Oxidative Status and Distribution of NADP-Dependent Isocitrate Dehydrogenase and Aconitate Hydratase in Rat Cardiomyocytes under Normal Conditions and during Ischemia

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Oxidative status of rat cardiomyocytes during ischemia induced by occlusion of the descending branch of the coronary artery was studied by the methods of Fe-induced chemiluminescence and spectrophotometry of primary and secondary lipid peroxidation product. The concentrations of low-molecular-weight antioxidants α-tocopherol and citrate and activities of NADP-dependent isocitrate dehydrogenase (EC 1.1.1.41) and aconitate hydratase (EC 4.2.1.3) were also measured. Ischemia was associated with intensification of free radical processes, increased antioxidant activity in subcellular fractions of the myocardium, activation of NADP-isocitrate dehydrogenase, accumulation of citrate, and inhibition of aconitate hydratase. Differential centrifugation, ion exchange chromatography on various ion exchangers, and electrophoresis in polyacrylamide gel revealed no redistribution of enzyme activity between the cytoplasmic and mitochondrial cardiomyocyte fractions during ischemia.

Key Words: *ischemia; cardiomyocytes; citrate; NADP-dependent isocitrate dehydrogenase; aconitate hydratase*

Oxidative stress resulting from the imbalance between hyperproduction of reactive oxygen species (ROS) and insufficiency of antioxidant systems is the leading pathogenic factor of ischemia. Recent studies of biochemical bases of the pathogenesis of cardiac ischemia were focused on the search for effective methods of antioxidant defense at the subcellular level [6,15]. In light of this, localization and interrelations between various antioxidant mechanisms of detoxification of H_2O_2, the main source of hydroxyl radicals, the most aggressive ROS, attracts special attention. It is known that H_2O_2 is utilized by glutathione reductase/glutathione peroxidase system of the mitochondrial matrix. Activity of this system depends on NADP concentration in cells [5]. In case of insufficiency of this system, H_2O_2 diffusion through the mitochondrial membrane into the cytoplasm is prevented by α-tocopherol, a nonenzyme component of the antioxidant system [1]. It is believed that the antioxidant function of α-tocopherol is realized only in the presence of low-molecular-weight acids, in particular, citrate [1]. Moreover, citrate forms chelate complexes with Fe^{2+} involved in H_2O_2 degradation to $OH^{\bullet}$ in Fenton's reaction. For elucidation of the mechanisms mediating the interaction between these antioxidant systems, we evaluated the intensity of free radical processes, content of α-tocopherol and citrate, and activity of aconitate hydratase (AH, citrate utilization) and NADP-dependent isocitrate dehydrogenase (NADP-IDH, NADPH generation) in subcellular fractions of rat cardiomyocytes under normal conditions and during ischemia.

MATERIALS AND METHODS

The experiments were carried out on 100 white male rats weighing 250-300 g. Myocardial ischemia was

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modeled by 45-min occlusion of the descending branch of coronary artery on the level of auricle [3]. Cytoplasmic and mitochondrial cardiomyocyte fractions were isolated from normal and ischemic rat myocardium by differential centrifugation [10]. Myocardium samples were homogenized in 3-fold volume of 50 mM Tris-HCl buffer (pH 7.6) with 0.3 M sucrose. The homogenate was filtered and centrifuged for 5 min at 3500*g* to remove undestroyed cell elements and organelle membranes and the supernatant was centrifuged for 15 min at 15,000*g*. The resultant supernatant (cytoplasmic fraction) and pellet (mitochondria) were used for determination of chemiluminescence parameters, contents of primary and secondary LPO products, α-tocopherol, and citrate, and activities of NADP-IDH and AH. Mitochondria were washed from sucrose, resuspended in a minimum volume of 50 mM Tris-HCl (pH 7.6) with 0.175 M KCl, and centrifuged in the above described regime. Cross contamination of subcellular fractions was evaluated by activity of marker enzymes succinate dehydrogenase (SDH), NAD-IDH (mitochondrial markers), and lactate dehydrogenase (cytosolic marker).

Oxidative status of normal and ischemic myocardium was evaluated by Fe^{2+}-induced chemiluminescence and by the content of primary and secondary LPO products diene conjugates (DC) and malonic dialdehyde (MDA) in subcellular fractions measured spectrophotometrically.

Chemiluminescence of the cytoplasm mitochondrial suspension from normal rats and animals subjected to ischemia was recorded on a BCL-0.6 biochemiluminometer equipped with special software. Chemiluminescence was induced by adding 0.2 ml 2% H_2O_2 to a cuvette containing 0.40 ml 0.125 M KH_2PO_4 (pH 7.4), 0.4 ml 0.01 mM $FeSO_4$, and 0.05 ml sample. Kinetic curve was recorded for 40 sec. The following parameters were analyzed: photosum of chemiluminescence, intensity of maximum flash (I_{max}), and slope of kinetic curve ($tg\alpha_1$) [4]. DC content was measured spectrophotometrically in heptene extract at λ=233 nm [8], MDA was determined by the reaction with 2-thiobarbituric acid (2-TBA). Antioxidant activity of subcellular myocardium fractions was evaluated by 2 independent methods: by the slope of chemiluminescence curve ($tg\alpha$) and spectrophotometrically by the concentration of the main endogenous antioxidant, α-tocopherol [7]. Citrate concentration was determined spectrophotometrically using a modified micromethod by qualitative reaction with thiocarbamide [2].

Distribution of enzyme activities was studied by differential centrifugation and ion exchange chromatography, and by polyacrylamide gel (PAAG) electrophoresis with subsequent specific reaction for NADP-IDH and AH. Enzyme activity in mitochondria was measured after solubilization with 1% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.6) [10]. NADP-IDH and AH activities were evaluated by NADP reduction and cis-aconitate formation, respectively, measured spectrophotometrically at λ=340 and λ=233 nm [9,12]. NADP-IDH activity was determined using 50 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM $MnCl_2$, 0.05 mM isocitrate, and 0.4 mM NADP. AH spectrophotometry was carried out in 50 mM Tris-HCl buffer (pH 7.8) containing 0.15 mM citrate. Protein content in samples was determined by the method of Lowry [11].

We used Sephadex G-25, G-150, carboxymethyl sephadex (Pharmacia), diethylaminoethyl and carboxymethyl cellulose (Whatman), Tris (Serva), isocitrate (Sigma), NADP (Reanal), and TBA (Merck), and some domestic reagents of chemical or analytical purity grade.

The experiments were carried out in 3 and 2 biological and analytical replications, respectively. The data were processed using Student's *t* test; the differences were significant at $p<0.05$.

RESULTS

DC content in the cytoplasmic and mitochondrial cardiomyocyte fractions from rats with experimental ischemia increased 9.1- and 4.3-fold, the content of MDA increased 5.4- and 2.7-fold, respectively, compared to the corresponding parameters in normal rats (Table 1). In ischemic rats DC/MDA ratio increased by 76 and 59% in the cytoplasmic and mitochondrial fractions, respectively. These data attest to intensification of LPO processes in cardiomyocyte cytoplasm during ischemia. The measurement of chemiluminescence parameters showed similar results (Table 1). In rats with experimental ischemia, the photosum and I_{max} increased 2.1- and 22.5-fold in the cytoplasmic fraction, and 1.9- and 7.9-fold in the mitochondrial fraction, respectively, compared to normal; $tg\alpha_1$ increased 20.1- and 16.8-fold in the cytoplasm and mitochondrial suspension, respectively. Thus, occlusion of the descending branch of the left coronary artery intensified free radical processes and LPO both in cytoplasmic and mitochondrial fractions of rat cardiomyocytes.

In cytoplasmic and mitochondrial fractions $tg\alpha_2$ 16.3- and 4.3-fold surpassed the normal (Table 1). The study of α-tocopherol distribution in subcellular fractions showed that in control rats it occurred primarily in the cytoplasm, while in experimental rats it was found primarily in the mitochondrial fraction (Table 1). The content of α-tocopherol in the mitochondrial fraction during ischemia increased 1.8-fold, while in the cytoplasmic fraction it decreased by 10% compared to normal. The antioxidant function of α-tocopherol in mitochondria is associated with direct recombination with ROS in hydrophilic regions of the membrane and

TABLE 1. Intensity of Free Radical Processes in Subcellular Fractions of Myocardium from Control Rats and Animals with Experimental Ischemia (*M*±*m*)

Parameter	Cytoplasmic fraction		Mitochondrial fraction	
	control	ischemia	control	ischemia
DC, μmol/g	0.56±0.03	5.07±0.21*	0.83±0.02	3.56±0.13*
MDA, μmol/g	0.89±0.06	4.83±0.15*	1.07±0.04	2.88±0.09
DC/MDA	0.63	1.11	0.78	1.24
α-Tocopherol, μmol/g	0.96±0.05	0.75±0.03*	0.86±0.04	1.33±0.05*
%	55.8±3.7	65.5±3.2	44.2±3.3	34.5±3.5
Citrate, μmol/g	0.233±0.040	0.344±0.010*	0.132±0.060	0.204±0.030
%	63.8±1.2	62.8±1.3	36.2±1.3	37.2±1.1
Photosum of slow flash, arb. units	2.62±0.20	5.40±0.50	2.43±0.47	9.5±0.2
$tg\alpha_1$	0.24±0.10	4.82±0.16	0.16±0.04	2.72±0.05
$tg\alpha_2$	-0.08±0.04	-1.30±0.09	-0.12±0.01	-0.52±0.03
I_{max}	0.15±0.05	3.37±0.07	0.19±0.07	1.49±0.05

Note. *p<0.05 compared to the control.

incorporation of its hydrophobic core (tocol) into the compact membrane architecture [1]. The obtained data point to subcellular specificity in mobilization of cardiomyocyte antioxidant systems during ischemia.

Study of citrate distribution in subcellular structures showed that this metabolite was present primarily in the cytoplasm (Table 1). During experimental ischemia citrate content increased 1.5- and 1.6-fold in the cytoplasm and mitochondria, respectively (Table 1). This ischemia-induced increase in citrate content probably plays a protective role due to its ability to chelate Fe^{2+} ions [6,15].

Differential centrifugation showed that NADP-IDH and AH in both normal and experimental rats were present primarily in cardiomyocyte cytoplasm (Fig. 1). The distribution of activities of cytoplasmic and mitochondrial marker enzymes showed that most organelles remained undestroyed after differential centrifugation. The cross contamination of cytoplasmic and mitochondrial fractions was 6±2 and 4±2%, respectively. The data on subcellular NADP-IDH and AH localization obtained by differential centrifugation and ion exchange chromatography on various exchangers suggest that high and low activity peaks corresponded to cytoplasmic and mitochondrial NADP-IDH and AH forms (Fig. 2). It can be assumed that mitochondrial and cytoplasmic fractions of rat myocardium contain one NADP-IDH and one AH isoform each. Ischemia 1.8- and 2.5-fold increased NADP-IDH activity in cardiomyocyte cytoplasm and mitochon-

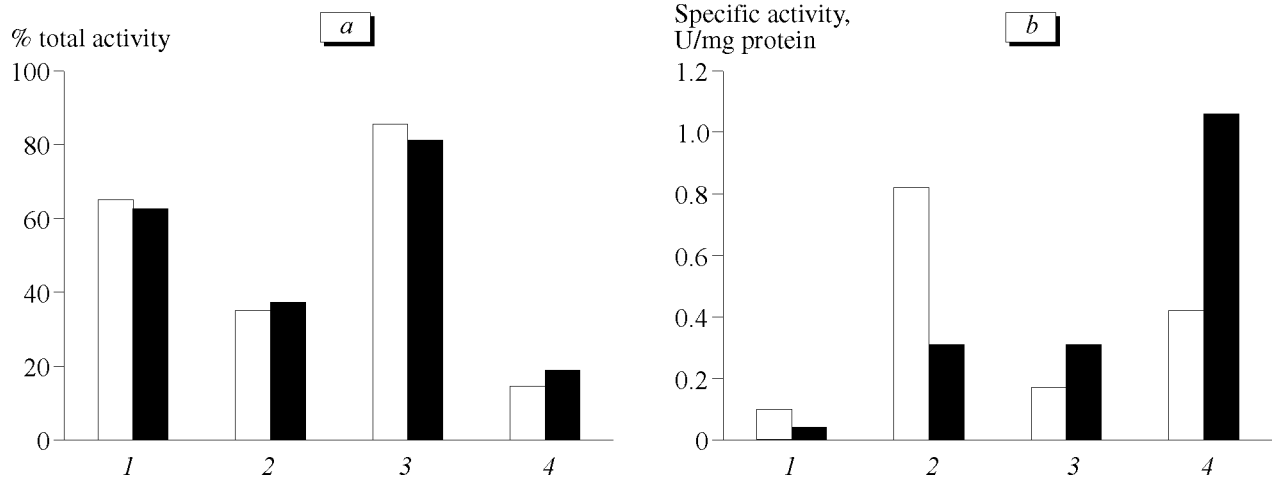

Fig. 1. Relative (*a*) and specific (*b*) activity of aconitate hydratase (*1*, *2*) and NADP-dependent isocitrate dehydrogenase (*3*, *4*) in cytoplasmic (*1*, *3*) and mitochondrial (*2*, *4*) fractions of myocardium from control rats (open bars) and animals subjected to experimental ischemia (dark bars); differential centrifugation.

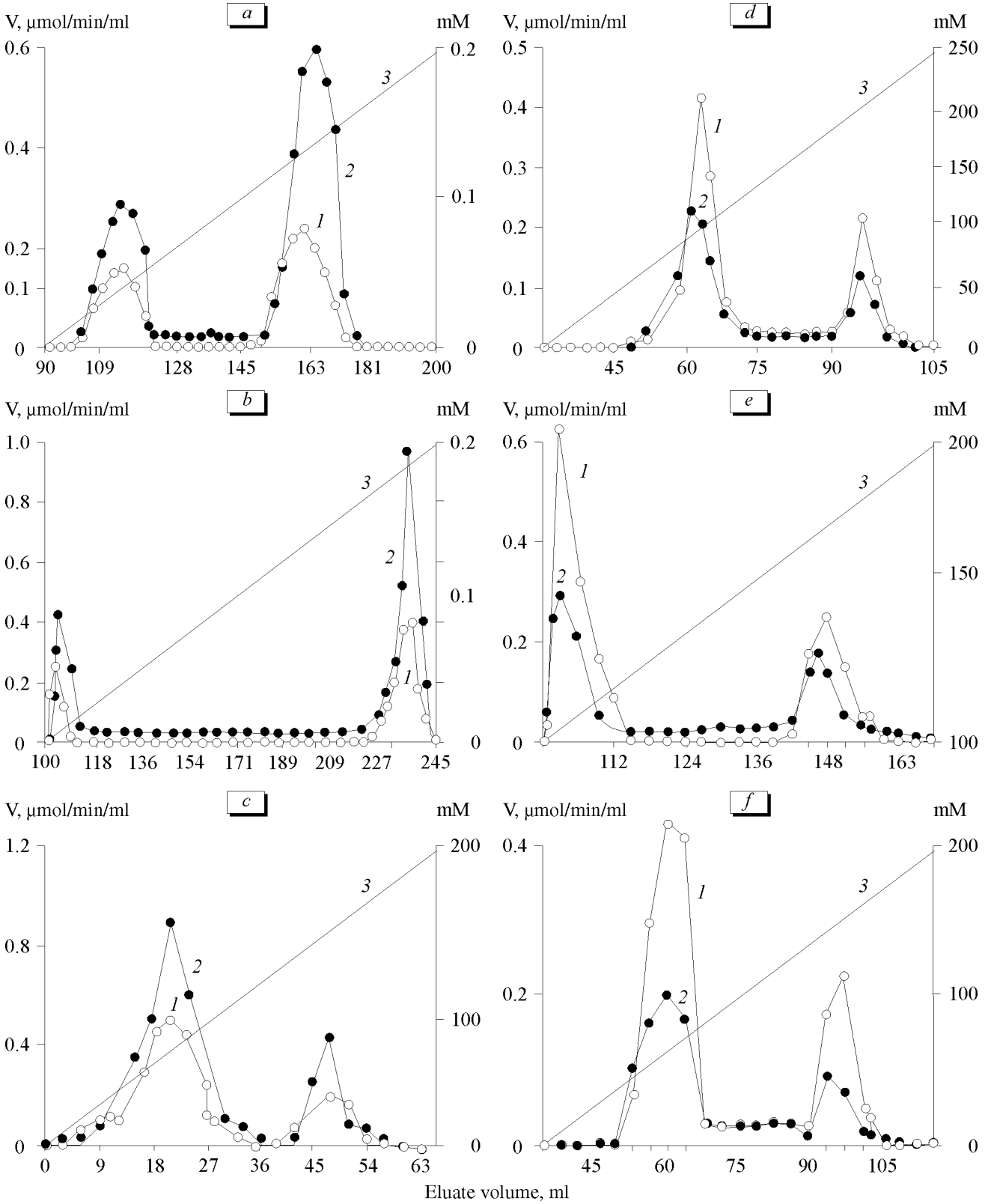

Fig. 2. Elution profiles of NADP-dependent isocitrate dehydrogenase (*a*-*c*) and aconitate hydratase (*d*-*f*) in cytoplasmic (first peak) and mitochondrial (second peak) myocardium fractions from control rats (*1*) and animals with experimental ischemia (*2*). *a*, *d*) chromatography on carboxymethyl sephadex, *b*, *e*) carboxymethyl cellulose, *c*, *f*) diethylaminoethyl cellulose. *3*) KCl concentration (right ordinate).

dria, respectively, whereas AH activity decreased 2.3- and 2.6-fold, respectively (Fig. 1, *b*). Electrophoresis revealed two NADP-IDH and AH isoforms in normal and ischemic myocardium. NADP-IDH from ischemic myocardium was characterized by more intensive protein band compared to the control. On the opposite, specific staining for AH revealed a weaker protein band in ischemic myocardium compared to the control. R_f values for enzymes from normal and ischemic myocardium did not differ (0.51 and 0.46 for mitochondrial and cytoplasmic NADP-IDH and 0.78 and 0.89 for mitochondrial and cytoplasmic AH, respectively).

As was shown earlier, NADP-IDH activity in the cytoplasmic and mitochondrial fractions of the brain and cerebellum increased by 26 and 43% and by 6 and 20%, respectively [9]. Previous studies demonstrated inhibition of AH activity in rat epithelium and lungs under conditions of intense ROS production induced by hypoxia, ischemia, and hyperoxia [9,12]. Some studies report hypoxia-induced impairment of citrate metabolism in the cytoplasmic fraction of rat brain [13]. These effects probably take place during ischemic lesion of functionally different mammalian tissues. Since in rat myocardium NADPH is produced not due to dehydrogenases of pentose-phosphate pathway, but mainly in NADP-IDH-catalyzed reaction [14], NADP-IDH in subcellular fractions of ischemic myocardium can play a role in maintaining glutathione reductase/glutathione peroxidase system of cardiomyocytes.

An interreflection probably exists between functioning of the antioxidant systems regulating the level of free radical processes via H_2O_2 detoxification by α-tocopherol and glutathione reductase/glutathione peroxidase complex and NADP-IDH and AH activity in ischemic rat cardiomyocytes. The decrease on AH activity and accumulation of citrate can potentiate the effect of α-tocopherol and decrease the rate of Fenton reaction due to Fe^{2+} chelation. At the same time, activation of glutathione reductase/glutathione peroxidase enzyme system occurring on minute 40 of ischemia can be associated with intense NADPH generation by NADP-IDH.

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