

## High Concentrations of Glucose can Activate or Inhibit Human Erythrocyte Aminolevulinatase Dehydratase *in vitro* Depending Exposure Time

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**Abstract:** Hyperglycemia can cause oxidative stress and inactivate susceptible enzymes. In the present investigation we examined whether short-term incubations (24 or 48 h) with high concentrations of glucose (10-200 mmol L<sup>-1</sup>) inhibit ALA-D from human erythrocytes. Incubations of erythrocytes for 24 h with glucose (10 up to 40 mmol L<sup>-1</sup>) resulted in an increase of  $\delta$ -ALA-D activity (P<0,001). Incubations of erythrocytes with 100 to 200 mmol L<sup>-1</sup> glucose for 48h inhibited  $\delta$ -ALA-D activity (P<0,001). DTT (2 mmol L<sup>-1</sup>) increased glucose-inhibited  $\delta$ -ALA-D activity 120%, but the activity did not return to the control level. These results indicated that the inhibitory effect of glucose depends on time of exposure and its concentration. A significant positive correlation was found between  $\delta$ -ALA-D activity and NPSH groups from erythrocytes incubated 24h with different glucose concentrations. (r=0.65, P<0.0001. In contrast to the results of 24 h, incubation of erythrocytes for 48 h with 100, 150 and 200 mmol L<sup>-1</sup> of glucose did not modify significantly the NPSH content from erythrocytes. Incubations of erythrocytes for 48h with increasing concentrations of glucose (100 to 200 mmol L<sup>-1</sup>) resulted in a significant concentration-dependent increase of TBARS content compared with control group. The TBARS content of erythrocytes incubated for 24 h with 5 (control), 10, 20, 30, 40 and 100 mmol L<sup>-1</sup> of glucose tend to increase as the concentration of glucose increased; however, the values were significantly higher than control only after incubation with 30, 40 and 100 mmol L<sup>-1</sup> of glucose. The results of this study indicate that the use of high concentrations of glucose (above 30 mmol L<sup>-1</sup>) for short periods is of little pathophysiological significance for the study of molecular mechanism underlining enzymes inhibition caused by glucose. Thus, further *in vitro* studies (using glucose concentrations lower than 30 mmol L<sup>-1</sup> for higher periods of cells exposure to glucose) are necessary to established whether or not *in vitro* incubation of erythrocytes with glucose can be considered a reliable model for the study of the toxicity of glucose to proteins under pathophysiological conditions.

**Key words:** Ebselen, sugar reducing,  $\delta$ -aminolevulinatase dehydratase ( $\delta$ -ALA-D), oxidative stress

### INTRODUCTION

Diabetic state is associated with increased oxidative stress and hyperglycemia is an important factor which facilitates overproduction of oxygen free radicals<sup>[1-17]</sup>. Pro-oxidative state can produce permanent chemical alterations in proteins and increase lipid peroxidation in a variety of models of hyperglycemia<sup>[17,18]</sup>. Exposure of macromolecules *in vitro* to glucose concentrations representative of hyperglycemia is considered a relevant model for studying the functional degenerations occurring in diabetes mellitus<sup>[13]</sup>. In line with this, the deleterious effects of hyperglycemia on the properties of physiologically abundant proteins such as hemoglobin, albumin and collagen have been extensively investigated<sup>[16]</sup>. Nonenzymatic glycation is the first of a series of reactions caused by hyperglycemia and this

process can promote *in vivo* and *in vitro* cross-linking of proteins<sup>[6,19]</sup>.

$\delta$ -Aminolevulinatase dehydratase ( $\delta$ -ALA-D) is an essential enzyme for aerobic organisms because participates in the biosynthesis pathway of tetrapyrrole molecules, which constitute prosthetic groups of physiologically significant proteins such as hemoglobin and cytochromes<sup>[20, 21]</sup>. This enzyme seems to be a good marker for oxidative stress due to its sulfhydryl nature, which renders the enzyme highly sensitive to the presence of pro-oxidant situations<sup>[8-10,22-25]</sup>. Of particular importance, the heme synthetic pathway is impaired in porphyria and a frequent coexistence of diabetes mellitus and porphyria disease has been reported in humans and experimental animal models<sup>[26,27]</sup>, which can be linked to inhibition of this enzyme found in diabetics<sup>[28,29]</sup>.

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The aim of this study was to investigate *in vitro* the effect of high concentrations of glucose on  $\delta$ -ALA-D activity and whether the activity of enzyme could be correlated with TBARS production and with non-protein -SH content from human erythrocytes. Although high concentrations of glucose represents an artificial situation and indicates a limitation of such sort of study, it has the advantage of providing rapid results that can guide further molecular approaches to investigate the role of hyperglycemia in the development of pathological complications of diabetes.

## MATERIALS AND METHODS

**Compounds:** 5-aminolevulinic acid, DL-dithiothreitol and malondialdehyde (MDA) were obtained from Sigma (St. Louis, MO., USA). Mono- and dibasic potassium phosphate, acetic acid, ortho-phosphoric acid, tris buffer (tris[hydroxymethyl]aminomethane), glucose, hydrogen chloride, trichloroacetic acid and sodium chloride were obtained from Merck (Rio de Janeiro, Brazil).

**Blood sample collection:** Blood samples from 20 healthy donors (10 mL each) were drawn in the fasting state and processed within 1h of collection. Samples were transferred to sterile polypropylene tubes containing heparin. For *in vitro* experiments the samples were centrifuged for 10 min at 1,500g and erythrocytes were washed three times with NaCl 0.9% and were suspended with NaCl 0,9% taking hematocrit 50% (1:1). Tubes containing 1.5 mL of cell suspension were incubated with different glucose concentrations at 37°C for 24 or 48 h. The incubation mixtures contained 10 mmol L<sup>-1</sup> Tris HCl (pH 7,4) and 400  $\mu$ g Gentamicin. The osmolarity of the solutions were adjusted to 300 mOsm by adding different concentrations of NaCl.

**Biochemical assays:**  $\delta$ -ALA-D activity was assayed by the method of Sassa *et al.*<sup>[30]</sup> by measuring the rate of product (porphobilinogen) formation except that 84 mmol L<sup>-1</sup> potassium phosphate buffer, pH 6.4 and 2.4 mmol L<sup>-1</sup> ALA were used. The reaction was started 10 min after the addition of the enzyme preparation by adding the substrate. Incubations were carried out for 1 h at 37°C. The reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of  $6.1 \times 10^4$  M<sup>-1</sup> for the Ehrlich-porphobilinogen salt. The reaction rates were linear with respect to time of incubation and added protein for all experimental conditions.

**Thiobarbituric acid reactive species assay:** Thiobarbituric acid reactive species (TBA-RS) were determined according to Ohkawa *et al.*<sup>[31]</sup>, with few modifications. Blood was precipitated with one volume of TCA (40%) and left on ice for 30 min before

centrifugation. TBARS were quantified by adding 1000  $\mu$ l of the supernatant fractions to the color reaction medium. The amount of TBA-RS produced was measured at 532 nm, using MDA to construct standard curves.

**Determination of erythrocytes sulfhydryl status:** Erythrocyte non-protein sulfhydryl groups were determined using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate) with few modifications. Blood was precipitated with 1 volume of 5% TCA. After centrifugation, the supernatant was neutralized with Tris-NaOH and non-protein sulfhydryl groups (NPSH) were quantified on these supernatant fractions. The sulfhydryl concentration was calculated against a standard curve of cysteine<sup>[32]</sup>.

**Statistical analysis:** Results are expressed as means  $\pm$  SEM. The results were evaluated by ANOVA (SPSS for Windows 8.0, SPSS 1998, Chicago, IL). When the ANOVA was significant, differences between groups were determined using Duncan's Multiple Range test (SPSS for Windows 8.0, SPSS 1998, Chicago, IL). Correlation coefficients were determined by linear regression analysis. Differences between groups were considered to be significant when  $P < 0.05$ .

## RESULTS

Incubations of erythrocytes for 24 h with increasing concentrations of glucose (10 up to 40 mmol L<sup>-1</sup>) in a significant increase of  $\delta$ -ALA-D activity ( $P < 0,001$ ; Fig. 1). In the presence of 100 mmol L<sup>-1</sup> glucose, enzyme activity return to control level. In contrast, incubations of erythrocytes with 100 to 200 mmol L<sup>-1</sup> glucose for 48h inhibited  $\delta$ -ALA-D activity, when compared with erythrocytes of control group (incubated with glucose 5 mmol L<sup>-1</sup>) ( $P < 0,001$ ). Involvement of cysteinyl groups in  $\delta$ -ALA-D inhibition caused by 48 h exposure to glucose was examined by testing the possible restoration effect of dithiothreitol (DTT) on this enzyme. Addition of DTT (2 mmol L<sup>-1</sup>) in the assay mixture increased  $\delta$ -ALA-D activity about 120%, but the activity did not return to the control level (Fig. 2). Taken together, these results indicate that the inhibitory effect of glucose depends on time of exposure and its concentration.

Since reduced cysteinyl residues are essential to  $\delta$ -ALA-D, we realized that the changes in erythrocyte  $\delta$ -ALA-D activity could be related to changes in the levels of non-protein -SH (NPSH). To test this hypothesis we determined the NPSH content after incubations of erythrocytes with different concentrations of glucose. NPSH content from erythrocytes incubated for 24 h increased significantly as the concentration of glucose increased from 5 to 30 mmol L<sup>-1</sup>. Thereafter, NPSH tended to return to control

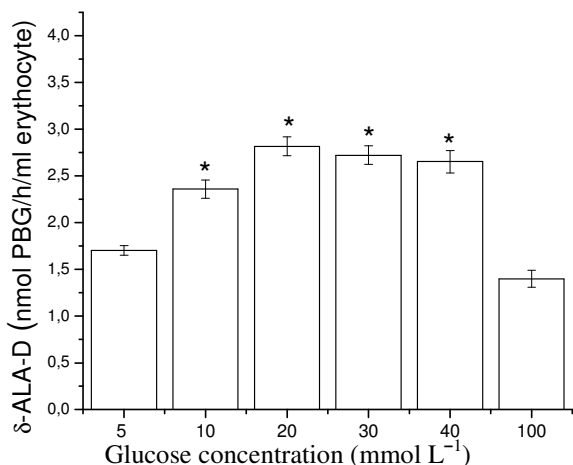


Fig. 1:  $\delta$ -ALA-D activity in erythrocytes incubated for 24 h with glucose (5 to 100 mmol L<sup>-1</sup>). Erythrocytes were incubated at 37°C. Results are expressed as means $\pm$ S.D. for six sample per group (P<0.001)

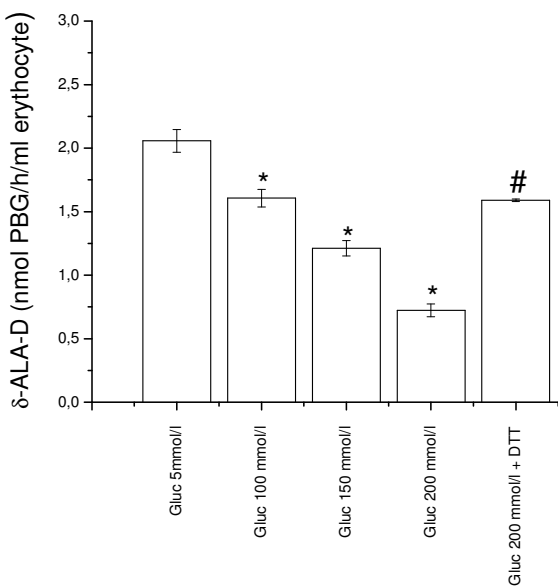


Fig. 2:  $\delta$ -ALA-D activity in erythrocytes incubated for 48 h with glucose (5 to 200 mmol L<sup>-1</sup>). After the pre-incubation of 48 h ALA-D from erythrocytes was determined either in the absence or the presence of 2 mmol L<sup>-1</sup> DTT. Erythrocytes were incubated at 37°C. Results are expressed as means $\pm$ S.D. for ten samples per group (\*P <0.001; # P <0.001)

levels (Fig. 3). A statistically significant positive correlation was found between  $\delta$ -ALA-D Activity and NPSH groups from erythrocytes incubated 24h with different glucose concentrations. ( $r=0.65$ ,  $P<0.0001$ ; Fig. 4). In contrast to the results of 24 h, incubation of erythrocytes for 48 h with 100, 150 and 200 mmol L<sup>-1</sup> of glucose did not modify significantly the NPSH content from erythrocytes (Fig. 5).

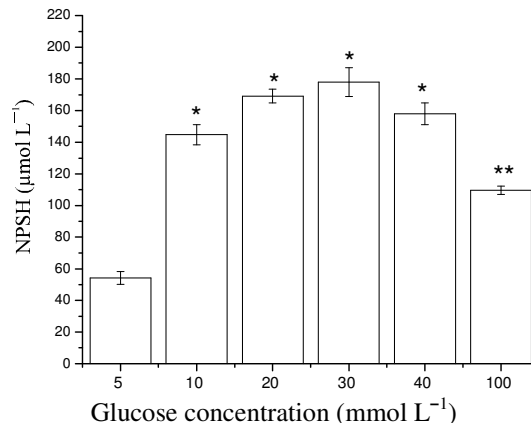


Fig. 3: Non-protein thiol groups (NPSH) from erythrocytes incubated 24 h at 37°C with different concentrations of glucose. Data are expressed as mean $\pm$ SEM for 6 samples per group. (\*P<0.001; \*\*P<0.01)

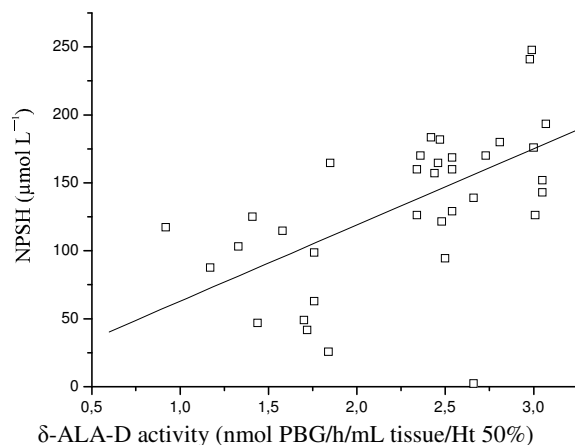


Fig. 4: Correlation between  $\delta$ -ALA-D activity and NPSH from erythrocytes incubated 24h with different glucose concentrations. Data are expressed as mean $\pm$ SEM for 6 samples per glucose concentration ( $r=0.65$ ,  $P<0.0001$ )

Incubations of erythrocytes for 48h with increasing concentrations of glucose (100 to 200 mmol L<sup>-1</sup>) resulted in a significant concentration-dependent increase of TBA-RS content compared with control group (Fig. 6). The TBA-RS content of erythrocytes incubated for 24 h with 5 (control), 10, 20, 30, 40 and 100 mmol L<sup>-1</sup> of glucose tend to increase as the concentration of glucose increased; however, the values were significantly higher than control only after incubation with 30, 40 and 100 mmol L<sup>-1</sup> of glucose (data not shown).

## DISCUSSION

Glycosilation of protein play a significant role in protein aging<sup>[7,13,33]</sup> and this reaction starts with the

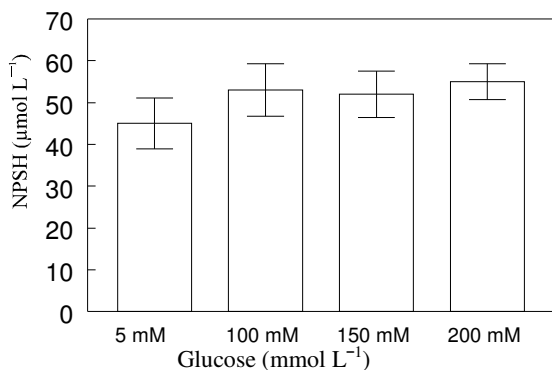


Fig. 5: Non-protein thiol groups (NPSH) from erythrocytes incubated 24 h at 37°C with different concentrations of glucose. Data are expressed as mean $\pm$ SEM for 5 samples per group. (\*P<0.001; \*\*P<0.01)

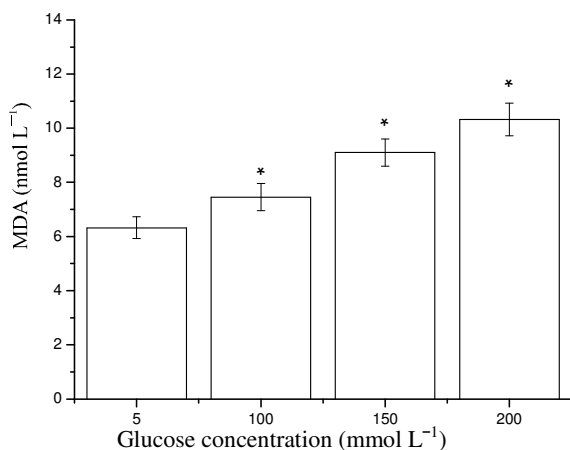


Fig. 6: TBARS content in erythrocytes incubated for 48 h at 37°C with glucose in the concentrations of 5  $\text{mmol L}^{-1}$  up to 200  $\text{mmol L}^{-1}$ . Data are expressed as mean $\pm$ SEM for 8 samples per group. \*(P<0.001)

reversible formation of a Schiff base between glucose and protein amino groups. Hyperglycemia can increase the production of reactive oxygen species, which contribute to cause permanent chemical alterations in proteins. In the present study, we showed that the exposure for 24 h to high concentrations of glucose (up to 40  $\text{mmol L}^{-1}$ ), contrary to our expectancy, caused a significant increase in  $\delta$ -ALA-D activity of erythrocytes. Probably, this was a consequence of production of low-molecular -SH containing molecules (glutathione) derived from reducing equivalents of NADPH formed in the pentose pathway. In fact, we found a positive correlation between ALA-D activity and NPSH groups in erythrocytes incubated for 24 h with glucose. The protecting effect of glucose decreased when the blood was incubated with 100  $\text{mmol L}^{-1}$  of glucose. The decrease in NPSH can be

due to the fact that elevated extra and intracellular glucose concentrations stimulate the polyol pathway, which depletes the intracellular NADPH levels<sup>[5, 34, 35]</sup>.

In addition, the decrease can be also a consequence of elevated production of free radicals, that was confirmed by an increase in erythrocyte TBARS. The formed radicals could interact either directly or indirectly with thiols, oxidizing them to disulfides<sup>[36-40]</sup>. In any case, the present results clearly indicate that care must be taken when using short-term *in vitro* experiments with high concentrations of glucose. The stimulation of  $\delta$ -ALA-D by pathologically relevant concentrations of glucose (10-30  $\text{mmol L}^{-1}$ ) possibly has no *in vivo* significance. In fact, data from our laboratory and other suggest that *in vivo* hyperglycemia is associated with  $\delta$ -ALA-D inhibition<sup>[8, 10, 28, 29]</sup>.

In contrast to the results of 24 h, exposure of erythrocytes for 48 h to non-physiological concentrations of glucose (100 to 200  $\text{mmol L}^{-1}$ ) caused  $\delta$ -ALA-D inhibition that was partially reversed by DTT. These results strongly suggest that cysteinyl residues of  $\delta$ -ALA-D are involved in the enzyme inhibition caused by high concentrations of glucose. These results are in sharp contrast with our laboratory studies showing that enzyme thiols are not involved in the inhibition of  $\delta$ -ALA-D after very short-term (up to 1 h) periods of exposure to extremely high concentrations of reducing sugars<sup>[41]</sup>. The molecular mechanism underlying  $\delta$ -ALA-D impairment in diabetes is still not completely understood, but may be caused either by glycation of the active site lysine residue involved in Schiff's base formation with the first  $\delta$ -ALA molecule or oxidation of essential reduced cysteinyl residues of the enzyme<sup>[8, 10, 19]</sup>. Over production of free radicals could be also contributing to the formation of adducts between the aldehyde group of glucose and the amino group of lysine in  $\delta$ -ALA-D. Taken together, these results may indicate that glucose inhibit the enzyme activity by two distinct mechanisms: 1) one involving the oxidation of cysteinyl residues and 2) another involving a formation of an intermediate between glucose and the lysil residue at the active site of the enzyme. Probably the oxidation of cysteinyl residues was mediated by glucose autoxidation and free radical production.

In conclusion, the results of the present investigation indicate that use of relatively high concentrations of glucose (above 30  $\text{mmol L}^{-1}$ , which corresponds to 540 mg% glucose and a blood concentration rarely found in treated diabetes) for short periods of exposition are of little pathophysiological significance for the study of molecular mechanism underlining enzymes inhibition caused by glucose. These results also indicate that authors must be careful in extrapolating *in vitro* findings to *in vivo* situations, fact that has occurred in the literature<sup>[18, 42]</sup>. Thus, although *in vitro* models can guide *in vivo* studies,

further and more detailed *in vitro* studies (using lower glucose concentrations than 30 mmol L<sup>-1</sup> with higher periods of cells exposure to glucose) are necessary to established whether or not *in vitro* incubation of erythrocytes with glucose can be considered a reliable model for the study of the toxicity of glucose to proteins under pathophysiological conditions.

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