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IMPACT OF CAPTOPRIL ON CARDIAC MITOCHONDRIAL FUNCTION IN AN *IN-VIVO* ANIMAL MODEL OF ATHEROSCLEROSIS

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Recently, we have demonstrated that a twelve-week treatment with hypercholesterolemic atherogenic diet induced profound alterations in mitochondrial energy metabolism and oxidative capacity of isolated cardiac muscle's mitochondria. The aim of the present study was to investigate the effects of treatment with an ACE inhibitor (captopril) on energy metabolism and oxidative capacity in cardiac muscle's mitochondria using an in-vivo animal model of experimental atherosclerosis. Mitochondrial oxidative capacity was followed-up during the course of twelve weeks of atherogenic hypercholesterolic diet. Fifty five male Chinchilla rabbits, were randomized to one of four groups: a control group (A, n=17) received physiological saline orally; experimental group (B, n=18) received atherogenic 2% hypercholesterolemic diet; group C (n=10) received the atherogenic diet and two hours later on the aqueous solution of captopril (10 mg/kg/day); group D (n=10) received the aqueous solution of captopril, only. Isolation of mitochondrial fraction of the heart was performed by the method of Tyler. The oxygen consumption rate was studied at different respiration phases: as basal, unstimulated (V_4) and as ADP-stimulated (V_3), and expressed as indices of respiratory control ratio (RCR) and ADP/O. Hypercholesterolemic atherogenic diet induced profound alterations 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 i.e. dramatic decrease in oxidative capacities). The sensibility of mitochondria to ADP (ADP/O) was also reduced (qualitative change) in rabbits treated with the atherogenic diet (group B) compared to controls (group A). Respiratory control ratio was not significantly different among the studied groups. These results indicate that hypercholesterolemic atherogenic diet impairs mitochondrial oxidative capacity without affecting coupling of oxidation and phosphorilation. Captopril treatment had only limited effects on alterations of oxidative capacity in hypercholesterolemic rabbits. The main characteristic of oxidative capacity, i.e. the maximal respiration rate (V_3), was not improved by captopril.

Key words: ACE inhibitor, heart, hypercholesterolemia, mitochondria

INTRODUCTION

Evidence from animal studies and clinical trials suggests that angiotensin II, synthesized locally by the action of angiotensin converting enzyme (ACE) within the arterial wall, may be important in the pathogenesis of coronary artery disease. Thus, ACE inhibition can partially restore endothelial function in the arteries of animals fed an atherogenic diet and can also protect against the neointimal proliferative response to endothelial balloon injury. In the SAVE trial, the mortality reduction observed in the group treated with captopril was accompanied by a 25% reduction in the risk of recurrent infarct expansion and remodeling. In the SOLVD studies, ACE inhibition was found to protect against ischemic end-points, patients randomized to enalapril showed a 23% reduction of the risk of unstable angina. Altogether, these studies permit to speculate that the progression of coronary arteries disease might be amenable to modification by ACE inhibition.

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 when compared with healthy controls in both the heart and the aorta (Corral-Debrinski *et al*.,1991; Corral-Debrinski *et al*., 1992, Knight-Lozano *et al*., 2002; Ballinger *et al*., 2002). Atherosclerotic lesions in brain microvessels from Alzheimer's (AD) patients and rodent AD models have significantly more mtDNA deletions and abnormalities (as is the case with 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 of 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 e.g. oxidative capacity of cardiac muscle's mitochondria in the course of hypercholesterolemic atherogenic diet.

MATERIAL AND METHODS

Animals and diets

Male *Chinchilla* strain rabbits, aged two months at the outset of the study were used. They were randomized into four experimental groups. In the course of twelve weeks of treatment, the rabbits received daily (orally) the following: physiologic saline (control, group A, n=17), atherogenic 2% cholesterol diet (*in vivo* animal model of atherosclerosis, group B, n=18), atherogenic diet and two hours after that an aqueous solution of captopril (10 mg/kg/day) (Sigma Chem. USA) (group C, n=10) and aqueous solution of captopril only (group D, n=10). The treatment was administered 7 days a week for twelve weeks. The so treated animals were used in further experimental procedures. 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

The 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 the Fridewald equation. In order to determine the atherogenic risk of lipid origin for all rabbits, two indexes were calculated: LDL/HDL-cholresterol 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 the animals were sacrificed and the mitochondrial fraction of the rabbit's heart was isolated by the method of Tyler (1979). Isolation buffer comprising sucrose (300 mM), Tris (20 mM), and EGTA (10 mM); pH 7.35 at 4^{o}C. Briefly, the first phase implies tissue homogenization, previously digested by proteases (collagenase, nagarase, Type XXII, EC 3.4.21.62), while differential centrifugation complies the second phase, thus at the end isolated mitochondria of the heart were precipitated at the bottom of the tubes. A subsample of the mitochondrial suspension was saved for protein determination by the method of Lowry *et al*., using bovine serum albumin as the standard (Lowry *et al*., 1974).

Measurement of Respiration Rate

Respiratory parameters of the cardiac mitochondrial population were studied. 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 ^{o}C. This respiration buffer containes 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. Before use the electrode was calibrated daily (Wise R., 1985). Mitochondrial oxidative phosphorilation was studied in GMC medium equilibrated with air at 30 ^{o}C, and continuously stirred. Glutamate (0.2 mol/L) and malate (0.1 mol/L) were used as substrates (unstimulated, basal respiration rate, state 4, V_4). As mitochondria consume oxygen, the electrode plots the curve of oxygen uptake. Mitochondrial respiration was calculated as the decrease in oxygen concentration after the addition of the mitochondrial suspension, assuming an initial oxygen concentration of 224 nmol/L. The typical 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 the 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 the effects on DNP-potentiated rate on mitochondrial respiration. At the end of each measurement, 1 mmol/L NaCN was added, which eliminated all increases in oxygen consumption.

Two indices, 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 was dissolved in edible oil. Biliary dog serum, glutamate, malate and all other reagents 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 Student's *t*-test (computer program Microsoft Excel Version 2000). A value of $p<0.05$ was considered statistically significant.

RESULT

The levels of LDL/HDL- and total cholesterol/ HDL-cholesterol indices during the treatment are presented in Figs. 1 and 2 and in Table 1. At the end of treatment

both indices were significantly higher in groups B and C than in groups A and D ($p<0.01$).

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

Prameters mmol*l^{-1}	At the outset of treatment		At the end of treatment	
	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.

Table 2. Cardiac muscle mitochondrial respiratory parameters at the end of the 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 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

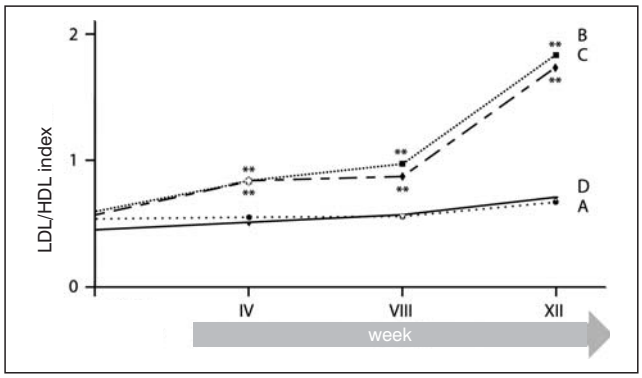

Figure 1. The influence of the treatment on LDL/HDL cholesterol index

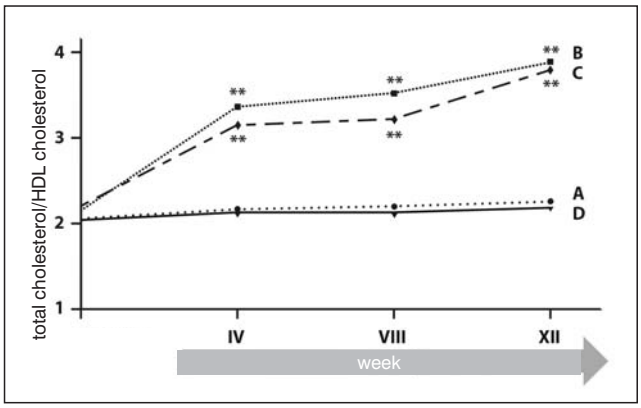

Figure 2. The influence of the treatment on total/HDL cholesterol index

The cardiac mitochondrial oxygen consumption rates are presented in Table 2 and Figures 3-6. Unstimulated oxygen consumption rate (State 4) of isolated rabbit's heart mitochondria was significantly lower in rabbits treated with atherogenic diet (group B) and rabbits receiving captopril (group D), than in the controls (Fig. 3).

The maximum oxygen consumption rate (State 3) was also significantly lower in groups B and D than in group A (Fig. 4).

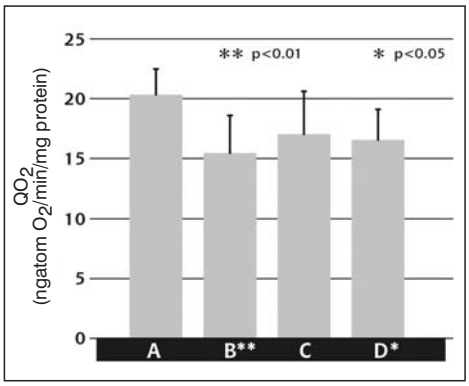

Figure 3. Unstimulated respiration rate (State 4) of isolated rabbit heart mitochondria

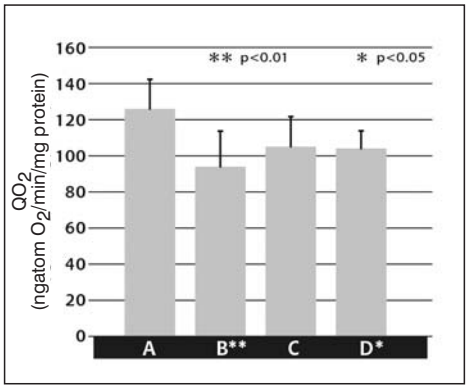

Figure 4. Maximal respiration rate (State 3) of isolated rabbit heart mitochondria

However, the ADP/O index of the isolated rabbit's heart mitochondria was significantly lower in group B than in group A, while in group D this index was not significantly different (Fig. 5). These results may suggest the presence of different mechanisms of oxygen consumption inhibition. Namely, with the used substrates the index was ADP/O=3, or higher. 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. Lipid peroxidation is the most commonly recognized one. Reduction of this index in group B coincides with our results that refer to antioxidative capacity of plasma and the determination of nitrite levels (data not presented). Since a fall of ADP/O index was not noted in group D, inhibition of mitochondrial respiration in this group is probably related to the cardioprotective effects of the 12-weeks treatment with ACE inhibitor.

Respiratory control ratio (RCR) was not significantly different among the groups. It ranged from 6.07 to 6.28 in all studied rabbits (Fig. 6). These results indicate the absence of uncoupling in the studied cardiac mitochondria. In group C, captopril added to the atherogenic diet resulted in a significant reduction of the mitochondrial oxygen consumption, compared to atherogenic treatment (Fig. 6).

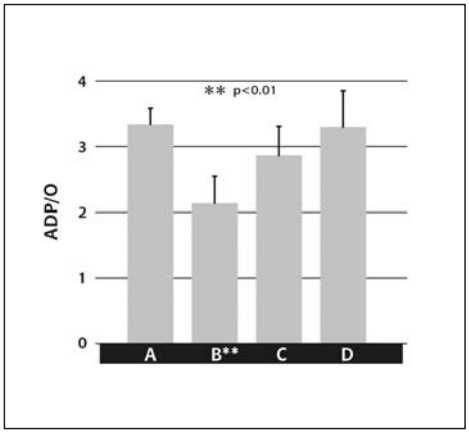

Figure 5. ADP/O index of isolated rabbit heart mitochondria

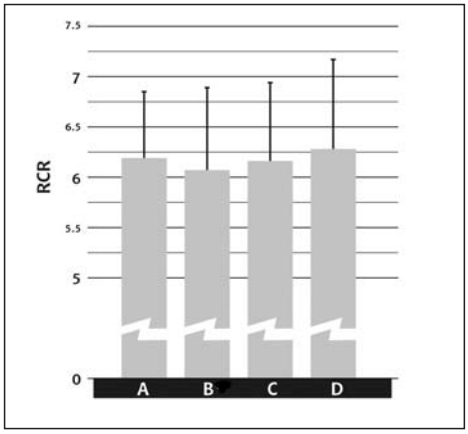

Figure 6. Respiratory control ratio (RCR) of isolated rabbit heart mitochondria

DISCUSSION

This study examined whether treatment with an inhibitor of ACE could improve multiple abnormalities in cardiac muscle mitochondria in hypercholesterolemic rabbits. The most significant finding in the current study is that captopril treatment had only limited effects on these abnormalities on oxidative capacity.

Twelve weeks of hyperlipemic atherogenic diet induced multiple abnormalities in the 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 the control animals.

The effects of ACE inhibitors on myocardial oxygen consumption were studied by Zang *et al.* (1997) as well, but in a different experimental design. They used isolated micro blood vessels and slices of cardiac muscle, using B2 receptor blockers (HOE 140), nitric oxide (NO) synthase inhibitors (L-NAME and L-NNA), as well as inhibitors of serine proteinase. They have found that respiration in cardiomyocytes was influenced by regulation of the locally produced kinine, mediated by endothelial NO production. In both experimental models, suppression of cardiac mitochondrial respiration was noted under the influence of captopril.

Cardiovascular diseases are often associated with energy deficit, and in many cases this is accompanied by lipid disorders such as hyperlipidemias and obesity (Christoffersen *et al.* 2003*)*. However, the nature of such a 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, the 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.

The ADP/O index was significantly lower in group B (2.07) than in group A (3.24). 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, lipid peroxidation being 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 the antioxidative capacity of plasma and the determined nitrite levels (Kojic, 2002). The fall of this index is probably related to irreversible 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 (reestablish redox homeostasis) (Ballinger *et al*., 2002).

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

mitochondrial respiration inhibition in physiological (cardioprotective) 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. These multiple abnormalities in cardiac muscle mitochondrial oxidative capacity may be responsible for functional alterations of the 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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UTICAJ KAPTOPRILA NA FUNKCIJU MITOHONDRIJA SRCA U *IN-VIVO* ANIMALNOM MODELU ATEROSKLEROZE

KOJIĆ ZVEZDANA i ŠĆEPANOVIĆ LJILJANA

SADRŽAJ

Kardiovaskularne bolesti su često povezane sa deficitom energije koji je u mnogim slučajevima udružen sa poremećajima statusa lipida, kao što su hiperlipidemija i gojaznost. Cilj ovog rada je bio da se ispita oksidativni kapacitet mitohondrija srca na kraju dvanaestonedeljne hiperholesterolemijske aterogene dijete. Ogled je izveden na trideset i pet mužjaka Činčila kunića. Kontrolna grupa (A, n=17), je dobijala peroralno fiziološki rastvor, dok je eksperimentalna grupa (B, n=18) dobijala aterogenu 2% hiperholesterolemijsku dijetu. 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 izučavan je i pomoću indeksa kontrole respiracije (RCR) i indeksa ADP/O.

Hiperholesterolemijska aterogena dijeta dovela je do velikih promena u energetskom metabolizmu i oksidativnom kapacitetu mitohondrija srca. Kod kunića koji su bili izloženi hiperholesterolemijskoj dijeti, uočena je značajno manja brzina bazalne, nestimulisane respiracije mitohondrija, kao i manja brzina maksimalne ADP-om stimulisane respiracije mitohondrija (kvantitativne promene) u odnosu na kontrolnu grupu. U ovoj grupi kunića, senzitivnost mitohondrija na ADP je bila manja (kvalitativne promene). Indeks kontrole respiracije nije se 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.