Depletion of Total Antioxidant Capacity in Type 2 Diabetes

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The purpose of the study was to examine the relationship between antioxidant depletion, glycemic control, and development of chronic complications in a controlled population of type 2 diabetic patients. Fifty age-matched type 2 diabetic patients receiving sulfonylureas but not insulin treatment were screened and assigned to two groups based on the presence or absence of proteinuria. A third group of normal subjects without diabetes were also enrolled in the study. All subjects in the three groups were Egyptians who were matched for body weight, and the two diabetic groups were also age-matched. Plasma glucose and fructosamine levels were higher in the two groups of diabetic patients versus the control group, but lipid peroxide levels were higher only in the patients with proteinuria. Compared with the control group, the total antioxidant capacity was depleted in the two diabetic groups, but the depletion was more severe in patients with proteinuria. Thus, the mean Trolox equivalent antioxidant capacity (TEAC) of the control group was 2.7 ± 0.45, versus 1.7 ± 0.5 ($P < .001$) in the patients without proteinuria. Furthermore, the TEAC measured in patients with proteinuria, who also had more diabetic complications, was lower (1.4 ± 0.5, $P < .001$) than the TEAC in patients without urinary protein. In conclusion, a depletion of the total antioxidant capacity is associated with a higher incidence of diabetic complications.

NUMEROUS STUDIES have shown that while different indices of free-radical damage increase, there is a decrease in the concentration of various individual antioxidant substances, indicating the presence of oxidative stress in diabetes.[1-8] It has also been suggested that there is a link between the development of microvascular and macrovascular diabetic complications and oxygen free-radical damage.[9-11] Many biochemical pathways, including glucose autoxidation, polyol and prostanoid biosynthesis, and protein glycation, which are associated with hyperglycemia, have been implicated in the increased free-radical production in diabetic subjects.[7,9,10] More recently, it has also been suggested that hyperglycemia may induce the development of diabetic complications through the activation of protein kinase C (PKC) isoenzymes.[12] Consistent with this hypothesis, it has been reported that free radicals can interfere with various biological processes by the activation of PKC.[13] Since hyperglycemia generates increased free-radical production,[3,4,7,9] a plausible mechanism exists by which it may initiate the cascade of events that result in the development of diabetic complications.[12] However, there is no consensus on either the cause of oxidative stress in diabetes or the role played by oxidative stress in diabetic complications. Whereas some investigators have suggested that oxidative stress has a causative role in the development of diabetic complications, others have proposed that oxidative stress may merely be a common consequence of tissue damage reflected in the appearance of complications.[1-11,14]

Evidence of oxidative stress in diabetes has been provided either by the increased levels of degradative products of reactive oxygen species (ROS),[1-5,11,14-16] deficiencies of specific antioxidants,[3,4,6,17] or reductions in total antioxidant activity in diabetic patients.[17] However, relationships between the antioxidant status, glycemic control, and the risk for development of chronic complications in individuals with diabetes are not completely clear. The purpose of the present study was to compare the total antioxidant capacity in nondiabetic individuals versus two groups of diabetic subjects whose metabolic syndrome is distinguished by the presence of proteinuria.[18]

SUBJECTS AND METHODS

Fifty Egyptian men and women with type 2 diabetes and 20 normal subjects without family history of the disease were enrolled in the study. Maiden names and family names were used to determine familial identities, and no first-degree relatives were present among the study participants. All study subjects were matched for body weight. The patients with type 2 diabetes were aged-matched and treated with oral sulfonylureas alone and were not receiving any other medications. After provision of informed consent, diabetic patients were screened for proteinuria with Albustix (Miles Labs, Elkhart, IN) and assigned to two groups, each with 25 subjects distinguished by the absence (group 1) or presence (group 2) of urinary protein. Proteinuria was confirmed after three consecutive positive Albustix tests. The presence of secondary complications in diabetic patients was examined at enrollment in the study. The examination included tests for peripheral vascular and neurological diseases, coronary heart disease (CHD) assessed by electrocardiogram, and retinopathy assessed by fundus examination. Ophthalmoscopy through dilated pupils was performed by the same individual. The characteristic features of the two patient groups and the controls are shown in Table 1. Following a 12-hour fast, blood samples were taken from all subjects. In addition, 2-hour postprandial blood samples were obtained from the diabetic patients. All blood samples were centrifuged immediately to obtain plasma aliquots that were either used immediately for glucose assay or frozen at −80°C until needed for other assays.

Biochemical Assays

Plasma glucose levels were determined by the glucose oxidase procedure performed with an autoanalyzer (Beckman, Fullerton, CA). Triglyceride, free fatty acid, and cholesterol levels were measured using assay kits (Wako Pure Chemical Industries, Richmond, VA). Plasma fructosamine, an index of glycemic control comparable to glycosylated hemoglobin,[19] was determined with an assay kit (Quimica Clinica

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Table 1. Characteristic of the Study Subjects (mean ± SD)

Characteristic	Control	Group 1 (no proteinuria)	Group 2 (proteinuria)
No. of subjects	20	25	25
Sex ratio (men/women)	14/6	2/23	9/16
Age (yr)	33 ± 6.7	46.9 ± 5.5	49.3 ± 7.0
BMI (kg/m²)	23.1 ± 1.3	24.8 ± 2.0	23.4 ± 2.0
Duration of diabetes (yr)	—	4.6 ± 2.5 (2 mo-7 yr)	8.2 ± 8.0 (2-20 yr)*
No. of patients with complications	—	7 Retinopathy	16 Retinopathy, 7 CHD

Abbreviation: BMI, body mass index.
*$P < .5$.

Tarragona, Spain). This assay is a colorimetric procedure in which Nitro Blue Tetrazolium is reduced by the action of fructosamine to form formazane.[19] Insulin levels were determined by a radioimmunoassay kit (Linco, St Louis, MO), and plasma lipid peroxide levels were estimated with a lipid peroxidation assay kit (Calbiochem, La Jolla, CA) that measures both malondialdehyde (MDA) and 4-hydroxyalkenal (4-HNE). Plasma total antioxidant capacity was estimated by the ABTS* [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolorization assay involving preformed ABTS* radical cation and using Trolox (Aldrich Chemicals, Milwaukee, WI) as a standard.[20] In this assay, ABTS is made to react with potassium persulfate in the absence (blank) or presence of standards and samples, and the absorbance reading is taken at 734 nm. The total antioxidant capacity in a sample is then assessed as the Trolox equivalent antioxidant capacity (TEAC). A value of 1 TEAC in a sample is defined as a concentration that is equivalent to 1 mmol/L Trolox, a water-soluble analog of alpha-tocopherol (vitamin E).

Data Analysis

The data are presented as the mean ± SD and were evaluated for statistical significance using an ANOVA computer program (GraphPad, San Diego, CA) for a comparison of the differences among the three study groups. Depending on the outcome of the test, Bonferroni correction was used to assess the significance of differences among the three groups. In all tests, a P value less than .05 was accepted as statistically significant.

RESULTS

The mean duration of diabetes was longer for group 2 patients with proteinuria ($P < .05$) versus group 1 patients without proteinuria (Table 1). The most significant diabetic complications in the patients were CHD and retinopathy. Group 1 patients without proteinuria had fewer complications (only seven retinopathies) than group 2 patients with proteinuria (Table 1). Group 2 had a total of 23 cases with retinopathy (16 cases) or CHD (seven cases). Table 2 summarizes the biochemical characteristics of the three groups. As expected, the mean fasting and postprandial plasma glucose levels were higher in the two diabetic groups than in the control subjects. These higher glucose levels corresponded to higher plasma fructosamine levels in both diabetic groups versus the control group. Oxidative stress was assessed in all three groups by lipid peroxide levels and total antioxidant capacity (Table 3). Compared with the control subjects, lipid peroxide levels were modestly elevated in group 1, but they were significantly higher in group 2. The total antioxidant capacity of plasma samples was significantly depleted for both groups of diabetic patients versus the control subjects. However, the antioxidant depletion was more pronounced in diabetic patients with proteinuria (group 2) versus those without proteinuria (group 1), suggesting that the total antioxidant capacity in diabetic patients may be a more sensitive indicator of oxidative stress than plasma lipid peroxide levels.

DISCUSSION

We have shown that compared with nondiabetic subjects, type 2 diabetics are subjected to severe oxidative stress identifiable as a depletion of the total antioxidant capacity in the face of increased levels of lipid peroxides, which are degradative products of ROS. Our data are consistent with previous studies that showed increased levels of various indices of ROS in individuals with diabetes,[1-5,8-11,14-16] as well as studies showing beneficial effects of antioxidant therapy on the metabolic condition of these patients.[1,3,9,10,21-23] Lipid peroxidation is a well-established mechanism of oxidative damage caused by ROS, and the measurement of the aldehydes MDA and 4-HNE provides a convenient index of lipid peroxidation.[16,24] Since the classic determination of MDA alone by the thiobarbituric acid assay is affected by many interfering agents and has poor reproducibility,[25] we measured MDA and 4-HNE levels in the present study and found them to be elevated in diabetic patients. However, this assay for lipid peroxides was not sensitive enough to differentiate the severity of oxidative damage in the two diabetic groups studied. In contrast, the newly developed total antioxidant capacity assay[20] appears to be a very sensitive method capable of measuring even subtle differences between the two conditions of the metabolic syndrome of type 2 diabetes distinguished by the presence of proteinuria.[18] The impressive

Table 2. Biochemical Measurements (mean ± SD)

Parameter	Control	Group 1	Group 2
FPG (mg/dL)	82.6 ± 8.5*	180 ± 54	191 ± 44.5
PPG (mg/dL)	102.5 ± 9.8*	228 ± 60	249 ± 45
Fasting insulin (μU/mL)	6.9 ± 3.6	13.6 ± 9.5	27.2 ± 43.5
Fasting FFA (mmol/L)	0.5 ± 0.17	0.8 ± 0.3	0.6 ± 0.3
Triglycerides (mg/dL)	111.7 ± 18.3	151.9 ± 61.5†	176 ± 58‡
Cholesterol (mg/dL)	162.5 ± 19.6	185 ± 43.5	217.7 ± 66.8‡
Fructosamine (mmol/L)	1.7 ± 0.9	2.7 ± 0.15†	3 ± 1.4‡

NOTE. Plasma glucose, fructosamine, and lipid levels were determined by standard colorimetric assays, and insulin levels were measured by radioimmunoassay.

Abbreviations: FPG, fasting plasma glucose; PPG, postprandial plasma glucose; FFA, free fatty acid.

*$P < .001$ *v* groups 1 and 2.

†$P < .05$ *v* control.

‡$P < .01$ *v* control.

Table 3. Assessment of Oxidative Stress (mean ± SD)

Parameter	Control	Group 1	Group 2
Lipid peroxides (μmol/L)	5.7 ± 1.3	10.3 ± 8.0	13.4 ± 7*
TEAC (mmol/L)	2.7 ± 0.5‡	1.7 ± 0.5	1.4 ± 0.5†

NOTE. Plasma lipid peroxides were measured spectrophotometrically by determination of MDA and 4-HNE levels. TEAC was determined using Trolox, the water-soluble analog of vitamin E, as a standard.

*$P < .001$ *v* control.

†$P < .001$ *v* group 1.

‡$P < .001$ *v* groups 1 and 2.

sensitivity of the total antioxidant capacity assay may be related to the fact that it not only measures the well-known antioxidants, such as vitamins C and E, beta-carotene, glutathione, cysteine, dihydrolipoate, ubiquinol, and polyphenols, but also determines the contributions to defense against oxidative damage by the lesser-known substances such as albumin, uric acid, bilirubin, and other molecules present in the body.[20,26] Indeed, it has been reported that there is an inverse correlation between the glycosylated hemoglobin level and total free-radical scavenging activity and that this association is primarily due to a similar correlation between the former and the uric acid level.[27]

There is evidence of an association between oxidative stress and the development of diabetic complications.[1,7-11,14,16,28] It is of interest that studies have shown proteinuria to be associated with increased morbidity and mortality in type 2 diabetes, primarily due to CHD.[18] In the present study, CHD as a diabetic complication was only observed in patients with proteinuria (group 2). In a recent study, when cardiovascular risk factors were considered with antioxidant levels, there was an association between antioxidant depletion and cardiovascular disease risk in type 2 diabetes.[29] Furthermore, it was previously shown that there is an association between an increase in the urinary albumin excretion rate and the prevalence of maculopathy and proliferative retinopathy.[30] More recently, it has been reported that oxidative stress is present in erythrocytes of patients with type 2 diabetes, and the intensity of oxidative stress appeared greater in patients with nephropathy than in those without this diabetic complication.[16] The data in the present study showing a higher incidence of diabetic complications in a group of diabetic patients with more severe depletion of total antioxidant capacity are consistent with these previous findings.

It has been suggested that the oxidation of lipids such as chylomicrons may play a causative role in the generation of ROS in diabetes.[15] This suggestion is consistent with the present study in which increases in plasma triglyceride and cholesterol were observed in group 2 diabetic subjects, who had significantly elevated plasma peroxide levels compared with control subjects. However, our findings do not necessarily prove a causative role for oxidative stress in the pathogenesis of diabetic complications, since it may also be argued that oxidative stress in the study subjects was attributable not only to the development of complications but also to the duration of diabetes. In conclusion, our study shows that the plasma total antioxidant capacity is depleted in type 2 diabetic patients. Furthermore, it appears that the measurement of total antioxidant capacity may be a reliable method for assessment of oxidative stress and could be useful in determining the risk for development of diabetic complications, consistent with findings in a previous study.[28]

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