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Short Report:

A Study on Proteolytic Enzyme Activity in the Erythrocytes of Diabetic Patients

BS Varashree, Assistant Professor,
P Gopalakrishna Bhat, Professor,
Department of Biochemistry, Kasturba Medical College, Madhav Nagar, Manipal- 576104, India.

Address For Correspondence:
BS Varashree,
Assistant Professor,
Department of Biochemistry,
Kasturba Medical College,
Madhav Nagar,
Manipal- 576104, India.
E-mail: varasuhas@yahoo.com

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Abstract:
The present study demonstrates the possibility of increased proteolytic activities in diabetic individuals. Proteolytic activity was measured by the amount of amino group released by the erythrocyte lysate of the diabetic individual using phenylhydrazine treated hemoglobin as substrate. The proteolytic activity in erythrocyte lysates against oxidatively damaged hemoglobin was significantly increased in diabetic individuals compared to controls ($p<0.001$).The result of this study indicates that in diabetic individuals, proteolytic enzymes degrade many oxidatively altered proteins preventing the accumulation of altered and damaged proteins in the cell.
Key Words: Oxidative stress; Proteolytic activity; Diabetes mellitus

Introduction:
Oxidative stress is a result of either overproduction of reactive oxygen radicals or decreased efficiency of inhibitory or scavenger systems. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity. Oxidants are balanced by the activities of enzymes and non-enzymes called antioxidants.(1) This vitally important defense system controls the production and elimination of oxidants and is essential in controlling the damage that occurs during oxidative stress. However, when the body is overwhelmed by increased production of oxidative agents and defense against these agents is decreased, the ensuing damage contributes to cellular derangements, cell injury, and death. Proteolytic enzymes are the second line of defense against the free radicals, which degrade and eliminate the damaged molecules. Cells maintain the quality and functional integrity of proteins by degradation and replacement of proteins damaged by oxidation and glycation. Proteolytic process preferentially degrades the oxidatively modified proteins. Oxygen radicals and other ROS cause modification of proteins. Although damage to protein substrate is likely to cause protein degradation, it is possible that oxygen radical might activate cellular proteases or damage protease inhibitors and promote indiscriminate proteolysis. Oxidant damage to proteins could result in changes in the secondary and tertiary conformation of proteins. These may lead to changes in protein function, chemical fragmentation and increased susceptibility to proteolytic attack.(2) Cell proteins with altered structures may also arise from post synthetic modifications including nonenzymatic glycosylation, spontaneous deamidation or reaction with free radicals and oxidants.(3) Exposure of normal human erythrocytes to oxygen radicals can induce oxidant damage to erythrocyte proteins especially hemoglobin. An enzyme system exists in erythrocytes which rapidly degrades the oxidatively damaged proteins.(4) Proteolytic enzymes degrade many oxidatively altered proteins, thus preventing the accumulation of altered and damaged proteins in the cell. The erythrocytes in diabetes are ill equipped to handle increased oxidant stress that it faces. Exposure of normal human erythrocytes to oxygen radicals can damage the erythrocyte proteins which lead to increased susceptibility to proteolytic attack. Studies have suggested an increased activity of erythrocyte proteolytic enzymes in degrading oxidant damaged hemoglobin in diabetes mellitus.(5) The present study was carried out to assess the activity of erythrocyte proteolytic enzymes in degrading oxidant damaged hemoglobin in diabetes mellitus when compared to normal individuals.

Materials and Methods:
Sample collection: The study group comprised of non-diabetic individuals and diabetic patients attending the Kasturba hospital, Manipal. Informed consent from the patients was obtained for the study. Patients were selected at random and no distinction was made between those with insulin dependent or non insulin dependent diabetes. The control group included fifty one non diabetic patients (mean age=54.47). The test group consisted of fifty three diabetic individuals (mean age=52.92); whose fasting glucose level was more than 126mg%. The diabetic status was assessed by estimating the fasting blood sugar (FBS) using glucose oxidase method. Proteolytic activity by the amount of amino group released by the erythrocyte lysate of the diabetic individual using phenylhydrazine treated hemoglobin as substrate.

Preparation of oxidatively damaged haemoglobin substrate:

Preparation of the hemolysate: Blood collected from a volunteer was centrifuged at 3000rpm for 8 minutes. The packed cells were lysed with 1.5 volumes of water. Then centrifuge at 16,000xg for 20 minutes. The hemolysate was dialyzed against 0.05M tris HCl buffer with 0.1mM EDTA (pH 8.3)

Preparation of phenylhydrazine treated hemoglobin (3): Oxidant damage to hemoglobin was induced by treating it with phenylhydrazine. The phenyl hydrazine-treated hemoglobin was adjusted to a final concentration of 50 mg/ml and stored at -20°C. The above oxidatively damaged hemoglobin was used as substrate for the proteolytic enzymes of the erythrocyte cell-free extracts prepared from the study populations.

Estimation of proteolytic activity in human erythrocytes:

Preparation of erythrocyte cell free extracts (6): The washed human erythrocytes were lysed in 1.5 volumes of freshly prepared 1mM DL- dithiothreitol centrifuged at 16,000xg for 20 minutes. The supernatant was dialyzed against 10 volumes of buffer using a membrane with molecular weight cut off of 12-14 kda. The dialysis buffer contained 20mM 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.

Proteolytic activity in human erythrocytes that degrades oxidatively damaged hemoglobin: The erythrocyte contains several proteolytic enzymes, some of which are known to degrade oxidatively damaged hemoglobin. 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. The end products of the degradation are smaller peptides, which are TCA soluble and can be measured as an increase in the number of free amino groups using spectrofluorimeter.(7) Estimation of free amino groups in erythrocyte lysates before incubation gives an indication of endogenous protein damage due to oxidative stress. Proteolytic activity in the cell-free extracts was measured as follows. Alanine was used as a standard for the estimation of amino groups released during proteolytic degradation of oxidatively damaged hemoglobin. Amino group concentrations in the TCA supernatants were calculated from the alanine standard graph. The difference in the amino group concentration before and after incubation was taken as a measure of proteolytic activity in the cell-free extract and was expressed as nanomoles per gram of hemoglobin concentration.

Statistical analysis: All values of analyzed parameters were ex-pressed 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=0.001 was considered statistically significant.

Results and Discussion:

The proteolytic activity was determined in the erythrocytes taken from both individuals with diabetes mellitus (test group) and normal healthy individuals (control group). Erythrocyte proteolytic activity was higher in cases (167.2±648 nmoles/gHb) than the controls (27.9±31.7 nmoles/gHb). FBS did not correlate with the erythrocyte proteolytic activity. The proteolytic activities in the diabetic erythrocytes were significantly higher when compared to the control group (p=0.001) (Table 1, Figure 1).The significantly increased proteolytic activity in the erythrocyte lysates of diabetic patients, when compared to normals, correlated with the other studies.(6)

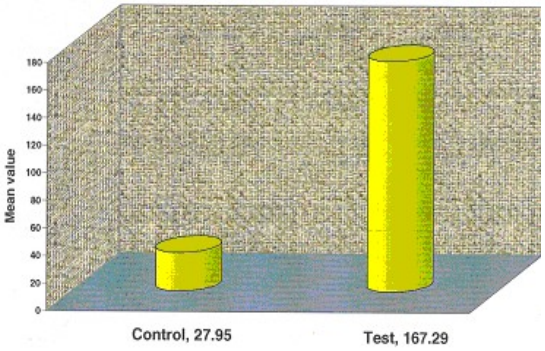

Figure 1: Proteolytic activities in the diabetic erythrocytes compared to the control group

Table 1: Proteolytic activities in the diabetic erythrocytes compared to the control group

Parameters	Mann- Whitney 'u' test	'p'value
Fasting blood sugar (mg %)	8.61	0.001
Proteolytic activity(nmoles/gHb)	6.24	0.001

The increased proteolytic activity suggests an increased ability of the erythrocytes to proteolytically degrade oxidant damaged hemoglobin in the diabetic patients when compared to hemolysates from normal individuals.

Reactive oxygen species are known to induce damage to the proteins. This leads to the alteration in the protein structure and function. In order to degrade these altered proteins the cells are equipped with proteolytic enzymes. The erythrocyte contains several proteolytic enzymes, some of which are known to degrade oxidatively damaged haemoglobin. Proteolytic enzymes degrade many oxidatively altered proteins preventing the accumulation of altered and damaged proteins in the cell**.** It has been shown that erythrocyte membrane proteins become susceptible to degradation by membrane bound serine protease activity after oxidative modification of the membranes.(8,9) 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.(10)To know if the proteolytic system could efficiently work as an antioxidant scavenging system, proteolytic activity of erythrocytes extracts was estimated using phenylhydrazine treated hemoglobin substrate, the enzymes in the erythrocyte degrade oxidatively damaged haemoglobin and simultaneously any other oxidatively damaged protein present in the erythrocyte lysate. The end products of the degradation are a number of smaller peptides that are TCA soluble and can be measured as an increase in the number of free amino groups. Estimation of free amino groups in the erythrocyte lysates before incubation gives an indication of endogenous protein damage due to oxidative stress. In the present study, the proteolytic activity in the erythrocyte lysates of diabetic patients was significantly increased when compared to normals, which correlated with the other studies.(6) Hyperglycemia greatly increased proteolytic activity as shown by the increase in the concentration of amino groups released. The erythrocytes of diabetic patients are capable of disposing off extra load of oxidant damaged hemoglobin. Degradation of oxidatively damaged hemoglobin is done by the proteolytic system present in the erythrocyte lysate.(4,11)

References:

1. Bruch CG, Pierce JD. Oxidative stress in critically ill patients. *American Journal of Critical Care* 2002;11:543-551
2. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002;82:48-56.

3. Fagan JM, Waxman L, Goldberg AL. Red blood cells contain a pathway for the degradation of oxidant damaged hemoglobin that does not require ATP or ubiquitin. *J of Biological Chemistry* 1986;261(13):5705-5713.
4. Raghothama C, Rao P. Increased proteolysis and oxidatively damaged hemoglobin in erythrocyte lysates in diabetes mellitus. *Clinica Chimica Acta* 1994;225:65-70.
5. Raghothama C, Rao P. Degradation of glycated hemoglobin by erythrocytic proteolytic enzymes. *Clinica Chimica Acta* 1996;245:201-208
6. Salo DC, Lin SW, Pacifici RE, Davies KJA. Superoxide dismutase is preferentially degraded by a proteolytic system from red blood cells following oxidative modification by hydrogen peroxide. *Free Radical Biol Metab* 1988;5:335-339.
7. Peterson GL. Determination of total protein. *Methods in enzymol* 1983;91:95-119
8. 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. *Biochem Biophys Acta* 1998;1374(1-2):47-55.
9. Fujino T, Watanabe T, Beppu M, Kikugawa K, Yasuda H. Identification of oxidized protein hydrolase of human erythrocytes as acylpeptide hydrolase. *Biochem Biophys Acta* 2000;1478(1):102-112.
10. 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):337-343.
11. Davies KJA, Goldberg AL. Proteins damaged by oxygen radicals are rapidly degraded by RBC extracts. *J Biol Chem* 1987;262:8227-8234.