Endocrine (2014) 47:146–151
DOI 10.1007/s12020-013-0136-3

ORIGINAL ARTICLE

Serum prolidase enzyme activity and oxidative stress levels in patients with diabetic neuropathy

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Received: 18 August 2013 / Accepted: 22 November 2013 / Published online: 18 December 2013

Abstract Previous studies have suggested that prolidase and nitric oxide (NO) regulate many processes, such as collagen synthesis and matrix remodeling. Oxidative stress plays an important role in the development of microvascular complications in diabetic patients. Data on serum prolidase activity in patients with diabetes mellitus or diabetic neuropathy (DN) are limited and conflicting. The aim of this study was to measure serum prolidase activity, NO, total antioxidant status (TAS), and malondialdehyde (MDA) levels in patients with DN. Forty-five patients with DN and 40 healthy controls were enrolled. Serum prolidase activity, TAS, MDA, and NO levels were determined. Serum MDA and NO levels were significantly higher in DN patients than controls ($p = 0.002$, $p = 0.001$, respectively), while prolidase activity and TAS levels were lower ($p = 0.003$, $p = 0.001$, respectively). Prolidase activity was negatively correlated with NO and MDA ($r = -0.911$, $p < 0.001$; $r = -0.905$, $p < 0.001$, respectively), while positively correlated with TAS ($r = 0.981$, $p < 0.001$) in DN patients. The current study is the first showing the decreased serum prolidase enzyme activity. Our results suggest that decreased collagen turnover may occur in DN patients, who have increased oxidative stress and increased NO levels. Decreased prolidase activity seems to be associated with increased NO levels and oxidative stress along with decreased antioxidant levels in DN. Therefore, decreased prolidase activity may play a role in pathogenesis of DN. Prospective clinical studies are necessary to confirm these findings.

Keywords Diabetic neuropathy · Nitric oxide · Total antioxidant status · Malondialdehyde · Prolidase enzyme activity

Abbreviations

ROS	Reactive oxygen species
DM	Diabetes mellitus
ECM	Extracellular matrix
NO	Nitric oxide
NOS	Nitric oxide synthase
MMPs	Metalloproteinase
DN	Diabetic neuropathy
TAS	Total antioxidant status
MDA	Malondialdehyde
NO_2^-/NO_3^-	Nitrite/nitrate
HbA1c	Glycosylated hemoglobin

Introduction

Diabetes mellitus (DM) is a common metabolic disorder with a high prevalence worldwide and a disorder characterized by

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hyperglycemia, which is associated with non-enzymatic glycation, increased oxidative stress, changes in antioxidant enzyme activity, and carbonyl stress [1]. Previous studies assessed that there is a strong relation between antioxidant enzyme activities and poor glycemic control in diabetes, which likely accounts for diabetic complications [2, 3]. DM patients are at increased risk of developing microvascular complications, such as diabetic retinopathy, neuropathy, and nephropathy. Neuropathy is one of the long-term microvascular complications of DM which is associated with high morbidity [4, 5].

Collagen is a major extracellular matrix (ECM) component [6]. Increased ECM turnover may have a pathophysiologic role in the progression of collagen biosynthesis in atherosclerosis and endothelial dysfunction [7]. One of the enzymes involved in collagen biosynthesis is prolidase [E.C.3.4.13.9]. Prolidase is a manganese-requiring iminodipeptidase that releases carboxy-terminal proline or hydroxyproline from oligopeptides, which is used for collagen resynthesis and cell growth [8]. It plays an important role in recycling proline for collagen biosynthesis [9]. It seems that prolidase enzyme activity may be a step-limiting factor in the regulation of collagen biosynthesis [10]. Several studies suggested that prolidase activity is affected by oxidative stress [11, 12].

Nitric oxide (NO) is generated by the nitric oxide synthase (NOS) enzyme family. NO is expressed in a wide range of mammalian cells, including macrophages, hepatocytes, and endothelial cells [13]. Moreover, NO is a hypothesized potential regulatory effector for prolidase and may regulate metalloproteinase (MMPs) activity [14]. Although prolidase is a special type of MMPs, it may be regulated by NO, because it catalyzes the terminal step in matrix breakdown [14].

It has been suggested that high blood glucose levels may delay cell proliferation, decrease collagen production, and increase collagen breakdown [15–17]. To our knowledge, there are few studies regarding the relationships between diabetic neuropathy (DN) and collagen structure deterioration [16–18]. Previous studies have assessed prolidase activity in clinical conditions. While some authors reported increased prolidase activity in clinical conditions [19–21], others report a decrease [22–25]. However, there is limited and conflicted information regarding serum prolidase activity in patients with DM or DN [24, 26].

The aim of this study was to measure serum prolidase activity, NO, total antioxidant status (TAS), and malondialdehyde (MDA) levels in patients with DN.

Materials and methods

The prospective study was conducted in the Departments of Endocrinology and Neurology of Medical Faculty of Yuzuncu Yil University. Forty-five patients with diabetic neuropathy and 40 controls were enrolled in this study. DM diagnosis was made according to the revised American Diabetes Association criteria [27].

The mean duration of diabetes was 33 ± 21 years. The mean duration of DN was 21 ± 17 years. All of the patients were receiving dual oral antidiabetic drugs. The study groups included normotensive patients, without previous antihypertensive medication.

All patients with diabetic neuropathy had symptomatic symmetric distal neuropathy (i.e., hypoactive or abolic deep tendon reflexes, reduced tactile, pinprick, and/or position sensation) with at least moderate severity of one or more of the typical symptoms (pain, burning, paresthesia, numbness, or cramps) in the lower extremities.

The diagnosis of DN was made in accordance with the criteria recommended by the San Antonio Conference. Diagnosis of DN was made according to these criteria when patients had two or more symptoms on neurologic examination (pain, burning, paresthesia, numbness, and cramps) and electrophysiologic investigation (slowed nerve conduction velocity) [28].

Control subjects underwent routine physical and laboratory evaluations to insure that none had DM, symptoms or signs of neuropathy, metabolic or endocrine disorders with potential involvement of the peripheral nervous system, a history of lumbosacral pathology affecting the tested side, hyperlipidemia, hypertension, coronary artery disease, psychiatric, metabolic, hepatic, or renal disease. Smokers and individuals who took potentially neurotoxic drugs or supplemental vitamins were not used as controls.

The study protocol was carried out in accordance with the Helsinki Declaration as revised in 2000. All participants were informed about the study protocol, and written consent was obtained from each subject.

Exclusion criteria

Exclusion criteria included congestive heart failure, acute myocardial infarction, coexistent illness (i.e., infections), diabetic macroangiopathic complications (i.e., coronary artery disease, peripheral vascular disease), hematuria, smoking, excessive alcohol intake, B12 deficiency, use of antioxidant drugs such as beta-blocking agents (carvediol, nebivolol), statins, vasoactive agents, diuretics, and vitamins.

Nerve conduction study

Nerve conduction studies were performed with standard electromyograhy equipment (Medelec Synergy). Motor conduction studies examined the median (recorded at the abductor pollicis brevis), ulnar (recorded at the abductor

digiti quinti), peroneal (recorded at the extensor digitorum brevis), and tibial (recorded at the adductor hallucis) nerves, bilaterally. Sensory nerve conduction studies were performed on the bilateral median, ulnar, sural, and peroneal superficial nerves using antidromic or orthodromic measurements. The F waves of median, ulnar, peroneal, and posterior tibial were assessed. An electrophysiologic diagnosis of DN was made if there were two or more abnormalities in the nerve conduction study.

Sample preparation

Blood samples were obtained in the morning following 12 h of fasting. They were collected into empty tubes and immediately stored on ice at 4 °C. The serum was then separated from the cells by centrifugation at 3,000 rpm for 10 min. Serum samples for the measurement of prolidase activity, TAS, NO, and MDA levels were stored at −20 °C until they were used.

Measurement of total antioxidant status

Serum TAS was determined spectrophotometrically, using an automated measurement method, developed by Erel [29]. The results are expressed as mmol Trolox Equiv./L.

Measurement of serum lipid peroxidation

Serum MDA levels were determined spectrophotometrically, using the modified thiobarbituric acid-reactive substance method by Yoshioka et al. [30]. The results were expressed as nmol/ml.

Determination of prolidase activity

Serum prolidase activity was determined spectrophotometrically according to the method of Myara et al. [31], which is based on the measurement of proline using Chinard's reagent [32]. The results are expressed as U/L.

Measurement of serum nitric oxide

Serum nitrite/nitrate (NO_2^-/NO_3^-) concentrations were determined spectrophotometrically with the Griess reaction, as described by Tracey et al. [33]. The results are expressed as μmol/L.

Other parameters

Serum glucose and glycosylated hemoglobin (HbA1c) were measured with commercial kits (Abbott®) in an autoanalyzer (Aeroset®, Germany). The $HbA1_c$ normal range was 4.7–6.3 %.

Statistical analysis

The results are expressed as the mean ± standard deviation. Nonparametric continuous variables were compared using the Mann–Whitney *U* test. Parametric variables were compared using the Student's *t* test. Correlation analyses were performed with Pearson's correlation test. Differences were regarded as significant at $p < 0.05$. Data were analyzed using the SPSS® for Windows statistical software (Version 11.0).

Results

The demographic and clinical data from the DN and control groups are shown in Table 1. There were no statistically significant differences between DN patients and controls in age, sex, and body mass index (all, $p > 0.05$) (Table 1).

Serum glucose levels were significantly higher in DN than in controls ($p < 0.004$). HbA1c levels were significantly higher in DN than in controls ($p < 0.001$) (Table 1).

Serum MDA and NO levels were significantly higher in DN patients than in controls ($p = 0.002$, $p = 0.001$, respectively), while prolidase activity and TAS levels were lower ($p = 0.003$, $p = 0.001$, respectively) (Table 2).

Table 1 Demographic and clinical data in patients with diabetic neuropathy and healthy controls

Parameters	Controls ($n = 40$)	Patients ($n = 45$)	p
Age (year)	50 ± 11	51 ± 9	0.231
Sex (female/male)	28/12	26/19	0.243
Glucose (mg/dl)	83.34 ± 17.08	206.68 ± 70.46	0.004
Body mass index (kg/m^2)	22.40 ± 1.24	22.32 ± 2.21	0.268
HbA1c (%)	5.39 ± 0.38	8.48 ± 2.30	0.001

Values are mean ± SD

Table 2 Prolidase activity, nitric oxide, oxidative stress in patients with diabetic neuropathy and healthy controls

Parameters	Controls ($n = 40$)	Patients ($n = 45$)	p
TAS (mmol Trolox Equiv./L)	7.25 ± 0.25	4.32 ± 0.22	0.001
MDA (nmol/mL)	7.11 ± 2.19	21.97 ± 4.17	0.002
NO (μmol/L)	50.15 ± 3.66	69.99 ± 4.33	0.001
Prolidase (U/L)	58.51 ± 2.03	40.07 ± 0.88	0.003

Values are mean ± SD

TAS total antioxidant status, *NO* nitric oxide, *MDA* malondialdehyde

Serum prolidase activity was negatively correlated with NO and MDA levels ($r = -0.911, p < 0.001$; $r = -0.905$, $p < 0.001$, respectively), while positively correlated with TAS levels ($r = 0.981$, $p < 0.001$) in DN patients.

Sensory or motor nerve conduction function was abnormal in 25 of the 45 diabetic polyneuropathy patients.

When patients with DN divided according to detected and undetected diabetic polyneuropathy as electrophysiologic, no differences were observed between in respect to serum prolidase activity, MDA, NO, and TAS levels (all $p > 0.05$).

Both diabetes and DN duration were not correlated with prolidase activity, MDA, NO, and TAS values in patients ($p > 0.05$).

Discussion

In the present study, we examined associations among prolidase activity; NO, MDA, and TAS levels; and DN severity in patients with type 2 DM. We found that DN patients had significantly lower serum prolidase activity and TAS levels than healthy controls. We also observed that serum NO levels and MDA levels are significantly higher in DN patients than in controls. In the present study, we found negative correlations between prolidase activity, and NO and MDA levels. To the best of our knowledge, this is the first report investigating serum prolidase activity along with NO and oxidative stress levels in DN patients.

DM is a major cause of peripheral neuropathy, commonly manifested as distal symmetric polyneuropathy [36]. DN, a major microvascular complication of DM, comprises disorders of peripheral nerve in people with diabetes. The prevalence of neuropathy in diabetic patients is about 30 %, whereas up to 50 % of patients will certainly develop neuropathy during their disease [34]. DN develops on a background of hyperglycemia and an entangled metabolic imbalance, mainly oxidative stress. DN is associated with risk factors for macrovascular disease. Several authors have observed reduced nerve perfusion and endoneurial hypoxia in both humans and animal models [35].

Collagen, the most abundant protein in the body, constitutes more than a quarter of all proteins and is essential for connective tissue maintenance. Collagen biosynthesis may require prolidase activity to breakdown collagen and intracellular proteins [36]. It is also a major ECM component [6]. Increased ECM turnover plays a major role in collagen biosynthesis progression in atherosclerosis, endothelial dysfunction [7]. MMPs are one of the most important enzyme classes that participate in the catabolism of ECM proteins. Additionally, oxidative stress levels are directly related to the inhibition of collagen production, and prolidase is considered to be a target enzyme of this process [37]. Several studies have suggested that MMPs are associated with oxidative stress [38, 39].

Prolidase and its regulation by NO may be important in the regulation of collagen turnover. NO has been shown to play a role in regulating collagen metabolism, and high NO concentrations are associated with increased collagen biosynthesis and modification [40, 41]. It has been demonstrated that NO end products are significantly higher in the serum of diabetic patients with neuropathy. Diabetes and the metabolic changes in peripheral nerves contribute to decreased NO production and diminished nerve blood flow [42].

We also evaluated serum NO levels in the study groups. We observed that serum NO levels are significantly higher in DN patients compared to healthy controls. Our results showed that chronic high glucose concentration induced the increase of plasma NO level in DN patients. It was reported that simulated hyperglycemia resulted in a significant down-regulation of eNOS expression and NO production by cultured human coronary endothelial cells [43, 44]. However, several studies have demonstrated increased NO production in diabetes [45, 46].

Reactive oxygen species (ROS) are highly reactive molecules that are formed in oxidative processes. ROS are considered to have an important role in the development of microvascular complications in patients with DM [47]. Hyperglycemia in diabetes is associated with increased glycation, oxidative stress, and nitrosative stress. Poor glycemic control in type 2 DM has also been associated with the depletion of protective serum antioxidant activity [48]. Lipid peroxides are thought to be formed by free radicals and may play an important role in the development of vascular disease [49]. In DM, there is an increased production of free radicals, which, in turn, promotes lipid peroxidation. MDA is formed as an end product of lipid peroxidation [49]. Several studies have demonstrated that there is a close association between oxidative stress levels and DN in animal and human studies [50, 51].

Data on serum prolidase activity in patients with DM or DN are limited and conflicting [24, 26]. Erbagci et al. [24] studied prolidase activity in type 2 diabetic subjects with and without osteoporosis, as determined by bone mineral density. They reported decreased serum prolidase activity in patients with type 2 DM compared with healthy controls. Also, they remarked that this result may be interpreted as evidence of decreased bone resorption. On the other hand, there is only one report regarding serum prolidase activity in DN patients in the literature [26]. In that study, Uzar et al. [26] reported increased serum prolidase activity and oxidative stress levels in DN patients. Decreased antioxidant levels also were reported by the same researchers [26]. However, we found decreased prolidase activity in DN patients. Our data clearly demonstrate that collagen

turnover is decreased in DN patients, and, thus, disagree with the previous publication [26].

The measurement of TAS was a useful test for prediction of oxidative status [29]. It is well known that oxidative stress can be defined as an increase in oxidants and/or a decrease in antioxidant capacity, and various oxidants and antioxidants have additive effects on the oxidative status [52]. Little is known about report regarding serum TAS levels in DN patients in the literature [26]. In that study, Uzar et al. [26] reported decreased serum TAS levels in DN patients. In the present study, we assayed the oxidative status of the study population using the TAS, an indicator of oxidative stress, which reflects the redox balance between oxidation and antioxidation [29]. Moreover, Uzar et al. [26] reported increased serum total oxidant status levels and oxidative stress index values in DN patients. In the present study, we found that DN patients had significantly lower serum TAS levels than healthy controls.

Our study has several limitations: first, it is a cross-sectional design. However, it is only a preliminary study investigating collagen metabolism and oxidative stress in DN by measuring serum prolidase activity, NO, and oxidative stress. Second, in the present study, a new group which has DM only without any complications could not be investigated. Third, the number of DN patients was small, and these observations must be confirmed in a larger sample of patients.

The current study is the first showing the decreased serum prolidase enzyme activity. Our results suggest that decreased collagen turnover may occur in DN patients, who have increased oxidative stress and increased NO levels. Decreased prolidase activity seems to be associated with increased NO levels and oxidative stress along with decreased antioxidant levels in DN. Therefore, decreased prolidase activity may play a role in pathogenesis of DN. However, further prospective clinical studies are necessary to confirm the pathophysiologic role of serum prolidase activity in DN.

Conflict of interest The authors stated that there are no conflicts of interest regarding the publication of this article.

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