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Regulation of mitochondrial NADP-isocitrate dehydrogenase in rat heart during ischemia

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Abstract

The changes in the regulation of at mitochondrial NADP-isocitrate dehydrogenase (NADP-ICDH) in a rat heart during have been analysed. Increase of enzyme activity in the cytosol and mitochondria of the heart ischemia was detected. Catalytic properties of the mitochondrial NADP-ICDH at norm and pathology have been compared on homogeneous enzyme preparations. Enzyme from the normoxic and ischemic heart showed the same electrophoretical mobility and molecular mass. Enzyme isolated from the ischemic heart mitochondria demonstrated higher activation energy and lower thermal stability. NADP-isocitrate dehydrogenase at the normoxic and ischemic conditions exhibited different K_m for substrates and regulatory behaviour in relation to ATP, ADP, 2-oxoglutarate, citrate, malate, reduced and oxidised glutathione. The inhibitory effect of the Fe^{2+} and H_2O_2 mixture associated with the generation of hydroxyl radicals was lower in the ischemic enzyme. We hypothesise that the specific features of regulation behaviour of NADP-ICDH from the ischemic tissues permits the enzyme to supply NADPH to the glutathione reductase/glutathione peroxidase system. (Mol Cell Biochem **294:** 97–105, 2007)

Key words: ischemia; oxidative stress; NADP-isocitrate dehydrogenase; enzyme regulation

Introduction

The role of free radical processes in the aetiology of ischemia and myocardial infarction is widely accepted [1–4]. It is considered that the reactive oxygen species (ROS) formed in mitochondrial and microsomal oxidative reactions contribute to the development of cardiomyocite damage. Mitochondria are a major cellular source of ROS. The generation of superoxide anion depends on the respiration rate and the reduced state of electron chain carriers support the formation of superoxide ion by one-electron transfer reactions [5, 6]. Additionally, defective oxidative phosphorylation [7], degradation of ATP, xanthine [8, 9] and Fe-binding proteins, and free Fe^{2+} accumulation [10–12] increase the generation of ROS in mitochondria under the ischemic conditions. The glutathione reductase/glutathione peroxidase system (GRGP-system) has been proposed as a main mechanism of ROS-level regulation [10]. This mechanism plays an important role in H_2O_2 detoxification and might prevent the formation of more aggressive hydroxyl radical by the Fenton reaction [11]. The activity of the GRGP-system primarily depends on NADPH levels needed for the reposition of the reduced glutathione [13].

Reaction catalysed by the NADP-dependent isocitrate dehydrogenase (NADP-ICDH) plays a key role in NADPH supply in rat cardiomyocites [14]. Using a NIH3T3 cell line, Jo *et al.* [15] showed that mitochondrial NADP-ICDH acted as an antioxidative enzyme. The two NADP-ICDH isoenzymes in mammalian tissues were shown to differ in their

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distribution, sub-cellular localisation and some molecular and catalytic properties [16–19]. Earlier we reported the properties of the ischemic cytoplasmic NADP-ICDH from a rat heart and showed that enzyme could serve as an alternative to the pentose phosphate pathway as a source of NADPH at pathology [20]. It has not been excluded that the NADPH formed by mitochondrial NADP-ICDH in oxidative decarboxylation of threo-Ds-isocitrate might be essential for NADPH supply of the myocardial GRGP-system. Although, little is known about the functioning of mitochondrial NADP-ICDH in the myocardium, the high catalytic activity and the specific mRNA expression indicates its regulation at gene level under specific conditions [21, 22]. The physiological role of NADP-ICDH in the heart mitochondria is a controversial subject at the present time. Besides the indications that mitochondrial NADP-ICDH may function as an antioxidant there are some evidences suggesting that this enzyme operates in reverse direction of the tricarboxylic acid cycle (TAC). It has been proposed that the reverse flux through NADP-ICDH is coupled with the transhydrogenase reaction and may play a significant role in fine regulation of the TAC activity and in the enhancement of energy production [23].

This study aims to characterise the catalytic properties and regulation of the mitochondrial NADP-ICDH from rat myocardium at normoxia and ischemia and to elucidate the role of the enzyme in cardiomyocites response to the ischemic oxidative stress.

Materials and methods

Materials

NADP, ADP, ATP and glutathione were obtained from Reanal, Hungary. D, L-Isocitrate (trisodium salt) and 2-oxoglutarate were from Sigma Chemical Company, London, U.K. Tris was supplied by Serva, Germany. CM-Cellulose was purchased from Whatman, London, U.K. CM-Sephadex, Sephadex G-25 and G-150 was from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest analytical grade available.

Experimental procedures

Healthy male rats of the species, *Rattus novergicus albino*, weighting 200–250 g maintained on identical diet were used in the experiments. Experimental myocardial ischemia was induced by occlusion of the rats left coronary artery for 45 min [24]. Control rats have been "sham" operated, by unilateral thoracotomia.

Cell-free extract was prepared by tissue homogenisation in 0.05 M Tris-HCl-buffer**,** pH 7.8 containing 1 mM EDTA, 0.3 M sucrose. Suspension was filtered through four layers of cheesecloth and centrifuged at 3,500 g for 5 min. The pellet was discarded and supernatant centrifuged at 15,000 g for 15 min. Final supernatant containing cytoplasm NADP-ICDH activity was discharged. Pellet with intact mitochondria was washed and centrifuged twice in a minimal volume of 0.05 M Tris-HCl buffer, pH 7.8 containing 0.175 M KCl. The pellet was dissolved in 0.05 M Tris-HCl buffer, pH 7.8 and used in the purification of mitochondrial NADP-ICDH.

The enzyme reaction was followed spectrophotometrically by measuring the rate of NADPH formation at 340 nm [16]. The reaction mixture consisted of 0.05 M Tris-HCl buffer, pH 7.8, 0.20 mM $MnCl_2$, 0.05 mM isocitrate, 0.40 mM NADP. Enzyme unit corresponds to the amount of enzyme, catalysing the formation of 1 μmol of NADPH per minute at 25°C. The protein content of samples was determined according to Lowry *et al.* [25], with modifications of Yu and Steek [26].

All purification procedures were carried out at 0 to + 4°C. After centrifugation and washing, the mitochondrial suspension was treated with 20% glycerol and 1 % Triton X-100 (v/v).

Contamination by low weight compounds was avoided by gel-filtration on a Sephadex G-25 column (1.5 × 20.0 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.8**,** containing 0.10 mM EDTA and 0.50 mM β-mercaptoethanol (buffer A). Fractions containing the highest NADP-IDH activity were joined together and applied to a CM-Cellulose column (0.5 × 13.0 cm) pre-equilibrated with buffer A. Elution of the enzyme during ion-exchange chromatography was performed with the same buffer containing stepwise or linear concentration gradients of KCl. Fractions possessing NADP-IDH activity were loaded onto a Sephadex G-150 column (2.0 × 45.0 cm), previously equilibrated with buffer A. The flow rate of elution was 35, 30 and 25 $ml \cdot h^{-1}$ on each step of purification, respectively. Homogeneity of this enzyme was monitored by electrophoresis in 7.5% polyacrilamide gel as described by Davis [27] and gels stained with Coomassie Blue R-250 [28].

Electrophoretical homogeneous mitochondrial NADP-IDH purified from control, normoxic (86.1-fold purified) and ischemia-induced (104.2-fold) myocardium was used to perform a comparative study of enzyme catalytic and regulatory properties. The molecular mass of NADP-IDH was determined in the presence or absence of isocitrate and Mn^{2+} ions at non-saturating and saturating concentrations by gel-filtration on Sephadex G-150 [29]. Kinetic parameters were determined by modulating the concentrations of isocitrate, NADP or Mn^{2+} ions in the presence of a saturating concentration of NADP (0.40 mM), isocitrate (0.05 mM) or $MnCl_2$ (0.20 mM), respectively.

The effect of temperature on the NADP-IDH activity was determined after 5 min-incubation of the purified enzyme in a

reaction mixture at a temperature range between 10 and 70°C. Stability of NADP-IDH was estimated by temperature incubation of an appropriated amount of purified enzyme (0.07 mg of protein per tube). At 20 min intervals, aliquots were withdrawn and analysed for enzyme activity. Protein denaturation has determined by spectrophotometer measurement of the magnitude of light diffusion value (τ) at 490 nm.

The enzyme regulation in the presence of adenine nucleotides, TAC intermediates, glutathione, Fe^{2+} ions and H_2O_2 was carried out. Co-operation between metabolite inhibitors, Fe^{2+} ions and H_2O_2, was estimated according to Kurganov [30]. Type and constants of NADP-ICDH inhibition (K_i) was determined according with Dixon and Kornish-Bouden methods [31].

The statistical significance of the experimental data was determined by analysis of variance followed by Student t-tests. Differences between values were considered significant if $P < 0.05$. The graphs were plotted using data processed with programs of linear and parabolic approximation.

Results and discussion

In our previous work [20], we have shown that 45 minutes of ischemia causes an enhancement of free radical processes and total antioxidative activity estimated by chemiluminescence in cytosol and mitochondria of a rat heart. A 1.5-fold increase of the α-tocopherol contents in ischemic mitochondria was also observed. These changes were accompanied by 2.5- and 1.8-fold increase of NADP-ICDH activity in ischemic mitochondria and cytosol [20, 32]. Nevertheless, the control of the free radical processes intensity and total antioxidative activity took place in the presented experiments. NADP-ICDH was isolated from the heart as a whole taking into consideration that during infarction the myocardium lesions were not limited to the zone of ischemia. Several works have shown changes in the non-ischemic zone, which were similar to changes at ischemic disease of heart, specifically in the activation of lipid peroxid oxidation [33].

The mitochondrial NADP-ICDH of ischemic tissue was purified by ion exchange chromatography on DEAE-Cellulose, CM-Cellulose, DEAE-Sepharose and CM-Sephadex using a linear gradient of 0–200 mM KCl. Fractions with higher enzyme activity were obtained by chromatographic separation in CM-and DEAE-Cellulose. During the elution from DEAE-cellulose columns the NADP-ICDH activity in 155 mM KCl, the fractions were poorly separated from the aconitate hydratase activity (data not shown). Purified NADP-ICDH from normoxic and ischemic mitochondria migrated through the CM-Cellulose columns as a single peak in the presence of 22 and 27 mM KCl, respectively, while the specific activity of enzymes from both mitochondria varied nearly 2.5 times. The enzyme specific activity from the mitochondria of normoxic and ischemic heart had 15.5 and 38.0 unit mg^{-1} protein, respectively. Other purification parameters were 86.1 and 105.6-fold purification or 3.5 and 4.4% yield, respectively (Table 1). Yield of NADP-ICDH active fractions during elution on the Sephadex G-150 column coincided with a single peak of total protein (data not shown). The analysis of purified NADP-ICDH preparations of both tissues revealed a single protein band with the same electrophoresis' mobility, R_f 0.43 (Fig. 1), which differed from the mobility of the cytosolic isoenzyme from the normoxic or ischemic rat heart, with R_f 0.51 [20]. The purified mitochondrial enzyme were electrophoretically homogenous and appeared be free of cytosolic NADP-ICDH contaminations. The mitochondrial enzyme from the ischemic heart exhibited an increase of 2.5-fold in the specific activity over the enzyme from normoxic tissue. This result could be related to the observed initial differences in enzyme activity of mitochondrial fractions from the normoxic and ischemic heart detected either by differential centrifugation or ion exchange chromatography. Ischemia-induced increase of NADP-ICDH activity in the mitochondria of a rat heart was consistent with earlier observations for NADP-ICDH from the rat brain cytosol fraction [18]. Correlation between ischemic impairment and rising of NADP-ICDH activity in the cell cytoplasm of a rabbit spinal cord was also reported [34]. Thus, the enhancement in NADP-ICDH activity could occur in different mammalian tissues under ischemic conditions. However, some earlier studies reported the changes in enzyme activity only under pathological conditions [16, 34]. In previous work, we compared the cytosolic enzyme features in normoxic and ischemic tissues and related them to the level of ROS production [20]. Despite of that, the role and features of rat heart mitochondrial NADP-ICDH functioning at ischemia is still far from being understood. In order to elucidate the mechanisms associated with the enzyme activity changes at ischemia, the molecular and catalytic properties of mitochondrial NADP-ICDH from the normoxic and ischemic rat heart were compared.

Vertebrate's NADP-IDH consists of two identical subunits, which are involved in an association-dissociation mechanism of the enzyme activity regulation [34]. The substrate plays a relevant role as modulator of the subunits association or dissociation, as described for the NADP-ICDH from pig liver [35]. However, in the present study, the same molecular mass values have been found in enzyme from normoxic and ischemic rat heart (77.6 ± 5.0 kDa). These values were the same in the presence as well as in the absence of substrates and cofactors. Therefore, NADP-IDH association-dissociation mechanisms cannot explain the changes in enzyme activity during ischemia. We suggest that the changes in activity of mitochondrial NADP-ICDH under ischemia could be associated with some structural or conformational modifications of the enzyme. The increase in substrate affinity associated with the

Table 1. Purification of NADP-Isocitrate dehydrogenase from the normoxic and ischemic rat myocardium

Purification step	Exp. conditions	Total activity (units)[a]	Total protein, (mg)[a]	Specific activity, (units/mg of protein)[a]	Total yield, (%)	Times purified (total)
Crude extract	Normoxia	8.86 ± 0.08	48.1 ± 1.68	0.18 ± 0.05	100.00	1.00
	Ischemia	17.15 ± 0.30	48.05 ± 1.75	0.36 ± 0.07	100.00	1.00
Mitochondria + X100 Triton X100	Normoxia	1.30 ± 0.05	3.1 ± 0.30	0.42 ± 0.03	14.67	2.33
	Ischemia	3.22 ± 0.07	3.05 ± 0.35	1.06 ± 0.04	18.78	2.94
G-25	Normoxia	1.26 ± 0.03	3.00 ± 0.55	0.42 ± 0.04	14.22	2.33
	Ischemia	3.10 ± 0.02	3.00 ± 0.40	1.03 ± 0.02	18.08	2.86
CM-cellulose	Normoxia	0.67 ± 0.02	0.24 ± 0.04	2.79 ± 0.01	7.56	15.50
	Ischemia	1.54 ± 0.06	0.23 ± 0.05	6.70 ± 0.02	8.98	18.61
G-150	Normoxia	0.31 ± 0.04	0.02 ± 0.02	15.5 ± 0.05	3.50	86.11
	Ischemia	0.76 ± 0.04	0.02 ± 0.01	38.0 ± 0.05	4.43	105.60

[a] The results are expressed as the means + S.D. derived from three experiments.

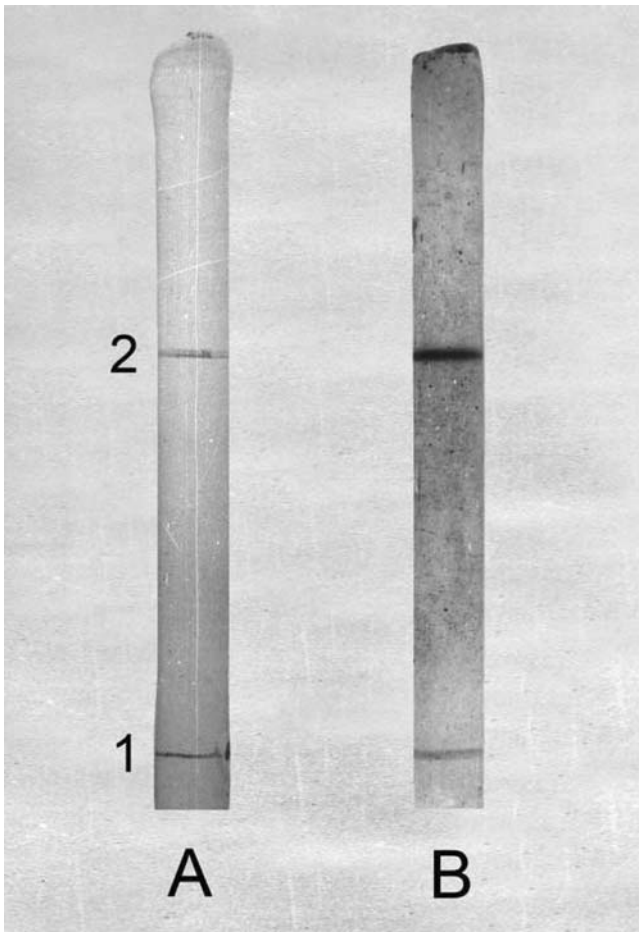

Fig. 1. Polyacrilamide gel electrophoresis of the NADP-isocitrate dehydrogenase from normoxic (a) and ischemic (b) rat heart mitochondria. *Band 1* shows a set of marker front. *Band 2* – NADP-isocitrate dehydrogenase. Proteins were stained with Coomassie Brilliant Blue R250.

changes of K_m for isocitrate and Mn^{2+} observed in NADP-ICDH under ischemia supports this supposition. The values for K_m of normoxic and ischemic NADP-ICDH to isocitrate, NADP and Mn^{2+} ions are presented in Table 2. Mitochondrial NADPH-ICDH from ischemic and normoxic heart had different temperature optimums, which were 40 and 50°C, respectively (Fig 2A). The Arrhenius plot for the enzyme incubation temperature at the intervals between 10 to 40°C showed a breakpoint at 30°C, resulting in activation energy values of 1820 kcal between 10 and 30°C and 5936 kcal between 30 and 40°C for ischemic NADP-ICDH (Fig. 2B). No breakpoint in the Arrhenius plot for the normoxic enzyme has been identified. The activation energy for the enzyme at the temperature interval between 10 and 50°C has 7756 kcal. Despite the lower initial activity, the purified normoxic NADP-ICDH showed higher thermostability, at 60–70°C, than at ischemic conditions. Faster enzyme inactivation and the increase of light diffusion are other parameters confirming these differences (Fig. 2C, D). These results allow us to conclude that mitochondrial NADP-ICDH from the normoxic and ischemic heart were the same enzyme, however, under ischemia the enzyme showed higher substrate affinity and lower temperature stability. Thus, temperature breakpoint, higher energy activation, lower temperature optimum and stability can indicate possible enzyme structure and conformational changes during the ischemia. Some data point out to the structural modifications of NADP-ICDH under oxidative stress. In particular, it was reported that mitochondrial NADP-ICDH contains reduced cysteinyl residues that regulate the enzyme activity [36, 37]. Their sulfohydryl groups are susceptible to modification by ROS, nitric oxid, lipid peroxidation products and S-glutathionylation [38–42]. Modification through glutathionylation of NADP-ICDH from the HEK293 cells and intact respiring rabbit heart mitochondria treated with oxidants may lead to a slight disruption of protein structure

Table 2. Kinetic proprieties of the purified mitochondrial NADP-Isocitrate dehydrogenase from the normoxic and ischemic rat heart

Parameters	Normoxia[a], μM	Ischemia[a], μM
K_m isocitrate	45 ± 5	$17 \pm 6^*$
K_m NADP	46 ± 5	46 ± 4
K_m Mn^{2+} ions	420 ± 15	$150 \pm 10^*$
K_i Fe^{2+} ions	50 ± 4 (2)	50 ± 3 (2)
K_i H_2O_2	450 ± 50 (3)	$1630 \pm 48^*$ (3)
K_i ($Fe^{2+} + H_2O_2$)	15 ± 2 (2)	$25 \pm 3^*$ (2)
K_i glutathione	150 ± 32 (3)	no*
K_i citrate	159 ± 29 (1)	$280 \pm 41^*$(1)
K_i 2-oxoglutarate	80 ± 26 (1)	$250 \pm 30^*$(1)

[a]The results are expressed as the means + S.D. derived from three experiments. (*) Statistical significant values, $P < 0.05$. In brackets is marked the type of inhibition: 1 – competitive; 2 – non-competitive; 3 – mixed.

Table 3. Effects of malate, succinate and fumarate on the activities of NADP-isocitrate dehydrogenase from the normoxic and ischemic rat myocardium

Metabolites	Concentration, mM	Activity of NADP-ICDH, normoxia	% from the control value[a] Ischemia
Malate	0.1	90 ± 4	$60 \pm 4^*$
	0.25	87 ± 4	$55 \pm 5^*$
	0.5	85 ± 5	$52 \pm 4^*$
	1	83 ± 7	$40 \pm 6^*$
	1.5	80 ± 5	$35 \pm 4^*$
Succinate	0.1	85 ± 6	80 ± 4
	0.25	80 ± 4	77 ± 6
	0.5	74 ± 5	74 ± 6
	1	70 ± 4	70 ± 5
	1.5	65 ± 4	65 ± 5
Fumarate	0.1	78 ± 4	72 ± 4
	0.25	74 ± 4	65 ± 3
	0.5	73 ± 5	65 ± 6
	1	70 ± 4	63 ± 5
	1.5	68 ± 3	60 ± 6

[a]The results are expressed as the means $\pm$ S.D. derived from 3–5 experiments. (*) Statistical significant values, $P < 0.05$.

[42]. It was proposed that this mechanism of activity regulation might prevent enzyme from further oxidation by ROS to its irreversible forms [42]. Moreover, the glutathionylated NADP-ICDH is more resistant to protease action and digestion, presumably through slight conformational changes of its molecules. However, it must be pointed out, that the glutathionylation can inhibit the mitochondrial NADP-ICDH, when the production of oxidants exceeds antioxidant capacity [42]. According to our data, mobilisation of the antioxidant system took place in rat heart mitochondria during occlusion of the left coronary artery for 45 minutes [32]. Therefore, NADP- ICDH may participate in the cellular defence system against oxidative damage by supplying NADPH to mitochondria needed for the regeneration of GSH. Increasing enzyme activity under the referred conditions may support this conclusion.

Our study of the influence of metabolites, which organelle content could be changed under ischemia, on the normoxic and ischemic enzyme behaviour showed that the concentrations of ATP and ADP can regulate the activity of NADP-IDH under ischemic conditions. It is known that the ATP degradation [13] related to breaking synthesis [8], transport [43] and ATP utilisation [44], and ADP accumulation leads to the decrease of ATP/ADP ratio, which is considered as an indicator of radical metabolic changes under ischemia. A non-reversible ischemic damage and cell necrosis of cardiomyocites was accompanied by a 50% decrease of initial ATP concentrations to 3.5 μM [4, 44]. We showed that the ATP increase from 3.5 to 250 μM caused a slight increase of enzyme activity in ischemic mitochondria ($118 \pm 3\%$, at 7 μM ATP) when an enzyme inhibition was observed at normoxia (Fig. 3A). At the same time, a slight inhibition by ADP was observed at concentrations ranging from 5.0 to 40.0 μM for normoxic and from 2.5 to 250 μM for ischemic enzyme (Fig. 3A). The absence of an ATP inhibition and a stronger inhibitory effect of ADP at the physiological concentrations under ischemia could reflect other metabolic functions of this enzyme under oxidative stress [4, 12, 45].

We also analysed the influence of some di- and tricarboxylic acids, including 2-oxoglutarate, citrate, malate, fumarate and succinate, on NADP-ICDH activity from both tissues. Inhibitory effects of these intermediates on NADP-ICDH were detected. But the effects of citrate, 2-oxoglutarate and malate were different for the enzyme from the normoxic and ischemic tissues. The 2-oxoglutarate behaved as a competitive inhibitor with respect to isocitrate. K_i for 2-oxoglutarate had values of 80 and 205 μM, for the normoxic and ischemic enzyme, respectively (Table 2). On the contrary, citrate (Table 2) and malate (Table 3) were stronger inhibitors of NADP-IDH under ischemia than at normoxia, while the fumarate and succinate had no significant effect on the enzyme activity (Table 3). These results may indicate that mitochondrial NADP-ICDH under ischemic conditions exhibited an abnormal catalytic behaviour. Interestingly, the strongest NADP-ICDH inhibitor at normoxia, 2-oxoglutarate, had lower inhibitory effect under ischemia. It should be taken into account that 2-oxoglutarate serves as the main nitrogen acceptor and it is a precursor of glutamate [18, 20]. Hence, under ischemia the changes of enzyme behaviour in relation to 2-oxoglutarate can stimulate the amino acids biosynthesis [46] and utilisation of accumulated the TAC intermediates [43]. At the same time it could be also proposed that the increase of the enzyme activity and substrate affinity under ischemia may promote the enhancement of NADPH supply for the GRGP-system, activity of which increases during oxidative stress [13, 47].

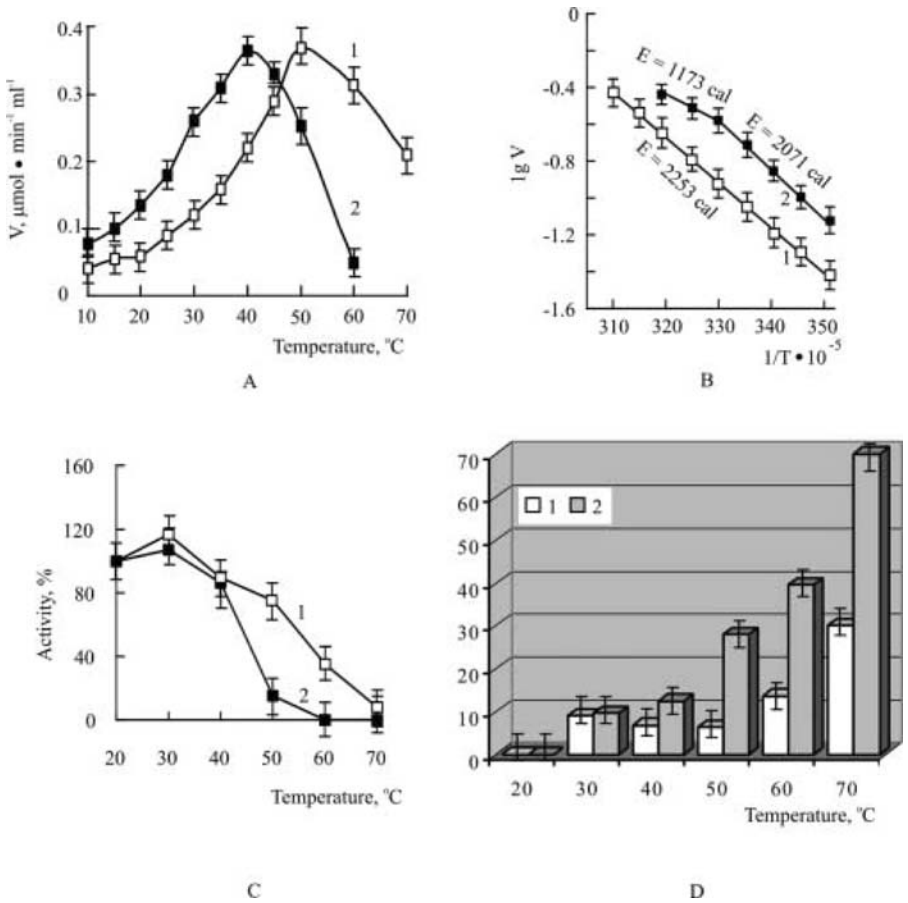

Fig. 2. The influence of temperature on NADP-isocitrate dehydrogenase activity from normoxic (1) and ischemic (2) rat heart mitochondria. **A.** Determination of the enzyme temperature optimum. Data are means ± S.D. ($n = 4$) **B.** Arrhenius plot of the data from 10°C to 50°C and 10°C to 40°C. Data are means ± S.D. ($n = 4$). **C.** Determination of the enzyme thermo-inactivation. Data are means ± S.D. ($n = 4$). **D.** Determination of the enzyme thermostability. Determination of the enzyme thermostability was estimated spectrophotometrically by measuring the magnitudes of light diffusion value (τ) of NADP-isocitrate dehydrogenase preparations at 490 nm. The results are expressed as the means + S.D. ($n = 4$).

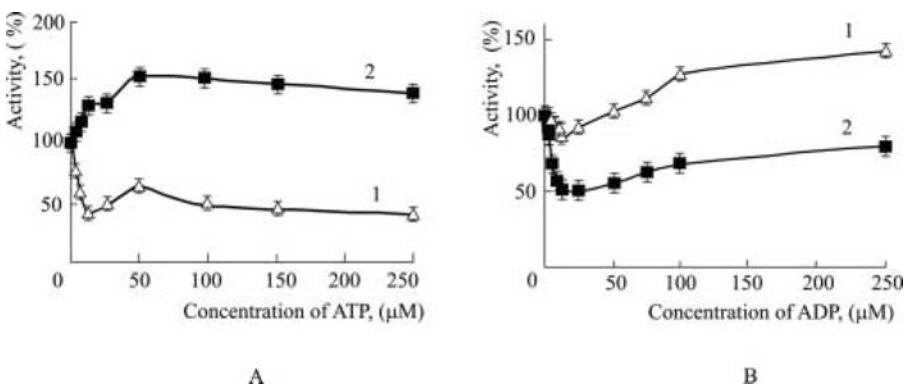

Fig. 3. The influence of ATP (A) and ADP (B) on NADP-isocitrate dehydrogenase activity from normoxic (1) and ischemic (2) rat heart mitochondria. The results are expressed as the means + S.D. derived from three experiments.

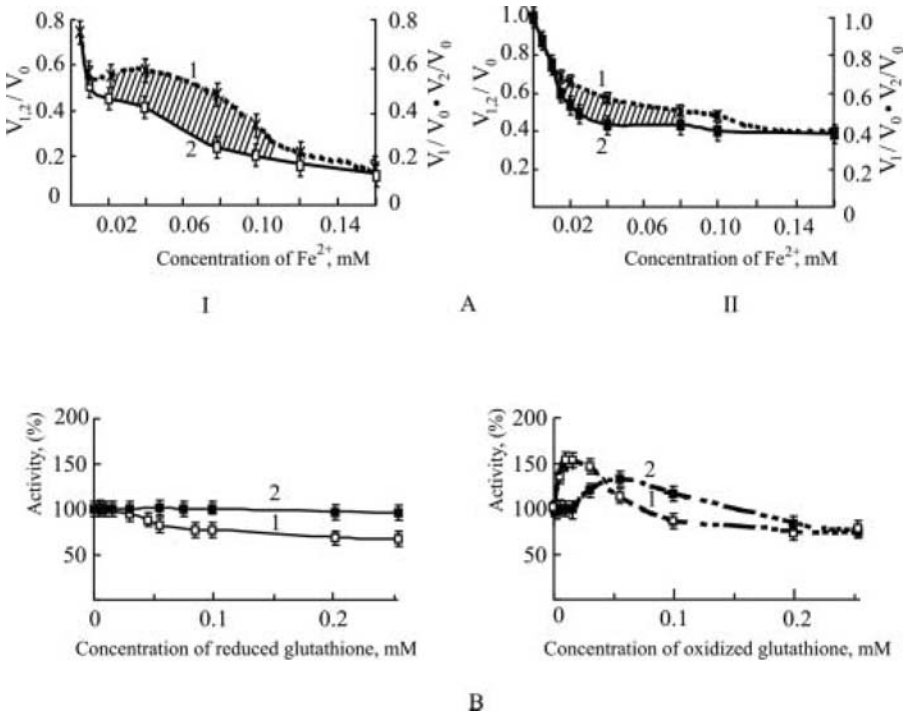

Fig. 4. The influence of Fe^{2+} and H_2O_2 mixture (A) and glutathione (B) on NADP-isocitrate dehydrogenase activity from normoxic (1) and ischemic (2) rat heart mitochondria. Determination of the type of Fe^{2+} and H_2O_2 co-operation during inhibition of NADP-isocitrate dehydrogenase from mitochondria of normoxic (I) and ischemic (II) rat heart was performed according to Kurganov. The enzyme activity assayed as a function of NADPH concentrations using 0.4 mM NADP, 0.2 mM $MnCl_2$ and 0.05 mM isocitrate in the presence of 0.10 mM H_2O_2. The 0.02–0.16 mM concentration range of Fe^{2+} ions were used. 1 – $V_{1,2}/V_0$ – ratio of the enzyme reaction velocity in the presence or absence of two inhibitors; $V_1/V_0 \times V_2/V_0$ – product of ratio of the enzyme reaction velocity at the presence of the first inhibitor (Fe^{2+} ions) and the second inhibitor (H_2O_2) to initial velocity. Data are means ± S.D. ($n = 5$).

The influence of substrates of the Fenton reaction on the NADP-ICDH activity was evaluated. Strong inhibition of NADP-ICDH by H_2O_2 in mitochondria of healthy rats was observed (Table 2). At the same time, the inhibitory effect of Fe^{2+} was not different at both normoxic and ischemic conditions. However, when combined, Fe^{2+} and H_2O_2 exhibited pronounced inhibitory effect at normoxia (lower K_i values) when compared with ischemia (higher K_i values) (Table 2). The analysis of interactions between these two inhibitors allow us to conclude that the NADP-ICDH inhibition by Fe^{2+} and H_2O_2 was characterised by a synergistic effect expressed in a lower extent under ischemic conditions (Fig. 4A). Fe^{2+} and H_2O_2 interactions lead to the formation of hydroxyl radical, an aggressive ROS species. Therefore, decrease of the NADP-ICDH activity can be associated with the enzyme oxidation through the carbonylation. This suggestion is consistent with the fact that protein carbonylation was a common biomarker of severe oxidative damage in proteins [48]. It cannot be excluded that regulation of the enzyme by Fe^{2+} and H_2O_2 might be connected with its participation in the NADPH generation for the GRGP-system. Under ischemia, the lowest enzyme repression in the presence of the components of the Fenton reaction can also increase NADPH level and activate the GRGP-system.

Under our experimental conditions the reduced glutathione inhibited NADP-ICDH from the normoxic heart mitochondria at the concentrations ranging from 5 to 100 μM, while had no inhibitory effect on the enzyme from ischemic mitochondria (Fig. 4B, Table 2). On the contrary, oxidised glutathione activated the NADP-IDH from the ischemic mitochondria at the concentrations ranging from 25 to 125 μM, whereas it had weaker effect on the enzyme from the normoxic tissue (Fig. 4B). NADP-ICDH activity increased in 10 ± 2% and 53 ± 2% in the presence of 10 μM oxidised glutathione at normoxia and ischemia, respectively. These data suggest that the reduced and oxidised glutathione have different regulatory effects on the activity of the mitochondrial NADP-IDH at the normoxic and ischemic conditions. The observed regulatory effects could be important in the mitochondrial NADP-ICDH and the GRGP-system interaction in a rat heart under increasing ROS production.

NADP-ICDH activation coupled with the increasing NADPH production due to particular kinetic and regulatory enzyme changes can lead to the growth of reduced/oxidized

glutathione ratio, which in turn is determined by the GP activation. This supposition remains in contradiction with the reverse metabolic flux through NADP-ICDH and its hypothesized role in the fine regulation of the TCA activity [23]. On the other hand, it is consistent with the suggestion of Jo *et al.* [15] based on evidence of antioxidant role of the enzyme obtained from N1H3T3 cells. It must be taken into account that in heart the mitochondrial NADPH/NADP ratio is more than 50 [49, 50], when in the N1H3T3 cells was less than 1 [23]. Nevertheless, under conditions of severe oxidative stress created in myocardium, during ischemia the main consequences of the TAC repression would be the decrease of NADH level and suppression of the electron transport. NADH availability can limit the activity of transhydrogenase to the enhancement of NADPH generation [23, 51]. Some other evidences related to the antioxidant role of the mitochondrial NADP-ICDH were also reported [39, 52, 53].

It cannot be excluded that changes in the NADP-ICDH behaviour under ischemia might have a regulatory significance for the functioning of the myocardial antioxidative GRGP-system. Higher catalytic activity, K_m, and differential sensitivity of NADP-ICDH to several metabolites can be explained on the grounds of adaptation strategies to the oxidative stress and tissue recovery.

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