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Influence of NF-κB inactivation with ammonium pyrrolidinedithiocarbamate on nitric oxide production in rat heart during metabolic syndrome

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Obesity has become an epidemic since almost 1/6th of the Earth's population is suffering from obesity and obesity-induced pathological states. One of the key events in the pathogenesis of obesity and its complications (metabolic syndrome, type II diabetes) is NF-κB activation. Therefore, searching for drugs affecting NF-κB activation during metabolic syndrome (MetS) is a perspective way of its treatment. The purpose of this study was to evaluate the influence of NF-κB inactivation with ammonium pyrrolidinedithiocarbamate on nitric oxide production, activity of arginases, concentration of nitric oxide metabolites, and hydrogen sulfide content in the rat heart during MetS modelling. We conducted our study on 24 mature male Wistar rats weighing 200-260 g, divided into 4 groups: control group, MetS group, ammonium pyrrolidinedithiocarbamate (PDTC) administration group, and PDTC+MetS group. MetS was induced by introducing of 20% fructose solution as the only source of water for 60 days. PDTC was administered intraperitoneally thrice a week at a dose of 76 mg/kg. In a 10% rat heart homogenate, we analyzed: total NO-synthase (NOS) activity, inducible (iNOS) and constitutive NO-synthase (cNOS) activities, nitrite and nitrate reductase activities, nitrite, peroxynitrite, nitrosothiols and hydrogen sulfide content. In PDTC+MetS group, iNOS activity decreased by 22.6% compared to MetS group; arginase activity decreased by 20.9%; activity of nitrate and nitrite reductases decreased by 24.3% and 32.3%, respectively. Nitrite concentration increased by 25.8%, while ONOO- levels decreased by 56.7% and nitrosothiol levels increased by 57.9%. H2S concentration decreased by 11.6%. The activation of transcription factor NF-κB during the development of metabolic syndrome leads to hyperproduction of nitric oxide due to excessive activity of inducible NO-synthase and nitrite reductases.

Key words: heart; metabolic syndrome; nitric oxide; NF-κB; ammonium pyrrolidinedithiocarbamate.

INTRODUCTION

With the development of material culture of human civilization, the populations of developed countries received almost unlimited access to food. In the USA, a person can go to the charitable establishment called "Food Bank" and take enough supply of nutrients to live a comfortable life. The nutritional quality of food provided by the "Food Bank" came into attention of researchers [1]. In most cases, the food received from the "Food Bank" is reach in carbohydrates and fats, promoting the Western type diet behavior among recipients of such charity. Scientific literature provides evidence that frequent or constant consumption of "Western type diet" is one of the factors leading to obesity [2].

Obesity in the modern world becomes an epidemic if we take into account that almost 1/6th of populace of Earth is suffering from obesity and obesity-induced pathological states [3]. One of the key events in pathogenesis of obesity and its complications (metabolic syndrome, type II diabetes) is activation of metabolic-induced inflammation (metaflammation) [4]. Metaflammation is a systemic process, which affects all organs and tissues and involves changes in polarization of tissue macrophages from M2 (anti-inflammatory) to M1 (pro-inflammatory) phenotype. Phenotype change of tissue macrophages is usually accompanied by excessive production of pro-inflammatory cytokines and

nitric oxide, driven by the inducible isoform of NO-synthase (iNOS) [5].

Nitric oxide (NO) is an extremely important gaseous mediator for physiology of the heart. A decrease in its production from constitutive isoforms of NO-synthase (cNOS), like endothelial NO-synthase, may lead to insufficient relaxation of blood vessels in the heart, which may eventually lead to ischemia and hypoxic damage to the heart muscle tissue [6]. On the other hand, under conditions of excessive production of NO in the tissues reaction of NO with superoxide (SAR) becomes possible resulting in formation of toxic peroxynitrite ($ONOO^-$), which can cause development of nitrosative stress by nitrosylation of organic components of the cell (mainly proteins in nature) [7].

Obesity is often seen as one of the components of more severe systemic process, namely, MetS. During obesity and metabolic syndrome, increased expression of iNOS is often observed, leading to heightened nitric oxide production [8, 9]. Excessive nitric oxide production and development of metaflammation during obesity and MetS is often caused by activation of transcriptional factor NF-κB [10]. Therefore, search for drugs affecting activation of transcriptional factor NF-κB during metabolic syndrome is a perspective way of its treatment.

The purpose of this study was to evaluate influence of NF-κB inactivation with ammonium pyrrolidinedithiocarbamate on nitric oxide production, activity of arginases, concentration of nitric oxide metabolites and hydrogen sulfide content in rat heart during metabolic syndrome modelling.

METHODS

We conducted our study on 24 mature male Wistar rats weighing 200-260 g, which were divided into 4 groups of 6 animals each. The first group was a control group. The second group is the experimental metabolic syndrome group (MetS group). Experimental MetS was reproduced by using a 20% fructose solution as the only source of water for 60 days [11]. The third group is the group of ammonium pyrrolidinedithiocarbamate (PDTC) administration group. PDTC administration was carried out according to the following scheme: PDTC was administered at a dose of 76 mg/kg of body weight intraperitoneally three times a week during 60 days starting from the first day of experiment [12]. The fourth group is the group of the combined effect of PDTC administration and reproduction of experimental MetS (PDTC+MetS group). Animals of this group received a 20% fructose solution as the only source of water for 60 days and were injected with PDTC at a dose of 76 mg/kg of body weight intraperitoneally three times a week during 60 days starting from the first day of experiment.

Animals were kept in the vivarium of the Poltava State Medical University under standard conditions. We worked with laboratory animals according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Law of Ukraine "On the protection of animals from cruelty" (21.02.2006) and General Ethical Principles of Animal Experimentation (First National Congress on Bioethics, 2001). All manipulations with laboratory animals were approved by the Bioethics Commission of the Poltava State Medical University (Record No. 206 from 24.06.2022).

The body-mass index (BMI) was calculated following the recommendations given by Novelli E.L. et al. [13]. Right after anesthesia, rats were weighted (g), and their body length (nose-to-anus, cm) was measured. The BMI was then calculated using the formula: body weight (g) / length^2 (cm^2) [13].

In order to evaluate the development of insulin resistance, we used the Triglyceride glucose-body mass index (TyG-BMI) and Metabolic score for insulin resistance (METS-IR) index [14].

The withdrawal of animals from the experiment was carried out under thiopental anesthesia

by taking blood from the right ventricle of the heart. The blood taken from the right ventricle of the heart was used for further biochemical studies of glucose and lipid metabolism. Another object of the study was a 10% homogenate of rat heart.

Total activity of NO-synthases (gNOS) was evaluated by increase in nitrite (NO_2^-) concentration after incubation of homogenized tissue samples for 30 min. at temperature 37°C in the incubation solution (3.1 ml) containing: 2.5 ml of 161 mmol/l tris-buffer (pH 7.4), 0.3 ml of 31 mmol/l L-arginine, 0.1 ml 32 μmol/l NADPH and 0.2 ml of 10% tissue homogenate [15].

In order to evaluate the activity of constitutive isoforms of NO-synthase (cNOS) we used the following procedure: 0.2 ml of 10% tissue homogenate was taken for analysis and was incubated for 60 min at t 37°C in incubation solution (3.3 ml) containing: 2.5 ml of 152 mmol/l tris-buffer (pH 7.4), 0.3 ml of 29 mmol/l L-arginine, 0.2 ml of 545 μmol/l aminoguanidine hydrochloride and 0.1 ml of 30 μmol/l NADPH. The activity of inducible isoform of NO-synthase (iNOS) was calculated by the formula: iNOS = gNOS-cNOS [15].

We used Griess-Ilosvay reagent for nitrite estimation (1% sulfanilic acid in 30% acetic acid and 0.1% 1-naphtylamine in the same solvent). The concentration of nitrites was measured using a spectrophotometer Ulab-101 (540 nm in cuvette with optical path length of 5 mm) [15]. Total activity of arginases was assessed by difference of L-ornithine concentration before and after incubation of 0.1 ml of 10% tissue homogenate in incubation solution (0.8 ml) containing 0.5 ml of 125 mmol/l phosphate buffer (pH 7.0), 0.2 ml of 6 mmol/l L-arginine. Evaluation of L-ornithine was performed after addition 0.1 ml of modified Chinard's reactive (2.5% ninhydrin on acidic mixture consisting of 2:3 60% orthophosphoric and ice acetic acids mixed at a ratio 6:4 with water) and 1.0 ml of ice acetic acid [15].

Nitrite reductase (NiR) activity was assessed by a decrease in nitrite content after 60 min at 37°C incubation of 0.2 ml of 10% tissue homogenate in incubation medium (2.3 ml) consisting: 1 ml of 87 mmol/l phosphate buffer (pH 7.0), 1 ml of 4.35 mmol/l sodium nitrite, and 0.1 ml of 61 μmol/l NADH. Nitrites content was measured before and after incubation [15]. Nitrate reductase (NaR) activity was assessed by decrease in nitrate content after 60 min at 37°C incubation of 0.2 ml of 10% tissue homogenate in incubation medium (2.3 ml) consisting: 1 ml of 87 mmol/l phosphate buffer (pH 7.0), 1 ml of 4.35 mmol/l sodium nitrate, and 0.1 ml of 61 μmol/l NADH. We immediately took aliquot 0.2 ml of mixture to measure initial nitrate content [15].

The concentration of peroxynitrites of alkali (Na^+, K^+) and alkali-earth (Ca^{2+}) metals was measured by using its reaction with potassium iodide under pH 7.0 in 0.2 mol/l phosphate buffer with the same pH. For this purpose, we took 0.1 ml of 10% tissue homogenate, which then was solved in 3.9 ml of 156 mmol/l phosphate buffer (pH 7.0) and 1 ml of 6 mmol/l potassium iodide (final volume of 5 ml) [15].

The concentration of low molecular weight S-nitrosothiols (S-NO) was determined by increase in nitrite concentration after 30 min incubation of 0.2 ml of 10% tissue homogenate in incubation solution (2.6 ml) containing: 2.0 ml of 154 mmol/l phosphate buffer (pH 7.0), 0.1 ml of 923 μmol/l sodium fluoride, and 854 μmol/l mercury chloride [15].

The concentration of hydrogen sulfide (H_2S) was estimated by amount of a color dye formed in reaction of H_2S with specific sulfide coloring reagent (0.4 g of N,N-dimethyl-p-phenylenediamin and 0.6 g of iron (III) chloride ($FeCl_3 \cdot 6H_2O$) dissolved in 100 ml of 6 mol/l HCl) [15].

In the blood, we studied the concentration of the following metabolic substances: glucose, triglycerides (TG), total cholesterol (TC), cholesterol from low-density lipoproteins (LDL-C), cholesterol from high-density lipoproteins (HDL-C). All the above mentioned substances were evaluated by respective assays produced by "Filisit Diagnostika" (Ukraine).

Obtained results were statistically processed in Microsoft Office Excel with extension Real

Statistics 2019 (free license by Charles Zaiontz). We used non-parametric Kruskal-Wallis ANOVA followed by pair-wise Mann-Whitney analysis for estimation of statistical significance of differences between studied groups. Differences were deemed statistically significant if $P < 0.05$. To avoid multiple comparisons error, we used Bonferroni correction method. Data in tables are represented as means (M) and standard error of mean (SEM).

RESULTS AND DISCUSSION

The induction of MetS development by addition of 20% fructose solution as the only source of drinking water to the ration of rats for 60 days led to development of hyperglycemia, hyperlipidemia, dyslipidemia and increase of the TyG-BMI and METS-IR indexes, which can be assumed as evidence of heightened insulin resistance (Table 1). Blockade of transcriptional factor NF-κB activation by PDTC administration on the background of MetS modelling decreased blood sugar level, triglycerides level, total blood cholesterol and low density lipoproteins levels, as well as lowered insulin resistance as evidenced by decrease of the TyG-BMI and METS-IR indexes.

The modelling of MetS was accompanied by following changes in the heart of rats: increase in total NOS activity, mainly due to increase in iNOS activity; increase in arginase-dependent L-arginine cleavage, as evidenced by increase of arginase activity by 29.9%; activation of nitrate-nitrite pathway of nitric oxide production, as evidenced by increase in NiR and NaR activities by 155.9% and 27.9%, respectively; domination of peroxynitrites formation from nitric oxide, as evidenced by increase in peroxynitrites concentration by 320.0% and decrease in nitrite and nitrosothiols concentrations by 60.0% and 21.9%, respectively; increase in hydrogen sulfide concentration by 16.5% (Table 2).

Increased activity of iNOS observed in our study in MetS group can be explained by increased expression of iNOS genes leading to elevated concentration of this enzyme in cardiomyocytes, or by change in polarization of macrophages present in the heart muscle towards M1 phenotype. However, both of these processes are mainly controlled by activation of transcriptional factor NF-κB [16, 17]. The possibility of fructose diet-induced activation of transcriptional factor NF-κB is proven by several other authors [18, 19]. Dependence of iNOS activity on activation of transcriptional

Table 1. Metabolic changes in the rat blood and insulin resistance indexes under conditions of metabolic syndrome (MetS) and ammonium pyrrolidinedithiocarbamate PDTC administration (M ± SEM, n = 6)

Parameters	Groups			
	Control	MetS	PDTC	MetS+PDTC
Glucose, mmol/l	3.89±0.06	8.24±0.11 *	3.58±0.04 */**	5.53±0.14 */**/***
Triglycerides, mmol/l	0.90±0.06	2.75±0.06 *	1.02±0.06 **	1.91±0.13 */**/***
Total cholesterol, mmol/l	1.18±0.01	1.77±0.02 *	1.20±0.04 *	1.48±0.02 */**/***
LDL-C, mmol/l	0.17±0.005	0.28±0.01 *	0.14±0.006 */**	0.20±0.01 */**/***
HDL-C, mmol/l	0.55±0.02	0.38±0.01 *	0.58±0.01 **	0.53±0.01 */**
Rat weight, g	212.8±2.9	244.8±2.1*	210.2±2.7 **	232.2±3.4 */**/***
BMI, g/cm^2	0.52±0.02	0.66±0.01*	0.53±0.01 **	0.57±0.01 */**/***
TyG-BMI index	40.91±1.93	64.65±0.88 *	42.24±0.83 **	51.79±1.16 */**/***
METS-IR index	5.61±0.05	6.79±0.02 *	5.64±0.04 **	6.25±0.06 */**/***

Note (here and in Table 2): *the data are statistically significantly different from the control group ($P < 0.05$). ** the data are statistically significantly different from the experimental MetS group ($P < 0.05$). *** the data are statistically significantly different from the group of PDTC administration ($P < 0.05$).

factor NF-κB explains the efficacy of PDTC in decreasing the heightened activity of iNOS during combined exposure of rats to both PDTC and MetS modelling [20].

Scientific literature provides evidence, that high fructose diet increases the expression of endothelial NOS (eNOS) genes, however, it blocks eNOS phosphorylation at serine 1177, which decreases enzyme activity [21].

An increase in arginase activity observed in our study in the MetS group coincides with the findings of Vratarić et al. [22], who demonstrated that after 9 weeks of a 20% fructose solution consumption expression of Arg-1 increased in the prefrontal cortex of mice. However, Shi et al. [23] showed in their study, that fructose overconsumption may lead to a decrease of arginase activity in liver cells due to depletion of manganese (Mn) content. Therefore, fructose influence on arginase activity may be specific to the type of affected cells.

Introduction of fructose rich diet according to our results led to an increase in animal weight (by 15.0%) and BMI (by 26.9%), which allows us to state about obesity development in MetS group. Obesity by itself can be a contributing factor to increased production of nitric oxide in MetS group [24]. However, despite increased total production of nitric oxide in heart tissue the bioavailability of NO to the heart blood vessels be reduced, as evidenced by decrease of cNOS activity observed in MetS group, which can potentially lead to vascular stiffening often observed during obesity [25].

Table 2. Production and metabolism of nitric oxide in the rat heart under conditions of MetS and PDTC administration (M ± SEM, n = 6)

Parameters	Groups			
	Control	MetS	PDTC	MetS+PDTC
Total NO-synthase activity, μmol/min per g of protein	1.62±0.02	2.38±0.03*	1.81±0.13 **	1.86±0.05 */**
Constitutive NO-synthase activity, μmol/min per g of protein	0.053±0.0002	0.047±0.003*	0.049±0.0001*/**	0.051±0.0002 */**/***
Inducible NO-synthase activity, μmol/min per g of protein	1.57±0.02	2.34±0.03*	1.76±0.13 **	1.81±0.05*/**
Arginase activity, μmol/min per g of protein	2.21±0.03	2.87±0.02*	2.20±0.03 **	2.27±0.01 **
Nitrate reductase activity, μmol/min per g of protein	5.27±0.28	6.74±0.67*	5.50±0.06	5.10±0.18 **
Nitrite reductase activity, μmol/min per g of protein	4.24±0.18	10.85±0.35*	7.06±0.10*/**	7.35±0.06 */**/***
Nitrite concentration, nmol/l	7.85±0.12	3.14±0.27*	4.31±0.37*/**	3.95±0.68 *
Peroxynitrite concentration, μmol/g	0.50±0.03	2.10±0.05*	0.57±0.03 **	0.91±0.02 */**/***
Concentration of nitrosothiols, μmol/g	0.73±0.03	0.57±0.03*	0.81±0.01 **	0.90±0.01*/**/***
Concentration of H_2S, μmol/g	10.34±0.21	12.05±0.37*	10.94±0.04 *	10.65±0.15 **

An increase in H_2S concentration in the MetS group may be considered as adoptive response to the development of oxidative-nitrosative damage to the rat heart caused by excessive fructose intake, because H_2S has a pronounced antioxidant and anti-inflammatory properties [26, 27].

We observed an activation of nitrate-nitrite pathway of nitric oxide synthesis under conditions of metabolic syndrome modeling. Administration of PDTC did not abolish this activation but reduced its activity. Fructose administration by itself is able to increase uric acid content in the body due to its activator effect on xanthine oxidase [28]. Xanthine oxidase is a part of xanthine oxidoreductase complex, which has a profound nitrate/nitrite reduction ability, which explains high activities of nitrite and nitrate reductases in the rat heart observed in our study [29]. Activation of transcriptional factor NF-κB can also contribute to increase of xanthine oxidase activity, with subsequent increase in nitrate/nitrite reduction, which explains partial efficiency of PDTC in lowering activity of nitrate-nitrite pathway of nitric oxide production during combined exposure of organism to PDTC and MetS [30]. Our results point to possible presence of stimulation ability of PDTC on activity of nitrite reductases, the exact mechanism of which should be studied separately.

Since PDTC administration on the background of MetS modelling decreased peroxynitrite concentration in the rat heart, we can assume that increased peroxynitrite concentration was caused primarily by activation of transcriptional factor NF-κB.

A decrease in nitrite content observed in the MetS, PDTC, and PDTC+MetS groups can be attributed to increased activity of nitrite reductases, which rapidly convert nitrites to nitric oxide. In the MetS group, the simultaneous decrease in nitrite and nitrosothiol content, along with an increase in peroxynitrite concentration, suggests that peroxynitrite formation is the dominant pathway for nitric oxide elimination during metabolic syndrome. The relationship between peroxynitrite and transcriptional factor NF-κB is complex: peroxynitrite formation can be stimulated by NF-κB activation [31], while preformed peroxynitrite can, in turn, induce NF-κB activation [32]. Since PDTC administration on the background of MetS modelling reduced peroxynitrite concentration in the rat heart, it is likely that the increased peroxynitrite concentrations were primarily driven by transcriptional factor NF-κB activation.

CONCLUSIONS

The activation of transcription factor NF-κB during the development of metabolic syndrome in the rat heart leads to hyperproduction of nitric oxide due to excessive activity of inducible NO-synthase and nitrite reductases. In this condition, the excessively produced nitric oxide is primarily utilized for peroxynitrite formation, increasing a threat of nitrosative stress development in the rat heart.

Administration of ammonium pyrrolidinedithiocarbamate is an effective approach for correcting excessive nitric oxide production in the rat heart during metabolic syndrome. It reduces the possibility of nitrosative stress development by lowering peroxynitrite concentration.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВПЛИВ ІНАКТИВАЦІЇ NF-КВ ПІРОЛІДИНДИТІОКАРБАМАТОМ АМОНІЮ НА ПРОДУКЦІЮ ОКСИДУ АЗОТУ В СЕРЦІ ЩУРІВ ПРИ МЕТАБОЛІЧНОМУ СИНДРОМІ

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Ожиріння набуває характеру епідемії, оскільки майже 1/6 населення Землі страждає від нього та спричинених ним патологічних станів. Однією з ключових подій у патогенезі ожиріння та його ускладнень (метаболічний синдром,

цукровий діабет 2-го типу) є активація NF-κB. Тому пошук препаратів, що впливають на активацію останнього при метаболічному синдромі, є перспективним шляхом лікування. Мета цього дослідження – оцінити вплив інактивації NF-κB піролідиндітіокарбаматом амонію на продукцію оксиду азоту, активність аргіназ, концентрацію метаболітів оксиду азоту та вміст сірководню в серці щурів підчас моделювання метаболічного синдрому. Дослідження проводили на 24 статевозрілих щурах-самцях лінії Вістар масою 200–260 г, розділених на 4 групи. До 1-ї контрольної групи ввійшли інтактні щури, до 2-ї – з метаболічним синдромом, щурам 3-ї групи вводили піролідиндітіокарбамат амонію, до 4-ї групи ввійшли тварини з метаболічним синдромом, яким вводили піролідиндітіокарбамат амонію. Метаболічний синдром індукували введенням 20%-го розчину фруктози як єдиного джерела води протягом 60 днів. Піролідиндітіокарбамат амонію вводили внутрішньоочеревинно тричі на тиждень у дозі 76 мг/кг. У 10%-му гомогенаті серця щурів досліджували загальну активність NO-синтази (NOS), активність індуцибельної (iNOS) і конститутивної NO-синтази (cNOS), активність нітрит- і нітратредуктази, вміст нітритів, пероксинітритів, нітрозотіолів і сірководню. У тварин 4-ї групи активність iNOS знизилася на 22,6% порівняно зі значеннями 2-ї групи. Активність аргінази знизилася на 20,9%, а нітрат- і нітритредуктаз – на 24,3 і 32,3% відповідно. Концентрація нітритів зросла на 25,8%, а $ONOO^-$ знизилася на 56,7% і нітрозотіолів збільшилася на 57,9%. Концентрація H_2S зменшилася на 11,6%. Активація NF-κB під час розвитку метаболічного синдрому призводила до гіперпродукції оксиду азоту внаслідок надмірної активності iNOS і нітритредуктаз.

Ключові слова: серце; метаболічний синдром; оксид азоту; NF-κB; піролідиндітіокарбамат амонію.

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