT 5 μM (30 min, in 37°C water bath). PSS was oxygenated (95% O_2/5% CO_2) for 30 min before use. After removal of adipose tissue, the PSS solution were dissolved in hexane (1:3 volume ratio), extracted and vortexed. Next, 1 mL water was added to the solution. In order to ensure that the concentration of PAME between the aqueous and the lipophilic phase was in equilibrium the samples were shaken by hand for 4 min. Thereafter, the phases were separated by centrifugation and the lipophilic hexane phase containing fatty acid methyl esters was removed and dried under nitrogen. The fatty acid methyl ester residues were re-dissolved in 50 μL hexane and transferred into an autosampler vial. Samples were analyzed by using a fully automated Agilent 7890A-5977B system equipped with a flame ionization detector. Peaks of re-dissolved PAME were identified by comparison with PAME standard and their nominal concentrations were determined (Yi et al., 2014; Siegert et al., 2017). (+) Fat masses were measured in rat aortic (2 mm) and human mesenteric artery (1 mm) rings ($n = 6$ each) to calculate magnitudes of effective [PAME] in the 20 or 5 mL myograph bath chambers, respectively.

Materials and Statistics

The composition of PSS (in mM) was 119 NaCl, 4.7 KCl, 1.2 KH_2PO4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, and 1.6 $CaCl_2$. The composition of 60 mM KCl solution (in mM) was 59 NaCl, 60 KCl, 1.2 KH_2PO4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, and 1.6 $CaCl_2$. All salts were purchased from Sigma Aldrich (Schnelldorf, Germany). XE991 was purchased from Tocris (Bristol, UK). 5-HT, phenylephrine (PE), 4-aminopyridine (4-AP), nordihydroguaiaretic acid (NDGA), 17-octadecynoic acid (ODYA), 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA) were purchased from Sigma Aldrich (Schnelldorf, Germany). PAME were purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

Data were analyzed by Prism version 5.0 (GraphPad Software, La Jolla, California, USA) and were shown as mean ± SD or mean ± SEM. Paired, unpaired Student's *t*-tests or one-way ANOVA were used as appropriate. In **Figures 1C, 2C**, statistical significance was determined by two-way ANOVA or repeated-measures two-way ANOVA, followed by Bonferroni *post-hoc* test, and using Prism 6 software. A value of $P < 0.05$ was considered statistically significant; n represents the number of arteries tested.

RESULTS

Contraction of Rat Aortas and Human Mesenteric Arteries With and Without PVAT Under K_v7 Channel Inhibition

We first investigated the role of K_v7 channels in the anti-contractile effects of PVAT in rat aortas. Rat aortic rings without PVAT [(−) fat] showed stronger contractions to 5-HT (relative increase, 60–100% between 2 and 10 μM 5-HT) than vessels

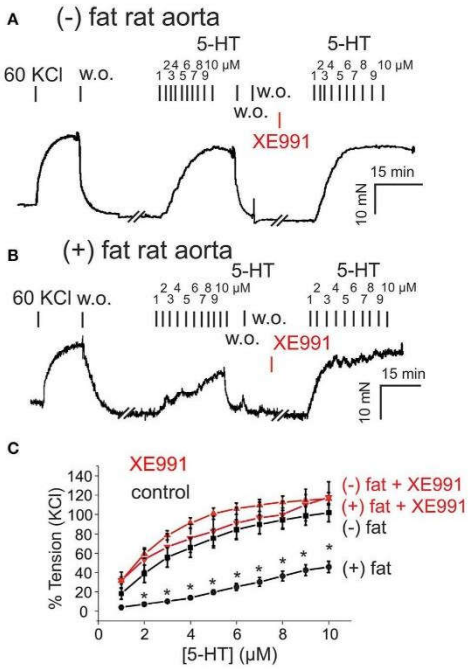

FIGURE 1 | Presence of anti-contractile effects of perivascular adipose tissue (PVAT) in rat aorta and their inhibition by XE991. **(A,B)** Original recordings showing inhibition of the anti-contractile effects of PVAT by XE991. Incubation of (–) fat aortic rings with 30 µM XE-991 **(A)** had no effect on the contractile response. Incubation of (+) fat aortic rings with 30 µM XE991 **(B)** increased the contractile response to 5-HT. **(C)** Concentration-response curves for 5-HT (1–10 µM) in (+) fat vessels in the presence ($n = 6$) and absence of XE991 ($n = 6$) and in (–) fat vessels in the presence ($n = 12$) and absence of XE991 ($n = 6$). $^*p < 0.05$ for (+) fat control vessels compared to all other groups each, 60 KCl, 60 mM KCl.

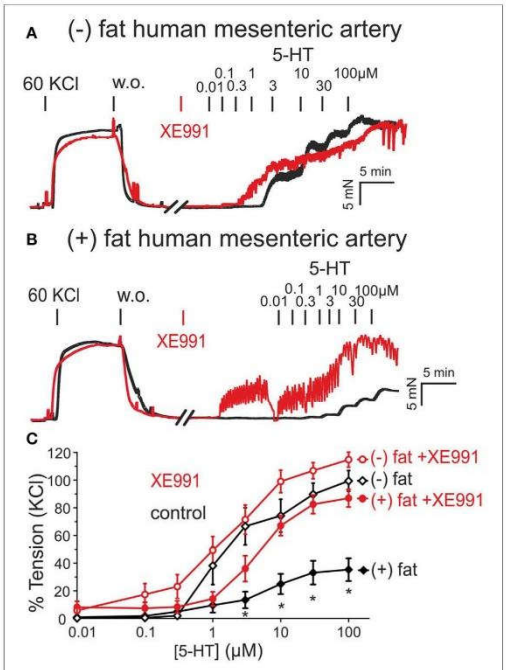

FIGURE 2 | Presence of anti-contractile effects of PVAT in human mesenteric arteries and their inhibition by XE991. **(A,B)** Original recordings showing inhibition of the anti-contractile effects of PVAT by XE991 (30 µM). Recordings are shown in the presence (red curves) and absence (black curves) of XE991. XE991 had no effects on 5-HT contractions in (–) fat rings **(A)**, but increased 5-HT contractions in (+) fat rings **(B)**. **(C)** Concentration-response curves for 5-HT (0.01–100 µM) in (+) fat vessels in the presence ($n = 13$) and absence of XE991 ($n = 15$) and in (–) fat vessels in the presence ($n = 10$) and absence of XE991 ($n = 10$). $^*p < 0.05$, $p < 0.05$ for (+) fat control vessels compared to all other groups each.

with PVAT [(+) fat] (**Figure 1**). Incubation of the vessels with the K_v7 channel inhibitor XE991 (30 µM, 30 min) inhibited the anti-contractile effects of PVAT mediated by transferable ADRF in rat aortic rings (**Figures 1B,C**, **Supplementary Figures 1**). XE991 slightly (<20%) increased contractions of (–) fat rat aortic rings in response to 5-HT, but there was no difference between contractions of (–) fat and (+) fat rings in response to 5-HT (**Figures 1A,C**). Contraction of (–) fat and (+) fat aortic rings obtained by 60 mM KCl containing PSS were not different (13.07 ± 1.29 mN, $n = 16$ vs. 10.81 ± 1.19 mN, $n = 14$, $P > 0.05$, respectively).

Similar results were observed in human visceral arteries. Human mesenteric arteries without PVAT [(–) fat] showed significantly stronger contractions in response to 5-HT than vessels with PVAT [(+) fat] (**Figure 2**). Incubation of the vessel rings with XE991 (30 µM, 30 min) inhibited the anti-contractile effects of PVAT (**Figures 2B,C**). XE991 did not affect 5-HT-induced contractions in (–) fat human mesenteric artery rings (**Figures 2A,C**).

Similar data were obtained when vessels were contracted with phenylephrine (**Supplementary Figures 2**). These data suggest that PVAT displays anti-contractile effects in both rat aortas and human mesenteric arteries mediated by XE991-sensitive K_v7 channels, and occur independently of the vasoconstrictor agonists used, i.e., serotoninergic or alpha-adrenergic agonists. Contraction of (–) fat and (+) fat human mesenteric arterial rings obtained by 60 mM KCl containing PSS were not different (19.46 ± 3.60 mN, $n = 10$ vs. 21.00 ± 2.15 mN, $n = 12$, $P > 0.05$, respectively).

PAME Relaxations and Effects of NDGA, ODYA, and AUDA

Exogenous PAME (EC_{50} ~1.4 µM; maximal relaxation E_{max} ~25%) was capable of producing relaxations of (–) fat rat aortas (**Figure 3**). PAME relaxations were not affected by removal

of the endothelium (**Figure 3C**). Pre-treatment of aortic rings with XE991 (30 μM, 30 min) prevented the relaxant effects of PAME (**Figures 3B,F**). PAME effects were not abolished by the K_v channel blocker 4-aminopyridine (4-AP 2 mM, 10 min) (**Figure 3D**) or NDGA (10 μM, 30 min), a lipoxygenases inhibitor (**Figure 3G**). The cytochrome P450 epoxygenase inhibitor 17-octadecynoic acid (ODYA 10 μM, 30 min) slightly inhibited PAME relaxations in rat aorta (**Figure 3E**). However, the soluble epoxide hydrolase inhibitor 12-(3-adamantan-1-ylureido)-dodecanoic acid (AUDA 10 μM, 30 min) did not increase PAME relaxations (**Figure 3H**), which is expected for involvement of P450 expoygenase mediators. Instead, it inhibited PAME relaxations implicating non-specific effects of ODYA in inhibiting PAME effects. PAME up to 10 μM failed to induce relaxations of (–) fat human mesenteric arteries (**Figure 4**). Pre-treatment of the vessels with XE991 (30 μM, 30 min) did not affect the lack of PAME effects (**Figures 4B,C**). Taken together, the results indicate that exogenous PAME at relatively high concentrations (EC_{50} >1 μM) can induce slight (E_{max} ~25%) relaxations in pre-contracted rat aortic rings, which are mediated by opening of K_v7 channels. In contrast, PAME is not a potent vasodilator (up to 10 μM) of human mesenteric arteries.

Role of PAME in ADRF-Containing Bath Solutions of Rat Aortas and Contribution of K_v7 Channels

To demonstrate that the intact aortic preparation releases ADRF which can abrogate vascular contraction by opening VSMC K_v7 channels (Tano et al., 2014; Gollasch, 2017), we performed bioassay experiments where we transferred aliquots of the bath solution from an intact donor preparation incubated in 2 μM 5-HT-containing solution to vessel preparations without PVAT, pre-contracted with 5-HT. This maneuver transferred the factor (Tano et al., 2014; Gollasch, 2017) released by either intact preparations or isolated perivascular adipose tissue (PVAT) (**Figure 5B**) to arteries without adipose tissue (Tano et al., 2014). Bath solutions from (+) fat rings incubated with 5-HT produced stronger relaxations than bath solutions from (+) fat rings incubated in PSS without 5-HT (**Figures 5A** right, **B**), indicating that ADRF release is increased by 5-HT. According to the proposed K_v7 channel mechanism (Tano et al., 2014; Gollasch, 2017), ADRF produced relaxations, which were inhibited by XE991 (30 μM) (**Supplementary Figures 1**). Next, we were interested in [PAME] in ADRF-containing bath solutions and whether PAME release from PVAT can be stimulated by 5 μM 5-HT. We found that 5-HT is capable to release endogenous

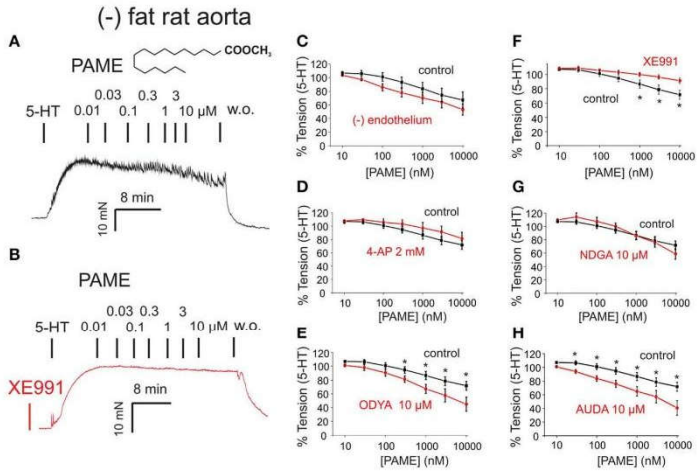

FIGURE 3 | Effects of palmitic acid methyl ester (PAME, 0.01–10 μM) on rat aorta. The effects are shown in the absence (control, black curves) and presence (red curves) of inhibitors of K_v channels [4-aminopiridine (4-AP) 2 mM] or XE991 30 μM, lipoxygenase (LOX 15,12,5) (nordihydroguaiaretic acid (NDGA) 10 μM), cytochrome P450 (CYP) [17-octadecynoic acid (17-ODYA) 10 μM], and soluble epoxide hydrolase (sEH) [12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA) 10 μM]. The effects were studied in the presence (+) and absence (–) of endothelium in 2 μM 5-HT precontracted (–) fat aortic rings. **(A,B)** Original recordings demonstrating the relaxant effect of PAME (0.01–10 μM) and inhibition of this effect **(B)** by pre-treatment of the vessels with 30 μM XE991. **(C)** Concentration-response curves for PAME in (–) fat aortic rings in the presence (Control) ($n = 8$) and absence (–) of the endothelium ($n = 6$). **(D)** Concentration-response curves for PAME in the absence ($n = 13$) and presence ($n = 7$) of 2 mM 4-AP in (–) fat aortic rings. **(E)** Concentration-response curves for PAME in non-treated ($n = 13$) and 10 μM 17-ODYA treated ($n = 6$) (–) fat aortic rings. *$p < 0.05$. **(F)** Concentration-response curves for PAME in the absence ($n = 6$) and presence ($n = 6$) of 30 μM XE991 in (–) fat aortic rings. *$p < 0.05$. **(G)** Concentration-response curves for PAME in non-treated ($n = 13$) and 10 μM NDGA ($n = 6$) treated (–) fat aortic rings. **(H)** Concentration-response curves for PAME in non-treated ($n = 13$) and 10 μM AUDA treated ($n = 6$) (–) fat aortic rings. Data are expressed as mean ± SEM. *$p < 0.05$.

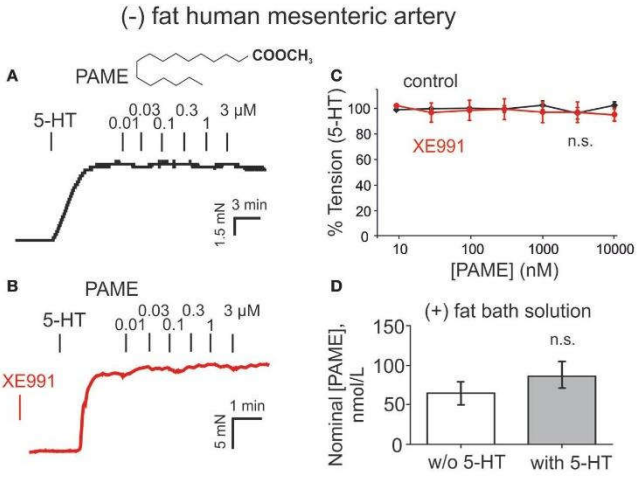

FIGURE 4 | Lack of palmitic acid methyl ester (PAME, 0.01–10 µM) to induce relaxation in human mesenteric artery rings and measurements of PAME in bath solutions from human (+) fat mesenteric artery rings. **(A,B)** Original recordings demonstrating lack of vasorelaxant effects of PAME (0.01–3 µM) in (–) fat rings. Effects of PAME are shown in the absence (control, black curves) and presence (red curves) of XE991. The effects were studied on 2 µM 5-HT pre-contracted (–) fat mesenteric artery rings. **(C)** Concentration-response curves for PAME in (–) fat rings in the presence (Control) (PAME 0.01–3 µM, $n = 12$; PAME 10 µM, $n = 8$) and absence (PAME 0.01–3 µM, $n = 12$; PAME 10 µM, $n = 8$) of XE991. **(D)** Production of PAME shows no difference between bath solutions from human visceral adipose tissue incubated in the presence ($n = 8$) and absence of 5-HT ($n = 8$). w/o, without; n.s., not significant.

PAME from rat aortic PVAT samples (**Figure 5C**). However, the PAME concentrations in 10 mL PSS solutions containing 3 g rat aortic PVAT samples were lower than 300 nM, indicating that effective PAME concentrations in myograph bath chambers are in the range of <1 nM (i.e., 500 times lower). **Figures 5C, 3A–H** show that [PAME] lower than 1 nM are unable to affect wall tension to produce relaxations of (–) rat aortas. These data indicate that PAME cannot be the transferable ADRF we seek.

Endogenously Released PAME Levels in Bath Solutions of Human Visceral Adipose Tissue Are Low and Not Controlled by 5-HT

Endogenously released nominal PAME levels were also detected in aliquots of bath-solutions containing 3 g human visceral adipose tissue in 10 mL PSS (**Figure 4D**). However, in contrast to the rat aorta (**Figure 5C**), PAME levels were even lower and 5-HT (5 µM) was unable to induce endogenous PAME release in the adipose tissue samples. These data indicate that 5-HT can induce PAME release from rat peri-aortic adipose tissue, but not from human visceral adipose tissue, where spontaneous PAME release is even lower and not controlled by 5-HT.

DISCUSSION

In the present study, we investigated the roles of K_v7 channels and PAME in PVAT regulation of arterial tone in human visceral mesenteric arteries. The major findings are that XE991-sensitive K_v (K_v7) channels are involved in the anti-contractile effects of PVAT on human mesenteric arteries, similarly to rat aortas. Furthermore, exogenous PAME displays properties of a PVRF in rat aorta, where it may contribute to paracrine PVAT regulation of arterial tone, but not in human mesenteric arteries. Our data indicate that PAME is not ADRF. Nevertheless, the data support previous findings (Lee et al., 2011) suggesting that fatty acids, particularly perivascular adipose tissue-derived methyl palmitate (PAME), can play a role in paracrine regulation of vascular tone and possibly in the pathogenesis of hypertension in rats, where deficiency or malfunction of K_v channels (Gálvez et al., 2006; Galvez-Prieto et al., 2008), particularly K_v7 channels (Jepps et al., 2011; Li et al., 2013; Zavaritskaya et al., 2013), have been suggested to be involved. Our results suggest that these effects could involve malfunctional K_v7 channels, independently of metabolism of endogenous lipid epoxides. Since the [PAME] released into bath media were exceptionally low, we conclude that PAME released from PVAT only in close proximity to VSMCs can regulate arterial tone in rat aorta.

PVAT Effects and K_v7 Channels

We demonstrated earlier that PVAT markedly attenuates the contractile response to 5-HT, phenylephrine and angiotensin II in aortic and mesenteric ring preparations of rats (Löhn et al., 2002; Verlohren et al., 2004). The data suggest a major role of the K_v7 family of K^+ channels as putative downstream targets of ADRF, which is a major PVRF released from PVAT to reduce arterial

tone (Zavaritskaya et al., 2013; Gollasch, 2017). This suggestion is supported by findings showing that XE991 (K_v7 blocker) inhibited the anti-contractile effects of PVAT in visceral arteries of rats and mice (Schleifenbaum et al., 2010; Tano et al., 2014) (this study). Data were obtained by two different vasoconstrictor agonists, namely 5-HT and phenylephrine, indicating that K_v7 channel targeting could be common mechanism for PVAT regulation of arterial tone. We employed XE991 at 30 μmol/L to ensure effective block of the K_v7 channels because native VSMC $K_v7.4$ and $K_v7.5$ channels are inhibited by this compound with IC_{50} of 5.5 and 65 μmol/L, respectively (Tykocki et al., 2017). The XE991 effects are unlikely mediated by inhibition of BK_{Ca} or $K_v7.1$ channels (Tsvetkov et al., 2016b, 2017). Our present results show that PVAT displayed anti-contractile effects in human mesenteric arteries. The anti-contractile effects were inhibited by XE991, supporting the idea that K_v7 channels are involved in PVAT regulation of arterial tone in humans. Data obtained on *Kcnq1*$^{-/-}$ mice (Tsvetkov et al., 2016b) suggest that these effects are mediated by K_v7 channels distinct from $K_v7.1$, i.e., most likely $K_v7.3$, $K_v7.4$ and/or $K_v7.5$, which are all expressed in mesenteric artery VSMCs from rats (Mackie et al., 2008; Jepps et al., 2011; Zavaritskaya et al., 2013), mice (Yeung et al., 2007; Tsvetkov et al., 2016b, 2017), and humans (Ng et al., 2011).

PAME Effects and K_v7 Channels

We found that PAME was capable to produce relaxations of rat aortas. These effects were inhibited by XE991. The effects were not inhibited 2 mM 4-AP, but see (Lee et al., 2011). In contrast, PAME at similar concentrations did not relax human mesenteric arteries. Together, these data suggest that PAME could contribute to PVAT relaxations by activating K_v7 channels in rat aorta, but not in human mesenteric arteries. The results are in line with the idea that 4-AP is not inhibiting K_v7 channels in rat aorta. We next explored the role of PAME and K_v7 channels in the anti-contractile effects of PVAT in rat aorta using a bioassay approach (Gálvez et al., 2006; Galvez-Prieto et al., 2008). In these experiments, we confirmed earlier findings indicating that 5-HT induces vessel relaxation by releasing a transferable vasoactive substance (ADRF) from PVAT into the bath solution (Maenhaut and Van de Voorde, 2011; Gollasch, 2012). As a negative control, we transferred aliquots of periadventitial fat solution in a similar fashion without 5-HT. We found that these aliquots produced less potent relaxations in rat aortas without PVAT suggesting that 5-HT is capable to stimulate the release of ADRF from PVAT. Moreover, inhibition of K_v7 channels in (–) fat aortic rings by XE991 disrupted these effects in our bioassay experiment. We previously demonstrated that ADRF effects occur without involvement of the endothelium (Gollasch, 2012). Thus, the present data indicate that 5-HT induces ADRF release from PVAT, which displays anti-contractile properties though activation of XE991-sensitive (K_v7) K_v channels in VSMCs. We next focused on PAME bioactivity and release under these conditions. This part of our study is important for understanding the role of PAME as putative ADRF and/or paracrine PVRF. We found that PAME (EC_{50} ~1.4 μM) only slightly relaxed rat aortas (E_{max} about 25%), whereas similar concentrations of PAME had no effects on human mesenteric arteries.

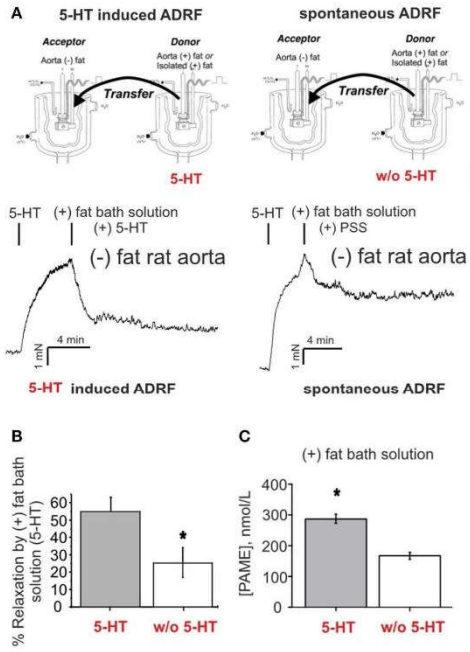

FIGURE 5 | Relaxation of (–) fat aortic rings induced by transfer of bath solutions from (+) fat rat aortic rings. **(A)** Original recordings demonstrating stronger relaxations of ADRF-containing bath solutions from (+) fat rings incubated with 5-HT than from (+) fat rings incubated in PSS without 5-HT. **(B)** Bath solutions from (+) fat rings incubated with 5-HT produced stronger relaxations than bath solutions from (+) fat rings incubated in PSS without 5-HT. Relaxation is expressed as a percentage of the steady-state tension (100%) obtained with 2 μM 5-HT. Data are expressed as mean ± SEM of 6 (left bar) and 5 rings (right bar) prepared from 6 and 5 animals, respectively. **(C)** Bath solutions from rat peri-aortic adipose tissue incubated in the presence 5-HT ($n = 8$) produced more PAME than bath solutions from rat peri-aortic adipose tissue incubated in the absence of 5-HT ($n = 8$). *$p < 0.05$.

PAME Source and Metabolism

Palmitic acid, or hexadecanoic acid in IUPAC nomenclature, is the most common saturated fatty acid found in plants, animals and humans. Together with stearic acid and oleic acid, palmitate acid belongs to the free fatty acids (FFAs), which play an important role as a source of energy for the body (Ibarguren et al., 2014). Endogenous PAME appears to play a role in modulation of the autonomic ganglionic transmission and vasodilatory effects of nitric oxide (NO) (Lin et al., 2008). In plants, palmitate acid can be metabolized through the lipoxygenase pathway (Osipova et al., 2010). However, there is no evidence that palmitate acid is metabolized through the lipoxygenase pathway in animals or humans. Although palmitate acid seems to have some effects on lipoxygenase and cyclooxygenase in platelets (Sakai et al., 1976), there

are no reports that this occurs in other mammalian cells. Consistently, it is not surprising that NDGA, a non-selective lipoxygenase inhibitor, failed to inhibit exogenous PAME relaxation in rat aorta in our study, suggesting no involvement of lipoxygenases metabolites in the PAME effects in the vasculature.

Dietary triacylglycerols with palmitic acid can reduce plasma phospholipid arachidonic and docosahexaenoic acids *in vivo* (Innis and Dyer, 1997). To rule out involvement of cytochrome P450 (CYP) metabolites in PAME relaxation in rat aorta, we tested the effects of a CYP epoxygenases and FFA ω-hydrolases inhibitor (ODYA), and a soluble epoxide hydrolase inhibitor (AUDA), which blocks breakdown and inactivation of CYP–derived active vasodilatory metabolites from arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid (Hercule et al., 2007, 2009). Since ODYA (10 μM) and AUDA (10 μM) did not produce reciprocal effects on PAME relaxations, we conclude that PAME is a vasodilator in rat aorta independently of metabolism of endogenous lipid epoxides.

PAME Released by PVAT

Most vessels possess some amount and type of PVAT, varying from mostly brown fat (thoracic aorta) to mixed brown and white fat (mesenteric vessels) (Watts et al., 2013). We were able to detect endogenously released PAME in bathing solutions of both rat peri-aortic and human visceral adipose tissues. Furthermore, 5-HT was capable to induce PAME release from rat peri-aortic adipose tissue, indicating a humoral active release process. However, the measured nominal concentrations of PAME were too low to explain transferable ADRF properties in both vascular preparations under study. Together, we suggest that PAME is actively released from PVAT and displays properties of relaxing factor in rat aorta, but not in human mesenteric arteries, where it may contribute as paracrine PVRF to PVAT regulation of arterial tone, independently of metabolism of endogenous lipid epoxides. It will be interesting to determine human mesenteric PVAT is unresponsive to PAME release and action because it is mostly white adipose tissue.

In conclusion, our studies implicate important roles of K_v7 channels in PVAT control of arterial tone in both rat aorta and human mesenteric arteries, which supports previous findings obtained on other, non-human arteries (Gollasch, 2017). Furthermore, our study highlights the potential role of PAME to contribute as paracrine PVRF to regulation of vascular contraction by opening K_v7 channels, at least in rats. Our study has translational implications since malfunction of PVAT/K_v7 channels has been proposed to contribute and to serve as therapeutic targets to improve vascular dysfunction in experimental obesity and hypertension (Rahmouni, 2014), but data on existence of this prototype of vasoregulation in human vessels were missing. Further studies are warranted to investigate PVRFs, K_v7 and other vascular potassium channels to develop new prevention and treatment strategies for cardiovascular disorders associated with obesity and hypertension.

AUTHOR CONTRIBUTIONS

All authors planned and designed the experimental studies. NW, AK, and GD performed the wire myography experiments. NW and MG drafted the article, and all authors, contributed to its completion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00583/full#supplementary-material

Supplementary Figure S1 | Original recordings demonstrating relaxation of (–) fat rat aortic rings by transfer of ADRF-containing bath solutions from (+) fat rat aortic rings **(A)**, and pre-treatment of (–) fat vessels with 30 μM XE991 can inhibit relaxation of transfer ADRF-containing bath solutions in (–) fat rat aortic rings **(B)**.

Supplementary Figure S2 | Presence of anti-contractile effects of perivascular fat in human mesenteric arteries and their inhibition by XE991. **(A,B)** Original recordings showing inhibition of the anti-contractile effects of perivascular fat by XE991 (30 μM). Recordings are shown in the presence (red curves) and absence (black curves) of XE991. XE991 had no effects on phenylephrine contractions in (–) fat rings **(A)**, but increased phenylephrine contractions in (+) fat rings **(B)**. **(C)** Concentration-response curves for phenylephrine in (+) fat vessels in the presence ($n = 23$) and absence of XE991 ($n = 18$) and in (–) fat vessels in the presence (closed circle, $n = 25$) and absence of XE991 ($n = 17$). $^*p < 0.05$ for (+) fat control vessels compared to all other groups each.

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The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone

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The anti-contractile effect of perivascular adipose tissue (PVAT) is an important mechanism in the modulation of vascular tone in peripheral arteries. Recent evidence has implicated the XE991-sensitive voltage-gated K_V (KCNQ) channels in the regulation of arterial tone by PVAT. However, until now the *in vivo* pharmacology of the involved vascular K_V channels with regard to XE991 remains undetermined, since XE991 effects may involve Ca^{2+} activated BK_{Ca} channels and/or voltage-dependent $K_V1.5$ channels sensitive to diphenyl phosphine oxide-1 (DPO-1). In this study, we tested whether $K_V1.5$ channels are involved in the control of mesenteric arterial tone and its regulation by PVAT. Our study was also aimed at extending our current knowledge on the *in situ* vascular pharmacology of DPO-1 and XE991 regarding $K_V1.5$ and BK_{Ca} channels, in helping to identify the nature of K^+ channels that could contribute to PVAT-mediated relaxation. XE991 at 30 μM reduced the anti-contractile response of PVAT, but had no effects on vasocontraction induced by phenylephrine (PE) in the absence of PVAT. Similar effects were observed for XE991 at 0.3 μM, which is known to almost completely inhibit mesenteric artery VSMC K_V currents. 30 μM XE991 did not affect BK_{Ca} currents in VSMCs. *Kcna5*$^{-/-}$ arteries and wild-type arteries incubated with 1 μM DPO-1 showed normal vasocontractions in response to PE in the presence and absence of PVAT. K_V current density and inhibition by 30 μM XE991 were normal in mesenteric artery VSMCs isolated from *Kcna5*$^{-/-}$ mice. We conclude that K_V channels are involved in the control of arterial vascular tone by PVAT. These channels are present in VSMCs and very potently inhibited by the KCNQ channel blocker XE991. BK_{Ca} channels and/or DPO-1 sensitive $K_V1.5$ channels in VSMCs are not the downstream mediators of the XE991 effects on PVAT-dependent arterial vasorelaxation. Further studies will need to be undertaken to examine the role of other K_V channels in the phenomenon.

Keywords: XE991, KCNQ channels, $K_V1.5$ channels, adipocyte-derived relaxing factor (ADRF), perivascular adipose tissue (PVAT), BK channels

INTRODUCTION

Over the past decade, various potassium (K^+) channels have been implicated as important players in the regulation of arterial vascular tone and its control by perivascular adipose tissue (PVAT). Opening of vascular smooth muscle cell (VSMC) K^+ channels causes K^+ efflux and membrane hyperpolarization, which leads to reduced Ca^{2+} influx though L-type $Ca_V1.2$ channels and consequently arterial relaxation (Nelson and Quayle, 1995). A variety of endogenous vasodilators, such as hypoxia, acidosis, as well as metabolites and autacoids (e.g., adenosine, prostacyclin) act as potent K^+ channel openers to produce relaxation (Sobey, 2001; Tano and Gollasch, 2014). Noteworthy, many of these substances produce relaxation by opening maxi Ca^{2+} activated (BK_{Ca}) K^+ channels in VSMCs (Bentzen et al., 2014). Only very few substances have been reported to relax vessels by opening arterial smooth muscle voltage-gated K_V channels (Tanaka et al., 2006; Park et al., 2015). Among them adenosine and atrial natriuretic peptide (ANP) act *via* activation of the KCNQ (K_V7) subfamily of K_V channels (Khanamiri et al., 2013; Stott et al., 2015).

Recent studies have demonstrated a paracrine role for PVAT to produce relaxation of arterial smooth muscle cells in a number of vascular beds (Lohn et al., 2002; Verlohren et al., 2004; Gao et al., 2005; Zavaritskaya et al., 2013). Certain adipokines, such as adiponectin (Weston et al., 2013), angiotensin-1 to 7 (Lee R. M. K. W. et al., 2011), methyl palmitate (Lee Y.-C. et al., 2011), and notably H_2S (Schleifenbaum et al., 2010) were recently proposed as potential perivascular-derived relaxing factors (PVRFs), which could mediate the anti-contractile properties of PVAT. The paracrine effects of PVAT involve the opening of K^+ channels, however, the identity of the K^+ channel subtype(s) involved is still a matter of debate (Tano et al., 2014).

Voltage-gated K_V channels of the KCNQ (K_V7) family have been proposed to play an important role in PVAT control of arterial tone. This conclusion is based on observations demonstrating that the anti-contractile effects of PVAT are inhibited by the pan KCNQ channel blocker XE991 at 30 μM or the pan K_V channel blocker 4-aminopyridine (2 mmol/L; Fésüs et al., 2007; Schleifenbaum et al., 2010; Lee Y.-C. et al., 2011; Zavaritskaya et al., 2013). XE991 is a widely used pan K_V7 channel blocker, which inhibits $K_V7.1$ homomeric or $K_V7.1$/KCNE channels (IC_{50} of ~0.8 μM and 11.1 μM, respectively; Wang et al., 2000), KCNQ2/3 channels (EC_{50} ~1 μM; Wang et al., 1998), KCNQ4 (EC_{50} ~5.5 μM; Søgaard et al., 2001), and KCNQ5 (EC_{50} ~65 μM; Schroeder et al., 2000). Noteworthy, XE991 can also inhibit other K_V channels, such as ERG (K_V11; EC_{50} ~110 μM) (Elmedyb et al., 2007) and $K_V1.2$/1.5, $K_V2.1$/$K_V9.3$ channels (~30% inhibition at 10 μM) in heterologous expression systems (Zhong et al., 2010).

However, it is unknown whether XE991 is indeed specific for vascular K_V channels *in situ*, and does not inhibit native BK_{Ca} channels. This is particularly relevant since BK_{Ca} channels have been proposed to play a role in PVAT control of arterial tone in other studies (Lynch et al., 2013; Weston et al., 2013), although studies using BK_{Ca} deficient mice gave opposing results (Fésüs et al., 2007). A recent study showed that K_V channels in VSMCs of mouse mesenteric arteries are very sensitive to XE991 (EC_{50} ~60 nM), suggesting that these channels may contribute to PVAT control of arterial tone (Schleifenbaum et al., 2010, 2014). A very recent study suggested that diphenyl phosphine oxide-1 (DPO-1) sensitive $K_V1.5$ channels could contribute to the K_V current in VSMC (Fancher et al., 2015).

Therefore, we tested whether $K_V1.5$ channels are involved in the control of arterial tone and its regulation by PVAT or not. Our study is also aimed at extending our current knowledge on the *in situ* vascular pharmacology of DPO-1 and XE991 regarding $K_V1.5$ and BK_{Ca} channels, in helping to identify the nature of K^+ channels that could contribute to PVAT-mediated relaxation.

METHODS

Mouse Model

We used *Kcna5*$^{-/-}$ mice as previously described (Pannasch et al., 2006). The mouse model was evaluated by RT-qPCR (Figure S1). Either litter- or age-matched (10–14 weeks old) male wild-type (129S6 background, previously known as 129SvEv-Ta) mice were used as controls. 250–300 g male Sprague Dawley rats were obtained from Charles River, Germany, Berlin. All experimental procedures were performed in accordance with the German legislation on protection of animals. Animal care followed American Physiological Society guidelines, and local authorities (Landesamt für Gesundheit und Soziales Berlin, *LAGeSo*) approved all protocols. Mice were housed in individually ventilated cages under standardized conditions with an artificial 12-h dark–light cycle with free access to water and food.

Wire Myography

First order mesenteric arteries were removed immediately after killing the mice or rats under inhalation anesthesia with isoflurane by cervical dislocation, quickly transferred to cold (4°C), oxygenated (95% O_2/5% CO_2) physiological salt solution (PSS) containing (in mmol/L) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, 1.6 $CaCl_2$), and dissected into 2 mm rings whereby perivascular fat and connective tissue were either intact [(+) PVAT or removed (−) PVAT] without damaging the adventitia. Each ring was positioned on two stainless steel wires (diameter 0.0394 mm) in a 5-ml organ bath of a Mulvany Small Vessel Myograph (DMT 610 M; Danish Myo Technology, Denmark). The organ bath was filled with PSS. The bath solution was continuously oxygenated with a gas mixture of 95% O_2 and 5% CO_2, and kept at 37°C (pH 7.4) (Verlohren et al., 2004; Fésüs et al., 2007). The mesenteric rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mm Hg by stepwise distending the vessel using LabChart DMT Normalization module. This normalization procedure was performed to obtain the passive diameter of the vessel at 100 mm Hg (Fésüs et al., 2007). The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display. After 60 min equilibration arteries were pre-contracted either with isotonic external 60 mmol/L KCl until a stable resting tension was acquired. The composition of 60 mM KCl (in mmol/L) was 63.7 NaCl, 60 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 11.1 glucose, and 1.6 $CaCl_2$. Drugs were

added to the bath solution if not indicated otherwise. Tension is expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60 mM KCl.

Isolation of Arterial VSMCs

VSMCs from mesenteric arteries were isolated as described (Gollasch et al., 1998; Plüger et al., 2000). Briefly, the arteries were isolated and quickly transferred to cold (4°C) oxygenated (95% O_2–5% CO_2) PSS. The arteries were cleaned, cut into pieces, and placed into a Ca^{2+}-free Hank's solution (in mmol/L): 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 $MgCl_2$, 1 mg/ml bovine serum albumin (BSA, Sigma, Taufkirchen), 10 glucose, and 10 HEPES (pH 7.4 with NaOH) containing 0.5 mg/ml papain (Sigma) and 1.0 mg/ml DTT for 50 min at 37°C. The segments then were placed in Hank's solution containing 1 mg/ml collagenase (Sigma, type F and H, ratio 30 and 70%, respectively) and 0.1 mmol/L $CaCl_2$ for 10 min at 37°C. Following several washes in Ca^{2+}-free Hank's solution (containing 1 mg/ml BSA), single cells were dispersed from artery segments by gentle triturating. Cells were then stored in the same solution at 4°C.

Electrophysiology

Voltage dependent potassium (K_V) currents and BK_{Ca} currents were measured in the conventional whole-cell configuration of the patch-clamp technique at room temperature as previously described (Gollasch et al., 1996; Essin et al., 2007; Schleifenbaum et al., 2014). Patch pipettes (resistance 3–5 MΩ) for recording K_V currents were filled with a solution containing (in mmol/L): 130 KCl, 1 $MgCl_2$, 3 Na_2ATP, 0.1 Na_3GTP, 10 HEPES, and 5 EGTA (pH 7.2; Yeung and Greenwood, 2005). Patch pipettes for recording BK_{Ca} currents contained (in mmol/L): 130 KCl, 1 $MgCl_2$, 3 Na_2ATP, 0.1 Na_3GTP, 10 HEPES, 5 EGTA, and 4.3 $CaCl_2$ (estimated $[Ca^{2+}]$ free, 10^{-6} mol/L; pH 7.2). The external bath solution contained (in mmol/L): 126 NaCl, 5 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 11 glucose and 10 HEPES (pH 7.2; Yeung and Greenwood, 2005). Holding potential was −60 mV. Whole cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) or an EPC 7 amplifier (List, Darmstadt, Germany) and digitized at 5 kHz, using a Digidata 1440A digitizer (Axon CNS, Molecular Devices), and pClamp software versions 10.1 and 10.2 (Schleifenbaum et al., 2014).

RT-qPCR

Total RNA was isolated from snap-frozen heart and aortae tissues with or without $K_V1.5$ by using the RNeasy RNA isolation kit (Qiagen, Hamburg, Germany) according to the manufacturer's instruction. Isolated RNA concentration was measured and RNA quality was tested by NanoDrop-1000 spectrophotometer (PeqLab, Erlangen, Germany). For the synthesis of cDNA, equivalent amounts of RNA (2 μg) were used and processed by a high capacity cDNA reverse transcription kit (Life Technologies GmbH, Darmstadt, Germany). Quantitative analysis of target mRNA expression was performed with real-time PCR using the relative standard curve method (Markó et al., 2016). TaqMan or SYBR green analysis was conducted according to the manufacturer's instructions, using an Applied Biosystems 7500 Sequence Detector (Life Technologies Corporation, Carlsbad, CA, USA). The expression level of the target genes was normalized by the expression of 18S. Primers for were synthesized by Biotez (Berlin, Germany) and the sequences are as follows: $K_V1.5$ Forward sequence: 5′-GCTACTTCGATCCCTTGAGAAAT-3′; Reverse sequence: AGTAGTACAAAATGCCATCGAAGCT, 18S Forward sequence: 5′-ACATCCAAGGAAGGCAGCAG-3′; Reverse sequence 5′-TTTTCGTCACTACCTCCCCG-3′.

Materials

All salts and other chemicals were obtained from Sigma-Aldrich (Germany) or Merck (Germany). All drugs were freshly dissolved on the day of each experiment according to the material sheet. The following concentrations of drugs were used: phenylephrine (Sigma-Aldrich) ranged from 0.01 to 100 μmol/L, 5-HT from 0.01 to 10 μM, DPO-1 (Tocris) 1 and 10 μmol/L, 100 nmol/L iberiotoxin (Sigma Aldrich). XE991 (Tocris) was applied at concentrations between 0.3 and 30 μM.

Statistics

Data represent mean ± SEM. EC_{50} values were calculated using a Hill equation: $T = (B_0 - Be)/(1 + ([D]/EC_{50})^n) + Be$, where T is the tension in response to the drug (D); Be is the maximum response induced by the drug; B_0 is a constant; EC_{50} is the concentration of the drug that elicits a half-maximal response (Bychkov et al., 1998). Curve fittings were done by Prism 6 software using non-linear regression. Statistical significance was determined by two-way ANOVA or repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test, and using Prism 6 software. In case of unbalanced data, this software uses analysis of "unweighted means" to compare groups. Extra sum-of-squares *F*-test was performed for comparison of concentration-response curves and their 95% confidence intervals (CI). *P*-values < 0.05 were considered statistically significant. n represents the number of independent arteries tested or the number of cells measured. All rings were obtained from at least 3 different animals.

RESULTS

Regulation of Arterial Tone by DPO-1 Sensitive $K_V1.5$ Channels

First, we examined the role of $K_V1.5$ channels in the regulation of arterial tone by alpha1 adrenoceptor ($alpha_1$-AR) stimulation. In this set of experiments, we used the $K_V1.5$ channel blocker DPO-1 at concentrations assumed to be specific and potent for $K_V1.5$ channel inhibition (Stump et al., 2005; Lagrutta et al., 2006; Regan et al., 2006). In the presence of 1 μM DPO-1, mesenteric artery rings without PVAT [(−) PVAT] displayed similar contractions in response to phenylephrine (PE) compared to non-treated (−) PVAT control rings (**Figures 1A,B**). The 95% CI for EC_{50} of control and DPO-1 treated rings were 1.21–1.79 μM and 0.78–1.40 μM, respectively. The anti-contractile effects of PVAT were also unchanged by 1 μM DPO-1: the 95% CI for EC_{50} of control (+) PVAT and 1 μM DPO-1 (+) PVAT treated rings were 3.99–8.14 μM and 4.99–10.05 μM, respectively. To further confirm the results obtained with the $K_V1.5$ channel inhibitor,

we performed similar experiments using mesenteric artery rings from *Kcna5*$^{-/-}$ mice. PE induced vasocontractions in (−) PVAT *Kcna5*$^{-/-}$ rings were not different from those observed in (−) PVAT *Kcna5*$^{+/+}$ rings (**Figures 1C,D**). Similarly, we observed PE induced vasocontractions in (+) PVAT *Kcna5*$^{-/-}$ rings, which were not different from those observed in (+) PVAT *Kcna5*$^{+/+}$ rings (**Figures 1C,D**). The 95% CI for EC_{50} of (−) PVAT and (+) PVAT arteries isolated from *Kcna5*$^{-/-}$ mice were 0.81–1.30 μM and 4.94–7.79 μM, respectively. Data are summarized in **Table 1**. Experiments on rat mesenteric arteries showed similar results: Cumulative dose-response curves in response to serotonin (5-HT) were similar in vessel rings in the absence or presence of DPO-1 (**Figure 1E**). Together, the results suggest that K_V1.5 channels do not play a functionally relevant role in the control of arterial tone by PVAT, α1-AR and 5-HT agonists, in both mouse and rat mesenteric arteries.

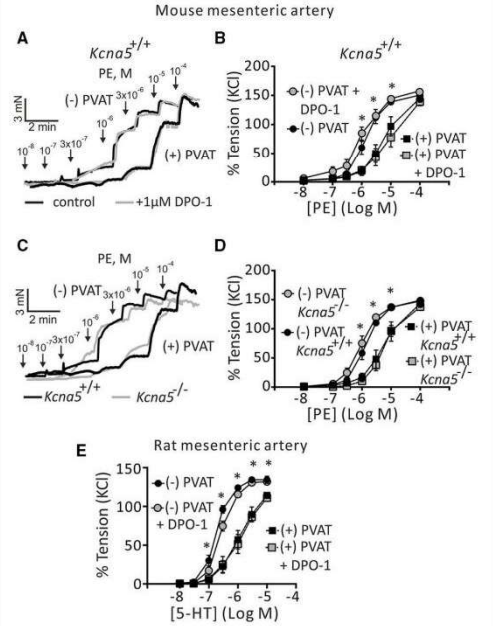

FIGURE 1 | Effects of pharmacological blockade and genetic deletion of K_V1.5 channels on regulation of arterial tone by perivascular adipose tissue (PVAT), phenylephrine (PE), and serotonin (5-HT). (A) Original traces showing the effects of 1 μM DPO-1 on PE-induced contractions in (−) PVAT and (+) PVAT mesenteric artery rings compared with control rings without DPO-1. **(B)** Concentration-response relationships for PE-induced contractions in *Kcna5*$^{+/+}$ (+) PVAT ($n = 8$) or (−) PVAT ($n = 9$) mesenteric arteries in the absence of DPO-1 or in *Kcna5*$^{+/+}$ (+) PVAT ($n = 8$) and (−) PVAT ($n = 9$) arteries after 30 min of pre-incubation with 1 μM DPO-1. *$p < 0.05$, for (−) PVAT vs. (+) PVAT or (−) PVAT + DPO-1 vs. (+) PVAT + DPO-1; repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test. **(C)** Original traces showing the effects of genetic deletion of *Kcna5* on PE-induced contractions in (−) PVAT and (+) PVAT mesenteric artery rings. **(D)** PE-induced contractions of (+) PVAT and (−) PVAT artery rings isolated from *Kcna5*$^{+/+}$ [(+) PVAT, $n = 8$; (−) PVAT, $n = 9$] and *Kcna5*$^{-/-}$ [(+) PVAT, $n = 12$; (−) PVAT, $n = 12$] mice. *$p < 0.05$, for (−) PVAT *Kcna5*$^{+/+}$ vs. (+) PVAT *Kcna5*$^{+/+}$ or (−) PVAT *Kcna5*$^{-/-}$ vs. (+) PVAT *Kcna5*$^{-/-}$. **(E)** Cumulative concentration-response relationships to 5-HT in (+) PVAT ($n = 7$) and (−) PVAT rat mesenteric arteries ($n = 6$) and the effects of 1 μM DPO-1 on (+) PVAT ($n = 6$) and (−) PVAT rat mesenteric arteries ($n = 8$). *$p < 0.05$, for (−) PVAT vs. (+) PVAT or (−) PVAT + DPO-1 vs. (+) PVAT + DPO-1; repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test.

DPO-1 Sensitive K_V Channels Distinct from K_V1.5 May Regulate Arterial Tone

Next, we studied putative non-K_V1.5 channel dependent effects by using higher concentrations of DPO-1. **Figure 2A** shows that 1 μM DPO-1 had no effects on basal tone of *Kcna5*$^{+/+}$ mesenteric artery rings with and without PVAT. Surprisingly, application of 10 μM DPO-1 resulted in a stable contraction of *Kcna5*$^{+/+}$ mesenteric arteries without but not with PVAT (**Figure 2B**). This effect remained stable over 30 min and was observed also on rings isolated from *Kcna5*$^{-/-}$ mice (**Figure 2C**). Thus, unexpectedly, inhibition of DPO-1 sensitive K_V channels distinct from K_V1.5 channels or other pathways could contribute to vascular tone in this preparation.

Effects of XE991 on K_V Currents, BK_{Ca} Currents and the Anti-Contractile Effects of PVAT

K_V currents were recorded in mesenteric artery VSMCs freshly isolated from *Kcna5*$^{+/+}$ and *Kcna5*$^{-/-}$ mice. We did not observe any difference between K_V current densities in *Kcna5*$^{+/+}$ and *Kcna5*$^{-/-}$ VSMCs. Moreover, K_V current inhibition by 30 μM XE991 was not different between *Kcna5*$^{+/+}$ and *Kcna5*$^{-/-}$ VSMCs (**Figures 3A,B**). 30 μM XE991 did not affect basal tone of mesenteric arteries prepared with or without PVAT (Figure S2).

In order to better understand the effects of XE991, we tested its actions on BK_{Ca} currents, potential mediators of the PVAT effect. VSMC *Kcna5*$^{+/+}$ BK_{Ca} currents were recorded in the absence and presence of 30 μM XE991. 100 nM iberiotoxin (a potent and highly selective BK_{Ca} channel inhibitor) was used as positive control. While 30 μM XE991 did not affect the BK_{Ca} current, iberiotoxin almost completely inhibited the BK_{Ca} current. These results are consistent with plasma membrane VSMC BK_{Ca} channel activity resistant to XE991 *in situ*, at concentrations up to 30 μM of XE991 (**Figures 3C,D**).

Additionally, we tested the effects of 30 μM and 0.3 μM XE991 on the paracrine effects of PVAT on arterial tone. We found that XE991 even at the low concentration abolished the anti-contractile effects of PVAT. Interestingly, application of 0.3 and 30 μM XE991 resulted in a similar reduction of the anti-contractile effects of PVAT (**Figures 4A,B**). The EC_{50} 95% CI values were 1.92–3.09 and 1.32–2.02 μM for (+) PVAT rings preincubated with 0.3 μM XE991 and 30 μM XE991, respectively; and 4.18–5.84 μM for (+) PVAT rings in the absence of XE991. (−) PVAT rings showed no difference, regardless of the absence or presence of 0.3 μM or 30 μM XE991. Data

TABLE 1 | EC_{50} and its confidence intervals.

Condition	Mouse background	Without PVAT			With PVAT		
		EC_{50} μM	95% confidence interval (CI)	*n*	EC_{50} μM	95% confidence intervals (CI)	*n*
Kcna5$^{+/+}$	129SVE-M	1.48	1.21–1.79	9	5.72	3.99–8.14	8
Kcna5 $^{-/-}$	129SVE-M	1.02	0.81–1.30	10	6.20	4.94–7.79	12
Kcna5$^{+/+}$ +1 μM DPO-1	129SVE-M	1.04	0.78–1.40	11	7.24	4.99–10.05	11
Control	C57BL/6	0.70	0.57–0.87	17	4.94	4.18–5.84	21
0.3 μM XE991	C57BL/6	0.48	0.30–0.75	5	2.43	1.92–3.09	13
30 μM XE991	C57BL/6	0.38	0.24–0.65	5	1.64	1.32–2.02	12

Data calculated from concentration-response curves to phenylephrine after normalization to maximum response from within each curve. For comparison of data and groups, see text and figures.

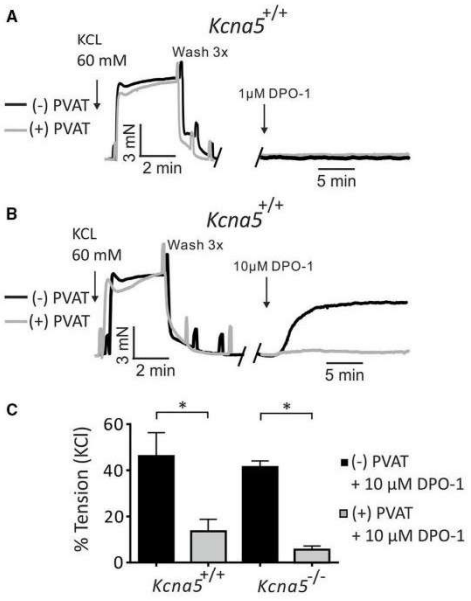

FIGURE 2 | Effects of 1 μM and 10 μM DPO-1 on basal arterial tone of mesenteric artery rings isolated from *Kcna5*$^{+/+}$ and *Kcna5* $^{-/-}$ mice. Original traces showing contraction of mesenteric (–) and (+) PVAT rings induced by 60 mmol/L KCl and by 1 μM (**A**) and 10 μM DPO-1 (**B**). (**C**) Vessel tension induced by 10 μM DPO-1. Tension is expressed as a percentage of KCl contractions. *Kcna5*$^{+/+}$ (–) PVAT, $n = 9$; (+) PVAT, $n = 13$. *Kcna5*$^{-/-}$ (–) PVAT, $n = 7$; (+) PVAT, $n = 9$. *$p < 0.05$, two-way ANOVA followed by Bonferroni *post hoc* test.

are presented in **Table 1**. Together, our data demonstrate that XE991 is a potent inhibitor of PVAT control of arterial tone at low concentrations similar to its potency of inhibiting K_V currents in VSMCs (Schleifenbaum et al., 2014). BK_{Ca} channels are however exempt from this inhibitory effect of XE991 (up to 30 μM).

DISCUSSION

Perivascular adipose tissue plays a potent anti-contractile role in the control of arterial tone along arterial segments of different vascular beds and species. The main findings of this study are threefold. First, XE991 inhibits the PVAT effect at nanomolar concentrations in mesenteric arteries of mice. Interestingly, similar concentrations were found in earlier studies to inhibit VSMC K_V currents (50% inhibition at 60 nM XE991) (Schleifenbaum et al., 2014). Second, *Kcna5*$^{-/-}$ mice exhibited normal VSMC K_V currents and arterial contractions in the absence and presence of PVAT, whose effects were insensitive to DPO-1. Third, the KCNQ channel blocker XE991 does not affect plasma membrane VSMC BK_{Ca} channels at concentrations, which inhibit the anti-contractile effects of PVAT. Together, the results of our current study implicate KCNQ-type K_V channels in the XE991-mediated inhibition of the PVAT effects. Simultaneously, we exclude BK_{Ca} as well as $K_V1.5$ channels as potential downstream candidates in this process.

$K_V1.5$ in Regulation of Arterial Tone

In recent studies, $K_V1.5$ channels have been shown to determine microvascular tone and the arteriolar response to vasoconstrictors in rat cerebral arteries (Chen et al., 2006; Fancher et al., 2015). Furthermore, $K_V1.5$ channels in the heart are essential in coupling myocardial blood flow to cardiac metabolism (Ohanyan et al., 2015). Moreover, hypertension is associated with altered expression of vascular $K_V1.5$ channels (Wang et al., 1997; Platoshyn et al., 2001; Cox and Rusch, 2002; Cox et al., 2008; Cidad et al., 2014). Therefore, $K_V1.5$ channels may represent interesting putative targets of PVAT and that raised the question of their potential involvement in the regulation of arterial tone by phenylephrine in mouse mesenteric arteries. Our results suggest that $K_V1.5$ channels are however not involved. In effect, the anti-contractile effects of PVAT were not different between *Kcna5*$^{-/-}$ and *Kcna5*$^{+/+}$ arteries. Additionally, the $K_V1.5$ channel inhibitor DPO-1 at 1 μM displayed no effect on vasocontractions in the absence and presence of PVAT. Notably, the mechanism of DPO-1 action ("open channel" blocker) might be a potential confounding factor, since $K_V1.5$ channels are activated at $V_{0.5}$ of −14 mV (Grissmer et al., 1994). However, the genetic approach had the

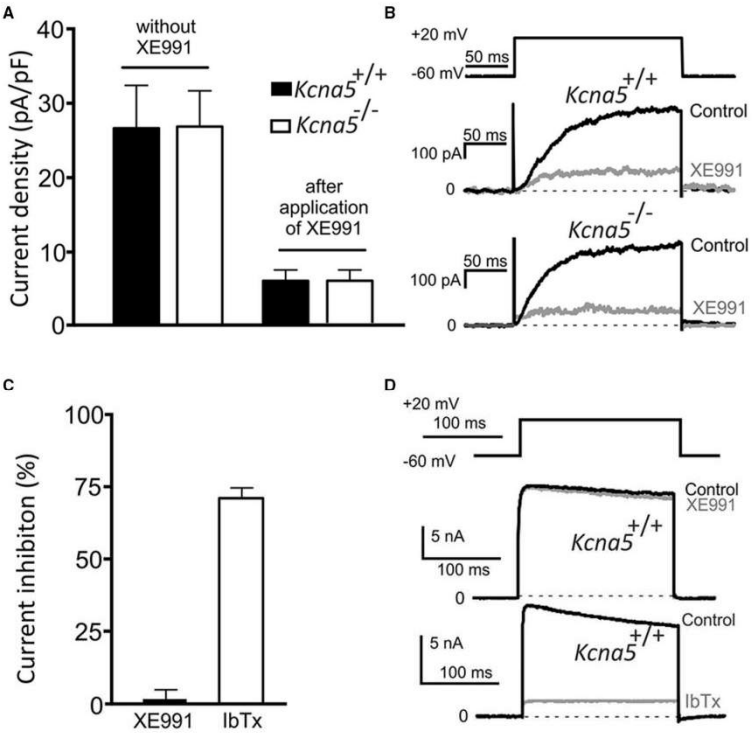

FIGURE 3 | Voltage dependent (K_V) K^+ currents and BK_{Ca} currents in freshly-isolated mesenteric artery vascular smooth muscle cells (VSMCs) from *Kcna5*$^{-/-}$ and *Kcna5*$^{+/+}$ mice. K_V currents before (−XE991) and after (+XE991) application of 30 μM XE991. Current densities of peak K_V currents **(A)** ($n = 6$ *Kcna5*$^{-/-}$ cells, $n = 10$ *Kcna5*$^{+/+}$ cells, before and after application of XE991, respectively); original recordings **(B)**. **(C)** Relative inhibition of peak BK_{Ca} current by iberiotoxin (IbTx) at 100 nM ($n = 5$) and XE991 at 30 μM ($n = 9$) in *Kcna5*$^{+/+}$ VSMCs, original recordings are represented in **(D)**.

advantage to study vascular effects in the absence of $K_V1.5$ channels avoiding possible confounders related to membrane potential-dependent drug mechanisms. Furthermore, we did not observe any significant differences in the K_V current density and inhibition by XE991 in *Kcna5*$^{-/-}$ and *Kcna5*$^{+/+}$ VSMCs. Therefore, we conclude that $K_V1.5$ channels have no apparent role in PVAT-dependent relaxation and are not the XE991 sensitive channels that contribute to this process. This conclusion is in line with our previous results obtained on cloned and heterologously expressed $K_V1.5$ alpha subunits in HEK293 cells (Schleifenbaum et al., 2014). In these experiments, 100 nM XE991 failed to block $K_V1.5$ currents. We also observed similar responses of arterial rings without PVAT to PE and 5-HT, regardless of genetic deletion of $K_V1.5$ alpha subunits or pharmacological blockade of $K_V1.5$ channels by DPO-1. To our knowledge, this is the first study to firmly establish that $K_V1.5$ channels are not involved in the regulation of arterial tone of systemic visceral arteries of mice and rats, at least in mesenteric arteries. Our conclusions substantiate the work of other groups, namely that patients with genetic mutations of *KCNA5* exhibit pulmonary arterial hypertension and arterial fibrillation but not systemic hypertension (Yang et al., 2009; Wipff et al., 2010; Machado et al., 2015). Together, our data questions the contribution of $K_V1.5$ channels in a number of small resistance arteries to peripheral arterial resistance. The findings are however not generalizable to all vascular beds as $K_V1.5$ channels were demonstrated to play important vasoregulatory functions in cerebral arteries and in *Gracilis* skeletal muscle arteries (Chen et al., 2006; Fancher et al., 2015).

DPO-1 Sensitive K^+ Channels and Pathways Distinct from $K_V1.5$ Involved in Regulation of Arterial Tone

DPO-1 was described as a specific $K_V1.5$ inhibitor at micromolar concentrations (Stump et al., 2005; Lagrutta et al., 2006; Regan et al., 2006). It exerts its inhibitory effects through binding

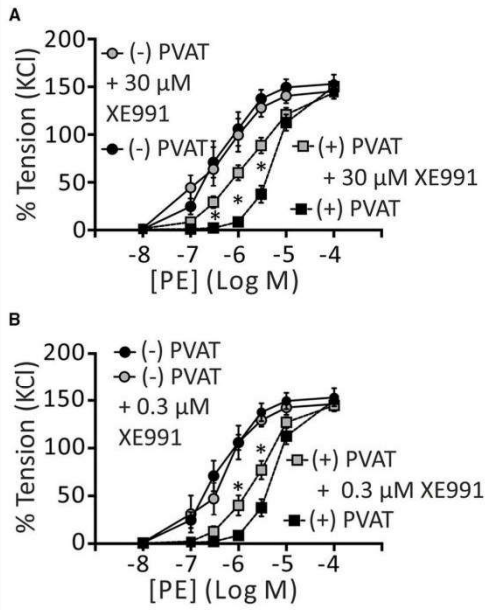

FIGURE 4 | Effects of XE991 on regulation of arterial tone by perivascular adipose tissue. Cumulative concentration-response curves to PE in the presence of 30 μM XE991 **(A)** and 0.3 μM XE991 **(B)**, expressed as a percentage of KCl contractions. (–) PVAT, $n = 17$; (+) PVAT, $n = 21$. (+) PVAT (+) 30 μM XE991, $n = 12$; (+) PVAT + 0.3 μM XE991, $n = 13$; (–) PVAT (+) 30 μM XE991, $n = 5$; (–) PVAT + 0.3 μM XE991, $n = 5$. *$p < 0.05$, repeated-measures two-way ANOVA, followed by Bonferroni *post hoc* test * in **(A)**, (+) PVAT vs. (+) PVAT + 30 μM XE991. *In **(B)**, (+) PVAT vs. (+) PVAT + 0.3 μM XE991. Experiments were performed on mouse mesenteric arteries.

with several key residues in the S5- pore loop-S6 domains, thus resulting in blockade of the open state of the $K_V1.5$ channel (Karczewski et al., 2009; Du et al., 2010). Other studies have suggested a DPO-1 preference for vascular $K_V1.5$ channels, though with limited selectivity (Fancher et al., 2015). In this study, DPO-1 (1–10 μM) inhibited the outward K^+ current in arterial smooth muscle cells from wild-type (*Kcna5*$^{+/+}$) mice and mice lacking the *Kcna5* gene; however, the inhibitory effect was much greater in cells from *Kcna5*$^{+/+}$ mice (Fancher et al., 2015). Subsequently, our data in **Figure 2** suggests that 10 μM DPO-1 induces contractions by inhibiting channels distinct from $K_V1.5$ channels. Those channels appear to be important for the regulation of resting arterial tone. Interestingly, DPO-1 is able to block $K_V1.3$ channel currents at EC_{50} of 3.1 μM in human T cells (Zhao et al., 2013). *Kcna3* mRNA expression is also observed in mouse mesenteric arteries (Fountain et al., 2004; Cidad et al., 2014). Although the ability of $K_V1.5$ and $K_V1.3$ to form heteromers (Kv1.5/Kv1.3) (Villalonga et al., 2007) impede the study of their specific roles in native tissues *in vivo*, it is intriguing to speculate that $K_V1.3$ channels could represent a putative target of PVAT regulation of arterial tone. Future studies are necessary to clarify their role.

Effects of XE991 on K_V channels, BK_{Ca} Channels and Paracrine PVAT Effects

In the mouse mesenteric arteries, *Kcnq1, Kcnq4,* and *Kcnq5* expression was demonstrated at the mRNA level (Yeung et al., 2007), whereas mRNA expression of *Kcnq2, Kcnq3* was not detectable or only at borderline low levels (Yeung et al., 2007; Schleifenbaum et al., 2014). We previously suggested that KCNQ type K_V channels are key players in the paracrine role for periadventitial adipose tissue in the regulation of arterial tone (Schleifenbaum et al., 2010; Zavaritskaya et al., 2013); based on mRNA expression levels (see above), KCNQ1, KCNQ4, and/or KCNQ5 channels are likely candidates. This suggestion is also based on the ability of 30 μM XE991 (pan KCNQ blocker) and 2 mmol/L 4-aminopyridine (pan K_V blocker) to block the anti-contractile effects of PVAT. Interestingly, KCNQ channel openers normalized reduced anti-contractile effects of PVAT in a rat model of hypertension (Zavaritskaya et al., 2013), which suggests therapeutic perspectives of KCNQ targeting in cardiovascular disease. It is thus imperative to better understand the actions of these compounds on the vasculature. In order to obtain more information about the potency of XE991 inhibition on PVAT-mediated anti-contractility, we performed experiments with a 100x lower concentration of XE991. The data show that this considerably reduced concentration still exerted an inhibitory impact on PVAT regulation of arterial tone (**Figures 4A,B**), while basal tone was unaffected in rings (+) or (−) PVAT (Figure S2). The incomplete inhibition observed, however suggests the possible involvement of additional K^+ channels in this process. Furthermore, our data demonstrates that VSMC plasma membrane BK_{Ca} channels are not involved in the effects of XE991 on PVAT regulation, since XE991 does not inhibit BK_{Ca} currents (this study). This is in line with our previous findings indicating that the paracrine effects of PVAT on arterial tone are normal in the presence of BK_{Ca} channel blockers or in arteries that lack BK_{Ca} beta1 channel subunits (Fésüs et al., 2007; Zavaritskaya et al., 2013).

Previous studies examined the ability of XE991 to inhibit heterologously expressed $K_V1.5$ alpha subunits. We found that 100 nM and 30 μM XE991 was unable to block monotetrameric $K_V1.5$ channels heterologously expressed in HEK293 or CHO cells (Zavaritskaya et al., 2013; Schleifenbaum et al., 2014). Interestingly, Zhong et al. found a small (∼20% at (+) 5 mV) inhibiting effect for 10 μM XE991 on heterologously expressed heterotetrameric $K_V1.2/K_V1.5$ and $K_V2.1/K_V9.3$ channel subunits (Zhong et al., 2010). The block of $K_V1.2/K_V1.5$ channels was voltage dependent, and evident only at voltages positive to -15 mV. Our present study contributes to the debate about the importance of accessory subunits for determining the pharmacological properties of vascular K^+ channels *in vivo*. Since regulatory $K_V$beta1.3 subunits can decrease the sensitivity of $K_V1.5$ channels to pharmacological inhibitors such as DPO-1 (Gonzalez et al., 2002; Arias et al.,

2007; Du et al., 2010), one could argue that DPO-1 is not a reliable tool to study $K_V1.5$ channels in native tissues. However, we believe that our pharmacological approach in combination with the *Kcna5*$^{-/-}$ mouse model firmly demonstrates that the XE991 sensitive regulation of arterial tone by PVAT regulation does not involve native vascular $K_V1.5$ channels.

CONCLUSION

In conclusion, our results demonstrate that $K_V1.5$ channels are not involved in the control of mesenteric arterial tone and its regulation by PVAT in mouse and rat mesenteric arteries. The nature of the 10 μM DPO-1 sensitive component is unclear, but is most likely related to non-specificity of this drug, for example in targeting vascular $K_V1.3$ and/or KCNQ channels *in situ*. Importantly, the inhibitory effects of XE991 on PVAT vasorelaxation are rather related to inhibition of KCNQ-type K_V channels than BK_{Ca} channels. These data unequivocally substantiate the hypothesis of different targets of perivascular relaxing factor(s), which employ distinct mechanisms to mediate an anti-contractile effect. Further studies should focus on the enhancement of these relaxing factors, as these will be beneficial for patients with cardiovascular diseases.

AUTHOR CONTRIBUTIONS

All authors planned and designed the experimental studies. DT and NW performed the wire myography experiments. MK and JT performed the electrophysiological experiments. DT and MG drafted the article, and all authors contributed to its completion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00335

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Publication II

Publication #3: Cystathionine γ-Lyase–Produced Hydrogen Sulfide Controls Endothelial NO Bioavailability and Blood Pressure.

István András Szijártó, Lajos Markó, Milos R. Filipovic, Jan Lj Miljkovic, Christoph Tabeling, Dmitry Tsvetkov, **Ning Wang**, Luiza A. Rabelo, Martin Witzenrath, André Diedrich, Jens Tank, Noriyuki Akahoshi, Shotaro Kamata, Isao Ishii, Maik Gollasch. Cystathionine γ-Lyase–Produced Hydrogen Sulfide Controls Endothelial NO Bioavailability and Blood Pressure. Hypertension. 2018 Jun; 71(6):1210-1217.
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My publications do not appear in the electronic version of my paper for reasons of data protection.

Publication III

9. Curriculum Vitae

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10. Complete list of publications

Original Publishing

Ning Wang, Artur Kuczmanski, Galyna Dubrovska and Maik Gollasch. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Front. Physiol., 2018 May; 9: 583. doi: 10.3389/fphys.2018.00583.

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Review

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Abstracts/Poster

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