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THE PREVENTIVE EFFECT OF EMBRYONIC ANTITUMOR MODULATOR (EATM) ON THE LEVEL AND ACTIVITY OF METALLOPROTEINS IN RATS WITH STRETOZOTOCIN-INDUCED DIABETES

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Abstract

The significant increase of the level and superoxide-producing and ferrihemoglobin-reducing activities of prooxidant metalloproteins (PM), fractions of NADPH oxidase or cytochrome b_{558} of acidic nature isolated from erythrocyte membranes, as well as from membranes, mitochondria, and nucleus of the spleen cells, is observed at streptozotocin-induced diabetes (SID) in rats. Simultaneously, the elevation in levels of cytochrome c isolated from spleen cells and decrease in levels of cytochrome b5 from cytosol of erythrocytes and superoxide-producing lipoprotein from blood serum, suprol, are observed. These changes occur in conditions of decreased levels of antioxidant metalloproteins Cu,Zn-SOD, Mn-SOD, catalase from cytosol of erythrocytes and spleen cells leading to characteristic disbalance of oxygen homeostasis and disturbance of physiological equilibrium between prooxidant and antioxidant metalloproteins. As a result, there is perturbation in metabolism of reactive oxygen species (ROS), while the exhaustion of the organism and the rate of mortality in animals increase.

Preventively administered Embryonic Antitumor Modulator (EATM) produces a definite antistressor-regulatory effect, approximating mentioned indices to the norm. EATM does not posses antioxidant activity in vitro, although its positive influence is possibly related to the increase of antioxidant metalloproteins level in vivo.

Keywords: Embryonic Antitumor Modulator (EATM), Streptozotocin, diabetes, metalloproteins.

INTRODUCTION

The mechanism of pathological changes in spreptozotocin-induced diabetes (SID) is conditioned by various factors. Streptozotocin (STZ) penetrating into the beta-cells of pancreas with glucose causes alkylation of DNA, the impairment of which induces activation of poly-adenosine diphosphate (poly-ADP) ribozilation process with depletion of cellular NAD+ and ADP.

Adenosine triphosphate (ATP) dephosphorylation increase under the influence of STZ leads to formation of a substrate for xanthine-xanthine oxidase and O2-, H_2O_2, and HO, resulting in denaturation of beta-cells causing their apoptosis [*Szkudelski T., 2001]. STZ suppresses oxidation of glucose [Bedoya F. et al., 1996*], biosynthesis and secretion of insulin [*Nukaysuka K. et al., 1990*]; it is donor of NO. that also damages beta cells and DNA [*Kroncke K. et al., 1995*].

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In diabetes mellitus (DM), the increase in levels of reactive oxygen species (ROS) due to the increased levels of isoforms of NADPH-oxidase Nox 4 and p22phox causes nephropathy and oxidative stress of renal cells [*Etoh T. et al., 2003; Luo Z. et al., 2010*], as well as endothelial dysfunction of pulmonary arteries [*Lopez-Lopez J. et al., 2008*].

These changes occur on the background of a significant reduction in the antioxidant status of the organism [*Jiao S. et al., 2007; Miyamoto A. et al., 2008*]. Decrease in activity of Nox2 by apotsinin, NADPH oxidase inhibitor, has a positive impact on the given indices in DM mice with increased insulin levels and decreased degree of destruction of beta-cells in the pancreas [*Xiang F. et al., 2010*]. Substances with antioxidant activity (SOD-mimetic complexes, D-pinitol from soybean oil, Pinica granatum flowers) also have a regulatory action in DM [*Bagri P. et al., 2009; Peixoto E. et al., 2009; Sivakumar S., Subramanian S., 2009*].

The specified exogenous protective factors regulate not only metabolism of reactive oxygen

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species (ROS), but also the immune system, functioning of which is closely connected to this metabolism [*Vignais P., 2002*].

From this point of view, the Embryonic Antitumor Modulator of Mkrtchyan (EATM) [*Mkrtchyan L., 2004*], which realizes its effect by stimulating the immune system of an organism [*Mkrtchyan L., 2009; 2010*], provides the neuroprotective action as well [*Enkoyan K., 2009*] and can be effective in DM.

The goal of this study was to determine the level of NADPH-dependent O2--producing [*Simonyan G et al., 2003*] and ferrihemoglobin (ferri Hb)-restoring activity [*Simonyan G. et al., 2006*] of prooxidant metalloproteins (isoforms of NADPH oxidase from erythrocyte membranes, blood serum, components of spleen cells as the immune system organ, as well as from suprol, cytochrome b5 and cytochrome c) and the activity of key antioxidant metalloproteins (total fraction of Cu,Zn-SOD and Mn-SOD, catalase) [*Simonyan M. et al., 1997; Simonyan G. et al., 2001; 2008*] in DM under conditions of preventively injected EATM.

MATERIAL AND METHODS

The experiments were conducted in white rats weighing 200-220 g; there were 12 rats in each group. DM was induced by one-time intraperitoneal (ip) injection of STZ (Sigma) at a dose of 55 mg/kg body weight of rats (Study Group 1). Study Group 2 animals were preliminary ip injected 1 mg EATM (prophylactic regimen) and in 7 days STZ was similarly injected to animals of Study Group 1. EATM was kindly provided by Professor L.N. Mkrtchyan.

The rats were decapitated under light ether anesthesia on day 21 of the experiment. Blood was stabilized by 0.2% sodium oxalate.

Isolation of cytochrome b_{558} isoform fractions from spleen:

Isoforms of NADPH oxidases (or isoforms of cyt b_{558} of acidic nature) of aqueous mixtures of nuclei, mitochondria and cell membranes (CM) were obtained by a licensed procedure without the use of a detergent for solubilization of these hemoproteins [Simonyan M. et al., 1997; Simonyan G. et al., 2006]. The spleens (4 g) were washed with physiological saline, weighed and homogenized in 0.25 M sucrose (1 g tissue in 10 mL sucrose) in a glass homogenizer with teflon pestle for 1 min at 4oC. Nuclei, mitochondria and cell membranes were precipitated by differential centrifugation, sediments were washed twice by sucrose solution (1:20 v/v) followed by centrifugation. Further precipitation of nuclei, mitochondria, and cell membranes were washed with water (1:50 v/v) and again centrifuged (10000 r/min, 10 min). Precipitates of nuclei, mitochondria, and CM after purification from traces of sucrose and other subtend water-soluble salts were mixed with water (1:5 v/v) and finally homogenized in an analogous regimen.

After centrifugation, the supernatants of total fraction of cyt b_{558} isoforms from nuclei, mitochondria, and cell membranes were subjected to ion-exchange chromatography on the column with cellulose KM-52 to remove traces of hemoglobin or other associated proteins of basic properties. Protein fractions not detaining on a column with KM-52 were subjected to further ion-exchange column chromatography with cellulose DE-52. After washing this column with water and then 0.01 M potassium phosphate buffer (PPB) pH 7.4, isoforms of cyt b_{558} of acidic nature were eluted from DE-52 column using 0.2 M PPB.

Isolation of cytochrome b_{558} isoform fractions from erythrocyte membranes (EM):

Fraction of NADPH oxidase (cytochrome b_{558} of acidic nature) was isolated by the similar method [Simonyan G. et al., 2006] by solubilization of a fraction of cytochrome b_{558} of acidic nature and ion-exchange chromatography on celluloses DE-52 and KM-52.

Isolation of suprol and isoform fractions of NADPH oxidase (extracellular cyt b_{558}) from blood serum:

Blood serum, purified from traces of erythrocytes and plasma serum cells for 4 days at 4°C, was incubated and dialyzed against water. After centrifugation of the dialysate (6000 rpm, 10 min) suprol (superoxide-producing high-density lipoprotein [Simonyan G. et al., 2001]) was precipitated (activated) with 0.05 M $FeCl_3$ and

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isolated by centrifugation. The precipitate of activated suprol fraction was dissolved in 0.04*M*l PPB. The fraction of extracellular cyt ***b***$_{558}$ was isolated by ion-exchange chromatography of this supernatant on Sephadex DEAE A-50 and eluted with 0.04 *M*l PPB. After 40-fold dilution of this eluate with water and ion-exchange chromatography on a column with celluose DE-52, the fraction of extracellular cyt ***b***$_{558}$ was eluted with 0.04 *M*l PPB.

The level of cyt ***b***$_{558}$ was determined by measuring the optical density at $\lambda = A_{530}$ (beta absorption band) characteristic for cyt ***b***$_{558}$. The specific content of cyt ***b***$_{558}$ (for $\lambda = A_{530}$) obtained from nuclei, mitochondria, and cell membranes of spleen or from EM or blood serum was determined per 1 *mL* solution of cyt b558 obtained from 1 *g* of spleen, 1 *mL* serum and 1 *mL* of erythrocytes.

Determination of NADPH-dependent O_2--producing activity of cyt b_{558} ***isoforms***:

NADPH-dependent O_2--producing activity of cyt ***b***$_{558}$ isoforms of acidic nature was determined by nitrotetrazolic blue (NTB) method by calculating the percentage of formazan formed at 560 *nm* as a result of NTB reduction with superoxide radicals. The amount of protein, which stimulates the formation of formazan by 50%, was considered the unit of NADPH-dependent O_2--producing activity of cyt ***b***$_{558}$.

Determination of ferriHb-restoring activity of cyt b_{558} ***isoforms***:

FerriHb-restoring activity of cyt (***b***$_{558}$) isoforms was determined using freshly obtained ferriHb of erythrocytes cytoplasm of rats with density of optical absorption (alpha-absorption band) at A_{565}= 0.8. Directly in quartz cuvettes of spectrophotometer 0.2 *mL* cyt ***b***$_{558}$ with λ at A_{530} = 0.3 was added to 3 *mL* of ferriHb solution. After stirring the reaction mixture, it was incubated under aerobic conditions for 15-16 hours at 30oC. Further, after repeated stirring the reaction mixture, the kinetics of recovery of ferriHb to ferroHb by measuring the decrease in density of alpha absorption band of ferriHb at 565 *nm* was determined; this decrease is in direct proportion to the formed ferroHb at A_{555}. The amount of protein causing a reduction of maximum optical absorption of alpha-band of ferriHb up to 0.05 during one hour at 20°C was considered a unit of ferriHb-restoring activity of cyt ***b***$_{558}$.

Isolation of cyt b$_5$ fraction, SOD, and catalase from the cytosol of erythrocytes and determination of SOD and catalase activity:

Self-precipitated erythrocytes were washed with saline (1:100 v/v) to remove traces of plasma cells and hemolyzed with water (1:10 v/v), the erythrocyte membranes (EM) were precipitated by centrifugation of hemolysate at pH 5.6 (6000 *rpm*, 15 *min*), the supernatant solution was dialyzed against water and after centrifugation the supernatant was subjected to ion-exchange column chromatography with cellulose DE-52. The total fraction of Cu,Zn-SOD and self- precipitated catalase was eluted with 0.04 *M*lPPB, while for cyt b5 0.2 *M*l PPB was used. The quantity of cyt b5 was determined by measuring the density at $\lambda = A_{525}$ (beta absorption band).

SOD activity was determined by NTB method measuring the optical absorption of formazan (at 560 *nm*). The amount of protein causing 50% reduction in values of optical absorption was considered a unit of SOD activity. Specific SOD activity was determined per 1 *mL* of erythrocytes.

Catalase activity was determined by permanganatometric titration of hydrogen peroxide solution in the absence or presence of catalase. One unit of catalase activity was defined to be the amount of protein, which caused the splitting of 0.1 *M*l hydrogen peroxide during 1 min at 20°C. The specific activity of catalase was calculated per 1 *mL* of erythrocytes.

Isolation of total fraction of Cu,Zn-SOD and Mn-SOD, as well as catalase and cyt c from the cytosol of spleen cells:

After separating the precipitats of nuclei, mitochondria and cell membranes of spleen the supernatant solution was dialyzed against water. The dialysate was centrifuged and subjected to ion-exchange chromatography first on a column with cellulose KM-52, from which cyt c was eluted with 0.2 *M*l PPB. The fraction of SOD and catalase did not suppress in this column, but was deposited on a column with cellulose DE-52, from which the total fraction of SOD

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Table 1.

EATM effect (%) on the levels of MPA in SID-rats (p<0.05; n = 6)

No.	MPA	DM (Study Group 1)	EATM + DM (Study Group 2)
1.	Extracellular *cyt* b_{558}	↑ 91.6 ± 7.1 (p<0.03)	↑ 66.7±5.9 (p<0.03)
2.	Isoforms of *cyt* b_{558} from EM	↑ 116.6 ± 14.1	↑ 62.5 ± 5.9
3.	Isoforms of *cyt* b_{558} from CMS	↑ 81.5 ± 7.0	↑ 46.7 ± 4.5
4.	Isoforms of *cyt* b_{558} from NSC	↑ 225.0 ± 31.3	↑ 137.1 ± 16.9
5.	Isoforms of *cyt* b_{558} from MSC	↑ 159.0 ±20.1 (p<0.01)	↑ 101.2 ± 8.8 (p<0.01)
6.	*Cyt* b_5 from cytosol of erythrocytes	↓ 40.0 ± 3.3	↓ 31.1 ± 2.8
7.	*Cyt* **c** from cytosol of spleen cells	↑ 80.0 ±6.5 (p<0.03)	↑ 25.1 ± 4.7 (p<0.03)

Table 2.

EATM effect (%) on the levels of NADPH-dependent O_2--producing activity of MPA in SID-rats (p<0.05; n=6)

No.	MPA	DM (Study Group 1)	EATM + DM (Study Group 2)
1.	Extracellular *cyt* b_{558}	↑ 45.3 ± 2.8 (p<0.01)	↑ 31.4 ± 3.1 (p<0.01)
2.	*Cyt* b_{558} from EM	↑ 110.0 ± 20.1	↑ 54.1 ± 5.0
3.	*Cyt* b_{558} from CMS	↑ 238.7 ± 49.3	↑ 158.2 ±21.4
4.	*Cyt* b_{558} from NSC	↑ 78.6 ± 6.1	↑ 56.5 ±4.9
5.	*Cyt* b_{558} from MSC	↑ 71.2 ± 4.9	↑ 39.1 ±3.3
6.	O_2^--producing activity of suprol	↑ 162.0±16.7 (p<0.03)	↑ 82.1 ±5.9 (p<0.03)

Table 3.

ATM effect (%) on the ferriHb-restoring activity of MPA in SID-rats (p<0.05; n=6)

No.	MPA	DM (Study Group 1)	EATM + DM (Study Group 2)
1.	Extracellular *cyt* b_{558}	↓ 42.1 ± 2.2	↓ 36.4 ± 3.3
2.	*Cyt* b_{558} from EM	↓ 60.0± 6.1	↓ 44.1 ± 5.0
3.	*Cyt* b_{558} from CMS	↓ 31.9 ± 3.0	↓ 25.4 ± 4.6
4.	*Cyt* b_{558} from NSC	↓ 29.3 ± 4.6	↓ 24.1 ± 3.3
5.	*Cyt* b_{558} from MSC	↓ 27.1 ± 3.2	↓ 24.0 ± 2.7

and catalase were eluted with 0.04 M PPB. SOD and catalase activity was determined by the method described above; the specific activity was estimated per 1 *g* of spleen.

Optical spectral measurements were carried out on spectrophotometer “Specord UV/VIS” (Germany) in 1 *cm* cuvette.

The statistical processing of the results was carried out by the well-known method of variation statistics of Student-Fisher by determination of reliability criterion p.

RESULTS AND DISCUSSION

At the final stage of DM (day 21 of the experiment) death of animals in Study Group 1 was 75% indicating to an essential breach of endocrine function of pancreas and intoxication of an organism. Under the influence of EATM, in Study Group 2, death of animals was reduced to 44%; moreover, there was a significant normalization of the appearance and behavior of animals, reduction of polyuria and polyphagia.

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Table 4.

EATM effect (%) on superoxide dismutase and catalase activity of MAA in SID-rats ($p<0.05$; n=6)

No.	MAA	DM (Study Group 1)	EATM + DM (Study Group 2)
1.	Fraction of Cu,Zn-SOD from the cytosol of erythrocytes	↓ 74.2 ± 8.1	↓ 57.5 ±8.0
2	Total fraction of Cu,Zn-SOD and Mn-COD from the cytosol of spleen cells	↓ 68.9 ±5.9 (p<0.01)	↓ 42.2 ± 4.6 (p<0.01)
3.	Fraction of catalase from cytasol of erythrocytes	↓ 43.7 ± 5.5	↓ 28.3 ± 4.9
4.	Fraction of catalase from the cytosol of spleen cells	↓ 42.1 ± 6.1	↓ 26.2 ±5,9

The above changes took place on a background of typical shifts in levels of

- metalloproteins of prooxidant activity (MPA): isoforms of acidic nature of NADPH oxidase or cyt $\boldsymbol{b_{558}}$ localized in blood serum, EM, cell membranes of spleen, and mitochondria of spleen cells, the nuclei of spleen cells, as well as cyt c, cyt b5 and suprol as superoxide-producing lipoprotein of serum; and
- metalloproteins of antioxidant activity (MAA): Cu,Zn-SOD and catalase from cytosol of erythrocytes, the total fraction of Cu,Zn-SOD and Mn-SOD, as well as fraction of catalase from cytosol of spleen cells (Table 1).

At streptozotocin-induced diabetes (SID), Study Group 1, ($\lambda = A_{530}$ beta absorption band), the level of NADPH oxidase (or cyt $\boldsymbol{b_{558}}$) significantly increases (from 81.5 to 225%) in the EM, cell membranes (CMS), mitochondria of spleen cells (MSC) and especially in nuclei of spleen cells (NSC). The level of cyt c from spleen cells (at $\lambda=A_{520}$) is being significantly increased, indicating the decrease of mitochondria stability at SID. As to MPA, only the level of cyt b5 from the cytosol of erythrocytes is being decreased.

As shown in Table 2, the O_2-- producing activity of NADPH oxidase fraction isolated from CMS increases sharply. The increase in this activity is more expressed in NADPH oxidase from EM, NSC, MSC, as well as from blood serum. In DM, the O_2--producing activity of suprol is being significantly reduced. The latter may be associated with increased lipid peroxidation of phospholipid residues of suprol produced by suprol of O_2- [*Simonyan R. et al., 2005*].

EATM has a positive effect, bringing the O_2- -producing activity of cyt $\boldsymbol{b_{558}}$ isoforms (or NADPH oxidases) of acidic nature with different cell localization to the norm.

FerriHb-restoring activity of these NADPH oxidases is considerably reduced in DM, especially for the NADPH oxidase of EM and for extracellular NADPH oxidase (Table 3), indicating to a significant breach of oxygen homeostasis. In this case, EATM virtually does not change the oxygen homeostasis (only ferroHb or oxyHb, but neither ferriHb nor metHb are capable to transport molecular oxygen to the cells).

As to MAA, it is noteworthy that in DM the activity of Cu,Zn-SOD fraction from cytosol of erythrocytes and spleen cells (the total fraction of Cu,Zn-SOD and Mn-SOD) is being decreased almost in the same range (Table 4). The catalase activity in cytosol of erythrocytes and spleen cells is similarly being reduced.

Discussion

The Embryonic Antitumor Modulator of Mkrtchyan (EATM) administered in a preventive mode exerts a regulatory effect by increasing the activity of key antioxidant enzymes. This effect of EATM can be stimulated by its prooxidant action.

The unique feature of EATM is that this combined preparation plays not only a positive antitumorous, neuroprotective role, but also manifests antidiabetic properties in DM through regulation of the level and activity of key metalloproteins of anti- and prooxidant activity: regulators of ROS metabolism.

These results indicate that at DM, there is a decrease in stability of EM, CMS, MSC, and es-

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pecially of NSC and enhancement of cleavage (releasing) of cyt b_{558} isoforms from the heterogeneous phase (from membranes) to a homogeneous phase (in solution). This is facilitated by increasing lipid peroxidation (LPO) of biomembranes at DM [*Bozturk O. et al., 2010; Kambij S., Sandhir R., 2010; Soman S. et al., 2010*].

This is confirmed by a significant decrease in the process of cyt b558 releasing from EM and stability increase of the latter under the influence of preparations with antioxidant activity (Cu,Zn-SOD, catalase, ceruloplasmin, as well as a synthetic analogue of proline-rich polypeptide: galarmin obtained from neurosecretory granules of hypothalamus) [*Simonayn R. et al., 2003*].

The increased level of serum (extracellular) cyt b558 is possibly associated with decreased stability of erythrocytes that occurs at various pathological conditions, especially in case of malignant tumors [Simonyan G. et al., 2005a;b]. The mechanism of cyt c levels increase in mitochondria of spleen cells at DM may be a result of LPO enhancement.

In Study Group 2, under the influence of introduced EATM, all the studied parameters are notably normalized (Table 1). The mechanism of this effect of EATM is not related to its antioxidant action: EATM does not possess SOD-mimetic or catalase-mimetic activity in vitro. It is more likely that such an effect of EATM is associated with stimulation of antioxidant systems in vivo.

The conclusion might be drawn: at DM there is not only an increase in NADPH oxidase activity in beta-cells of the pancreas, but also in the EM, cell membranes, mitochondria and nuclei of spleen cells on the background of decreased activity of antiradical defense system causing a breach of oxygen homeostasis and creating an imbalance between the MPA and MAA, thus breaching the metabolism of these metalloproteins with corresponding oxidative damage of blood and spleen due to ROS production, as a result of which pancreas is affected and animal death is registered. EATM introduced in a preventive mode has a certain regulatory effect, preserves the life of animals with experimental diabetes by preventing the destruction of pancreas [*Vahedian V., Aghajanova Y., 2010*].

Thus, Embryonic Antitumor Modulator (EATM) has a preventive effect in an experimental diabetes mellitus as well. This universal quality of EATM is obviously conditioned by its ability to modulate the general adaptive mechanisms engaged in development of various pathological processes.

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