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Sympathetic sudomotor disturbance in early type 1 diabetes mellitus is linked to lipid peroxidation

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Abstract

The present study was performed to determine whether increased lipid peroxidation, as assessed from malondialdehyde (MDA) excretion, is associated with deterioration in peripheral nerve function in early type 1 diabetes mellitus. These parameters were measured annually for 3 years in 36 patients who entered the study less than 2 years after the diagnosis of diabetes. Malondialdehyde excretion was 1.51 ± 0.20 μmol/g creatinine in the controls, and 2.43 ± 0.21, 2.39 ± 0.22, and 1.93 ± 0.21 μmol/g creatinine at the first, second, and third evaluations, respectively ($P < .005$). The increased MDA was seen only in the female participants. Malondialdehyde excretion was increased in those with high vs low hemoglobin A_{1c} across all years ($P < .05$). Malondialdehyde excretion correlated negatively with sudomotor function below the waist. The mean sweat production from the 3 evaluations correlated with mean MDA excretion across all years in the proximal leg ($r = -0.42$, $P < .005$) and distal leg ($r = -0.40$, $P < .01$). Below the waist, sweating correlated with MDA ($r = -0.40$, $P < .01$) as did total sweat ($r = -0.38$, $P < .01$). The response amplitudes of the peroneal nerves correlated negatively with MDA excretion (for the mean values at the second 2 evaluations, $P < .005$, $r = -0.45$). Tests of sensory function correlated inconsistently with MDA excretion. In summary, lipid peroxidation, as assessed from malondialdehyde excretion, is associated with sudomotor dysfunction in early diabetes.

1. Introduction

Lipid peroxidation has been linked to peripheral nerve dysfunction in experimental animals [1], and recent evidence has shown that this may be the mechanism by which hyperglycemia and oxidative stress damage peripheral nerves in man. We have observed that nitrosative stress is increased in early diabetes and that the latter correlates with lipid peroxidation as assessed from plasma 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) [2]. Moreover, both nitrosative stress (as assessed from plasma nitrite and nitrate [NOx]) and 8-iso-$PGF_{2\alpha}$ showed negative correlations with sympathetic sudomotor activity [3]. To further document the association between lipid peroxidation and sudomotor dysfunction we measured the excretion of malondialdehyde (MDA) in a group of recently diagnosed patients with type 1 diabetes mellitus whose peripheral nerve function was characterized in a 3-year longitudinal study.

2. Research design and methods

2.1. Patients

There were 37 patients (10 males, 27 females) with type 1 diabetes mellitus enrolled 2 to 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 >2 drinks per day) were excluded from the study. All patients were taught to monitor their glucose levels at home and to adjust their insulin doses as necessary to maintain optimal glycemic control. Hemoglobin A_{1c} (HbA_{1c}) was measured 1 to 4 times a year for 3 years. Thirty-six patients underwent 3 annual evaluations; 1 male patient withdrew after the second year.

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Table 1
Clinical characteristics of patients

	Diabetic	Healthy control subjects
n (M/F)	37 (10/27)	41 (14/27)
Age (y)	20.3 (10-40)[a]	21.0 (10-42)[b]
Disease duration at initial evaluation (mo)	10.4 (2-22)	

[a] At diagnosis.
[b] At time of study.

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 AM, and insulin adjustments were made as needed. Patients were administered a weight-maintaining diet containing 130 mEq sodium daily for 3 days before the collection of blood and urine. The diet did not contain fish, which might increase the excretion of MDA [4], or lettuce, spinach, or celery, which are rich in nitrates and might interfere in the measurement of NOx [5].

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 protocol was approved by the institutional review board of the West Virginia University Hospital, and informed consent was obtained.

2.2. Peripheral nerve testing

2.2.1. Large fiber somatosensory function

Nerve conduction studies were performed with a TD-20 TECCA electromyograph (TECCA, Pleasantville, NY). Skin temperature was measured and 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.

2.2.2. Small fiber somatosensory function

Quantitative sensory testing was used to assess small and thinly myelinated A Δ fibers, which convey cold sensation, and C fibers, which convey heat [6]. 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 small thermal stimuli both above and below the detection threshold. The hot and cold stimuli were delivered so that the largest stimulus (1-second duration) led to a 1° change in temperature. The smallest stimulus (0.1 second) led to a 0.1° change in temperature. Specific thermal thresholds were then determined by a microprocessor-controlled forced choice technique (Neurolink, East Lyme, CT).

2.2.3. Cardiovascular autonomic function: beat to beat variation with deep breathing

Patients were studied in the supine posture after relaxing for 10 minutes. Heart rate was monitored while they breathed slowly (5 seconds inspiration/5 seconds expiration) and deeply for 5 minutes. The difference between the maximum and minimum instantaneous heart rates (maximum − minimum) reflects the integrity of the parasympathetic innervation of the heart [7].

2.2.4. Cardiovascular autonomic function: heart rate response to the Valsalva maneuver

The heart rate was monitored electrocardiographically while the patients were supine and instructed to expire into a sphygmomanometer until a pressure of 40 mm Hg was maintained for 20 seconds. The Valsalva ratio was calculated by dividing the maximal instantaneous heart rate during the maneuver by the minimal heart rate observed after release [7].

2.2.5. 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 [8]. Respiration was monitored so that spurious frequency spectra resulting from slow breathing or sighing could be eliminated. High-frequency spectra (0.15-0.40 Hz) indicate parasympathetic cardiac innervation.

2.2.6. Sympathetic function

We tested the sympathetic modulation of renin processing by measuring the ratio to inactive renin [9]. We assessed norepinephrine production from vanillylmandelic acid excretion [10]. Sudomotor function was assessed by the quantitative sudomotor axon reflex test [11]. Cardiac sympathetic function was assessed from the intermediate-frequency (0.04-0.15 Hz) power spectral analysis of heart rate [8].

2.3. Biochemical measurements

2.3.1. Hemoglobin A_{1c}

Hemoglobin A_1 was measured by agar gel electrophoresis [12]. The reference range for the nondiabetic population was 4.7% to 7.3%. We converted the data to an estimated

Table 2
Changes in HbA_{1c} in patients with good vs poor glycemic control

	Good control (%)	Poor control (%)
First evaluation	5.6 ± 0.1	8.0 ± 0.3
Second evaluation	6.4 ± 0.2*	8.4 ± 0.3
Third evaluation	6.3 ± 0.2*	8.5 ± 0.1

* $P < .01$, different from the first evaluation.

HbA_{1c} by multiplying our data by a conversion factor 6.05% (the upper limit of normal for control subjects in the Diabetes Control and Complications Trial) divided by 7.3% (the upper limit of normal for the HbA_1 in our study). The resulting ratio was 0.829.

2.3.2. Malondialdehyde excretion

The thiobarbituric acid derivative of MDA was formed by heating an acidified aliquot of urine as described by Kosugi et al [13]. Three hundred microliters of acetic acid (5.2 N) were added to 900 μL of urine in the presence and absence of standard (MDA bis-dimethyl acetal, Aldrich Chemicals, Milwaukee, WI). Twenty-five microliters of butylated hydroxytoluene (0.88% in glacial acetic acid) was added. Then, 150 μL of 0.1% thiobarbituric acid (dissolved in 0.05 N NaOH) was added and the mixture was heated to 100°C for 15 minutes.

The thiobarbituric acid adduct was then extracted into 2 mL butanol, evaporated under nitrogen at 37°C [14], purified by high-performance liquid chromatography using a Shodex column (RSpak KC -811, JM Science, Grand Island, NY), and then heated to 60°C using H_3PO_4 (0.1%) as mobile phase. Quantitation was performed spectrophotometrically at 532 nm.

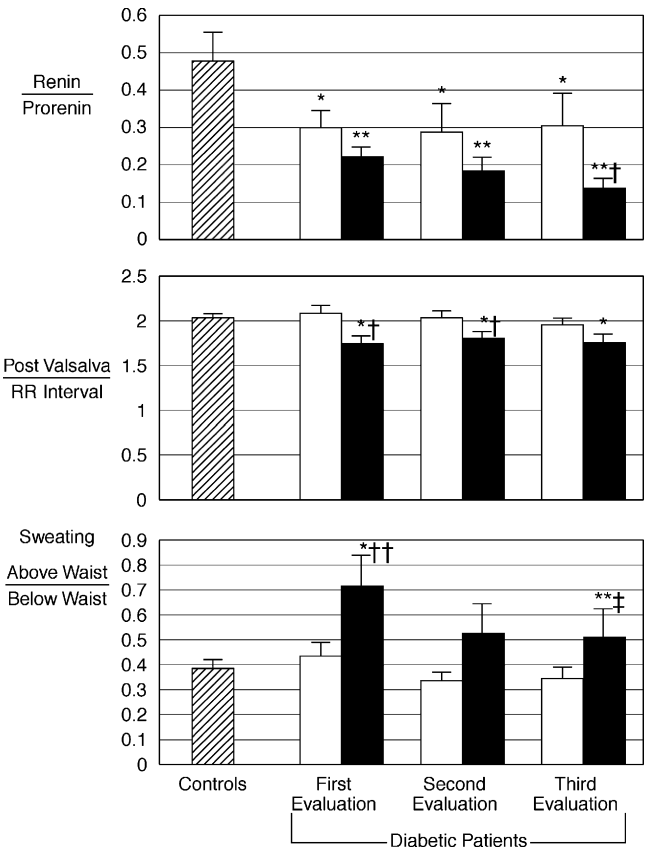

Fig. 1. Effect of glycemic control on sympathetic function in early diabetes. Mean results ± SE are presented for patients whose $HgbA_{1c}$ values were below (open bars) or above (closed bars) the median for the group, respectively, versus the control subjects (hatched bars). *Different from controls. $P < .05$; **$P < .01$; †$P < .01$; †different from patients with low HgbA $P < .05$; ††$P < .01$; ‡diabetic patients with high vs low $HgbA_{1c}$ across all years were different. $P < .01$. Reprinted with permission from Hoeldtke RD. Nitrosative stress in early type 1 diabetes. Clin Auton Res 2003;13:406-21.

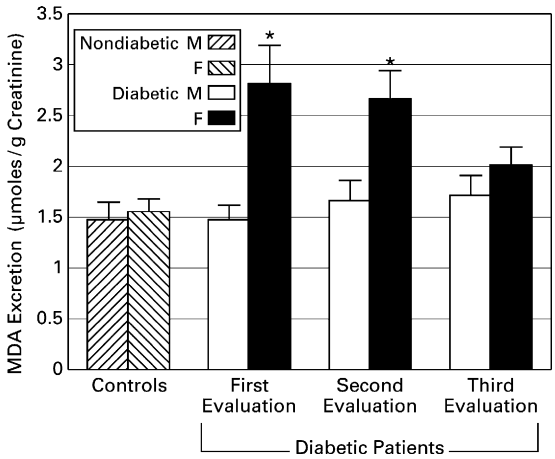

Fig. 2. Effect of sex on MDA excretion. Data represent mean μmol MDA excreted/g creatinine ± SE. To convert MDA to micrograms, multiply by 164.2. *$P < .001$, different from the nondiabetic females.

Nitrite and nitrate, 8-iso-$PGF_{2\alpha}$, vanillylmandelic acid, and prorenin/renin were assayed as previously described [3,9].

2.4. Statistical analysis

Analysis of variance was used to test differences between diabetic patients and control subjects and differences between years in the longitudinal study [15]. Association between biochemical parameters and peripheral nerve function was assessed using regression analysis [16].

3. 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. The median HbA_{1c} at the first, second, and third evaluations were 6.3%, 7.21%, and 7.50%, respectively. 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 with good glycemic control had the same age and sex distribution as those with poor control. Glycemic control deteriorated between the first and second years of the study, but showed little deterioration between years 2 and 3 (Table 2).

Multiple abnormalities in sympathetic function were observed in the diabetic patients. The renin-to-prorenin ratio, an index of the sympathetic innervation of the kidneys, was decreased in the diabetic patients at the first evaluation and deteriorated significantly in those with worse glycemia, as previously reported [3,9]. In addition, there was a relative increase in sudomotor responses above the

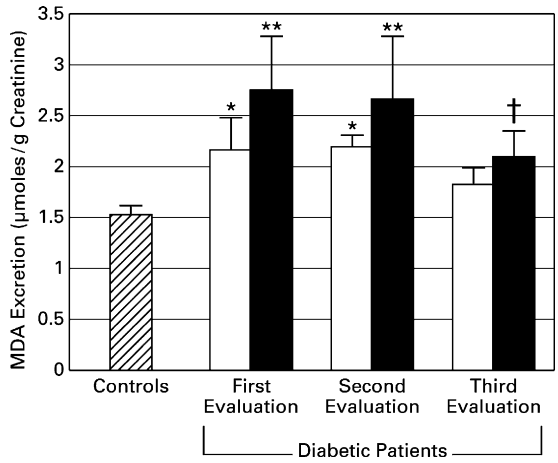

Fig. 3. Effect of glycemic control on MDA excretion. Data represent μmol MDA excreted/g creatinine ± SE. To convert MDA to micrograms, multiply by 164.2. *$P < .05$, different from controls; **$P < .005$; †$P < .05$, MDA excretion was increased in those with high vs low HbA_{1c} across all years.

waist and a relative decrease in sudomotor responses below the waist in the poorly controlled patients, a typical response to sympathetic injury (Fig. 1). The heart rate response to the Valsalva maneuver was decreased in the poorly controlled

Table 3
Effect of glycemic control on MDA excretion (μmol/g creatinine)

HbA_{1c} tertile	Lowest	Middle	Highest
First evaluation	2.21 ± 0.56	2.07 ± 0.52	3.17 ± 0.56
Second evaluation	1.63 ± 0.34	2.09 ± 0.33	3.15 ± 0.36**
Third evaluation	1.74 ± 0.26	1.61 ± 0.29	2.28 ± 0.25
Mean of 3 evaluations	1.89 ± 0.24	2.05 ± 0.26	2.56 ± 0.25*

To convert MDA to micrograms, multiply by 164.2.
* $P < .05$, greater than in the other tertiles.
** $P < .01$.

patients and the excretion of vanillylmandelic acid was decreased at the third evaluation [3].

Malondialdehyde excretion was 1.51 ± 0.20 μmol/g creatinine in the controls, and 2.43 ± 0.21, 2.39 ± 0.22, and 1.93 ± 0.21 μmol/g creatinine in the diabetic patients at the first, second, and third evaluations, respectively ($P < .005$). The increase in MDA excretion was observed only in the diabetic females (Fig. 2). Malondialdehyde excretion correlated with the HbA_{1c} at the first ($P < .01$, $r = 0.39$) and second evaluations ($P < .025$, $r = 0.37$) and for the pooled data from all evaluations ($P < .025$, $r = 0.34$). Diabetic patients were categorized as having good or poor control if their HbA_{1c} was below or above the median for the group at each evaluation as described above. This analysis revealed that at the first and second evaluations the

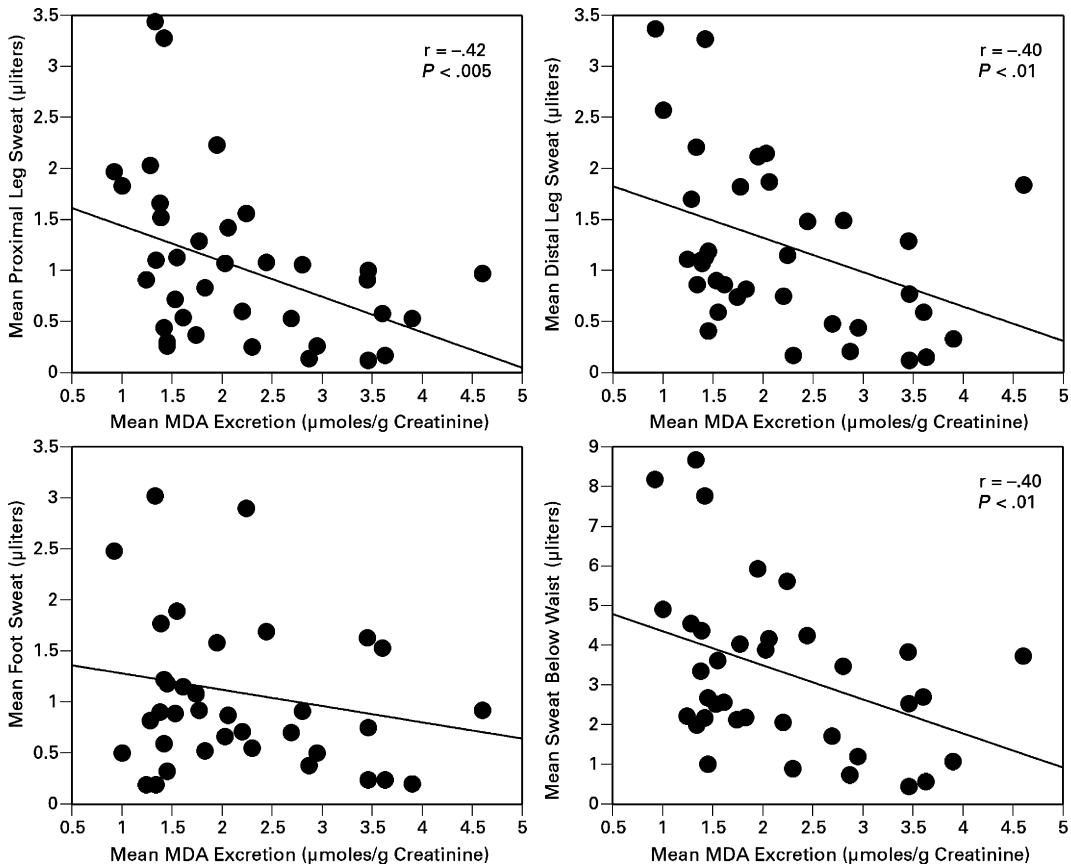

Fig. 4. Mean MDA excretion vs mean sweat production. The mean MDA excretion from the 3 evaluations was plotted against the mean sweat production at each site for each diabetic patient. To convert MDA to micrograms, multiply by 164.2.

MDA excretion in the poorly controlled patients was nearly double that in control subjects (Fig. 3). Diabetic patients with poor control had higher MDA excretion than those with good control (Fig. 3, $P < .05$). We also categorized patients each year into HbA_{1c} tertiles and confirmed that those in the highest tertile had the greatest MDA excretion, although this trend was only significant in year 2 and for the pooled data (Table 3). Malondialdehyde excretion correlated with the insulin doses of the patient ($r = 0.4$, $P < .01$), but only at the time of the second evaluation.

Sweat production correlated negatively with MDA excretion at multiple sites (Fig. 4). The mean sweat production at each site was analyzed vs the mean MDA excretion for each patient. Malondialdehyde excretion correlated negatively with sweat production in the forearm ($r = -0.34$, $P < .025$), proximal leg ($r = -0.42$, $P < .005$), and distal leg ($r = -0.40$, $P < .01$). A similar trend was seen in the foot, but the changes were not significant (Fig. 4). Malondialdehyde excretion correlated with sweating below the waist ($r = -0.40$, $P < .01$) and total sweat ($r = -0.38$, $P < .01$). The ratio of sweating above the waist to sweating below the waist tended to correlate with MDA excretion, but this was not significant ($P = .055$). We corrected for the effect of sex on MDA excretion by calculating sex-specific z scores and reanalyzing the sweating data. The correlations observed were very similar to those observed for the data without the sex correction. The MDA excretion z scores correlated positively with the ratio of sweating in the forearm to sweating in the foot at the third evaluation ($r = 0.36$, $P < .025$). Sex differences in sweating could not explain the correlation between MDA excretion and sudomotor function because there was no difference in sweating between the diabetic males and females (although there was greater sweating in the nondiabetic males vs the nondiabetic females) [17].

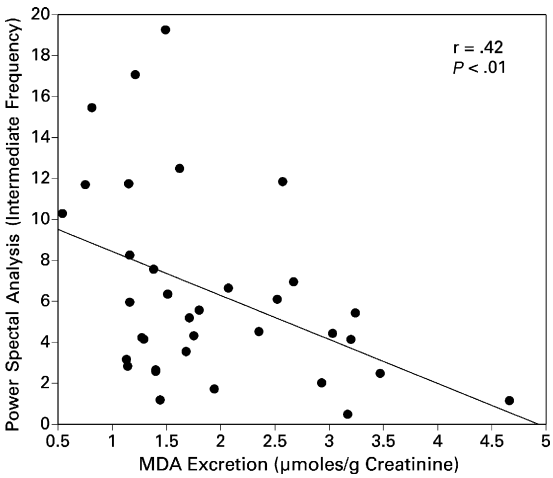

Fig. 5. Mean MDA excretion vs intermediate-frequency power spectral analysis. Data represented were gathered at the third evaluation. To convert MDA to micrograms, multiply by 164.2. The intermediate-frequency power spectral analysis is a measure of sympathetic function.

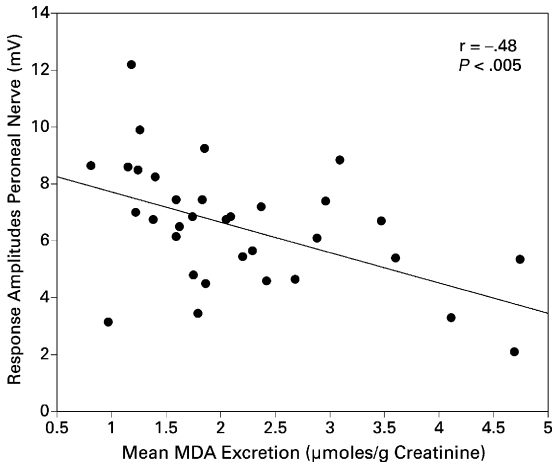

Fig. 6. MDA excretion vs peroneal nerve response amplitudes The mean results for the second and third evaluations are represented. To convert MDA to micrograms, multiply by 164.2.

Malondialdehyde excretion did not correlate with other tests of sympathetic function such as the renin/prorenin, vanillylmandelic acid excretion, or the Valsalva ratio. Malondialdehyde excretion, however, showed a negative correlation ($r = -0.42$, $P < .01$) with the intermediate frequency (0.04-0.15 Hz) of power spectral analysis, a measure of the sympathetic modulation of heart rate variability, at the third evaluation (Fig. 5). A weak correlation ($r = -0.28$, $P = .053$) was observed between MDA excretion and high-frequency power (0.15-0.40 Hz), a measure of parasympathetic modulation of heart rate variability, at the third evaluation. Malondialdehyde excretion did not correlate with beat to beat variation with deep breathing or the mean circular resultant, alternative measures of parasympathetic control of heart rate [18].

The response amplitudes of the peroneal nerves correlated with MDA excretion at the second evaluation ($r = -0.52$, $P < .0025$) and the third evaluation ($r = -0.36$, $P < .025$) (Fig. 6). Malondialdehyde excretion correlated with the response amplitudes in the median nerve only at the second evaluation ($r = -0.39$, $P < .025$). The response amplitudes in the ulnar nerve did not correlate with MDA excretion. Motor nerve conduction velocities did not correlate with MDA excretion. Malondialdehyde excretion did not correlate with any test of large fiber sensory function. The detection of a cold stimulus in the feet was better in those with high MDA excretion at the second evaluation ($P < .025$) contrary to expectations, but this pattern was not seen in the upper extremities or at other evaluations. The detection of heat did not correlate with MDA at any site at any evaluation.

4. Discussion

Oxidative stress is linked to lipid peroxidation in the central nervous system [19], peripheral nervous system [1], retina [20], and kidney [21]. Most of the data supporting this

relationship have been gathered in experimental animals. We have, however, recently reported that in type 1 diabetes mellitus, chronic hyperglycemia leads to oxidative stress, nitrosative stress, and increased lipid peroxidation, as assessed from 8-iso-$PGF_{2\alpha}$, and that the latter has an adverse effect on peripheral nerve function, especially sudomotor function [3]. Chronic hyperketonemia probably plays a contributory role [22]. There are problems, however, with using 8- iso-$PGF_{2\alpha}$ as an index of lipid peroxidation in patients with diabetes. Davi et al [23] originally reported that 8-iso-$PGF_{2\alpha}$ was increased in diabetic patients, but other groups have failed to confirm this [24]. We found that 8-iso-$PGF_{2\alpha}$ was increased in poorly controlled patients, but actually suppressed in the well-controlled patients and normal when the data from the well controlled and poorly controlled patients were combined. Moreover, the correlations between 8-iso-$PGF_{2\alpha}$ and sudomotor function we described were observed only in the feet and in some instances of borderline significance [3]. Plasma NOx, an indicator of nitrosative stress showed stronger negative correlations with sudomotor function and, in this regard, reinforced the isoprostane data. Nitrite and nitrate, and 8-iso-$PGF_{2\alpha}$ correlated well with one another [2], showed a similar sex distribution [3], and had similar effects on sudomotor function. These results were consistent with many experimental studies indicating that nitrosative stress is linked to lipid peroxidation [25] and that the latter has neurotoxic effects [26]. This is an attractive hypothesis because the formation of lipid peroxides in nerve membranes has adverse effects on their fluidity, electrical conductivity, and function. This has been difficult to test, however, because lipid peroxides are formed locally in cell membranes, are rapidly degraded, and are difficult to assess in vivo.

To extend our previous studies of the biochemical correlates of peripheral nerve injury in early diabetes we measured the excretion of MDA, a by-product of multiple free radical–initiated lipid peroxidation pathways [27]. Although a variety of lipid peroxides are increased in diabetes [27] the measurement of MDA is of special interest because the experimental literature [1,26] as well as the clinical literature [27-29] linking neuropathy to lipid peroxidation have focused on MDA. We observed that MDA excretion was increased, especially in the poorly controlled patients, and the effect was much more evident in females. The effects of glycemia and sex on MDA were similar to previously reported effects of these factors on NOx and 8-iso-$PGF_{2\alpha}$ in this cohort [2,3]. Others have observed this sex effect on nitrate biosynthesis [30] and 8-iso-$PGF_{2\alpha}$ [31], but we are unaware of previous data indicating an effect of sex on MDA. These results, taken together, do not support the described antioxidant effect of endogenous estrogen [32], and we are unable to explain the effect of sex on these biochemical measures of oxidative stress. This confounding factor cannot explain the negative correlations between MDA excretion and sudomotor function because the latter were observed even after we converted the MDA data to sex-specific z scores. These results reinforce previous studies of this cohort in which we demonstrated negative correlations between NOx and 8-iso-$PGF_{2\alpha}$ and sudomotor function. Moreover, we observed negative correlations between MDA excretion and response amplitudes of the peroneal nerves (Fig. 6). Thus, we interpret these data to support experimental data, indicating that nerve damage in diabetes is linked to lipid peroxidation [1,26]. Our results indicate this mechanism plays a role in the initiation of peripheral nerve dysfunction, before the onset of symptoms or even subclinical neuropathy. We are unable to explain, however, why there was no evidence of increased MDA in the diabetic males who may be even more vulnerable to the adverse consequences of lipid peroxidation than are females. Impotence is frequently the first symptom of neuropathy, and Tuncayengin et al [28] have recently reported that diabetic patients with erectile dysfunction have higher penile MDA than impotent nondiabetic patients. It appears that in males, abnormalities in lipid peroxidation may take place locally in vulnerable tissues yet not be quantitatively important enough in the whole patient to affect MDA excretion.

Although we have documented that multiple metabolic processes linked to oxidative stress have adverse effects on sudomotor function, such correlations were not evident in most tests of somatosensory nerve function. Our analysis of data for NOx and 8-iso-$PGF_{2\alpha}$ also suggested that sympathetic sudomotor dysfunction was especially vulnerable to the effects of oxidative stress [3]. We suspect that oxidative stress would have similar effects on somatosensory function if we had followed our patients longer [29]. Although this represents a limitation of this study, we specifically elected to focus on early diabetes because patients with chronic disease frequently have multiple confounding problems, such as hyperlipidemia and hypertension, each of which might cause oxidative stress and compromise our analysis of its relation to hyperglycemia.

What is the clinical significance of these findings? We have no evidence to support our assumption that the sudomotor disturbances we describe lead to sympathetic neuropathy. None of the patients complained of decreased sweating, hyperhidrosis, or orthostatic intolerance during the course of this study. Sympathetic nerve injury is nevertheless worrisome in early diabetes because it could eventually predispose patients to iatrogenic hypoglycemia. Recent evidence suggests that clinical hypoglycemia unawareness is largely the result of disturbances in the activation of sympathetic neurons [33,34].

Finally, the results of this study have therapeutic implications. Because oxidative stress and lipid peroxidation begin to damage the peripheral nervous system during the first few years of diabetes, this is the ideal time to initiate antioxidant trials even in asymptomatic patients. This is feasible. α-Lipoic acid, for example, is nontoxic, prevents

neuropathy in rats [35], and suppresses oxidative stress clinically [36]. Previous clinical trials with α-lipoic acid have been promising but failed to affect neurophysiologic parameters [37]. Although these results suggest this agent may be ineffective in man, we believe the disappointing results stem from the fact that the studies were performed in patients with chronic disease [37]. Nevertheless, most published trials have indicated that this agent suppresses the symptoms of diabetic neuropathy slightly [38]. Vitamin E has similarly had beneficial effects on renal hyperfiltration and retinal blood flow in early diabetes [39]. Future antioxidant trials should be designed to prevent complications, and be initiated early in diabetes, perhaps within the first few years of diagnosis.

In summary, MDA excretion was increased in patients with poorly controlled recent-onset type 1 diabetes mellitus. Malondialdehyde excretion varied inversely with sudomotor function and peroneal nerve response amplitudes. These results suggest that hyperglycemia stimulates oxidative stress and lipid peroxidation in early diabetes and these metabolic processes are damaging to peripheral nerves.

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