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ORIGINAL PAPER

The Role of Oxidative Stress in Diabetic Neuropathy: Generation of Free Radical Species in the Glycation Reaction and Gene Polymorphisms Encoding Antioxidant Enzymes to Genetic Susceptibility to Diabetic Neuropathy in Population of Type I Diabetic Patients

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Abstract Diabetic neuropathy (DN) represents the main cause of morbidity and mortality among diabetic patients. Clinical data support the conclusion that the severity of DN is related to the frequency and duration of hyperglycemic periods. The presented experimental and clinical evidences propose that changes in cellular function resulting in oxidative stress act as a leading factor in the development and progression of DN. Hyperglycemia- and dyslipidemia-driven oxidative stress is a major contributor, enhanced by advanced glycation end product (AGE) formation and polyol pathway activation. There are several polymorphous pathways that lead to oxidative stress in the peripheral nervous system in chronic hyperglycemia. This article demonstrates the origin of oxidative stress derived from glycation reactions and genetic variations within the antioxidant genes which could be implicated in the pathogenesis of DN. In the diabetic state, unchecked superoxide accumulation and resultant increases in polyol pathway activity, AGEs accumulation, protein kinase C activity, and hexosamine flux trigger a feed-forward system of progressive cellular dysfunction. In nerve, this confluence of metabolic and vascular disturbances leads to impaired neural function and loss of neurotrophic support, and over the long term, can mediate apoptosis of neurons and Schwann cells, the glial cells of the peripheral nervous system. In this article, we consider AGE-mediated reactive oxygen species (ROS) generation as a pathogenesis factor in the development of DN. It is likely that oxidative modification of proteins and other biomolecules might be the consequence of local generation of superoxide on the interaction of the residues of L-lysine (and probably other amino acids) with α-ketoaldehydes. This phenomenon of non-enzymatic superoxide generation might be an element

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of autocatalytic intensification of pathophysiological action of carbonyl stress. Glyoxal and methylglyoxal formed during metabolic pathway are detoxified by the glyoxalase system with reduced glutathione as co-factor. The concentration of reduced glutathione may be decreased by oxidative stress and by decreased in situ glutathione reductase activity in diabetes mellitus. Genetic variations within the antioxidant genes therefore could be implicated in the pathogenesis of DN. In this work, the supporting data about the association between the $-262T > C$ polymorphism of the catalase (CAT) gene and DN were shown. The $-262TT$ genotype of the CAT gene was significantly associated with higher erythrocyte catalase activity in blood of DN patients compared to the $-262CC$ genotype ($17.8 \pm 2.7 \times 10^4$ IU/g Hb vs. $13.5 \pm 3.2 \times 10^4$ IU/g Hb, $P = 0.0022$). The role of these factors in the development of diabetic complications and the prospective prevention of DN by supplementation in formulations of transglycating imidazole-containing peptide-based antioxidants (non-hydrolyzed carnosine, carcinine, *n*-acetylcarcinine) scavenging ROS in the glycation reaction, modifying the activity of enzymic and non-enzymic antioxidant defenses that participate in metabolic processes with ability of controlling at transcriptional levels the differential expression of several genes encoding antioxidant enzymes inherent to DN in Type I Diabetic patients, now deserve investigation.

Keywords Advanced glycation Maillard reaction · α-Dicarbonyl compounds · Superoxide anion radical production · Diabetic neuropathy · Catalase promoter polymorphisms · Glutathione · Gene encoding glutathione *S*-transferase · Therapeutic treatment of diabetic neuropathy · Imidazole-based Dipeptide Mimetics

Disease is the retribution of outraged Nature.
Hosea Ballou.

Introduction

Diabetes has reached epidemic proportions in the Western world. In the United States, 17 million individuals have diabetes, greater than 6 % of the population [1]. The morbidity and mortality of diabetes is due to the development of both macrovascular and microvascular complications [2]. Macrovascular complications including myocardial infarction, stroke, and large vessel peripheral vascular disease are 2–4 times more prevalent in individuals with diabetes. The underlying common factor in macrovascular complications is the ability of the diabetic condition to accelerate atherogenesis.

Diabetes-specific microvascular disease is a leading cause of blindness, renal failure, and nerve damage; diabetes-accelerated atherosclerosis leads to increased risk of myocardial infarction, stroke, and limb amputation. Four main molecular mechanisms have been implicated in glucose-mediated vascular damage. All seem to reflect a single hyperglycaemia-induced process of overproduction of superoxide by the mitochondrial electron transport chain [2]. While macrovascular complications are common among diabetics, diabetes-specific microvascular complications will eventually affect nearly all individuals with diabetes [3]. Retinal microvascular dysfunction in diabetes is a major component of diabetic retinopathy. Diabetic retinopathy is the most common cause of adult blindness in the United States. Ninety percent of diabetics present evidence of retinopathy within 15 years of disease onset and approximately 25,000 new cases of diabetes-related blindness are reported per year [4–6]. Diabetes is also the leading cause of renal failure in the United States, accounting for 40 % of new cases each year [7]. Greater than half of all patients with diabetes develop neuropathy, a progressive deterioration of nerves resulting in peripheral and autonomic nerve dysfunction. Around 10 % of these cases of neuropathy are associated with abnormal sensations and pain [8]. The quality and distribution of pain are variable, although descriptions of burning pain in the hands and feet are commonly reported. Like other neuropathic pain states, painful diabetic neuropathy (DN) has an unknown pathogenesis and, in many cases, is not alleviated by nonsteroidal anti-inflammatory drugs or opiates [8]. The incidence of neuropathy increases with duration of diabetes and is accelerated by poor control [9]. As a result, DN is the most common cause of nontraumatic amputations and autonomic failure [10, 11]. In his or her lifetime, a diabetic patient with neuropathy has a 15 % chance of undergoing one or more amputations [12]. In this article, we wished to evaluate, in chronic DN, the role of oxidative injury as a function of generation of reactive oxygen species (ROS) during the formation of advanced glycation end products (AGEs) which are a heterogeneous group of molecules formed from the non-enzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. Glycation of cytoskeletal proteins, through structural or functional changes of the nerve fibers, has been involved in the pathogenesis of DN [13–16]. Furthermore, increased AGE accumulation has been described in the cytoskeletal and myelin protein extracts of the sural and peroneal nerves of human subjects, distributed in the cytoplasm of endothelial cells, pericytes, axoplasm and Schwann interstitial collagens and basement membranes of the perineurium cells of both myelinated and unmyelinated fibers correlated with the myelinated fiber loss [17, 18]. Antioxidant enzymes reduce enhanced oxidative stress in the peripheral

nerve. Overall, this article demonstrates the origin of oxidative stress derived from glycation reactions and genetic variations within the antioxidant genes which could be implicated in the pathogenesis of DN.

Peripheral neuropathy, commonly manifested as distal symmetrical polyneuropathy, is a diabetic complication that contributes to mortality in most cases. A variety of genetic and non-genetic factors are considered to play a role in the pathogenesis of DN. Enhanced oxidative stress altering nerve blood supply, nerve structure, and endoneural metabolism represents an important factor predisposing to peripheral nerve damage and dysfunction in diabetic conditions [19]. The pathogenesis of PDN involves multiple mechanisms. Recent clinical trials indicate that the severity of DN is inversely correlated with the level of patient glycemic control. The findings obtained in 1999–2003 support the role of previously established mechanisms such as increased aldose reductase activity, non-enzymatic glycation or glyco-oxidation, activation of protein kinase C (PKC), enhanced oxidative stress, impaired neurotrophic support, and reveal the importance of new downstream effectors of oxidative injury [19, 20]. Each pathway becomes perturbed as a direct or indirect consequence of hyperglycemia-mediated superoxide overproduction by the mitochondrial electron transport chain. Either inhibition of superoxide accumulation or euglycemia restores the metabolic and vascular imbalance and blocks both the initiation and progression of complications [2, 20, 21].

Investigations on biopsy material from patients with mild to severe neuropathy show graded structural changes in nerve microvasculature including basement membrane thickening, pericyte degeneration, and endothelial cell hyperplasia. Arterio-venous shunting also contributes to reduced endoneurial perfusion. These vascular changes strongly correlate with clinical defects and nerve pathology. Vasodilator treatment in patients and animals improves nerve function. Early vasa nervorum functional changes are caused by the metabolic insults of diabetes, the balance between vasodilation and vasoconstriction is altered. Vascular endothelium is particularly vulnerable, with deficits in the major endothelial vasodilators, nitric oxide, endothelium-derived hyperpolarising factor, and prostacyclin. Hyperglycemia- and dyslipidemia-driven oxidative stress is a major contributor, enhanced by AGE formation and polyol pathway activation [22]. In the diabetic state, unchecked superoxide accumulation and resultant increases in polyol pathway activity, AGEs accumulation, PKC activity, and hexosamine flux trigger a feed-forward system of progressive cellular dysfunction. In nerve, this confluence of metabolic and vascular disturbances leads to impaired neural function and loss of neurotrophic support, and over the long term, can mediate apoptosis of neurons and Schwann cells, the glial cells of the peripheral nervous system [23–25]. Decreases in nerve growth factor (NGF), neurotrophin-3 (NT-3), ciliary neurotrophic factor, and insulin-like growth factor (IGF-I) in nerves from animals with experimental diabetes are well documented and correlate with the presence of neuropathy [26–28].

The mechanisms underlying oxidative stress in chronic hyperglycemia and the development of neuropathy have been examined in animal models [25]. This oxidative stress is associated with the development of apoptosis in neurons and supporting glial cells and so could be the unifying mechanism that leads to nervous system damage in diabetes [23, 24]. This article explores the evidence for oxidative stress as a significant mediator in the development of DN as well as the potential for prevention of complications through rigorous antioxidant therapy. Neurons not only are lost in diabetes, but their ability to regenerate is also impaired, particularly the small-caliber nerve fibers [29]. Understanding and the ability to intervene in oxidative stress, therefore, may both prevent neuron degeneration and promote regeneration [30].

One renown unifying mechanism of nervous system injury in diabetes lies in the ability of both metabolic and vascular insults to increase cellular oxidative stress and impair the function of mitochondria [24, 31]. Recent studies have supported this hypothesis, including in vivo and in vitro measurement of oxidative stress in sensory neurons as well as neuronal protection by antioxidants. In vitro, application of 10–20 mm glucose to dorsal root ganglia neurons leads to production of $O_2^{-\cdot}$ and H_2O_2 that leads to lipid oxidation and neuronal death. This glucose-induced death is prevented by IGF-I, in part through decreased ROS production [23]. Further evidence comes from feeding mice with a high-glucose diet. In this case, the mice experience hyperglycemia that leads to free radical production and oxidative stress [32]. Glucose at elevated concentrations undergoes non-enzymic reactions with primary amino groups of proteins to form glycated residues called Amadori products. Glycation is the non-enzymatic reaction of glucose, alpha-oxoaldehydes, and other saccharide derivatives with proteins, nucleotides, and lipids. After a series of dehydration and fragmentation reactions, the Amadori products are converted to stable covalent adducts known as AGEs [33]. These reactions are catalyzed by transition metal ions. Treatment of diabetic rats with a transition metal chelator can prevent diabetes-induced nerve conduction deficits [34]. Glycation of proteins is directly related to the concentration of glucose and therefore is produced through poor glycemic control. A number of common foods contain AGEs that can increase the AGE-induced stress in diabetic patients and promote nephropathy [35].

AGEs bind to a cell surface receptor known as receptor for AGE (RAGE), a multiligand member of the Ig superfamily. This binding initiates a cascade of signal

transduction events involving p44/p42 MAPKs, nuclear factor-κB, p21Ras, and other intermediates [36, 37]. Interaction of AGEs with RAGE induces the production of ROS through a mechanism that involves localization of prooxidant molecules at the cell surface [38] and a key role for activated NADPH oxidase [39].

AGEs are covalently formed and found to accumulate with aging, atherosclerosis, and diabetes mellitus, especially associated with long-lived proteins such as collagens [40, 41] and nerve proteins [42, 43]. It was suggested that the formation of AGEs not only modifies protein properties, but also induces biological damage in vivo [44–48]. The formation of α-dicarbonyl compounds seems to be an essential step for the cross-linking reaction, which leads to the formation of AGEs. To elucidate the mechanism for the cross-linking reaction, we studied the reaction between three-carbon α-dicarbonyl methylglyoxals and amino acid. We found that three types of free radical species were generated, and their structures were identified by EPR spectroscopy and other methods. These radicals are a cross-linked radical cation (the methylglyoxal dialkylimine radical cation or its protonated cation), the methylglyoxal radical anion, and the superoxide radical anion (which formed in the presence of oxygen molecules). Therefore, AGEs in vivo are products of the combined processes of glycation and oxidative modification. The results of this article suggest that the formation of α-ketoaldehydes or deoxyglucosones is a critical step that leads to protein cross-linking, formation of radical cation sites on the cross-linked proteins, and generation of radical counteranions. $O_2^{-\cdot}$ and H_2O_2 generated from radical counteranions can initiate free radical chain reactions including lipid peroxidation (LPO). This kind of reaction by long-lived glycated protein may contribute to the increased peroxidation of lipids and may also contribute to accelerating oxidative modification of vascular wall lipid in diabetes and atherosclerosis.

Oxidative stress occurs when the balance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products is skewed in the favor of the former.

In the peripheral nerve, antioxidant mechanisms include a number of cytosolic and lypophilic low-molecular weight and enzymatic antioxidants [49]. The key enzymatic scavengers are superoxide dismutase [EC 1.15.1.1], catalase [EC 1.11.1.6], glutathione peroxidase [EC 1.6.4.2.], glutathione reductase [EC 1.11.1.9], and glutathione *S*-transferases [EC 2.5.1.18]. The antioxidant enzymes reduce oxidative stress through the inactivation of highly toxic free oxygen radicals and peroxides and, therefore, play an important protective role in the pathogenesis of DN. In animal model of diabetes, reduced glutathione (GSH) and GSH-containing enzymatic scavengers (glutathione peroxidase (GSH-Px) and reductase) were found to be decreased in the peripheral nervous system [50], while catalase levels have been elevated in the peripheral nerve [51]. Several studies have reported reduced activity of both GPH-Px and catalase in blood of type I diabetic patients with vascular complications [52, 53]. The decreased activity of enzymatic scavengers can result from the non-enzymatic glycation and modification with glycoxidation products followed by inactivation of the modified enzyme [54, 55].

However, genetic variations within the antioxidant enzymes, particularly those which influence enzymatic activity and gene expression, could be also responsible for quantitative changes in the activity and expression profiles of antioxidant enzymatic scavengers in nerve tissue and hence contribute to genetic susceptibility for DN.

In this study, in line with the experimental assay of the free radical species generated in the late stage of the glycation reaction in the model system of interaction of L-Lysine with dicarbonyl glycating compounds, we also clinically evaluate whether variations within genes encoding important antioxidant enzymes, such as catalase (CAT), glutathione peroxidase 1 (GPX1), and glutathione *S*-transferase M1 (GSTM1) and T1 (GSTT1), could contribute to genetic susceptibility to DN in type I diabetes.

Materials and Methods

Experimental Design

Superoxide dismutase from bovine erythrocytes, methylglyoxal, L-lysine, 1,1,3,3-tetraethoxypropane, nitro blue tetrazolium (NBT), lucigenin, and other reagents produced by Sigma (USA) were used in the work. Malondialdehyde was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane as described in [56].

EPR spectra were recorded at room temperature in an E-109E spectrometer (Varian, USA). Recording settings were as follows: microware power 20 mW, microware frequency 9.15 GHz, high frequency modulation amplitude 0.2 mT. Spectrum recording was started 1 min after the mixing of reaction components. The reaction mixture (120 μl) was introduced into PTFE 22 gas-permeable capillaries (Zeus Industrial Products, USA). The capillaries were placed into a quartz tube for continuous nitrogen or air flow during the measurement. EPR spectra were simulated by SimFonia software (Bruker, Germany). The EPR signal of the stable synthetic free radical diphenylpicrylhydrazine was used as a standard [57].

Generation of superoxide anion radical ($O_2^{-}\cdot$) was detected using two independent methods: reduction of nitro blue tetrazolium by the superoxide and $O_2^{-\cdot}$-induced

Table 1 Clinical characteristics of type 1 diabetic patients with (DN +) and without (DN−) diabetic neuropathy

Characteristic	DN+ ($n = 216$)	DN− ($n = 250$)	*P*
Male/female ratio	112/104	136/114	0.58[a]
Age (years)	25.2 ± 7.7	24.1 ± 8.5	0.095
Duration of diabetes (years)	1.9 ± 0.8	2.1 ± 0.6	0.067
SCV (m/s)	36.8 ± 1.9	46.9 ± 1.1	0.0019
SNAP (μV)	12.3 ± 1.5	14.7 ± 1.3	0.023
MCV (m/s)	36.0 ± 1.5	45.7 ± 1.2	0.0022
CMAP (mV)	4.9 ± 0.7	6.1 ± 0.6	0.054
VPT (V)	28.8 ± 6.2	11.5 ± 3.3	0.00092
TPT (°C)	9.1 ± 2.5	2.7 ± 1.1	0.0057
GSH (μmol/l)	51 ± 17	69 ± 18	0.0009
Catalase activity (IU × 10^4/g Hb)	15.3 ± 2.8	13.2 ± 2.7	0.011
GSH-Px activity (IU/g Hb)	20.1 ± 4.4	22.6 ± 4.7	0.14
Glutathione *S*-transferase activity (IU/g Hb)	36.2 ± 5.2	35.5 ± 5.6	0.24

Nerve conduction velocity (SCV) and sensory nerve action potential amplitude (SNAP) in the sural nerve and motor nerve conduction velocity (MCV) and compound muscle action potential amplitude (CMAP) in the peroneal nerve were measured using surface electrodes and the MS92a EMG machine (Medelec Limited, Old Woking, Surrey, U.K.) for surface stimulation and recording. Quantitative sensory testing (QST) included assessment of vibration and thermal perception. A vibration perception threshold (VPT) test was applied using a Biothesiometer (Bio-Medical Instrument Co., Newbury, Ohio, USA). Temperature perception threshold (TPT) was determined using a thermo-esthesiometer (Hokushin Seiki Kogyo, Japan) on the top of the middle finger of both hands. All the QST procedures were done bilaterally in triplicate. The mean of three readings was taken as a perception threshold

Data are mean ± SE

[a] χ^2 test (df = 1); other data are compared using the one-way ANOVA test

chemiluminescence of lucigenin. The kinetics of accumulation of NBT reduction product, formazan, was determined by absorption at 560 nm in a Hitachi-557 spectrophotometer (Japan) at 25 °C. The reaction was initiated by adding 10 mM methylglyoxal or 10 mM MDA to the medium containing 100 μM NBT and 10 mM L-lysine in 100 mM carbonate buffer, pH 9.5. Chemiluminescence was measured by a Lum-5773 chemiluminometer (Russia) in medium containing 20 μM lucigenin, 15 mM L-lysine, and 15 mM methylglyoxal in 100 mM K,Na-phosphate buffer, pH 7.8. Measurements were performed at 37 °C under continuous stirring of the reaction medium. Statistical treatment of the data was performed using Student's *t*-criterion.

Clinical Design

Patients

The work described in this article has been carried out in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)* for experiments involving humans: http://www.wma.net/en/30publications/10policies/b3/index.html; EU Directive 2010/63/EU.

Patients were recruited in the Department of Neurology of the I.M. Sechenov Medical Academy (Moscow). They represented unrelated Russian individuals who lived in Moscow and the Moscow region. Informed consent was obtained from all subjects prior to participation in the study. The research protocol was carried out according to principles of the Helsinki Declaration and approved by the Ethics Committee of the Medical Academy.

The separate study design enrolled a total of 466 patients with diabetes of short duration (less than 3 years). Type 1 diabetes (T1D) was diagnosed in the clinical setting according to the American Diabetes Association diagnostic criteria [58]. DN patients were selected according to recommendations of the San Antonio conference on DN [59] by presence of one or more typical syndromes (pain, numbness, cramps), diminished heart rate variation with deep breathing, abnormal quantitative tests for tactile, vibration, thermal warming and cooling thresholds, and reduced nerve conduction velocity [49]. The case group (DN+) included 216 patients with clinical DN, whereas the control group (DN−) included the remaining 250 individuals with no neuropathy. None of the patients had been treated with antioxidants. Patients with causes of neuropathy others than diabetes (i.e., chronic alcohol abuse), truncal neuropathy, neurodegenerative disorders such as Parkinson's disease and others, and ischemic-related diabetic foot disease were excluded from this study. Clinical and medicinal/biochemical characteristics of the patients selected are shown in Table 1.

Clinical Measurements

The standard clinical measurements are described in details in a separate design study [49]. In brief, the deep breathing test assessed parasympathetic nerve function. In the deep breathing test, six maximal expirations and inspirations are performed continuously during 1 min in the supine position during the recording of a continuous electrocardiogram. Sensory nerve conduction velocity (SCV) and sensory nerve action potential amplitude (SNAP) in the sural nerve and motor nerve conduction velocity (MCV) and compound muscle action potential amplitude (CMAP) in the peroneal nerve were measured using surface electrodes and the MS92a EMG machine (Medelec Limited, Old Woking, Surrey, U.K.) for surface stimulation and recording. Quantitative sensory testing (QST) included assessment of vibration and thermal perception. A vibration perception threshold (VPT) test was applied using a Biothesiometer (Bio-Medical Instrument Co., Newbury, Ohio, USA). Temperature perception threshold (TPT) was determined using a thermo-esthesiometer (Hokushin Seiki Kogyo, Japan) on the top of the middle finger of both hands. All the QST procedures were done bilaterally in triplicate. The mean of three readings was taken as a perception threshold.

Antioxidant Determination Assays

Reduced gluthatione (GSSG) in erythrocyte hemolysates was quantified as described by Bentler et al. [60–62]. Antioxidant enzyme activities (CAT, GSH-Px, and glutathione *S*-transferase) in erythrocyte hemolysates have been measured according to Bentler [61].

DNA Analysis with a Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from whole-blood samples pre-treated with proteinase K (Fermentas, Vilnius, Lithuania) using extraction with phenol and chloroform [49].

For each gene studied, polymorphic regions were amplified using a polymerase chain reaction (PCR) performed according the following design.

The polymerase chain reaction, now widely used in research laboratories and doctor's offices, relies on the ability of DNA-copying enzymes to remain stable at high temperatures. No problem for *Thermus aquaticus*, the sultry bacterium from Yellowstone that now helps scientists produce millions of copies of a single DNA segment in a matter of hours. In nature, most organisms copy their DNA in the same way. The PCR mimics this process, only it does it in a test tube. When any cell divides, enzymes called polymerases make a copy of all the DNA in each chromosome. The first step in this process is to "unzip" the two DNA chains of the double helix. As the two strands separate, DNA polymerase makes a copy using each strand as a template. The four nucleotide bases, the building blocks of every piece of DNA, are represented by the letters A, C, G, and T, which stand for their chemical names adenine, cytosine, guanine, and thymine. The A on one strand always pairs with the T on the other, whereas C always pairs with G. The two strands are said to be complementary to each other. To copy DNA, polymerase requires two other components: a supply of the four nucleotide bases and something called a primer. DNA polymerases, whether from humans, bacteria, or viruses, cannot copy a chain of DNA without a short sequence of nucleotides to "prime" the process, or get it started. So the cell has another enzyme called a primase that actually makes the first few nucleotides of the copy. This stretch of DNA is called a primer. Once the primer is made, the polymerase can take over making the rest of the new chain. A PCR vial contains all the necessary components for DNA duplication: a piece of DNA, large quantities of the four nucleotides, large quantities of the primer sequence, and DNA polymerase. The polymerase is the Taq polymerase, named for *T. aquaticus*, from which it was isolated. The three parts of the polymerase chain reaction are carried out in the same vial, but at different temperatures. The first part of the process separates the two DNA chains in the double helix. This is done simply by heating the vial to 90–75 °C (about 165 °F) for 30 s.

The modified PCR cocktail contained [49]: 67 mM Tris–HCl, pH 8.8, 16.7 mM ammonium sulfate, 1.0 mM magnesium chloride, 0.1 % Tween-20, 10 % dimethyl sulfoxide, 0.2 mM each dNTP, 5 pmol of each primer, 100 ng of genomic DNA, and 1.0 unit of *Taq* DNA polymerase (Fermentas) in a total volume of 20 µl. PCR was run on a GeneAmp® PCR System 9600 (Applied Biosystems, Foster City, California, USA) at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C to detect the 1167C > T dimorphism of the CAT gene and polymorphic markers within the GSTT1 and GSTM1 genes, or 65 °C to determine the −262T > C substitution of the CAT gene and 197Pro > Lys polymorphism of the GPX1 gene, for 30 s and extension at 72 °C for 1 min, with final extension at 72 °C for 7 min. To identify the 1167C > T polymorphism of the CAT gene and 197Pro > Leu dimorphism of the GPX1 gene, a PCR product was further digested with BstXI and BstDEI restriction enzyme, respectively [49, 62]. The −262T > C polymorphism of the CAT gene was characterized using digestion with *Sma*I as described by Folsberg et al. [63]. Polymorphic markers within the GSTT1 and GSTM1 genes have been simultaneously detected using a multiplex PCR [64]. PCR products and digestion fragments were analyzed electrophoretically in 2 % agarose gel with ethidium bromide [49].

Statistical Analysis

For statistical analysis, the Statgraphics Plus for Windows version 3.1 software (Statistical Graphic Corp.,Rockwille, Madison, USA) was used. The ANOVA test was applied for comparisons between groups. The χ^2 test was used to compare allele and genotype frequencies in control subjects and diabetic patients complicated with DN. Odds ratios (OR) and 95 % confidence interval (95 % CI) were calculated to assess the strength of relationship between the polymorphic marker and DN [49]. A P value of <0.05 was considered statistically significant.

Results

Mechanism of Superoxide Formation in the Late Stage of the Glycation Reaction in the Model System of Interaction of L-Lysine with Dicarbonyl Glycating Compounds

For the comparative study of the interaction of L-lysine with carbonyl compounds, we used the major secondary product of lipid peroxidation (MDA) and its isomer α-ketoaldehyde (α-oxoaldehyde)—methylglyoxal. Increased formation of the very reactive dicarbonyl compound MGO (methylglyoxal), one of the side-products of glycolysis, and MGO-derived AGEs seem to be implicated in the development of diabetic vascular complications.

In mammals, methylglyoxal is formed in the course of glycolysis and on autooxidation of glucose [65–67]. L-Lysine was used because this amino acid is one of the main targets of the action of active carbonyl compounds in protein molecules [65–69]. Amino acids react with methylglyoxal to form AGEs. This reaction is suspected to produce free radicals. These free radicals are (1) the cross-linked radical cation, (2) the methylglyoxal radical anion as the counterion, and (3) the superoxide radical anion produced only in the presence of oxygen [70]. The results indicate that dicarbonyl compounds cross-link free amino groups of protein by forming Schiff bases, which donate electrons directly to dicarbonyl compounds to form the cross-linked radical cations and the methylglyoxal radical anions. Oxygen can accept an electron from the radical anion to generate a superoxide radical anion, which can initiate damaging chain reactions [70]. The data of ROS generation during the glycation reaction have been investigated by our group. Figure 1 shows the results of EPR spectroscopic study of the products of L-lysine reactions with methylglyoxal and MDA. The data presented in this figure demonstrate that free radical intermediates are formed under anaerobic conditions in the reaction of L-lysine with methylglyoxal but not with MDA (Fig. 1a, spectra *1* and *3*). The EPR spectrum recorded during the reaction of L-lysine with methylglyoxal has a multicomponent hyperfine structure [71].

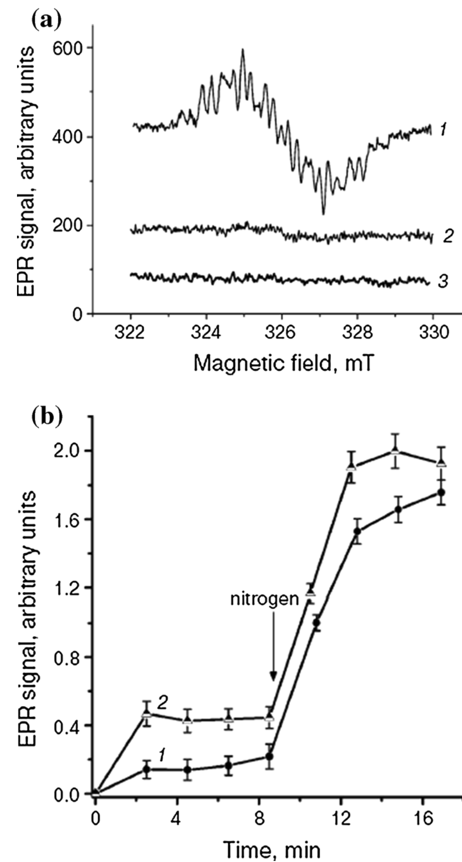

Fig. 1 (**a**) EPR spectra of free radical intermediates of the reaction between L-lysine and dicarbonyl compounds. The reaction medium contained 160 mM L-lysine and 160 mM methylglyoxal (spectra *1* and *2*) or 160 mM MDA (spectrum *3*) in K,Na-phosphate buffer (0.2 M, pH 7.8). EPR signals were registered 4 min after mixing the components under aeration (spectrum *2*) or under nitrogen (spectra *1* and *3*). (**b**) Effect of aeration and SOD on the kinetics of accumulation of free radical intermediates recorded by EPR. The reaction medium contained: (*1*) 160 mM L-lysine and 160 mM methylglyoxal in 0.2 M K,Na-phosphate buffer, pH 7.8; (*2*) the same as (*1*) + 400 SOD units

Previously, in work [70], such EPR spectrum was recorded in reaction mixture containing L-alanine and methylglyoxal. In this work, using C^{13}- and N^{15}-substituted and deuterated L-alanine derivatives it has been shown that the EPR spectrum is a superposition of signals of methylglyoxal anion radical ($MG^{-\cdot}$) and Schiff base cation radical (dialkylimine) appearing on the interaction of methylglyoxal with the amino acid. Based on this, we suggest that the EPR spectrum observed in our experiments is also a superposition of signals of $MG^{-\cdot}$ and the cation radical of methylglyoxal dialkylimine with lysine.

It is important to note that only trace quantities of free radical intermediates were registered under aeration of the

reaction mixture (Fig. 1, spectrum *2*) [71]. Substitution of air for nitrogen after incubation of methylglyoxal and L-lysine mixture under aerobic conditions results in a significant (nearly by an order of magnitude) increase in the level of free radicals, supposedly dialkylimine and methylglyoxal (Fig. 1b). It is significant that under these conditions the content of free radical intermediates increases on addition of superoxide dismutases (SOD) to the reaction mixture (Fig. 1b, curve *2*) [71]. The effect of SOD might be due to the fact that this enzyme removes the superoxide radical generated in the tested model system. Indeed, the data obtained in work [64] indicate that $O_2^{-\cdot}$ is formed by single-electron oxygen reduction by methylglyoxal semidione in accordance with the reaction:

$$MG^{-\cdot} + O_2 \rightarrow MG + O_2^{-\cdot}. \qquad \text{(reaction1)}$$

Our model system has also demonstrated that $O_2^{-\cdot}$ is intensively generated on the interaction of L-lysine with methylglyoxal in carbonate buffer, pH 9.5. Superoxide formation was assessed by the accumulation of formazan on NBT reduction (Fig. 2a). The accumulation of formazan under these conditions might not depend on $O_2^{-\cdot}$, since it is probable that NBT is reduced by other intermediates of L-lysine reaction with methylglyoxal. Nevertheless, reasoning from the fact that SOD significantly (more than 4 times) inhibited the formation of formazan under the above conditions, one can state that the most part of NBT is reduced under the action of $O_2^{-\cdot}$ (Fig. 2a). However, only insignificant generation of superoxide radical was observed on the interaction of L-lysine with MDA (Fig. 2b), supported in Ref. [71]. The rate of reaction of amino groups with methylglyoxal becomes lower on increasing acidity of the medium [72]. It is concluded that the primary step in the reaction involves the formation of a Schiff base linkage between the lysine side chain and methylglyoxal. These findings reaffirm the concept that, by the formation of Schiff bases, aldehydes can act as electron acceptors in charge transfer interactions with proteins [72].

The application of chemiluminescence as a method more sensitive than NBT reduction [73] revealed the formation of $O_2^{-\cdot}$ in the mixture of methylglyoxal with L-lysine at pH 7.8 (Fig. 3), i.e., under conditions close to physiological. SOD under these conditions almost completely inhibits the chemiluminescence of lucigenin, which is evidence of the dependence of this process on the presence of superoxide anion radical (Fig. 3, curve *2*).

The decrease in concentration of free radicals recorded by EPR in aerated reaction medium is probably not associated with inhibition of their formation. Indeed, with nitrogen purging, the content of free radical intermediates reaches its maximum in 8 min after the mixing of reaction components, but after the gas medium is replaced by air the level of EPR-revealed free radicals quickly drops (Fig. 4a).

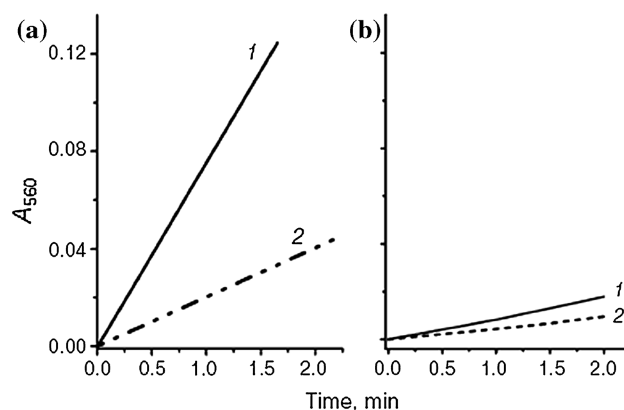

Fig. 2 Effect of SOD on kinetics of formazan formation during the reaction of L-lysine with methylglyoxal (a) or MDA (b). The reaction medium contained: (*1*) 100 mM carbonate buffer, pH 9.5, 10 mM L-lysine, and 10 mM methylglyoxal or MDA; (*2*) the same as (*1*) + 120 SOD units

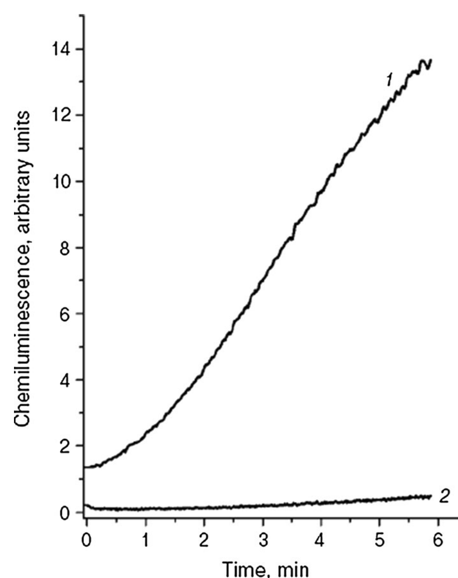

Fig. 3 Effect of SOD on superoxide-dependent chemiluminescence of lucigenin. The reaction medium contained: (*1*) 100 mM K,Na-phosphate buffer, pH 7.8, 20 μM lucigenin, 15 mM L-lysine, 15 mM methylglyoxal; (*2*) the same as (*1*) + 120 SOD units

Under these experimental conditions, SOD reliably reduced the rate of decline of EPR signal intensity during aeration (Fig. 4a, curve *2*). In 2 min, after the increase in oxygen concentration in the medium containing L-lysine and methylglyoxal, it is impossible to reveal there free radical intermediates (Fig. 4a, curve *1*). Nevertheless, the EPR spectrum containing five components of hyperfine structure and a g-factor equal to 2.0042 were recorded on aeration of the reaction medium in the presence of SOD (Fig. 4b, spectrum *2*). According to the literature data, the characteristics of the EPR spectrum presented in Fig. 4b (spectrum *2*) correspond to the signal of the *cis*-form of methylglyoxal anion radical. This fact confirms the above

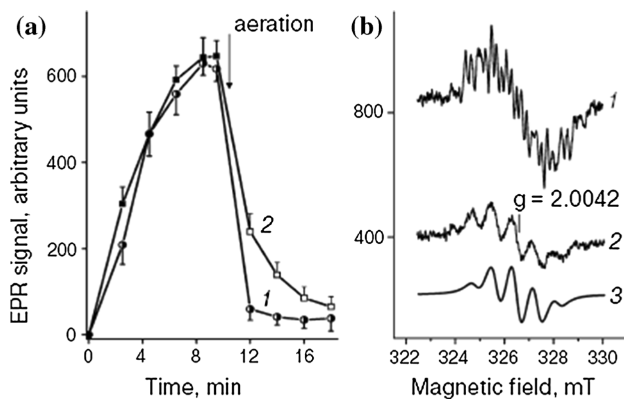

Fig. 4 Effect of oxygen and SOD on the level of free radical derivatives of methylglyoxal and dialklylimine. (**a**) Decrease under aeration conditions of the level of $MG^{-\cdot}$ and dialkylimine cation radical in the absence (*1*) and presence of SOD (*2*). Reaction medium composition is the same as in Fig. 3b. (**b**) EPR spectrum of SOD-containing reaction medium (400 U/ml) 8 min after the mixing of lysine and methylglyoxal. EPR spectra were recorded under nitrogen purging (*1*); the same sample 2 min after the beginning of aeration (*2*); simulation of the spectrum of methylglyoxal anion radical (*3*). Closed squares on curve *2* (**a**) correspond to EPR signals analogous to spectrum *1* (**b**); open squares correspond to the signal analogous to spectrum *2* (**b**)

assumption that the free radical intermediates of L-lysine reaction with methylglyoxal are $MG^{-\cdot}$ and the cation radical of dialkylimine. Thus, molecular oxygen seems to interact directly with the free radical derivatives of methylglyoxal and dialkylimine, and the products formed in this reaction are not registered by EPR (Fig. 4a), see also for support, Ref. [71]. However, SOD protects the anion radical of methylglyoxal under aerobic conditions, which points to the possibility of $MG^{-\cdot}$ elimination under the effect of superoxide. Indeed, it has been established that in aqueous media $O_2^{-\cdot}$ reduces some organic radicals [74] and catalyzes protonation and disproportionation of nitrobenzene anion radical [71]. By analogy, it can be supposed that superoxide radical interacts with the protonated semidione of methylglyoxal, reducing it in accordance with the reaction:

$$MG^{\cdot}(H) + O_2^{-\cdot} \rightarrow \text{hydroxyacetone} + O_2. \qquad \text{(reaction2)}$$

Clinical Features of Patients with DN

When the groups of patients were compared, significant differences were observed between all characteristics used to evaluate nerve function except for CMAP (Table 1). Therefore, these parameters could represent strong and helpful criteria for the selection of diabetic patients with neuropathy.

Levels of reduced glutathione (GSH) were significantly decreased in blood of DN patients compared to non-complicated individuals ($P < 0.001$). This could result from enhanced peroxidation indicating development of accelerated oxidation stress in DN patients [49]. Significant changes in activity of antioxidant enzymes were found only for catalase, whose activity was increased in complicated subjects ($P < 0.05$) (Table 1).

Polymorphisms of Antioxidant Enzymes and Oxidative Stress

Among the polymorphisms tested, significant differences were shown only for the −262T > C nucleotide substitution of the CAT gene (Table 2). The −262C allele was associated with higher risk of DN in the population of patients sample (OR 1.98, *P* corrected = 0.002). Carriage of the −262T > C molecular variant of the CAT gene was found to be correlated with catalase activity in erythrocytes. Higher enzyme activity was detected in blood of DN patients carrying the −262TT genotype compared to those who had the −262CC genotype (17,800 IU/g Hb vs 13,500 IU/g Hb, $P < 0.002$) (Table 3). Additionally, diabetic individuals without complications carrying the −262TT and −262CT genotypes had significantly higher levels of the reduced glutathione than T1D patients homozygous for −262CC (Table 3). These observations, therefore, suggest a protective role of the −262T CAT allele against rapid development of the oxidative stress in T1D.

For the 1167C > T polymorphism of the CAT gene, no significant correlation with enzyme activity and blood levels of reduced gluatathione was found (Table 3). Proline-to-leucine amino acid change in codon 197 of the GPX1 showed no significant relationship to total glutathione peroxidase activity and GSH levels in blood of both complicated and non-complicated patients (Table 3). Similar results have been obtained for the +/null polymorphism of the GSTT1 gene encoding glutathione *S*-transferase θ_1, which showed no significant association with total glutathione *S*-transferase activity nor with reduced concentration of glutathione in blood of the patients (Table 3). However, the “+” allele of the GSTM1 encoding glutathione *S*-transferase μ_1 was shown to be significantly associated with increased total GST activity as well as with elevated GSH levels in both complicated and non-complicated diabetic subjects (Table 3).

Evidence for Oxidative Stress in DN

One unifying mechanism of nervous system injury in diabetes lies in the ability of both metabolic and vascular insults to increase cellular oxidative stress and impair the function of mitochondria [75, 76]. Recent studies have supported this hypothesis, including in vivo and in vitro measurement of oxidative stress in sensory neurons as well as neuronal protection by antioxidants. In vitro, application

Table 2 Allele and genotype frequencies of polymorphisms tested within antioxidant enzyme genes in type 1 diabetic patients with (DN +) and without (DN−) diabetic neuropathy

Marker	Allele/genotype	DN+ ($n = 216$)	DN− ($n = 250$)	χ^{2a}	*P*	OR [95 % Cl]
1167C > T CAT	Allele C (%)	347 (80.3)	390 (78.0)	0.76	0.38	
	Allele T (%)	85 (19.7)	110 (22.0)			
	Genotype CC (%)	141 (65.3)	146 (58.4)			
	Genotype CT (%)	65 (30.1)	98 (39.2)	5.32	0.07	
	Genotype TT (%)	10 (4.6)	6 (2.4)			
−262T > C CAT	Allele T (%)	186 (43.1)	266 (53.2)	9.55	0.002	0.70 [0.54–0.90]
	Allele C (%)	*246 (56.9)*	*234 (46.8)*			*1.98 [1.53–2.58]*
	Genotype TT (%)	53 (24.6)	96 (38.4)			0.52 [0.35–0.78]
	Genotype TC (%)	80 (37.0)	74 (29.6)	10.27	0.0058	
	Genotype CC (%)	83 (38.4)	80 (32.0)			
197Pro > Leu GPX1	Allele Pro (%)	362 (83.8)	411 (82.2)	0.42	0.52	
	Allele Leu (%)	*70 (16.2)*	*89 (17.8)*			
	Genotype ProPro (%)	151 (69.9)	167 (66.8)			
	Genotype ProLeu (%)	60 (27.8)	77 (30.8)	0.53	0.77	
	Genotype LeuLeu (%)	5 (2.3)	6 (2.4)			
+/null GSTT1	Allele "+" (%)	272 (63.0)	330 (66.0)	0.93	0.33	
	Allele "null" (%)	160 (37.0)	170 (34.0)			
+/null GSTM1	Allele "+"	154 (25.6)	156 (31.2)	0.15	0.15	
	Allele "null"	278 (64.4)	344 (68.8)			

[a] χ^2 test: df = 1 for allele frequency comparisons; df = 2 for genotype frequency comparisons

of 10–20 mm glucose to dorsal root ganglia neurons leads to production of $O_2^{-\cdot}$ and H_2O_2 that leads to lipid oxidation and neuronal death. This glucose-induced death is prevented by IGF-I, in part through decreased ROS production [77]. Further evidence comes from feeding mice with a high-glucose diet. In this case, the mice experience hyperglycemia that leads to free radical production and oxidative stress [78]. There is a close correlation between oxidative stress in diabetes and the development of complications. In type 1 diabetic patients, oxidative stress is evident within a few years of diagnosis before the onset of complications. As the disease progresses, antioxidant potential decreases, and plasma lipid peroxidation products increase depending upon the level of glycemic control [79]. Type 2 diabetic patients have increased lipid peroxidation compared with age-matched control subjects, as well as decreased plasma GSH and GSH-metabolizing enzymes and antioxidant potential, all of which relate directly to the rate of development of complications [80–82]. Similarly, oxidative stress is linked to preclinical features of disease, such as vascular endothelial activation that can lead to atherosclerosis [83]. The early increase of oxidative stress in diabetes is more pronounced in women and may account for increased cardiovascular disease in female patients [84].

Animal studies using pharmacological and genetic approaches revealed important roles of increased aldose reductase, PKC and poly(ADP-ribose) polymerase activities, advanced glycation end products and their receptors, oxidative-nitrosative stress, growth factor imbalances, and C-peptide deficiency in both painful and insensate neuropathy [85]. This review describes recent achievements in studying the oxidative stress in pathogenesis of DN and the generation systems of ROS derived from the glycation reactions and developing potential pathogenetic molecular targets and pharmaceutical tools for further treatments.

Several ROS are normally produced in the body to perform specific functions. Superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) are three free radical ROS that are not only essential for normal physiology, but are also believed to accelerate the process of aging and to mediate cellular degeneration in disease states. These agents besides their direct oxidation actions together produce highly active singlet oxygen, hydroxyl radicals, and peroxynitrite that can attack proteins, lipids, and DNA.

Possible sources of oxidative stress and damage to proteins in diabetes include free radicals generated by autoxidation reactions of sugars and sugar adducts to protein and by autoxidation of unsaturated lipids in plasma and membrane proteins. The oxidative stress may be amplified by a continuing cycle of metabolic stress, tissue damage, and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger

Table 3 Activities of antioxidant enzymes and levels of reduced glutathione in carriers of different genotypes of the CAT, GPX1, GSTT1, and GSTM1 genes

Marker	Allele/genotype	CAT activity (IU × 10^4/g Hb)		P[a]	GSH (π mol/L)		P[a]
		DN+	DN−		DN+	DN−	
1167C > T CAT	CC	15.4 ± 2.6	13.5 ± 2.9	0.061	48 ± 18	67 ± 17	0.002
	CT	14.7 ± 3.3	12.7 ± 2.5	0.064	56 ± 14	72 ± 19	0.063
	TT	15.6 ± 2.7	13.7 ± 2.5	0.082	52 ± 17	65 ± 20	0.021
−262T > C CAT	TT	17.8 ± 2.7[b]	13.8 ± 2.7	0.0022	54 ± 16	74 ± 19[c]	0.0033
	TC	15.4 ± 2.5	13.4 ± 2.7	0.059	52 ± 18	70 ± 18[d]	0.004
	CC	*13.5 ± 3.2*	*12.3 ± 2.8*	0.26	47 ± 16	62 ± 16	0.0068
		GSH-Px activity (IU/g Hb)					
197Pro > Leu GPX1	ProPro	20.5 ± 4.5	22.9 ± 4.9	0.067	52 ± 16	71 ± 16	0.002
	ProLeu	19.1 ± 4.2	22.0 ± 4.3	0.027	47 ± 18	65 ± 22	0.0054
	LeuLeu	*19.6 ± 3.9*	*22.0 ± 4.5*	0.11	55 ± 19	67 ± 21	0.019
		GSH activity (IU/g Hb)					
+/null GSTT1	+	36.9 ± 5.5	36.1 ± 5.3	0.38	55 ± 18	73 ± 18	0.00066
	Null	35.0 ± 4.7	34.3 ± 6.2	0.41	48 ± 16	67 ± 18	0.0012
−/null GSTM1	+	39.4 ± 5.3[e]	39.0 ± 5.8[f]	0.46	59 ± 17[g]	78 ± 19[h]	0.0017
	Null	34.4 ± 5.1	33.9 ± 5.5	0.47	46 ± 17	65 ± 17	0.0003

Data are mean ± SE

[a] One-way ANOVA test

[b] CAT activity in DN+ patients homozygous for −262TT vs. DN+ patients homozygous for −262CC, $P = 0.0017$

[c] GSH level in DN+ patients homozygous for −262TT vs. DN+ patients homozygous for −262CC, $P = 0.019$

[d] GSH level in DN+ patients heterozygous for −262TC vs. DN+ patients homozygous for −262CC, $P = 0.039$

[e] GST activity in DN+ patients with "+" allele vs. DN+ patients with null allele, $P = 0.0013$

[f] GST activity in DN− patients with "+" allele vs. DN− patients with null allele, $P = 0.0014$

[g] GSH level in DN+ patients with "+" allele vs. DN+ patients with null allele, $P = 0.0062$

[h] GSH level in DN− patients with "+" allele vs. DN− patients with null allele, $P = 0.0057$

systems, which further exacerbate the oxidative stress. Such investigation and assay studies may lead to therapeutic approaches for limiting the damage from glycation and oxidation reactions and for complementing existing therapy for treatment of the complications of diabetes.

Free amino groups of proteins react slowly with reducing sugars such as glucose by the glycation or Maillard reaction to form poorly characterized brown fluorescent compounds. This process is initiated by the condensation reaction of reducing sugars with free amino groups to form Schiff bases, which undergo rearrangement to form the relatively stable Amadori products [86, 87]. The Amadori products subsequently degrade into α-dicarbonyl compounds, deoxyglucosones [88]. These compounds are more reactive than the parent sugars with respect to their ability to react with amino groups of proteins to form cross-links, stable end products called advanced Maillard products or AGEs.

The molecular structures of some AGEs have been identified as pentosidines [89–93], pyrrole derivatives [94], pyrazine derivatives [95, 96], and N^{ε}-carboxymethyllysine [97–101]. In the presence of molecular oxygen, the formation of these products from sugars is catalyzed by transition metal ions via glycoxidation, which oxidizes Amadori products to N^{ε}-carboxymethyllysine [97, 98], and the autoxidation of glucose, which produces superoxide radical anions ($O_2^{-\cdot}$), H_2O_2, and α-ketoaldehydes [102–106]. The major pathways of glycation reaction-mediated damage to macromolecules therefore involve both nonoxidative and oxidative processes. Their individual contributions to biological damage, however, are not well understood.

The formation of α-dicarbonyl compounds seems to be an important step for cross-linking proteins in the glycation or Maillard reaction. To elucidate the mechanism for the cross-linking reaction, we studied the reaction between a three-carbon α-dicarbonyl compound, methylglyoxal, and amino acids. Our former results showed that this reaction generated yellow fluorescent products as formed in some glycated proteins [107]. In addition, a few types of free radical species were also produced, and their structures were determined by EPR spectroscopy. These free radicals

are (1) the cross-linked radical cation, (2) the methylglyoxal radical anion as the counterion, and (3) the superoxide radical anion produced only in the presence of oxygen [70]. The generation of the cross-linked radical cations and the methylglyoxal radical anions does not require metal ions or oxygens. These results indicate that dicarbonyl compounds cross-link free amino groups of protein by forming Schiff bases, which donate electrons directly to dicarbonyl compounds to form the cross-linked radical cations and the methylglyoxal radical anions. Oxygen can accept an electron from the radical anion to generate a superoxide radical anion ($O_2^{-\cdot}$), which can initiate damaging chain reactions.

Analysis of the literature data [67, 69, 70] suggests a sequence of biological reactions resulting in the formation of free radicals on the interaction of amino acids with carbonyl compounds during the advanced glycation and lipid peroxidation (LPO) reactions (Fig. 5a). The presented scheme shows that dialkylimine is a Schiff base, which is a product of interaction of methylglyoxal carbonyl groups with two L-lysine molecules. As a result of reaction of dialkylimine with one more molecule of α-ketoaldehyde, the Schiff base cation radical and methylglyoxal semidione ($MG^{-\cdot}$) are formed, respectively (Fig. 5a). The absence of noticeable quantities of free radical products in the medium with L-lysine and MDA might be due to low reactivity of Schiff bases formed on their interaction. In fact, in contrast to the dialkylimine formed by methylglyoxal and L-lysine, the dialkylimine being an MDA derivative has no coupled double bonds (Fig. 5a) [71]. This peculiarity of chemical structure seems to reduce the ability of dialkylimines to participate in reactions of single-electron oxidation/reduction. The findings make it possible to substantially supplement the previously proposed mechanism of superoxide generation on modification of amino acids under the action of methylglyoxal [70]. According to this mechanism, the reactions presented in Fig. 5a result in the formation of $MG^{-\cdot}$,which further reduces oxygen to $O_2^{-\cdot}$ (see Results, reaction 1).

We assume that $O_2^{-\cdot}$ is not only formed but also seems to be utilized in reactions with the involvement of $MG^{-\cdot}$ (Fig. 5a) [71]. Besides, superoxide is probably produced on oxidation by oxygen of both methylglyoxal semidiones and dialkylimine cation radicals (free radicals of the Schiff bases of lysine and methylglyoxal) (Fig. 5b) [71]. These assumptions explain the effect of oxygen on the kinetics of accumulation and dissipation of free radicals emerging in the mixture of L-lysine and methylglyoxal. The work [70] has shown that accumulation of methylglyoxal semidione and Schiff base cation radical depends only insignificantly on oxygen concentration. According to our data, this fact may be associated with application of L-alanine as amino acid in the cited work and by the high pH value of the reaction medium (carbonate buffer, pH 9.5). It should be noted that our model system (phosphate buffer, pH 7.8) is much closer to physiological conditions. Thus, it is most likely that oxidative modification of proteins and other biomolecules might be the consequence of local generation of superoxide on the interaction of the residues of L-lysine (and probably other amino acids) with α-ketoaldehydes. This phenomenon of non-enzymatic superoxide generation might be an element of autocatalytic intensification of pathophysiological action of carbonyl stress. Glycation, generation of advanced glycosylation end products (AGEs), and formation of protein carbonyl groups play important roles in aging, diabetes, its secondary complications, and neurodegenerative conditions. Glyoxal and methylglyoxal are detoxified by the glyoxalase system with reduced glutathione as co-factor. The concentration of reduced glutathione may be decreased by oxidative stress and by decreased in situ glutathione reductase activity in diabetes mellitus. A reduced concentration of reduced glutathione may predispose diabetic patients to oxidative damage and to alpha-oxoaldehyde-mediated glycation by decreasing the in situ glyoxalase I activity [108]. More research is required to understand the role of glycation in the development of DN.

DN probably arises from a combination of microvascular and neuronal deficits. Nerve dysfunction in diabetes is associated with increased oxidative stress. Oxidative stress can contribute significantly to these deficits and may be a direct result of hyperglycemia. Brief postprandial peaks in plasma glucose are sufficient to generate hyperglycemic oxidative stress. In contrast, acute glucose deprivation also causes apoptosis of peripheral neurons through a mechanism that at least partially involves oxidative stress [109].

Different models of diabetes have produced conflicting data regarding increases or decreases in antioxidant enzymes. It has been demonstrated that active oxygen species induce antioxidant enzyme expression in some tissues, and this phenomenon is considered proof of an existing oxygen-dependent toxicity. In cultured vascular endothelial cells, glucose-induced oxidative stress leads to increased mRNA for antioxidant enzymes for a period of 2 week [110].

The total radical antioxidant potential assay clearly demonstrates that diabetic patients have lower antioxidant defenses and that total antioxidant potential is a better indication of antioxidant status than examination of individual antioxidants [111]. Measures of individual antioxidants often do not correlate with glucose levels [112]. In both clinical diabetes and experimental in vivo and in vitro models, antioxidant potential correlates with the degree of glycemic control and decreases with prolonged diabetes [113, 114].

Multiple logistic regression analysis of biochemical and clinical variables in diabetic patients was performed to

Fig. 5 (**a**) Scheme of biological reactions of L-lysine with methylglyoxal (glycating agent) and malondialdehyde (secondary molecular lipid peroxidation product). (**b**) Scheme of superoxide generation on the interaction of molecular oxygen with free radical intermediates of methylglyoxal reactions with L-lysine

identify those associated with the presence of diabetic complications (retinopathy, neuropathy, and nephropathy) [108]. The presence of diabetic complications correlated positively with duration of diabetes and patients age and negatively with the concentration of reduced glutathione in erythrocytes [108]. In our study, decreased levels of

glutathione in blood of DN patients (Table 1) could result from enhanced oxidative stress in blood vessels. These observations are consistent with results of other studies reporting significant reduction of GSH in the peripheral nerve and blood of rats with experimental DN [115] and patients with diabetic microangiopathy [116]. Peripheral nerve, which requires exogeneous glutathione due to the deficiency of its own γ-glutamyl-cysteine synthetase, an enzyme involved in GSH biosynthesis, is particularly sensitive to oxidative stress [108, 117]. Thus, depletion of reduced glutathione could represent an important characteristic for the estimation of oxidative stress in diabetes and late diabetic complications because of the unique role of the altered ratio between reduced and oxidized forms of glutathione in molecular mechanisms of DN [117].

Significantly higher activity of catalase in blood of DN patients compared to individuals without complications (Table 1) is likely to reflect a homeostatic response of the organism to high glucose-induced oxidative stress in short-term diabetes [118]. This is supported both by the observations showing an increase in serum catalase activity in diabetic patients recently affected with microvascular complications [107] and also the elevated levels of catalase mRNA in the peripheral nerve of rats with experimentally induced DN [115].

Folsberg et al. [63] reported a positive association between the −262T molecular variant of catalase and higher transcriptional activity of the CAT gene promoter that could explain significantly increased levels of erythrocyte catalase in Swedish individuals with the −262TT and −262CT genotypes compared to subjects homozygous for −262CC. We also observed a significant association between the −262TT genotype of CAT and higher activity of the enzyme in DN patients (Table 3). This is likely to result from the increased transcriptional activity of the −262T CAT promoter variant providing higher levels of catalase mRNA compared to DN patients, who are homozygous for −262CC. This suggests a protective role of the −262T molecular variant of catalase against increased oxidative stress in type 1 diabetes and rapid development of DN.

For the GSTM1 gene, a significant relationship between the "+" allele and high total activity of glutathione *S*-transferase was found, while the null allele was associated with decreased GST activity (Table 3). A null GSTM genotype results from gene deletion [119]. Approximately 50 % of the Caucasian population is deficient for GSTM1. The +/null polymorphism of GSTM1 is usually associated with different intracellular concentrations of the enzyme, which are extremely low in individuals with the null GSTM1 genotype [119]. However, some investigators reported earlier on a relationship between +/null GSTM polymorphism and the enzyme activity, with high activity for the "+" allele and low activity for the null allele [120]. This correlation could result from different levels of the enzyme observed depending on genotype of carriage.

Here we report finding an association between −262T > C polymorphism of the CAT gene and DN. Both superoxide dismutase and catalase represent phase I antioxidant enzymes metabolizing toxic chemicals (superoxide and peroxides) to less toxic products further detoxified by phase II enzymes, such as glutathione peroxidase and glutathione *S*-transferase, into metabolites which can be easily excreted from the body. In our studies, we found evidence for relationships between phase I enzymes and DN that could underline a unique role of phase I enzymatic scavengers in DN pathogenesis.

Oxidative stress is associated with the development of apoptosis in neurons and glial cells and so could be the unifying mechanism that leads to nervous system damage in diabetes. The intensive studies have supported this hypothesis, including in vivo and in vitro measurement of oxidative stress in sensory neurons and dorsal route ganglion. There are several polymodal pathways that lead to oxidative stress in the peripheral nervous system in chronic hyperglycemia.

In this article, we consider AGE-mediated ROS generation as an important pathogenesis factor in the development of DN. Increased non-enzymic glycation alters the function and structures of various macromolecules in tissues causing basement membrane thickening, demyelination, and impaired axonal transport as a result of glycation of myelin, tubulin, and neurofilaments. Extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with their cellular receptors. Plasma proteins modified by AGE precursors bind to AGE receptors on endothelial, mesengial cells, and macrophages, inducing receptor-mediated production of ROS [120]. Interaction of AGE with RAGE activates the pleiotropic transcription factor NF-κB, which causes pro-inflammatory changes in gene expression. The transgenic and knockout animal models have strengthened the concept that the AGE-RAGE interaction plays a crucial role in the development and progression of DN. Also the fact that agents that modify the AGE formation process improve some of the symptoms of DN supports the role of AGE formation in its pathogenesis [121]. In this work, the supporting data about the association between the −262T > C polymorphism of the CAT gene and DN were shown. The −262TT genotype of the CAT gene was significantly associated with higher erythrocyte catalase activity in blood of DN patients compared to the −262CC genotype $(17.8 \pm 2.7 \times 10^4\ \mathrm{IU/g\ Hb}\ \mathrm{vs.}\ 13.5 \pm 3.2 \times 10^4\ \mathrm{IU/g\ Hb},\ P = 0.0022)$.

Conclusion

DN represents the main cause of morbidity and mortality among diabetic patients. The presented experimental and clinical evidences propose that changes in cellular function resulting in oxidative stress act as a leading factor in the development and progression of DN. The mechanisms of neuronal injury converge upon oxidative stress and this appears as a therapeutic target in pharmaceutical intervention, particularly using transglycating peptide-based antioxidants (non-hydrolyzed carnosine, D-carnosine, carcinine, *n*-acetylcarcinine) scavenging ROS in the glycation reaction, modifying the activity of enzymic and non-enzymic antioxidant defenses. The presented therapeutic strategies feature the latest universal antioxidant management strategies of diabetes and diabetic complications with transglycating (site-specific de-glycation) agents to help the establishment effectively and maximize the productivity of diabetes control. Presumably, these therapeutic peptide-based compounds are endowed with ability of controlling at transcriptional levels differential expression of genes encoding antioxidant enzymes inherent to DN in Type I Diabetic patients. The actives of the cited peptidomimetics are promising for creation of topical, systemic, and oral formulations of multiple-protective therapeutics for prevention or treatment of diabetic complications, including DN. Specifically, via their antioxidant, transglycating and chaperone-like activities, the included in the oral patented formulation of non-hydrolyzed carnosine compounds, synergistically control diabetic and microangiopathy complications with glucose lowering medication types, delay oxidation damage, downregulate inflammatory cytokines, and enhance anticoagulant activity in diabetic patients [122, 123]. The developed oral formulations of non-hydrolyzed carnosine or carcinine provide the effects of noninsulin antidiabetic drugs added to metformin therapy on glycemic control, weight gain, and hypoglycemia in type 2 diabetes. The cumulative data recently published support the multiple roles of the imidazole-containing therapeutic dipeptides as the potent protective agents for delaying the onset and progression of type 2 diabetes preventing the diabetic deterioration and complications development [122, 123].

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Conflict of interest The authors report no conflict of interest in this work. The authors bear primary responsibility for accuracy of made statements and employment of the described products and for the content and writing of the paper.

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