Hydroperoxides in Plasma Are Reduced by Intensified Insulin Treatment

A randomized controlled study of IDDM patients with microalbuminuria

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OBJECTIVE — An association between reactive oxygen species and diabetic micro- and macrovascular complications has been proposed. In the present study, we have examined the effect of an improved blood glucose control on plasma levels of hydroperoxides in patients with IDDM.

RESEARCH DESIGN AND METHODS — Subjects included 30 young IDDM patients with microalbuminuria who were randomized to receive either continuous subcutaneous insulin infusion (CSII) by a portable insulin pump (n = 15) or conventional insulin treatment (CIT) (n = 15) for 24 months. Plasma levels of hydroperoxides were measured by the ferrous oxidation with Xylenol Orange, version 2 (FOX2) assay. This method measures total lipid hydroperoxides and, unlike other methods, does not suffer from extraction losses.

RESULTS — The mean HbA_{1c} level was lower in the CSII group at the end of the study than in the CIT group: (mean [95% CI]) 8.6 (8.1–9.1) vs. 9.6 (9.0–10.3)%, respectively ($P < 0.002$). The level of plasma hydroperoxides was very similar at the start of the study but was significantly lower in the CSII group compared with the CIT group at the end of the study: 2.9 (2.1–3.7) vs. 4.3 (3.2–5.4) μmol/l, respectively ($P < 0.02$). In the CSII group, hydroperoxides were reduced by 31% from baseline ($P < 0.001$), whereas there was no change in levels of hydroperoxides in the CIT group. Mean hydroperoxide levels correlated with mean HbA_{1c} during the study (r = 0.39, $P < 0.04$). Hydroperoxide levels were associated with the levels of microalbuminuria (r = 0.45, $P < 0.02$).

CONCLUSIONS — This study provides support for the hypothesis that hyperglycemia is an important factor in the generation of hydroperoxides, and, thus, reactive oxygen species, in the circulation of IDDM patients.

IDDM is postulated to be associated with increased oxidative stress, which may contribute to micro- and macrovascular late complications (1,2). One putative explanation for how diabetes can induce oxidative stress is that glucose can generate reactive oxygen species (3).

In the presence of free metal ions, glucose can undergo a process called autoxidation (4). In vitro, this process has been shown to generate toxic free radicals and hydroperoxide (4), which mediates endothelial cell dysfunction (5). Hyperglycemia has been shown to augment membrane lipid peroxidation of rat glomeruli in vitro (6). LDLs in patients with poorly controlled type 1 diabetes have an increased susceptibility to LDL oxidation, leading to reduced antioxidant defense (7), but this is not the case during good blood glucose control (8). Dietary supplementation with the antioxidant vitamin E was recently reported to prevent the early changes associated with diabetic renal dysfunction in rats with diabetes (9).

A number of studies have assessed circulating lipid peroxidation products as an index of oxidative stress in patients with NIDDM (10–15), but there have been few studies in IDDM patients (16,17). Most investigations of oxidative stress in diabetes have measured thiobarbituric acid reactive substances (TBARS). This simple assay measures substances other than parameters of lipid peroxidation and is affected by the lipid content of the sample (18,19).

In the present study, the ferrous oxidation with Xylenol Orange, version 2 (FOX2) assay, coupled with the selective hydroperoxide reductant triphenylphosphine, was used to determine levels of lipid hydroperoxides (20,21). This method measures total lipid hydroperoxides and, unlike other methods, does not suffer from extraction losses (20). We have previously shown elevated levels of hydroperoxides in plasma from patients with IDDM with poor glycemic control compared with healthy subjects (20,21).

In the present study, we have examined whether improved glycemic control by intensified insulin treatment can reduce the plasma lipid hydroperoxide levels in young subjects with IDDM and early nephropathy. In addition, we have studied the relationship between hydroperoxide levels and HbA_{1c}, as well as the urinary albumin excretion rate (UAER).

RESEARCH DESIGN AND METHODS

Subjects

There were 371 patients with IDDM between the ages of 10 and 30 years and with a diabetes duration of >5 years that

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Abbreviations: AGE, advanced glycation end product; CIT, conventional insulin treatment; CSII, continuous subcutaneous insulin infusion; FOX2, ferrous oxidation with Xylenol Orange, version 2; HPLC, high-performance liquid chromatography; TBARS, thiobarbituric acid reactive substances; UAER, urinary albumin excretion rate.

were screened for persistent microalbuminuria. Of the 45 patients found to have persistent microalbuminuria, 33 patients agreed to take part in a prospective randomized study concerning the importance of improved blood glucose control in adolescents and young adults (22). Persistent microalbuminuria was defined as a UAER of 15–200 μg/min in at least two of three overnight urine samples taken during a 12-month period. Patients gave their written consent after having received thorough information. The protocol was approved by the Regional Ethics Committee.

Study design

Patients were randomized to either continuous subcutaneous insulin infusion (CSII) by a portable insulin pump or conventional insulin treatment (CIT) (multiple injections or two to three injections per day) and stratified using the following factors in order of priority: mean 1-year prestudy HbA_{1c}, duration of diabetes, median 1-year prestudy UAER, age, and sex. Randomization and stratification was done by computer, and the statistician performing it had no knowledge of the patients' identities or modes of treatment. The patients were followed regularly by the same investigator (H.-J.B.) at the outpatient clinic at 2-month intervals during the 24-month study period.

There were 3 patients who did not follow the study protocol and were therefore taken out of the study. Kidney biopsies were taken at baseline and at the end of the study in 18 of the 30 patients to investigate the effects of improved blood glucose control on changes in diabetic glomerulopathy. Results of this study have been published earlier (23,24). None of the 30 patients had proliferative retinopathy. One patient had blood pressure levels higher than 140/90 mmHg. Although initially hypertensive (150/98 mmHg), this patient's blood pressure declined to 145/85 mmHg after 6 months without antihypertensive treatment.

Preparation of plasma

Nonfasting blood samples were collected by venipuncture into sampling vials (10 ml) containing heparin. Plasma was obtained by centrifugation at 2,000*g* for 10 min at room temperature. The samples were stored at −70°C until analysis.

Clinical laboratory measurements

HbA_{1c} was analyzed with high-performance liquid chromatography (HPLC) (Diamat; Biorad, Richmond, CA). Normal range was 4.3–6.1%, with an interassay coefficient of variation of 3%. UAER was measured in timed overnight urine samples at 2-month intervals. The albumin concentration was measured by immunoturbidimetry in samples kept at 4°C from 1 to 3 days. The interassay coefficient of variation was 4.7% in the range of 10–50 mg/l. Blood pressure was measured sitting with a conventional mercury sphygmomanometer after a 10-min rest. Diastolic pressure was recorded after the disappearance of the Korotkoff V sound.

Total serum cholesterol, HDL cholesterol, and triglyceride concentrations were measured in nonfasting blood samples by enzymatic methods (25,26). LDL cholesterol was calculated using the Friedewald equation (27). No triglyceride concentrations were >4.0 mmol/l.

Measurement of indices of lipid peroxidation

Hydroperoxide. Total plasma hydroperoxide content was determined using the FOX2 assay (for lipid hydroperoxide) (20,28). Briefly, 90-μl aliquots of plasma were transferred into six (1.5 ml) microcentrifuge vials, and 10 μl of methanol was added to the remaining three vials. This generated blank and test samples, respectively. All vials were then vortexed and incubated at room temperature for 30 min before the addition of 900 μl FOX2 reagent with mixing. After incubation at room temperature for a further 30 min, the vials were centrifuged at 12,000*g* at 25°C for 10 min. Absorbance of the supernatant was then determined at 560 nm. Lipid hydroperoxide content in the plasma samples was determined as a function of the mean absorbance difference of samples with and without elimination of hydroperoxide by triphenylphosphine. The coefficient of variation for individual plasma samples using this method is typically <5% (20,28). Inclusion of butylated hydroxytoluene in plasma samples did not influence the measurements. As previously shown, this method is not influenced by diurnal, fasting, or nonfasting conditions (20). In native plasma, >65% of the total hydroperoxide content is confined to LDL, 17% to VLDL, and 11% to HDL cholesterol (29). Glucose and lipids do not influence the hydroperoxide assay, as shown by the lack of correlation between these parameters in patients with type 2 diabetes and control subjects (20,21).

TBARS. Malondialdehyde and related aldehydes (TBARS) in whole samples were determined by mixing of plasma samples (100 μl) with 0.67% thiobarbituric acid (1,000 μl) and 20% trichloroacetic acid (500 μl), followed by incubation at 100°C for 20 min. The absorbance of the supernatant was monitored at 532 nm after centrifugation at 12,000*g* for 5 min. The concentration of lipid peroxidation products was calculated as malondialdehyde equivalents using the extinction coefficient for the malondialdehyde-thiobarbituric acid complex of $1.56 \times 10^5\ mol^{-1} \cdot cm^{-1}$ (30). Inclusion of butylated hydroxytoluene in plasma samples/reagents did not influence the measurements.

α-Tocopherol (vitamin E) analysis. 200 μl of plasma was mixed with 500 μl of ethanol (containing 50 ng/ml of γ-tocopherol as an internal standard), total lipids were partitioned with hexane, and the solvent was subsequently dried under a stream of nitrogen. The residue was then redissolved in 200 μl acetonitrile. HPLC separation was performed on a Hypersil-ODS column (10 cm × 5 mm, particle size 5 μmol/l; Chrompack, Bergen op Zoom, The Netherlands). Tocopherols were monitored fluorometrically (emission: 295 nm; excitation: 340 nm) (20).

Data analysis

Comparisons between groups were analyzed by two-tailed Student's *t* test, or by Mann-Whitney U test when the data did not show a normal distribution. UAER data were logarithmically transformed before statistical analysis. Differences between baseline and follow-up were compared using a paired Student's *t* test or Wilcoxon's test. The relationship between variables was assessed by Spearman's rank correlation. The two treatment groups were combined in the analysis of the correlations between mean levels of hydroperoxides and HbA_{1c} during the study, since the mode of insulin administration by itself is not thought to influence the hydroperoxide levels. Results are expressed as means (95% CI). The level of significance was set at $P < 0.05$. Calculations were performed using the Number Cruncher Statistical System (Kaysville, UT).

RESULTS

— The clinical characteristics at baseline of the participating patients are given in Table 1.

HbA_{1c}

From baseline to the end of the study, HbA_{1c} in the CSII group fell from 10.1

Table 1—*Baseline patient characteristics after randomization in the CSII group and the CIT group*

	CSII group	CIT group
n	15	15
Sex (F/M)	8/7	7/8
Age (years)	18 (14–29)	19 (17–29)
Duration (years)	10 (5–18)	12 (8–16)
HbA_{1c} (%)	10.1 (8.1–12.6)	9.8 (7.9–12.2)
UAER (μg/min)	25 (15–131)	32 (15–194)
Systolic blood pressure (mmHg)	118 (94–142)	124 (114–150)
Diastolic blood pressure (mmHg)	80 (56–92)	84 (74–98)

Data are *n* or median (range) except UAER data, which are means (range) of three measurements made in the year preceding the study.

(9.2–11.1) to 8.6 (8.1–9.1)% ($P < 0.002$, Wilcoxon), whereas there was no significant change in HbA_{1c} in the group receiving CIT, 9.9 (8.8–11.0) vs. 9.6 (9.0–10.3)% (Table 2). The HbA_{1c} levels were also lower in the CSII group at the end of the study compared with those of the CIT group, 8.6 (8.1–9.1) vs. 9.6 (9.0–10.3)%, respectively ($P < 0.002$, Student's *t* test).

Hydroperoxides

Plasma hydroperoxides fell by 31% in the CSII group from baseline to the end of the study: 4.2 (3.7–4.8) vs. 2.9 (2.1–3.7) μmol/l, respectively ($P < 0.001$, Student's *t* test). There was no change in plasma hydroperoxide levels in the CSII group after 12 months of treatment, despite a trend toward improved glycemic control. There were no significant changes in hydroperoxides in the CIT group from the start to the end of the study: 4.4 (3.8–4.9) vs. 4.3 (3.2–5.4) μmol/l, respectively (Fig. 1). At 24 months, the hydroperoxide levels were 33% lower in the CSII group compared with the CIT group: 2.9 (2.1–3.7) vs. 4.3 (3.2–5.4) μmol/l, respectively ($P < 0.02$, Mann-Whitney *U* test).

TBARS, total cholesterol, LDL cholesterol, α-tocopherol, insulin, and smoking

There were no significant changes in TBARS, triglycerides, total cholesterol, or LDL cholesterol during the study. Plasma α-tocopherol levels in the CSII and CIT groups were similar at the start of the study: 7.8 (6.1–9.5) and 8.1 (6.8–9.4) μmol/l, respectively. At the end of the study, there was a slight nonsignificant increase in plasma α-tocopherol levels in the CSII group of 18% and in the CIT group of 35%. There were no significant differences between the CSII group and the CIT group regarding insulin dose at the start of the study: 0.99 (0.90–1.08) vs. 0.94 (0.86–1.02) $U \cdot kg^{-1} \cdot day^{-1}$, respectively (NS). A small nonsignificant decrease in the insulin dose of 0.04 $U \cdot kg^{-1} \cdot day^{-1}$ was seen from the start to the end of the study in the CSII group, whereas the CIT patients decreased the insulin dose by 0.03 $U \cdot kg^{-1} \cdot day^{-1}$ (NS). Table 2 summarizes the clinical characteristics of the CSII and CIT groups during the study.

There were no differences in cigarette consumption between the CSII group (10 smokers) and the CIT group (8 smokers) at the start of the study. There was no cross-sectional association between smoking and plasma hydroperoxides. In a logistic regression analysis, there was no effect of changes in smoking habits on changes in plasma hydroperoxide levels.

Hydroperoxides versus HbA_{1c} and UAER

A significant association was found between mean HbA_{1c} levels and mean hydroperoxide levels during the study: $r = 0.39$, $P < 0.04$ when the CSII and CIT groups were combined. As shown in Table 3, there was also a significant cross-sectional correlation between peroxides and UAER: $r = 0.45$, $P < 0.02$. In a follow-up measurement of microalbuminuria (median of three measurements) 5 years after the study was initiated, there was a correlation between hydroperoxide at the start of the study and UAER 5 years after: $n = 30$, $r = 0.39$, $P < 0.04$. UAER did not change significantly in the CSII or the CIT groups from baseline, 45 (15–76) and 41 (16–67) μg/min, respectively, to the end of the study at 24 months, 64 (11–117) and 55 (15–95) μg/min, respectively.

There were no significant correlations between hydroperoxides and total cholesterol, triglycerides, α-tocopherol, blood glucose, or HbA_{1c} at the baseline of the study (Table 3).

Table 2—*Change in HbA_{1c}, TBARS, α-tocopherol, total cholesterol, and LDL cholesterol in the CSII group (n = 15) and the CIT group (n = 15)*

	0 months	12 months	24 months
HbA_{1c} (%)			
CSII	10.1 (9.2–11.1)	8.8 (8.0–9.7)	8.6 (8.1–9.1)*†
CIT	9.9 (8.8–11.0)	10.2 (9.0–11.4)	9.6 (9.0–10.3)
TBARS (μmol/l)			
CSII	2.4 (1.9–2.8)	2.1 (1.8–2.3)	2.4 (2.1–2.7)
CIT	2.6 (2.3–3.0)	2.2 (2.0–2.4)	2.4 (2.1–2.6)
α-Tocopherol (μg/ml)			
CSII	7.8 (6.1–9.5)	8.0 (6.7–9.2)	9.2 (7.7–10.6)
CIT	7.5 (6.4–8.7)	7.8 (6.5–9.2)	10.1 (7.5–12.7)
Total cholesterol (mmol/l)			
CSII	5.1 (4.5–5.7)	4.6 (4.2–5.1)	4.9 (4.3–5.6)
CIT	5.3 (4.6–5.9)	5.3 (4.7–5.9)	5.2 (4.6–5.8)
Triglycerides (mmol/l)			
CSII	1.2 (1.0–1.5)	1.0 (0.8–1.2)	1.1 (0.9–1.4)
CIT	1.4 (1.0–1.8)	1.4 (0.7–2.0)	1.3 (0.9–1.6)
LDL cholesterol (mmol/l)			
CSII	3.2 (2.76–3.8)	2.9 (2.5–3.3)	3.1 (2.6–3.7)
CIT	3.2 (2.7–3.6)	3.4 (2.8–4.1)	3.4 (2.7–4.0)

Data are means (95% CI). For α-tocopherol at 24 months, CSII *n* = 14 and CIT *n* = 13. *$P < 0.002$ vs. baseline; †$P < 0.02$ vs. CIT group.

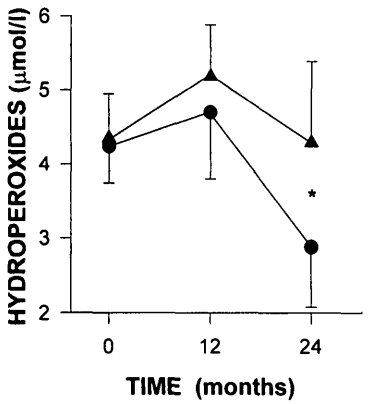

Figure 1—*Plasma hydroperoxide levels (mean, 95% CI) during the study in the CIT group* (n = 15) (▲) *and the CSII-group* (n = 15) (●). *In the CSII group, a fall was seen from 0 to 24 months* ($P < 0.001$). *$P < 0.02$, CIT vs. CSII at 24 months.*

CONCLUSIONS — This study is the first to report that improved glycemic control reduces oxidative stress as assessed by plasma hydroperoxide levels in patients with IDDM receiving intensified insulin treatment with CSII. In addition, we have found a significant association between plasma lipid hydroperoxides and microalbuminuria and between the mean levels of HbA_{1c} and hydroperoxides over 2 years.

There are several putative mechanisms by which intensified insulin treatment can influence the hydroperoxide levels. First, improved blood glucose control, as observed in our intervention group, can also influence the antioxidant enzymes. This has been shown by Oda et al. (31), who found that superoxide dismutase was decreased in NIDDM patients and that the enzyme was inactivated by in vitro glycation. In contrast to this, Pieper et al. (32) showed increased levels of catalase and Cu,Zn-superoxide dismutase in diabetic animals as a sign of chronic increase in hydroperoxides. This increase in antioxidant enzymes is normalized by insulin treatment, which restores normoglycemia (33). Second, as proposed by Wolff and Dean (3), glucose can oxidize (autoxidative glycosylation) when catalyzed by transition metals and form hydrogen peroxides and reactive dicarbonyl sugars.

Third, hyperglycemia leads to modifications of proteins by nonenzymatic glycation (1,34), forming advanced glycation end products (AGEs), which are sources of free radical production (1). One product of both oxidation and nonenzymatic glycation of protein is carboxymethyllysine, a structurally defined AGE. Because carboxymethyllysine has been shown to be the quantitatively most important AGE epitope formed in vitro, one could speculate whether the pathological effects of AGEs are mediated in part by increased oxidative stress, as indicated in a recent paper by Schleicher et al. (35). It remains to be seen, however, whether reduced levels of plasma hydroperoxides can also diminish tissue or serum levels of AGEs such as carboxymethyllysine.

Finally, there is a potential antioxidant effect of insulin-stimulating nitric oxide production (36,37), since nitric oxide has been shown in vitro to possess both pro- and antioxidative properties, depending on the levels of other reactive oxygen species (38). In the present study, however, insulin levels were the same at the start of the study and did not change in the two groups during the study. We, therefore, think it is unlikely that insulin is the cause of the reduced plasma hydroperoxide levels observed in the CSII group.

The significant association between the mean HbA_{1c} during the study and the mean hydroperoxide levels supports the notion that hyperglycemia is important for the generation of oxygen species. On the other hand, there was no cross-sectional relationship between hydroperoxides and blood glucose or HbA_{1c}, which is in accordance with previous studies with IDDM (39), NIDDM, and control subjects (20,21).

Part of the effect of reduced mean blood glucose on the development and progression of diabetic late complications might be brought about by a reduction in plasma hydroperoxides. It is, therefore, of interest that there was a significant cross-sectional association between hydroperoxides and UAER. In animal experiments, treatment with high doses of an antioxidant (d-α-tocopherol) normalized the glomerular filtration rate and the filtration fraction and kept the UAER threefold lower in the treatment group compared with untreated rats with diabetes (9). The aim of the present study was not to investigate whether a reduced plasma hydroperoxide level can prevent the progression of microalbuminuria. However, with a >10% reduction in HbA_{1c}, as observed in the CSII group, one would expect a long-term risk reduction of progression to proteinuria of ~37%, which was found in the Diabetes Control and Complications Trial (40).

Hydroperoxides are the initial stable products formed during peroxidation of unsaturated lipids such as fatty acids and cholesterol. The FOX2 technique, used in our study, measures all classes of hydroperoxides in plasma and requires no plasma lipid extraction step. It, therefore, does not suffer from extraction losses, as occurs with other methods (20). The levels of plasma hydroperoxides measured in the present study are similar to those measured by the same FOX2 technique and by other precise methods in NIDDM patients (20) and, recently, in IDDM patients (39).

Santini et al. (39) have recently shown that hydroperoxide levels from frozen plasma measured by the same method used by us are increased more than threefold in patients with IDDM without microvascular

Table 3—*Cross-sectional analysis of the whole group* (n = 30) *before intervention*

	TBARS	α-Tocopherol	Cholesterol	Triglycerides	HDL cholesterol	Glucose	HbA_{1c}	UAER
Hydroperoxides	−0.15	0.20	−0.05	0.04	−0.15	0.22	0.22	**0.45**
TBARS		0.09	−0.02	0.06	0.17	0.10	−0.16	−0.28
α-Tocopherol			**0.64**	0.31	0.34	−0.16	0.11	−0.02
Cholesterol				**0.65**	0.26	0.06	**0.41**	0.13
Triglycerides					−0.02	0.30	0.34	0.25
HDL cholesterol						0.12	0.06	−0.15
Glucose							**0.49**	0.26
HbA_{1c}								0.31

Analysis of UAER is based on the mean of two measurements (at −2 and 0 months). $r > 0.36$ (shown in bold) is significant ($P < 0.05$).

complications, compared with carefully matched control subjects. It is, therefore, of interest that in the present study we show in a prospective controlled manner that intensified insulin treatment can reduce the increased hydroperoxide levels in IDDM patients.

There were no differences in plasma TBARS levels in either the CSII or the CIT group in this study. Moreover, we did not find any association between either UAER or HbA_{1c} and TBARS. This may be because of the poor specificity of method or, alternatively, that an improved blood glucose does not influence the free oxygen radicals measured by the TBARS assay (18,19).

There was a nonsignificant increase in α-tocopherol in both the intervention group and the control group at the end of the study. α-Tocopherol contributes only 5% to the total radical antioxidant parameter (41). Hence, one would not expect that a small increase in α-tocopherol would influence the hydroperoxide levels. The increase found may be due to a change in dietary habits. However, as reflected in the α-tocopherol levels, the subjects in this study did not receive vitamin E supplementation. Because there were no changes in total cholesterol, triglycerides, or α-tocopherol levels during the study, we did not adjust plasma peroxides for these variables as has been suggested earlier (21).

This study provides further support for the hypothesis that hyperglycemia is an important factor in the generation of hydroperoxides in the circulation of patients with IDDM. Further, we have shown that there is an association between plasma hydroperoxide levels and microalbuminuria. Thus, oxidative status may possibly influence the progression of diabetic nephropathy in man, as has recently shown in the diabetic rat (9).

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