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Effect of oxidative stress on angiotensin II-induced smooth muscle contractile activity of urinary bladder from fructose fed rats

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The persistent hyperglycemia in the diabetes and metabolic syndrome causes a generation of reactive oxygen species (ROS) and can seriously violate the oxidative homeostasis. This could affect the oxidative sensitive signal transduction pathways, thus contributing to the pathogenesis of some later complications as is the smooth muscle dysfunction. The purpose of this study was to examine the effects of fructose intake on the oxidative homeostasis and on Angiotensin II (AngII) – induced motility of the urinary bladder. Mature Wistar rats were randomly divided into two groups (9 rats per group): control group (drinking tap water) and fructose-drinking group (15% fructose, dissolved in tap water). The duration of the experiment was 12 weeks. In the end of the experimental period, strips from urinary bladder were prepared and influenced by AngII. The curves of contractions were analyzed and the parameters of the contractile process were calculated. Detection of the oxidative status was performed by the evaluation of ascorbate radicals, ROS production and lipid peroxidation in tissue homogenates from liver, kidneys and blood. The plasma glucose and some parameters of lipid metabolism were registered. The developed metabolic disturbances decreased force parameters, changed the time profile characteristics, and reduced the speed of AngII-stimulated urinary bladder contraction. The oxidative imbalance was clearly demonstrated by the elevated levels of NO• and reactive oxygen radicals. Metabolic and oxidative disturbances as a result of fructose-fed diet modified the smooth muscle contractile activity and led to a smooth muscle dysfunction.

Keywords: Angiotensin II, fructose, oxidative imbalance, urinary bladder

INTRODUCTION

The metabolic syndrome (MetS) is a cluster of clinical and biochemical features that include abdominal obesity, insulin resistance and dyslipidemia. It is well known that MetS is associated with the increased risk of developing type 2 diabetes (T2D) [1]. Nowadays, the growing incidence of disorders in many smooth muscle organs is frequently observed in patients suffering from T2D and MetS. The lower urinary tract symptoms (LUTS) are among the first significant complications connected with the progression of the mentioned diseases [2]. LUTS are found to be common and chronic, with over 50% of the patients suffering from some form of urinary bladder dysfunction (urinary incontinence, detrusor underactivity or detrusor overactivity, etc.) [2, 3]. The changes in the bladder contractility during T2D may be due to a variety of factors, including alterations in innervation, changes in signaling between urothelium and smooth muscle or within the smooth muscle itself [2]. Interestingly, MetS is considered as an independent risk factor of bladder dysfunction.

According to Lee [3], LUTS are positively associated with MetS with or without diabetes. This raises the question for the role of the persistent hyperglycemia and the other biochemical features as the underlying factors for the development of LUTS even before the actual manifestation of the diabetes. The persistent hyperglycemia causes a generation of reactive oxygen species (ROS) and can seriously violate the oxidative homeostasis. Oxidative stress (OS) is defined as an imbalance between ROS production and the antioxidant activity in cell and plasma, resulting in accumulation of oxidative products. The role of OS in many pathological conditions such as MetS and diabetes is well-recognized [4, 5]. The excess accumulation of oxidative products could affect the oxidative sensitive signal transduction pathways, thus contributing to the pathogenesis of the metabolic disorders as is the smooth muscle dysfunction. On the other hand, the metabolic disturbances are connected with the activity of circulated and local rennin–angiotensin systems (RAS) which are one of the main regulators of

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smooth muscle tone and contractility. As a main effector of RAS, Angiotensin II (AngII) has various actions, many of them affecting the activity of the visceral smooth muscles of the urogenital tract [6]. Moreover, a crosstalk between AngII and the insulin resistance [1, 7], as well as RAS and the lipid metabolism [8] has been reported. The established role of RAS in diabetes and its interaction with insulin signaling and some other aspects of metabolism [9], focused our interest towards the effects of AngII on diabetic visceral smooth muscles. Despite the observed development of diabetic smooth muscle dysfunction in a number of organs, the information about how MetS and T2D change urinary bladder response to Ang II is still insufficient.

The purpose of this study was to examine the effects of the fructose diet on the oxidative metabolic homeostasis and on Ang II – induced motility of the urinary bladder of Wistar rats.

EXPERIMENTAL

Animals and sample preparation

All of the experiments were carried out according to the Guide for the Care and Use of Laboratory Animals and the guidelines of the Council Directive 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes. The animal experiments were approved by the Commission of Ethics at the Medical Faculty of Trakia University, Stara Zagora.

Mature male Wistar rats, weighing 250-300 g, housed in polycarbonate wire floor cages in controlled conditions (12 h light/dark cycles), temperature of 18–23°C and humidity of 40–60%, were divided into two groups (9 rats per group): control group (healthy animals) and fructose group (animals drinking 15% fructose dissolved in tap water and injected after 14 days with streptozotocin 20 mg/kg i.p.). The duration of the experiment was 12 weeks. At the end of the experimental period the animals were anesthetized with Nembutal 50 mg/kg i.p. and exsanguinated. Fresh blood (8-10 ml) was collected directly from the heart in cold EDTA-containers (5 ml Monovette, Germany). After centrifugation of the blood (4000 rpm at 4°C for 10 min), several samples of plasma (200 µl) from each animal were stored at 4°C until further assay was done. The liver and kidneys were immediately isolated and washed in cold saline (4°C). After homogenization and addition of solvents the samples were centrifuged at 4000 rpm at 4°C for 10 min and 300 µl of supernatant from each sample were stored at -4°C until further assay was done. The urinary bladder was dissected out and transferred immediately in cold Krebs solution (3°C). The composition of Krebs solution, the preparation of the urinary bladder strips and the recording of mechanical activity were as it was previously described [10].

Chemicals and drugs

AngII (Sigma-Aldrich, Germany) was solubilized in bidistilled water. Streptozotocin (Sigma-Aldrich, Germany) was dissolved in cold 0.1 M citrate buffer, pH 4.5. Dimethyl sulfoxide (DMSO), N-tert-butyl-alpha-phenylnitrone (PBN), 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide (Carboxy-PTIO.K), PBS and all reagents for the preparation of Krebs solution were purchased from Sigma-Aldrich Chemie GmbH, Germany. All other chemicals used in this study were of analytical grade.

Biochemical and EPR analyses

The biochemical analyses were performed on a UV–VIS spectrophotometer-400 (THERMO Sci., RS232C, Stratagene, USA). Total cholesterol and triglycerides were estimated using a commercially available diagnostic kit (AM- 2035- KA, 2017). Blood glucose levels were measured by Medisign mm 810 glucometer (Empecs Medical Device Co. Ltd., China). The electron paramagnetic (EPR) measurements were performed on an X-band EMXmicro spectrometer (Bruker, Germany) with settings: 3505 g centerfield, 6.42 mW microwave power, 5 g modulated amplitude, 1-5 scans. All experiments were made in triplicate.

Estimation of plasma lipid peroxidation

The method of estimation of lipid peroxidation of thiobarbituric acid (TBA), which measures malondialdehyde (MDA)-reactive products, was used [11]. In brief, 0.5 mg of fresh spleen-tissues, 1 ml of physiological solution, and 1 ml of 25% trichloroacetic acid were mixed and centrifuged at 7,000 rpm for 20 min. 2 ml of protein-free supernatant with 0.5 ml of 1% TBA (prepared in 0.025 M NaOH) were added to the reaction mixture. The resultant mixture was then subjected to 95°C in a water bath for 1 h. A pink-coloured chromogen complex was formed, readable at 532 nm.

Estimation of plasma antioxidant enzymes

The activities of superoxide dismutase (SOD) and catalase (CAT) were determined using the method described by Sun *et al.* [12] and by Aebi [13], respectively.

Estimation of plasma EPR ex vivo evaluation of nitric (NO•) radicals

Plasma NO• radicals were studied by the methods of Yoshioka *et al.* [14] and Yokoyama *et al.* [15], adapted for EPR estimation of the spin-adduct formed between carboxy-Ptio.K and generated radicals. EPR settings were as follows: 3505 G centerfield, 6.42 mW microwave power, 5G modulation amplitude, 75G sweep width, 2.5×102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Estimation of plasma EPR ex vivo evaluation of ascorbate radicals (Asc•)

The Asc• levels in organ homogenates were studied according to Buettner and Jurkiewicz [16] with slight modifications, adapted for EPR estimation. EPR settings were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1×104; modulation amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scan per sample.

Estimation of cellular ex vivo evaluation of ROS production

Liver and kidneys tissue homogenates (100 mg) and 100 µl of plasma were homogenized with 900 µl of 50 mM spin-trap PBN dissolved in DMSO using sonication at one cycle for 2 min. After 5 min of ice incubation, the suspension was centrifuged at 4000 rpm at 4ºC for 10 min. Supernatants were transferred into cold Eppendorf tubes and immediately analysed. The real-time formation of ROS production in the supernatant was estimated according to methods described earlier [17] with some modifications [18]. EPR settings were as follows: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1× 106; modulation amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92 s, 5 scans per sample.

Analysis of the contractile activity

After the equilibration, the preparations from urinary bladder (n=9 for each group) were influenced by AngII (1 µmol). The mechanical activity was digitized and recorded using ISOSYS-Advanced 1.0 software (Experimetria, Ltd., Hungary). The conversion of the data for later analysis was performed with KORELIA-Processing and KORELIA-Dynamics programs [19, 20]. The duration of the interval for analysis of the tonic contraction was defined from the beginning of the contraction, until the amplitude fell to 50%. The amplitude of contraction, the integrated force of contraction (AUC) and following time-parameters [21]: half-contraction time (T_{hc}); contraction time (T_c); half-relaxation time (T_{hc}); contraction plus half-relaxation time (T_{chr}), were examined.

Statistical analysis

EPR spectral processing was performed using Bruker Win-EPR and SimFonia Software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student *t*- test to determine significant differences among data groups. The results were expressed as mean ± standard error (SE). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Biochemical analysis

As it can be seen from Table 1, the levels of blood glucose were significantly increased in the experimental group compared to the controls. The same tendency was observed about the levels of plasma lipids. There were significant differences between the two groups with exception of the levels of total cholesterol. The most significant difference was observed in the levels of triglycerides in the fructose group, which concentration was twice as high compared to controls (Table 1).

Table 1. Plasma glucose and lipids concentration.

Parameter	Control group (n=9)	Fructose group (n=9)
Glucose (mmol/l)	9.84±0.91	12.59±0.62*
Total Cholesterol (mmol/l)	1.33±0.04	1.24±0.08
LDL (mmol/l)	0.43±0.04	0.28±0.05*
HDL (mmol/l)	0.54±0.02	0.43±0.04*
Triglycerides (mmol/l)	0.81±0.06	1.73±0.36*
Total Cholesterol/ HDL ratio	2.51±0.08	2.92±0.14*
Triglycerides/ HDL ratio	1.54±0.13	3.74±0.6*

**vs* controls, $p < 0.05$

Estimation of the oxidative stress

The levels of oxidative stress (Table 2) were measured by investigation of the antioxidant activity of SOD and CAT, the concentration of MDA and EPR analysis of free radicals. In regard to the antioxidant activity of SOD and CAT there were observed no statistically significant differences between the two investigated groups. On the other hand, there was a significant decrease in the levels of MDA in the plasma, liver and kidneys in the experimental group compared to the control one. The EPR analysis of free radicals demonstrated big differences in both plasma and tissue homogenates. Measured Asc• in all samples were significantly decreased in the experimental group compared to control. Exactly the opposite statistically significant differences were observed in the levels of NO•. Concerning the levels of ROS in plasma, there were no statistical differences between both groups. The levels of ROS were significantly decreased in the livers from the experimental group, while in kidneys there was a significant increase compared to the control group.

Smooth muscle contraction

Fig.1 represents a graphical visualization of the contractile process of AngII-induced urinary bladder activity in the different groups. The amplitude of the contractions of the bladders from the fructose group was significantly lower than that of the control one (0.98±0.08 g *vs*. 1.74±0.22 g). There was no significant difference between both investigated groups regarding AUC (106.53±10.7 gs *vs.* 121.13±13.7gs). The investigation of time parameters demonstrated a significant delay in developing urinary bladder contraction of the animals from the fructose group (T_{hc} – 24.9±4.6 s *vs*. 13.3±1.7 s; T_c – 60.6±10.4 s *vs.* 33±3.2 s; T_{chr} – 134.3±23.4 s *vs.* 83±7 s).

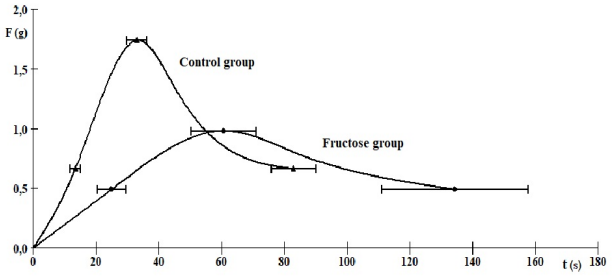

Fig. 1. Graphical visualization of the contractile process of Ang II-induced urinary bladder activity in the different groups (control and fructose, n=9).

DISCUSSION

The goal of the present study was to examine the effects of the fructose diet on the oxidative metabolic homeostasis and on AngII – induced urinary bladder motility. In our experiment, the fructose drinking rats showed a statistically significant worsening of both glucose and lipid profile.

Table 2. Levels of oxidative stress in plasma and tissue homogenates.

\		Controls (n=9)	Fructose group(n=9)
SOD (IU/gHb)	Plasma	467.1±32	392.3±56
	Liver	660.8±48	603.5±20
	Kidneys	479.7±28	507.8±18
CAT (IU/gHb)	Plasma	63845±4852	72364±6123
	Liver	53775±4823	63840±5982
	Kidneys	74849±5584	67977±5122
MDA (µmol/l)	Plasma	3.06±0.52	1.41±0.2*
	Liver	2.76±0.35	1.58±0.18*
	Kidneys	3.32±0.46	1.00±0.12*
ROS (Arb. Units)	Plasma	18.12±2.05	17.19±1.98
	Liver	18.43±1.82	11.64±1.3*
	Kidneys	17.96±1.96	28.59±3.2*
NO• (Arb. Units)	Plasma	7.01±0.8	9.17±0.95*
	Liver	6.66±1.1	13.74±2.1*
	Kidneys	12.28±2.3	16.1±2.5
Asc• (Arb. Units)	Plasma	0.74±0.08	0.07±0.01*
	Liver	0.61±0.08	0.05±0.01*
	Kidneys	2.62±0.18	0.12±0.01*

**vs* controls, $p < 0.05$

SOD – superoxide dismutase; CAT – catalase; MDA – malondialdehyde; ROS – reactive oxygen species; NO• – nitric radicals; Asc• – ascorbate radicals.

In this regard, particularly indicative were the total cholesterol/HDL-ratio and triglycerides/HDL-ratio, which are recently considered to be more significant markers for metabolic risk than the absolute values themselves [22]. At the same time, the low levels of MDA in the fructose group are impressive, and are not in accordance with the classical conception for the oxidative stress constellation (high levels of lipid peroxidation and ROS). In the studies of some authors [23, 24], although not statistically significant, inversely proportional relationship between the high triglyceride levels and the low MDA concentration was reported. In our study, the lower MDA levels in the plasma and tissue homogenates of fructose drinking rats are probably associated with the 2-fold higher triglyceride levels in the experimental group, rather than directly indicating the lipid peroxidation level. As more specific and more accurate, EPR methods reliably reveal the levels of oxidative stress. In these tests, the levels of Asc• radicals were statistically lower in all of the samples from the experimental group, thus clearly showing the depletion of the stores for ascorbic acid. On the other hand, ROS and NO• were significantly increased in most of the samples, which indicates the presence of oxidative stress in the experimental group. These results are in agreement with the majority of the authors describing the primary role of OS in the development of MetS and T2D [4, 5].

OS causes alterations on proteins, lipids and DNA, thus leading to organ dysfunction. Regarding the smooth musculature, there are controversial data about the contractility (enhanced or decreased) of the urinary bladder in diabetic animals, depending on the different design of the studies (diabetes induction methodology, used species, etc.), but the registered reduced contractility to muscarinic agonists is reported as a common result [2]. The observed and described by us reduced contractile responses to AngII are also in support to the stated above. Obviously, the metabolic disturbances and OS seriously affect the reaction of the urinary bladder to contractile agents. The excessive formation of ROS, which leads to OS and disrupted cell calcium signaling machinery, is probably the main etiological factor for this disturbance [3]. According to Wang *et al.* [25], the accumulation of glycation end products is likely to play a central role in the development of the bladder dysfunction. Moreover, many diabetic patients with bladder dysfunction have suffered from metabolic perturbations [3]. Many authors conclude that there is a relationship between the duration of diabetes and the bladder dysfunction [3, 25, 26]. With longer periods of diabetes-induced hyperglycemia the bladder characteristics progress toward decompensation and underactivity. This decompensated phase is probably a result from the polyuria and the hyperglycemic-induced metabolic disturbances and OS [25, 27].

In a conclusion, in our study the oxidative imbalance was clearly demonstrated by the elevated levels of NO• and reactive oxygen radicals. Metabolic and oxidative disturbances as a result of fructose diet modified the smooth muscle contractile activity and led to a urinary bladder dysfunction.

Disclosure of interest: *The authors declare that they have no conflict of interest.*

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