RESEARCH REPORT

Hyperbaric oxygenation affects acetylcholine-induced relaxation in female diabetic rats

Dragan Manojlović, MD [1]; Ana Stupin, MD [2,3]; Zrinka Mihaljević, PhD [2]; Anita Matić, PhD [2]; Helena Lenasi, MD [4]; Ines Drenjančević, MD [2]

[1] Department of Surgery, General Hospital Vukovar, Vukovar, Croatia

[2] Department of Physiology and Immunology, Faculty of Medicine University Josip Juraj Strossmayer Osijek, Osijek, Croatia

[3] Department of Pathophysiology, Physiology and Immunology, Faculty of Dental Medicine and Health Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

[4] Institute of Physiology, Faculty of Medicine University of Ljubljana, Ljubljana, Slovenia

CORRESPONDING AUTHOR: Ines Drenjancevic – *ines.drenjancevic@mefos.hr*

ABSTRACT

We aimed to assess the effects of intermittent hyperbaric oxygenation (HBO_2 at 2 bars for 120 minutes a day for four successive days) on acetylcholine-induced vasorelaxation (AChIR) in female Sprague-Dawley (SD) rats (N=80) that were randomized into four groups: healthy controls (CTR); diabetic rats (DM); and control and diabetic rats that underwent hyperbaric oxygenation (CTR+HBO and DM+HBO), respectively. AChIR was measured in vitro in aortic rings, with/without L-NAME, MS-PPOH, HET0016 or indomethacin. mRNA expression of eNOS, iNOS, COX-1, COX-2, thromboxane A synthase 1 (TBXAS1), CYP4A1, CYP4A3 and CYP2J3 was assessed by qPCR. Systemic oxidative stress and plasma antioxidative capacity were determined with the thiobarbituric acid-reactive substances (TBARS) and the ferric reducing ability of plasma (FRAP) assays, respectively. There was no significant difference in AChIR among experimental groups of rats. In CTR and DM group of rats, AChIR was mediated by NO and EETs pathway, while in the CTR+HBO and DM+HBO groups, NO-pathway prevailed. iNOS expression was upregulated in the DM group compared to CTR, while HBO_2 upregulated eNOS in CTR group and TBXAS1 in DM group of rats. In both, CTR and DM group of rats, the sensitivity to ACh in the presence of L-NAME or in the presence of MSPPOH was significantly decreased compared to the response to ACh in the absence or presence of indomethacin or HET0016. DM and DM+HBO rats had increased TBARS compared to their respective controls. In conclusion, HBO_2 presumably alters vasorelaxation in response to ACh from NO-EETs mediated pathways to solely NO-pathway, without affecting oxidative status of DM rats.

KEYWORDS: hyperbaric oxygen; diabetes; endothelium; 20-HETE; epoxyeicosatrienoic acids; female rats; nitric oxide; acetylcholine

INTRODUCTION

Diabetes mellitus (DM) has been strongly associated with development and progression of cardiovascular disease (CVD); e.g., diabetic patients exhibit increased incidence of myocardial infarction and stroke, as well as peripheral ulcerations due to tissue hypoxia caused by insufficient perfusion. Among other possible mechanisms, this tissue underperfusion could be explained by altered vascular reactivity of both micro- and macrocirculation [1-4]. Along with altered smooth muscle vascular cell (SMVC) function and advanced glycation of proteins, the disbalance of endothelial mediators (e.g., vasoconstrictors versus vasodilators), as well as increased production of reactive oxygen species (ROS) have been proposed to contribute to diabetic vascular dysfunction, although the exact metabolic pathways and signaling mechanisms are still not fully elucidated [1-3, 5-9]. While most studies have confirmed an attenuated acetylcholine (ACh)-induced and nitric oxide (NO)-dependent vasodilation in diabetic models [1,7,10,11], the contribution of other endothelial vasoactive mediators is less well investigated. Besides NO, the arachidonic acid (AA) metabolites released from the endothelium play an important role in the regulation of vascular tone. The enzymes cyclooxygenase-1 and -2 (COX-1, -2) metabolize AA to prostaglandin G_2 and prostaglandin H_2. These in turn may be converted to various prostaglandins: e.g., prostacyclin (PGI_2), which exhibits vasodilator effect, or to thromboxanes (TXA_2, TXB_2), which act as vasoconstrictors. Under the action of various specific cytochrome P450 (CYP450) enzymes, AA could either be metabolized in epoxidation reactions to epoxyeicosatrienoic acids (EETs), which act as potent vasodilators, or by ω-hydroxylase to 20-hydroxyeicosa-

tetraenoic acid (20-HETE) which cause vasoconstriction [12-14]. Some studies reported involvement of 20-HETE in diabetes-induced vascular dysfunction, while others suggested EETs to be protective against streptozotocin-induced diabetic nephropathy [15] and coronary artery disease [16]. Recently we reported that in male diabetic rats, ACh-induced relaxation (AChIR) of aortic rings was mediated mainly by NO, with contribution of CYP450-produced vasodilators, possibly EETs [17]. Still, the data on CYP450 metabolites and their role in DM remain scarce and inconclusive [2,18,19], especially in the female sex.

One of the adjunct therapies for DM that is particularly efficient in treatment of diabetic ulcerations has been hyperbaric oxygen (HBO_2) therapy [20-22]. HBO_2 increases tissue oxygenation, but more interestingly, it has been implicated in benefiting vascular reactivity by enhancing relaxation of isolated aortic rings and reducing infarct size of isolated hearts [20-22]. Also, upregulation of eNOS, endothelial nitric oxide synthase 3 (NOS), was demonstrated in rat cerebral microvascular endothelial cells under the influence of HBO_2 [20-22]. Some of the metabolites of CYP450 enzymes have been proposed to act as a putative oxygen sensor correlating with tissue pO_2) [23,24]. Thus, it is tempting to speculate that the CYP450 metabolites-dependent vasodilation and/or expression of CYP450 enzymes might possibly be altered by HBO_2 (especially in DM) [20-22]. Indeed, earlier study by our research group has shown upregulation of CYP2J and CYP2C enzymes that catalyze the epoxygenation of AA into EETs in aortic rings of streptozotocin-induced diabetic male rats exposed to intermittent HBO_2 treatment [20-22,25]. In light of available data we hypothesized that HBO_2 might interfere with the endothelium-dependent vasodilation as well as with the synthesis or sensitivity to 20-HETE and/or EETs which, in turn, could have an important effect in restoring vascular function in diabetes.

The aim of the present study was to assess the potential effect of intermittent HBO_2 on vascular reactivity in streptozotocin-induced diabetic female rats with special focus on the role of CYP450 metabolites. We aimed to investigate whether HBO_2 treatment induces changes in vascular reactivity to ACh in isolated aortic rings of diabetic female rats, and to elucidate if potential changes might be due to altered involvement of CYP450 metabolites. To do so, we have performed functional vascular reactivity experiments in the presence and absence of specific CYP450 inhibitors and measured messenger RNA (mRNA) expression of various CYP450 isoforms, respectively.

MATERIALS AND METHODS

Experimental animals

The animals were raised at the animal care of the Faculty of Medicine Josip Juraj Strossmayer University of Osijek, Croatia, which is a registered and certified user/breeder of mice and rats for educational and scientific purposes. All experimental procedures conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985). The experiments were approved by the Ethics committee of the Ethics Committee Faculty of Medicine, University of Osijek.

A total of 80 female Sprague-Dawley (SD) rats were divided into four groups (n=20 per group):

1) controls (CTR);
2) diabetic rats (DM);
3) controls that underwent the HBO_2 protocol (CTR+HBO);
4) diabetic rats that underwent the HBO_2 protocol (DM+HBO).

Fifteen rats from each group (n=15 per group) were used for functional aortic ring reactivity experiments, and five animals from each group (n=5 per group) were used for the measurement of mRNA expression of enzymes catalyzing vasoactive mediators' synthesis in thoracic aorta tissue. All rats were housed in pairs in shoebox-style cages, with free access to standard rat chow and tap water, and maintained on a 12:12-hour light:dark cycle. Rat weight was measured at the end of the study protocol.

A Type 1 DM model was induced by intraperitoneal streptozotocin (60mg/kg) injection at the age of six weeks. A OneTouch Ultra (LifeScan, Inc., Milpitas, California, U.S.) glucometer and the tail-cut method were used to measure blood glucose levels one week after the streptozotocin injection and again on the day of the experiment (at the age of 12 weeks). All animals that did not develop diabetes at one week after the injection (minimum blood glucose cutoff at the age of seven weeks was 15 mmol/L), or without a confirmed diabetes again at 12 weeks were euthanized and not used in further experiments.

Hyperbaric oxygen treatment

Under the HBO_2 protocol, rats from the HBO_2 groups were treated in a hyperbaric chamber (containing carbon dioxide/CO_2 adsorbent) with 100% oxygen (using a pressure of 2 bar) for 120 minutes a day for four successive days (with the addition of 15 minutes for compression phase and 15 minutes for decompression phase) during

four consecutive days (Recompression Chamber for Experiments 110L, Djuro Djakovic, Aparati d.d., Slavonski Brod, Croatia) [17].

Surgery, aortic rings acquisition and assessment of aortic ring reactivity to acetylcholine

General procedures for aortic rings experiments were done according to the protocol already described in our laboratory [17,26]. A day after HBO_2 treatment (at the age of 12 weeks, after a six-week DM duration) the aortic rings experiments were conducted. The rats were anesthetized with a combination of ketamine (75mg/kg) and midazolam (2.5mg/kg), and decapitated with a guillotine. The descending thoracic aorta was dissected from the connective tissue, placed in an oxygenated modified Krebs-Henseleit solution, and cut into short segments (rings) of about 3-4 mm in length (N=4 rings per n=1 thoracic aorta). These rings were then mounted in tissue bath chambers containing Krebs-Henseleit solution (maintained at 37°C) that was oxygenated with 95% O_2/5% CO_2 compressed gas mixture. Passive tension for each ring was set at 2.0 grams (g). Intactness of endothelium was tested by precontracting the rings with 10^{-7} molar concentration (M) (final concentration) noradrenaline (NA) and inducing relaxation with 10^{-5} M ACh. Rings that failed to relax were not used for further studies. After the initial test for vessel viability and endothelial integrity, maximal contraction was induced with 60 mM KCl + 10^{-7} NA.

After this phase, in the AChIR protocol aortic rings were precontracted with 10^{-7} M NA for five minutes, and cumulative concentration-response curves to ACh were obtained by adding ACh of increasing concentration to achieve the final bath concentration of 10^{-9} to 10^{-5} M ACh. AChIR protocol was used in the absence and in the presence of the corresponding inhibitor:

1) the eNOS inhibitor, nitro-L-arginine methyl ester (L- NAME, $3x10^{-4}$ M);
2) non-selective COX-1 and COX-2 inhibitor, indomethacin (10^{-5} M);
3) the selective EETs epoxidation inhibitor, N-(methyl sulfonyl)-2-(2-propynyloxy)-benzenehexanamide MS-PPOH (10^{-5} M) that inhibits the formation of arachidonate 11,12-epoxides by CYP4A2 and CYP4A3 enzymes; and
4) the selective inhibitor of 20-HETE formation, N-hydroxy-N'-(4-n-butyl-2-methylphenyl)-formamidine (HET0016, 10^{-5} M) in tissue bath.

Before adding any increasing concentration of ACh, aortic rings were incubated for 10 minutes with the corresponding inhibitor. The amount of relaxation was expressed as the percentage of the remaining contraction of the NA-induced vasoconstriction.

Oxidative stress measurements

Blood samples were collected from the decapitation site, centrifuged at 3,500 rpm for 10 minutes, and serum samples were stored at -80 °C. Experiments were performed according to the protocol that was already described in our laboratory [27]. As a direct indicator of oxidative stress, the spectrophotometric thiobarbituric acid reactive substances (TBARS) method was used for measuring the products of lipid peroxidation with malondialdehyde (MDA) as standard (μmol l–1 MDA). The products bind to a thiobarbituric acid (TBA) at low pH. Since the method is non-specific because other substances also bind to a TBA (including proteins), trichloroacetic acid (TCA) was first added to the sample to precipitate the proteins, and after that the supernatant was used for the measurements [28]. The absorbance of the sample was measured at 572 and 532 nm.

Antioxidant capacity was assessed using the ferric reducing ability of plasma assay (FRAP). Fe^{3+}-TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) is reduced to Fe^{2+}-TPTZ in the presence of antioxidants; blue discoloration occurs. The absorbance of the sample was measured at 593 nm (Nanophotometer P300 UV/VIS, IMPLEN), using trolox as a standard (mmol trolox) [29].

mRNA expression of enzymes catalyzing vasoactive mediators in rat aorta

Quantitative real-time PCR was used to detect expression levels of eNOS and inducible nitric oxide synthase (iNOS), COX-1, COX-2 and thromboxane A synthase 1 (TBX-AS1), CYP4A1, CYP4A3 and CYP2J3 in all four groups of female SD rats. An aorta sample for each rat was placed in RNA later (Qiagene, Germany) and stored at –80 °C. RNA isolation from tissue homogenate was made with TRI reagent (Life Technologies, U.S.) according to the protocol used/developed by Chomczynski and Sacchi [20]. Isolated and dissolved RNA was stored at -80°C. RNA integrity was checked on 1% agarose gel, and concentration was measured with NanoDrop 1000 spectrophotometer. cDNA was made with the High Capacity Reverse Transcriptase kit (Applied Biosystems, U.S.) under conditions determined by the protocol. Gene expression was normalized to the expression of the housekeeping gene HPRT and further analyzed. Expression determination of all genes was measured on the real-time PCR Bio Rad CFX96.

Table 1. Sequences of custom-made primers for determination of mRNA expression

mRNA	Primer sequence	PCR product length (bp)	Annealing temperature (°C)
HPRT	For -5'-GAAAGAACGTCTTGATTGTTGAAGATAT-3' Rev - 5'-GAGAGGTCCTTTTCACCAGCAA-3'	129	59
COX-1	For - 5`-TCCTGTTCCGAGCCCAGTT-3` Rev - 5`-GCCAGTGATAGAGGTGGTTGAAT-3`	69	61
COX-2	For - 5`GAAAGAAATGGCTGCAGAGTTGA 3` Rev - 5`GCAGGGCGGGATACAGTTC 3`	71	63
TBXAS1	For - 5'-CTGAGGAAGTTGGGCATCAGA-3' Rev - 5'-CCTGGCGGAAAAACATCAA-3'	70	57
eNOS	For - 5`-CGAACAGCAGGAGCTAGAGG-3` Rev - 5`-GAGGTGGATCTCTCCTGGGT-3`	211	64
iNOS	For - 5`-TGGTGAGGGGACTGGACTTT-3` Rev - 5`-CCAACTCTGCTGTTCTCCGT-3`	101	63
CYP2J3	For - 5'-CCTTTCTGTTCCTGGCTGATTT-3' Rev - 5'- AGGCCCTGGCGGGTAGT-3'	62	60
CYP4A1	For - 5'-GTTCTACCTGCAAAGGCAATGG-3' Rev - 5'-TGCCCAAAGAACCAGTGGAA-3'	78	60
CYP4A3	For - 5'-TCTCAGGGAGCAAAACACGA-3' Rev - 5'-CAACAGGAGCAAACCATAACCA-3'	134	59

eNOS- endothelial nitric oxide synthase; iNOS- inducible nitric oxide synthase; COX-1- cyclooxygenase 1; COX-2- cyclooxygenase 2; TBXAS1- thromboxane A synthase 1; CYP2J3- cytochrome P450 2J3; CYP4A1- cytochrome P450 4A1; CYP4A3- cytochrome P450 4A3.

Custom-made primers (Primer 3 software) used for gene expression measurements of eNOS and iNOS, COX-1, COX-2, TBXAS1, CYP4A1, CYP4A3 and CYP2J3 and HPRT1 are shown in Table 1.

Reagents

NA, ACh, L-NAME and indomethacin were purchased from Sigma-Aldrich. Ketamine and midazolam were obtained from Pfizer. Streptozotocin was purchased from Sigma-Aldrich. The CO_2 adsorbent Drägersorb 800 Plus was produced by Dräger, Lübeck, Germany. The Krebs-Henseleit solution (composition: 113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \times 7H_2O$, 22 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 2.5 mM $CaCl_2 \times 2H_2O$, 0.026 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4) was prepared from EDTA purchased from Sigma-Aldrich, $CaCl_2 \times 2H_2O$ and $NaHCO_3$ from Merck KGaA, Darmstadt, Germany, with the rest of the chemicals purchased from Kemika, Zagreb, Croatia. MS-PPOH and HET0016 were gifts from John R. Falck, Southwestern Medical Center, Dallas, Texas, U.S. The chemicals used to determine the oxidative stress were thiobarbituric acid (TBA; Sigma-Aldrich, U.S.), trichloroacetic acid (TCA; Panreac, Europe) and 1,1,3,3-tetramethoxypropane (TMP; Sigma-Aldrich).

Statistical analysis

All data are summarized as means ± SEM. Two-way ANOVA tests and Bonferroni post hoc tests were used to test differences in ACh-induced relaxation among groups. Half maximal effective concentration (LogEC50) ACh values were compared by One-Way ANOVA and Tukey post hoc tests. One-Way ANOVA and Tukey post hoc tests were used to measure the difference in body weight, blood glucose, oxidative stress level and gene expression among groups. A probability of $P \leq 0.05$ was considered to be statistically significant. SigmaPlot, version 11.2 (Systat Software, Inc., Chicago, Illinois, U.S.) and GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, California, U.S.) were used for statistical analysis and for the graphic presentation of the obtained results.

Table 2. Body weight, blood glucose and oxidative stress level of 12-week-old female rats (n=20 per group)

parameters	CTR	DM	CTR+HBO	DM+HBO
body weight, g	237 ± 6	179 ± 14 *	240 ± 5	209 ± 5 †‡
blood glucose, mmol/L	6.5 ± 0.2	32.5 ± 0.5 *	7.2 ± 0.2	28.7 ± 0.9 †
TBARS, μmol MDA	0.65 ± 0.10	1.03 ± 0.03*	0.94 ± 0.01	1.08 ± 0.04 † §
FRAP, mM Trolox	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01

Data are presented as mean ± SEM. n - number of rats; CTR - control; DM - diabetes mellitus; HBO - hyperbaric oxygenation; TBARS - thiobarbituric acid reactive substances; MDA - malondialdehyde; FRAP - ferric reducing ability of plasma.
* P<0.05 CTR vs. DM; † P<0.05 CTR+HBO vs. DM+HBO; ‡ P<0.05 DM vs. DM+HBO; § DM+HBO vs. CTR

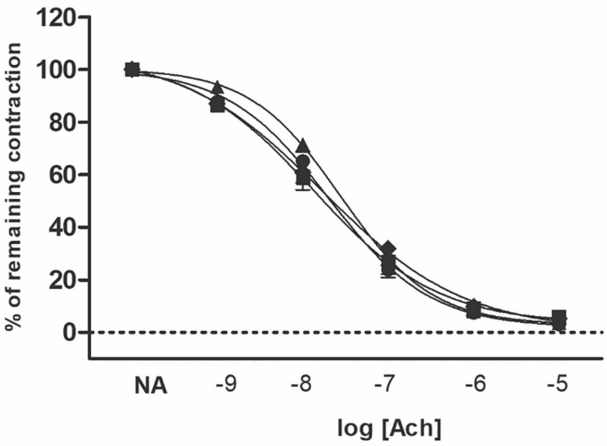
RESULTS

Body weight, blood glucose level and oxidative stress of experimental animals (at the age of 12 weeks)

Body weight, blood glucose level and oxidative stress of all experimental animals are presented in Table 2. Diabetic rats (DM) had lower body weight compared to controls (CTR), which was expected due to untreated DM. Also, diabetic rats that underwent HBO_2 (DM+HBO) treatment had lower body weight compared to healthy controls that underwent HBO_2 (CTR+HBO). Diabetic rats that underwent HBO_2 (DM+HBO) had higher body weight compared to DM rats (Table 2). As expected, blood glucose level was significantly increased in both DM and DM+HBO rats compared to their corresponding controls, which confirmed successful development of Type 1 diabetes model in our experimental protocol (Table 2). DM rats had increased TBARS compared to CTR rats (Table 2). Also, TBARS were significantly increased in DM+HBO rats compared to CTR+HBO (Table 2). Additionally, DM+HBO rats had significantly increased TBARS compared to CTR group of rats. There was no significant difference in ferric reducing ability of plasma assay (FRAP) among experimental groups of rats (Table 2).

Mechanisms of acetylcholine-induced relaxation of isolated rat aortic rings

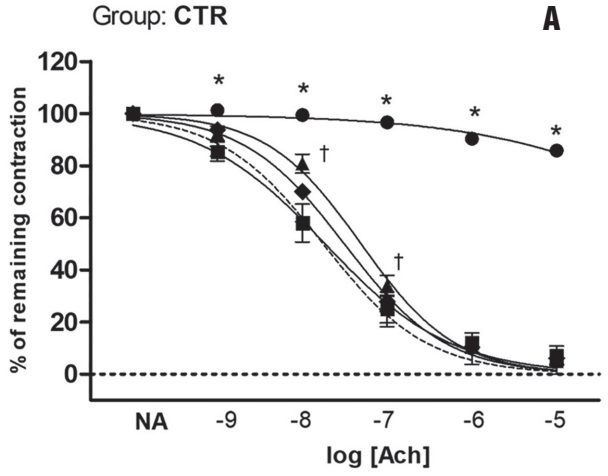
Figure 1 presents the baseline AChIR of isolated rat aortic rings in all experimental groups of SD rats. There were no significant differences in the magnitude of AChIR of isolated rat aortic rings among groups. Also, half maximal effective concentration of ACh (LogEC50) (the sensitivity to ACh) was not significantly different among experimental groups of rats (table within Figure 1).



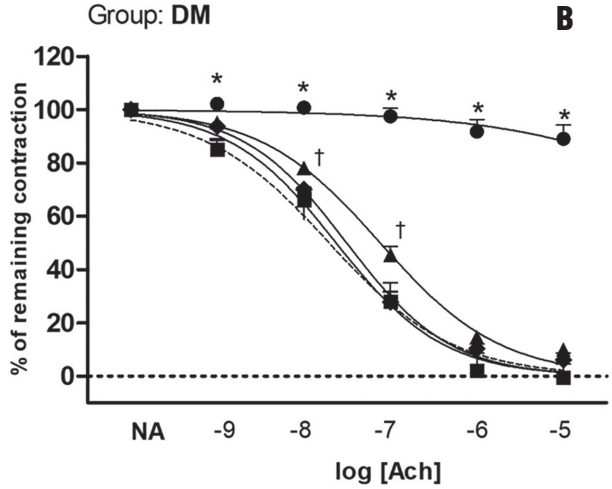
	LogEC50	EC50 (molL-1)
● control (N=20)	-7.776	1.676E-8
■ DM (N=20)	-7.872	1.343E-8
▲ CTR+HBO (N=20)	-7.552	2.807E-8
◆ DM+HBO (N=20)	-7.761	1.735E-8

Figure 1. ACh induced relaxation (AChIR) of isolated rat aortic rings in CTR, DM, CTR+HBO and DM+HBO rats.

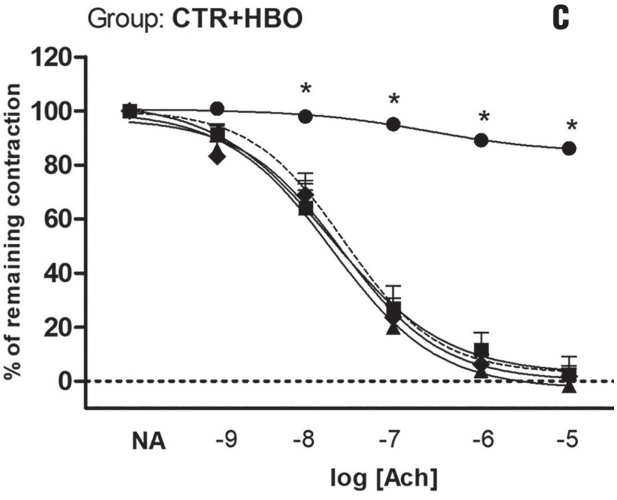
There was no significant difference in AChIR among the experimental groups of rats. There was no difference in the sensitivity to ACh among the groups (table within figure). LogEC50 values (shown in corresponding tables) were compared by one-way ANOVA test.
ACh concentration 10^{-9} to 10^{-5} molL-1. N- number of aortic rings. EC50 (molL-1)- half maximal effective concentration presents concentration of ACh (molL-1) which induces a response halfway between the baseline and maximum.



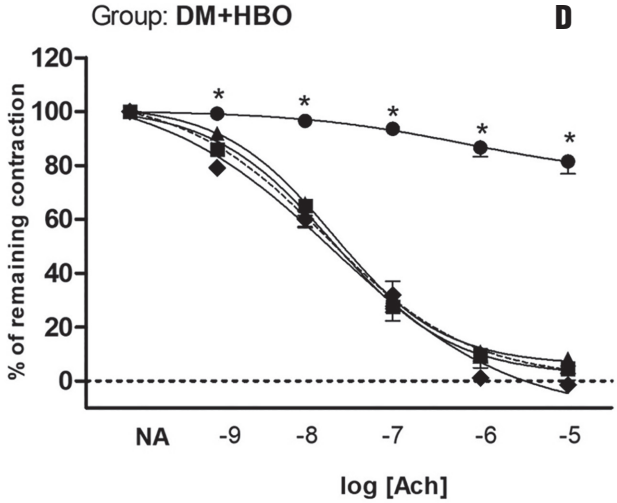
	LogEC50	EC50 (molL-1)
--- baseline (N=20)	-7.776	1.676E-08
● L-NAME (N=17)	-6.303	4.976E-07 ‡
■ INDO (N=16)	-7.950	1.222E-08
▲ MSPPOH (N=11)	-7.355	4.415E-08 §
◆ HET0016 (N=12)	-7.616	2.421E-08



	LogEC50	EC50 (molL-1)
--- baseline (N=20)	-7.872	1.343E-08
● L-NAME (N=16)	-6.552	2.803E-07 ‡
■ INDO (N=17)	-7.542	2.869E-08
▲ MSPPOH (N=11)	-7.248	5.652E-08 §
◆ HET0016 (N=7)	-7.632	2.334E-08



	LogEC50	EC50 (molL-1)
--- baseline (N=20)	-7.552	2.807E-08
● L-NAME (N=16)	- 6.600	2.515E-07 ‡
■ INDO (N=10)	-7.711	1.944E-08
▲ MSPPOH (N=8)	-7.692	2.033E-08
◆ HET0016 (N=9)	-7.638	2.303E-08



	LogEC50	EC50 (molL-1)
--- baseline (N=20)	-7.761	1.735E-08
● L-NAME (N=16)	-6.150	7.086E-07 ‡
■ INDO (N=16)	-7.693	2.029E-08
▲ MSPPOH (N=12)	-7.677	2.106E-08
◆ HET0016 (N=14)	-7.732	1.851E-08

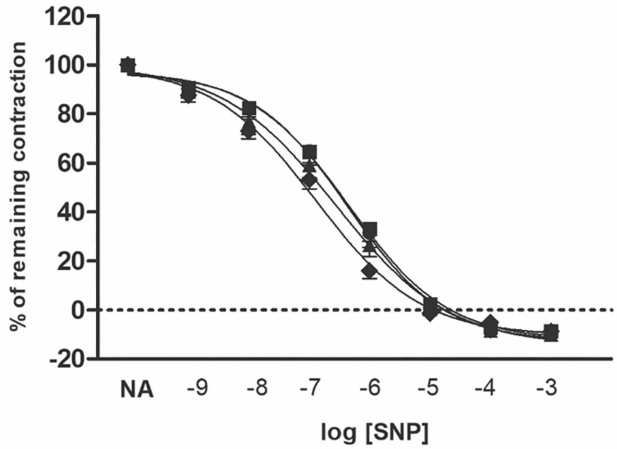
Figure 2. Mechanisms of acetylcholine induced relaxation (AChIR) response of isolated rat aorta rings in CTR (Panel A), DM (Panel B), CTR+HBO (Panel C) and DM+HBO rats (Panel D).

The presence of L-NAME and MS-PPOH significantly reduced AChIR of isolated rat aortic rings in CTR group (Panel A), and DM group of rats (Panel B). Indomethacin and HET0016 administration did not have any significant effect on AChIR in any group, while MS-PPOH administration did not have any significant effect on AChIR in both CTR+HBO (Panel C) and DM+HBO groups (Panel D). Data were compared by two-way ANOVA and Bonferroni post hoc tests. *~ continued on next page*

Potential mechanisms mediating AChIR response of isolated rat aortic rings in experimental groups of rats are presented in Figure 2. In the CTR group the presence of L-NAME and MS-PPOH significantly reduced the AChIR, while INDO (COX-1 and -2 inhibitor) and HET0016 (20-HETE formation inhibitor) administration did not have any significant effect on AChIR in the CTR group of rats (Figure 2, Panel A). Similar results were obtained in the DM group of rats (Figure 2, Panel B). In both CTR and DM groups of rats, analysis of aortic ring sensitivity to ACh demonstrated that the sensitivity to ACh in the presence of L-NAME and in the presence of MSPPOH was significantly decreased compared to the response to ACh alone or in the presence of indomethacin or HET0016 (tables within Panels 2A and 2B). In the CTR+HBO and DM+HBO groups, only the presence of L-NAME, but not MS-PPOH, INDO or HET0016, significantly reduced the AChIR (Figure 2, Panels C and D). Furthermore, in the CTR+HBO and DM+HBO groups, the sensitivity to ACh was significantly lower in the presence of L-NAME compared to the basic response or the response to ACh in the presence of indomethacin, MS-PPOH or HET0016 (Figure 2, tables within Panels 2C and 2D).

Sodium nitroprusside-induced relaxation of isolated rat aortic rings

Figure 3 presents the relaxation of aortic rings in response to SNP (SNPIR), an endothelium-independent NO donor in all experimental groups of SD rats. There were no significant differences in the SNPIR among groups. Also, the sensitivity to SNP was not significantly different between experimental groups of rats (table within Figure 3).



	LogEC50	EC50 (molL-1)
● control (N=9)	-6.357	4.391E-7
■ DM (N=18)	-6.558	2.764E-7
▲ CTR+HBO (N=11)	-6.346	4.505E-7
◆ DM+HBO (N=16)	-6.884	1.307E-7

Figure 3. SNP induced relaxation (SNPIR) of isolated rat aorta rings in CTR, DM, CTR+HBO and DM+HBO rats.

There was no significant difference in SNPIR between the experimental groups of rats. Also, there was no difference in the sensitivity to SNP between experimental groups of rats (table within figure). LogEC50 values (shown in corresponding tables) were compared by one-way ANOVA test. SNP concentration 10^{-9} to 10^{-3} molL-1. N- number of aortic rings. EC50 (molL-1)- half maximal effective concentration presents concentration of SNP (molL-1) which induces a response halfway between the baseline and maximum.

mRNA expression of enzymes catalyzing vasoactive mediators in rat aorta

mRNA expression of iNOS in rat aortic tissue was significantly increased in DM compared to CTR group of rats (Table 3). eNOS mRNA expression was significantly increased in CTR+HBO compared to CTR rats, while TBXAS1 mRNA expression was significantly increased in DM+HBO compared to DM rats (Table 3). COX-1, COX-2, CYP2J3, CYP4A1 and CYP4A3 mRNA expression in thoracic aorta did not significantly differ among the groups (Table 3).

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The sensitivity to ACh in the presence of L-NAME and MS-PPOH was significantly decreased compared to response to ACh alone or in the presence of Indomethacin or HET0016 in both CTR and DM groups of rats (tables within Panel A and Panel B). Sensitivity to ACh in the presence of L-NAME was significantly decreased compared to response to ACh alone or in the presence of other inhibitors in both CTR+HBO and DM+HBO groups of rats (tables within Panel C and Panel D). LogEC50 values were compared by one-way ANOVA followed by Holm-Sidac pairwise multiple comparison. Statistically significant ($p < 0.05$) AChIR in the presence of L-NAME (*) or MS-PPOH (†) compared to baseline ACh response. ‡ $P<0.05$ L-NAME vs. baseline, Indomethacin and MS-PPOH; § MS-PPOH vs. baseline and Indomethacin. N- number of aortic rings. EC50 (molL-1)- half maximal effective concentration presents concentration of ACh (molL-1) which induces a response halfway between the baseline and maximum.

Table 3. Relative mRNA expression of eNOS, iNOS, COX-1, COX-2, TBXAS1, CYP2J3, CYP4A1 and CYP4A3; to HPRT1 in thoracic aorta tissue (n=5 per group)

parameters	CTR	DM	CTR+HBO	DM+HBO
eNOS	0.10 ± 0.01	0.15 ± 0.03	0.16 ± 0.02 §	0.17 ± 0.04
iNOS	1.0E-3 ± 0.3E-3	0.03 ± 0.02 *	2.0E-3 ± 1.0E-3	8.0E-3 ± 2.0E-3
COX-1	0.13 ± 0.01	0.13 ± 0.04	0.14 ± 0.02	0.09 ± 0.02
COX-2	0.16 ± 0.06	0.12 ± 0.08	0.12 ± 0.04	0.09 ± 0.03
TBXAS1	1.12 ± 0.15	1.17 ± 0.11	1.23 ± 0.09	1.67 ± 0.11 ‡
CYP2J3	2.64 ± 0.30	1.93 ± 0.43	2.80 ± 0.23	1.75 ± 0.24
CYP4A1	0.06 ± 0.02	0.05 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
CYP4A3	1.4E-6 ± 2.5E-7	0.8E-6 ± 2.8E-7	1.7E-6 ± 2.5E-7	1.0E-6 ± 1.9E-7

Data are presented as mean ± SEM. n - number of rats; CTR - control; DM - diabetes mellitus; HBO - hyperbaric oxygenation; eNOS - endothelial nitric oxide synthase; iNOS - inducible nitric oxide synthase; COX-1 - cyclooxygenase 1; COX-2 - cyclooxygenase 2; TBXAS1 - thromboxane A synthase 1; CYP2J3 - cytochrome P450 2J3; CYP4A1 - cytochrome P450 4A1; CYP4A3 - cytochrome P450 4A3. * $P < 0.05$ CTR vs. DM; ‡ $P<0.05$ DM vs. DM+HBO; § CTR vs. CTR+HBO

DISCUSSION

Abundant evidence shows that diabetic patients are at high risk for development of CVDs (e.g., coronary heart disease, stroke, peripheral arterial disease, cardiomyopathy, congestive heart failure), and that cardiovascular complications are now the leading causes of diabetes-related morbidity and mortality [30]. It is well established that endothelial dysfunction accompanies DM, and manifests in form of vascular complications that are evident in both macro- and microcirculation [31]. Still, even though vascular alterations are commonly described in patients and experimental animals with diabetes, endothelium-dependent relaxation (assessed by ACh-induced vascular relaxation of isolated rat aortic rings) has been shown to be either unaffected, attenuated or even augmented in experimental diabetic animals [32,33].

Salient finding of the present study is that six weeks of Type 1 DM in female SD rats did not affect the magnitude of the AChIR of isolated aortic rings; however, HBO_2 affected the mechanisms of AChIR. Furthermore, even though DM did not affect the AChIR in female rats, oxidative stress level was significantly increased in both the DM and DM+HBO groups of diabetic rats when compared to controls. These results indicate very early changes in oxidative balance in DM, apparently before measurable functional vascular impairment could be identified. Finally, HBO_2 effects on the mechanisms of AChIR are not related to the level of oxidative stress in any group of female rats.

Increased oxidative stress underlies impaired vascular reactivity in many cardiometabolic diseases, including DM [17,34-37]. However, in the present study increased oxidative stress in the DM group of rats did not affect the mechanisms of ACh-induced vascular relaxation (Table 2, Figure 1). It is known that acute HBO_2 (i.e., single exposures up to 3 ATA) can enhance production of reactive oxygen species (ROS) and increase TBARS [38,39]. Our results show elevated levels of TBARS in both diabetic groups compared to their respective controls, while intermittent HBO_2 did not additionally increase the level of oxidative stress, which is consistent with our previous results [17,25,38].

A number of independent animal and human studies on diabetes reported impaired vascular function in various vascular beds and vessel calibers in terms of enhanced vascular responsiveness to vasoconstrictors and attenuated response to vasodilators [1-4]. For example, a study by Bhwardai, et al. demonstrated that after eight weeks of Type 1 DM, the AChiR of isolated aortic rings was attenuated, accompanied by a decrease in aortic and serum nitrite/nitrate concentrations and impaired aortic endothelial integrity [34]. Furthermore, aortic superoxide levels were increased, together with increased serum lipid peroxidation levels and hyperglycemia [34]. As mentioned, in the present study the mechanisms mediating aortic rings relaxation to ACh have been altered by HBO_2 (in both CTR and DM rats), and the AChIR following HBO_2 was no longer dependent on EETs. Since we did not

observe any significant HBO_2-induced changes in mRNA expression of isozymes that produce EETs, it is possible that HBO_2 affects vascular sensitivity to EETs, rather than affecting its synthesis, which could be concluded from the findings presented in Figure 2 (tables within Panels A and B). Furthermore, the sensitivity to ACh in the presence of L-NAME was also significantly decreased compared to basic response or response to ACh in the presence of indomethacin or MS-PPOH or HET0016 in both CTR+HBO and DM+HBO groups (Figure 2, tables within Panels C and D). However, in the present study, the contribution of 20-HETE or COX-1 and COX-2 metabolites to AChIR was not observed. Potential limitation of the study may be that only mRNA expression of enzymes of interest was performed, and the changes in mRNA do not necessarily provide information on enzyme activities. However, not even enzyme activities must relate to some functional changes, because the quantity of the protein present in the tissue of interest is important, and that may depend on the regulation of expression. Correlations among the mRNA, protein expression, enzyme activity and observed physiological or clinical findings are often weak or not uniform [40-42]. Thus, these kinds of data have to be interpreted cautiously.

Due to untreated disease, the body weight of DM rats was significantly decreased compared to all other groups of rats. Significant increase in blood glucose levels in DM and DM+HBO rats also confirms that DM was properly induced, as expected (Table 2). Although animals exhibited significant symptoms of untreated DM (polydipsia, polyphagia, polyuria), the six-week duration of DM did not affect the magnitude of the AChiR, which was surprising on one hand. However, our findings are partly in accordance with the study of Brown, et al., which demonstrated that DM had little effect on the AChiR of the aortic rings from male and female rats affected with DM for eight weeks and slightly attenuated SNP-induced vasorelaxation, but exhibited some effect on myocyte function, e.g., prolonged duration and maximal velocity of myocardial contraction and relaxation duration in both genders [43]. In the study by Hopfner, et al. the AChIR responses were attenuated in the 14-week group, but not in the two-week group of diabetic rats, while the endothelium-independent (SNP-evoked) responses remained unchanged [44]. This was similar to our results.

Development of changes in vascular reactivity in DM has a temporal manner [44-46]. For example, Pieper, et al. described a three-phase response to AChIR in diabetic male rats. They first saw an enhancement of vascular relaxation at 24 hours after induction of DM; further normalization of vasorelaxation after one and two weeks of DM; and finally, an impaired relaxation in response to ACh eight weeks after induction of DM in the aortic rings model [45]. A temporal manner of the development of vascular impairment could also explain the preserved AChIR in our study at six weeks of DM duration. On the other hand, our experiment was designed to elucidate potential effects of HBO_2 treatment on the mechanisms of AChIR as early as possible, even before manifested vascular complications. Thus, the present results demonstrated that the mechanisms mediating AChIR were affected by HBO_2, although the magnitude of the response (relaxation to ACh) was not affected either by DM or by HBO_2. In such an early phase of DM we failed to demonstrate that the EETs pathway was robustly involved in the vascular relaxation in HBO_2 groups. Some other studies have shown that in female rats in an early DM phase, i.e., at two weeks of DM, there was a modulation of thromboxane A2 production, but no change in the NO system (in its production or in its metabolic pathway, such as phosphodiesterase isoform activities) was observed. At four weeks of DM duration, a reduction in NO activity was superimposed, and the activity of phosphodiesterase was reduced, while the production of vasodilatory prostaglandins was increased, possibly as a compensatory mechanism to maintain normal vascular reactivity [46]. This seems to be specific to female sex [38], since it opposes our findings in male diabetic rats, treated by the same protocol, where all three vasodilator pathways were affected by DM [17].

In the present study, HBO_2 and DM seem to shift the mechanisms of vasorelaxation toward a NO-dependent pathway. The expression profile of enzymes involved in vasoreactivity supports this conclusion, since there was a significant increase in eNOS gene expression in CTR+HBO rats, compared to CTR rats, while iNOS gene expression was significantly increased in DM rats compared to CTR rats. Similar observations were found in the aortas of the Goto-Kakizaki (GK) rat model of genetic Type 2 DM, which exhibited an increased protein expression of eNOS and a decrease in the level of its co-factor tetrahydrobiopterin (BH4). Since GK rats had impaired relaxation to ACh with a significantly enhanced superoxide production and decreased NO bioavailability, the observed increased eNOS protein expression may be of compensatory nature [35]. Our results are in agreement with these observations. However, all changes observed in our study are modest compared to changes in male

DM rats undergoing HBO_2 [17], which might possibly be due to previously observed enhanced ACh-induced vasorelaxation in female compared to male rats (38). In the present study, the relaxation in response to SNP was preserved in all groups (Figure 3), suggesting that a six-week duration of DM and HBO_2 per se do not adversely affect vascular smooth muscle cell responsiveness to NO.

CONCLUSION

The present study suggests that HBO_2 alters the mechanisms of endothelium-dependent vasorelaxation to ACh in female rats very early in the development of Type 1 DM. These effects are slightly different from the previously assessed mechanisms of AChIR in male rats [17]. Since vasorelaxation to endothelium-dependent and -independent stimuli is preserved after six weeks of DM, NO pathway may be upregulated to compensate for decreased sensitivity to both NO and CYP450 vasodilator metabolites of arachidonic acid in DM.

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Conflict of interest statement

The authors declare no conflict of interest exists with this submission.

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