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Amelioration of glucose induced hemolysis of human erythrocytes by vitamin E

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ABSTRACT

Cells under aerobic condition are always threatened with the insult of reactive oxygen species, which are efficiently taken care of by the highly powerful antioxidant systems of the cell. The erythrocytes (RBCs) are constantly exposed to oxygen and oxidative stress but their metabolic activity is capable of reversing the injury under normal conditions. *In vitro* hemolysis of RBCs induced by 5, 10 and 20 mM glucose was used as a model to study the free radical induced damage of biological membranes in hyperglycemic conditions and the protection rendered by vitamin E on the same. RBCs are susceptible to oxidative damage, peroxidation of the membrane lipids, release of hemoglobin (hemolysis) and alteration in activity of antioxidant enzymes catalase and superoxide dismutase. The glucose induced oxidative stress and the protective effect of vitamin E on cellular membrane of human RBCs manifested as inhibition of membrane peroxidation and protein oxidation and restoration of activities of superoxide dismutase and catalase, was investigated.

Thiobarbituric acid reactive substances are generated from decomposition of lipid peroxides and their determination gives a reliable estimate of the amount of lipid peroxides present in the membrane. Vitamin E at 18 μg/ml (normal serum level) strongly enhanced the RBC resistance to oxidative lysis leading to only 50–55% hemolysis in 24 h, whereas RBCs treated with 10 and 20 mM glucose without vitamin E leads to 70–80% hemolysis in 24 h. Levels of enzymic antioxidants catalase, superoxide dismutase and nonenzymic antioxidants glutathione showed restoration to normal levels in presence of vitamin E. The study shows that vitamin E can protect the erythrocyte membrane exposed to hyperglycemic conditions and so a superior antioxidant status of a diabetic patient may be helpful in retarding the progressive tissue damage seen in chronic diabetic patients.

1. Introduction

The red blood cells are constantly exposed to oxidative injury, but their metabolic activity is capable of reversing the injury under normal conditions. Following an increased oxidative stress when content of active oxygen species exceeds the cellular antioxidant capacity, irreversible damage to the erythrocytes occurs, resulting in their ultimate loss by hemolysis and removal from circulation [1]. Elevated levels of glucose in the medium or blood are known to cause membrane damage and cell death of erythrocytes. However the biochemical mechanism of this damage is not known. Similar to the non enzymatic glycosylation of haemoglobin leading to formation of glycosylated haemoglobin in diabetics, glycosylation of cell membrane proteins leading to formation of advanced glycosylation end products may lead to irreversible crosslinking of membrane proteins which may contribute to loss of elasticity and increased membrane osmotic fragility [2].

Diabetes is associated with various microvascular and macrovascular complications and oxidative stress is one of the main causes of diabetic complications. Free radicals play an important role in pathogenesis of tissue damage in many clinical disorders. Oxygen free radicals are capable of damaging biomolecules of all classes including nucleic acids, proteins, lipids, lipoproteins, carbohydrates and connective tissue macromolecules. Normally, there is a balance between tissue oxidant and antioxidant activity, the latter achieved by the antioxidant scavenger system which includes enzymes like superoxide dismutase, catalase, glutathione peroxidase, antioxidant vitamins C, A, E and carotenoids and free other free radical scavengers like glutathione [3–5].

Vitamin E is a chain breaking antioxidant. It appears to be the first line of defense against peroxidation of polyunsaturated fatty acids contained in cellular and sub-cellular membrane phospholipids [6]. In RBCs vitamin E constitutes an important membrane antioxidant protecting the cell against oxidative damage. In the present study, *in vitro* oxidative hemolysis of human red blood cell (RBCs) was used as a model to study the free radical induced damage of biological membranes due to glucose and the protective effect of vitamin E.

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The hemolysis of RBCs was studied by treating with 5, 10 and 20 mM glucose to mimic normal (90 mg/dl) and diabetic (180 and 360 mg/dl) levels of glucose respectively with and without vitamin E supplementation. There was an increase in release of hemoglobin (hemolysis) in a concentration dependent manner when RBCs were incubated with glucose which was considerably reduced when incubation mixture was supplemented with vitamin E. RBCs are susceptible to oxidative damage, resulting in peroxidation of the membrane lipids which was monitored by levels of thiobarbituric acid reactive substances (TBARS). The levels of TBARS shows an increase in presence of glucose but the increase was lower when the RBCs were incubated with vitamin E and glucose. Levels of antioxidant enzymes catalase, superoxide dismutase and GSH are measure of the status of antioxidants under oxidative stress. Vitamin E at normal serum concentrations inhibited the oxidative damage induced by glucose by maintaining the antioxidant levels. Na^+K^+ ATPase and aspartate transaminase (AST) levels were also monitored to check the damage to the plasma membrane of the RBC.

2. Materials and methods

In the study, all experiments were performed on heparinised healthy human venous blood collected from volunteers.

Epinephrine and reduced glutathione was purchased from Sigma Chemicals, St. Louis, MO, USA. All other chemicals used were of highest analytical grade and solvents were of Qualigen grade.

2.1. Preparation of RBCs

The blood samples were centrifuged for 10 min at 2500 rpm and the supernatant (plasma and buffy coat) was discarded. Washing was done in normal saline three times and the RBC's pelleted by centrifugation at 2500 rpm for 10 min in a refrigerated centrifuge. The final pellet was then diluted to a volume in the ratio 1:10 with phosphate buffered saline, pH 7.4 [2].

2.2. Hemolysis

Three milliliters of washed and diluted RBCs was incubated with an equal amount of PBS, 5, 10 and 20 mM of glucose to mimic normal (90 mg/dl) and diabetic (180 and 360 mg/dl) blood glucose levels. Vitamin E at concentrations of 18 μg/ml (normal serum levels) was added. The tubes were incubated for 24 h in the shaker at 37 °C. Penicillin was the antibiotic of choice. The percentage of hemolysis was calculated using 100% hemolysis brought about by adding equal quantity of distilled water. Vitamin E control was also maintained. After the incubation period, the sample was centrifuged and the supernatant was read at 540 nm for hemolysis [6]. The hemolysate was further used for estimation of glutathione (GSH), TBARS, aspartate transaminase, catalase and superoxide dismutase and the membrane was used for assay of Na^+K^+ ATPase.

2.3. Membrane lipid peroxidation

Membrane lipid peroxidation was determined by the thiobarbituric acid (TBA) reactivity of malonaldehyde (MDA) and expressed as nanomoles/mg protein [2]. 0.2 ml of lysate was mixed thoroughly with 0.8 ml of phosphate buffered saline (pH 7.4) and 0.025 ml of butylated hydroxytoluene solution (8.8 g/L). After addition of 0.5 ml of 30% trichloroacetic acid, the samples were placed on ice for 2 h and then centrifuged at 2000*g* for 15 min. One ml of supernatant was mixed with 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% TBA in 0.05 N NaOH. The samples were placed in boiling water for 15 min, cooled to room temperature, and the absorbance was determined at 532 nm. 1,1,3,3-Tetramethoxypropane was used as standard. Values were expressed as nanomoles/mg protein.

2.4. Membrane and lysate protein

Membrane and lysate protein was estimated by the method of Lowry et al. [7].

2.5. The level of reduced glutathione in erythrocytes

The level of reduced glutathione in erythrocytes was determined by the method of Beutler [8] based reduction of Ellman's reagent by –SH groups to form 5,5′dithiobis(2-nitrobenzoic acid) with development of a relatively stable yellow color when reacted with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and was read at 412 nm.

2.6. Catalase activity

Catalase (EC.1.11.1.6) activity was determined according the protocol of Aebi [9]. The reaction mixture contained 40 mM H_2O_2 in a 50 mM phosphate buffer pH 7.0, and 0.1 ml pure enzyme in a total volume of 3 ml. Catalase (CAT) activity was assayed as monitoring the decrease in absorption at 240 nm when hydrogen peroxide was added to the sample. It is expressed in micromoles of H_2O_2 decomposed/min/mg protein.

2.7. Activity of SOD

Activity of SOD (EC.1.15.1.1) was determined by the method of Misra and Fridovich [10].

Three milliliters of the reaction mixture consisted of 1.5 ml of 100 mM carbonate buffer, pH 10.3; 0.01 ml of 30 mM EDTA (ethylenediaminetetraacetic acid), suitable aliquot of enzyme preparation and water to make up the volume to 2.94 ml. The reaction was started by addition of 0.06 ml of 15 mM epinephrine. Change in absorbance was recorded at 480 nm for 1 min at 15 s intervals. Control consisting of all the ingredients, except enzyme preparation, was run simultaneously. One unit of enzyme activity has been defined to cause 50% inhibition of auto-oxidation of epinephrine present in the assay system by 1 ml enzyme preparation.

2.8. Na^+K^+ ATPase

Na^+K^+ ATPase (EC 3.6.1.37) was measured by the method of Bonting [11] as the difference in inorganic phosphate (P)liberated from ATP in the presence and in the absence of 1000 pM ouabain. The assay medium consisted of 100 mM NaCl, 20 mM KCI, 6 mM MgCl, 10 mM imidazole and 6 mM ATP. The temperature of incubation was 37 °C and the samples were incubated for 15 min. The results were expressed as micromoles of P liberated per milligram of protein per minute.

2.9. Aspartate transaminase

Aspartate transaminase (EC 2.6.1.1) was assayed by the method of Reitman and Frankel [12]. 0.2 ml of lysate with 1 ml of substrate (aspartate and α-ketoglutarate, in phosphate buffer pH 7.4) was incubated for a hour. one milliliters of DNPH solution was added to arrest the reaction and kept for 20 min in room temperature. After incubation 10 ml of 0.4 N NaOH was added and absorbance was read at 540 nm. Activities expressed as IU/mg protein.

Results are expressed as mean ± SD of $n = 5$ in each group. Statistical analysis was done using the unpaired students "*t*" test.