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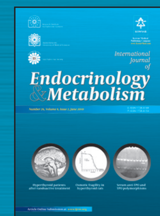

Oxidative stress and proteolytic activity in erythrocytes of diabetic patients

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ABSTRACT

Background: High blood glucose levels induce oxidative stress and decrease antioxidant defenses.
Objectives: The present study aims to assess the association, if any, between increase in proteolytic enzyme activities and oxidative stress in diabetic subjects.
Patients and Methods: Fifty-one non diabetic patients (33 men, 18 women, mean age 54.47 year) and 53 diabetic patients (39 men, 14 women, mean age 52.92year) were included in the study. Diabetic status was assessed by the fasting blood sugar (FBS) using glucose oxidase method. Oxidant stress was measured by estimating erythrocyte malondialdehyde (MDA) in terms of thiobarbituric acid reacting substance (TBARS). Proteolytic activity was estimated by the amount of amino groups released by the erythrocyte lysate of the diabetic individual using phenylhydrazine treated hemoglobin.
Results: Erythrocyte MDA was higher in diabetics (cases) (4.7±1.7 nmoles/gHb) than in controls (3.3±2.2 nmoles/gHb) (p=0.001). Erythrocyte proteolytic activity was also higher in cases (167.2±648 nmoles/gHb) than the controls (27.9±31.7 nmoles/gHb).
Conclusions: Both erythrocyte lipid peroxidation and the proteolytic activity in the erythrocyte lysates of diabetic patients was significantly higher in cases than in controls. Diabetes is associated with a significant increase in TBARS, an index of oxidant stress. Proteolytic enzymes degrade many oxidatively altered proteins preventing the accumulation of altered and damaged proteins in the cell.

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Background

Free radicals and their derivatives, which exist in living tissues at low concentrations are capable of damaging cellular components and may contribute to various diseases. Of all biomolecules, lipids seem to be more susceptible to the damaging effects of ROS (1). Anti oxidants are substances that are able to compete with other oxidizable substrates at relatively low concentrations and to thus significantly delay or inhibit the oxidation of these substrates (2). Antioxidants prevent free radical mediated injury. The primary antioxidant defense system includes vitamin E, vitamin A, glutathione, uric acid, superoxide dismutase, catalase, and peroxidases, whereas the secondary antioxidant defense system includes lipolytic enzymes, phospholipase, proteolytic enzymes, proteases, peptidases, endonucleases and exonucleases (2). Oxidative stress, a measure of the steady level of reactive oxygen or oxygen radicals in the biologic system, is a result of either overproduction of reactive oxygen radicals or decreased efficiency of inhibitory or scavenger systems (3). Free radicals have become increasingly implicated in human disease (3, 4). In patients with diabetes mellitus, there is an increase in the level of oxidative stress (5), which is a common pathway linking diverse mechanisms for the pathogenesis of diabetes due to

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hyperglycemia. Increased intracellular glucose metabolism enhances mitochondrial superoxide generation. Glycation of tissue proteins and other macromolecules and excess production of polyol compounds from glucose are among the mechanisms thought to produce tissue damage from chronic hyperglycemia (6). Formation of ROS, associated with impairment of ß –cell function in type 2 diabetes mellitus (7), has been implicated in both pancreatic ß- cell dysfunction and cell death, caused by autoimmune attack and the action of cytokines in type I diabetes mellitus.

Red blood cells (RBCs) are susceptible to oxidative damage as result of the high polyunsaturated fatty acid content of the membrane and high cellular concentration of oxygen and hemoglobin. They are a constant source of superoxide radicals owing to the well-known tendency of hemoglobin to autooxidize (8). High levels of glucose can produce permanent chemical alteration in proteins and increase lipid peroxidation in a variety of experimental models of hyperglycemia. Studies have shown that lipid peroxidation levels were significantly higher in the RBCs of diabetic rats than in controls (9). Data shows that serum and erythrocyte MDA levels were increased in diabetic patients (10).

An enzyme system exists in erythrocytes which rapidly degrades oxidatively damaged proteins (11). Proteolytic enzymes are the second line of defense against the free radicals, which degrade and eliminate the damaged molecules. The proteolytic process preferentially degrades oxidatively modified proteins. The erythrocytes in individuals with diabetes are ill equipped to handle increased oxidant stress; studies have suggested there is increased activity of erythrocyte proteolytic enzymes in degrading oxidant damaged hemoglobin in diabetes mellitus (12).

Objectives

The aim of the present study was to determine the extent of lipid peroxidation and proteolytic enzyme activity in the erythrocytes of diabetic patients and to assess the association between increase in proteolytic activities and oxidation stress.

Patients and Methods

Sample collection

The study group comprised of nondiabetic individuals and diabetic patients attending the Kasturba hospital, Manipal. Informed consent from the patients was obtained for the study. Patients were randomly selected, with no distinction being made between those with insulin dependent or non insulin dependent diabetes; the case group consisted of fifty three diabetic individuals (14 females and 39 males, with fasting glucose levels > 126 mg/dL, and controls included 51 nondiabetic persons (18 females and 33 males). The mean age of controls was 54 ± 12 and that of cases was 52 ± 12 years. Blood samples (2ml) were collected by venepuncture into tubes containing 3.6 mg EDTA and stored at 4°C.

Erythrocyte malondialdehyde was estimated within 24 hours of blood collection. The hemolysates prepared from the above blood samples were stored at 25°C and proteolytic activities were measured within 2 weeks.

Chemicals

Ethylenediaminetetraacetic acid (EDTA; 99.5%), 2-thibarbitursae (thiobarbituric acid), N-(2-hydroxyethyl), and 2-mercaptoethanol were obtained from MERCK. HEPES (N-2 hydroxye ethyl piperazine-N'-2-ethanesulfonic acid was obtained from Sigma. Butylated hydroxyl toluene (BHT), dl-dithiothreitol, phthaldialdehyde (o-phthalaldehyde), and benzene were obtained from SISCO. Malondialdehyde (MDA) was prepared from1, 1, 3, 3-tetraethoxy propane (97%), from ALDRICH. Dialysis tubing was obtained from Thomas scientific, USA. All other reagents were of analytical grade.

1) Assay of proteolytic activity in the erythrocytes (11)

1-1) Preparation of oxidatively damaged haemoglobin substrate

1-1-1) Preparation of the hemolysate

Blood samples were centrifuged at 3000 rpm for 8 minutes. The plasma and buffy coat were discarded. The packed cells were lysed with 1.5 volumes of water, then centrifuged at 16,000xg for 20 minutes. The hemolysate was dialyzed against 0.05M tris HCl buffer with 0.1mM EDTA (pH 8.3)

1-1-2) Preparation of phenylhydrazine treated hemoglobin (11)

Oxidant damage to hemoglobin was induced by treating it with phenylhydrazine for 3 hours; this treatment oxidatively modifies the hemoglobin (13).

1-2) Estimation of proteolytic activity in erythrocytes

1-2-1) Preparation of erythrocyte cell free extract(14)

Washed erythrocytes were lysed in 1.5 volumes of freshly prepared 1mM DL- dithiothreitol centrifuged at 16,000 xg for 20 minutes;- supernatant was dialyzed for 16 hours against 10 volumes of buffer using a membrane with molecular weight cut off of 12- 14 kda. The dialysis buffer contained 20 mM Na_2HPO_4 and NaH_2PO_4 (pH 7.8), 20% v/v glycerol and 0.5 mM DTT. The dialyzed cell free extracts were used for proteolytic activity estimation.

1-3) Proteolytic activity in erythrocytes

The erythrocyte contains several proteolytic enzymes, some of which are known to degrade oxidatively damaged hemoglobin (11). Under the experimental conditions used in this study, when a sample of erythrocyte

lysate is incubated with phenylhydrazine treated hemoglobin at 37°C, the enzymes in the erythrocyte degrade oxidatively damaged hemoglobin and simultaneously any other oxidant damaged protein present in the erythrocyte lysate as well. The end products of the degradation are a number of smaller peptides, which are TCA soluble and can be measured as an increase in the number of free amino groups (15). Estimation of free amino groups in erythrocyte lysates before incubation gives an indication of endogenous protein damage caused by oxidative stress. Proteolytic activity in the cell-free extracts was measured using the method used by Raghothama, *et al.* (11) and was expressed as nanomoles per gram of hemoglobin concentration of free amino groups in the erythrocyte lysates.

Estimation of MDA

MDA content of erythrocytes was estimated as thiobarbituric acid reactive substances using thespectrophotometric method as described by Jain, *et al.* (16). The MDA value was calculated from the MDA standard graph and expressed as nanomoles/gram of hemoglobin.

Statistical analysis

Data are expressed as Mean ± SD. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS/PC; SPSS-13, Chicago, USA). The results were analyzed statistically using students unpaired ‘t’ test, Mann Whitney ‘U’ test and Karl Pearson correlation test; $p < 0.05$ was considered statistically significant.

Results

Parameters of erythrocyte oxidative stress which were estimated included malondialdehyde (MDA) and the proteolytic activity of the erythrocytes. In the diabetics, the MDA levels were significantly higher when compared to the control group ($p = 0.001$). The proteolytic activities in the diabetic erythrocytes were significantly higher when compared to the control group ($p = 0.001$) *(Table 1).* Thus there is an increased ability of the erythrocytes to proteolytically degrade oxidant damaged hemoglobin in the diabetic patients when compared to hemolysates from normal individuals. In the diabetic cases, the fasting blood sugar did not correlate with erythrocyte MDA and erythrocyte proteolytic activity. Also in the diabetic cases, there was no correlation of erythrocyte MDA with erythrocyte proteolytic activity.

Table 1. Indicators of oxidative stress in the erythrocytes of controls and cases

	Controls (No. = 51)	**Cases** (No. = 53)
Fasting blood sugar (mg %)	96.2 ± 14.8 [a]	189.2 ± 65.1 [b]
MDA (nmoles/gHb)	3.38 ± 2.23	4.79 ± 1.78 [b]
Proteolytic activity (nmoles/gHb)	27.9 ± 31.8	167.3 ± 65 [c]

[a] Mean ± SD
[b] p: 0.001
[c] p: 0.001, in comparison with controls

Discussion

In the present study erythrocyte lipid peroxidation increased in diabetic patients, a finding in agreement with those of several other studies (17-22), that documented elevated levels of lipid peroxidation products in diabetic subjects. The exact mechanism by which the elevated blood glucose leads to membrane lipid peroxidation is not known. Some studies have shown that glucose can enolize and then reduce the molecular oxygen to give α-keto aldehydes, hydrogen peroxide and ROS, (16) which causes peroxidative breakdown of phospholipid fatty acids and accumulation of MDA.

ROS are known to induce damage to the proteins, which leads to alteration in protein structure and function (23). In the present study there was an increase in the proteolytic activity in the erythrocyte lysates of diabetic patients. Hyperglycemia greatly increased proteolyitc activity as shown by the increase in the concentration of amino groups released. The erythrocytes of diabetic patients are capable of disposing off the extra load of oxidant damaged hemoglobin. Degradation of oxidatively damaged hemoglobin is done by the proteolytic system present in the erythrocyte lysate (11, 24, 25). It has been shown that erythrocyte membrane proteins become susceptible to degradation by membrane bound serine protease activity after oxidative modification of the membranes (26, 27). Studies have demonstrated that in human erythrocytes oxidized hemoglobin is cleaved into peptides by a high molecular mass proteinase identified as a member of the multicatalytic proteinase family (25).

In conclusion, oxidative stress is observed in hyperglycemia which results in lipid peroxidation and the increased activity of the proteolytic enzymes.

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Conflict of interest

None declared.

References

1. Wolff SP, Garner A, Dean RT. Free radicals, lipids and protein degradation. *Trends Biochem Sci.* 1986;**11**(1):27-31.
2. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med.* 1992;**119**(6):598-620.
3. Halliwell B. Free radicals and antioxidants: a personal view. *Nutr Rev.* 1994;**52**(8 Pt 1):253-65.
4. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and x-irradiation: a mechanism in common. *Science.* 1954;**119**(3097):623-6.
5. Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev.* 1994;**74**(1):139-62.
6. Ahmed RG. The physiological and biochemical effects of diabetes on the balance between oxidative stress and antioxidant de-

fense system. *Med J Islamic World Acad Sci.* 2005;**15**(1):31-42.
7. Fridlyand LE, Philipson LH. Does the glucose-dependent insulin secretion mechanism itself cause oxidative stress in pancreatic beta-cells? *Diabetes.* 2004;**53**(8):1942-8.
8. Gonzalez G, Celedon G, Escobar M, Sotomayor C, Ferrer V, Benitez D, et al. Red cell membrane lipid changes at 3,500 m and on return to sea level. *High Alt Med Biol.* 2005;**6**(4):320-6.
9. Kakkar R, Kalra J, Mantha SV, Prasad K. Lipid peroxidation and activity of antioxidant enzymes in diabetic rats. *Mol Cell Biochem.* 1995;**151**(2):113-9.
10. Jeremy JY, Breimer LH, Thompson CS, Mikhailidis DP. Elevated lipid peroxidation levels in red blood cells of streptozotocin-treated diabetic rats. *Metabolism.* 1991;**40**(4):442-3.
11. Raghothama C, Rao P. Increased proteolysis of oxidatively damaged hemoglobin in erythrocyte lysates in diabetes mellitus. *Clin Chim Acta.* 1994;**225**(1):65-70.
12. Raghothama C, Rao P. Degradation of glycated hemoglobin by erythrocytic proteolytic enzymes. *Clin Chim Acta.* 1996;**245**(2):201-8.
13. Jain SK, Hochstein P. Generation of superoxide radicals by hydrazine: Its role in phenylhydrazine-induced hemolytic anemia. *Biochimica et Biophysica Acta (BBA)-General Subjects.* 1979;**586**(1):128-36.
14. Salo DC, Lin SW, Pacifici RE, Davies KJ. Superoxide dismutase is preferentially degraded by a proteolytic system from red blood cells following oxidative modification by hydrogen peroxide. *Free Radic Biol Med.* 1988;**5**(5-6):335-9.
15. Peterson GL. Determination of total protein. *Methods Enzymol.* 1983;**91**:95-119.
16. Jain SK, McVie R, Duett J, Herbst JJ. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. *Diabetes.* 1989;**38**(12):1539-43.
17. Selvaraj N, Bobby Z, Koner BC, Das AK. Reassessing the increased glycation of hemoglobin in nondiabetic chronic renal failure patients: a hypothesis on the role of lipid peroxides. *Clin Chim Acta.* 2005;**360**(1-2):108-13.
18. Komosinska-Vassev K, Olczyk K, Olczyk P, Winsz-Szczotka K. Effects of metabolic control and vascular complications on indices of oxidative stress in type 2 diabetic patients. *Diabetes Res Clin Pract.* 2005;**68**(3):207-16.
19. Turk HM, Sevinc A, Camci C, Cigli A, Buyukberber S, Savli H, et al. Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 diabetes mellitus. *Acta Diabetol.* 2002;**39**(3):117-22.
20. Memisogullari R, Taysi S, Bakan E, Capoglu I. Antioxidant status and lipid peroxidation in type II diabetes mellitus. *Cell Biochem Funct.* 2003;**21**(3):291-6.
21. Hartnett ME, Stratton RD, Browne RW, Rosner BA, Lanham RJ, Armstrong D. Serum markers of oxidative stress and severity of diabetic retinopathy. *Diabetes Care.* 2000;**23**(2):234-40.
22. Peerapatdit T, Sriratanasathavorn C. Lipid Peroxidation and Antioxidant Enzyme Activities in Erythrocytes of Type 2 Diabetic Patients. *J Med Assoc Thai.* 2010;**93**(6):682-93.
23. Ahmed N, Babaei-Jadidi R, Howell SK, Thornalley PJ, Beisswenger PJ. Glycated and oxidized protein degradation products are indicators of fasting and postprandial hyperglycemia in diabetes. *Diabetes Care.* 2005;**28**(10):2465-71.
24. Davies KJ, Goldberg AL. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *J Biol Chem* 1987;**262**(17):8227.
25. Sacchetta P, Battista P, Santarone S, Di Cola D. Purification of human erythrocyte proteolytic enzyme responsible for degradation of oxidant-damaged hemoglobin. Evidence for identifying as a member of the multicatalytic proteinase family. *Biochim Biophys Acta.* 1990;**1037**(3):337-43.
26. Fujino T, Ishikawa T, Inoue M, Beppu M, Kikugawa K. Characterization of membrane-bound serine protease related to degradation of oxidatively damaged erythrocyte membrane proteins. *Biochim Biophys Acta.* 1998;**1374**(1-2):47-55.
27. Fujino T, Watanabe K, Beppu M, Kikugawa K, Yasuda H. Identification of oxidized protein hydrolase of human erythrocytes as acylpeptide hydrolase. *Biochim Biophys Acta.* 2000;**1478**(1):102-12.