Endogenous Protective Mechanisms in Remodeling of Rat Heart Mitochondrial Membranes in the Acute Phase of Streptozotocin-Induced Diabetes

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Short title:

Remodeling of Heart Mitochondrial Membranes in Acute Diabetes

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Summary

The aim of present study was to investigate functional and physical alterations in membranes of heart mitochondria that are associated with remodeling of these organelles in acute phase of streptozotocin-induced diabetes and to elucidate the role of these changes in adaptation of the heart to acute streptozotocin-induced diabetes (evaluated 8 days after single dose streptozotocin application to male Wistar rats). Action of free radicals on the respiratory chain of diabetic-heart mitochondria was manifested by 17 % increase ($p<0.05$) in oxidized form of the coenzyme Q_{10} and resulted in a decrease of states S3 and S4 respiration, the respiratory control index, rate of phosphorylation (all $p<0.01$) and the mitochondrial transmembrane potential ($p<0.05$), but the ADP/O ratio decreased only moderately ($p>0.05$). On the contrary, membrane fluidity and the total mitochondrial Mg^{2+}-ATPase activity increased (both $p<0.05$). In diabetic heart mitochondria, linear regression analysis revealed a reciprocal relationship between the increase in membrane fluidity and decrease in trans-membrane potential ($p<0.05$, $r = 0.67$). Changes in membrane fluidity, transmembrane potential, Mg^{2+}-ATPase activity and the almost preserved ADP/O ratio appear as the manifestation of endogenous protective mechanisms participating in the functional remodeling of mitochondria which contributes to adaptation of the heart to diabetes.

Key words: diabetic rat, heart mitochondria, oxidative phosphorylation, mitochondrial membrane fluidity, mitochondrial transmembrane potential

Introduction

Heart in rats with acute streptozotocin-induced diabetes is characterized by altered metabolism (Kucharská *et al.* 2000, Stebelová *et al.* 2006, Volkovová *et al.* 1993, 1997, Ziegelhöffer *et al*. 1996, 1997, 2003a), changes in performance and susceptibility to additional pathological impulses such as ischemia etc. (Andelová *et al.* 2006, Ravingerová *et al.* 2000, 2003) and by functional remodeling of their sarcolemmal (Ziegelhöffer *et al*.1997) and mitochondrial membranes (Ferko *et al*. 2006a). Goals of the present study are: i) to investigate the changes induced by acute diabetes and/or remodeling in physical properties of mitochondrial membranes (such as the transmembrane potential and fluidity of membrane lipids) in relation to the capability of remodeled cardiac MIT to utilize oxygen and to synthesize ATP; ii) to check whether, and to what extent may be the formation of conjugated dienes in mitochondrial membrane lipids co-responsible for the changes associated with remodeling of diabetic heart mitochondria (MIT); iii) to elucidate the role that the observed changes may play in endogenous protective mechanisms securing the adaptation of heart to streptozotocin-induced diabetes.

Methods

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1985) as well as with the rules issued by the State Veterinary and Alimentary Administration of the Slovak Republic, basing on piece of legislation No 289/2003 of the Slovak Parliament.

Male Wistar rats (9-11 weeks old, 220±20g b. wt.) were kept on 12/12 light/dark regimen, they were fed with standard pellet diet and had free access to water. Animals were made diabetic with a single dose of streptozotocin (55 mg/kg, i.p.). Diabetic state of the rats

was controlled by estimation of glucose (BIO-LA-TEST, Glucose GOD 250, Pliva-Lachema, Brno, Czech Republic), and glycohemoglobin (Burrin *et al.* 1980) in the blood, as well as cholesterol (Watson 1960) and triacyglycerols (Fossati and Prencipe 1982) in the serum. Serum insulin was determined by commercial RIA kit (Linco Researech USA).

Isolation of mitochondria (MIT)

Hearts damped with small volume of ice-cold isolation solution (IS, containing in $mmol.l^{-1}$: 180 KCl, 4 EDTA and 1 % bovine serum albumin, pH=7.4) were cut into small pieces with scissors, transferred to a teflon/glas homogenizer together with 20 ml of IS containing in addition protease (Sigma P 6141) 2.5 $mg.g^{-1}$ w. wt. (heart) and homogenized gently for 2-3 min. After centrifugation at 1000x g for 10 min the protease containing supernatant, together with a part of MIT which were in a direct contact with the protease, was discarded. Pellet was resuspended in the same volume of IS but without protease, again homogenized and spunned down as previously. This supernatant containing now predominantly MIT which were not in direct contact with protease, was spunned down at 5000 x g for 15 min. The pellet containing MIT was again resuspended in albumin-free IS containing only 180 $mmol.l^{-1}$ KCl, 4 $mmol.l^{-1}$ EDTA and the final MIT fraction was spunned down at 5000 x g for 15 min. The isolation procedure was performed at 4 ºC.

Mg^{2+}-dependent and 2, 4-dinitrophenol (DNP) -stimulated ATPase (the total MIT ATPase) was assessed by estimation of P_i liberated from ATP splitting (Ziegelhöffer *et al.* 1997). Contamination of the isolated MIT fraction by other subcellular membranes was tested *via* estimation of ATPase activities characteristic for the presence of sarcolemma (Na^+,K^+-ATPase) and sarcoplasmic reticulum (Mg^{2+}-Ca^{2+}-ATPase) in the absence and presence of their specific inhibitors ouabain and thapsigargin (Máleková *et al.* 2006). Oxygen consumption by isolated mitochondria was estimated by means of a Clark oxygen electrode

(Gvozdjaková *et al.* 1984). Mitochondrial coenzyme Q_{10} (CoQ_{10}) was determined by HPLC using the method of Lang *et al.* (1986). Membrane fluidity was assessed as the degree of fluorescence anisotropy using the fluorescent dye DPH (1, 6-diphenyl-1, 3, 5-hexatriene). Transmembrane potential of the mitochondria was monitored by confocal microscopy using carbocyanine (N, N' - di (3 - trimethylammoniumpropyl) thiadicarbocyanine tribromide) as a fluorescent indicator; it was indicated as the fluorescence intensity ratio between 680 nm and 570 nm (aggregates/monomers) of the carbocyanine (Waczulíková *et al.* 2007).

Statistics

Results are given as means ± SEM. Statistical significances were ascertained by using the Student's two-tailed test for unpaired observations. Only corrected $p<0.05$ values were considered as significant.

Chemicals

If not specified differently in the text, all reagents and chemicals applied in the study were of analytical grade. Streptozotocin, EDTA and TRIS as well as the other chemicals, if not specified elsewhere, were purchased from Sigma-Aldrich USA.

Results

Diabetic state of experimental animals was confirmed by 258 % elevation of blood glucose and 89 % elevation in the total hemoglobin content. In addition, a 292 % rise of triacylglycerols and a 62 % increase of cholesterol in the serum were also observed in comparison with controls. On the other hand, serum levels of insulin were decreased by 53 % indicating an impairment of its secretion (Table 1).

The degree of contamination of MIT preparation by membranes of the sarcolemma and sarcoplasmic reticulum, estimated by presence of their marker enzymes amounted to 0.84 % and 1.59 %, respectively (data not shown).

Due to a disconcert in unison of components of the electron-oxygen transport system (Tretter *et al.* 2004) free radicals induced a slight but significant ($p<0.05$) increase in content of the oxidized form of CoQ_{10} and slowed down the flow of electrons in the respiratory chain. These perturbations diminished the oxygen consumption states 3 and 4 as well as the respiratory control index (RCI) and the rate of phosphorylation (all $p<0.05$ or more), but they were not enough powerful to cause an uncoupling of oxidative phosphorylation, documented by a non-significant decrease ($p>0.05$) ADP/O ratio, or to damage to mitochondrial Mg^{2+}-ATPase (the reversely working ATP synthase). On the other hand, the activity of the latter enzyme became slightly but significantly increased ($p<0.05$, Table 2).

Remodeling of the MIT in diabetic hearts also involved a significant ($p<0.05$) increase in fluidity of the mitochondrial membrane (Fig. 1.). At the same time the MIT from diabetic animals exhibited significantly ($p<0.001$) lower transmembrane potential (Fig. 2.) as compared with MIT from control animals. Linear regression analysis of the latter findings revealed significant association ($p<0.05$, $r=0.67$) between the increase of membrane fluidity and decrease of transmembrane potential. Testing the stability of transmembrane potential in control and diabetic heart MIT by gradual increase of the extramitochondrial concentration of K^+ ions (Fig. 3.) revealed that although the MIT from diabetic hearts have markedly lower transmembrane potential, they possess higher capability to keep it stable.

Discussion

The aim of the present study was to examine alterations characterizing the diabetes-induced remodeling of cardiac MIT and to identify those changes that are related to

endogenous protection or adaptation to the disease itself. This task required an experimental protocol enabling to investigate exclusively the processes related to diabetes. However, it is well known, that similarly like in men, experimental diabetes is also accompanied by numerous complications. In many cases these spontaneously appearing complications may exert additive or amplifying effect on the alterations caused by the diabetes itself. For these reasons we focused our attention to the acute phase of diabetes when the disease was already fully developed (Table 1), but the diabetes accompanying complications were still not present in a measurable extent.

Due to disturbances in function of the respiratory chain the diabetic heart is not capable to utilize oxygen properly. Consequently, cardiac cells experience a situation resembling hypoxia (Juránek and Bezek 2005, Ziegelhöffer *et al.* 2005) in spite of the fact that the hypoxia is not caused by a decrease in tissue pO_2. This state is known as pseudo-hypoxia. Because the high availability of oxygen does not allow a switch over to anaerobic energy production, cardiac cells would be suffering energy deficiency. To cope with this problem the diabetic heart is forced to activate endogenous protective mechanisms which are involved in functional remodeling of MIT (Ferko *et al.* 2006a). This remodeling requires changes in both chemical and physical properties of MIT membranes (Ferko *et al.* 2006b, Ziegelhöffer *et al.* 2005, 2007) coupled with a break in oxygen sensing of cardiac cells (Holotňáková *et al*. 2007).

The remodeling is usually understood as structural and functional deviations from the normal state that are caused by some pathological stimuli. Nevertheless, it was already demonstrated that not all remodeling-associated deviations from normal function are noxious. Some may belong to endogenous protective mechanisms and represent compensatory or even adaptation changes alleviating the effect of the given pathology (Ziegelhöffer *et al*. 1997, 2002, Waczulíková *et al.* 2002).

The increase observed in amount of the oxidized form of CoQ_{10} and the decrease in state 3 and state 4 oxygen consumption, as well as in respiratory control index and in the rate of phosphorylation (Table 2) could be considered without any doubt as pathological deviations. However, it was already demonstrated that in acute diabetes, the strength of the above mentioned damage may be considerably counteracted by augmented transfer of energy from cardiac MIT to the cytoplasma (Ziegelhöffer *et al*. 2005). The latter compensatory process is based on significant increase in the number of functionally still integrated substrate and energy transition pores (also termed as contact sites) in the mitochondrial membrane (Ziegelhöffer-Mihalovičová *et al*. 1997) and it is accompanied with an increase in fluidity of the mitochondrial membrane (Ziegelhöffer 2005). The observed increase in Mg^{2+}-ATPase activity, is also contributing to the stabilization of coupling between the oxidation and phosphorylation (Ziegelhöffer *et al*. 2002, 2003b).

Our discovery of tight association between the fluidity and transmembrane potential of diabetic heart MIT does not inform only about the mutual interrelationship between these two physical variables but it also stresses their exclusive regulatory role in processes located in mitochondrial membranes. In the present case, their functional remodeling enables the diabetic heart to resist the pseudo-hypoxia. The observation of more stabile transmembrane potential in diabetes-remodeled heart MIT is in concert with the latter notion.

It may be summarized that the increase in membrane fluidity and in mitochondrial Mg^{2+}-ATPase activity, as well as the stabilization of transmembrane potential in heart MIT, which are associated with acute functional remodeling of these organelles in streptozotocin-induced diabetes participate in endogenous protective mechanisms alleviating the effect of the disease. The presented new information may be helpful in search for such a treatment of diabetes that would utilize the endogenous protective mechanisms in the myocardium.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Table 1. *Levels of glucose and content of glycohemoglobin in the blood, levels of triacylglycerols, cholesterol and insulin in serum of rats with acute (8 days) streptozotocin diabetes.*

	Controls	**Diabetes**
Glucose [mmol x l^{-1}]	5.13 ± 0.15	18.35 ± 0.92*
Glycohemoglobin [%Hb]	4.11 ± 0.13	7.78 ± 1.06*
Triacylglycerols [g x l^{-1}]	1.21 ± 0.09	4.74 ± 0.35*
Cholesterol [g x l^{-1}]	1.70 ± 0.11	2.76 ± 0.13*
Insulin [ng x ml^{-1}]	1.04 ± 0.15	0.49 ± 0.09*

*Data are means ± S.E.M. from 15 experiments, * $p<0.01$ control vs. diabetic rats (Student's two-tailed test for unpaired observations).*

Table 2. *Changes in functional properties of rat heart mitochondria induced by diabetes-caused remodeling.*

Variable	Results		p
	Controls	**Diabetes**	
CoQ_{10} oxidized form (mmol x mg^{-1} prot.)	0.472 ± 0.016	0.559* ± 0.017	< 0.05
$QO_2(S_3)$-glutamate (nAtO x mg^{-1} prot. x min^{-1})	151.31 ± 4.31	111.86 ± 3.93	< 0.05
$QO_2(S_3)$-succinate (nAtO x mg^{-1} prot. x min^{-1})	238.64 ± 12.14	188.72 ± 8.51	< 0.05
$QO_2(S_4)$-glutamate (nAtO x mg^{-1} prot. x min^{-1})	35.7 ± 1.04	25.26 ± 1.13	< 0.05
$QO_2(S_3)$-succinate (nAtO x mg^{-1} prot. x min^{-1})	132.33 ± 5.28	112.72 ± 3.93	< 0.05
Respiratory control index - glutamate	5.375 ± 0.261	4.415 ± 0.228	< 0.05
Respiratory control index - succinate	1.8 ± 0.039	1.67 ±0.028	< 0.05
Phosphorylation rate - glutamate (nmol ATP x mg^{-1} prot. x min^{-1})	362.66 ± 13.04	262.97 ± 12.54	< 0.05
Phosphorylation rate - succinate (nmol ATP x mg^{-1} prot. x min^{-1})	322.89 ± 12.97	243.46 ± 12.47	< 0.05
ADP/O ratio - glutamate (nmol ADP x $nAtO^{-1}$)	2.505 ± 0.261	2.405 ± 0.228	N.S.
ADP/O ratio - succinate (nmol ADP x $nAtO^{-1}$)	1.36 ± 0.228	1.31 ± 0.028	N.S.
Mg^{2+}-ATPase (µmol P i x g^{-1} prot. x h^{-1})	65.79 ± 1.12	71.26* ± 1.21	< 0.05

Data are means ± S.E.M. from 15 experiments. Statistical evaluation of control vs. diabetic rats was done using the Student's two-tailed test for unpaired observations. N.S. – not significant. Diabetes-induced increase variables is indicated by asterisks. All other variables were decreased in diabetic hearts.

Fig. 1. *Fluorescence anisotropy of DPH in the mitochondrial membrane. Data are means ± S.E.M. of 7 experiments.*

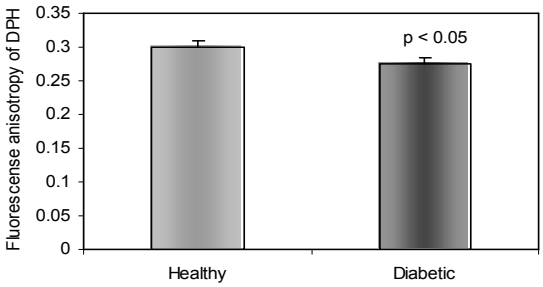
Fluorescense anisotropy of DPH
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
p < 0.05
Healthy
Diabetic

Fig. 2. *Carbocyanine fluorescence intensity ratio – aggregates/monomers (680 nm/570 nm) in isolated heart mitochondria from healthy and diabetic rats. Data are means ± S.E.M. of 7 experiments.*

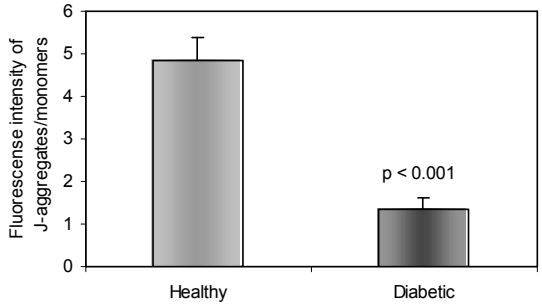

Fig. 3. *Effect of KCl on stability of mitochondrial trans-membrane potential in healthy and diabetic rat hearts.*