Nitrosative Stress, Uric Acid, and Peripheral Nerve Function in Early Type 1 Diabetes

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The present study was performed to determine whether nitric oxide overproduction is associated with deterioration in peripheral nerve function in type 1 diabetes. We measured peripheral nerve function and biochemical indicators of nitrosative stress annually for 3 years in 37 patients with type 1 diabetes. Plasma nitrite and nitrate (collectively NO_x) were 34.0 ± 4.9 μmol/l in the control subjects and 52.4 ± 5.1, 50.0 ± 5.1, and 49.0 ± 5.2 in the diabetic patients at the first, second, and third evaluations, respectively ($P < 0.01$). Nitrotyrosine (NTY) was 13.3 ± 2.0 μmol/l in the control subjects and 26.8 ± 4.4, 26.1 ± 4.3, and 32.7 ± 4.3 in the diabetic patients ($P < 0.01$). Uric acid was suppressed by 20% in the diabetic patients ($P < 0.001$). Composite motor nerve conduction velocity for the median, ulnar, and peroneal nerves was decreased in patients with high versus low NTY (mean Z score −0.522 ± 0.25 versus 0.273 ± 0.22; $P < 0.025$). Patients with high NO_x had decreased sweating, and those with suppressed uric acid had decreased autonomic function. In conclusion, nitrosative stress in early diabetes is associated with suppressed uric acid and deterioration in peripheral nerve function. ***Diabetes*** **51:2817–2825, 2002**

Although hyperglycemia has been proven to cause peripheral nerve dysfunction in patients with diabetes, the biochemical mechanisms for this effect are poorly understood (1,2). Recent studies in experimental animals have indicated that hyperglycemia stimulates the production of nitric oxide, which reacts with superoxide anion to form peroxynitrite, which is damaging to the endothelium (3) and perineurium (4). Nitric oxide is unstable and cannot be directly measured, but its production can be estimated from its stable breakdown products nitrite and nitrate (collectively NO_x) (5). Peroxynitrite is also unstable and difficult to measure directly, but its formation can be estimated by measuring the nitrotyrosine component of protein (6). We measured these biochemical markers of nitrosative stress to determine whether they are increased in human diabetes and have an impact on peripheral nerve function. In addition, we measured 8-isoprostaglandin F2α (8-iso-PGF2α) (7), an isoprostane reflective of lipid peroxidation and the activity of inducible nitric oxide synthase (iNOS) (8). We also measured uric acid, which is an endogenous antioxidant and scavenger of peroxynitrite (9).

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8-iso-PGF2α, 8-isoprostaglandin F2α; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; HPLC, high-performance liquid chromatography; iNOS, inducible nitric oxide synthase; NTY, nitrotyrosine; TY, tyrosine; VMA, vanillylmandelic acid.

RESEARCH DESIGN AND METHODS

Patients. Thirty-seven patients (10 males, 27 females) with type 1 diabetes were enrolled 2–22 months after diagnosis in a longitudinal study of peripheral nerve function (Table 1). Patients with symptoms of neuropathy, other systemic illnesses, or excessive alcohol consumption (an average of more than two drinks per day) were excluded. All patients were taught to monitor their glucose levels at home and to adjust their insulin doses as necessary to maintain optimal glycemic control. HbA_{1c} was measured one to four times a year for 3 years. Thirty-six patients underwent three annual evaluations; one patient withdrew after the second year.

The diabetic patients were admitted to beds designated for research at West Virginia University Hospital to control their dietary intake, activity, and glucose before and during the annual autonomic function testing. Glucose was monitored before each meal and snack and at 3:00 A.M, and insulin adjustments were made as needed. All patients were administered a standard weight-maintaining diet containing 130 mEq sodium daily for 3 days before the collection of blood and urine; the diet did not include foods with high nitrite content (celery, lettuce, or spinach).

Autonomic function tests were also performed in 41 age- and sex-matched healthy control subjects to provide a basis of comparison with the diabetic patients. The control subjects were also admitted to the hospital, administered the same diet, and subjected to the same restrictions.

The research was approved by the Institutional Review Board of West Virginia University Hospital, and informed consent was obtained.

Peripheral Nerve Testing

Large fiber somatosensory function. Nerve conduction studies were performed with a TD-20 TECCA electromyograph (TECCA Corp., Pleasantville, NY). Skin temperature was maintained above 31°C. Motor nerve conduction velocities, compound action potentials, distal latencies, and F-wave latencies were measured in the median, ulnar, and peroneal nerves. Sensory nerve amplitudes and latencies were measured in the median, ulnar, and sural nerves.

Small fiber somatosensory function. Quantitative sensory testing was

TABLE 1
Clinical characteristics of patients

	Diabetic Patients	Healthy control subjects
n (M/F)	37 (10/27)	41 (14/27)
Age (years)	20.3 (10–40)*	21.0 (10–42)†
Disease duration at initial evaluation (months)	10.4 (2–22)	—

Data are means (ranges) unless noted otherwise. *Age at diagnosis. †Age at testing.

TABLE 2
Nitrosative stress in early diabetes

	Control subjects	Diabetic patients		
		First evaluation	Second evaluation	Third evaluation
NO_x (μmol/l)	34.0 ± 4.9	52.4 ± 5.1*	50.0 ± 5.1*	49.0 ± 5.2*
NTY (μmol/l)	13.3 ± 2.0	26.8 ± 4.4†	26.1 ± 4.3†	32.7 ± 4.3*
TY (mmol/l)	43.7 ± 5.6	40.0 ± 5.2	41.5 ± 3.7	42.1 ± 4.0
NTY/TY ($\times 10^{-3}$)	0.339 ± 0.12	0.774 ± 0.13*	0.692 ± 0.11*	0.872 ± 0.14*

Data are means ± SE. *P <0.01, †P <0.05 vs. control subjects.

used to assess small and thinly myelinated Aδ fibers, which convey cold sensation, and C fibers, which convey heat (10). The hot and cold stimuli were applied to the dorsal aspect of the feet and the wrist, and the participants were asked to distinguish between progressively small thermal stimuli. Specific thermal thresholds were then determined by a microprocessor-controlled forced choice technique (Neurolink, East Lyme, CT).

Cardiovascular autonomic function: beat-to-beat variation with deep breathing. Patients were studied in the supine posture after relaxing for 10 min. Heart rate was monitored while they breathed slowly (5 s inspiration/5 s expiration) and deeply for 5 min. The difference between the maximum and minimum instantaneous heart rates (maximum – minimum) reflects the integrity of the parasympathetic innervation of the heart (11).

Cardiovascular autonomic function: heart rate response to the Valsalva maneuver. The heart rate was monitored while the patients were supine and instructed to expire into a sphygmomanometer until a pressure of 40 mmHg was maintained for 20 s. The Valsalva ratio was calculated by dividing the maximal instantaneous heart rate during the maneuver by the minimal heart rate observed after release (11).

Cardiovascular autonomic function: power spectral analysis. Instantaneous heart rate was measured with a Hokanson electrocardiograph monitor, which allows each R-R interval to be recorded into a computer program (DE Hokanson, Bellevue, WA). Power spectral analysis was performed using the fast Fourier transform (12). Respiration was monitored so that spurious

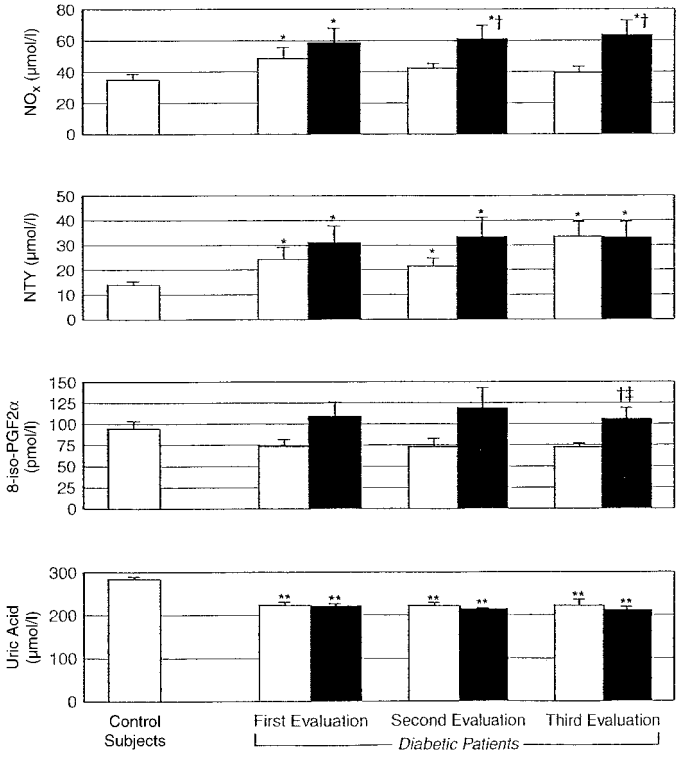

FIG. 1. Effects of glycemic control on nitrosative stress and serum uric acid. □, well-controlled patients; ■, poorly controlled patients. *P < 0.05 vs. control subjects; **P < 0.01, †P < 0.05 vs. patients in good control; ‡P < 0.01, for patients in good control across all years versus patients in poor control.

TABLE 3
Effect of sex on NO_x and 8-iso-PGF2α

	Control subjects		Diabetic patients					
			First evaluation		Second evaluation		Third evaluation	
	M	F	M	F	M	F	M	F
NO_x (μmol/l)	42.1 ± 4.7	29.7 ± 2.8*	36.6 ± 5.3†	57.7 ± 8.2‡	38.6 ± 5.2†	54.2 ± 6.7‡	36.3 ± 5.5†	53.3 ± 7.2‡
8-iso-PGF2α (pmol/l)	91.1 ± 19	93.2 ± 13	46.5 ± 14	88.7 ± 13	59.0 ± 10	105 ± 18	75.4 ± 14	90 ± 9.6§

Data are means ± SE. *P < 0.02 vs. male control subjects; †P < 0.025 vs. female diabetic patients; ‡P < 0.01 vs. female control subjects; §P < 0.05 for diabetic females versus diabetic males across all years.

TABLE 4
Effect of diabetes on uric acid excretion

	Control subjects	Diabetic patients		
		First evaluation	Second evaluation	Third evaluation
Serum uric acid (μmol/l)	280 ± 9.9	218 ± 7.2*	215 ± 9.5*	214 ± 8.0*
Uric acid excretion				
mmol/day	2.35 ± 0.14	2.45 ± 0.16	2.64 ± 0.11	2.73 ± 0.14†
mmol/g creatinine	2.03 ± 0.09	2.14 ± 0.10	2.38 ± 0.11†	2.06 ± 0.10
Fraction excretion of uric acid (%)	5.93 ± 0.33	8.08 ± 0.56‡	9.60 ± 0.56‡	7.68 ± 0.45‡
Creatinine clearance (ml/min)	103 ± 5.6	101 ± 5.5	98.7 ± 4.6	127 ± 6.5†

Data are means ± SE. *$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$ vs. control subjects.

low-frequency spectra resulting from slow breathing or sighing could be eliminated. High-frequency spectra (0.15–0.40 Hz) indicate parasympathetic cardiac innervation.

Sympathetic function. We tested the sympathetic modulation of renin processing by measuring the ratio of renin to inactive renin (13,14). We assessed norepinephrine production from vanillylmandelic acid (VMA) excretion (13,15). Sudomotor function was assessed by the quantitative sudomotor axon reflex as previously described (16).

Biochemical measurements

HbA$_{1c}$. HbA_{1c} was measured by agar gel electrophoresis (17). The reference range for the nondiabetic population was 4.7–7.3%.

Renin and prorenin. Active renin was measured as the rate of conversion of renin substrate to angiotensin I by plasma collected in EDTA (18). Total renin (active plus inactive) was prepared in a separate 1-ml aliquot of plasma by preincubating the latter for 1 h with 1 mg trypsin from porcine pancreas (Sigma, St. Louis, MO). Total and active renin were then assayed by determining angiotensin I via radioimmunoassay using ^{125}I-labeled angiotensin I (INCStar, Stillwater, MN) (19). Prorenin was calculated as the difference between total and active renin. To avoid the confounding effect of ovarian prorenin, blood sampling was rescheduled for women who were menstruating at the time of their annual evaluations (13).

VMA. Urinary VMA was measured by high-performance liquid chromatography (HPLC) and coulometric detection using isoVMA as an internal standard (20).

NO$_x$. Serum NO_3 was converted to NO_2 by nitrate reductase, which was quantitated by the Griess reaction using an enzyme-linked immunosorbent assay (ELISA) method (5).

Nitrotyrosine. Plasma proteins were precipitated with acetone and then digested with pronase. 3-Nitrotyrosine (NTY) and tyrosine (TY) were measured with HPLC and detected electrochemically (CoulArray; ESA, Chelmsford, MA) (6).

8-Iso-PGF2α. 8-Iso-PGF2α was measured by an ELISA method (7) using a kit from Cayman Laboratories (Ann Arbor, MI).

Uric acid. Uric acid was oxidized in the presence of uricase to form hydrogen peroxide, which was measured photometrically (21).

Statistical analysis. ANOVA was used to test differences between diabetic patients and control subjects and differences between years in the longitudinal study (22). Association between biochemical parameters and peripheral nerve function was assessed using regression analysis (23).

RESULTS

No vascular complications or symptoms of neuropathy developed in the diabetic patients during the course of this study. One patient developed hypertension and one patient withdrew from the study after the second evaluation. Twenty of the 37 patients maintained glycemic control within American Diabetes Association guidelines (HbA_{1c} <1% above the upper limit of normal for the nondiabetic population). Patients were stratified each year as to whether their glycemic control was good or poor by determining whether their average HbA_{1c} was below or above, respectively, the median of the average HbA_{1c} determinations for all patients at that evaluation. Patients in good glycemic control had the same age and sex distribution as those in poor control.

NO_x concentrations were higher in the diabetic patients at the first (52.4 ± 5.1 μmol/l), second (50.0 ± 5.1 μmol/l), and third (49.0 ± 5.2 μmol/l) evaluations than in the control subjects (34.0 ± 4.9 μmol/l) ($P < 0.025$) (Table 2). NO_x was elevated in the diabetic patients with high HbA_{1c} compared with control subjects ($P < 0.01$ at each evaluation) but nearly normal in the well-controlled diabetic patients (Fig. 1). NO_x was higher in the female diabetic patients than in the female control subjects ($P < 0.01$) or the male diabetic patients ($P < 0.025$) (Table 3). NO_x was no different in the male diabetic versus control subjects. NO_x was positively correlated with creatinine clearance at the time of the third evaluation ($P < 0.01$).

NTY was 13.3 μmol/l in the control subjects and approximately double in the diabetic patients (Table 2). Tyrosine was similar in the diabetic versus control subjects, and the NTY/TY ratio was accordingly increased. The increased NTY was observed in the male as well as the female patients with diabetes. Those in poor control had a larger increase in NTY (Fig. 1). We detected no chlorotyrosine, an indicator of myeloperoxidase activity (an alternative source of NTY), in patients or control subjects (24).

8-Iso-PGF2α was not increased in the diabetic patients compared with control subjects. Nevertheless, 8-iso-PGF2α was higher in the poorly controlled versus the well-controlled diabetic patients (Fig. 1). There was a strong correlation between 8-iso-PGF2α and NO_x in the diabetic patients at the first ($P < 0.05$), second ($P < 0.001$),

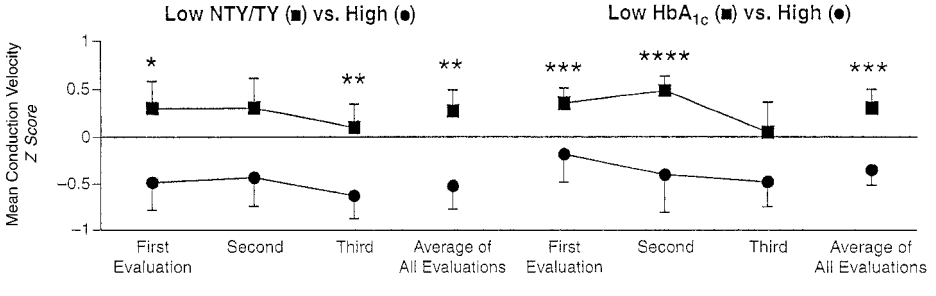

FIG. 2. NTY/TY, glycemic control, and motor nerve conduction velocity *Z* scores. *Z* scores were calculated from conduction velocities for the median, peroneal, and ulnar nerves, and the mean *Z* score was calculated at each evaluation. **P* < 0.05, ***P* < 0.025 vs. high NTY/TY; ****P* < 0.05, *****P* < 0.025 vs. high HbA_{1c}.

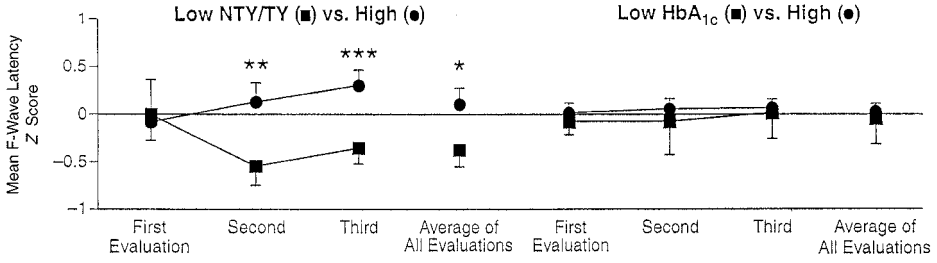

FIG. 3. NTY/TY, glycemic control, and motor nerve F-wave latencies. *Z* scores were calculated for F-wave latencies for the median, peroneal, and ulnar nerves, and the mean score was calculated at each evaluation. *$P < 0.05$, **$P < 0.025$, ***$P < 0.01$ vs. low NTY/TY.

and third ($P < 0.001$) evaluations. Sex-specific *Z* scores for NO_x and 8-iso-PGF2α (see below) were similarly correlated. The diabetes-related sex difference described for NO_x was also seen for 8-iso-PGF2α (Table 3). 8-Iso-PGF2α and NO_x showed similar correlations with physiological parameters. Both correlated negatively with creatinine clearance at the time of the third evaluation ($P < 0.01$ for NO_x, $P < 0.001$ for 8-iso-PGF2α). Both parameters correlated negatively with sudomotor function (see below).

Serum uric acid was suppressed in the diabetic patients, and the differences from the control subjects were highly significant at each time point ($P < 0.001$) (Table 4). Serum uric acid was decreased in males and females and in well-controlled as well as poorly controlled patients (Fig. 1). Nevertheless, there was a negative association between HbA_{1c} and serum uric acid that approached significance at the second evaluation ($P = 0.065$) and was significant at the third evaluation ($P < 0.025$). The fractional excretion of uric acid was increased in the diabetic patients, and total excretion was increased at the time of the third evaluation. Uric acid excretion correlated with creatinine clearance in the diabetic patients at the first ($P < 0.001$), second ($P < 0.01$), and third ($P < 0.05$) evaluations.

We observed a number of negative associations between the biochemical indicators of nitrosative stress and peripheral nerve function. The NTY/TY ratio was associated with decreased motor nerve conduction velocity (Table 5; Fig. 2) and increased F-wave latencies (Table 5; Fig. 3). The association of NTY with conduction velocities was comparable to that with HbA_{1c} (Fig. 2). There was also a negative association ($P < 0.025$) between mean motor nerve conduction velocity *Z* scores and mean NTY/TY (Fig. 4). NTY did not correlate, however, with sensory or cardiovascular autonomic function.

NO_x and 8-iso-PGF2α both showed a negative correlation with sudomotor function. To assess the effects of NO_x, we categorized patients each year as to whether their NO_x levels were above or below the median for the group at that time point. We observed that patients with high NO_x had decreased sweating below the waist and an increase in the ratio of sweating above the waist to below the waist, a typical profile in patients with sympathetic nerve injury (Fig. 5). The diabetes-related sex differences in NO_x cannot explain the decreased sudomotor function in the patients with high versus low NO_x. We observed no sex differences in sudomotor function in patients with diabetes (even though nondiabetic males sweat more than nondiabetic females). To further address any potential sex effect, we calculated sex-specific *Z* scores for NO_x and 8-iso-PGF2α and plotted these against sudomotor function. Regression analysis of the NO_x *Z* scores versus sweating confirmed negative associations ($P < 0.025$ at the second and third evaluations) (Fig. 6). There were similar but weaker associations between NO_x and 8-iso-PGF2α *Z* scores and sweating (Fig. 6). Neither NO_x nor 8-iso-PGF2α correlated with sensory function.

NTY, NO_x, and 8-iso-PGF2α did not correlate with cardiovascular autonomic function. We found, however, that performance on some of the cardiovascular and other autonomic function tests correlated with the suppression of uric acid. To assess this finding, we categorized each diabetic patient according to whether his or her uric acid level was above or below the sex-specific median uric acid level at that evaluation. We observed that the diabetic patients with suppressed uric acid had decreased ratios of active renin to inactive renin (prorenin) ($P < 0.01$) and decreased VMA excretion ($P < 0.025$) (Fig. 7). The ratio of sweating above the waist to sweating below the waist,

TABLE 5
Nitrosative stress and somatosensory function

	First Evaluation		Second Evaluation		Third Evaluation		Average of all Evaluations	
	Low NTY/TY	High NTY/TY	Low NTY/TY	High NTY/TY	Low NTY/TY	High NTY/TY	Low NTY/TY	High NTY/TY
Conduction velocity (m/s)								
Median	57.7 ± 1.1	53.3 ± 1.2*	56.2 ± 1.2	54.1 ± 1.2	55.6 ± 0.96	53.7 ± 1.0	56.7 ± 0.73	53.7 ± 0.83*
Ulnar	57.1 ± 1.6	55.2 ± 1.8	57.0 ± 1.3	55.6 ± 1.3	57.6 ± 1.2	54.2 ± 1.3†	57.2 ± 1.0	55.1 ± 1.2‡
Peroneal	48.1 ± 1.2	46.4 ± 1.25	49.7 ± 1.5	45.7 ± 1.4†	47.4 ± 1.1	45.6 ± 1.1	48.8 ± 0.95	45.7 ± 1.1
Mean conduction velocity *Z* score	0.300 ± 0.28	−0.484 ± 0.30†	0.304 ± 0.31	−0.431 ± 0.31	0.102 ± 0.24	−0.628 ± 0.25‡	0.273 ± 0.22	−0.522 ± 0.25‡
F-wave latencies (ms)								
Median	25.1 ± 0.70	25.7 ± 0.73	24.9 ± 0.57	26.7 ± 0.52‡	25.5 ± 0.44	27.2 ± 0.45*§	25.3 ± 0.51	26.6 ± 0.48†
Ulnar	27.0 ± 0.87	26.7 ± 0.91	25.6 ± 0.55	26.9 ± 0.50†	25.7 ± 0.49	28.3 ± 0.51§	26.1 ± 0.57	27.1 ± 0.53
Peroneal	48.6 ± 1.7	49.0 ± 1.7	44.9 ± 1.5	49.8 ± 1.4*	46.5 ± 1.5	48.3 ± 1.5	46.0 ± 1.2	49.0 ± 1.1
F-wave latency *Z* scores	0.0013 ± 0.27	−0.077 ± 0.29	−0.544 ± 0.21	0.132 ± 0.20‡	−0.349 ± 0.17	0.307 ± 0.16*	−0.370 ± 0.18	0.109 ± 0.17*

Data are means ± SE and are results from motor nerves. *$P < 0.01$, †$P < 0.05$, ‡$P < 0.025$, §$P < 0.001$ vs. low NTY/TY.

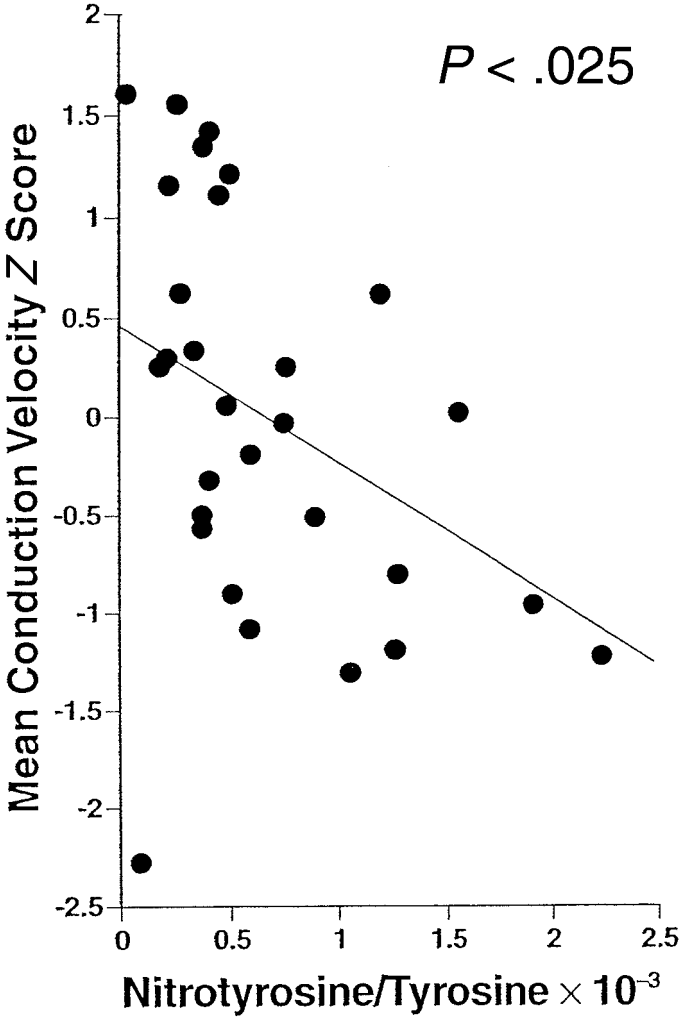

FIG. 4. Mean NTY/TY versus mean motor nerve conduction velocity *Z* score. Each data point represents the mean *Z* score for nine tests (three nerves × 3 years) plotted against the mean of the three NTY/TY determinations (one at each evaluation).

which increases with sympathetic nerve injury (21) was accordingly elevated in the diabetic patients with suppressed uric acid ($P < 0.025$). High-frequency power spectra, an indication of cardiac parasympathetic activity, were decreased in the patients with suppressed versus normal uric acid ($P < 0.0025$) (Fig. 6). A similar pattern was observed for the beat-to-beat variation with deep breathing ($P < 0.025$), although the diabetic patients as a whole were no different from control subjects. The heart rate response to the Valsalva maneuver was the only test of autonomic function that did not correlate with the uric acid status of the patient.

DISCUSSION

Previous data from this cohort have documented that hyperglycemia had a demonstrable impact on peripheral nerve function (25,26), although the mechanism for this effect was unclear. Recent reports that peroxynitrite is toxic to the endothelium (3) and perineurium (4) prompted this analysis of the effects of chronic hyperglycemia on nitric oxide metabolism and uric acid, a scavenger of peroxynitrite. Our data revealed that nitrosative stress was greater in poorly controlled patients (Fig. 1),

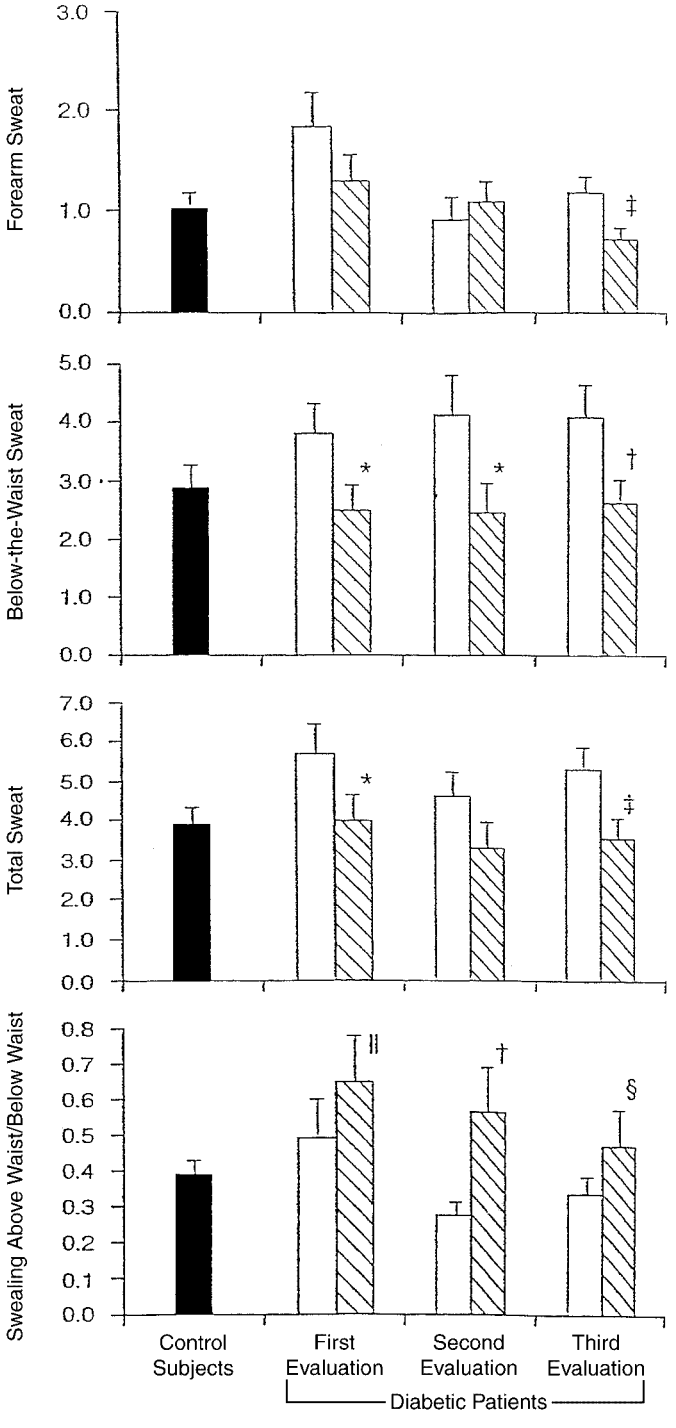

FIG. 5. Sudomotor function in patients with high versus low NO_x. ■, control subjects; □, diabetic patients with low NOx; ▨, diabetic patients with high NO_x. Data are means ± SE of sweat produced (μl). *$P < 0.05$ vs. patients with low NO_x; †$P < 0.025$ vs. patients with low NO_x; ‡$P < 0.01$ vs. patients with low NO_x; §$P < 0.05$, diabetic patients with high versus low NO_x across all years; ‖$P < 0.05$ vs. control subjects.

and HbA_{1c} had a negative association with uric acid ($P < 0.025$ at the third evaluation). Thus our results support our presumption that hyperglycemia was the stimulus to nitrosative stress and prompted this analysis of the relationship between nitric oxide overproduction and peripheral nerve function.

Nitric oxide overproduction in diabetes has been documented in several animal (27) and clinical (28,29) studies.

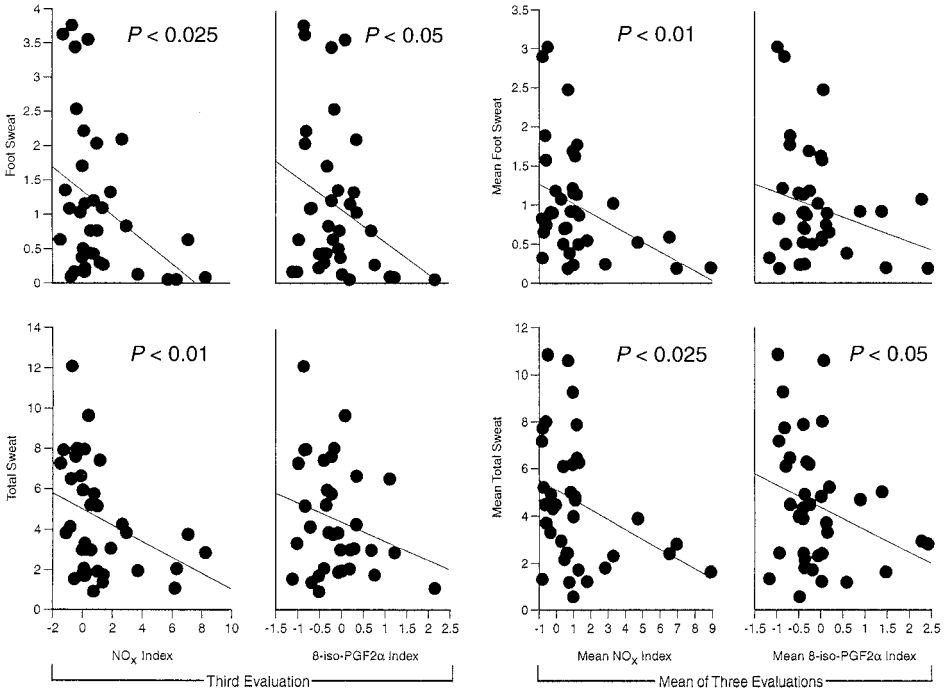

FIG. 6. Associations between the NO_x index and the 8-iso-PGF2α index and sweating. Sex-specific *Z* scores were calculated for NO_x and 8-iso-PGF2α and plotted against sweating. The sudomotor data are not corrected for sex because there were no sex-related differences in sweating in the diabetic patients (even though there were sex differences in control subjects). The averages of the NO_x indexes and the 8-iso-PGF2α indexes were plotted against the averages of the sudomotor responses from the three evaluations. The association between the 8-iso-PGF2α index and total sweat at the third evaluation approached statistical significance ($P = 0.06$). The association between mean 8-iso-PGF2α and mean foot sweat for all evaluations approached significance ($P = 0.065$).

This appears contradictory to the abundant experimental data indicating that nitric oxide is a beneficial endothelium-derived vasodilating factor that is deficient in diabetes (3,30). Nitric oxide production by the endothelium is regulated by endothelial nitric oxide synthase (eNOS) and may respond differently to chronic hyperglycemia than does nitric oxide produced elsewhere. Nitric oxide in macrophages, monocytes, epithelial cells, vascular smooth muscle, hepatocytes, and many other tissues of the body is synthesized by iNOS, which is the most important source of nitric oxide in the whole patient. The gene expression of iNOS is mediated by nuclear factor κB (NFκB) (31) which, in turn, is activated by hyperglycemia and oxidative stress (32,33). Accordingly, Pitre et al. (4) reported immunochemical evidence of increased perineural iNOS in diabetic rats, and Coppey et al. (3) reported that NTY staining of the endothelium in diabetic rats was associated with failure of acetylcholine-induced nitric oxide release and suppressed vasodilation (3). Thus we postulate that the negative associations between NTY and motor nerve function (Figs. 2–4) reflect peroxynitrite-induced endothelial damage and ischemia (3). In addition, peroxynitrite may be directly toxic to peripheral nerves. Although there are only limited animal data supporting this theory (4,34), it is well recognized that peroxynitrite is toxic to the central nervous system (35).

Stimulation of iNOS and nitric oxide overproduction also enhances lipid peroxidation. The formation of lipid peroxides in nerve membranes has adverse effects on fluidity, electrical conductivity, and function. The synthesis of 8-iso-PGF2α is a measure of oxidative stress and lipid peroxidation and is linked to iNOS activity. iNOS-deficient mice, for example, have decreased nitric oxide production and decreased 8-iso-PGF2α (8). The strong correlation we observed between NO_x and 8-iso-PGF2α ($P < 0.001$ at the second and third evaluations) is consistent with these experimental data. The similar diabetes-related sex differences for NO_x and 8-iso-PGF2α (Table 3) that we and others (29) have observed further supports this concept. We therefore interpret the negative associations between NO_x and sudomotor function to signify that increased nitric oxide stimulates lipid peroxidation, which in turn has adverse effects on sudomotor nerves. The similarity of the negative associations between NO_x/sudomotor function and 8-iso-PGF2α/sweating is consistent with this interpretation (Fig. 6).

Our data also indicate that nitrosative stress is associated with decreased uric acid, and the latter was negatively associated with autonomic function (Fig. 7). Uric acid is a peroxynitrite scavenger, so its suppression may reflect this metabolic interaction (9). Reciprocal changes in NTY and uric acid provide evidence that peroxynitrite overproduction is a dominant metabolic process even in patients with recent-onset diabetes and no overt complications. The suppression of uric acid was associated with multiple changes in autonomic function. The ratio of renin to inactive renin, an index of the integrity of the sympathetic nerves innervating the kidneys (13,14), was decreased in the patients with suppressed uric acid. The patients with suppressed uric acid also had a redistribution of sudomotor responses, another measure of sympathetic dysfunction (25), and slightly decreased VMA (Fig. 7). Power spectral analysis of heart rate variability revealed evidence of decreased parasympathetic function in patients with suppressed uric acid. Patients with suppressed uric acid had worse performance on five of six measures of autonomic function than patients with normal uric acid. The suppression of uric acid is probably a compensatory response to oxidative stress. This implies that uric acid is functioning as an antioxidant in vivo. The antioxidant properties of uric acid have been demonstrated in vitro (36) and documented in birds (37). Pathologic analyses of brain tissue of patients with Alzheimer's disease have revealed reciprocal relationships between uric acid and NTY, which has been interpreted to mean uric acid acts as a peroxynitrite scavenger and is neuro-

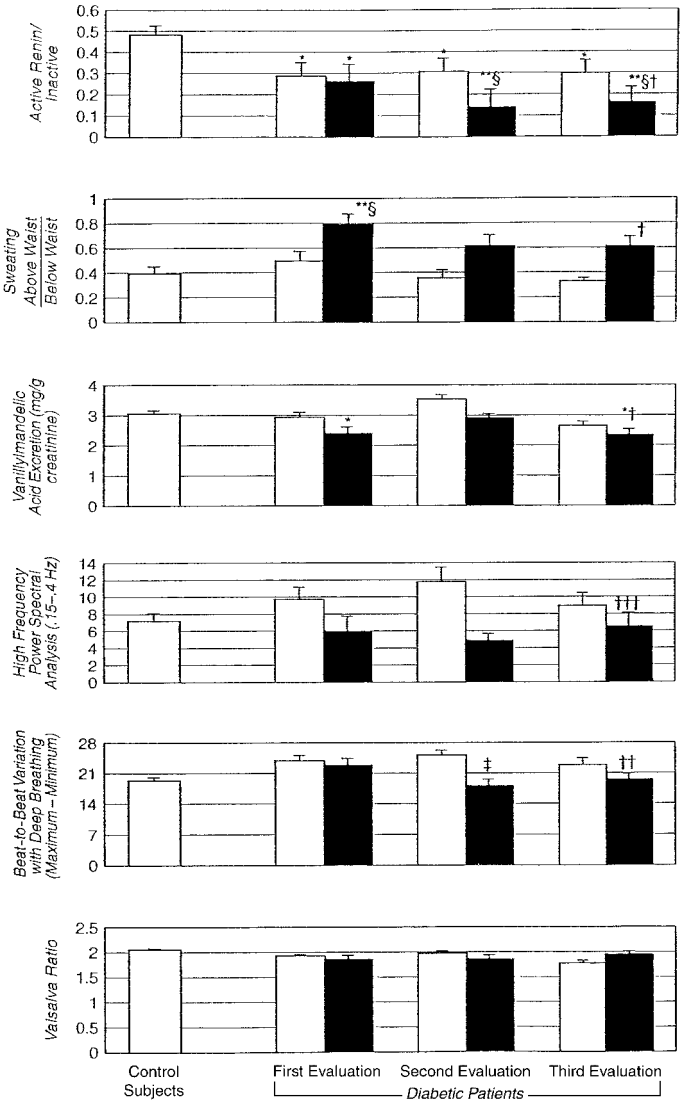

FIG. 7. Uric acid and autonomic function. Mean results ± SE are illustrated for patients categorized according to whether their uric acid was below the sex-specific median uric acid level at that evaluation. □, normal uric acid; ■, suppressed uric acid. *$P < 0.05$, **$P < 0.01$ vs. control subjects; †$P < 0.025$, diabetic patients with suppressed uric acid were different across all years from those with normal uric acid and different from control subjects; ††$P < 0.025$, †††$P < 0.0025$, diabetic patients with suppressed versus normal uric acid were different across all years; ‡$P < 0.025$, §$P < 0.01$ vs. patients with normal uric acid.

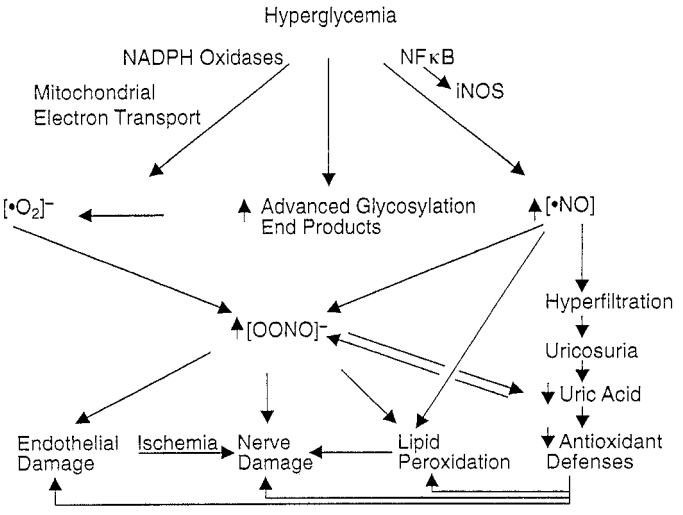

FIG. 8. Relationship between nitrosative stress and neuropathy. [˙O_2]– superoxide anion; [˙NO] nitric oxide; [OONO]–, peroxynitrite.

protective (35). Our data, gathered in vivo, are consistent with this concept.

Why should diabetes lead to suppression of uric acid? Multiple mechanisms need to be considered, and nitric oxide overproduction probably plays a central role either directly or by indirect renal mechanisms (Fig. 8). It is likely that uric acid is degraded or metabolized when it scavenges peroxynitrite (9). Accordingly, the suppression of serum uric acid occurred early, at the first patient evaluation, when there was minimal uricosuria (Table 4). This indicates that direct effects of peroxynitrite excess are the most important cause of uric acid suppression, at least initially. Indirect renal effects appear to play a contributory role. At the time of the third evaluation, uricosuria was documented, which would be expected to aggravate the uric acid deficit, limit the scavenging of peroxynitrite, and increase the latter, which should further suppress uric acid, thus completing a vicious cycle (Fig. 8). Nitric oxide overproduction is a critical component in this indirect mechanism, since it mediates hyperfiltration. This was initially reported by Chiarelli et al. (28) and confirmed by us (NO_x correlated with creatinine clearance [$P < 0.01$] at the time of the third evaluation). The increase in glomerular filtration coupled with an increase in filtration fraction for uric acid eventually leads to uricosuria (Table 4). The loss of this endogenous antioxidant and peroxynitrite scavenger is disadvantageous and may exacerbate multiple diabetic complications. Nitric oxide overproduction has been demonstrated in early diabetic nephropathy (28), and increased urinary 8-iso-PGF2α (38), increased renal NTY (39), and suppressed serum uric acid (40) have been documented in patients with more advanced disease. Previous reports of an association between autonomic dysfunction and nephropathy (41,42) may therefore reflect the fact that both of these complications are linked to overproduction of reactive oxygen species and nitric oxide.

Although we have observed associations between motor nerve conduction velocities and F-wave latencies and NTY, no such associations with motor or sensory response amplitudes were observed. Insofar as changes in conduction velocity reflect the integrity of the myelin sheath, and response amplitudes reflect the viability of the axon, our results are consistent with the traditional teaching that segmental demyelination is an early event in diabetic neuropathy (43).

Although nitric oxide overproduction has been previously reported in patients with diabetes, this is a complex and poorly understood phenomenon. Our simplified interpretation (Fig. 8) does not take into account the multiple potential mechanisms for formation of reactive oxygen species in patients with diabetes, nor did we address the possibility that eNOS may generate both superoxide anions and nitric oxide (and therefore peroxynitrite) (44). Similarly, we did not take into account the possibility that nitric oxide overproduction represents a response (rather than a stimulus as we depicted) to lipid peroxidation. None of these considerations refutes our main hypothesis,

namely, that nitrosative stress in diabetes has adverse effects on peripheral nerve function in humans. There are no previous clinical data to suggest this, but the theory is plausible, since nitrosative stress is linked to oxidative stress and there are multiple animal studies implicating the latter in experimental diabetic neuropathy (3,30). Nitrosative stress and oxidative stress in concert lead to peroxynitrite formation and lipid peroxidation, which synergistically compromise ATP synthesis and damage mitochondria (45), decrease cellular viability (46), and promote apoptosis (47). Unfortunately, hyperglycemia stimulates the synthesis of reactive oxygen intermediates in multiple tissues and subcellular locations, and it is uncertain which is the most important or suppressible with antioxidants (48). Nevertheless, small clinical trials have indicated that vitamin E has beneficial effects on somatosensory (49) and autonomic (50) function in diabetic patients. Our data indicate that oxidative stress and nitrosative stress have detectable adverse effects on peripheral nerve function within the first few years of diabetes, and therefore, it may be possible to prevent them with interventions introduced early in those patients who are unable to maintain normoglycemia.

Finally, there are a number of limitations to this study. First of all, we have no evidence for our assumption that the biochemical evidence of nitrosative stress we detected in the systemic circulation was reflective of the metabolic environment of the nerves or their vascular supply. Second, we are unable to explain why motor nerve and autonomic function were affected by nitrosative stress but sensory nerve function was not.

In summary, we have evidence that nitric oxide overproduction occurs in patients with poorly controlled type 1 diabetes and leads to increased peroxynitrite and lipid peroxidation and suppressed uric acid. These metabolic changes are associated with detectable adverse effects on peripheral nerve function even in patients who have been exposed to hyperglycemia only a few years.

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