Published in final edited form as:

Exp Clin Endocrinol Diabetes. 2009 February ; 117(2): 72–77. doi:10.1055/s-2008-1078710.

Nitrosylated Proteins in Monocytes as a new Marker of Oxidative-Nitrosative Stress in Diabetic Subjects with Macroangiopathy

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Abstract

Background—Peroxynitrite plays an important role in the pathogenesis of diabetic complications. Nitrosylated protein expression in peripheral blood monocytes reflects intracellular peroxynitrite injury, and thus could be a marker of higher diagnostic and prognostic value than plasma nitrotyrosine level. The purpose of this pilot study was to assess if peripheral blood monocytes of diabetic subjects accumulate nitrosylated proteins, and if nitrosylated protein expression correlates with blood glucose control, variables of lipid profile, C-reactive protein concentration (a marker of inflammation), and differs in patients with and without diabetic macrovascular and microvascular complications.

Methods—Nitrosylated protein expression in peripheral blood monocytes (Western blot analysis) was assessed in 31 subjects with diabetes mellitus (29 Type 2, 2 Type 1; 20 males, 11 females; mean age 66 years). The presence of microangiopathy was defined by retinopathy, albumin excretion, and/or neuropathy, and macroangiopathy by carotid plaques, a history of myocardial infarction, and/or stroke.

Results—Diabetic subjects accumulated significant amounts of nitrosylated proteins in peripheral blood monocytes. Nitrosylated protein expression positively correlated with body weight, blood glucose, HbA_{1c}, and plasma C-reactive protein concentrations in the whole cohort as well as in subjects with diabetic macroangiopathy.

Conclusions—Monocyte nitrosylated protein expression is a new biomarker of metabolic control and inflammation in diabetic subjects with macroangiopathy. A more detailed assessment of diabetic microvascular complications in a larger group of patients is needed to determine if this variable can be employed as a biomarker of the presence, severity, and progression of diabetic neuropathy, retinopathy, and nephropathy.

Keywords

nitrosylated proteins; diabetes mellitus; plasma glucose; HbA_{1c}; lipids

Introduction

Growing evidence suggests that enhanced oxidative-nitrosative stress, and, in particular, increased production of the potent oxidant peroxynitrite (a product of superoxide anion radicals with nitric oxide) is a characteristic feature of both experimental and clinical diabetes mellitus [1]. Peroxynitrite causes numerous cytotoxic effects i.e., 1) protein nitration and nitrosylation; 2) DNA single-strand breakage and base modification; 3) activation of poly(ADP-ribose) polymerase with resultant changes in transcriptional regulation and gene expression; 4) changes in cell signaling; 5) mitochondrial dysfunction; and, in extreme cases, 6) induction of necrosis and apoptosis [1]. Accumulation of nitrotyrosine, a footprint of peroxynitrite-induced injury, has been identified in vascular endothelium [1–3], myocardium [1,2,4], retina [5,6], kidney [7], peripheral nerve [8–14], spinal cord [13], dorsal root ganglion neurons [9–14] and *vasa nervorum* [14,15] of streptozotocin-diabetic rats and mice, Zucker fatty and Zucker diabetic fatty rats, as well as leptin-deficient (ob/ob) and high fat diet-fed mice. These findings suggest the presence of peroxynitrite cytotoxicity at both early and advanced stages of Type 1 and Type 2 diabetes, and, furthermore, at the prediabetic stage. Enhanced nitrosative stress has also been documented in circulation [16–18], cutaneous microvasculature [19], myocardium [20], and kidney [21] of human subjects with diabetes mellitus.

Recent studies suggest an important role of peroxynitrite in the pathogenesis of diabetic vascular dysfunction [1–3], peripheral [8,10–12,14] and autonomic [22], and retinopathy [23]. A pharmacological approach with a new class of agents, peroxynitrite decomposition catalysts [1–3,8,10–12,14], revealed an association between intracellular nitrotyrosine accumulation and development of diabetic complications. These new findings provide rationale for evaluation of intracellular nitrotyrosine abundance in easily accessible biological materials (e.g., peripheral blood monocytes, skin) of human subjects with diabetes mellitus as a potential new biochemical biomarker of the presence, severity, and the rates and development and progression of chronic diabetic complications. The purpose of this pilot study was to assess if peripheral blood monocytes of diabetic subjects accumulate nitrosylated proteins, and if nitrosylated protein expression correlates with blood glucose control, variables of lipid profile, C-reactive protein concentration (a marker of inflammation), and differs in patients with and without diabetic macrovascular and microvascular complications.

Materials and Methods

The study was performed in accordance with the principles of the Declaration of Helsinki and was reviewed and approved by the Dresden University Hospital institutional Review Board. Patients gave written informed consent before any study-related procedure.

Human subjects

The study was performed in 31 patients with diabetes mellitus (20 males, 11 females, mean age 66 years). 29 patients had noninsulin-dependent (Type 2) and 2 patients insulin-dependent (Type 1) diabetes. The presence of microangiopathy was defined by evidence of retinopathy (examination by an ophthalmologist), and/or nephropathy (albuminuria plus ≥ 2.6 mg/mmol creatinine in 24-h urine) or neuropathy (the vibration threshold $< 6/8$ in a tuning fork test). The presence of macroangiopathy was defined by plaques in the carotids (ultrasound investigations), a history of myocardial infarction, and/or stroke. Body weights and systolic and diastolic blood pressure were registered upon enrollment. Blood samples were obtained by standard venipuncture techniques in a fasting state and used for determination HbA1c, plasma glucose, lipid profile including serum total cholesterol, HDL,

LDL, and triglycerides, and plasma C-reactive protein by routine clinical chemistry procedures, as well as for isolation of peripheral blood monocytes.

Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality, and were purchased from Sigma Chemical Co., St. Louis, MO. Mouse monoclonal anti-nitrotyrosine antibody was purchased from Upstate, Lake Placid, NY. Secondary horse antimouse peroxidase-labelled antibody was purchased from Vector Laboratories, Burlingame, CA.

Peripheral blood monocyte isolation and assessment of nitrosylated proteins by Western blot analysis (Fig. 1)

Monocytes have been isolated according to a protocol obtained from Dr. George L. King laboratory. 8ml of blood have been collected in a Vacutainer cell preparation tube containing a polymer that acts as a density gradient to separate mononuclear cells from the whole blood (Becton, Dickinson, and Company), with sodium citrate. The samples have been centrifuged at 1500–1800 RCF for 30 min, to separate monocytes from plasma and red blood cells. The monocytes sediment in a layer just above the polymer matrix. Then the upper layers have been drawn off, and monocytes collected into a 15ml centrifuge tube. PBS has been added to fill the tube, and the monocyte samples washed by centrifugation at 1500–1800 g for 15 min. Supernatants have been discarded, and monocyte pellets washed with PBS two more times. After this, monocyte pellets have been frozen and shipped to Pennington Biomedical Research Center for nitrotyrosylated protein analysis. Monocyte samples have been transferred to an extraction buffer (1–2/5 v/v) containing 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 % Triton X-100; 5 mM EDTA; 50 mM NaF; and the protease/phosphatase inhibitors leupeptin (10 µg/ml), aprotinin (20 µg/ml), benzamidine (10 mM), phenylmethylsulfonyl fluoride (1 mM), sodium ortho-vanadate (1mM), and homogenized on ice. The homogenates have been sonicated (3 × 5 s) and centrifuged at 14 000 g for 20 min. All the aforementioned steps have been performed at 4 ° C. The lysates (40–100 µg protein) have been mixed with equal volume of 2× sample-loading buffer containing 62.5 mM Tris-HCl, pH 6.8; 2 % sodium dodecyl sulfate; 5 % β-mercaptoethanol; 10 % glycerol and 0.025 % bromophenol blue, and fractionated in 5–17 % SDS-PAGE in an electrophoresis cell (Mini-Protein III; Bio-Rad Laboratories, Richmond, CA). Electrophoresis has been conducted as described [24]. Gel contents have been electrotransferred (250 mA, 2 h) to nitrocellulose membranes using Mini Trans-Blot cell (Bio-Rad Laboratories, Richmond, CA) and western transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; and 20 % (v/v) methanol). Free binding sites have been blocked in 5 % (w/v) nonfat dry milk in 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05 % Tween 20, for 1 h, after which antinitrotyrosine antibody has been applied for 2 h. Then affinity purified secondary antibodies conjugated to horseradish peroxidase have been applied for 1 h. After extensive washing, protein bands detected by the antibodies have been visualized with the BM Chemiluminescence Blotting Substrate (POD) (Roche, Indianapolis, IN). Nitrosylated protein expression has been quantified by densitometry (Quantity One 4.5.0 software, Bio-Rad Laboratories, Richmond, CA). Membranes have then been stripped in the 62.5 mM Tris-HCl, pH 6.7 buffer containing 2 % SDS and 100 mM β-mercaptoethanol, and reprobed with β-actin antibody. Nitrosylated protein expression data have been normalized for β-actin and quantified using a commercial nitrosylated protein standard (individual quantitation for each Western blot). The data have been expressed in µg nitrosylated proteins per relative unit (RU) of monocyte β-actin.

Statistical analysis

The results are expressed as means ± standard deviations. Individual between-group comparisons have been made using the unpaired two-tailed Student's t-test or Mann-

Whitney rank sum test where appropriate. Significance was defined at $p < 0.05$. Correlation analyses have been performed according to Pearson.

Results

Baseline characteristics

Baseline characteristics of diabetic subjects enrolled in the present study are given in Table 1.

Nitrosylated protein expression in peripheral blood monocytes and its correlation with variables of metabolic control

Diabetic subjects accumulated significant amounts of nitrosylated proteins in peripheral blood monocytes (Fig. 2). Nitrosylated protein expression displayed a large (~50-fold) variability among individual diabetic subjects (minimum: 0.02, maximum: 1.0 μg nitrosylated proteins $\times \text{ml}^{-1}$ of peripheral blood monocytes). Nitrosylated protein expression positively correlated with body weights ($r = 0.372$, $p < 0.039$, Fig. 2) in the whole cohort. It also positively correlated with glycemia i.e., HbA_{1c} concentration ($r = 0.479$, $p < 0.006$, Fig. 3) and plasma glucose concentration ($r = 0.480$, $p < 0.006$, Fig. 4), as well as with the inflammatory marker, C-reactive protein, concentration ($r = 0.428$, $p < 0.016$, Fig. 5). Further analysis identified similar correlations between nitrosylated protein expression and the afore-mentioned variables in diabetic subjects with macro-, but not those with microangiopathy (Table 2). Note, that after exclusion of two outliers with the values of 0.82 and 1.0 μg nitrosylated proteins per RU of monocyte β -actin, correlations between monocyte nitrotyrosylated protein content and body weight and blood glucose concentrations in either total cohort or diabetic subjects with macroangiopathy disappeared, whereas correlations between nitrotyrosine levels and HbA_{1c} and C-reactive protein concentrations were not affected. Nitrosylated protein expression did not correlate with age, duration of diabetes, or blood pressure in either whole cohort, or diabetic subjects with macro- or microangiopathy.

Nitrosylated protein expression in peripheral blood monocytes and the presence of diabetic macro- or microangiopathy

Nitrosylated protein expression did not differ in diabetic subjects with and without macroangiopathy (0.27 ± 0.32 and 0.16 ± 0.13 μg nitrosylated proteins per RU monocyte β -actin, respectively, $p > 0.05$). In a similar fashion, nitrosylated protein expression did not differ in diabetic subjects with and without microangiopathy (0.12 ± 0.08 and 0.25 ± 0.27 μg nitrosylated proteins per RU monocyte β -actin, respectively, $p > 0.05$).

Discussion

It has previously been reported that plasma nitrotyrosine concentrations are increased in subjects both Type 1 and Type 2 diabetes compared with non-diabetic controls [16–18]. In Type 1 diabetic subjects, plasma nitrotyrosine concentrations correlated with glycemic control and endothelial dysfunction [25]. In type 2 diabetic subjects, plasma nitrotyrosine concentrations displayed correlations with plasma glucose concentrations, but not with total plasma antioxidant capacity or glycosylated haemoglobin concentration [16]. Interestingly, production of nitrotyrosine and its levels in circulation directly correlated with postprandial hyperglycemia [17]. Postprandial nitrotyrosine accumulation in circulation has been counteracted by a number of pharmacological agents including the rapid and short-acting insulin secretagogue mitiglinide [26], the amylin substitute pramlintide [27], as well as a statin and angiotensin II receptor blocker therapy [28].

In contrast to circulatory nitrotyrosine content which is significantly increased after a food intake [17], fluctuates with changes in dietary status [29], and is easily amenable to correction [29], intracellular nitrotyrosine content is rather difficult to change. In a recent experimental study, institution of the six month-long period of good glycemic glucose control after the six month-long period of poor glycemic control did not result in a significant reduction of retinal nitrotyrosine concentrations or the number of nitrotyrosine-positive retinal capillary cells in streptozotocin-diabetic rats [6]. Furthermore, the inability to correct retinal nitrotyrosine concentration was associated with the failure to prevent formation of acellular capillaries [6], and, in contrast, an antioxidant treatment that corrected retinal nitrosative stress, prevented diabetes-induced background retinopathy [30]. The latter indicates that intracellular nitrosylated proteins accumulating in tissue-sites for diabetic complications are relatively stable, and participate in a metabolic memory, a phenomenon playing an important role in development and progression of diabetic complications [6,31]. These findings, together with increasing experimental evidence for the important role of nitrosative stress in diabetic endothelial and myocardial dysfunction [1–3], peripheral and autonomic neuropathy [8,10,11,13,14,22], and retinopathy [23], suggest that intracellular nitrosylated protein abundance in easily accessible biological materials e.g., peripheral blood monocytes, may appear a reliable predictor of severity and progression of diabetic complications.

In the present study, diabetic subjects accumulated identifiable and highly variable amounts of intracellular nitrosylated proteins in peripheral blood monocytes. Monocyte nitrosylated protein expression correlated with glycemic control, consistent with other experimental [6] and clinical [16–18] reports. Furthermore, identification of a positive correlation between monocyte nitrosylated protein abundance and C-reactive protein concentration is also in line with a recent study demonstrating 1) an important role of the ubiquitin-proteasome system overactivity in the inflammatory process, and 2) increased monocyte ubiquitin, p50, and p65 levels and superoxide generation, as well as higher proteasome 20S levels, in human subjects with Type 2 diabetes [32]. Of interest, correlations between nitrotyrosylated protein expression and glycemia as well as C-reactive protein concentrations are present in a subgroup of diabetic subjects with macro-, but not microangiopathy. Recently, it has been demonstrated that both nitrotyrosine and superoxide production and inflammatory response are increased in carotid plaques of subjects with Type 2 diabetes and atherosclerosis compared with the group with atherosclerosis only [32]. These data, in combination with the findings of the present study, suggest that nitrosylated protein expression in peripheral blood monocytes, a new marker of oxidative stress and a correlate of inflammatory response, may be used to predict severity and progression of diabetic macroangiopathy. Note, that there was a trend towards an increased nitrosylated protein expression in diabetic subjects with macroangiopathy, compared to those without this complication. A larger study may be needed for identification of statistically significant difference between the two groups.

Recent experimental studies have demonstrated the role for nitrosative stress in diabetic microvascular complications (reviewed in [1]). Clinical studies revealed direct correlations between increased plasma nitrotyrosine content and endothelial dysfunction [25,28] as well as redistribution of sudomotor responses, an early sign of sympathetic nerve dysfunction, in patients with Type 1 diabetes [33]. Furthermore, a production of NO, a precursor of peroxynitrite, was drastically reduced by the peroxisome proliferator-activated receptor gamma agonist, pioglitazone, an agent that markedly improved endothelium-dependent vasodilatation in subjects with Type 2 diabetes [34]. Whereas the lack of any differences between monocyte nitrosylated protein abundance in diabetic subjects with and without microangiopathy was disappointing, it should be taken into consideration that the present pilot study was performed in a small (n = 31) group of diabetic subjects and did not include any detailed assessment of diabetic microvascular complications. In a recent neuropathy

study [35], that included 61 Type 1 and 124 Type 2 diabetic patients and a comprehensive assessment of diabetic neuropathy, plasma antioxidant capacity for peroxynitrite (assessed by the pholasin test) correlated with the neurological impairment score of the lower limbs (NIS-LL) in Type 1 diabetic patients only. Note, however, that another study from the same group, that included 62 diabetic patients without peripheral diabetic neuropathy and without cardiac autonomic neuropathy, 105 diabetic subjects with peripheral diabetic neuropathy but without cardiac autonomic neuropathy, and 22 diabetic subjects with both peripheral and cardiac autonomic neuropathies, did not reveal any correlations between plasma antioxidant capacity for peroxynitrite and a number of individual measures of nerve function in patients with both types of diabetes, and the authors had to perform the multiple linear regression analysis to identify the lag time for peroxynitrite disappearance from circulation as an independent factor associated with NIS-LL [36]. In addition, several clinical trials suggest that pathogenetic therapies improve some measures of peripheral nerve function, without having a statistically significant effect on others [37–41]. In particular, a 52-week multicenter placebo-controlled double-blind parallel group study of the aldose reductase inhibitor fidarestat [37] in 279 patients with diabetic neuropathy, demonstrated beneficial effect of treatment on five out of six measures of motor function, whereas improvement of sensory function was not significant. Apparently, a larger study with a detailed characterization of diabetic neuropathy, rather than measurements of a single variable i.e., a vibration perception threshold, is needed to evaluate monocyte nitrosylated protein expression as a potential marker of the presence and severity of the disease. In a similar fashion, a more detailed characterization of other complications is needed to evaluate if monocyte nitrosylated protein expression can be used as a marker of the presence, severity and progression of diabetic retinopathy and kidney disease.

In conclusion, the present pilot study has demonstrated that diabetic subjects accumulate different amounts of nitrosylated proteins in peripheral blood monocytes and that monocyte nitrosylated protein abundance correlates with body weights and indices of metabolic control and inflammation in the whole cohort and subjects with macro-, but not microangiopathy. A more detailed assessment of diabetic microvascular complications in a larger groups of patients is needed to determine if this variable can be employed as a biomarker of the presence, severity, and progression of diabetic neuropathy, retinopathy, and nephropathy in future studies and clinical trials.

Acknowledgments

The study was supported in part by the American Diabetes Association Research Grant 7-05-RA-102 and the National Institutes of Health Grant DK 071566-01 (both to I.G.O.).

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Fig. 1. Representative Western blot analyses of nitrosylated protein expression in peripheral blood monocytes of individual diabetic subjects.

Fig. 2.

Correlation between body weights and nitrosylated protein abundance (μg nitrosylated proteins per RU monocyte β -actin) in the whole cohort of diabetic subjects. r -correlation coefficient.

Fig. 3.
Correlation between HbA_{1c} and nitrosylated protein abundance (μg nitrosylated proteins per RU monocyte β-actin) in the whole cohort of diabetic subjects. r—correlation coefficient.

Fig. 4. Correlation between blood glucose and nitrosylated protein abundance (μg nitrosylated proteins per RU monocyte β -actin) in the whole cohort of diabetic subjects. r -correlation coefficient.

Fig. 5. Correlation between C-reactive protein and nitrosylated protein abundance (μg nitrosylated proteins per RU monocyte β -actin) in the whole cohort of diabetic subjects. r -correlation coefficient.

Table 1

Baseline data

Variable	Mean $\pm$ SD
body weight (kg)	85.8 $\pm$ 12.8
HbA1C (%)	6.8 $\pm$ 0.81
plasma glucose (mmol/L)	8.22 $\pm$ 2.07
BP syst (mm Hg)	136.8 $\pm$ 8.2
BP diast (mm Hg)	82.6 $\pm$ 6.2
total cholesterol (mmol/L)	4.8 $\pm$ 1.2
triglycerides (mmol/L)	3.1 $\pm$ 4.1
HDL cholesterol (mmol/L)	1.24 $\pm$ 0.54
LDL cholesterol (mmol/L)	2.40 $\pm$ 0.80
C-reactive protein (mg/L)	4.40 $\pm$ 4.17

BP blood pressure

Table 2

Coefficients of correlations between nitrosylated protein abundance and metabolic variables in the subcohorts of diabetic subjects with and without macroangiopathy

Variable	With macro angiopathy (n 14)	Without macro-angiopathy (n 17)
body weight (kg)	$r = 0.609$; $p = 0.021$ *	$r = 0.135$; $p > 0.05$
HbA1C (%)	$r = 0.659$; $p = 0.010$ *	$r = 0.239$; p n.s.
blood glucose (mmol/L)	$r = 0.741$; $p = 0.002$ *	$r = 0.048$; p n.s.
C-reactive protein (mg/L)	$r = 0.581$; $p = 0.029$ *	$r = 0.156$; p n.s.

r – correlation coefficient.

* $p < 0.05$