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ORIGINAL ARTICLE

Oxidative stress in non-insulin-dependent diabetes mellitus (NIDDM) patients

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Abstract Diabetes mellitus is a metabolic disorder characterized by hyperglycemia. The oxidative stress in diabetes was greatly increased due to prolonged exposure to hyperglycemia and impairment of oxidant/antioxidant equilibrium. Proteins and lipids are among the prime targets for oxidative stress. In the present study, the oxidative stress was evaluated in 55 diabetic patients and 40 healthy subjects by measuring the levels of protein oxidation, lipid peroxidation and some enzymatic and nonenzymatic antioxidants. The oxidative products of protein (PCG) and lipid peroxidation (MDA) and nitric oxide levels in plasma of NIDDM patients were significantly increased. However, the levels of enzymatic (GPx, SOD, catalase in RBC) and nonenzymatic (β-carotene, retinol, vitamin C & E and uric acid) antioxidants of RBC showed a significant decrease in NIDDM patients compared to normal subjects. Serum protein analysis by polyacrylamide gel electrophoresis (PAGE) showed the significant difference in the ceruloplasmin, transferrin, albumin, retinal binding protein, etc. in diabetic patients compared to healthy controls. In conclusion, the results suggest that increased protein oxidation, lipid peroxidation and NO levels, decreases the levels of enzymatic and nonenzymatic antioxidants and playing a major role in diabetic complications.

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Keywords Diabetes · Non-insulin-dependent diabetes mellitus (NIDDM) · Protein oxidation · Lipid oxidation · Antioxidant status · Oxidative stress

Introduction

Oxidative stress is known to be a component of molecular and cellular tissue damage mechanism in a wide spectrum of human diseases. Diabetes is associated with a number of metabolic alterations and principal among these is hyperglycemia. Hyperglycemia in diabetic patients can increase the oxidative stress by several mechanisms, including glucose autooxidation, nonenzymatic protein glycation and activation polyol pathway. In the pathological events, the increased free radical activity is suggested to play an important role in the lipid peroxidation and protein oxidation of cellular structures causing cell injury and is implicated in the pathogenesis of vascular disease in type I and type II diabetes [1, 18, 36, 56]. In vitro studies show that the action of reactive oxygen species (ROS) results in the formation of carbonyl groups in proteins and lipid peroxides in lipids. Lipid peroxidation and protein carbonyl group content was used as a marker of oxidative stress. Therefore, the evaluation of protein carbonyl group content in plasma is respected marker of free radical activity [6, 17]. A variety of natural antioxidants exist to scavenge oxygen free radicals and prevent oxidative damage to biological membranes. One group of these antioxidants is enzymatic (intracellular), which include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. In addition to enzymatic antioxidants, others are natural antioxidants; these are derived from natural sources by dietary intake and include vitamins A, C & E and carotenoids. Also, numerous small molecules are synthesized or

produced within the body that have antioxidant property (e.g. glutathione and uric acid) [31, 36]. These antioxidants appear to act cooperatively in vivo to provide greater protection to the organism against free radical damage than that could be provided by any single antioxidant acting alone.

Several studies have been carried out to evaluate the free radical induced lipid peroxidation and the antioxidants in diabetic patients. Many of these studies assessed only individual antioxidants. Controversial reports have been reported concerning the antioxidant status in diabetic patients [2, 8, 33, 41, 54]. Hence, the present study has been undertaken to evaluate oxidative stress in NIDDM diabetic patients by measuring the levels of oxidative products—protein carbonyls, lipid peroxidation (MDA) and some enzymatic and nonenzymatic antioxidants.

Subjects and methods

We studied a total of 95 subjects, among them 55 patients who fulfilled the World Health Organization Criteria for NIDDM and remaining 40 subjects were recruited from the community and used as healthy control subjects. All blood samples were collected after night for 12–14 h with their prior consent. These patients were being treated with oral hypoglycemic agents, had no other medications including vitamin supplements, other than having usual diet.

The total plasma proteins were measured by biuret method, plasma albumin was measured by dye-binding method using bromocresol green [10]. Protein oxidation was estimated by measuring protein carbonyl groups (PCGs) by Levine method [27] in plasma as a marker of free radical activity. Equal volumes of plasma and 2,4-dinitrophenylhydrazine were mixed and incubated at 37°C for 60 min. Subsequently, the protein was precipitated with 20% trichloroaceticacid. The protein precipitate was washed with ethanol:ethylacetate and dissolved in 1 ml of 6 M guanidine HCl at 60°C. The absorbance was measured in a spectrophotometer at 360 nm against blank. The molar extinction coefficient of DNPH = 22×10^{-3} per M/cm was used to calculate the concentration of PCGs in the given sample and the results were expressed as nmole carbonyl/mg of protein [45]. The lipid peroxidation in terms of malondialdehyde (MDA) was estimated by using TBARS method and MDA concentration calculated using an molar extinction coefficient of 1.56×10^2 per M/cm and expressed as μmol/l [45].

A measure of 5 ml of blood was collected in heparinized tubes at 8.00 a.m. after an overnight fast and immediately centrifuged at $1{,}500 \times g$ for 15 min at 4°C. Plasma and pelleted RBC were separated and stored in eppendorf tubes in ice for analysis. The activities of SOD, GPx and catalase in RBC were measured according to the methods of Beutler [4], Flohe and Gunzler [14] and Renu et al. [44]. Vitamin A, β-carotene, and ascorbic acid were measured spectrophotometrically determined by Carr–Price reaction, DNP method [45]. Nitric oxide estimated in plasma by the method of Moshage et al. [35]. Plasma vitamin E was measured as described by Ramaswamy [43]. Briefly, absolute ethyl alcohol and xylene were added to plasma samples and then centrifuged for 10 min. Equal volumes xylene and α, α′-dipyridyl reagent were mixed and absorbance measured at 460 nm and vitamin E content was expressed as μmol/l. Electrophoresis was carried out for separation of plasma proteins on 10% polyacrylamide slab gels, according to the method of Laemmli [26].

Statistical analysis

Statistical analysis was performed by GraphPad InStat software. Subjects with NIDDM were compared with healthy controls. Means and standard error of means were calculated and statistical significance was tested by one-way ANOVA. The strength of association between pairs of variables was assessed by Pearson correlation coefficient. The level of significance was set at $P < 0.05$.

Results

The biochemical particulars of the study subjects, NIDDM and healthy controls were depicted in Table 1. The patients' chosen for the study had fasting glucose levels more than 3-fold higher than normal subjects. The state of glycemic control was evaluated from fasting plasma glucose and blood HbA_{1C}. All the patients were under treatment with hypoglycemic agents. All hypertensive subjects were well treated with antihypertensive drugs, and there were no significant differences in systolic and diastolic blood pressures between the groups. The plasma lipid profile of NIDDM patients showed significantly elevated levels of triglycerides, total as well as VLDL- and LDL-cholesterol.

The TBARS (lipid peroxidation) levels increased by 3.0-fold in diabetics compared to healthy controls (Table 2). The protein oxidation (PCG) measured as carbonyl content in NIDDM subjects was significantly increased by 4-fold and the plasma total protein, albumin and A/G ratio decreased compared to normal subjects. However, the levels of nitric oxide in plasma were doubled in diabetics compared to normals and play a major role in the complications of diabetes. The activities SOD, GPx and catalase in RBC showed significant decrease in NIDDM patients compared to normal (Table 3). The levels of

Table 1 Biochemical characteristics of normal and NIDDM patients

Serial no	Clinical data	Normals	NIDDM
1	Number	40	55
2	Age (years)	40–60	45–80
3	Body mass index (kg/m^2)	21.5 ± 2.4	25.4 ± 3.0
4	Blood glucose (mg/dl)	80 ± 12	242 ± 76[a]
5	Serum total protein (g/dl)	7.15 ± 0.96	6.06 ± 0.6
6	Serum albumin (g/dl)	4.04 ± 0.45	3.20 ± 0.6
7	A:G ratio	1–2	0.96 ± 0.4
8	Triglycerides (mg/dl)	120 ± 34	253 ± 82[a]
9	Cholesterol (mg/dl)	172 ± 31	258 ± 63[a]
10	VLDL-cholesterol (mg/dl)	23 ± 6.6	64 ± 15[a]
11	LDL-cholesterol (mg/dl)	105 ± 14	181 ± 34[a]
12	HDL-cholesterol (mg/dl)	39 ± 6	32 ± 8
13	Blood urea (mg/dl)	35 ± 8	41 ± 10
14	Serum creatinine (mg/dl)	0.96 ± 0.23	1.22 ± 0.42
15	Glycosylated Hb (%)	6.12 ± 0.3	12.5 ± 3.2[a]

Results are expressed as mean ± SD

[a] $P < 0.05$ against the control values

Table 2 Effect of NIDDM on the levels of oxidative stress parameters

Serial no	Oxidative parameter	Normals	NIDDM
1	Lipid peroxidation (TBARS) (μmol/l)	58 ± 10	231 ± 32[a]
2	PCG (nmol/mg of protein)	3.82 ± 0.5	12.2 ± 2[a]
3	Nitric oxide (μmol/l)	35 ± 5.4	58 ± 6.5[a]

Results are expressed as mean ± SD

[a] $P < 0.05$ against the control values

antioxidant natural vitamins—retinol, β-carotene, vitamins C and E were significantly decreased in plasma by 14–45%, respectively, in NIDDM patients compared to controls. It is pertinent to note that the diabetes mellitus is a chronic metabolic disorder, which may be associated with the imbalance between protective effect of antioxidants and increased free radical production.

Discussion

Oxidative stress is thought to be increased in a system where the rate of free radical production is increased and/or the antioxidant mechanisms are impaired. In recent years, the oxidative stress-induced free radicals have been implicated in the pathology of IDDM and NIDDM patients [1, 2, 21, 57]. The present study was evaluated the oxidative stress by measuring the plasma levels of oxidant and antioxidants in the patients suffering from NIDDM. Hyperglycemia in diabetic patients can increase the levels

Table 3 Effect of NIDDM on the blood levels of antioxidants

Serial no	Antioxidant	Normals	IDDM
1	Vitamin A (retinol) (μmol/l)	3.24 ± 0.65	1.75 ± 0.52[a]
2	β-carotene (μmol/l)	3.86 ± 0.34	2.23 ± 0.4[a]
3	Vitamin C (ascorbic acid) (μmol/l)	78 ± 18	41 ± 10[a]
4	Vitamin E (α-tocopherol) (μmol/l)	21.4 ± 1.8	16.2 ± 1.2[a]
5	Glutathione peroxidase (GPx) (U/mg)	52 ± 5	40 ± 4.8[a]
6	Superoxide dismutase (SOD) (U/mg)	1620 ± 80	1340 ± 98[a]
7	Catalase (nmol H_2O_2 oxidised/min/ml)	266 ± 35	198 ± 33[a]
8	Uric acid (mmol/l)	0.14 ± 0.05	0.12 ± 0.02[a]

Results are expressed as mean ± SD

[a] $P < 0.05$ against the control values

of free radicals through glucose autooxidation, nonenzymatic posttranslational modification of proteins resulting from chemical reaction between glucose and primary amino groups of proteins—glycation and also through polyol pathway and protein kinase activation [3, 16, 50, 53]. The increase in protein oxidation and lipid peroxidation as reflected by increase in plasma levels of PCGs and MDA in diabetics in the present study are in accordance with previous finding of that hyperglycemia induces overproduction of oxygen free radicals in diabetes (Tables 1, 2) [38, 42, 52, 55, 58]. To our knowledge, there are only few reports in the literature concerning plasma PCG levels in NIDDM patients. Oxidized proteins constitute a substantial fraction of the catalytically inactive or less active forms of enzymes, which may have direct metabolic consequences [9, 20, 24, 29, 30, 57]. We also analyzed the total plasma proteins on polyacrylamaide gel electrophoresis under native conditions for observing the changes in individual protein component. In native PAGE (not shown), the diabetic patients showed decreased levels of transferrin, retinol binding protein (RBP), albumins and heptoglobulins. The variability was noticed in GC globulin fractions compared to controls. However, the thickness of bands of β-lipoproteins, ceruloplasmin and ceruloglobulin were significantly higher than those of the control group.

Nitric oxide (NO) has emerged as one of the most important molecules released from the endothelium and a variety of other tissues. Nitric oxide is a free radical that can act as a neurotransmitter and in a paracrine or autocrine manner to produce diverse cellular responses, both beneficial and detrimental. Superoxide neutralizes NO, and the peroxynitrite formed is a source of hydroxyl radicals that can cause endothelial damage [12, 37]. Increasing evidence

suggests that increased oxidative stress and changes in nitric oxide (NO) formation or activity play a major role in the complications of diabetes with decreased antioxidants.

Non-enzymatic antioxidants would affect lipid peroxidation by scavenging free radicals to produce a less reactive species. Enzymatic antioxidants that promote inactivation of inorganically derived free radicals include SOD, catalase and GPx [25, 29, 49, 57]. The relationship between hyperglycemia and oxygen free radicals is supported by our results demonstrating an association between blood levels of glucose and enzymatic antioxidants such as SOD and GPx. The decreased erythrocyte SOD activity in our diabetic patients also supports the hypothesis of free radical mediated injury in diabetes.

In the present study, we also observed on native PAGE (figure not shown), the plasma levels of ceruloplasmin in NIDDM were significantly higher than those of controls. The same results were reported by Diamon et al. [11] in type II diabetes; although the serum ceruloplasmin levels did not correlate with the blood HbA_1C levels in the all type 2 diabetic groups. However, others [19, 22, 51] reported a significant reduction in the plasma ceruloplasmin in type I diabetic patients and Wilson disease. Selenium-dependent GPx works in parallel with SOD, playing an important role in the reduction of H_2O_2 in the presence of reduced glutathione (GSH) and also protects cellular proteins and membranes against oxidative damage. In the present study, the GPx activity was decreased marginally compared to controls. However, in the published literature, the GPx response to diabetes has been conflicting. Diabetes has been reported to be associated with increased [39], decreased [2, 9] or unchanged [13, 23]. In lieu with GPx, the catalase activity was also decreased in diabetes compared to control subjects (Table 3). The low GPx activity could be directly explained by either low GSH content [28] or enzyme inactivation under severe oxidative stress [7].

A significant difference in the mean plasma total antioxidant status was observed in NIDDM patients (Table 3). A low level of vitamin E is associated with increased incidence of diabetes and studies showed that people with diabetes have decreased levels of antioxidants [5, 18, 34, 40, 42, 47]. People with diabetes may also have greater antioxidant requirements because of increased production of free radicals in hyperglycemia [32, 37, 48]. Non-enzymatic antioxidants delay or inhibit the oxidative process. Enhanced oxidation stress increases the need for lipid soluble antioxidants, such as α-tocopherol, vitamin C, vitamin A and β-carotene. A significantly lower mean plasma concentration of these vitamins was found in our diabetic patients compared to healthy controls (Table 3). β-carotene, an effective radical trapping antioxidant behavior only at low physiological oxygen pressures [15]. Reduced plasma and tissue levels of ascorbic acid in human have been reported to be associated with diabetes [32]. A significant negative correlation coefficient between blood glucose and vitamin C has been observed. The biochemical evidence of ascorbate deficiency in the presence of diabetes could due to its impaired tubular reabsorption [46], increased oxidation [22] or impaired regeneration from its oxidized state.

In conclusion, a high level of glucose hampers the activity of enzymatic antioxidants, decreases the levels of nonenzymatic antioxidants, who vitamins decreases chemically-induced diabetes and consequently increases the oxidative stress. The increased oxidative stress in patients with type II diabetes may predispose to the development of cardiovascular complications. We propose that diabetic patients may have elevated requirement for antioxidants. Supplementation with vitamin or dietary free radical scavengers such as vitamins C and E have a potential role in boosting antioxidant-related defenses and may be important in mitigating long-term complications in patients with diabetes.

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