Physiological Research Pre-Press Article

Diabetes converts arterial regulation by perivascular adipose tissue from relaxation into H_2O_2-mediated contraction

R. Emilova[1#], D. Z. Dimitrova[2#], M. Mladenov[3], N. Hadzi-Petrushev[3], T. Daneva[4], P. Padeshki[5], R. Schubert[6], M. Chichova[7], L. Lubomirov[8], D. Simeonovska-Nikolova[7], H. Gagov[7]

[1]Cytogenetics Laboratory, University Paediatric Hospital, Medical University, Sofia, Bulgaria, [2]Bulgarian Academy of Sciences, Institute of Biophysics and Biomedical Engineering, Sofia, Bulgaria, [3]University of Skopje Sts. Cyril and Methodius, Faculty of Natural Sciences and Mathematics, Institute of Biology, Skopje, Macedonia, [4]Bulgarian Academy of Sciences, Institute of Biology and Immunology of Reproduction, Sofia, Bulgaria, [5]National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria, [6]Ruprecht-Karls-University Heidelberg, Medical Faculty Mannheim, Department of Cardiovascular Physiology, Mannheim, Germany, [7]Sofia University St. Kliment Ohridski, Faculty of Biology, Sofia, Bulgaria, [8]Institute of Vegetative Physiology, University of Cologne, Germany.

[#] R.E. and D.Z.D. contributed equally to this work as first authors.

Corresponding author

H. Gagov, Faculty of Biology, Sofia University St. Kliment Ohridski, 8 Dragan Tsankov blvd, 1164 Sofia, Bulgaria. E-mail: hgagov@abv.bg

Summary

This study aims to reveal the reason for the increased force of 5-hydroxytryptamine-induced contraction of endothelium-denuded skeletal muscle arteries of diabetic rats in the presence of perivascular adipose tissue (PVAT). Our data on rat gracilis arteries show that i) PVAT of skeletal muscle arteries of healthy and diabetic rats releases hydrogen peroxide (H_2O_2), ii) higher concentrations of 5-hydroxytryptamine increase the production of H_2O_2 in PVAT; iii) an enhanced PVAT production of H_2O_2 is the main, if not the only, reason for the

sensitization of arterial contraction to 5-hydroxytriptamine-induced contraction in diabetes and iv) endothelium antagonizes the effect of PVAT-derived H_2O_2.

Keywords

Artery * Regulation * Reactive oxygen species * Adipose tissue * Serotonin

Short title Perivascular adipose tissue contracts by H_2O_2

Introduction

Perivascular adipose tissue (PVAT) encloses most of the systemic blood vessels (Cinti 2002). It has been demonstrated that PVAT releases many paracrine vasodilators and vasoconstrictors (Gollasch 2012, Orinovo 2015). PVAT also regulates smooth muscle cell proliferation and migration, trans-endothelial migration of monocytes, apoptosis of neutrophils, the inflammatory reaction of pro- or anti-inflammatory adipokines and cytokines (Rajsheker *et al.* 2010), pro- and anti-oxidant mechanisms (Gil-Ortega *et al.* 2014) and others. Hydrogen sulfide (H_2S) has a significant impact on the predominantly relaxing influence of PVAT on arteries isolated from healthy animals, including rat gracilis artery (a. gracilis) (Zavaritskaya *et al.* 2013, Schleifenbaum *et al.* 2010). Thus, H_2S seems to be an important, if not the main adipocyte-derived relaxing factor (ADRF), the physiological activator of voltage gated potassium channels of the K_{v7} (KCNQ channels) in arteries (Gollasch 2012, Zavaritskaya *et al.* 2013, Schleifenbaum *et al.* 2010, Köhn *et al.* 2012). The vasodilatory regulation by PVAT is lost or reversed to constriction by diabetes (Emilova *et al.* 2015, Houben *et al.* 2012), obesity, metabolic syndrome (Greenstein *et al.* 2009), and cardiovascular disease (Houben *et al.* 2012). It is obvious that pathologic conditions attenuate

the PVAT-evoked vasodilatory regulation of vascular tone, but the mediator or mediators of these PVAT effects, in particular the PVAT-dependent constriction in diabetes, are still to be investigated (Emilova *et al.* 2015, Houben *et al.* 2012).

Diabetes influences negatively the entire vascular wall to a different extent - mostly the endothelium (Greenstein *et al.* 2009), and to a lesser extent - the smooth muscle layer (Ding and Triggle 2010), while changes in sympathetic innervation vary from minor in short-term diabetes (Damon 2011) to severe neuroaxonal dystrophy, an axonopathy of terminal axons and synapses in long-term type 1 diabetes (Schmidt *et al.* 2004). Reactive oxygen species (ROS) participate in diabetic vascular dysfunction (Nicolls *et al.* 2007), in particular in the impaired regulation of arterial tone (Erdei *et al.* 2007) and in agonist-induced contractions (Kobayashi and Kamata 2002). Hydrogen peroxide (H_2O_2) is a relatively stable, plasma membrane permeable, and a non-radical form of ROS possessing moderate oxidant activity (Rodriguez-Martinez *et al.* 1998). Several studies propose an important role of H_2O_2 in the regulation of arterial responses – that of both constriction and dilation - in physiological and pathophysiological conditions (Erdei *et al.* 2007). In rats with STZ-induced diabetes, the elevated production of H_2O_2 mediates endothelium-dependent contractions of the femoral artery (Shi *et al.,* 2007), which leads to a selective loss of endothelium-dependent vasodilation of the thoracic aorta (Kobayashi and Kamata 2002) and modulates angiotensin-II-induced contractions of mesenteric arteries (Chin *et al.* 2007). Therefore, the aim of our study is to test the hypothesis that H_2O_2 is the mediator of the reversed PVAT regulation of skeletal muscle artery contraction in STZ-treated diabetic rats.

Methods

All experimental procedures were conducted in accordance with the Guiding Principles for Care and Use of Laboratory Animals approved by the Bulgarian Center for Bioethics and are in accordance with the International Guiding Principles for Biomedical Research Involving Animals. In this study, native and endothelium-denuded ring preparations of rat a. gracilis with or without PVAT were used. The regulatory role of PVAT was explored under three different conditions – in 5.5 mM glucose-containing physiological salt solution (PSS, healthy rats, control conditions), in 20 mM glucose-PSS (healthy rats, supra-physiological glucose concentration) and in the same 20 mM glucose-PSS for artery preparations from diabetic rats.

Diabetes was induced in male Wistar rats (8-10 weeks old, supplied by ERBOJ-Slivnitza, Bulgaria) by a single intraperitoneal injection of STZ (80 mg/kg body weight). A STZ application solution based on a citrate buffer (pH=4.5) was prepared immediately prior to treatment. Blood glucose levels were measured by a glucometer (Rapid Diagnostic PVT Ltd., Delhi, India) at the end of the first week after the induction of diabetes and again just prior to the experiments. Animals with blood glucose levels above 20 mM (360 mg/dl) were considered diabetic.

Male rats were used six to nine weeks after STZ treatment. The gracilis arteries were dissected and transferred to cold PSS (4^{o}C). The PVAT and connective tissue of these preparations were either removed, or left intact. Otherwise, all artery preparations were treated according to the same protocol. The effect of PVAT signaling is directed to the artery smooth muscle layer (Emilova *et al.* 2015). For this reason, we used mainly endothelium-denuded a. gracilis preparations. The presence or absence of the endothelium was confirmed by the appearance or lack of relaxation in 10^{-5} M acetylcholine of 60 mM KCl-contracted arteries. Isometric vessel contractions were measured with a Dual Vessel Myograph (DMT 410M, Aarhus, Denmark). The organ bath was filled with PSS containing (in mM): 119 NaCl,

4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 Mg_2SO_4, 1.6 $CaCl_2$, 5.5 or 20 glucose. The bath solution was continuously exposed to a gas mixture of 95% O_2 and 5% CO_2, and kept at 37ºC, pH=7.4. Arterial contractions are expressed as the percentage of the steady-state tension (100%) obtained with isotonic PSS containing 60 mM KCl, where 54 mM of NaCl were replaced with 54 mM KCl. Increasing concentrations of 5-hydroxytryptamine (5-HT) from 10^{-10} to 10^{-5} M were applied to induce gradual constriction of the circular 2 mm long artery rings. In experiments with catalase, three cumulative applications of 5-HT (10^{-10} – 10^{-5} M) were used. First and third 5-HT applications were control measurements of artery tensions in catalase-free PSS. Before starting the second 5-HT addition, the vascular rings were incubated with catalase or vehicle (H_2O) for 15 minutes. Forces of contractions are presented as a percentage of 60 mM KCl-elicited tensions. Data obtained in the presence of catalase were compared with these from the first 5-HT application. All drugs were added into the organ bath. The salts and drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The H_2O_2 release was determined according to the method of Pick and Keisari (1980). In brief, to measure the effect of 5-HT at concentrations of 10^{-7} M or 10^{-5} M on H_2O_2 release, PVAT was dissected from the region of the femoral artery (a. femoralis) including the a. gracilis from control and diabetic animals and incubated with PSS, containing phenol red and horseradish peroxidase (PRS), for 30 min at 37ºC. At the end of the incubation, the supernatants were centrifuged for 5 min at 200 g, at 4ºC. NaOH (1 M) was then added and the extinction of the samples was determined at 600 nm against reference samples containing PRS and NaOH without PVAT. The H_2O_2 content was calculated using a standard curve, with the same batch and concentration of PRS and NaOH to which H_2O_2 was added at known final concentrations. All experiments were performed in duplicate and the results were expressed as nM H_2O_2/min/g PVAT.

The data obtained from the isometric contraction measurements were analysed using

the statistical software SPSS 16.0. Usually the number of rats (N) is half the number of experiments (n). In some series, the N/n ratio is higher due to failed measurements that required additional experiments with vessels from other animals. The results are given as means±S.E.M. from six or more separate experiments. The statistical significance was determined using the Student t-test for unpaired samples to assess the significance between two groups. For H_2O_2 assay data, one-way ANOVA Kruskal-Wallis tests, followed by Dunn's multiple comparison post hoc tests were performed. Differences were considered significant when $p < 0.05$. GraphPad Prism6 was used to calculate pEC_{50} of 5-HT contractions.

Results

At the time of treatment the body weights of control and STZ-treated rats were comparable (173±9 g and 178±6 g). Two weeks after STZ treatment, PVAT was transformed from its native, multiple pearl-like structures into a thin, brown-colored tunic that seemed functionally inactive because its presence did not affect the 5-HT-induced contraction in diabetic a. gracilis (data not shown). After about 4 weeks, the normal appearance of PVAT was recovered and this was the reason to wait for more than a month after STZ treatment before starting the experiments with diabetic animals. Six to nine weeks after STZ-treatment the rats gained weight that was significantly lower (228±9 g, $p < 0.05$) compared to healthy controls (289±12 g). STZ-diabetic animals developed polyuria and a specific appearance of their fur - separate bristle hairs.

Data for PVAT relaxations (significant at 10^{-8}, 10^{-7} and 10^{-6} M) of control a. gracilis and PVAT-mediated contractions (significant at 10^{-6} and 10^{-5} M) of diabetic vessels are presented in Table 1. The presence of PVAT did not change E_{max} of control artery

preparations, estimated as the force of contractions obtained in the presence of 10^{-5} M 5-HT, while E_{max} significantly increased in PVAT-containing diabetic vessels.

Figure 1 presents data on the effect of catalase (1000 U/ml) on 5-HT-induced contractions obtained from endothelium-denuded a. gracilis preparations without PVAT from healthy rats in the presence of physiological (Fig. 1a) and supra-physiological glucose concentrations (Fig. 1b), and from diabetic rats (Fig. 1c). Artery preparations from animals with diabetes developed weaker contractions at 10^{-7} M 5-HT in the presence of catalase compared to its absence (Fig. 1c, $p < 0.01$). In the presence of catalase, 5-HT-induced contractions of vessels from healthy animals kept in normal and supra-physiological glucose concentrations, and from diabetic rats were the same (Fig. 1d).

In the presence of PVAT, the addition of catalase did not affect the concentration dependence of 5-HT-induced force of contraction of endothelium-denuded preparations from healthy animals, neither in physiological nor in supra-physiological glucose concentrations (Fig. 2a, b). In contrast, in diabetic a. gracilis rings with PVAT, the presence of catalase strongly decreased the force of contraction at all tested concentrations of 5-HT above 10^{-8} M (Fig. 2c). Thus, the catalase-induced difference in the force of contraction was much more pronounced in PVAT-containing diabetic arteries (Fig. 2c) compared to PVAT-free ones. In the same artery preparations with preserved endothelium this effect was not significant (Fig. 2c, triangles). Catalase also decreased significantly the contractions of PVAT-free preparations from STZ-treated rats but only at 10^{-7} M 5-HT (121.2±6.5% for PVAT-free versus 70.1%±9.7% for PVAT-controls, $p < 0.01$, Fig 1c). This increase of the force of contraction of PVAT-free vessels probably depends on H_2O_2 produced in the smooth muscle layer of a. gracilis. The presence of catalase eliminated the difference in the force of contractions of preparations from healthy and diabetic animals (Fig. 1d and 2d). In the absence of catalase, the calculated pEC_{50} of 5-HT-induced contractions in healthy a. gracilis

preparations with PVAT incubated in 5.5 mM or 20 mM glucose-containing PSS is lower in vessels with PVAT compared to PVAT-free vessels (Table 2). On the other hand, pEC_{50} of 5-HT-induced contractions of diabetic vessels free of PVAT is not different from that of vessels with PVAT. The addition of catalase eliminates the difference of the pEC_{50} in supra-physiological glucose concentration. Catalase decreases the pEC_{50} of 5-HT-induced force of contractions of diabetic a. gracilis with or without PVAT. In the presence of PVAT, however, this effect was much more pronounced (Table 2, last row).

The H_2O_2 production by PVAT in the presence of 10^{-7} or 10^{-5} M 5-HT was studied using PVAT isolated from the region of a. gracilis and a. femoralis of healthy and diabetic rats (Fig. 3a). Only the amount of PVAT of the a. gracilis area was very small; therefore, we also used PVAT from the adjacent a. femoralis. In healthy animals, the production of H_2O_2 by PVAT was higher at 10^{-5} M 5-HT compared to 10^{-7} M 5-HT ($p < 0.01$). PVAT isolated from vessels of diabetic rats also released more H_2O_2 at 10^{-5} M 5-HT compared to 10^{-7} M 5-HT ($p < 0.05$). The amount of H_2O_2 released from diabetic PVAT was larger compared to that of healthy PVAT at both 5-HT concentrations studied (for both 5-HT concentrations, $p < 0.001$). The observed effect of catalase (Fig. 2c) suggested an involvement of H_2O_2 in the contraction-enhancing effect of PVAT in diabetic animals. Therefore, we tested the influence of externally applied H_2O_2 on vessel preparations without PVAT from healthy and diabetic rats. In the presence of 60 mM KCl-containing PSS, which was used to induce a moderate contraction of the a. gracilis, the subsequent addition of increasing concentrations of H_2O_2 from $1*10^{-6}$ M to $33*10^{-6}$ M gradually enhanced their force of contraction (Fig. 3b, $p < 0.001$ and $n = 6$ for time control versus H_2O_2 in preparations from healthy animals and $p < 0.01$, $n = 6$ for time control versus H_2O_2 in preparations from diabetic rats). This effect was reversed into a decreased force of contraction at concentrations of 10^{-4} M and $3.3*10^{-4}$ M H_2O_2 (Fig. 3b, $p < 0.001$). Thus, a small concentration step from 33 µM to 100 µM is sufficient to change

the effect of H_2O_2 dramatically. Importantly, the relaxing potency of H_2O_2 above $33*10^{-6}$ M was smaller in diabetic as compared to healthy arteries ($p < 0.001$, $n = 6$), which suggests a suppression of relaxing H_2O_2 signaling in diabetic arteries.

Discussion

The novel findings of this study are: i) PVAT of skeletal muscle arteries of healthy and diabetic rats releases H_2O_2, ii) 5-HT increases the production of H_2O_2 in PVAT and iii) an enhanced H_2O_2 formation by PVAT is the main, if not the only, reason for the sensitization of a. gracilis to 5-HT in contractions.

A. gracilis preparations with PVAT from diabetic rats contract more strongly in the presence of moderate and high concentrations of 5-HT compared to those from healthy animals. This finding could be explained with several mechanisms – a smaller release of dilating mediator from PVAT, which most probably is H_2S (Zavaritskaya *et al.* 2013, Emilova *et al.* 2013), an enhanced vasoconstrictor release from PVAT, or both of them in vessels from diabetic animals. The first possibility – a loss or a vast decrease of PVAT-derived H_2S as the main reason for the observed phenomenon was not experimentally supported. The addition of DL-propargyl glycine, a selective inhibitor of cystathionine gamma-lyase, the main producer of H_2S in vascular walls, decreased contractions of a. gracilis preparations from diabetic rats in the presence of moderate and higher 5-HT concentrations (Emilova *et al.* 2015). Thus, the data suggest that a contractile mediator is responsible for the observed PVAT-dependent sensitization of diabetic a. gracilis contractions.

The relation of contractions and H_2S production in PVAT of diabetic a. gracilis (Emilova *et al.* 2015) and the intrinsic imbalance between pro- and anti-oxidant mechanisms (Gil-Ortega *et al.* 2014) leading to oxidative stress in diabetes (Nicolls *et al.* 2007) suggest

that PVAT may release H_2O_2 as a vasoconstrictor. H_2S can influence the bio-availability of H_2O_2. H_2S inhibits H_2O_2-mediated mitochondrial dysfunction by increasing the expression levels of antioxidant enzymes (Wen *et al.* 2013, Kimura 2014), by recovering glutathione level decreased by oxidative stress, by decreasing Ca^{2+} influx and by a ROS scavenging effect (Kimura 2014). The participation of adventitial H_2O_2 in vasoconstriction was tested using catalase. Catalase converts H_2O_2 into water and O_2, and thus eliminates the paracrine effects of H_2O_2. In the presence of catalase 5-HT-induced contractions of PVAT-containing artery preparations from diabetic rats are significantly suppressed if compared to the same reactions in enzyme-free conditions. Catalase eliminates the difference of concentration-dependent 5-HT contractions of PVAT-containing a. gracilis preparations between diabetic and healthy rats. The presence of catalase decreases the pEC_{50} of 5-HT-induced contractions in both PVAT-free and PVAT-containing vessels from healthy animals. Additionally, catalase creates a significant difference between these two groups due to a moderate decrease of pEC_{50} in PVAT-containing preparations and a vast decrease of pEC_{50} in PVAT-free vessels. The effects of catalase on PVAT-free arteries suggest that some H_2O_2 could be produced in the smooth muscle layer of a. gracilis. The vigorous decrease of E_{max} and pEC_{50} in PVAT-containing preparations caused by catalase suggests that PVAT dominates as a producer of the mediator sensitizing the diabetic vascular bed to 5-HT. Different pEC_{50} values of diabetic vessels with PVAT and without PVAT, both kept in catalase-containing PSS, suggest the release of a vasodilator from PVAT. Its physiological influence, however, is concealed by H_2O_2.

This hypothesis for the important role of PVAT-derived H_2O_2 as a signal molecule in diabetes is supported by the previously established increased activity of NADPH oxidases in vascular walls (which are membrane-bound enzymes generating H_2O_2 in hypoxia, atherosclerosis, vascular injury and experimental diabetes), as well as by many pro- and anti-

inflammatory factors, hormones and other mediators (Lassègue *et al.* 2012). NADPH oxidases and mitochondrial electron transport chains are the major routes for ROS production in a wide variety of cells, including adipocytes (Morrow 2003, Rolo and Palmeira 2006). Diabetes increases the formation of superoxide in mitochondria (Rolo and Palmeira 2006), which together with increased infiltration of inflammatory cells (Surmi and Hasti 2008) may cause the observed significant elevation of H_2O_2 production in PVAT.

Application of H_2O_2 leads to two opposite effects on rat a. gracilis: lower concentrations (1-33 μM) induce gradual constrictions and concentrations above 33 μM – considerable relaxations of control and very large relaxations of diabetic vessels. It is known that H_2O_2 signaling can either enhance or suppress vascular contractility. Thus, in endothelium-denuded arterioles from type 2 diabetic mice, exogenously administered H_2O_2 evokes constriction by stimulating the synthesis of thromboxane A_2 in the vascular wall, while the same vessels from healthy animals dilate (Erdei *et al.* 2007). The increased production of constrictor prostaglandins in smooth muscle cells is mainly due to cytosolic phospholipase A_2 activation and enhanced expression of cyclooxygenase-2 (Korbecki *et al.* 2013). In our experiments, the presence of catalase decreased the force of contraction of endothelium-denuded a. gracilis. This result suggests that PVAT and the smooth muscle layer release H_2O_2 in amounts that are below the transition concentration between 33 μM and 100 μM necessary to induce vasodilation. Similarly, Shi et al. (2007) report that application of 30 μM exogenous H_2O_2 induces maximal contraction of endothelium-denuded femoral artery.

In conclusion, H_2O_2 is an important PVAT-derived vasoconstrictor of a. gracilis of rats with STZ-induced diabetes, which transforms the adventitial modulation of 5-HT-induced contractions from predominantly relaxing into constrictive. This regulation, however, is minimized by vascular endothelium. Thus, diabetes seems to convert the regulatory role of PVAT from protecting to pathogenic by inducing a pro-inflammatory phenotype of PVAT

similarly to atherosclerosis and environmental factors such as a high-fat diet and tobacco smoke (Omar *et al.* 2014). Figure 3c presents a summary of PVAT common mediators and their paracrine regulations of skeletal muscle a. gracilis isolated from healthy (Zavaritskaya *et al.* 2013, Schleifenbaum *et al.* 2010, Emilova *et al.* 2015) and diabetic rats (this study).

Acknowledgements

This study was supported by Sofia University St. Kliment Ohridski, Sofia, Bulgaria, Projects 24/2013 and 20/2015, and by Ministry of Education and Science, Bulgaria, Project BG 051PO001-3.3.05-0001, No. 2-680/12.07.2013.

Conflict of Interest

There is no conflict of interest.

References

CHIN LC, ACHIKE FI, MUSTAFA MR: Hydrogen peroxide modulates angiotensin II-induced contraction of mesenteric arteries from streptozotocin-induced diabetic rats. *Vascul Pharmacol* **46**: 223-228, 2007.

CINTI S: Adipocyte differentiation and transdifferentiation: plasticity of the adipose organ. *J Endocrinol Invest* **25**: 823–835, 2002.

DAMON DH: Vascular-dependent effects of elevated glucose on postganglionic sympathetic neurons. *Am J Physiol Heart Circ Physiol* **300**: H1386-H1392, 2011.

DEBRECZENI B, GARA E, VERESH Z, MARKI A, RACZ A, MATICS R, HAMAR J, KOLLER A: Hydrogen peroxide via thromboxane A_2 receptors mediates myogenic response of small skeletal muscle veins in rats. *Clin Hemorheol Microcirc* **54**: 393-407, 2013.

DING H, TRIGGLE CR: Endothelial dysfunction in diabetes: multiple targets for treatment. *Pflugers Arch* **459**: 977-994, 2010.

EMILOVA R, DIMITROVA D, GEORGIEV V, DANEVA T, GAGOV H: Cystathionine gamma–lyase as a regulator of resistance artery contraction under normal and hyperglycemic conditions. *Bulg J Agric Sci* **19**: 175–177, 2013.

EMILOVA R, DIMITROVA D, MLADENOV M, DANEVA T, SCHUBERT R, GAGOV H: Cystathionine gamma-lyase of perivascular adipose tissue with reversed regulatory effect in diabetic rat artery. *Biotechnol Biotechnol Eq* **29**: 147-151, 2015.

ERDEI N, BAGI Z, EDES I, KALEY G, KOLLER A: H_2O_2 increases production of constrictor prostaglandins in smooth muscle leading to enhanced arteriolar tone in Type 2 diabetic mice. *Am J Physiol Heart Circ Physiol* **292**: H649-H656, 2007.

GIL-ORTEGA M, CONDEZO-HOYOS L, GARCÍA-PRIETO CF, ARRIBAS SM, GONZÁLEZ MC, ARANGUEZ I, RUIZ-GAYO M, SOMOZA B, FERNÁNDEZ-ALFONSO MS: Imbalance between pro and anti-oxidant mechanisms in perivascular adipose tissue aggravates long-term high-fat diet-derived endothelial dysfunction. *PLoS One* **9**: e95312, 2014.

GOLLASCH M: Vasodilator signals from perivascular adipose tissue. *Br J Pharmacol* **165**: 633–642, 2012.

GREENSTEIN AS, KHAVANDI K, WITHERS SB, SONOYAMA K, CLANCY O, JEZIORSKA M, LAING I, YATES AP, PEMBERTON PW, MALIK RA, HEAGERTY AM: Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* **119**: 1661–1670, 2009.

HOUBEN AJ, ERINGA EC, JONK AM, SERNE EH, SMULDERS YM, STEHOUWER CD: Perivascular Fat and the Microcirculation: Relevance to Insulin Resistance, Diabetes, and Cardiovascular Disease. *Curr Cardiovasc Risk Rep* **6**: 80-90, 2012.

KIMURA H: Production and physiological effects of hydrogen sulfide. *Antioxid Redox Signal* 20: 783-793, 2014.

KOBAYASHI T, KAMATA K: Modulation by hydrogen peroxide of noradrenaline-induced contraction in aorta from streptozotocin-induced diabetic rat. *Eur J Pharmacol* **441**: 83–89, 2002.

KORBECKI J, BARANOWSKA-BOSIACKA I, GUTOWSKA I, CHLUBEK D: The effect of reactive oxygen species on the synthesis of prostanoids from arachidonic acid. *J Physiol Pharmacol* **64**: 409-421, 2013.

KÖHN C, SCHLEIFENBAUM J, SZIJÁRTÓ IA, MARKÓ L, DUBROVSKA G, HUANG Y, GOLLASCH M: Differential effects of cystathionine-γ-lyase-dependent vasodilatory H_2S in periadventitial vasoregulation of rat and mouse aortas. *PLoS One* **7**: e41951, 2012.

LASSÈGUE B, SAN MARTÍN A, GRIENDLING KK: Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res* **110**: 1364-1390, 2012.

MORROW J: Is a oxidative stress a connection between obesity and atherosclerosis. *Arterioscler Tromb Vasc Biol* **23**: 368–370, 2003.

OMAR A, CHATTERJEE TK, TANG Y, HUI DY, WEINTRAUB NL: Proinflammatory phenotype of perivascular adipocytes. *Arterioscler Thromb Vasc Biol* **34**: 1631-1636, 2014.

ORIOWO MA: Perivascular Adipose Tissue, Vascular Reactivity and Hypertension. *Med Princ Pract* 24 *Suppl* **1**: 29-37, 2015.

PICK E, KEISARI Y: A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J Immunol Methods* **38**:161-170, 1980.

RAJSHEKER S, MANKA D, BLOMKALNS AL, CHATTERJEE TK, STOLL LL, WEINTRAUB NL: Crosstalk between perivascular adipose tissue and blood vessels. *Curr Opin Pharmacol* **10**: 191-196, 2010.

RODRIGUEZ-MARTINEZ MA**,** GARCIA-COHEN EC, BAENA AB, GONZALEZ R, SALAICES M, MARIN J: Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanism involved. *Br J Pharmacol* **125**: 1329–1335, 1998.

ROLO AP, PALMEIRA CM: Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. *Toxicol Appl Pharmacol*. **212**: 167-178, 2006.

SCHLEIFENBAUM J, KÖHN C, VOBLOVA N, DUBROVSKA G, ZAVARIRSKAYA O, GLOE T, CREAN CS, LUFT FC, HUANG Y, SCHUBERT R, GOLLASCH M: Systemic peripheral artery relaxation by KCNQ channel openers and hydrogen sulfide. *J Hypertens* **28**: 1875-1882, 2010.

SCHMIDT RE, DORSEY DA, BEAUDET LN, PARVIN CA, ZHANG W, SIMA AA: Experimental rat models of types 1 and 2 diabetes differ in sympathetic neuroaxonal dystrophy. *J Neuropathol Exp Neurol* **63**: 450-460, 2004.

NICOLLS MR, HASKINS K, FLORES SC: Oxidant stress, immune dysregulation, and vascular function in type I diabetes. *Antioxid Redox Signal* **9**: 879-889, 2007.

SEARLS YM, LOGANATHAN R, SMIRNOVA IV, STEHNO-BITTEL L: Intracellular Ca^{2+} regulating proteins in vascular smooth muscle cells are altered with type 1 diabetes due to the direct effects of hyperglycemia. *Cardiovasc Diabetol* **9**:8, 2010.

SHI Y, SO KF, MAN RY, VANHOUTTE PM: Oxygen-derived free radicals mediate endothelium-dependent contractions in femoral arteries of rats with streptozotocin-induced diabetes. *Br J Pharmacol* **152**: 1033-1041, 2007.

SURMI BK, HASTY AH: Macrophage infiltration into adipose tissue: initiation, propagation and remodeling. *Future Lipidol* **3**: 545-556, 2008.

WEN YD, WANG H, KHO SH, RINKIKO S, SHENG X, SHEN HM, ZHU YZ: Hydrogen sulfide protects HUVECs against hydrogen peroxide induced mitochondrial dysfunction and oxidative stress. *PLoS One* **8**: e53147, 2013.

ZAVARITSKAYA O, ZHURAVLEVA N, SCHLEIFENBAUM J, GLOE T, DEVERMANN L, KLUGE R, MLADENOV M, FREY M, GAGOV H, FÉSÜS G, GOLLASCH M, SCHUBERT R: Role of KCNQ channels in skeletal muscle arteries and periadventitial vascular dysfunction. *Hypertension* **61**: 151-159, 2013.

Table 1. Effect of PVAT on 5-HT-induced contractions of a. gracilis of healthy and diabetic rats, expressed as % of contractions in 60 mM KCl.

Group / log[5-HT] (M)	-9	-8	-7	-6	-5	n	N
5.5 mM glucose+PVAT	1.7±0.2	3.7±1.0**	51.6±3.0 ***	108.0±3.7*	131.6±3.1	28	16
5.5 mM glucose–PVAT	2.2±0.4	33.7±2.4*	97.9±3.6	130.6±3.2	136.2±3.5	27	15
20 mM glucose+PVAT	1.0±0.3	6.6±1.1*	47.3±2.7 **	110.6±2.6*	138.6±2.1	19	11
20 mM glucose–PVAT	1.1±0.3	27.4±2.6	88.8±5.3	125.8±3.2	137.8±3.4	21	12
STZ + PVAT	2.2±0.6	16.6±4.3	105.3±8.0	171.6±6.7*	189.4±7.6**	8	5
STZ – PVAT	1.7±0.3	24.8±6.8	121.2±6.5	149.2±6.8	157.3±9.9	9	5

PVAT-containing versus PVAT-free preparations are compared. Values are expressed as mean±S.E.M, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n = number of experiments and N = number of animals per group. Data are presented as mean±S.E.M. E_{max} values for all conditions are not different compared to the contraction induced by a single application of 10^{-5} M 5-HT.

Table 2. pEC_{50} of 5-HT-induced contractions of rat a. gracilis with or without PVAT and catalase.

Series	pEC_{50} for 5-HT	-PVAT	+PVAT	n	N
5.5 mM glucose	pEC_{50}	7.47 ± 0.12	6.73 ± 0.15 ***	10	5
5.5 mM glucose	pEC_{50}+catalase	7.15 ± 0.11	6.90 ± 0.11 **	10	5
20 mM glucose	pEC_{50}	7.26 ± 0.08	6.69 ± 0.11 ***	6	3
20 mM glucose	pEC_{50}+catalase	6.93 ± 0.11	6.88 ± 0.11 n/s	6	3
STZ-treated	pEC_{50}	7.37 ± 0.11	7.20 ± 0.12 n/s	6	3
STZ-treated	pEC_{50}+catalase	6.88 ± 0.12	6.43 ± 0.12 **	6	3

PVAT-containing versus PVAT-free preparations are compared. Values are expressed as mean±S.E.M., ** $p<0.01$, *** $p<0.001$, n = number of experiments and N = number of animals per group.

Figure legends

Fig. 1. Effect of catalase on 5-HT induced contractions of PVAT-free a. gracilis preparations in physiological (**a**, n = 6; p > 0.05, non-significant (n/s)) and supra-physiological glucose concentrations (**b**, n = 6; p > 0.05, n/s) as well as in diabetic conditions (**c**, n = 6; ****** p < 0.01). Open symbols – absence of catalase; closed symbols – presence of 1000 U/ml catalase. **d)** Normalized contractions of PVAT-free a. gracilis preparations in the presence of catalase at different 5-HT concentrations (n = 6; p > 0.05, n/s).

Fig. 2. Effect of catalase on 5-HT induced contractions of PVAT-containing a. gracilis preparations in physiological (**a**, n = 6; p > 0.05, n/s) and supra-physiological glucose concentrations (**b**, n = 6; p > 0.05, n/s) as well as in diabetic conditions (**c**, n = 6; ****** p < 0.01). Open symbols – absence of catalase; closed symbols – presence of 1000 U/ml catalase. Triangles in **c**) illustrate the effect of preserved endothelium on 5-HT induced contractions of PVAT-containing preparations. **d)** Normalized contractions PVAT-containing a. gracilis preparations in the presence of catalase at different 5-HT concentrations (n = 6; p > 0.05).

Fig. 3. H_2O_2 production by PVAT in the presence of 10^{-7} M 5-HT (blank bars) or 10^{-5} M 5-HT (shaded bars) from healthy (Control) and diabetic (STZ) rats (**a**, n= 6; * p < 0.05, ** p < 0.01). Effect of H_2O_2 on contractions of a. gracilis preparations induced by 60 mM KCl-containing PSS from healthy (open circles) and diabetic (closed squares) rats (**b**, n= 6; *** p < 0.001). Representative diagram with common mediators of PVAT participating in a. gracilis regulation in health and diabetes (**c**).

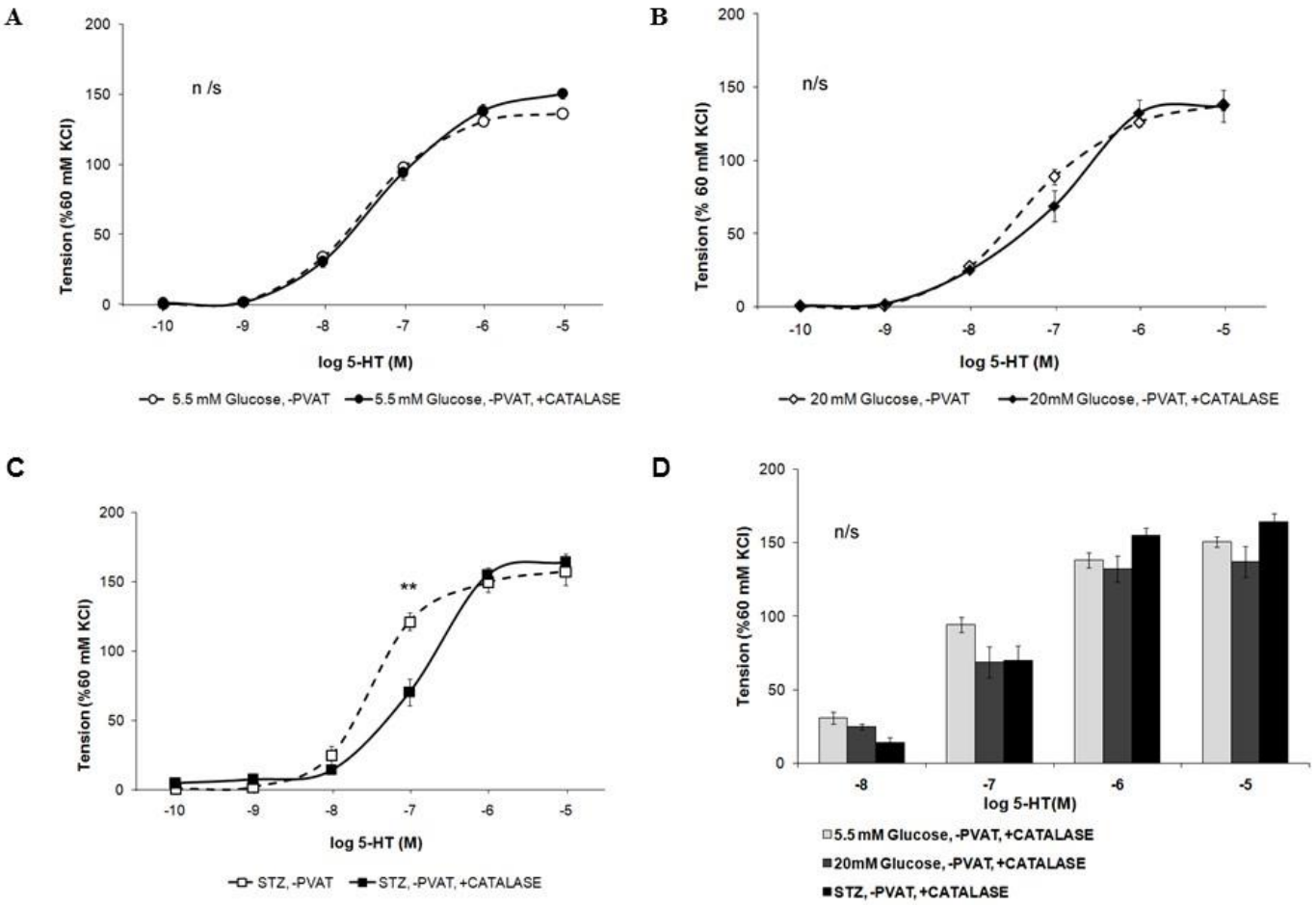

Figure 1,
Emilova et al., 2016

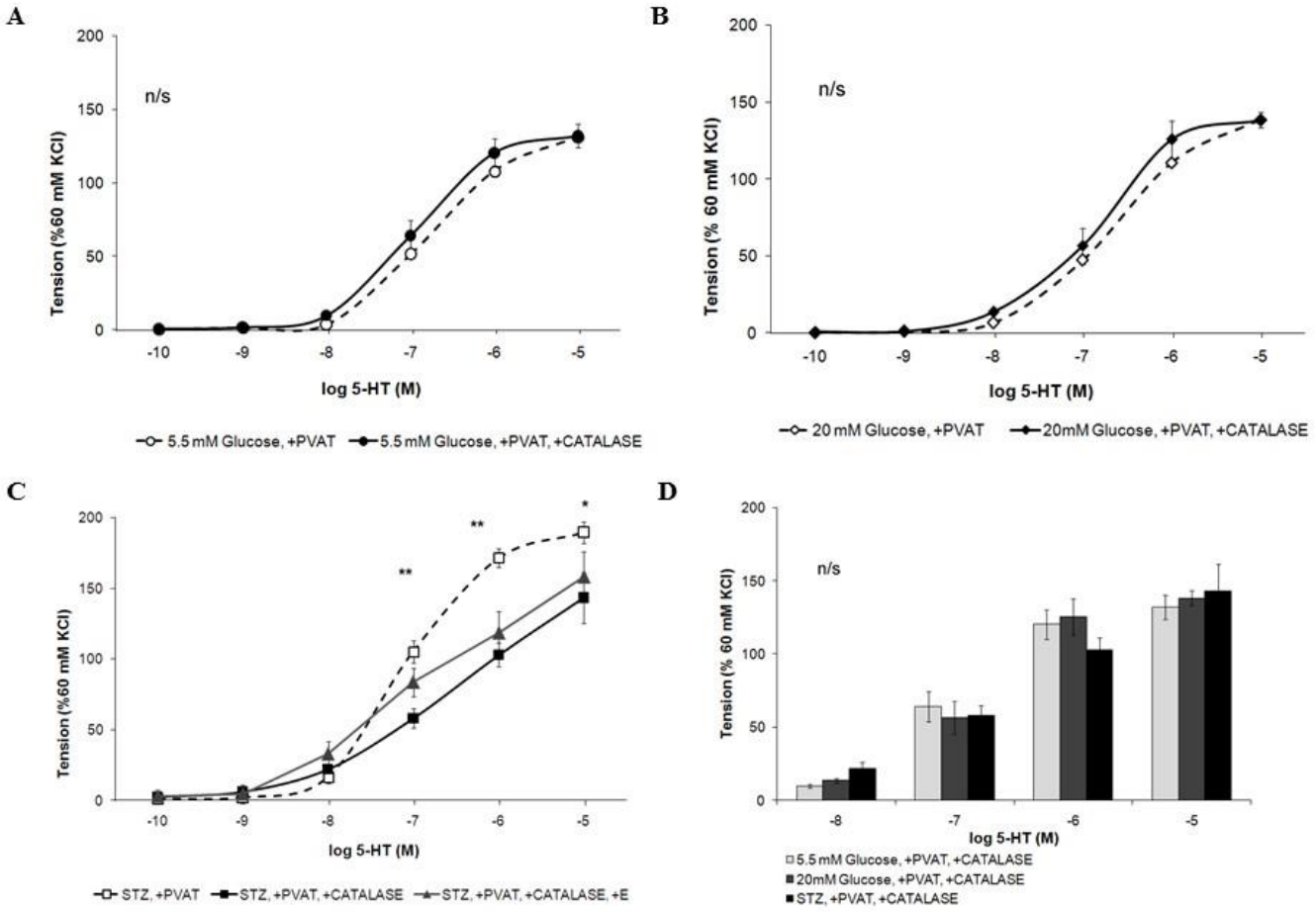

Figure 2,
Emilova et al., 2016

Figure 3,
Emilova et al., 2015