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ORIGINAL RESEARCH

Antioxidative effects of proteoglycans of embryonic genesis in streptozotocin-induced diabetic rats

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Introduction: It is well accepted that oxidative stress plays a significant role in the pathogenesis of diabetes mellitus. The objective of this study was to investigate the effect of proteoglycans of embryonic genesis (PEG) on concentrations and activity of prooxidative and antioxidative metalloproteins in streptozotocin (STZ)-induced diabetes in rats.

Methods: Study groups were as follows: vehicle control group (Group 1), STZ-induced diabetes (55 mg/kg, intraperitoneal injection [Group 2]), STZ-induced diabetes with prophylactic injection of PEG (0.5 mg/kg intraperitonealy injected) 1 week prior to STZ injection (Group 3). The following prooxidative metalloproteins were studied: levels of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) isoforms (extracellular Nox [eNox]) in serum; erythrocyte membranes; and spleen cell membranes, nucleus and mitochondria, as well as serum levels of superoxide-producing lipoprotein (suprol); cytochrome (cyt) b_5 from cytosol of erythrocytes; and cyt c from spleen cell cytosol. The antioxidative metalloproteins, particularly superoxide dismutase and catalase from erythrocyte and from spleen cell cytosol were studied.

Results: Results demonstrated the significant ($P < 0.05$) increase in the level and activity of NADPH-dependent, O_2^--producing eNox activity in Group 2 in comparison with the control Group and decrease of the ferrihemoglobin-reducing activities of these Nox, as well as a significant increase in O_2^--producing activity of suprol. In Group 2, there was a significant elevation of the level of cyt c, and decreased cyt b_5 level, as well as inhibition of superoxide dismutase and catalase activity.

Conclusion: The prophylactic injection of the PEG demonstrated overall antioxidative effect, with prevention of changes in prooxidative markers.

Keywords: proteoglycans of embryonal genesis (PEG), streptozotocin-induced diabetes, antioxidant, prevention

Introduction

Mechanisms of pathological change in streptozotocin (STZ)-induced diabetes is conditioned by various factors. The STZ penetrating into the beta-cells of a pancreas along with glucose causes alkylation of DNA, impairment of which induces activation of the poly-adenosine diphosphate-ribosylation process with depletion of cellular NAD^+ and ATP. Increased dephosphorylation of adenosine triphosphate under the influence of STZ leads to the formation of a substrate for xanthine–xanthine oxidase and of O_2^-, H_2O_2, and HO·, resulting in denaturation of beta-cells that causes apoptosis.[1] STZ suppresses the oxidation of glucose[2] and the biosynthesis and secretion of insulin,[3] serving as an NO· donor, which also damages the beta-cells and DNA.[4]

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In STZ-induced diabetes, the increase of the level of reactive oxygen species (ROS), due to the elevation of the levels of isoforms of nicotinamide adenine dinucleotide phosphate (NADPH oxidase (Nox), causes nephropathy and oxidative stress of renal cells,[5,6] as well as endothelial dysfunction of pulmonary arteries.[7] This, in turn, leads to a significant decrease of the antioxidant status of the organism.[8,9] A decrease of the activity of Nox by apocynin (the inhibitor of the Nox) plays a positive role in mice with STZ- induced diabetes by elevating insulin levels and decreasing the degree of destruction of beta-cells in the pancreas.[10] Medications with antioxidant activity (SOD-mimetic complexes, such as D-pinitol from soybean oil and *Punica granatum* flowers) also have regulatory action in STZ- induced diabetes.[11–13]

The specified exogenous protective factors regulate not only the metabolism of ROS, but also the immune system, operation of which is closely connected to this metabolism.[14] From this perspective, proteoglycans of embryonal genesis (PEG), also known as the embryonic anti-tumor modulator of Mkrtchyan,[15] which effectively stimulate the organism's immune system[16,17] and have been shown to have a neuro-protective action,[18] may also be effective in STZ-induced diabetes, providing an anti-stressor effect.

The aims of this study were: (1) to determine the level of NADPH-dependent, O_2^--producing[19] and ferrihemoglobin (ferriHb)-reducing activity[20] of Nox from erythrocyte membranes (EM), from serum, and from spleen cell membranes (SCM), nuclei (SCN) and mitochondria (SCMI); (2) to determine the level and O_2^--producing activity of superoxide-producing lipoprotein (suprol); (3) to determine the levels of cytochrome (cyt) c and cyt b_5; (4) to determine the activity of key antioxidant enzymes-metalloproteins Cu, Zn-superoxide dismutase (SOD), Mn-SOD, and catalase;[21–23] and (5) to determine the possible protective role of PEG if administered in a prophylactic (preventive) regimen in rats with STZ-induced diabetes.

Materials and methods

The experiments were carried out on white wild-type male rats weighing 200–220 g, with 12 rats in each group. Control group animals (Group 1) were injected with a one-time intraperitoneal (ip) 0.5 mL water as vehicle. Diabetes was induced in Group 2 animals with a one-time ip injection of STZ (Sigma-Aldrich, St Louis, MO) at a dose of 55 mg/kg body weight. In Group 3, animals received an ip injection of PEG (0.5 mg/kg body mass), and 7 days later received an ip injection of STZ (55 mg/kg) (prophylactic regimen). PEG (the embryonic anti-tumor modulator) was kindly provided by Professor LN Mkrtchyan. The rats were decapitated under phenobarbital anesthesia (40 mg/kg) after 21 days. Blood was stabilized by 0.2% sodium oxalate.

Isolation of fractions of the Nox isoforms from the cell components of the spleen

The fractions of the Nox isoforms from SCN, SCMI, and SCM were obtained as described earlier without use of detergent for solubilization of these hemoproteins.[24] After washing the spleen tissue (up to 4 g) with physiologic solution, homogenization in 0.25 M sucrose (1 g tissue in 10 mL sucrose) in a glass homogenizer with teflon pestle for 1 minute at 4°C was conducted.

SCN, SCM, and SCMI were precipitated by differential centrifugation. Sediments were washed twice with sucrose solution (1:20) then centrifuged (10,000 *g*, 10 minutes). For further precipitation, nuclei, mitochondria, and cell membranes were washed with water (1:50) and again centrifuged (10,000 *g*, 10 minutes). Purified from traces of sucrose and other impurities, water-soluble precipitates of SCN, SCM, and SCMI were mixed with water (1:5) and finally homogenized in an analogous manner. After solubilization of the protein fractions[23] and centrifugation of these mixtures, the supernatants were separately subjected to ion-exchange chromatography on the column of cellulose CM-52, equilibrated by 0.004 M potassium phosphate buffer (PPB) pH 7.4 to remove traces of hemoglobin or other associated proteins of the basic character. Protein fractions not detained on a column with CM-52 were subjected to ion-exchange chromatography on the column of DE-52 cellulose, and the Nox from SCN, SCM, and SCMB were eluted using 0.2 M PPB.

Isolation of Nox from EM

The Nox from EM were isolated by the same solubilization method, centrifugation (14,000 *g*, 15 minutes) and ion-exchange chromatography of the supernatant on celluloses CM-52 and DE-52, from which the Nox was eluted using 0.4 M PPB.[20]

Isolation of suprol and Nox from blood serum

After incubation of the blood serum (for 4 days at 4°C) and purification from traces of erythrocytes and plasma cells, the serum was dialyzed against water. After precipitation and activation of suprol with 0.05 M $FeCI_3$ for the production of O_2^-, suprol precipitate was isolated by centrifugation at 6000 g for 10 minutes.[22] The fraction of Nox was isolated from

the supernatant, after its ion-exchange chromatography on the Sephadex DEAE A-50 (Pharmacia, Stockholm, Sweden) (the Nox eluted with 0.04 M PPB). After 20-fold dilution of this eluate with water and ion-exchange chromatography on a column of cellulose DE-52, the fraction of Nox from blood serum (extracellular Nox or eNox) was also eluted with 0.04 M PPB.

The concentration of the Nox isoforms was determined by measuring the optical absorption density at A_{530} nm (beta absorption band) characteristic for the Nox. Specific content was determined for the Nox isolated from 1 g of spleen, 1 mL of serum, and 1 mL of erythrocytes.

Determination of NADPH dependent O_2^--producing activity of Nox isoforms

NADPH-dependent O_2^--producing activity of Nox isoforms was determined by nitrotetrazolium blue (NTB) method at 560 nm, the percentage of formazan formed was calculated as a result of the reduction of NTB by superoxide radicals.[19] The unit of NADPH-dependent, O_2^--producing activity of Nox was considered the amount of mg of the enzyme protein, which stimulates the formation of formazan by 50%.

Determination of ferriHB-reducing activity of Nox isoforms

FerriHb-reducing activity of the Nox isoforms was determined using the freshly obtained ferriHb from rat erythrocyte cytoplasm by measuring the optical absorption density (alpha absorption band) at 565 nm = 0.8 optical units. Directly in quartz cuvettes of spectrophotometer, the 0.2 mL of Nox with A_{530} = 0.3 optical units was added to 3 mL of ferriHb solution. After stirring, the reaction mixture was incubated under aerobic conditions for 15–16 hours at 30°C. Further, after remixing the reaction mixture, the kinetics of reduction of ferriHb to ferroHb by measuring the decrease of the absorption density of ferriHb at 565 nm was determined (this decrease is in direct proportion to the formed ferroHb measured at 555 nm). The amount of protein causing a decrease of the optical absorption density of ferriHb up to 0.05 during 1 hour at 20° was considered the unit of ferriHb-restoring activity of Nox.

Isolation of the fractions of SOD, catalases, and cyt b_5 from cytosol of erythrocytes, and determination of SOD and catalase activity

After washing out self-precipitated erythrocytes with normal saline (1:100 vol/vol) from traces of plasma cells and hemolysis with water (1:10 vol/vol), the EM were precipitated by centrifugation of hemolysate at pH 5.6 (6000 g, 15 minutes); thereafter the supernatant solution was dialyzed against water and, after centrifugation, the supernatant was subjected to ion-exchange column chromatography with cellulose DE-52 (equilibrated with the 0.002 M PPB). The total fraction of Cu,Zn-SOD, and catalase was eluted with 0.04 M PPB, and cyt b_5 was eluted with 0.2 M PPB. The amount of cyt b_5 was determined by measuring the optical absorption density at 525 nm (beta absorption band).

The SOD activity was determined by NTB method measuring the optical absorption density of formazan (at 560 nm). The unit of SOD activity was considered the amount of mg of the enzyme protein causing 50% decrease of optical absorption density of formazan.

The catalase activity was determined by permanganometric 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 that caused splitting of 0.1 M hydrogen peroxide in 1 minute at 20°.[24]

Table 1 The change of the specific levels (specific optical absorption density) of MPA in study groups

MPA	Units of optical density		
	Group 1	Group 2	Group 3
Extracellular Nox from blood serum	0.15 ± 0.02	0.28 ± 0.03, $P = 0.01$	0.25 ± 0.02, $P = 0.04$
Nox from EM	0.45 ± 0.07	0.97 ± 0.09, $P = 0.02$	0.73 ± 0.06, $P = 0.02$
Nox from SCM	1.20 ± 0.1	2.17 ± 0.04, $P = 0.01$	1.76 ± 0.08, $P = 0.01$
Nox from SCN	0.70 ± 0.05	2.25 ± 0.10, $P = 0.031$	0.96 ± 0.04, $P = 0.02$
Nox from SCMI	0.18 ± 0.02	0.46 ± 0.03, $P = 0.01$	0.36 ± 0.03, $P = 0.01$
cyt b_5 from cytosol of erythrocytes	0.09 ± 0.01	0.054 ± 0.003, $P = 0.02$	0.053 ± 0.003, $P = 0.04$
cyt c from cytosol of spleen cells	0.08 ± 0.006	0.14 ± 0.02, $P = 0.04$	0.10 ± 0.02, $P = 0.01$

Notes: Data are expressed as mean ± SD. Significance accepted at $P < 0.05$; n = 16 in each group.
Abbreviations: MPA, prooxidative metalloproteins; EM, erythrocyte membrane; SCM, spleen cell membrane; SCN, spleen cell nuclei; SCMI, spleen cell mitochondria; cyt, cytochrome.

Table 2 NADPH-dependent, O_2^--producing activity of Nox and suprol in study groups

MPA	Units of optical density/mg protein		
	Group 1	Group 2	Group 3
Extracellular Nox from blood serum	25.1 ± 2.3	36.4 ± 4.1, $P = 0.04$	32.9 ± 3.0, $P = 0.03$
Nox from EM (suprol)	12.9 ± 1.6	27.1 ± 5.0, $P = 0.04$	19.8 ± 2.7, $P = 0.03$
Nox from SCM	14.1 ± 1.5	47.7 ± 6.1, $P = 0.01$	36.4 ± 7.0, $P = 0.03$
Nox from SCN	12.2 ± 1.4	21.8 ± 3.1, $P = 0.03$	19.1 ± 2.0, $P = 0.04$
Nox from SCMI	12.1 ± 0.7	20.7 ± 2.8, $P = 0.01$	16.8 ± 2.3, $P = 0.01$

Notes: Data are expressed as mean ± SD. Significance accepted at $P < 0.05$; n = 16 in each group.
Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; suprol, superoxide-producing lipoprotein; MPA, prooxidative metalloproteins; EM, erythrocyte membrane; SCM, spleen cell membrane; SCN, spleen cell nuclei; SCMI, spleen cell mitochondria.

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

Spleen cell cytosols were dialyzed against water and centrifuged, and the supernatant subjected to ion-exchange chromatography on the column with cellulose CM-52, from which cyt c was eluted with 0.2 M PPB.[23] Fractions of SOD and catalase do not absorb on this column, but are deposited on a column with cellulose DE-52, from which the total fraction of SOD and catalase was eluted with 0.04 M PPB. The SOD and catalase activities were determined by the method described above, and the specific activity was estimated for 1 g of spleen.

All optical spectral measurements were carried out on a SPECORD® UV/VIS spectrophotometer (Analytik Jena AG, Jena, Germany) in a 1 cm cuvette. The statistical processing of the results was carried out by Student's *t*- and Fisher's exact tests by definition of reliability criterion *P*. A *P*-value of ≤0.05 was considered significant.

Results

Changes in the level of metalloproteins of prooxidant activity (isoforms of Nox, localized in the blood serum, EM, SCN, SCM, and SCMI, as well as cyt c, cyt b_5, and suprol) and metalloproteins of antioxidant activity (Cu,Zn-SOD and catalase from erythrocyte and cytosol and the total fraction of Cu,Zn-SOD, Mn-SOD, and catalase from spleen cell cytosol) were observed as described below and in Tables 1–4.

In STZ-induced diabetes (Group 2), the level of Noxes was significantly increased (from 81.5% to 225%) in EM, SCM, SCN, and SCMI. The level of cyt c was significantly increased in spleen cells, indicating the decrease of the stability of mitochondria in the STZ-induced diabetes, although the level of cyt b_5 from the cytosol of erythrocytes was decreased (Table 1).

In Group 3, under the influence of prophylactically injected PEG, Nox in the EM, SCM, SCN, and SCMI were mainly saved near to the normal values (Table 1).

As shown in Table 2, the $O2^-$-producing activity of suprol and Nox, isolated from blood serum, EM, SCN, SCM, and SCMI of rats with experimental diabetes, increased sharply.[30]

The PEG had a protective effect, retaining the O_2^--producing activity of isoforms of the Noxes and suprol near to the norm.

FerriHb-reducing activity of all Nox studied was considerably decreased in STZ-induced diabetes, especially for the Nox from EM and eNox (Table 3), indicating a significant disruption of oxygen homeostasis. In this case, PEG basically prevents the change of the oxygen homeostasis.

Table 3 The specific ferriHb-reducing activity of Nox in study groups

MPA	Units of optical density/mg protein		
	Group 1	Group 2	Group 3
Extracellular Nox from blood serum	14.6 ± 1.3	8.45 ± 0.7, $P = 0.01$	9.3 ± 0.6, $P = 0.02$
Nox from EM	10.7 ± 0.3	4.3 ± 0.06, $P = 0.04$	3.3 ± 0.08, $P = 0.01$
Nox from SCM	8.6 ± 0.2	5.8 ± 0.3, $P = 0.02$	6.4 ± 0.5, $P = 0.02$
Nox from SCN	10.1 ± 0.5	7.1 ± 0.2, $P = 002$	7.6 ± 0.3, $P = 0.01$
Nox from SCMI	16.1 ± 2.8	11.7 ± 2.0, $P = 0.01$	13.8 ± 2.2, $P = 0.03$

Notes: Data are expressed as mean ± SD. Significance accepted at $P < 0.05$; n = 16 in each group.
Abbreviations: ferriHB, ferrihemoglobin; MPA, prooxidative metalloproteins; EM, erythrocyte membrane; SCM, spleen cell membrane; SCN, spleen cell nuclei; SCMI, spleen cell mitochondria.

Table 4 The changes of specific SOD and catalase activity in the erythrocyte and spleen cytosol in study groups

MAA	Units of optical density/mg protein		
	Group 1	Group 2	Group 3
Fraction of Cu,Zn-SOD from the cytosol of erythrocytes	304.8 ± 50.7	78.6 ± 6.9, $P = 0.04$	129.5 ± 13.7, $P = 0.04$
Total fraction of Cu,Zn-SOD and Mn-COD from the cytosol of spleen cells	210.5 ± 21.5	65.4 ± 8.7, $P = 0.01$	121.6 ± 16.4, $P = 0.01$
Fraction of catalase from the cytosol of erythrocytes	2100.0 ± 201.7	1182.3 ± 123.9, $P = 0.04$	1505 ± 140.6, $P = 0.01$
Fraction of catalase from the cytosol of spleen cells	430.0 ± 42.8	248.9 ± 20.6, $P = 0.03$	319.3 ± 40.3, $P = 0.01$

Notes: Data are expressed as mean ± SD. Significance accepted at $P < 0.05$; n = 16 in each group.
Abbreviations: SOD, superoxide dismutase; MAA, antioxidative metalloproteins.

As for antioxidative metalloprotein, we can see that in STZ-induced diabetes the activity of the Cu,Zn-SOD fraction from the erythrocyte cytosol and the activity of the total fraction of Cu,Zn-SOD and Mn-SOD from the spleen cells were decreased in almost the same range (Table 4).

The catalase activity in the erythrocyte and spleen cytosols was also definitely decreased. The prophylactic administration of PEG (Group 3) resulted in increased activity of these key antioxidant enzymes and decreased NADPH-dependent O_2^--producing activity of Noxes and O_2^--producing activity of suprol, indicating its antioxidant effect.

Discussion

To the authors' knowledge, this is the first study investigating the effects of this novel PEG preparation on the model of diabetes mellitus. The obtained results reveal that in STZ-induced diabetes a decrease in stability of EM, SCM, SCN, and SCMI and enhancement of releasing of isoforms of Nox from heterogeneous phase (from membranes) to the homogeneous phase (in solution) took place, resulting in increased lipid peroxidation in biomembranes.[25–27] This is confirmed by a significant decrease in the process of Nox release from EM and increased stability of the latter under the influence of drugs with antioxidant activity (Cu,Zn-SOD, catalase, and ceruloplasmin, as well as a synthetic analog of the proline-rich polypeptide galarmin from neurosecretory granules of the hypothalamus).[28]

The increased level of eNox is possibly associated with the decreased erythrocyte stability. This phenomenon is observed in various pathological states, especially with malignant tumors.[29,30] The mechanism of increasing the level of cyt c from SCM in STZ-induced diabetes can be connected with the decreased mitochondria stability.

In Group 3, the mechanism of PEG-induced normalization of Nox is not related to its antioxidant action (PEG does not possess SOD-mimetic or catalase-mimetic activity in vitro). It is more likely that the effect of PEG is associated with increase of the levels of antioxidant systems in vivo.

The uniqueness of PEG is that the combined preparation plays not only a positive antitumor[17] and neuroprotective[18] role, but also an antidiabetic role by regulating the level and activity of key anti- and prooxidative metalloproteins – the regulators of ROS metabolism.

Thus, the obtained results, as well as our previous results,[31] indicate that PEG reveals a prophylactic (preventive) effect in experimental diabetes mellitus. This universality of the drug is potentially conditioned by its ability to modulate the general adaptive mechanisms operating on the molecular level at the initial stages of the pathological process. More studies are necessary to evaluate the potential short- and long-term side effects of PEG.

Disclosure

The authors report no conflicts of interest in this work.

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