Oxidative Stress and Antioxidant Defense in Relation to the Severity of Diabetic Polyneuropathy and Cardiovascular Autonomic Neuropathy

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OBJECTIVE — Oxidative stress resulting from enhanced free-radical formation and/or a defect in antioxidant defenses has been implicated in the pathogenesis of experimental diabetic neuropathy. The objective of this study was to evaluate plasma levels of various biomarkers of oxidative stress in diabetic subjects in relation to the presence or absence of polyneuropathy (PN) and/or cardiovascular autonomic neuropathy (CAN).

RESEARCH DESIGN AND METHODS — Plasma 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), superoxide anion ($O_2^{\cdot -}$) generation, lag phase to peroxidation by peroxynitrite ($ONOO^-$), vitamin E–to–lipid ratio, and vitamin C were measured in nonsmoking diabetic patients without PN and CAN (PN^-/CAN^- group; $n = 62$), in a group with PN but without CAN (PN^+/CAN^- group; $n = 105$), in those with both PN and CAN (PN^+/CAN^+ group; $n = 22$), and in healthy control subjects ($n = 85$).

RESULTS — All three markers of oxidative stress were significantly increased, and both markers of antioxidant defense were decreased in the PN^+/CAN^- group compared with the control group (all $P < 0.05$). PN^-/CAN^- subjects showed a significant increase compared with control subjects for 8-iso-$PGF_{2\alpha}$, $O_2^{\cdot -}$, and $ONOO^-$ and a decrease for the vitamin E–to–lipid ratio (all $P < 0.05$). In the PN^+/CAN^- group, a significant increase compared with the PN^-/CAN^- group was noted for $O_2^{\cdot -}$, whereas the vitamin E–to–lipid ratio and vitamin C were reduced (all $P < 0.05$). No significant differences were noted between the PN^+/CAN^- and PN^+/CAN^+ groups for each of the five markers of oxidative stress. In multivariate models, $O_2^{\cdot -}$ and $ONOO^-$ were independently associated with neuropathic deficits, but diabetes duration and triglyceride levels were also independent determinants.

CONCLUSIONS — Oxidative stress is enhanced in diabetic patients before the development of PN but to an even higher degree in those with PN, without further significant increase in relation to superimposed autonomic neuropathy. However, apart from oxidative stress, diabetes duration and triglyceride levels are also related to the severity of PN.

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Abbreviations: CAN, cardiovascular autonomic neuropathy; MNCV, motor nerve conduction velocity; NIS-LL, Neuropathy Impairment Score of the Lower Limbs; $O_2^{\cdot -}$, superoxide anion; $ONOO^-$, peroxynitrite; PGF, prostaglandin F; PN, polyneuropathy; SIN-1, 3-morpholino-sydnonimine HCl; TPT, thermal perception threshold; VPT, vibration perception threshold.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Diabetic polyneuropathy (PN) affects ~30% of the hospital-based population and 20% of community-based samples of diabetic patients. The most important etiologic factors are poor glycemic control, diabetes duration, and height, with possible roles for hypertension, age, smoking, hypoinsulinemia, and dyslipidemia (1). Hyperglycemia can induce oxidative stress via glucose autooxidation and the subsequent formation of advanced glycation end products, disruption of the polyol pathway, altered eicosanoid metabolism, and decreased antioxidant defenses (2,3). Enhanced oxidative stress in turn activates the nuclear redox-sensitive transcription factor κB, which upregulates genes such as cytokines, adhesion molecules, endothelium-1, and procoagulant tissue factor (4). These events may underlie the development of the chronic diabetes complications.

There is a growing body of evidence to support the notion that oxidative stress is the biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats. It has been shown (5) that Cu-prozinc superoxide dismutase activity is decreased in sciatic nerves from streptozotocin-induced diabetic rats. Glutathione content and glutathione peroxidase activity are also diminished in sciatic nerves from diabetic rats (6,7). Nerves of diabetic rats show lower amounts of vitamin E compared to control animals (8). Lipid peroxidation products such as malondialdehydes or conjugated dienes are elevated in diabetic sciatic nerves (5,8). Treatment of diabetic rats with insulin or antioxidants is associated with improved nerve function (9,10).

Because there is no information from clinical studies whether there is enhanced oxidative stress in diabetic patients with PN or autonomic neuropathy, we hypothesized that if oxidative stress is relevant in the pathogenesis of diabetic neuropathies, increased levels of its various mark-

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ers in plasma, including impaired antioxidant capacity, might be detected in patients with various manifestations and severities of these diabetic neuropathic complications.

RESEARCH DESIGN AND METHODS

— The diabetic subjects were recruited from the German Diabetes Clinic of the German Diabetes Research Institute at the Heinrich Heine University, Düsseldorf. Inclusion criteria were type 1 or type 2 diabetes according to the World Health Organization/American Diabetes Association criteria and age >18 years. Informed consent was obtained from all subjects eligible to participate in the study after the procedures involved were fully explained. Exclusion criteria were: *1*) neuropathy other than that of diabetic origin; *2*) smokers or ex-smokers <1 year; *3*) use of antioxidants (vitamin C, vitamin E, α-lipoic acid, β-carotene, probucol, carvedilol, and iron chelators) or prooxidants (primaquine and iron) within the last 3 months; *4*) peripheral arterial disease (intermittent claudication or nonpalpable foot pulses); *5*) history of coronary heart disease, myocardial infarction, heart failure (New York Heart Association class III–IV), or cardiac pacemaker; *6*) any medication that may adversely influence autonomic function; *7*) neurological diseases (e.g., Parkinson's disease, multiple sclerosis), and *8*) blood glucose levels >400 mg/dl and/or ketonuria.

Healthy volunteers ($n = 85$) were recruited among the staff of the German Diabetes Research Institute and from an ophthalmologist's practice in Langenfeld, Germany. None of the control subjects were diabetic, smokers, on any special diet, taking antioxidants/prooxidants, and/or taking medication related to any chronic disease at least 6 months before taking part in this study. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Assessment of peripheral nerve function

Electrophysiological tests, thermal discrimination, and vibration perception thresholds (VPTs) were performed as previously described (11). Motor nerve conduction velocity (MNCV) was measured in the median and peroneal nerves, whereas sensory nerve conduction velocity was determined in the median and sural nerves, at a skin temperature of 33–34°C, using surface electrodes (Sapphire; Medelec, Woking, U.K.). Quantitative sensory testing was evaluated by VPT at the second metacarpal bone and medial malleolus using the method of limits (Vibrameter; Somedic, Stockholm, Sweden) and by thermal perception thresholds (TPTs) including warm and cold thresholds at the thenar eminence and dorsum of the foot using the method of limits (Path-Tester; Tönnies, Germany). Neurological examination was performed using the Neuropathy Impairment Score of the Lower Limbs (NIS-LL) (12) and the Neuropathy Symptom Score (13). Criteria for diagnosis of diabetic distal symmetric PN were similar to those proposed by Dyck et al. (12), including slowing in peroneal MNCV and/or sural sensory nerve conduction velocity, elevated malleolar VPT, increased warm and/or cold TPT on the foot, NIS-LL ≥2, and Neuropathy Symptom Score ≥3.

Cardiovascular autonomic function tests

Autonomic reflex tests based on heart rate variability were performed using a NeuroDiag II computer system (Dr. Vetter, Baden-Baden, Germany) as previously described (14). The systolic blood pressure response to standing was performed using a Dynamap 1846 SX monitoring system (Critikon, Norderstedt, Germany). We have previously suggested the following seven indexes to be included in the test battery: the coefficient of the R-R interval variation at rest; spectral power in the low- and mid-frequency bands; the mean circular resultant of vector analysis during deep breathing; the maximum/minimum 30:15 ratio to standing up; the Valsalva ratio; and the postural change in systolic blood pressure. Definite cardiovascular autonomic neuropathy (CAN) was defined as three or more abnormalities among these seven indexes (15).

Retinopathy assessment

Color retinal photographs were taken after pupillary dilatation using a CR3-45NM nonmydriatic retinal camera (Canon, Tokyo, Japan) and were judged by an experienced examiner.

Nephropathy assessment

Urinary albumin excretion rate was determined from 12-h overnight samples collected on 3 consecutive days using the immunonephelometric technique (Array Protein System; Beckman, Fullerton, CA). Diabetic nephropathy was defined as a median urinary albumin excretion rate of ≥20 μg/min computed from the three samples.

Laboratory measurements

Glycemic control. HbA_{1c} was measured using the high-performance liquid chromatography technique (Diamat; BioRad, Munich, Germany). The normal range for our laboratory is 4.2–6.2%. Capillary blood glucose was measured by a hexokinase-based method (Boehringer, Mannheim, Germany). Mean blood glucose was computed from at least five values obtained over 24 h.

Blood collection and plasma preparation. After a 14-h fast, blood was collected by venipuncture into 10-ml sampling vials containing NH_4 and 2.7-ml vials containing EDTA (final concentration of 0.1%). Plasma was obtained by centrifugation at 1,500*g* at room temperature for 10 min. Samples of plasma from EDTA vials were immediately stored at −85°C for subsequent analysis (vitamins and antioxidant capacity). For F_2-isoprostane analysis, aliquots (1 ml) of plasma from vials containing NH_4 were combined with 10 μl of chain-breaking antioxidant butylated hydroxytoluene at a final concentration of 25 μmol/l and stored at −85°C until analysis.

Reagents. Butylated hydroxytoluene, α-tocopherol, γ-tocopherol, ascorbic acid, ascorbic oxidase, *o*-phenylenediamine, sodium acetate, *N,O-bis*(trimethylsilyl)trifluoro-acetamide, pentafluorobenzylbromide, and diisopropylethylamine were purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Prostaglandin F $(PGF)_2$ standards were obtained from SPI Bio (Massy, Cedex, France). Aminopropyl (NH_2) cartridges were from Waters (Milford, MA). Pholasin, adjuvant K, and 3-morpholinosydnonimine HCl (SIN-1) were kindly provided by Dr. Jan Knight (Knight Scientific, Plymouth, U.K.). All other general-purpose chemicals and organic solvents were of analytical grade (BDH, Poole, U.K., or Sigma, Poole, U.K.).

Markers of oxidative stress

8-iso-$PGF_{2\alpha}$ analysis. 8-iso-$PGF_{2\alpha}$ was analyzed as previously described using the following procedures (16,17): alka-

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line hydrolysis, total lipid extraction, NH_2-chromatography, pentafluorobenzyl derivatization, trimethylsilyl ether derivatization, and gas chromatography–mass spectrometry/negative-ion chemical ionization on a Hewlett Packard 5890 GC linked to a VG70SEQ (ThermoFinnigan, Herts, U.K.).

Vitamin E analysis. α-Tocopherol was measured by high-performance liquid chromatography using a Gilson pump model 305 coupled with a Gilson Fluorimeter model 121 (Gilson, Middleton, WI). Tocopherols were separated using acetonitrile/water (80/20 [vol/vol]) at a flow rate of 0.7 ml/min and the signal monitored (λ_{EX295} and λ_{EX340} nm) (18). The vitamin E–to–lipid ratio was defined as vitamin E divided by the sum of cholesterol and triglyceride levels.

Vitamin C analysis. Plasma samples (400 μl) were mixed with 200 μl metaphosphoric acid and proteins removed by centrifugation at 12,000*g* for 5 min. Aliquots (50 μl) of deproteinized plasma were transferred into microcentrifuge tubes (1.5 ml) and 930 μl of sodium acetate buffer. A standard curve was constructed by incubating increasing concentrations of ascorbic acid (0, 10, 20, 30, 40, 50, 60, and 100 μmol/l) with ascorbate oxidase (10 μl) and *o*-diphenylenediamine (10 μl). The concentrations of total ascorbate were obtained by intrapolating the fluorometric reading from the standard curve (19).

Assessment of plasma antioxidant capacity. *Peroxynitrite*. One hundred microliters of phosphate buffer (50 mmol/l; pH 7.4) containing pholasin (1.7 μg/ml) were pipetted into a microcuvette, and plasma or buffer for control (5 μl) was added. The reaction was initiated by adding SIN-1 (2 μl of 2 mg/ml in water) and the light emission measured continuously at 5-min intervals until the maximum reading was obtained. The signal was recorded on a 1250 LKB luminometer (LKB Wallace, San Francisco, CA). Antioxidant capacity was expressed as the time at which maximum light was emitted.

Superoxide anion. One-hundred microliters of phosphate buffer (50 mmol/l; pH 7.4) containing pholasin (0.5 μg/ml) and adjuvant K (50 μl/ml) were pipetted into a microcuvette, and plasma or buffer for control (5 μl) was added. Five microliters of xanthine (50 mmol/l) were then added and the reaction initiated by the addition of 20 μl of xanthine oxidase (0.5 units/ml phosphate buffer). The light signal was recorded over a period of 5 min on a 1250 LKB luminometer. Antioxidant capacity was expressed as the counts (millivolts) at maximum light emission.

Statistical analysis

Continuous data were expressed by the arithmetical mean ± SE. Differences between groups were analyzed using the *t* test for two independent samples or the Mann-Whitney *U* test. Qualitative data were given as absolute or relative frequencies, which were analyzed by the Fisher's exact test. Linear regression analysis was used to study associations between variables. Multiple linear regression analysis using forward stepping, with NIS-LL as the dependent variable, was performed to examine whether the markers of oxidative stress are independently associated with the severity of PN as assessed by the NIS-LL. Independent variables in the original model included age, sex, BMI, diabetes type, diabetes duration, height, HbA_{1c}, albuminuria, triglycerides, HDL and LDL cholesterol, retinopathy, and hypertension. Each of the five markers of oxidative stress was added to the original model in five separate models. The level of significance was set at $\alpha = 0.05$.

RESULTS — According to the aforementioned definitions, 62 patients had no evidence of either PN or CAN (PN^-/CAN^- group), 105 patients had PN but no CAN (PN^+/CAN^- group), and 22 patients had both PN and CAN (PN^+/CAN^+ group). The demographic, clinical, and laboratory data of the diabetic groups and healthy control group ($n = 85$) are shown in Table 1. The PN^-/CAN^- subjects were significantly younger than those in the other diabetic groups, whereas the control subjects were younger than the PN^+/CAN^- patients ($P < 0.05$). BMI was significantly higher in the PN^-/CAN^- and PN^+/CAN^- groups compared with the control group ($P < 0.05$). In the PN^+/CAN^- group, a higher percentage of men was noted when compared with the PN^-/CAN^- group ($P < 0.05$). The percentage of type 1 diabetic patients was lower in the PN^+/CAN^- than in the PN^-/CAN^- group ($P < 0.05$). Diabetes duration was shorter in the PN^-/CAN^- group than in the other diabetic groups, being longest in PN^+/CAN^+ subjects ($P < 0.05$). Cholesterol, LDL cholesterol, creatinine, and white blood cell count as well as the rates of nephropathy and hypertension were higher in the PN^+/CAN^- group than in the PN^-/CAN^- group ($P < 0.05$). The rates of nephropathy, retinopathy, and hypertension were higher in the PN^+/CAN^+ than in the PN^-/CAN^- group ($P < 0.05$), whereas only retinopathy was more frequent in the PN^+/CAN^+ than in the PN^+/CAN^- group ($P < 0.05$). HbA_{1c}, mean blood glucose, uric acid, rates of patients receiving insulin, and HDL cholesterol, triglyceride, and fibrinogen levels were similar in the three diabetic groups studied.

The results of the plasma markers of oxidative stress in the four groups studied are shown in Table 2. Five, four, and three markers were significantly altered in the PN^+/CAN^-, PN^-/CAN^-, and PN^+/CAN^+ groups, respectively, when compared with the control group ($P < 0.05$). Superoxide anion ($O_2^{\bullet -}$) generation was significantly enhanced, and both the vitamin E–to–lipid ratio and vitamin C were significantly diminished in the PN^+/CAN^- group as compared with the PN^-/CAN^- group ($P < 0.05$).

Single linear regression analyses revealed moderate associations between nerve function parameters and markers of oxidative stress, such as an inverse relationship between the cold TPT on the thenar eminence and vitamin E–to–lipid ratio ($r = -0.23$, $P < 0.05$), an inverse correlation between NIS-LL and vitamin E–to–lipid ratio ($r = -0.20$, $P < 0.05$), and an association between NIS-LL and $O_2^{\bullet -}$ generation ($r = 0.21$, $P < 0.05$).

To assess whether oxidative stress was an independent determinant of diabetic PN, multiple linear regression analysis using forward stepping was performed with NIS-LL as the dependent marker of the severity of PN. Independent variables entered into the model included age, sex, BMI, diabetes type, diabetes duration, height, HbA_{1c}, albuminuria, triglycerides, HDL and LDL cholesterol, retinopathy, and hypertension. The only factors that were independently associated with NIS-LL were diabetes duration and triglyceride level ($P < 0.05$). The results of further modeling in which the markers of oxidative stress were added to the original model are shown in Table 3. When $O_2^{\bullet -}$ generation was added to the original model, superoxide, diabetes duration, and triglycerides were independent factors associated with NIS-LL ($P <$

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Table 1—*Demographic, clinical, and laboratory data of the control and diabetic groups*

	Control	PN^-/CAN^-	PN^+/CAN^-	PN^+/CAN^+
n	85	62	105	22
Age (years)	47.5 ± 1.9	43.0 ± 2.0	57.9 ± 1.2*†	54.4 ± 2.9†
BMI (kg/m²)	25.1 ± 0.4	27.7 ± 0.6*	28.2 ± 0.5*	26.7 ± 1.0
Sex (M/F) (%)	42/58	36/64	57/43†	54/46
HbA_{1c} (%)	5.7 ± 0.06	9.1 ± 0.2*	9.6 ± 0.2*	9.8 ± 0.3*
Mean blood glucose (mmol/l)	—	10.1 ± 0.36	10.7 ± 0.27	11.0 ± 0.61
Uric acid (μmol/l)	—	291 ± 11.9	315 ± 11.9	321 ± 17.8
Type 1/type 2 diabetes (%)	—	50/50	24/76†	36/64
Insulin treatment (%)	—	74	73	91
Diabetes duration (years)	—	7.5 ± 0.9	12.2 ± 1.0†	18.5 ± 2.0†‡
Cholesterol (mmol/l)	5.80 ± 0.14	5.28 ± 0.16	5.78 ± 0.11†	5.54 ± 0.22
HDL cholesterol (mmol/l)	1.61 ± 0.05	1.34 ± 0.05*	1.27 ± 0.05*	1.29 ± 0.12*
LDL cholesterol (mmol/l)	3.70 ± 0.13	3.19 ± 0.13*	3.60 ± 0.10†	3.24 ± 0.27
Triglycerides (mmol/l)	1.27 ± 0.09	1.85 ± 0.32*	1.97 ± 0.10*	2.24 ± 0.31*
Creatinine (μmol/l)	69.0 ± 1.8	65.4 ± 1.8	71.6 ± 2.7†	75.1 ± 9.7
Fibrinogen (μmol/l)	—	8.3 ± 0.3	9.1 ± 0.4	9.7 ± 0.7
White blood cells (counts/μl)	—	5,811 ± 186	6,448 ± 172†	6,027 ± 348
Nephropathy (%)	—	18	35†	55†
Retinopathy (%)	—	19	34	62†‡
Hypertension (%)	—	27	59†	55†

Data are means ± SE, unless noted otherwise. *$P < 0.05$ vs. control; †$P < 0.05$ vs. PN^-/CAN^-; ‡$P < 0.05$ vs. PN^+/CAN^-.

0.05). When the lag time for peroxynitrite ($ONOO^-$) production was added, this variable remained as the only significant independent factor associated with NIS-LL ($P < 0.05$). Adding vitamin C resulted in retinopathy being the only factor left in the model ($P < 0.05$). When the vitamin E–to–lipid ratio was added to the original model (excluding triglycerides and HDL and LDL cholesterol), diabetes duration and type 2 diabetes constituted the factors independently associated with NIS-LL ($P < 0.05$). Finally, when 8-iso-$PGF_{2\alpha}$ was added to the original model, diabetes duration and triglycerides remained in the model as independent factors associated with NIS-LL ($P < 0.05$).

CONCLUSIONS — The results of this study provide evidence for an independent association between various plasma markers of oxidative stress and the severity of diabetic PN as assessed by the NIS-LL. The only other independent variable consistently associated with the NIS-LL was the duration of diabetes. However, increased oxidative stress was also evident, albeit to a lesser degree, in diabetic patients in whom PN and autonomic neuropathy were excluded. Patients in whom CAN was superimposed on PN did not show evidence of further enhancement of oxidative stress, although these patients had the longest duration of diabetes and highest rates of comorbidities such as nephropathy, retinopathy, and hypertension, suggesting that despite the high burden of multiple chronic complications, oxidative stress is maximized at somewhat earlier stages.

In this study, support for the proposal that lipid peroxidation is increased in diabetic subjects is provided by assessing plasma F_2-isoprostanes. The F_2-isoprostanes are a family of PGF_2-like compounds produced during peroxidation of arachidonic acid by a mechanism independent of the cyclooxygenase pathway. Of these, 8-iso-$PGF_{2\alpha}$ has received the most attention because it is one of the major products and has been shown to be biologically active. F_2-isoprostanes are formed in situ, whereas arachidonic acid is esterified to phospholipids and released by the action of phospholipase A_2. Therefore, 8-iso-$PGF_{2\alpha}$ determination is a more specific index of lipid peroxidation than measurements of malondialdehyde or lipid hydroperoxides by routine laboratory techniques (20,21).

Table 2—*Plasma markers of oxidative stress in the healthy control group and diabetic groups*

	Control	PN^-/CAN^-	PN^+/CAN^-	PN^+/CAN^+
n	85	62	105	22
8-iso-$PGF_{2\alpha}$ (nmol/l)	0.76 ± 0.09*	1.20 ± 0.19†	1.17 ± 0.11	1.15 ± 0.36
$O_2^{\cdot -}$ (mV)	2.56 ± 0.22*	3.31 ± 0.34†‡	4.20 ± 0.30§	5.17 ± 0.66‖
$ONOO^-$ lag time (min)	49.5 ± 1.4*	44.0 ± 1.3†	42.3 ± 1.1	40.5 ± 2.6‖
Vitamin E–to–lipid ratio ([μmol/l] · $[mmol/l]^{-1}$)	4.42 ± 0.15*	3.96 ± 0.20†	3.16 ± 0.11§	3.52 ± 0.33‖
Vitamin C (μmol/l)	57.5 ± 2.6*	61.1 ± 2.8	50.4 ± 2.4§	51.8 ± 5.4

Data are means ± SE. *$P < 0.05$ for control vs. PN^+/CAN^-; †$P < 0.05$ for PN^-/CAN^- vs. control; ‡$P < 0.05$ for PN^-/CAN^- vs. PN^+/CAN^+; §$P < 0.05$ for PN^+/CAN^- vs. PN^-/CAN^-; ‖$P < 0.05$ for PN^+/CAN^+ vs. control.

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Table 3—*Multivariate models identifying factors independently associated with the NIS-LL*

Variables added to original model/independent factors	Associations with NIS-LL when included in model		
	β	SE (β)	*P*
Original model			
Diabetes duration	0.137	0.053	0.010
Triglycerides	0.014	0.006	0.015
Original model + superoxide			
Superoxide	0.445	0.180	0.015
Diabetes duration	0.142	0.055	0.011
Triglycerides	0.012	0.006	0.041
Original model + peroxynitrite			
Peroxynitrite	−0.124	0.052	0.019
Original model + vitamin C			
Retinopathy	2.691	1.074	0.013
Original model + vitamin E–to–lipid ratio			
Diabetes duration	0.157	0.057	0.007
Type 2 diabetes	2.541	1.177	0.032
Original model + 8-iso-$PGF_{2\alpha}$			
Diabetes duration	0.144	0.057	0.013
Triglycerides	0.015	0.006	0.019

Vitamin E is an important lipid-soluble, chain-breaking antioxidant that protects against free radical damage in tissues and biological fluids (22). We found that the vitamin E–to–lipid ratio was significantly lower in the diabetic subjects without neuropathy compared with control subjects. Moreover, the vitamin E–to–lipid ratio was markedly lower in the diabetic subjects with PN compared with those without. This is confirmed by the finding that the plasma vitamin E–to–lipid ratio was inversely related to either TPT or NIS-LL.

Antioxidant capacity is a global measure that takes into account all known and unknown antioxidant activities present in a sample as well as their interaction. In this study, we utilized a new approach that is based on the capacity of a sample to scavenge free radicals and oxidants in the presence of the unique photoprotein pholasin, the luciferin found in bioluminescent mollusk *Pholas dactylus*. Pholasin emits light in the presence of different systems that are capable of generating free radicals and certain oxidants (23). In this study, plasma samples were assessed for their antioxidant properties against $O_2^{\cdot -}$ and $ONOO^-$. $O_2^{\cdot -}$ was produced by the oxidation of xanthine by the enzyme xanthine oxidase. $ONOO^-$ is formed by the reaction between superoxide and nitric oxide (NO), released simultaneously and continuously from a solution of SIN-1: $O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$. Indeed, total plasma antioxidant capacity as determined by the lag time for $ONOO^-$ and capability of the sample to quench $O_2^{\cdot -}$ were the two independent determinants of the severity of PN in the multivariate models.

Our results extend beyond the recent notion that nitrosative stress resulting from $ONOO^-$ overproduction is increased in diabetic patients. Nitrotyrosine, which has been used as an indirect marker of $ONOO^-$ generation, is increased in plasma from type 1 and type 2 diabetic patients (24–26). In patients with early type 1 diabetes, increased nitrotyrosine levels were associated with a deterioration in composite MNCV in the median, ulnar, and peroneal nerves after 3 years (25). Our results demonstrate that a shortened lag time to $ONOO^-$ production is an independent risk factor for the severity of diabetic PN. This is compatible with the notion that $ONOO^-$ is toxic to the endothelium and perineurium (25). A recent immunohistochemistry study (27) in sural nerve biopsies from patients with different PNs, including that of diabetic origin, showed that *N*ε-carboxymethyllysine, a major advanced glycation end product resulting from glycoxidation and lipid peroxidation, was detected in the perineurium as well as epineurial, perineurial, and endoneurial vessels.

One limitation to this study is the measurement of oxidative stress markers in plasma rather than in nerve tissue. Therefore, our results have to be interpreted with some caution because we cannot exclude the possibility that plasma concentrations do not adequately mirror the situation in the nerve. This problem could be theoretically addressed in a nerve biopsy study, wherein, however, it would be difficult for ethical reasons to include healthy subjects and diabetic patients without neuropathy. However, there is some evidence from experimental studies suggesting that changes in systemic rather than endoneurial oxidative stress may lead to nerve dysfunction (28). A second limitation is the differences in some demographic variables between the groups studied; but in view of the results confirmed by the multivariate models, we believe that the observed associations are independent and valid. A third limitation is the relatively low number of patients in the group with both PN and CAN compared with the other three groups. Because $O_2^{\cdot -}$ was highest and $ONOO^-$ lag time was shortest in the group with both PN and CAN, the possibility of a type II error cannot be completely ruled out.

In conclusion, we have used an array of assays to evaluate the impact of oxidative stress on various degrees and manifestations of diabetic neuropathy. We have found that assessment of plasma antioxidant nutrients or total antioxidant capacity was superior to measurement of plasma 8-iso-$PGF_{2\alpha}$ as a marker of oxidative stress. Moreover, we have shown that oxidative stress is more pronounced in diabetic subjects with PN compared with those without this complication, without further significant enhancement in relation to superimposed autonomic neuropathy. However, other independent determinants of PN, such as the duration of diabetes and triglyceride levels, have to be taken into account as possible confounding factors. These results provide a rationale toward improved patient selection for clinical trials using antioxidants in diabetic neuropathy.

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