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MOLECULAR MECHANISMS OF CARDIOPROTECTION OF N-ACETYLCYSTEINE AND LOSARTAN IN STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES MELLITUS

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Abstract

Diabetic cardiomyopathy (DC) decreases the life quality of patients with diabetes mellitus (DM) and often comes in a fatal outcome. DC is a complicated multiple myocardial dysfunction, and oxidative stress has an initial role in disease development. The study of the level of lipoperoxidation, as well as total antioxidant status, is necessary for the understanding of molecular mechanisms of cardioprotection. The research aimed to detect molecular mechanisms of cardioprotection of antioxidant N-acetylcysteine (NAC) and angiotensin II type 1 receptor blocker losartan (LOS) in case of streptozotocin-induced type 1 DM in rats. It was found that NAC (1,5 g/kg) and LOS (20 mg/kg) both have antioxidant activity due to increasing the level of unsaturated fatty acids (increase in the content of linoleic by 2,5 times and omega 3 linoleic acid in 2,3 times, $p<0,05$) and reduction of arachidonic acid content (by 1,4 times, $p<0,05$), which indicates a decrease in lipoperoxidation (2,2-fold decrease content of thiobarbiturate reactive substances, $p<0,05$) as well as an increase in the system of endogenous antioxidant defense of myocardium tissue (2,5 times increase in glutathione level; 1,6 and 1,7 times in the activity of superoxide dismutase, $p<0,05$). NAC more efficiently redistributed stearinic acid, increasing its content (by 1,3 times, $p<0,05$), indicating membrane-protective properties of myocardiocytes, and losartan – by the 2,7-fold increase of minor saturated fatty acids (myristic, pentadecanic, heptadecanic) ($p<0,05$). It was proved the antiproliferative action of NAC due to decreasing expression of MMP-2 protein by 20% ($p<0.01$).

Keywords: *type 1 diabetes mellitus, streptozotocin, cardioprotection, N-acetylcysteine, losartan, fatty acids, antioxidant, lipoperoxidation, mmp-2 expression*

Introduction

Diabetes mellitus (DM) is the most widespread endocrinological pathology that spreads dramatically. The WHO has predicted that by the 2025 the amount of patients with DM will rich up to 380 million [1] Furthermore, diabetic complications, including diabetic cardiomyopathy (DC) decrease life quality of patients and often comes in fatal outcome.

DC is a complicated multiple myocardial dysfunction, and oxidative stress (OS) has initial role in disease development, especially in activation of general pathways: direct toxic action on cardiomyocytes, DNA and protein damage, apoptosis, enhance of advanced glycation end products, activation of polyol pathway, renin-angiotensin-aldosterone activation, matrix metalloproteases (MMPs) etc. [2-7]. Furthermore, MMP-2 involves in cardiovascular diseases development, and usually works as mediator of ventricular remodelling and systolic dysfunction [8,9].

Lately, the cardioprotective effect of antioxidant N-acetylcysteine (NAC) in experimental studies has been actively discussed [10, 11]. Blocking the renin-angiotensin-aldosterone system seems to be essential in case of DM, perspective is treatment with angiotensin II type 1 receptor blockers [12,13]. Moreover, in our previous research we studied redox-dependent mechanism of cardioprotection of NAC and losartan (LOS) associated with reducing generation of superoxide radicals in aorta cells of diabetic animals [14].

The study of level of lipoperoxidation as well as total antioxidant status is necessary for understanding of molecular mechanisms of cardioprotection.

The aim of the research was to detect molecular mechanisms of cardioprotection of NAC and LOS in case of streptozotocin-induced DM in rats.

Methods

Text Wistar rats (200–250 g) were procured at our institution animal house facility in Bogomolets National Medical University (Kiev, Ukraine) and housed in an airconditioned room at a temperature of 25±2 °C and relative humidity from 45 % to 55 % under 12-h light: 12-h dark cycle. The animals had free access to food pellets except when starvation was required. Water was provided ad libitum. The experimental protocol was approved by Law of Ukraine N 3447-IV about "Animal protection from cruel treatment" and in accordance with the rules and guidelines of the Council Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes [15].

Type 1 DM (DM1) was induced by intraperitoneal injection of streptozoticin (STZ, Sigma, USA) at the dose of 50 mg/kg dissolved in 0.1 M citrate buffer pH 4.5 [16]. After 72 hours of STZ injection, the blood glucose level was measured with the using of a glucometer Accu-Chek Performa Nano (Roche Diagnostics, Germany). Only rats, which had blood glucose level higher then 15mmol/L were used for the study.

Animals were randomly divided into 5 groups: 1 st –Intact control (n=6; intact rats); 2nd – DM1 (n=7; untreated diabetic control rats supplemented with 0.9 % normal saline per os); 3rd – NAC (n=8; rats wit DM1 treated with N-acetylcysteine at a dose 1.5g/kg per os); 4th – LOS (n=7; rats with DM1 treated with losartan at a dose 20mg/kg per os as reference drug). One week after induction of diabetes investigated pharmacological agents were administrated to diabetic treated groups for 4 weeks.

Fatty acid (FA) composition of myocardium tissues was determined by a gas chromatography. FAs peaks were identified by comparing the retention time of standard FA peaks. Quantitative evaluation of FA lipids was performed by the normalization of the peak areas of methyl derivatives of FA and determination of their composition in percent [17]. Determination of markers of OS – thiobarbituric acid reactive substances (TBARS), reduced glutathione (G-SH), catalase activity (CAT) and superoxide dismutase activity (SOD) was performed in tissue homogenates of the heart (1:10 w/v). The intensity of lipoperoxidation was determined by the accumulation of TBARS due to the formation of a colored trimethine complex in the reaction with 2-thiobarbituric acid spectrophotometrically [18]. The state of antioxidant status of myocardial tissue was determined by the level of G-SH, CAT and SOD. The

level of G-SH was determined by the degree of staining of the thio-nitrophenyl anion in the Elman reaction at 412 nm spectrophotometrically [19]. Determination of CAT activity was performed according to the method [20]. SOD activity was evaluated by the degree of inhibition of the rate of autooxidation of adrenaline in an alkaline environment, followed by measurement of the optical density at 340 nm spectrophotometrically [21].

Expression of MMP-2 protein was determined according to the western blot analysis. Electrophoresis of extract of heart tissue protein in polyacrylamide gel with sodium dodecyl sulfate was performed by the method of U. K. Laemmli [22] with ThermoScientific P8DS device (USA). The number of protein samples was normalized to the level of control protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma, USA). Antibodies specific for MMP-2 (Invitrogen, USA) in 1:1000 dilution, antibodies specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma, USA) - 1: 5000 for the investigation were used.

The data was expressed as mean ± standard error of mean (SEM). One way analysis of variance (ANOVA) was applied to test the significance of difference between average parameters of different groups and multiple comparisons were determined by post hoc Dunnetts test. The statistical analysis was performed using IBM SPSS Statistics Base version 22.0 (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

After 4 weeks of treatment with NAC and LOS we observed such changes in FAs composition of lipids of myocardium tissues. Among the minor FAs, NAC resulted in 2.2 fold increase of amount of myristic FA (C14:0), LOS resulted in 2.8-fold increase of C14:0 compared to DM1 ($p<0.05$). Similar changes were observed in pentadecanoic acid (C15:0). During 4 weeks of NAC and LOS treatment, 2,2-fold and 2,7-fold increase in C15:0 was shown respectively, compared to DM1 ($p<0.05$). 3.1 fold increase in heptadecanoic acid (C17:0) was observed under the action of both NAC and LOS ($p<0.05$). Significant changes in increasing of stearinic acid (C18:0) were observed in NAC group only compared to diabetic control group (see Table 1). Thus, the sum total of saturated FA (SFA) significantly increased in NAC group, compared to DM1 group ($p<0.05$). Among the quantitative ratio of unsaturated FA (UFA), 1.8 – fold increase in percentage of linoleic acid (C18:2) were shown for both NAC and LOS groups compared to DM1 ($p<0.05$). Significant changes in oleic acid percentage were not observed. Conversion in polysaturated FA (PUFA) was also observed. Thus, 2.3-fold increase of linolenoic acid (C18:3) were common for NAC and LOS groups ($p<0.05$). Additionally, the percentage of arachidonic acid (C20:4) was significantly decreased in myocardium tissues by1.4 time less than in DM1 group ($p<0.05$) (see Table 1).

Administration of both NAC and LOS was accompanied with significant decrease amount of lipoperoxidation products in myocardium tissues, especially in 2.2 fold decrease of TBARS compared to diabetic control (DM1) ($p<0.01$). Treatment with NAC and LOS caused in the 2.5 increase G-SH level compared to DM1 ($p<0.01$). Exposure to NAC caused 60% elevation of enzymatic activity of SOD ($p<0.01$), exposure to LOS caused similar changes, in 70% increase of SOD activity ($p<0.01$) compare to DM1. CAT activity was restored equally in both groups NAC and LOS. Significant changes between NAC and LOS group were not observed (see Table 2).

Moreover, it was determined changes in MMP-2 protein expression in experimental groups. Exposure to NAC only showed statistically significant changes compared to diabetic control group DM1: the level of MMP-2 protein expression decreased by 1.2 times less than in DM1 ($p<0.05$). Additionally statistically significant changes were found between NAC and LOS group (see Figure 1).

Discussion

During the 4 weeks of experimental DM1 treatment with NAC and LOS was associated with increasing level of minor SFA (C14:0, C15:0, C17:0) compared to diabetic rats. Administration of LOS was specified in minor SFA redistribution indicating increase the total sum of SFA ($p<0.05$). In our study NAC treatment was associated with increasing content of C18:0 indicating the stabilization of myocardial cell membranes and enhancing the energy capacity of the myocardium, due to the

properties of stearinic acid as a source of energy in cells [23]. High values of SFA content indicate that NAC and LOS effectively act against OS as well as activation of lipoperoxidation process in myocardial cells. Nevertheless, Nawrocki A. et al. indicated slight changes in the composition of miramistinic, palmitic, stearic and linolenic acids in the myocardium of rats with STZ-induced DM [24]. Statistically significant increase in the content of linoleic acid (2.5 times) secondary to linolenoic (omega-3) acid ($p<0.05$) indicated an increase in the elasticity of cardiomyocyte membranes in case of NAC and LOS supplementation in rats with experimental DM1. Thus, positive changes in the composition of SFA and UFA in the myocardial tissue of rats treated with NAC and LOS indicate the activation of the antioxidant system in myocardial tissues, and a decrease in the amount of arachidonic FA indicates inhibition of lipoperoxidation process in myocardial tissue. Obtained results confirm the mechanism of antioxidant defense of NAC and LOS in myocardium tissues of rats with experimental DM1. It was determined that both NAC and LOS reduced processes of lipoperoxidation by 2.2 times and activated the endogenous antioxidant defense system by 2.5 times ($p<0.01$).

The next step was to determine expression of matrix-degrading enzymes (matrix metalloproteinases), which are involved in the regulation of cellular function of cardiomyocytes [25]. In our study we found significant changes in lowering of MMP-2 protein expression of heart tissue in rats only in case of NAC, indicating a decrease in myocardial remodeling processes. LOS had not such effects on MMP-2 expression in our study. Nevertheless, B. Dong et al. pointed out that LOS activated ACE2 overexpression and thereby inhibited myocardial collagen accumulation due to decreased MMP-2 expression, improved left ventricular function [26]. Wang L. et al. associated a reduction in myocardial fibrosis due to blocking of TGF-β1 protein expression, inhibition of the JAK/STAT system, in case of high doses of LOS, but authors studied the model of experimental type 2 DM [12].

Conclusion. The knowledge of molecular mechanisms of cardioprotective action of NAC and LOS was deepened. We have found that NAC and LOS provide antioxidant activity due to increasing the level of UFA (increase in C18:2 and C18:3 content), reduction of C20:4 content which indicates a decrease in lipoperoxidation process (by the content of TBARS) as well as an increase in the system of endogenous antioxidant defense of myocardium tissue (increase in G-SH and SOD activity). New data of the membrane-stabilizing effect of NAC and LOS were also indicated. NAC more efficiently redistributed stearinic acid, increasing its content that point out the membrane-protective properties of myocardiocytes, and LOS– more efficiently increased minor SFA (myristic, pentadecanic, heptadecanic). It was proved antiproliferative action of NAC resulted by decreasing the expression of MMP-2 protein.

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Table 1. Changes in fatty acids levels

Fatty acid, %	Conditions of the experiment			
	Intact control (n=6)	DM1 (n=7)	NAC (n=8)	LOS (n=7)
C14:0	0.32±0.03	0.20±0.01#	0.44±0.04*	0.57±0.02*
C15:0	0.34±0.03	0.20±0.01#	0.43±0.04*	0.54±0.03*
C16:0	22.37±1.46	13.92±0.90#	17.48±0.60 #	15.33±0.90#
C17:0	0.33±0.03	0.14±0.02#	0.44±0.04*	0.46±0.03*
C18:0	13.23±0.77	9.60±0.23#	11.97±0.34*	10.22±0.35#
C18:1	16.83±1.08	15.15±0.25	15.08±0.23	15.38±0.43
C18:2	30.78±0.52	29.38±0.80	38.78±2,40*	40.00±1.28*#
C18:3	0.32±0.03	0.21±0.01#	0.49±0.02*	0.52±0.04*
C20:4	17.85±2.20	25.87±2.98	18.05±2,70	17.87±1.81
Σ SFA	36.58±2.01	24.06±1.07#	30.76±0.78*#	27.12±0.84#
Σ UFA	65.78±2,37	70.61±2.85	72.40±2.82	73.77±0.88
Σ PUFA	48.95±2.68	55.46±2.96	57.32±2.86	58.39±0.68

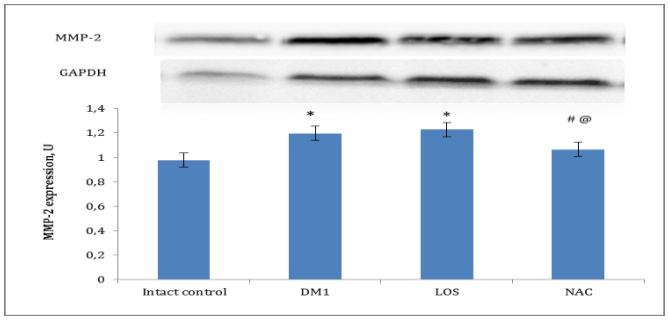
*p<0.05 significant difference compared to intact control, #p<0.05 significant difference compared to diabetic control group (DM1)

Table 2. Effects of N-acetylcysteine and losartan on oxidative stress markers in myocardium in case of experimental DM1

Markers	Conditions of the experiment			
	Intact control (n=6)	DM1 (n=7)	NAC (n=8)	LOS (n=7)
TBARS, mcM/mg of tissue	1.70±0.10	3.20±0.10#	1.44±0.13*	1.40±0.05*
G-SH, mcM/mg of tissue	0.44±0.01	0.20±0.01#	0.47±0.02*	0.51±0.02*
SOD, %	25.0±0.6	10.2±1.0#	16.2±0.8*#	17.5±1.2*#
CAT, mcat/ mg	28.0±1.3	42.6±1.7#	26.4±0.5*	27.2±1.8*

*p<0.01 significant difference compared to diabetic control group (DM1), #p<0.01 significant difference compared to intact control

Figure 1. Effects of N-acetylcysteine and losartan on MMP-2 expression levels in myocardium in case of experimental DM1



*p<0.01 significant difference compared to intact control, #p<0.01 significant difference compared to diabetic control group (DM1), @p<0.01 significant difference compared to LOS