Catalytic Characteristics of NADP-Isocitrate Dehydrogenase from the Liver of Rats in Health and after Injection of TNF-α and Melatonin Treatment

L. N. Tsvetikova, T. N. Popova, and T. I. Rakhmanova

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Activity of NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) increases and the catalytic characteristics of the enzyme isolated from the liver of rats are changed under conditions of apoptosis induction in comparison with these characteristics in health. Injection of melatonin induced a trend to normalization of kinetic parameters of catalytic activity and of some regulatory characteristics of the enzyme.

Key Words: *rat; liver; apoptosis; NADP-dependent isocitrate dehydrogenase; melatonin*

Free radical oxidation is intensified in apoptosis disorders, which results in disease development [3,5,10]. Stimulation of receptors on various cells by TNF-α causes an elevation of intracellular levels of reactive oxygen species because of reduction of transmembrane potential [7,8].

An important role in antioxidant defense is played by NADP-dependent isocitrate dehydrogenase (NADP-IDH), catalyzing reduction of $NADP^+$ to NADPH, essential for the functioning of the glutathione reductase/glutathione peroxidase antioxidant system [11].

Melatonin is involved in biorhythm synchronization, antistress defense, and can act as a trap of hydroxyl radical, singlet oxygen, and nitrogen oxide [1,2].

We studied the kinetic and regulatory characteristics of NADP-IDH from rat liver in health, in TNF-α induced apoptosis, and after injection of melatonin under conditions of apoptosis induction.

MATERIALS AND METHODS

The study was carried out on laboratory male rats (*Rattus rattus* L.; 150-200 g) kept under standard vivarium conditions. The animals were intraperitoneally injected with actinomycin D (20 μg/kg) for apoptosis induction and after 20 min with TNF-α (1 μg/kg) [12]. Melatonin was injected intraperitoneally (2 mg/kg) 3 times at 3-h intervals after apoptosis induction. The protective function of melatonin was studied 12 h after TNF-α injection.

The animals were divided into 3 groups. Group 1 controls (n=16) were kept under standard vivarium conditions. Group 2 (n=10) rats were injected with TNF-α. Group 3 (n=8) rats were injected with melatonin according to the above protocol after apoptosis induction. The animals were sacrificed 12 h after the toxicant injection at the peak of hepatocyte cytolysis.

Activity of NADP-IDH was measured spectrophotometrically (λ=340 nm). The medium for NADP-IDH activity measurements was as follows: 50 mM Tris-HCl buffer (pH 7.8) with 1.5 mM isocitrate, 2 mM $MnCl_2$, 0.25 mM NADP, and 0.1 mM EDTA. The amount of the enzyme catalyzing the formation of 1 μM reaction product within 1 min at 25°C was taken for a unit of enzyme activity.

Isolation and purification of NADP-IDH was carried out according to the protocol including protein fractionation by ammonium sulfate, gel filtration on Sephadex G-25, ion exchange chromatography on DEAE cellulose, and chromatography in a column

Voronezh State University, Russia. ***Address for correspondence:*** tsvn@front.ru. L. N. Tsvetikova

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packed with Toyopearl HW-65. All stages of enzyme purification were carried out at 0-4°C.

The molecular weight of the enzyme was evaluated by Toyopearl HW-65 gel chromatography using a calibration curve plotted using elution of marker proteins (phosphorylase *b*, BSA, ovalbumin, and carboanhydrase).

Electrophoresis of purified enzyme preparation was carried out by Davis' method. Universal silver nitrate staining of proteins in gel was used [9].

The resultant enzyme preparations were used in comparative analysis of the kinetic characteristics and regulation of NADP-IDH activity in health and under conditions of apoptosis induced by TNF-α, and under conditions of melatonin treatment during apoptosis development.

Experiments were repeated 8 times (biological) and 2-4 times (analytical). The results were compared with the control. The significance of differences was evaluated by Student's *t* test. The data were processed by standard statistical methods, the differences were considered significant at $p<0.05$.

RESULTS

Activity of NADP-IDH in liver homogenates was significantly (1.5 times) higher in rats injected with TNF-α than in intact animals. Injection of melatonin after apoptosis induction led to reduction of NADP-IDH activity in comparison with animals injected with TNF-α (Table 1). It seems that NADP-IDH stimulation contributes to the development of defense response to intensification of free radical oxidation processes. Inhibition of enzyme activity after melatonin injection can be due to the hepatoprotective effect of this substance [1].

In order to clear out the relationship between changes in NADP-IDH activity and the regulatory mechanisms of the enzyme functioning, NADP-IDH was isolated by our method [4] from the liver of control rats and animals injected with TNF-α and with melatonin after apoptosis induction. Enzyme preparations with 96-, 76-, and 84.2-fold purification degree and specific activities 8.6, 10.6, and 10.9 unit/mg protein were obtained, the output being 13.1, 12.1, and 10.5%, respectively.

Electrophoresis in PAAG has shown that NADP-IDH preaparions isolated from the liver of rats were homogeneous in the fractions with the maximum activity after Toyopearl HW-65 gel chromatography. Electrophoretic mobilities for NADP-IDH from the livers of control and experimental rats coincided (Rf=0.55).

The molecular weight of liver NADP-IDH from intact rats, rats with apoptosis and those treated with melatonin, evaluated by gel filtration though a column packed with Toyopearl HW-65 was 112±5.8 kDa. Hence, the molecular weight of the enzyme did not change during the development of apoptosis and melatonin treatment. This indicates that changes in NADP-IDH activity after apoptosis induction by TNF-α and injection of the hormone were most likely not caused by realization of the associative/dissociative mechanism of activity regulation.

The physicochemical and kinetic parameters of catalytic effect of the enzyme isolated from the livers of control and experimental rats were determined (Table 2).

Affinity of the enzyme isolated from the liver of rats injected with TNF-α for the coenzyme and cofactor increased (Michaelis' constant (Km) decreased by 2.5 and 1.3 times, respectively) in comparison with the normal level, while affinity for the substrate virtually did not change. The Km values for NADP suggest higher efficiency of NADPH regeneration under conditions of the cellular energy equivalents deficiency because of apoptosis development. This also can indicate the realization of the physicochemical mechanism of adaptation, aimed at the maintenance of the cellular free radical homeostasis under conditions of disease. The Km for $NADP^+$ and Mn^{2+} for NADP-IDH, isolated from the liver of rats injected with melatonin after apoptosis induction, increased by 1.6 and 1.3 times, respectively, in comparison with the parameter in apoptosis, while the affinity for the substrate virtually did not change, presumably because of reduced intensity of free radical processes because of antioxidant effect of this compound.

Study of the relationship between the velocities of isocitrate dehydrogenase reaction and hydrogen ion concentrations showed highest activity of

TABLE 1. Activity of NADP-IDH Isolated from the Liver of Rats in Health, after Injection of TNF-α, and Melatonin Injection after Apoptosis Induction (*M*±*m*)

Parameter	Normal value	TNF-α injection	Melatonin injection after apoptosis induction
Specific activity, U/mg protein	0.09±0.01	0.14±0.01*	0.13±0.01*
Specific activity, %	100	156*	145*

Note. *$p<0.05$ compared to normal value.

TABLE 2. Kinetic Parameters of NADP-IDH from Rat Hepatocytes in Health, after Injection of TNF-α, and Melatonin Injection after Apoptosis Induction (*M*±*m*)

Group	Km values, mM			pH_{opt}	pK_1	pK_2
	isocitrate	$NADP^+$	Mn^{2+}			
1 (control)	0.12±0.01	0.15±0.02	0.49±0.02	7.8	7.1	8.6
2 (TNF-α)	0.13±0.01	0.06±0.02*	0.39±0.01*	7.5	6.8	8.3
3 (melatonin after apoptosis induction)	0.12±0.01	0.13±0.01	0.50±0.03$^+$	7.6	6.9	8.3

Note. $p<0.05$ compared to: *group 1, $^+$group 2. pK_1 and pK_2: acid dissociation constants for carboxyl group and protonated amino group of amino acid. pK corresponds to pH value at 50% protein dissociation.

TABLE 3. Inhibition Constants of NADP-IDH Isolated from the Liver of Rats in Health, after Injection of TNF-α, and Melatonin Injection after Apoptosis Induction (*M*±*m*)

Intermediate	Experiment conditions	Ki, mM	Inhibition type
2-OG	Health	0.23±0.01	Competitive
	Injection of TNF-α	0.49±0.01*	Competitive
	Melatonin injection after apoptosis induction	0.112±0.01*	Competitive
OA	Health	1.72±0.06	Competitive
	Injection of TNF-α	0.75±0.02*	Competitive
	Melatonin injection after apoptosis induction	1.18±0.09*	Noncompetitive

Note. *$p<0.05$ compared to normal value.

NADP-IDH from rat liver in health, apoptosis, and apoptosis+melatonin treatment at pH 7.2-8.2. The optimum pH for NADP-IDH activity in health is 7.8 (Table 2). For the enzyme activity from the liver of rats injected with TNF-α this value is 7.5, presumably because of adaptation reaction to acidosis associated with the development of oxidative stress. Importantly that these trends in the difference of optimal pH in health and apoptosis induction are in line with published data on NADP-IDH functioning in health and free radical oxidation stimulation during the development of experimental toxic hepatitis [6]. Injection of the hormone against the background of pathology development shifts this parameter by 0.1 unit towards the optimum pH (control group). These shifts are presumably due to the antioxidant effects of melatonin reducing the intensity of free radical oxidation and hence, the degree of acidosis.

We detected the inhibitory effect of 2-oxoglutarate (2-OG) and oxaloacetate (OA) on the enzyme isolated from the liver of control rats and of animals injected with TNF-α (Table 3). The inhibition was competitive for isocitrate. The development of apoptosis was associated with reduction of OA inhibition constant (Ki) by 2.3 times in comparison with the normal value. The 2-OG Ki increased by 2.1 times. Injection of melatonin to animals with induced apoptosis led to shifts in 2-OG and OA Ki values towards the control value, which can also be a manifestation of antioxidant effects of melatonin.

Citrate stimulates NADP-IDH: activation constant values were 0.2, 0.7, and 0.5 mM for the enzyme in health, apoptosis, and melatonin treatment, respectively.

It seems that NADP-IDH characteristics and activity regulation under conditions of apoptosis development can serve as a physicochemical mechanism of adaptation nature, aimed at the maintenance of cell homeostasis during disease development. Modification of some regulatory characteristics of the enzyme during apoptosis development can presumably promote the increase of intracellular NADPH level, which suggests a greater contribution of this enzyme to the cellular antioxidant defense system.

Injection of melatonin leads to shifts in some parameters of the enzyme's catalytic effect towards the normal values, which can be associated with a reduction of free radical processes intensity due to the antioxidant effect of this compound.

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