Protein oxidation biomarkers in plasma of type 2 diabetic patients

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Objectives: To evaluate oxidative stress and the extent of oxidation of plasma proteins in type 2 diabetic patients.

Design and methods: Study was carried out on blood from 31 diabetic patients of both sexes (mean age = 58 ± 7; duration of diabetes 12 ± 5 years) and healthy age and sex matched normal subjects. Biomarkers of protein oxidation; plasma protein carbonyls (PCO), advanced oxidation protein products (AOOPs) and -SH group and free radical scavenging capacity of plasma was measured.

Results: PCO and AOOPs levels were significantly (P<0.005) higher in diabetic patients in comparison to healthy volunteers. Reduced free radical scavenging capacity (P<0.001) and -SH group (P<0.05) was observed in plasma of type 2 diabetic patients.

Conclusions: Our data suggest that diabetics are susceptible to protein oxidation. Oxidative modulation of proteins due to reduced radical scavenging activity of plasma patients may be one of the reasons of altered physiological processes in type 2 diabetic patients.

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Introduction

Numerous theoretical premises and experimental or clinical studies point to the participation of oxidative stress in diabetic pathogenesis and its late vascular complications [1]. Hyperglycemia is one of the most important factors that are responsible for oxidative stress and production of ROS in diabetes. Proteins are likely to be major targets of ROS, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants.

Attack of ROS modifies amino acid; lysine, arginine, proline, and histidine residues generating carbonyl moieties and action of chlorinated oxidants, mainly hypochlorous acid and chloramines, produced by myeloperoxidase in activated neutrophils, forms dityrosine containing cross-linked protein products known as advanced oxidation protein products (AOPPs), both of which have been identified as an early marker for oxidative stress and are used as a measure of protein damage [2,3]. Protein sulfydryl groups (–SH) is a good reflection of excess free radical generation, since the conformation of albumin is altered, allowing –SH groups to be oxidized [4].

In the present study we report the extent of oxidative stress in type 2 diabetic patients in terms of markers of protein oxidation; plasma protein carbonyls, AOPPs and –SH group. We also correlate the radical scavenging capacity of plasma with protein oxidation.

Materials and methods

Selection of subjects

The criteria for selection of type 2 diabetic patients were the same as reported earlier [5]. Mean age (58 ± 7) years, fasting plasma glucose level (10.2 ± 2.3) mmol/L, body mass index (BMI) (27 ± 4) kg/m², total plasma cholesterol (5.4 ± 1.3) mmol/L, and duration of diabetes was (12 ± 5) years. Briefly, blood from 31 diabetic patients (19 men, 12 women) was taken after informed consent has been obtained from all patients. None of the patients had high blood pressure or microalbuminuria. Care was also taken to exclude patients who had a family history of hypertension.

The control group consisted of 31 healthy volunteers, age and sex matched with diabetic subjects, mean age (56 ± 8) years, fasting plasma glucose level (4.7 ± 0.8) mmol/L, BMI (24.8 ± 3.8) kg/m², total plasma cholesterol (5.3 ± 1.3) mmol/L. None of the controls was affected by hypertension. Care was taken to select control subjects with no family history of diabetes mellitus or hypertension (two generation). None of the patients studied were receiving any hormonal treatment including insulin. All volunteers (diabetic patients and normal subjects) were informed about the nature of the study; the subjects gave written consent for the use of their blood samples for scientific study. The protocol of study was in conformity with the guidelines of the Allahabad University Institutional Ethical Committee.

Collection of blood, isolation of plasma and determination of PCO

Venous blood was obtained by venipuncture in heparin. Plasma was obtained by centrifuging the blood at 1800×g for 10 min at
Plasma protein carbonyls (PCO) content was measured according to procedure of Levine et al. [2]. PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and after the DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 4 mL of an ethanol/ethyl acetate mixture (1:1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution and the absorbance was measured at 370 nm, using the molar extinction coefficient of DNPH, $e = 22000 \text{ M}^{-1} \text{ cm}^{-1}$ and result was expressed in nmol/L of plasma.

**Determination of plasma AOPPs level**

Determination of AOPPs in plasma was based on spectrophotometric detection according to Witko-Sarsat et al. [3]. Briefly, 200 mL of plasma (diluted 1:5 with phosphate-buffered saline (PBS)) as test, 200 mL of chloramines-T solution (0-100 μmol/L) for calibration and 200 mL of PBS as blank were applied. 10 μL of 1.16 M potassium iodide and 20 μL of acetic acid were added and absorbance at 340 nm was measured immediately. Concentration of AOPPs was expressed as μmol/L of chloramine-T equivalents.

**Estimation –SH group**

Plasma –SH group was estimated according to Kitajima’s method [6], based on the ability of the –SH group to reduce 5, 5′-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product whose OD is measured at 412 nm. The concentration of the –SH groups is expressed as μmol/L of plasma.

**Estimation of radical scavenging capacity of plasma**

Radical scavenging capacity of plasma was estimated by DPPH• reduction assay as described by Janaszewska et al. [7]. Briefly 0.1 mL of plasma in phosphate buffered solution (10 μM, pH 7.4) was incubated in the methanolic solution of DPPH• (0.1 mM). Absorbance at 517 nm was measured after 30 min of incubation with vigorous shaking. The free radical DPPH• scavenging (i.e. reduction) activity was calculated.

![Fig. 1](image-url)
from the equation: Activity [% of DPPH reduction] = [(A–Ax)/A]×100%, where A is the absorbance of DPPH solution with methanol, Ax is the absorbance of a DPPH solution with plasma.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Statistical differences were analyzed with Student’s t-test; and the differences were considered to be significant when P<0.05.

Results

We observed a significant (P<0.005) increased level of PCO in diabetic patients in comparison to healthy control group (91.28±30.32 vs. 66.65±18.36) [Fig. 1a]. Same pattern of result in diabetics was also seen in the case of AOPPs level (89.51±36.46 vs. 63.64±25.23; P<0.005) [Fig. 1b]. The concentration of plasma –SH group in diabetic patients was reduced in comparison to healthy volunteers (336.37±116.28 vs. 466.64±102.47; P<0.001; Fig. 1c). Diabetic patients had lower plasma radical scavenging capacity than healthy controls of same age group (23.28±13.32 vs. 33.65±18.36; P<0.05; Fig. 1d).

Discussion

PCO provide a global index of protein oxidation involving the side chains of several amino acid residues. They represent the stable end-product generated upon formation of transient radical species, such as chloramines and nitrogen/carbon radicals, which are induced by oxidant stimuli. However, direct oxidation of amino acid side chain is not the only way through which carbonyl groups can be formed in proteins. In fact, glycation may induce formation of protein carbonyls, such as ketoamine derivatives, thus generating reactive radicals and perpetuating a vicious cycle [2].

AOPPs can be formed in vitro by exposure of serum albumin to hypochlorous acid (HOCl). In vivo, plasma concentration of AOPPs closely correlate with levels of dityrosine, a hallmark of oxidized protein, and pentosidine, a marker of protein glycoxidation tightly related to oxidative stress. Thus, AOPPs might be formed during oxidative stress by reaction of plasma proteins with chlorinated oxidants, and have been considered as novel markers of oxidant-mediated protein damage [3]. The oxidation of plasma –SH group, termed as thiol stress is quantitatively the major manifestation of protein oxidation. Plasma –SH group may serve an antioxidant function by scavenging oxidants that initiate peroxidation.

Increased PCO as well as AOPPs level in diabetic patients underlie the importance of the protein conformational changes in the pathogenesis of diabetic nephropathy [8]. Furthermore, the inverse relation of PCO and AOPPs with plasma–SH group in diabetics underlies the close relationship between the degree of ROS-mediated protein damage because it has been reported that –SH group play a prominent role in antioxidant reactions, and also in reactions of catalysis, regulation, electron transport and those preserving the correct structure of proteins [9].

Inverse relation of plasma –SH group and radical scavenging capacity of the plasma with PCO and AOPPs concentration in diabetic patients is a direct evidence of increased protein oxidation under conditions of elevated oxidative stress during diabetes, this may be correlated with altered glucose homeostasis and other late vascular complications in diabetes. Our results suggest that protein oxidation in type 2 diabetic patients might be promoted by an insufficient counter-regulation of the antioxidant system in the event of increased glycoxidation/ glycation. The antioxidant system may therefore play a role in protecting against glycocalyx damage by glycemia-induced oxidative stress. This study brings out the close relationship between antioxidant capacity and protein oxidation in type 2 diabetic patients (Fig. 2).

Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, therefore structural changes in proteins are considered to be among the molecular mechanisms leading to diabetic complications [10]. A clear negative correlation was observed by Cakatay [11] between serum albumin levels and HbA1c, and a positive correlation between serum AOPPs level and HbA1c concentration in type 2 diabetic patients. The correlation between serum albumin and HbA1c levels is an indicator of a close relation between protein oxidation and glycation in type 2 diabetic patients. A recent study has shown that both glycative and oxidative stress are increased in the poor glycemic control diabetic group compared with controls, contributing to the development of diabetic complications [12]. In conclusion, the present study demonstrates that oxidative stress in diabetics lead to increased oxidation of proteins which may affect many physiological functions.

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Kanti Bhooshan Pandey is a Senior Research Fellow of Council of Scientific & Industrial Research (CSIR), India.

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