Original Article

Insulin Reduces Eryptosis in Thalassemic Red Blood Cells

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Abstract

Eryptosis, a form of programmed cell death in red blood cells, is triggered by oxidative stress or energy depletion. Modulation of mechanisms regulating energy supply or oxidative stress impacts eryptotic behavior of red blood cells. In thalassemic blood cells enhanced eryptosis causes anemia. Insulin enhances the flux of glucose by stimulating glycolysis to produce ATP and NADH in human erythrocytes. Thalassemic red blood cells are prone to undergo eryptosis leading to anemia. This paper gives evidence that the stimulatory effect of insulin leads to a protection of oxidative-stress-induced eryptosis, especially in red blood cells of thalassemic donors. Oxidative stress in thalassemic red blood cells was induced by *tert*-butylhydroperoxide (tBOOH) with or without supplementing the media with insulin. Phosphatidylserine exposure, measured as annexin V-binding, was used as a marker for eryptosis in thalassemic red blood cells. Insulin significantly decreased ($P<0.05$) the percentage of phosphatidylserine exposing red blood cells in thalassemic red blood cells.

Keywords : Eryptosis, Thalassemia, Insulin, Oxidative stress

Introduction

Thalassemia is an inherited autosomal recessive disease that involves the decreased and defective production of hemoglobin in red blood cells. In thalassemia, the genetic defect results in reduced rate of hemoglobin synthesis and to the formation of abnormal hemoglobin molecules. Thalassemic red blood cells are subjected to oxidative stress leading to a shortened life span of the red blood cells eventually causing anemia1. Oxidative stress triggers eryptosis in red blood cells. Eryptosis is similar to apoptosis in nucleated cells. This mode of cell death is characterized by phosphatidylserine (PS) exposure at the outer membrane leaflet. Eryptosis is controlled by the activity of cation channels. Initially, non-selective cation channels can be opened by oxidative stress leading to an increased cytoplasmic Ca^{2+} concentration. Subsequently, the high intracellular Ca^{2+} activity stimulates Ca^{2+}-sensitive "Gardos" K^+ channels leading to an efflux of K^+ and Cl^- and osmotic cell shrinkage. Finally, PS is exposed by the activation of a Ca^{2+}-sensitive scramblase and PS-exposing cells are recognized by macrophages that engulf and degrade the affected cells [2-5]. In addition to direct oxidative stress, energy

depletion is a potent inducer of eryptosis. Via the activation of protein kinase C (PKC), Ca^{2+}-permeable cation channels are opened and trigger eryptosis[6, 7].

Since several studies have suggested that insulin may protect nucleated cells from apoptosis by decreasing oxidative stress in different cell lines, we speculated if insulin also impacts eryptosis. Red blood cells express a receptor for insulin and react on insulin with increased glycolysis and phosphorylation of tyrosine residues of several proteins[8, 9]. These effects occur through phosphofructokinase (PFK) activation, the key regulator enzyme of glycolysis[9]. We postulated that increasing of glycolysis by insulin might block cell death in red blood cells as well. In addition, we studied if this is also true for thalassemic erythrocytes since they are especially endagered by oxidative stress-induced eryptosis. This knowledge could be exploited to reduce PS-exposure and to extend the lifetime of these cells.

Materials and methods

Purification of thalassemic red blood cells

Fresh EDTA blood samples were depleted of white blood cells by Ficoll-Hypaque (Lymphoprep™, NYCOMED AS, Norway) centrifugation. After centrifugation for 10 min at 1000 g, plasma and buffy coat were removed, and the red blood cells were washed three times with isotonic phosphate-buffered saline (PBS), pH 7.4.

Insulin treatment and eryptosis induction

Purified thalassemic erythrocytes were suspended in Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES / NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$ at 0.3% hematocrit. They were treated with or without 10 mM insulin and incubated at 37 °C for 3 h. Subsequently, tBOOH was added to a final concentration of 0.1 mM to induce eryptosis. All samples were incubated at 37 °C for 17 h and phosphatidylserine exposure was measured by FACS.

Measurement of phosphatidylserine exposure:

The annexin V-FITC apoptosis detection kit I (BD Biosciences) was used to measure PS exposure. Briefly, cells were washed once with PBS (1X PBS Solution contains 10 mM Phosphate Buffer, 137 mM Sodium Chloride, and 2.7 mM Potassium Chloride.Adjust pH to 7.4, autoclave and store at RT) and twice with binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), and were resuspended in 100 µL binding buffer at a concentration of $1x10^6$cells/ml. Then, 5 µL annexin V were added and the cells were incubated at room temperature for 15 minutes in the dark. After incubation, cells were resuspended in 400 µL of binding buffer and measured by flowcytometry (FACS Calibur ; BDBiosciences, Heidel berg, Germany). Annexin fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

The different amounts of eryptotic cells of insulin treated and non-insulin treated red blood cells were compared with Student's t-test. A *P*-value of <0.05 was considered to be a significant difference.

Results

Oxidative stress, induced by *tert*-butylhydroperoxide (tBOOH), led to PS exposure at the erythrocyte surface as detected by FACS analysis utilizing annexin-V binding, which has thus been added to identify suicidal (i.e., eryotic) erythrocytes[4]. Regarding thalassemic erythrocytes,

*t*BOOH addition increased the amount of eryptotic cells from 1.27 % in untreated samples to 79.1% in tBOOH-treated samples in absence of 10 mM insulin. In presence of 10 mM insulin, tBOOH increased the amount of eryptotic cells from 1.26% to 56.21% **(Figure 1a).**

The effect of insulin on oxidative stress-induced eryptosis testing was induced by tBOOH in the absence or presence of 10 mM insulin. Insulin decreased the percentage of annexin V-binding thalassemic erythrocytes significantly ($P<0.05$). The presence of 10 mM insulin decreased the percentage of eryptotic thalassemic erythrocytes from 83.0 ± 4.73% to 51.83 ± 13.57% (n=6) **(Figure 1b).**

Discussion

The effect of insulin decreased the percentage of PS-exposing erythrocytes. It is well klnown, that glucose depletion induces eryptosis with the involvement of protein kinase C (PKC)[6]. Blocking of eryptosis by insulin as described here is probably the result of an enhanced glucose flux with an increased turnover rate of NAD/ NADH and an increased level of ATP[9], which is an inhibitor of PKC. Thus, we postu late that insulin reduces eryptosis by two mechanisms: First, by enhanced production of ATP and the resulting inhibition of PKC. Second, by the increased amount of NADH generated by glycolysis which can serve as an essential

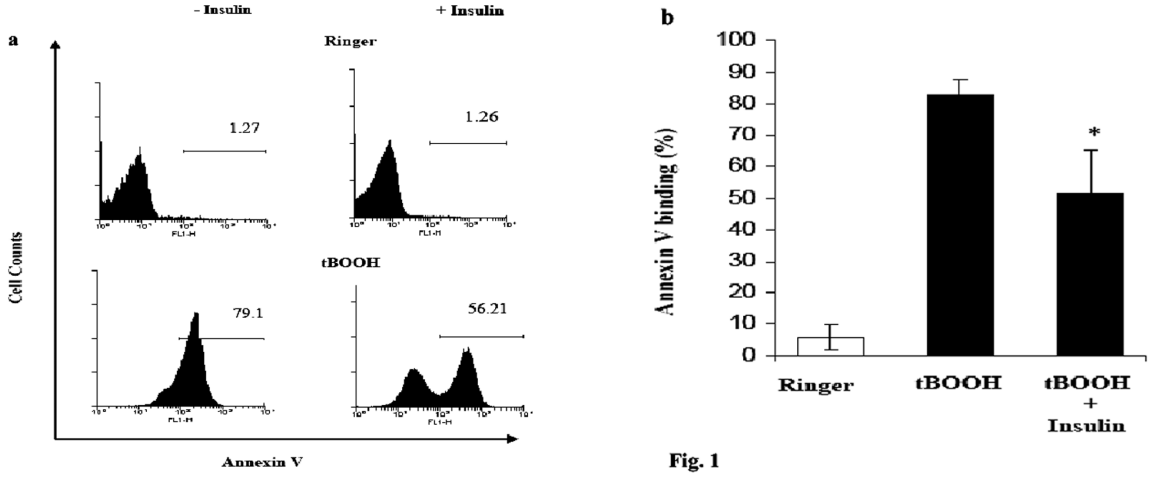

Fig. 1

Figure 1: Stimulation of phosphatidylserine exposure by tert-butylhydroperoxide (tBOOH) in the presence and absence of insulin. **a** Histrogram of erythrocyte annexin V bindind in a representative experiment of thalassemic erythrocytes incubated for 17 h in the absence (upper panels) or presence (lower panels) of tert-butylhydroperoxide (tBOOH) in the absence (left panels) or presence (right panels) of 10 mM insulin. **b** Arithmetic means ? SEM (n=6) of the pecentage annexin- V-binding erythrocytes after incubation for 17 h in the absence (Ringer, open bar) or presence (grey or black) of tBOOH (0.1 mmM) in absence (grey bar) or presence (black bar) of insulin (10 mM) .* P<0.05, Significance to decreased the percentage of annexin V-binding thalassemi-cerythrocytes to presence of insulin (10 mM).

cofactor to reduce methemoglobin and thus reduces oxidative strees[10]. On the other hand, activation of glycolysis by insulin reduces the amount of glucose metabolized by the pentose phosphate pathway and reduces the amount of NADP/ NADPH turnover. NADPH is an important factor to reduce oxidative stress by maintaining the reduced glutathione pool in the cell[11]. Obviously, this effect is of minor importance, since insulin clearly blocks oxidative stress-induced eryptosis. In conclusions, the present study thus discloses a novel effect of insulin on the induction of eryptosis. Insulin is a widely used nutrient and drug, which may well be employed in the treatment or prevention of anemia. This might be of special interest to reduce some negative effects of thalassemic diseases since the life time of thalassemic diseases since the life time of thalassemic red blood cells is shortened by increased oxidative stress.

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