

Effect of Sulodexide on Vascular Responses and Liver Mitochondrial Function in Diabetic Rats

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Summary

This study investigates the effects of long-term treatment with sulodexide (SLX) on norepinephrine (NE)-induced contractions, acetylcholine(Ach)-induced relaxations, acute cyclooxygenase blockade by diclofenac (DIC) in isolated femoral arteries (FA) and the parameters of oxidative phosphorylation in liver mitochondria. 15-weeks old Wistar rats were divided into four groups: control (C; injected with saline solution), treated control (C+SLX), diabetic (DM) and treated diabetic (DM+SLX). Diabetes was induced with a single i.v. dose of streptozotocin (STZ) 45 mg.kg⁻¹. SLX was administered i.p., at dose 100 IU.kg⁻¹ daily for 5 weeks. Vascular responses of isolated femoral arteries were measured using Mulvany-Halpern myograph. Respiratory function of the mitochondria was determined using voltamperometric method on oxygraph Gilson. In diabetic rats the amplitude of maximal response to NE was elevated. DIC pretreatment decreased the amplitudes of NE-induced contractions in all groups of rats. SLX treatment decreased sensitivity of FA to NE and caused higher relaxatory responses to Ach in C and DM. Oxygen consumption and phosphorylation rates ([QO₂(S₃)], [QO₂(S₄)] and (OPR)) and respiratory control ratio (RCR) were decreased in the mitochondria of DM rats. Mitochondria of C rats were not affected with SLX treatment. Administration of SLX in DM rats was associated with increase of RCR, other parameters were not affected. Our findings suggest that SLX treatment might be associated with vasculoprotective effects during diabetes and improvement of mitochondrial function.

Key words

Vascular response • Diabetes mellitus • Sulodexide • Endothelium
• Oxidative phosphorylation

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Introduction

Micro- and macrovascular diseases are currently the major causes of morbidity and mortality in patients with type 1 and type 2 diabetes mellitus (DM) (De Vriese *et al.* 2000). The vascular endothelium is a multifunctional organ and is critically involved in modulating vascular tone, cellular adhesion, thrombogenesis, smooth muscle cell proliferation and vessel wall inflammation. Thus, endothelial function is important for the homeostasis of the body and its dysfunction is associated with several pathophysiological conditions including DM (Sena 2013). On this basis, the vascular endothelium has emerged as a therapeutic target, where the intent is to improve systemic metabolic state by improving vascular function. Therapy that improves endothelial function has the potential to simultaneously benefit both vascular and metabolic disease (Mather 2013). The therapeutic approach to DM should also be focused on the reduction of the risk factors for cardiovascular diseases as hypertension and dyslipidemia using antihypertensives, hemorrheologics and hypolipidemics. Another therapeutic approach consists in targeting metabolic improvements by augmenting insulin vascular function. Several nonpharmacological and pharmacological

approaches have been demonstrated to improve or reverse endothelial dysfunction, although their effect is never selective and usually also targets one or more traditional cardiovascular risk factors (Versari *et al.* 2009). The endothelial glycocalyx, layer composed by proteoglycans covering luminal surface of vascular endothelium forms a barrier against atherosclerotic stimuli (Broekhuizen *et al.* 2010). Shredding of glycocalyx is an essential initial step in the pathophysiology of microvascular complications especially in conditions as diabetes mellitus. Treatment with glycosaminoglycans has been shown to prevent or recover the damaged glycocalyx and several of its consequences (Fрати Munari *et al.* 2014). The favourable effects of sulodexide (SLX), a mixture of heparin-like and dermatan fractions, has been evaluated on certain types of vasculature in various experimental models of endothelial damage. Sulodexide has been shown to significantly decrease the number of desquamated endothelial cells *in vitro* (Kristová *et al.* 2000) and to improve endothelium-dependent relaxation of mesenteric arteries (Kristová *et al.* 2008) and reverse morphological alterations in intima and adventitia of aortic artery (Vásquez *et al.* 2010) in streptozotocin-induced diabetes. We therefore aimed to examine the effects of SLX on endothelial function of femoral arteries in the model of experimental streptozotocin-induced diabetes in rats. It is well known that DM represents a metabolic burden for the liver. Chronic hyperglycemia causes an overproduction of free radicals *via* auto-oxidation of glucose, non-enzymatic protein glycation and enhanced flux of glucose through the polyol pathway. In DM liver mitochondria exhibit morphological and functional abnormalities (Huang *et al.* 2001). Since SLX is being metabolized in the liver (Marchi *et al.* 1994) and there are no research data dealing with *in vivo* effects of SLX on mitochondrial function in liver, we decided to study whether long-term administration of SLX affects respiratory function and energy production in the liver mitochondria in the healthy and diabetic rats.

Materials and Methods

Chemicals

Potassium chloride, potassium dihydrogen phosphate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonate acid (HEPES) and ethylenedinitrilotetraacetic acid disodium salt (Titriplex III), were obtained from

Merck, adenosine-5-diphosphate sodium salt (ADP), streptozotocin, sodium chloride, magnesium sulphate, sodium hydrogencarbonate, ethylenediaminetetraacetic acid, calcium chloride, ascorbic acid and glucose were obtained from Sigma, sulodexide (Vessel Due F, inj 600 LSU amp.) was obtained from Alfa Wasserman.

Animals

15 weeks old Wistar male rats (300-370 g) were obtained from facility Dobrá voda, SAV. They were housed under standard laboratory conditions (temperature 23 ± 1 °C, 12-h light-dark cycle, controlled air humidity, standard pelleted diet and tap water *ad libitum*). The experiment was performed according to Guide for the Care and Use of Laboratory Animals (1985), European Guidelines on Laboratory Animal Care and was approved by the Local Ethics Committee on Animal Experiments and State veterinary institute.

Experimental procedure

Blood glucose and body weight were measured at the beginning of the experiment. Animals were randomly divided into four groups. The first group was an untreated control (C), the second group was treated with sulodexide (C+SLX, 100 UI/kg/day). Diabetes was induced with single dose of streptozotocin ($45 \text{ mg}\cdot\text{kg}^{-1}$ of body weight dissolved in $0.1 \text{ mol}\cdot\text{l}^{-1}$ citrate buffer, pH 4.5) administered intravenously *via* the tail vein. Rats with *ad libitum* access to food were after 48 h characterized by the levels of blood glucose measured by Bayer Contour TS meter. Animals with blood glucose concentration $>16 \text{ mmol}\cdot\text{l}^{-1}$ were considered diabetic, and were included in the study. Third group was injected with physiological solution (DM) and the fourth group was treated with sulodexide (DM+SLX, 100 UI/kg/day). Treatment with sulodexide started on the seventh day after induction of diabetes and lasted for 5 weeks. Thereafter, blood glucose levels were determined in same conditions as at the beginning of the experiment. The animals were sacrificed by intraperitoneal application of thiopental ($50 \text{ mg}\cdot\text{kg}^{-1}$).

Measurement of vascular responses of femoral arteries

Isolated femoral arteries with intact endothelium were cut into 2 mm long segments and mounted in the chamber of Mulvany Halpern isometric myograph. The myograph chamber was filled with modified Krebs-Ringer solution (in $\text{mmol}\cdot\text{l}^{-1}$: NaCl 119, KCl 4.7, MgSO_4 25, KH_2PO_4 1.2, glucose 11.1,

CaCl₂ 2.5, EDTA 0.03 at 37 °C, gased with 95 % O₂ and 5 % CO₂). Rings were normalized upon standard procedures (Angus and Wright 2000) to achieve their basal tone and after 30 min incubated with depolarising solution (in mmol.l⁻¹: K⁺ 124 and Na⁺ 2.5) to provide maximal depolarisation-induced contraction followed by washout period to achieve constant basal tone. Further contractions were provided with cumulative applied doses of norepinephrine (NE; in mol.l⁻¹: 3.10⁻⁷, 10⁻⁷, 3.10⁻⁶, 10⁻⁶, 3.10⁻⁵, 10⁻⁵, 3.10⁻⁴ and 10⁻⁴). Relaxations were induced with single dose of acetylcholine (ACH; 3.10⁻⁶ mol.l⁻¹). The same protocol was used in the presence of diclofenac (DIC; 0.01 mol.l⁻¹, incubation time 15 min).

Isolation and measurement of mitochondrial function

Mitochondria were isolated from freshly excised livers by differential centrifugation as described by Hogeboom (1955), with some modification. The isolation medium was prepared according to Sammut *et al.* (1998), with the following modification (in mmol.l⁻¹): mannitol 225, sucrose 75 and Titriplex III 0.2. Liver was minced in the isolation medium (pH 7.4 at 4 °C) and homogenized using a teflon-to-glass homogenizer. The homogenate was centrifuged at 700 g for 10 min, the supernatant was decanted and centrifuged at 5600 g for 10 min. The mitochondrial pellet was washed twice with isolation medium. The resulting pellet was resuspended in the same medium to a final protein concentration of 20-40 mg.ml⁻¹. All procedures were performed at 4 °C. Proteins were determined according to Lowry *et al.* (1951).

Respiratory function of the mitochondria was determined using the voltamperometric method on oxygraph Gilson 5/6 H (USA) using Clark oxygen electrode at 30 °C. The incubation medium was prepared as described by Rouslin and Millard (1980) with a modification: in mmol.l⁻¹: HEPES 12.5, KCl 122, KH₂PO₄ 3, Titriplex III 0.5 and 2 % dextran. Glutamate/malate at 2.5 mmol.l⁻¹ was used as a NAD substrate. For assessing stimulated oxygen consumption, 500 nmol of ADP was added. The following parameters of oxidative phosphorylation were determined in the liver mitochondria: respiratory control ratio (RCR), coefficient of oxidative phosphorylation (ADP:O), the rate of oxygen uptake by the mitochondria stimulated with ADP – state 3 [QO₂(S₃)], the rate of basal oxygen uptake by mitochondria without ADP – state 4

[QO₂(S₄)] and the oxidative phosphorylation rate (OPR) (Estabrook 1967).

Statistical analysis

Data are expressed as arithmetical mean ± SEM. Statistical analyses were performed with one- and two-way ANOVA tests, Student's t-test and *post-hoc* Bonferroni test. P<0.05 was considered as a significant difference.

Results

The weight of the rats in all groups at the beginning of the experiment was not significantly different. At the end of the experiment, rats in the diabetic group had significantly lower body weight compared to the healthy groups (P<0.05). Concentration of glucose increased significantly in both diabetic groups. SLX treatment did not affect postprandial glycemia in diabetic rats. In control rats, SLX treatment resulted in significantly lower increase of glycemia compared to untreated controls (Table 1).

Vascular responses of femoral arteries

The administration of cumulative concentrations of norepinephrine to femoral arteries elicited concentration-response curves which were similar in vessels obtained from control rats, and control rats treated with sulodexide (Fig. 1A). In diabetic rats, the norepinephrine-induced dose-response curves were significantly shifted to the left (P<0.05), the amplitude of contraction elicited by the highest dose of NE (10⁻⁴ mol.l⁻¹) and sensitivity to NE were significantly enhanced (both P<0.05) compared to control rats (Fig. 1B). Diabetic rats were characterized by a steeper slope of dose-response curves, while treatment with SLX significantly reduced the slope of the curves (P<0.05). The norepinephrine-induced concentration-response curves were almost abolished in control groups (P<0.001) and significantly diminished in diabetic groups of rats (P<0.001) following pretreatment of vessels with diclophenac. The amplitude of contraction elicited by the highest dose of NE (10⁻⁴ mol.l⁻¹) was not affected by SLX treatment significantly (P>0.05), although sensitivity to norepinephrine (pEC₅₀) was significantly decreased compared to untreated diabetic rats (Fig. 1, Table 1).

Table 1. The body weights, glucose concentrations and pharmacodynamic parameters of norepinephrine and acetylcholine concentration response curves determined in femoral arteries of control and diabetic rats.

	C (n=10)	C+SLX (n=9)	DM (n=13)	DM+SLX (n=14)
Weight (1) (g)	322.6 ± 5.8	330.7 ± 6.3	332.2 ± 5.2	322.8 ± 4.1
Weight (2) (g)	370.4 ± 7.5	377 ± 9.4	255 ± 9.2 ^{a,b}	240.2 ± 12.5 ^{a,b}
Weight gain (%)	12.8 ± 0.6	12.1 ± 0.4	-31.9 ± 2.2 ^{a,b}	-37.9 ± 3.3 ^{a,b}
Glycemia (1) (mmol.l ⁻¹)	4.9 ± 0.1	5.2 ± 0.1	21.9 ± 1.6 ^{a,b}	22.7 ± 1.5 ^{a,b}
Glycemia (2) (mmol.l ⁻¹)	5.3 ± 0.2	5.3 ± 0.2	32.2 ± 0.9	32.9 ± 0.5
NE (3.10 ⁻⁴ mol.l ⁻¹) induced maximal contraction (mN.mm ⁻¹)	2.03 ± 0.21	1.53 ± 0.19 ^a	5.11 ± 0.27 ^{a,b}	5.37 ± 0.25 ^{a,b}
pEC ₅₀ (log mol.l ⁻¹)	4.23 ± 0.12	4.20 ± 0.14	5.08 ± 0.05 ^{a,b}	4.78 ± 0.19 ^{a,b,c}
Slope	1.03 ± 0.28	1.03 ± 0.33	1.57 ± 0.04 ^{a,b}	1.16 ± 0.03 ^{a,c}
ACH (3.10 ⁻⁵ mol.l ⁻¹) induced relaxation of NE-precontracted vessels (%)	80.06 ± 4.39	87.22 ± 6.45	63.24 ± 6.10 ^b	64.27 ± 2.55 ^b
ACH (3.10 ⁻⁵ mol.l ⁻¹) induced wall tension decrease (mN.mm ⁻¹)	1.63 ± 0.17	1.26 ± 0.15	3.10 ± 0.17 ^{a,b}	3.15 ± 0.18 ^{a,b}
E _{max} reduction elicited by DIC (%)	93.55 ± 0.97	88.63 ± 3.06	63.01 ± 2.34 ^{a,b}	80.20 ± 1.51 ^{a,c}

Values presented as arithmetic mean ± SEM, C – control group, C+SLX – control group treated with sulodexide, DM – diabetic group, DM+SLX – diabetic group administered with sulodexide, ACH – acetylcholine, DIC – diclofenac, NE – norepinephrine, pEC₅₀ – negative logarithm of EC₅₀ (half maximal effective concentration). Glycemia and weight were measured 48 h after application of STZ (1) and before sacrifice (2). Statistical significance: ^aP<0.05 vs. C, ^bP<0.05 vs. C+SLX, ^cP<0.05 vs. DM.

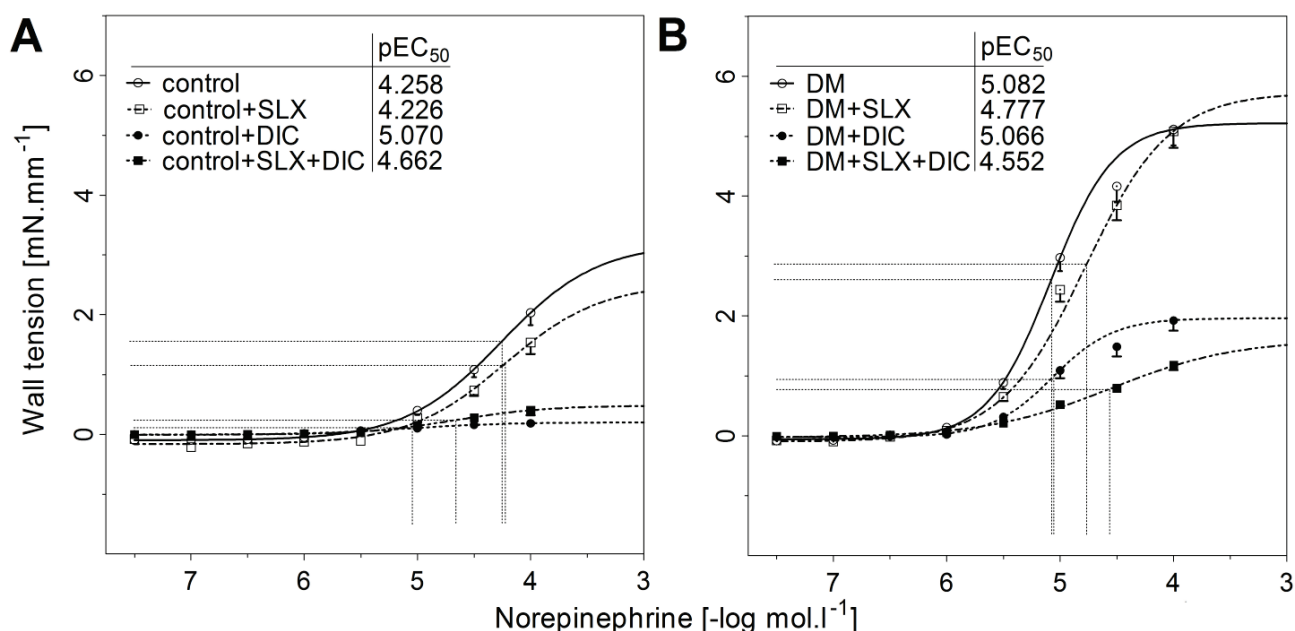


Fig. 1. Norepinephrine concentration-response curves of femoral arteries of healthy rats (A) and diabetic rats (B) recorded under normal condition and after blockade of cyclooxygenase by diclofenac (DIC, 0.01 mol.l⁻¹). The thin dotted lines represent pEC₅₀, data are expressed as arithmetic mean ± SEM from 9-13 rings isolated as described in Methods. Curves were adjusted with non-linear regression function.

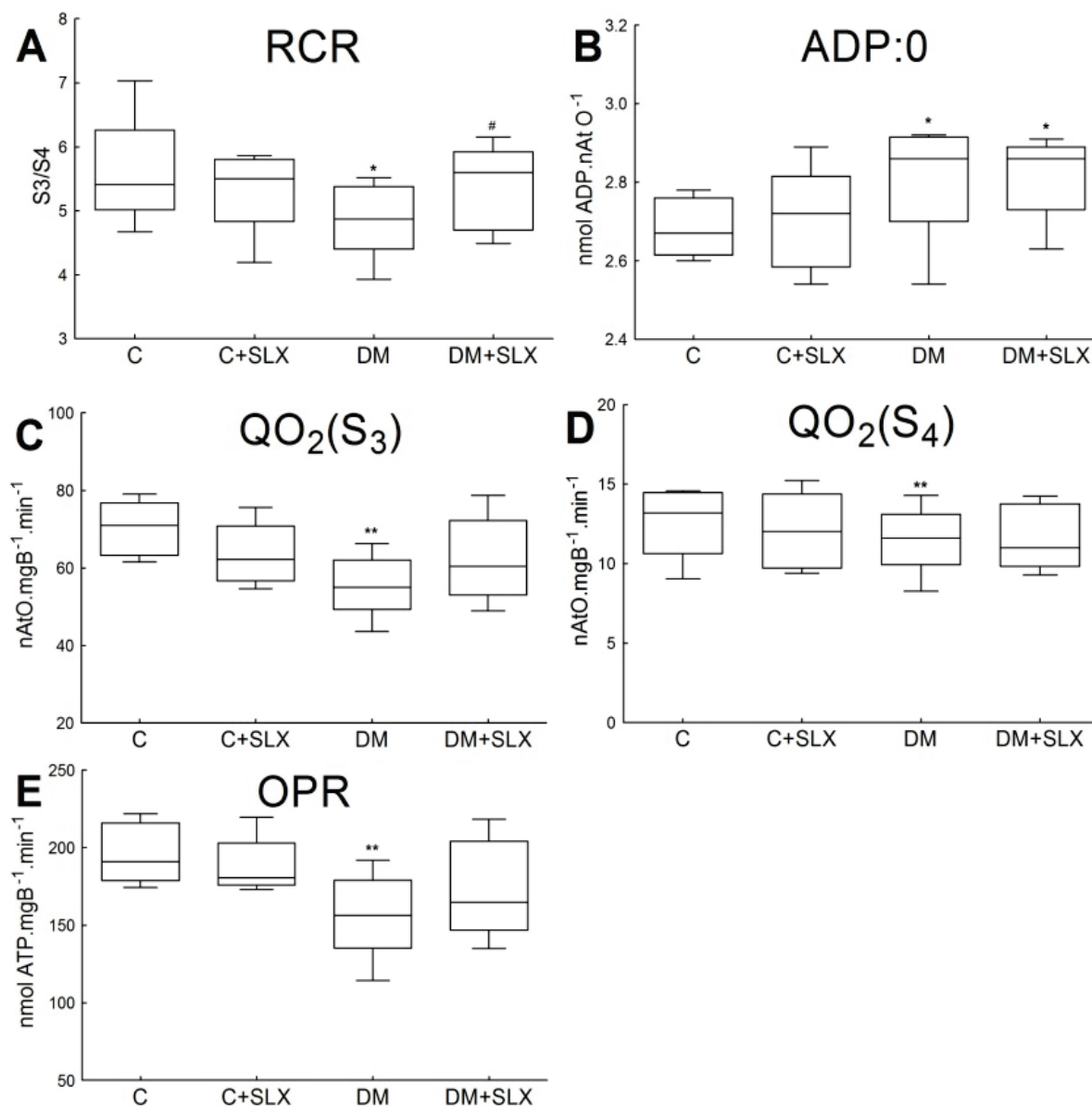


Fig. 2. Parameters of mitochondrial function following SLX treatment. RCR – respiratory control ratio (**A**), ADP:0 – coefficient of oxidative phosphorylation (**B**), $QO_2(S_3)$ – ADP stimulated respiration rate (**C**), $QO_2(S_4)$ – basal respiration rate (**D**), OPR – oxygen phosphorylation rate (**E**). C – control group, C+SLX – control group administered with sulodexide, DM – diabetic group, DM+SLX – diabetic group administered with sulodexide. Data are expressed as median (horizontal line), lower and upper quartile (open boxes) and minimal and maximal values (whiskers). The statistical significance was tested using the Student's t-test. Statistical significance: * $P < 0.05$ vs. C; ** $P < 0.01$ vs. C; # $P < 0.05$ vs. DM.

Acetylcholine ($3 \cdot 10^{-5} \text{ mol} \cdot \text{l}^{-1}$) decreased the maximal amplitude of contraction of femoral arteries induced by highest concentration of NE ($10^{-4} \text{ mol} \cdot \text{l}^{-1}$) by 80 % in control group and by 87 % in SLX-treated group, respectively. In diabetic groups, acetylcholine decreased the maximal amplitude of contraction of femoral arteries by 63 % in untreated and by 64 % in treated group, respectively. The statistical difference between treated and untreated groups was not significant ($P > 0.05$; Table 1).

Assessment of mitochondrial function

All evaluated parameters of oxidative phosphorylation in the liver mitochondria of diabetic rats were significantly decreased in comparison to control values (RCR: $P < 0.05$, $QO_2(S_3)$, $QO_2(S_4)$ and OPR: $P < 0.01$) except of the coefficient ADP:0, which was significantly increased compared to healthy rats ($P < 0.05$, Fig. 2). The measured parameters of liver mitochondria of control rats were not affected by SLX treatment. In diabetic rats, the coefficient RCR was found to be

significantly increased in rats treated with SLX compared to untreated diabetic rats ($P < 0.05$). Parameters of oxygen consumption rate in state 3, state 4, OPR and ADP:0 were not significantly affected by SLX treatment.

Discussion

It is well known fact, that vascular functions are altered by DM. Even short-term hyperglycemia can lead to augmented contractile responses, which has been confirmed also in our experiment. Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds of different animal models of diabetes and in humans with type 1 and type 2 diabetes. This may arise from several mechanisms: decreased production or enhanced inactivation of nitric oxide (NO), impairment of its diffusion to the underlying smooth muscle cells or enhanced production of EDCFs (endothelium-derived constricting factors) (De Vriese *et al.* 2000). Various drugs (Taddei *et al.* 2002, Grassi *et al.* 2005, Beckman 2004) have been demonstrated to improve or reverse endothelial dysfunction. SLX has been also found to reverse the damage of endothelium caused by DM, lower the number of circulating endothelial cells and improve endothelium-dependent relaxation of mesenteric arteries (Kristová *et al.* 2008). Many authors have described the favourable effects of various glycosaminoglycans on the integrity of endothelium (Broekhuizen *et al.* 2010, Połubińska *et al.* 2013, Vásquez *et al.* 2010). In our experiment, SLX treatment was able to significantly reduce augmented vascular responses, non-significantly restore endothelium-dependent vasodilation and decrease the sensitivity of arteries to norepinephrine. However, the mechanism how SLX might exert its vasoprotective action is not precisely known. We conclude that SLX has the potential to reverse vascular impairment resulting from DM, perhaps if administered for a longer time or together with appropriate anti-diabetic treatment.

Endothelial functions are dependent on the presence of well-preserved glycocalyx, which can be damaged by various stimuli like abnormal shear stress, ROS (reactive oxygen species) and hyperglycemia (Nieuwdorp *et al.* 2006). Glycocalyx alterations are involved in the pathogenesis of atherosclerosis and diabetic vascular complications (Masola *et al.* 2014) and can result in endothelial dysfunction, enhanced vascular permeability (Becker *et al.* 2010) and excessive activation of coagulation (Frati Munari 2013). There are evidences, that glycocalyx plays an important role in the

release of NO as a response to the increased shear stress (Mochizuki *et al.* 2003). Morphofunctional integrity of the matrix of the connective tissue, mainly composed of glycosaminoglycans is therefore essential for the functional efficiency of endothelium. It has been previously described, that SLX treatment partially restores endothelial glycocalyx during DM (Broekhuizen *et al.* 2010). *In vitro* supplementation with glycosaminoglycans reverses hyperglycemia-induced impairment of permeability of the endothelium (Gouverneur *et al.* 2008). Moreover, SLX increases the expression of eNOS and decreases concentration of endothelin-1 (Tiurenkov *et al.* 2012). It is well known, that chronic hyperglycemia reduces NO bioavailability due to oxidizing cofactors of the nitric oxide synthase, consequently leading to the decreased NO production (Masha *et al.* 2011). Antioxidant activity of glycosaminoglycans has been described both in animal (Albertini *et al.* 2000) and human studies (Bilinska *et al.* 2009). Inhibition of excessive ROS production, increased expression of eNOS and protection of endothelial glycocalyx may all play an important role in reversing endothelial dysfunction under pathological conditions.

To investigate the participation of the cyclooxygenase in norepinephrine-induced contractions, we measured contractions in the presence of cyclooxygenase inhibitor diclofenac. Surprisingly, reduction of E_{max} after blockade of the cyclooxygenase was significantly smaller in diabetic rats compared to healthy controls. Moreover, when comparing diabetic groups, this reduction was significantly higher in rats treated with SLX. To date, there is no satisfactory information about the influence of glycosaminoglycans on expression or activity of cyclooxygenase family enzymes. One study found that hyaluronan causes overexpression of cyclooxygenase resulting in subsequent TXA_2 production (Sun *et al.* 2001). This mechanism, however, would not explain the difference in the vascular responses after the blockade of the cyclooxygenase in our experiment.

To the date we were unable to find any experimental work assessing the effect of SLX on mitochondrial functions or parameters of oxidative phosphorylation. Given their essential function in aerobic metabolism, mitochondria are intuitively of interest in regard to the pathophysiology of diabetes. Impaired mitochondrial functions during diabetes have been observed both in human subjects (Kelley *et al.* 2002) and animal models (Marciniak *et al.* 2014). Our results show

that mitochondria isolated from livers of rats with induced DM exert reduced rates of oxygen consumption in both state 3 and state 4, decreased oxygen phosphorylation rate and respiratory control ratio compared to healthy rats. These results indicate decreased capacity of the respiratory chain and reduced rate of ATP synthesis in mitochondria in diabetic conditions, which is consistent with findings of diabetic models (Cheshchevik *et al.* 2011) and human DM studies (Koliaki and Roden 2013). Liver mitochondria isolated from diabetic rats treated with SLX showed increased RCR, the marker of integrity of mitochondria. Values of $QO_2(S_3)$ and OPR were not statistically different in treated diabetic rats compared to untreated rats, although we observed a clear trend of improvement of these parameters. We consider this result as important because it indicates that long-term administration of SLX might inhibit the impairment of respiration chain function and mitochondria energy

caused by diabetes.

In conclusion, we found augmented contractile responses and decreased endothelium-dependent relaxation responses in diabetic rats. SLX treatment significantly diminished the sensitivity of arteries to norepinephrine, non-significantly enhanced impaired endothelium-mediated relaxation responses and partially reversed the impairment of the capacity of the respiratory chain and improved rate of ATP synthesis in liver mitochondria in conditions of experimental diabetes.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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