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CARDIAC MUSCLE'S MITOCHONDRIAL DYSFUNCTION IN HYPERCHOLESTEROLEMIC RABBITS

KOJIĆ ZVEZDANA, POPOVIĆ NADA, ŠĆEPANOVIĆ LJILJANA and STEFANOVIĆ BRANISLAVA

School of Medicine, Belgrade

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Cardiovascular diseases are often associated with energy deficit and in many cases this is also accompanied by lipid disorders such as hyperlipidemias and obesity. The aim of the study was to check mitochondrial oxidative capacity in the course of twelve weeks atherogenic hypercholesterolic diet. Thirty five Chinchilla rabbits, male, were randomized to one of two groups: a control group (A, n=17) received (per os) physiological saline; experimental group (B, n=18) received atherogenic 2% hypercholesterolemic diet. Isolation of the mitochondrial fraction of the heart was done by the method of Tyler. The oxygen consumption rate was studied in different respiration phases: as basal, unstimulated (V_4) and as ADP-stimulated (V_3), and expressed as indices: respiratory control ratio (RCR) and ADP/O. Hypercholesterolemic atherogenic diet induced profound perturbations in mitochondrial energy metabolism and oxidative capacity. Basal oxygen consumption rate without ADP (V_4) and the maximal ADP-stimulated respiration rate (V_3) showed a marked reduction (quantitative changes); sensibility of mitochondria to ADP (ADP/O) was also reduced (qualitative change) in rabbits treated by atherogenic diet (group B) compared to controls (group A). Respiratory control ratio was not significantly different among the groups. These results indicate that hypercholesterolemic atherogenic diet impairs mitochondrial oxidative capacity without affecting coupling of oxidative and phosphorilative processes.

Key words: atherosclerosis, heart, hypercholesterolemia, mitochondria

INTRODUCTION

Numerous factors can increase cardiovascular diseases (CVD) risk. It is not yet clear whether they act to alter cellular function in a similar or dissimilar fashion. A shared feature among them is increased oxidative stress (Holland *et al*., 1996; Ohara *et al*., 1993) and it may be no coincidence that many also appear to cause cardiovascular mitochondrial damage and/or dysfunction (Ballinger SW, 2005).

Several lines of evidence suggest that an association exists among CVD development, mitochondrial damage, and function. It has been shown that CVD patients have increased mitochondrial (mt) DNA damage in both the heart and the aorta when compared with healthy controls (Corral-Debrinski *et al*., 1991; Corral-Debrinski *et al*., 1992; Knight-Lozano *et al*., 2002; Ballinger *et al*., 2002). Atherosclerotic lesions in the brain microvessels from Alzheimer's (AD) patients and rodent AD models have significantly more mtDNA deletions and abnormalities (as do the endothelium and perivascular cells), suggesting that the mitochondria within the vascular wall can be the central targets for oxidative stress-induced damage (Aliev *et al*., 2002). Chronic ischemia increases both mtDNA deletions in human heart tissue (Corral-Debrinski *et al*., 1992) and cardiac mitochondrial sensitivity to inhibitors of cellular respiration (Brookes *et al*., 2001). *Ex vivo* studies in the rat heart have shown that ischemia reduces myocardial oxidative phosphorilation capacities (Duan *et al*., 1989). Using a mouse model for myocardial infarction (MI), it was found that previous MI is associated with increased reactive oxygen species (ROS) and decreased mtDNA copy number, mitochondrial-encoded gene transcripts, and related enzymatic activities (complexes I, III, and IV). However, nuclear-encoded genes (complex II) and citrate synthase are unaffected (Ide *et al*., 2001). Cardiotoxic ROS generators increase mtDNA deletions and lipid peroxidation in the myocardial mitochondria; overexpression of mitochondrial antioxidants prevents these effects and increases cardiac tolerance to ischemia (Chen *et al*., 1998). Decreased vascular superoxide dismutase-2 (SOD2)-specific activities have been associated with increased exposure to CVD risk factors (Knight-Lozato *et al*., 2002) and increased susceptibility to ischemia/reperfusion-mediated cardiac damage and resistance to cardiac preconditioning (Asimakis *et al*., 2002). Moreover, deficiencies in mitochondrial antioxidants and/or regulatory proteins (uncoupling proteins – UCPs) that modulate mitochondrial oxidant production have been shown to promote the onset of CVD *in vivo*, consistent with the notion that mitochondrial-generated oxidants can play a contributory role in atherogenesis (Ballinger *et al*., 2002; Blanc *et al*., 2003). Likewise, overexpression of mitochondrial antioxidants and/or UCPs has been shown to protect against the effects of ischemia/ reperfusion and oxidative stress (Chen *et al*., 1998; Teshima *et al*., 2003; Bienengraeber *et al.*, 2003).

Cardiovascular diseases are accompanied by hyperlipidemic states, however, the cellular mechanisms by which exposure to lipids leads to deleterious effects remains unclear. The purpose of this study was to examine the respiratory chain complexes i.e. oxidative capacity of cardiac muscle mitochondria in the course of a hypercholesterolemic atherogenic diet.

MATERIAL AND METHODS

Animals and diets

Chinchilla strain rabbits, male, two months old at the outset of the study were used. In the course of twelve weeks of treatment, the rabbits received daily

(orally) either physiologic saline (control, group A, n=17) or atherogenic 2% cholesterol diet (animal model of experimental atherosclerosis) (atherogenic diet, group B, n=18). The investigation conformed to the *Guide for the Use and Care of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Measurment of plasma lipids

Total cholesterol, HDL-cholesterol and triglyceride levels in the rabbit plasma were measured by enzymatic "end point" kinetic spectrophotometric method (commercial kit Randox, Great Britain). The value of LDL-cholesterol was calculated using Fridewald equation. In order to determine the atherogenic risk of lipid origin for all rabbits, two indices were calculated: LDL/HDL-cholesterol and total cholesterol/HDL cholesterol. Cardiovascular risk of lipid origin was calculated for each group of rabbits in two intervals, at the outset and at the end of the treatment.

Mitochondrial preparation

After 12 weeks of treatment, animals were sacrificed and the mitochondrial fraction of the rabbit's heart was isolated applying the method of Tyler (1979). Isolation buffer consists of sucrose (300 mM), Tris (20 mM), and EGTA (10 mM); pH 7.35 at 4°C. Briefly, the first phase implies tissue homogenization, previously digested by protease (collagenase, nagarase, Type XXII, EC 3.4.21.62). The procedure for differential centrifugation complies the second phase, so that at the end pure isolated heart mitochondria remain precipitated at the bottom of the tubes, to be used for subsequent investigations. A subsample of the mitochondrial suspension is saved for protein determination by the method of Lowry *et al*. (1974), using bovine serum albumin as the standard.

Measurement of Respiration Rate

Oxygen consumption rate by cardiac muscle mitochondria was measured by Clark oxygen electrode (Biological Oxygen Monitor, model 5300, Yellow Springs Instrument Co., USA) in 3 ml Kreb's solution buffered with 10 mmol/L HEPES-NaOH, pH 7.4, at 30°C. This respiration buffer consists of sucrose (300 mM), KCl (50 mM), KH_2PO_4 (5 mM), $MgCl_2$ (1 mM), EGTA (5 mM) and Tris (20 mM), pH 7.35. The electrode was calibrated daily before use (Wise, 1985). Mitochondrial oxidative phosphorilation was studied in GMC medium equilibrated with air at 30°C, with continuous stirring. Glutamate (0.2 mol/L) and malate (0.1 mol/L) were used as substrates (unstimulated, basal respiration rate, state 4, V_4). As the mitochondria consume oxygen, the electrode plots the curve of oxygen uptake. Mitochondrial respiration was calculated as the decrease rate of oxygen concentration after the addition of mitochondrial suspension, assuming an initial oxygen concentration of 224 nmol/L. The set observation time for respiration measurements was 6 minutes. Five runs were conducted with each mitochondrial sample at a dilution of 1.5 to 2 mg of protein per milliliter. Oxidative

activity was expressed as nanomoles of consumed oxygen per minute per mg of protein of thick mitochondrial suspension.

The main characteristic of mitochondrial oxidative capacity is the maximal respiration rate (state 3, V_3). To estimate maximal respiration rate, adenosine diphosphate (ADP, 1 μL of 0.1 M) was added. For mitochondrial coupling, 1 μL of biliary dog serum was added during the same run to test the respiratory response. The mitochondrial uncoupler dinitrophenol (DNP) was added in order to examine effects on the DNP-potentiated rate of mitochondrial respiration. At the end of each measurement, 1 mmol/L NaCN was added, which eliminated all increases in oxygen consumption.

Two indexes, ADP/O and Respiratory Control Ratio ($RCR=V_3/V_4$), were calculated and expressed as the mean of the five replicate runs with each sample.

Drugs and Chemicals

Crystalline cholesterol was purchased from Galenica, USA, and it was dissolved in edible oil. Biliary dog serum, glutamate, malate and all other substances were purchased from Sigma Chemical Co., USA.

Statistical Analysis

Data were reported as means ± standard deviation. Differences of O_2 consumption in the mean values were analyzed by use of Student's *t*-test (computer program Microsoft Excel Version 2000). A value of $p<0.05$ was considered statistically significant.

RESULTS

The levels of total cholesterol, triglyceride, LDL-cholesterol and HDL-cholesterol levels in rabbit plasma in the course of twelve weeks of treatment by atherogenic diet are presented in Table 1. In group B, at the end of treatment, except HDL-cholesterol, all of these values were significantly higher compared to the control group ($p<0.01$).

Table 1. Lipid concentrations in rabbit plasma in the course of hypercholesterolemic diet

Parameters	At the outset of treatment		At the end of treatment	
mmol*l^{-1}	Group A (n=17)	Group B (n=18)	Group A (n=17)	Group B (n=18)
Total cholesterol	0.78 ± 0.06	0.75 ± 0.05	0.92 ± 0.11	4.32 ± 0.72**
Trygliceride	0.28 ± 0.05	0.29 ± 0.04	0.42 ± 0.05	1.07 ± 0.13**
LDL-cholesterol	0.18 ± 0.02	0.18 ± 0.03	0.24 ± 0.03	2.08 ± 0.21**
HDL-cholesterol	0.27 ± 0.03	0.24 ± 0.02	0.25 ± 0.03	0.24 ± 0.02

Values represent mean ± SD. Statistically significant differences: *p<0.05; **p<0.01 vs. group A.

LDL/HDL- and total cholesterol/ HDL-cholesterol indices during the treatment are presented in Figure 1. At the end of treatment both of these indices were significantly higher in group B than in group A ($p<0.01$).

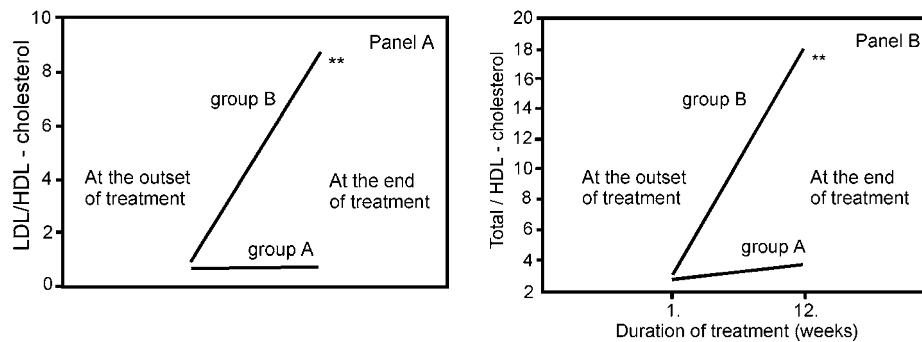

Figure 1. The effects of hypercholesterolemic atherogenic diet on lipid indices: LDL/HDL-cholesterol (panel A) and total/HDL-cholesterol (panel B). Statistically significant differences: **$p<0.01$ vs. group A

The cardiac mitochondrial oxygen consumption rates are presented in the first two columns of the Table 2. Basal, unstimulated (V_4) and maximal, ADP-stimulated oxygen consumption rate (V_3) were significantly lower in group B, treated by 2% hypercholesterolemic atherogenic diet than in group A ($p<0.05$). Ratio ADP/O was also significantly lower in group B (presented in the third column, $p<0.05$). Coupling of oxidation and phosphorylation processes was present in mitochondria of both groups (there was no statistically significant difference among the values of respiratory control ratio, fourth column).

Table 2. Cardiac muscle mitochondrial respiratory parameters at the end of twelve weeks treatment by hypercholesterolemic diet

Group	V_4	V_3	ADP/O	RCR
Control – A (n=17)	20.3 ± 2.1	126 ± 16	3.2 ± 0.2	6.2 ± 0.4
Experimental – B (n=18)	15.4 ± 3.0*	93 ± 20*	2.1 ± 0.4*	6.0 ± 0.8

Unstimulated (V_4) and maximal (V_3) respiration rate of isolated rabbit's heart mitochondria are expressed as nanomoles of consumed oxygen per minute per mg of protein of thick mitochondrial suspension. ADP/O index and respiratory control ratio (RCR).
The values are means ± S.D. of 5-8 experiments (control n=17, experimental n=18).
*$p<0.05$ vs. the control group.

DISCUSSION

The most significant finding in the current study is that twelve week hyperlipemic atherogenic diet induced multiple abnormalities in cardiac muscle energy metabolism and mitochondrial oxidative capacity. These abnormalities are: basal oxygen consumption rate without ADP (V_4) and the maximal ADP-stimulated respiration rate (V_3) showed a marked reduction (quantitative changes); sensibility of mitochondria to ADP (ADP/O) was also reduced (qualitative change) in comparison to control animals.

Cardiovascular diseases are often associated with energy deficit, and in many cases this is also accompanied by lipid disorders such as hyperlipidemias and obesity (Christoffersen *et al*., 2003). However, the nature of such deficit is still unclear. Since in the heart most of the energy is produced by mitochondria, structural and functional changes derived from, or caused by metabolic disorders, could compromise the energetic status of the organ. In fact, alterations in cellular and mitochondrial membrane composition have been described to affect not only electrical properties of the heart, but also energy production (Pepe, 2002).

In group B, basal and maximal respiration rates showed a marked reduction (24% and 26% respectively) evidencing decreased oxygen capacity in tested mitochondria. The simultaneous decrease in these two parameters suggests a diminished amount of mitochondria or a general mitochondrial dysfunction. Such a reduction in the number of mitochondria is frequently associated with aging. Tissues obtained from aged animals and from animals with developed atherosclerosis, not only showed a reduced number of mitochondria, but also display changes in mitochondrial structure, such as swelling, shortening of the cristae and matrix vacuolization (Feder *et al.* 1993). These changes were associated to an increased generation of superoxide anion and hydrogen peroxide, and also to a decline of energy production.

ADP/O index, was also significantly lower in group B than in group A. With the used substrates in group A ADP/O index was 3.24, but in group B it was 2.07. Reduction of this index suggests that in the studied mitochondria, in addition to the process of oxidative phosphorilation, other oxygen consuming processes also take place. In recent available literature lipid peroxidation is the most commonly recognized one (Droge, 2002; Mital *et al*., 2002). Reduction of this index in group B also coincides with our results that refer to antioxidative capacity of plasma and determination of nitrite levels (Kojic Z, 2002). The fall of this index is probably related to irreversibile inhibition of respiration due to enhanced peroxinitrite formation (Ballinger *et al*., 2004). There is, however, evidence that at moderate concentrations superoxide anion and related reactive oxygen species play an important role as regulatory mediators in signaling processes (re-establish redox homeostasis) (Ballinger *et al*., 2002).

Respiratory control ratio (RCR) was not significantly different among the groups. It ranged 6.07 to 6.28 in all studied rabbits. These results indicate absence of uncoupling in the studied mitochondria of the rabbit heart. These results may contribute to elucidation of complex relationships in the course of

mitochondrial respiration inhibition in physiological (cardioprotection) and patophysiological (atherogenesis) conditions.

In conclusion, in line with our previous work, in the present study, twelve weeks treatment by hypercholesterolemic, atherogenic diet led to quantitative changes in mitochondrial function. Hyperlipecholesterolemia also induced qualitative changes in mitochondrial oxidative capacity, namely decreased ADP sensitivity. For these multiple abnormalities in cardiac muscle mitochondria oxidative capacity may be the responsible for functional alterations of heart during coronary atherosclerosis. Decreased oxidative capacities could be the basis for lower oxygen utilization and exercise capacity in heart failure.

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Address for correspondence:
Doc. Dr Zvezdana Kojić
Institute of Physiology
School of Medicine, University of Belgrade
Visegradska 26, 11000 Belgrade
Serbia & Montenegro
e-mail: zvezdanak@med.bg.ac.yu

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DISFFUNKCIJA MITOHONDRIJA SRCA U TOKU HIPERHOLESTEROLEMIJE KOD KUNIĆA

KOJIĆ ZVEZDANA, POPOVIĆ NADA, ŠĆEPANOVIĆ LJILJANA
i STEFANOVIĆ BRANISLAVA

SADRŽAJ

Kardiovaskularne bolesti su često povezane sa deficitom energije, a ovo je u mnogim slučajevima udruženo sa poremećajima lipidnog statusa, kao što su hiperlipidemije i gojaznost. Cilj ovog rada je bio da ispita oksidativni kapacitet mitohondrija srca na kraju dvanaestonedeljne hiperholesterolemijske aterogene dijete. Ogled je izveden na 35 Činčila kunića, mužjaka, koji su, po slučajnom izboru, bili svrstani u jednu od dve grupe. Kontrolna grupa (A, n=17), je dobijala peroralno fiziološki rastvor dok je eksperimentalna grupa (B, n=18) dobijala aterogenu 2% hiperholesterolemijsku dijetu. Mitohondrijalna frakcija srca izolovana je metodom po Tyler-u. Potrošnja kiseonika izučavana je u različitim fazama respiracije: u fazi bazalne, nestimulisane respiracije mitohondrija (V_4) i u fazi maksimalne respiracije mitohondrija, kada su one stimulisane dodavanjem ADP-a (V_3). Oksidativni kapacitet mitohondrija je analiziran i pomoću dva indeksa: indeks kontrole respiracije (RCR) i kao indeks ADP/O.

Hiperholesterolemijska aterogena dijeta dovela je do velikih oštećenja u energetskom metabolizmu i u oksidativnom kapacitetu mitohondrija srca. Kod kunića koji su tretirani hiperholesterolemijskom dijetom, u odnosu na kontrolnu grupu kunića, uočena je značajno manja brzina bazalne, nestimulisane respiracije mitohondrija, kao i manja brzina maksimalne ADP-om stimulisane respiracije mitohondrija (kvantitativne promene). U ovoj grupi kunića senzitivnost mitohondrija na ADP je bila manja (kvalitativne promene). Indeks kontrole respiracije se nije značajno razlikovao između grupa. Ovi rezultati ukazuju da hiperholesterolemijska aterogena dijeta smanjuje oksidativni kapacitet mitohondrija a da pri tome ne utiče na "kuplovanost" mitohondrija tj. na vezu između procesa oksidacije i fosforilacije.