Functional Remodeling of Heart Mitochondria in Acute Diabetes: Interrelationships between Damage, Endogenous Protection and Adaptation

M. Ferko[1], A. Gvozdjaková[2], J. Kucharská[2], J. Mujkošová[1], I. Waczulíková[3], J. Styk[1], T. Ravingerová[1], B. Ziegelhöffer-Mihalovičová[1] and A. Ziegelhöffer[1]

[1] *Institute for Heart Research, Slovak Academy of Sciences, Bratislava, Slovakia. E-mail: usrdmife@savba.sk*
[2] *Laboratory of Pharmacobiochemistry, IIIrd Department of Internal Medicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia*
[3] *Division of Biomedical Physics, Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovakia*

Abstract. Rats with streptozotocin-diabetes develop mechanisms of endogenous protection (MEP) that participate actively in functional remodeling of cardiac sarcolemma. Remodeling of sarcolemma is a sign of damage but it also protects the cells of the diabetic heart (DH) against additional energy disbalance due to excessive Ca^{2+} entry. Since yet, cardiac mitochondria (MIT) were investigated predominantly from the aspect of damage only.

Aims of the present study were: i) to distinguish between acute diabetes-induced changes in function of rat heart MIT which clearly belong to damage from those that reflect the MEP and participate in functional remodeling of the MIT; ii) elucidate the significance of MEP-induced changes in heart MIT for cardiac energetics. Acute diabetes (8 days) was induced in adult male Wistar rats by streptozotocin (STZ, 65 mg·kg^{-1} i.p., single dose). On the day 8 after STZ administration, the diabetic animals exhibited 300–330 % increase in blood glucose, triacylglycerols and cholesterol as well as 89.6 % increase in glycohemoglobin (all $p < 0.01$). The blood level of insulin dropped by 53 % ($p < 0.02$). State 3 and state 4 oxygen consumptions of DH MIT were decreased against the controls, leading to drop of the respiratory control index (17.9 and 7.3 %) and oxidative phosphorylation rate (OPR, 27.5 and 24.6 %; all $p < 0.003$–0.02). These effects of damage yielding in strained energy balance of the acute DH were partially alleviated by MEP. The latter involved temporary preservation of the ADP : O ratio, with participation of elevated MIT Mg^{2+}-ATPase activity as well as increased formation of MIT substrate and energy transition pores (both $p < 0.05$). Hence, the energy

Correspondence to: Attila Ziegelhöffer, Institute for Heart Research, Slovak Academy of Sciences, Dúbravská cesta 9, P.O.Box 104, 840 05 Bratislava 45, Slovakia
E-mail: usrdzigy@savba.sk

disbalance of the acute DH was finally manifested in 13 % loss in its AMP content only ($p < 0.05$). Results indicate that MIT in STZ-DH are functionally remodeled. Defective O_2 consumption by MIT renders molecular changes suggestive of a mild hypoxic state but an increase in Mg^{2+}-ATPase activity and facilitated energy delivery from MIT to the cytoplasm indicate the presence of MEP acting in the MIT and alleviating the effect of decreased oxidative energy production in the acute DH.

Key words: Streptozotocin diabetes — Heart mitochondria — Myocardial energetics — Mitochondrial Mg^{2+}-ATPase — Endogenous protection

Introduction

It is well documented, that diabetes mellitus is a disease leading to serious disorganization of the metabolism. Correspondingly, alterations concern the processes not only in the myocardium *in toto*, but also in subcellular organels and particularly in membrane structures such as sarcolemma, sarcoplasmic reticulum, and mitochondria (MIT) (Nagano and Dhalla 1991; Rodrigues and McNeill 1992; Somogyi et al. 2001; Pierce et al. 2003). If all molecular and structural alterations, described in the myocardium in acute as well as in chronic stage of diabetes, acted simultaneously and indeed had the impact attributed to each of them, the diabetic heart (DH) would have little chance to keep working at all. But the heart works and this testifies for the fact that not all diabetes-caused changes have unambiguously pathologic character. Some among them may belong to mechanisms of endogenous protection (MEP) – an organized resistance against the disease-triggered metabolic and structural perturbations. MEP may partly mitigate the pathologic changes with a series of compensatory and adaptation processes, occurring in the DH and prolonging its survival (Tribulová et al. 1996; Ziegelhöffer et al. 1996, 1997; Ravingerová et al. 2000a, 2001). All this yields in a sensitive, dynamic equilibrium state termed as structural and functional remodeling of the heart and is characterized by slow, but permanent shifting of the equilibrium state toward the diabetic cardiomyopathy. Therefore, a good knowledge of MEP is essential for taking advantage of them in protection of the diabetic heart (Gøtzsche 1991; Ziegelhöffer et al. 1997, 1999; Zhang et al. 2002).

Since changes resulting from MEP appear concomitantly with the pathologic ones and both are triggered by diabetes, it is important to recognize and distinguish them as well as learn their pattern and outcome. From this aspect, acute diabetes seems to provide a relatively clear and suitable model, since the advanced stages of diabetes are usually accompanied with numerous, not directly diabetes-induced complications. Namely, the latter may either appear as additional alterations or interfere with the diabetes-induced changes making the cause-to-consequence relationships unclear.

In previous studies, our attention was predominantly focused to investigation of the MEP acting in cardiac cell membranes and to nailing down the role of remodeled cardiac sarcolemma in securing the heart function in rats with acute

streptozotocin (STZ)-diabetes (Ziegelhöffer et al. 1998, 2003; Ravingerová et al. 2000b; Ziegelhöffer-Mihalovičová et al. 2003). In present study, we focused our interest on elucidation of nature and meaning of the molecular mechanisms involved in acute diabetes-induced remodeling of function of the rat heart MIT.

Materials and 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 § 37 (6), piece of legislation No. 488/2002 of the Slovak Parliament.

Experiments were performed on adult male Wistar rats. Animals were kept on 12/12 light/dark regimen, they were fed with standard pellet diet and had free access to water.

Induction and control of diabetes

Diabetes was induced in rats (220–240 g b.w.) by intraperitoneal administration of STZ in a single dose of 65 $mg \cdot kg^{-1}$ b.w. Manifestation of the disease was followed daily by estimation of glucosuria using Gluko Phan® stripes (Pliva-Lachema, Brno, Czech Republic) and confirmed at termination of the experiment by estimation of glucose (Bio-La-Test, Glucose GOD 250, Pliva-Lachema) and glycohemoglobin (Burrin et al. 1980) in the blood, as well as cholesterol (Watson 1960) and triacyglycerols (Fossati and Prencipe 1982) in serum. Serum insulin was determined by commercial RIA kit (Linco Research, USA). A group of age- and weight-matched rats, kept parallel to the diabetic animals, served as controls. The animals were sacrificed by cervical dislocation on the 8^{th} day after STZ application. Hearts were quickly excised, cooled down and washed free of blood with ice-cold saline. After removal of vessels and fat, hearts were weighed and used either for preparation of MIT (estimation of oxygen consumption, parameters of oxidative phosphorylation, Mg^{2+}-ATPase activity, etc.), further for estimation of energy transition pores (contact sites) in MIT membranes and of the content of adenine nucleotides in cardiac tissue.

Isolation of MIT

Hearts damped with small volume of ice cold isolation solution (IS; containing in $mmol \cdot l^{-1}$: 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 $mg \cdot g^{-1}$ of wet weight (heart) and homogenized gently for 2–3 min. After centrifugation at $1000 \times g$ for 10 min, the protease containing supernatant together with a part of MIT being in 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 × *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 × *g* for 15 min. The isolation procedure was performed at 4 ℃.

Functional parameters of MIT

Oxygen consumption by the MIT

Oxygen consumption by isolated MIT was estimated by means of a Clark oxygen electrode, polarized to 8 V (Oxygraph Gilson, USA). MIT oxidized glutamate and succinate as nicotinamide dinucleotide (NAD, oxidized form)-dependent and flavin adenine dinucleotide (FAD, oxidized form)-dependent substrates in the presence of rotenone as an inhibitor of flow of electrons from complex I to complex III in the respiratory chain (Bailey and Cunningham 2002). Measurements were performed in a 1.6 ml chamber with magnetic stirring, heated to 30 ℃. The incubation medium contained (in mmol·l^{-1}): 3.0 KH_2PO_4, 120 KCl, 0.5 EDTA, 12.5 HEPES and 5.0 of either glutamate or succinate as well as 2 % dextran, 10 μl rotenone (1 mg·ml^{-1} in 96 % ethanol) and it was adjusted to pH = 7.2 by Tris-HCl (Rouslin and Millard 1980). Reaction was started by addition of MIT (1 mg·ml^{-1}). Tracings represented the oxygen consumption in basic condition in nanoatoms of oxygen *per* mg of protein *per* min (nAtO × mg^{-1} prot. × min^{-1}) i.e., in the presence of endogenous substrate and ADP only (state 4 respiration or QO_2(S4)).

Parameters of oxidative phosphorylation

Oxidative phosphorylation was assessed as the ADP : O (nmol : nAt) ratio i.e., as the amount of oxygen utilized in state 3 respiration (QO_2(S3)). This was measured in the presence of 500 nmol·l^{-1} concentration of exogenous ADP added subsequently after termination of measurement of the state 4 respiration. Besides QO_2(S4), QO_2(S3) and ADP : O, the following characteristics of oxidative phosphorylation were calculated: the respiratory control index (RCI) – i.e., the ratio of QO_2(S3) : QO_2(S4) and the oxidative phosphorylation rate (OPR) – velocity of the oxidative phosphorylation (nAtO × mg^{-1} prot. × min^{-1}).

CoQ_{9-ox} and CoQ_{10-ox} content of the MIT

Content of the oxidized isoforms of coenzyme Q (CoQ_{9-ox} and CoQ_{10-ox}) in the MIT was assessed by means of HPLC (Beckmann Gold, Germany) using a Sepharon SGX C18 column (250 × 4 mm i.d., particle size 7 μm; Tessek, Czech Republic). Mobile phase was composed of methanol-acetonitril-ethanol in a ratio of 6 : 2 : 2, flow rate 1 ml·min^{-1}. Sample injection volume was 20 μl. Analysis was performed at room temperature (Kucharská et al. 1996). Concentration of coenzymes was detected spectrophotometrically at $\lambda = 275$ nm using external standards (Sigma).

Mg-dependent MIT ATPase

Activities of the oligomycin-sensitive Mg^{2+}-dependent ATPase of isolated MIT were estimated in 1ml of incubation medium containing (in $mmol \cdot l^{-1}$): 250 imidazol buffer, pH 7.4; 40 $MgCl_2$; 40 ATP-Tris; 50–70 μg of MIT protein ($\sim$1 $\mu g \cdot \mu l^{-1}$). Nevertheless, the activity obtained in this way would be referring only to the part of MIT with leaky membranes since the membranes of intact MIT are impermeable to Mg^{2+}. Hence, to obtain the total Mg-ATPase activity, all MIT membranes should be made leaky. Therefore, when estimating the total Mg^{2+}-dependent activity of the enzyme by splitting of ATP, the incubation medium contained in addition also 0.1 $mmol \cdot l^{-1}$ 2,4-dinitrophenol (2,4-DNP). After 10 min preincubation at 37 ℃, the reaction was started with addition of ATP and terminated (after 20 min) by 1 ml of ice-cold 12 % trichloroacetic acid. Enzyme activities were measured by estimating the amounts of orthophosphate (P_i) liberated by ATP splitting and expressed in mmol P_i *per* g of MIT protein *per* h. Protein amount was estimated according to Lowry et al. (1953) using bovine serum albumin as a standard. Inorganic phosphorus was determined using the method of Taussky and Shorr (1953).

Purity of the MIT preparation

Purity of isolated MIT preparation was tested by estimation of ATPase activities characteristic for sarcolemma (Na^+,K^+-ATPase) and sarcoplasmic reticulum (Mg^{2+},Ca^{2+}-ATPase) in the absence and presence of their specific inhibitors.

Briefly: for estimation of the sarcolemmal Na^+,K^+-ATPase together with the total MIT Mg^{2+}-ATPase, the basic medium for the latter enzyme, described sub "Mg-dependent MIT ATPase", was completed with 0.1 ml 100 $mmol \cdot l^{-1}$ NaCl and 0.1 ml 20 $mmol \cdot l^{-1}$ KCl. Thus, the Na^+,K^+-ATPase activity was manifested as the difference between P_i splitted in the presence of Na^+, K^+ and Mg^{2+} and in the presence of the Mg^{2+} alone. The validity and reliability of the Na^+,K^+-ATPase activity estimation was also verified via selective inhibition of the enzyme by addition of 0.1 ml 0.1 $mmol \cdot l^{-1}$ ouabain.

For estimation of sarcoplasmic reticular Mg^{2+},Ca^{2+}-ATPase the basic medium for MIT ATPase was completed with 0.1 ml 0.1 $mmol \cdot l^{-1}$ $CaCl_2$. In further steps, the way of estimation was similar to that of the sarcolemmal Na^+,K^+-ATPase with the exception that instead of ouabain 0.1 ml 100 $nmol \cdot l^{-1}$ thapsigargin was applied as a specific inhibitor for verification of reliability of the Mg^{2+},Ca^{2+}-ATPase estimation.

Similarly as in the case of MIT Mg^{2+}-ATPase, the ATPase activities of the sarcolemma and sarcoplasmic reticulum were estimated spectrophotometrically at 700 nm, by measuring the amount of P_i liberated by ATP splitting and utilizing the already described techniques for measurements of inorganic phosphate and protein specific activities of ATPases were expressed in $\mu mol\ P_i \times mg^{-1}$ prot. $\times h^{-1}$.

Substrate and energy transition pores (SETP or contact sites) in MIT membranes

SETP were assessed by cytochemical detection of octameric form of the MIT creatine phosphokinase (Biermans et al. 1989). The method is based on reduction of thiocarbamylnitro blue-tetrazolium chloride salt in the presence of lactate and glucose-6-phosphate dehydrogenase. Thin sections of embedded tissue slices were examined in electron microscope. SETP formation was quantified stereologically (Baddeley et al. 1986) as the ratio of SETP surface to MIT surface. The testing grid was superimposed over the electronmicrographs and the ratio of intersections of cycloids with SETP and intersections of cycloids with MIT membranes was counted (for more details see Baddeley et al. 1986).

Adenine nucleotides in myocardial tissue

After fast thoracotomy in thiopental anaesthesia hearts were removed using the freeze clamp technique (Wollenberger et al. 1960). The frozen tissue was weighed and pulverized in liquid nitrogen and after extraction with 10 % $HClO_4$ (10 $ml \cdot g^{-1}$) spunned down at 5000 × g at 4 °C for 10 min. Supernatant was neutralized with 5 $mol \cdot l^{-1}$ K_2CO_3. Resulting $KClO_4$ was removed by filtration and the extract was used for analysis of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) content by means of HPLC (Beckmann Gold, Germany) using a Separon SGX C18 column (250 × 4 mm i.d., particle size 7 μm; Tessek, Czech Republic). A Beckmann Ultrasphere ODS column (45×4.6 mm i.d., 5 μm) was applied as precolumn. Mobile phase was composed of 0.1 $mol \cdot l^{-1}$ KH_2PO_4, 8 $mmol \cdot l^{-1}$ tetrabutylammonium hydrogen sulfate in 10 % methanol, pH = 6, flow rate 1 $ml \cdot min^{-1}$. Sample injection volume was 20 μl. Analysis was performed at room temperature.

Chemicals

All reagents and chemicals applied in the study were of analytical grade. STZ, EDTA and Tris as well as the other chemicals, if not specified elsewhere, were purchased from Sigma-Aldrich USA. Inorganic chemicals as well as ATP, ADP and AMP were from SERVA or Merck (Germany).

Statistics

Results are given as mean ± SEM. Statistical significances were ascertained by means of Student's two- tailed test for unpaired observations with Bonferroni's correction or by multiple comparisons ANOVA. Only corrected values $p < 0.05$ were considered as significant.

Results

On the day 8 after STZ administration, rats developed diabetes manifested in significant ($p < 0.01$) increase in glucose, triacylglycerols and cholesterol as well as

Table 1. Levels of glucose, triacylglycerols, cholesterol, insulin and the content of glycohemoglobin (in % of the total hemoglobin (Hb) content) in the blood of rats with acute (8 days) STZ-diabetes

	Healthy rats	Diabetic rats	Significances
Glucose ($mmol \cdot l^{-1}$)	5.33 ± 0.16	17.80 ± 0.89	$p < 0.01$
Triacylglycerols ($g \cdot l^{-1}$)	1.25 ± 0.1	4.63 ± 0.32	$p < 0.01$
Cholesterol ($g \cdot l^{-1}$)	1.76 ± 0.12	2.7 ± 0.12	$p < 0.01$
Glycohemoglobin (% Hb)	4.05 ± 0.13	7.68 ± 1.02	$p < 0.01$
Insulin ($ng \cdot ml^{-1}$)	1.04 ± 0.15	0.49 ± 0.09	$p < 0.02$

Results are means ± S.E.M. from 15 experiments. Statistical evaluation – control *vs.* diabetic rats using the two way ANOVA and Tukey's test for multiple comparisons. For further details see Materials and Methods.

decrease ($p < 0.02$) in insulin levels in the blood (Tab. 1). This was accompanied by glucosuria amounting to 20–25 $mol \cdot l^{-1}$ (not shown). Another marker of diabetes, the content of glycohemoglobin in the blood was also elevated significantly ($p < 0.01$).

Preparation of heart MIT used in the present study had to fulfill two criteria: i) it should contain sufficient number of intact MIT to secure proper estimation of oxidative phosphorylation; ii) it should be little contaminated by other subcellular organels. From the latter aspect, the suitability of preparation was investigated by estimation of marker ATPase activities of organels, which may participate in contamination.

Mean activity of the total MIT Mg^{2+}-ATPase in 10 tested preparations was 37.66 ± 1.09 $\mu mol\ P_i \times mg^{-1}$ prot. $\times\ h^{-1}$. The activity of Na^+,K^+-ATPase was estimated as an additional increase in the MIT Mg^{2+}-ATPase activity obtained by following addition of Na^+ and K^+ ions to the basic medium, and it amounted to 0.46 $\mu mol\ P_i \times mg^{-1}$ prot. $\times\ h^{-1}$. The proportion of Na^+,K^+-ATPase activity to that of the MIT Mg^{2+}-ATPase amounted by 0.84 %. This number represents the median value of the range between 0.68–1.47 % and indicates the percentage of contamination of the MIT fraction by sarcolemma.

The activity of Mg^{2+}-Ca^{2+}-ATPase indicating the presence of sarcoplasmic reticulum membranes in isolated MIT preparation amounted to 0.72 $\mu mol\ P_i \times mg^{-1}$ prot. $\times\ h^{-1}$. This value was obtained as the difference between the activity of the MIT Mg^{2+}-ATPase estimated in the absence and presence of Ca^{2+} ions in a concentration providing optimal stimulation of the Mg^{2+},Ca^{2+}-ATPase activity. The observed increase in ATPase activity against the Mg^{2+}-ATPase activity amounted to 1.59 %. This number represents the median value of the range between 0.94–2.01 % and indicates the percentage of contamination of the MIT fraction by sarcoplasmic reticulum.

In comparison with isolated MIT from healthy control hearts, MIT from the DH exhibited significantly ($p < 0.01$) decreased oxygen consumption in state 4 as

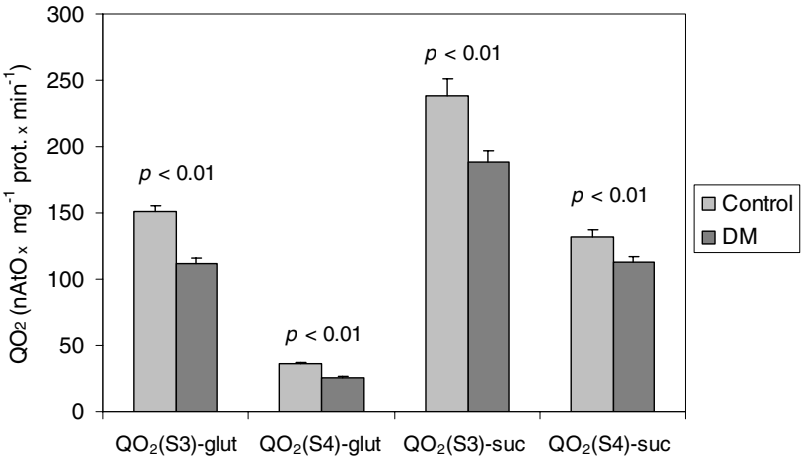

Figure 1. Glutamate and succinate-dependent state 3 and state 4 oxygen consumption (QO_2) by isolated mitochondria (MIT) from healthy control and acute diabetic hearts. QO_2(S3)-glut and QO_2(S4)-glut – state 3 and state 4 oxygen consumption with glutamate as substrate; QO_2(S3)-suc and QO_2(S4)-suc – state 3 and state 4 oxygen consumption with succinate as substrate. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

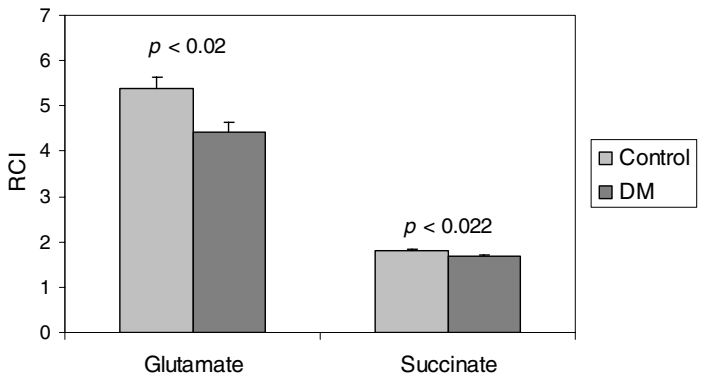

Figure 2. Respiratory control index (RCI) of MIT isolated from healthy control and acute diabetic hearts estimated with glutamate and succinate as substrates. Values of RCI are expressed as the ratio of the $QO_2(S3) : QO_2(S4)$. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

well as in state 3 for both glutamate as well as succinate as substrates (Fig. 1). Calculation of respiratory control indexes revealed that in MIT from DH, the de-

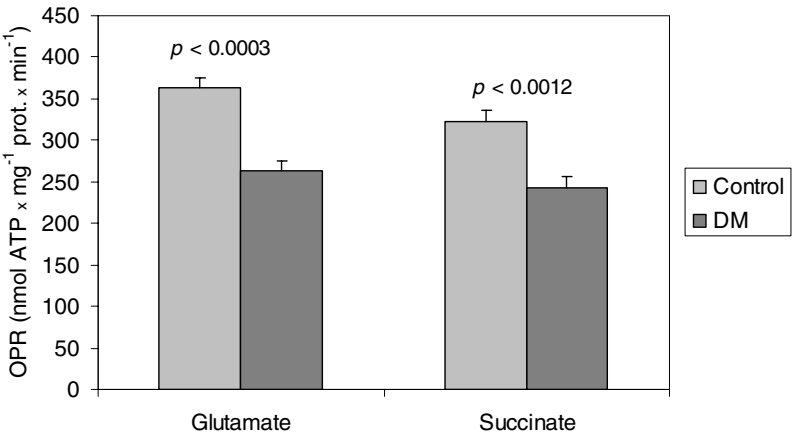

Figure 3. Values of oxidative phosphorylation rate (OPR) of MIT isolated from healthy control and acute diabetic hearts estimated with glutamate and succinate as substrates. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

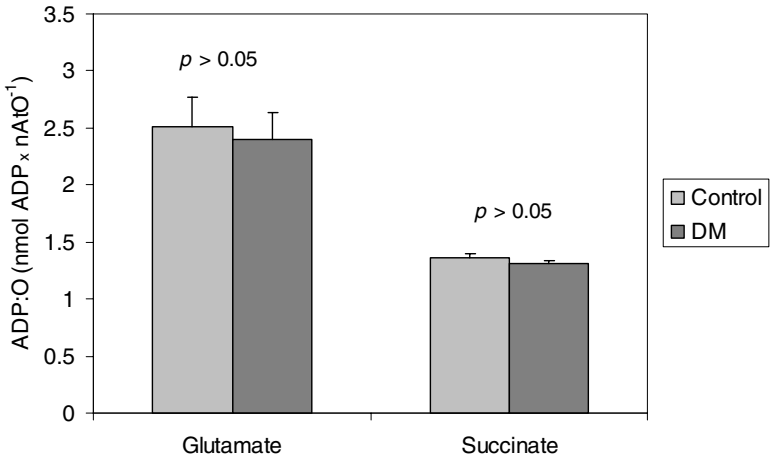

Figure 4. Values of oxidative phosphorylation (ADP : O) of MIT isolated from healthy control and acute diabetic hearts estimated with glutamate and succinate as substrates. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

crease in QO_2(S3) exceeded that in QO_2(S4). This yielded in significant ($p < 0.02$) depression of RCI values for glutamate and succinate as well (Fig. 2) and pointed to slowdown of the electron-flow in the respiratory chain of the DH MIT. Further

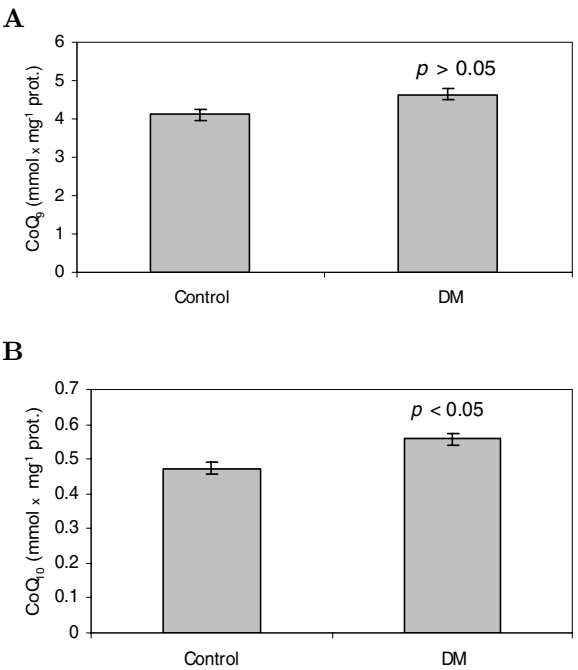

Figure 5. Acute STZ-diabetes induced changes in contents of oxidized forms of coenzymes Q_9(CoQ_9; panel A), and Q_{10} (CoQ_{10}; panel B) in the respiratory chain of isolated heart MIT. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

manifestation of diabetes-induced perturbations in function of the respiratory chain is a significant depression in the OPR for both glutamate ($p < 0.0003$) and succinate ($p < 0.0012$), respectively (Fig. 3). 12.9 and 18.4% increase in oxidized forms of CoQ_9 and CoQ_{10} (Fig. 4A and B) in MIT of hearts with acute DH indicates that action of free radicals may also be involved in hypofunction of the respiratory chain and decrease in OPR. Nevertheless, only the shift in CoQ_{10} was statistically significant ($p < 0.05$).

Interestingly, the depression of OPR, indicating a decrease in oxidative production of ATP, failed to be associated with a serious decrease in the ADP : O ratio ($p > 0.05$, Fig. 5) suggesting the presence of some mechanisms of endogenous protection. MIT Mg^{2+}-ATPase also might be involved in these mechanisms since the activity of this enzyme in DH MIT was increased significantly ($p < 0.05$, Fig. 6). Moreover, in acute DH, the amount of SETP in MIT membranes was also found significantly increased ($p < 0.05$, Fig. 7). The content of AMP was significantly lowered in diabetic hearts ($p < 0.05$, Fig. 8), but the total adenine nucleotide content as well as ATP and ADP contents were decreased only non-significantly. These results indicated that the acutely diabetic myocardium experiences a slight

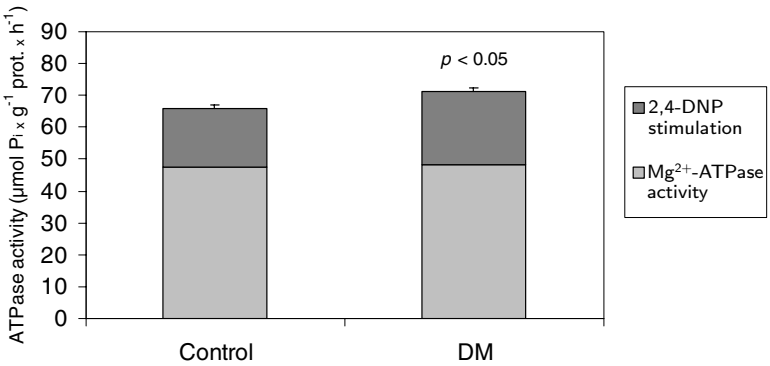

Figure 6. Basic and 2,4-dinitrophenol (2,4-DNP)-stimulated Mg^{2+}-ATPase activity of MIT isolated from hearts of healthy control and acute diabetic rats. The light part of columns – the basic Mg^{2+}-ATPase activity; the light plus dark parts of columns – the total Mg^{2+}-ATPase activity; dark part of columns – the 2,4-DNP-stimulated portion of the total Mg^{2+}-ATPase activity. Results are means ± S.E.M. from 15 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

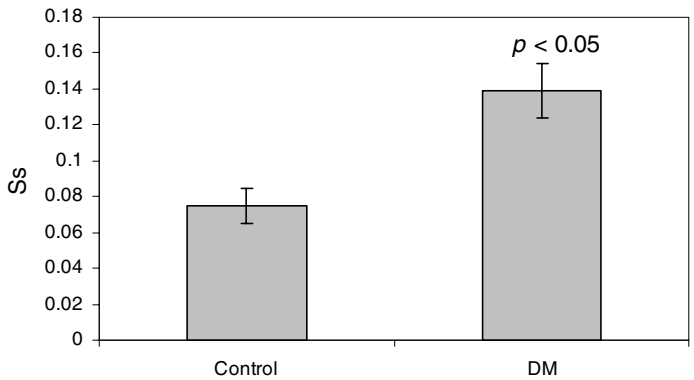

Figure 7. Substrate and energy transition pores (SETP) formation in MIT isolated from hearts of healthy control and acute diabetic rats. SETP were assessed by cytochemical detection of the octameric form of the MIT isoform of creatine phosphokinase and quantified stereologically. Results are means ± S.E.M. from 6 experiments and are expressed in surface density of MIT (Ss). Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

but constant degree of energy deficiency that may in a great part originate in depletion of the total adenine nucleotides (ΣAN) content. Evaluation of the ATP/ADP, ADP/AMP and ATP/AMP ratios amounting to 4.88, 2.83 and 11.13 in cardiac tissue of healthy controls and 4.66, 2.28 and 10.63 in the diabetic myocardium,

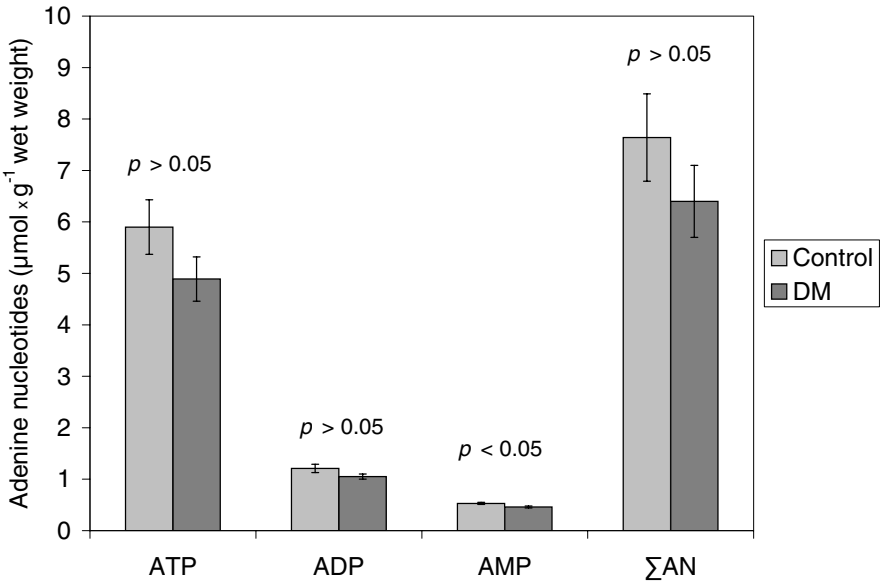

Figure 8. Levels of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and the total of adenine nucleotides (ΣAN) in cardiac tissue of healthy control and acute diabetic rats. Results are means ± S.E.M. from 7 experiments. Statistical evaluation – control *vs.* diabetic rat MIT (DM). For more details see Materials and Methods.

respectively, reveal a slowed down resynthesis of ATP from ADP and also from AMP in the DH. This is indicated by the fact that 4.9% of the total ATP content remains broken down on the ADP level and 4.5% on the AMP level and testifies for slight perturbations present not only in oxidative phosphorylation but also in the adenylate kinase reaction.

Discussion

Parallel to destructive processes, pathologic impulses such as diabetes also trigger mechanisms of endogenous protection – MEP. However, the changes induced by MEP in cardiovascular system are still little known. Since these changes are inadequately distinguished from diabetes-caused perturbations they become mostly suppressed by the treatment together with the pathological alterations.

It was well documented that the hyperglycemia, induced in rats by a single dose of STZ, reaches close to maximal levels as early as 24 h after administration of the drug and remains more or less stable for the following 8 days (Gøtzsche 1991). In general agreement with numerous of other authors, our earlier studies also confirmed that an 8 days lasting hyperglycemia is long enough for full development

of acute diabetes in rats, however, with effects of the accompanying complications still absent (Ziegelhöffer et al. 2002). In concert with previous data, in the present study the metabolic status of rats (Tab. 1) clearly confirms the presence of typical diabetes-induced metabolic alterations on the 8^{th} day after STZ administration.

Damage to oxidative energy production in the diabetic heart was already described by several authors long time ago (Kuo et al. 1983; Pierce and Dhalla 1984) and since that it was confirmed repeatedly by many others. Nevertheless, the information in this respect concerns predominantly the more advanced, chronic states of the disease. Moreover, the findings also seem to depend on the experimental model and methodology applied.

In contrast to results of investigators working with isolated MIT *in vitro*, Seymour and Brosnan (1991) investigated the oxidative energy production of DH MIT *in situ*, using isometric-perfused isolated rat heart (6 weeks of STZ-diabetes) and nuclear magnetic resonance spectroscopy. They found no evidence that either the aerobic energy metabolism in normoxic conditions or the energy metabolism in ischemic situation, are in any way abnormal in the diabetic heart. Today, this controversy may be at least partly explained by the knowledge about differences in free radical production by isolated MIT and by the MIT *in situ*. Experiments revealed that the respiratory chain is a major source of free radicals and the intensity of their production depends on which of the respiratory complexes are inhibited, on the substrates used to fuel the respiration and on membrane potential of the MIT. All these variables differ to some extent in isolated state and *in situ*. Some people believe, that the isolation of MIT and manipulation with isolated MIT may represent stimuli for free radical production and may aggravate or even induce pathological alterations. However, in MIT *in situ*, the treatment-induced alterations may stay absent. This view is supported by the finding that in contrast to isolated MIT, depolarization of MIT *in situ* does not influence the MIT free radical production (Tretter and Adam-Vizi 2004). Assuming all advantages and disadvantages, in present study we decided to use isolated MIT simply for making our results comparable with the prevailing part of other studies on function of MIT in the DH.

The decrease in oxygen consumption (Fig. 1) and the values of the RCI (Fig. 2) registered in our experiments documented that DH MIT are less capable to utilize oxygen with glutamate and succinate as well. The outcome of this disturbance is a decrease in OPR (Fig. 3) representing at the same time a lowered capacity of the oxidative phosphorylation to produce ATP. From pathophysiological point of view, this state may be termed as pseudo-hypoxia and in part resembles the situation experienced by the heart in mild hypoxia, but with a considerable decrease in tissue pO_2 and the accompanying switch over to lactate production being absent (Ziegelhöffer et al. 2005). Hence, it may be also anticipated that like in hypoxia, enhanced production of superoxide and peroxynitrite radicals (Andelová et al. 2005; Juránek and Bezek 2005; Važan et al. 2005) may in this state also occur in heart MIT (Tretter and Adam-Vizi 2004). The observed elevation in oxidized form of the CoQ_{10} (Fig. 5B) seems to justify such an assumption.

There is a circular argumentation about whether changes in enzyme activities and metabolism amounting 15 to 20% may be of real biological importance. To our opinion it is difficult to obtain a general answer to this question. The discussed differences between production and effects of radicals in isolated heart MIT and the MIT *in situ* seem to support this view. But as it concerns our findings of 12.9 and 18.4% increase in oxidized CoQ_9 and CoQ_{10}, the small but significant increase in MIT Mg^{2+}-ATPase activity and relatively small shifts in tissue content of adenine nucleotides and their mutual ratio, these seem to be significant at least from the point of view that they represent changes well fitting to a plausible sequence of events with either demonstrated or already known real consequences for the metabolism.

MIT Mg^{2+}-ATPase is an equivalent of the ATP-synthase estimated in reversed reaction and in the literature it is indicated by numerous synonyms. MIT Mg^{2+}-ATPase activity estimated by ATP splitting in presence of DNP is often referred to as: the Mg^{2+}-dependent and DNP-stimulated MIT ATPase (Cerreijó-Santaló 1967).

Perturbations in function of the respiratory chain and capacity of OPR, demonstrated in our studies in the DH, were not accompanied by any significant depression in efficacy of coupling of the oxidation with phosphorylation represented by the ADP : O ratio (Fig. 4). The significant increase in the oligomycin-sensitive MIT Mg^{2+}-ATPase activity (Fig. 6) seems to be in concert with the latter finding. This enzyme complex is playing at least three different roles: i) it acts as a proton translocating ATPase or proton pump involved in generation of the transmembrane proton gradient; ii) it utilizes the energy from the transmembrane proton gradient to phosphorylation of ADP to ATP, i.e. it is coupling the function of the respiratory chain to energy generation (Mitchell 1976); iii) by utilizing (splitting) intraMIT ATP it is also securing the demands of intraMIT energy requiring processes (Wang 1983). In respect to functions sub i) and ii), the elevated activity of the MIT ATPase observed in the present study may at least offer a support to preservation of the transmembrane proton gradient as well as the coupling of oxidation to phosphorylation demonstrated by preserved ADP : O (Ziegelhöffer 2005; Ziegelhöffer et al. 2005). On the other hand, the preserved ADP : O ratio practically excludes the possibility indicated sub iii) that the observed increase in Mg^{2+}-ATPase activity may serve for an increased breakdown of intraMIT ATP (Belisle and Kowaltowski 2002) since this would lead to uncoupling of oxidation from phosphorylation.

It has to be mentioned, that the membranes of intact MIT are impermeable to Mg^{2+} ions. Hence, Mg^{2+}-ATPase activity should be estimated in conditions, when all MIT have opened membranes and this may be achieved by addition of 2,4-DNP. Thus, the difference between Mg^{2+}-ATPase activity estimated in the absence and presence of 2,4-DNP also indicates the amount of intact MIT present in the given preparation. Interestingly, preparations of DH MIT always exhibited higher degree of intactness than MIT from healthy hearts, probably as the result of more stable membranes.

The slowdown of ATP synthesis in diabetic cardiomyocytes is also alleviated by augmented transfer of ATP to the cytosole *via* an increased amount of SETP in the MIT (Fig. 7). It was well documented that SETP in MIT membranes may be responsible for high capacity transfer of energy from MIT to the cytosole. SETP formation is an important mechanism belonging to MEP, triggered by prolonged calcium transients and increasing the availability of ATP in the diabetic heart cells (Ziegelhöffer-Mihalovičová et al. 1997; Ziegelhöffer et al. 2002).

Common consequence of functional remodeling of the MIT, i.e. keeping the production and intracellular transport of energy as high as possible and the remodeling of the sarcolemma, reducing the energy requirements by limited entry of extracellular Ca^{2+} as much as possible (Gøtzsche 1991; Ziegelhöffer et al. 1998 and 2002), is that the acute DH experience only mild energy deficiency.

It may be concluded that the acute diabetic heart suffers on pseudo-hypoxia with the energy production slowed down, but this is in part mitigated with MEP involving increased Mg^{2+}-ATPase (ATP-synthase) activity as well as facilitated transfer of ATP through the MIT membrane to the cytosole. Remodeled MIT function represents a genuine part of MEP and serve to preservation of energy equilibrium in the acute diabetic heart.

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