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In vitro inactivation of glucose-6-phosphate dehydrogenase from human red blood cells by acrolein: A possible biomarker of exposure

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SUMMARY

We have investigated the possibility of utilizing glucose-6-phosphate dehydrogenase (G6PD) as a macromolecular (biological) marker of acrolein exposure. The result showed a dose-dependent inactivation of the erythrocyte G6PD in situ or as a purified enzyme from human erythrocytes or yeast. Amino acid analysis on the chemically modified yeast G6PD showed a formation of a lysine adduct which is probably linked to the inactivation.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme distributed in all cells that catalyzes the first step in the hexose monophosphate pathway. It produces NADPH necessary for reactions of various biosynthetic pathways and for maintaining glutathione in its reduced form (GSH) [1]. Because GSH plays a vital role in the detoxification of hydrogen peroxide, the integrity of cell membranes and other cellular components depends strongly on G6PD activity which is especially important in erythrocytes with limited alternative means for producing NADPH and hence GSH, and therefore, more prone to oxidative damage and hemolysis [1,2].

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Acrolein is an industrial chemical intermediate and a ubiquitous product of combustion found in factory air, kitchens, cigarette smoke, direct and sidestream (passive). It is a highly irritating toxic unsaturated aliphatic aldehyde, found to be mutagenic to a number of species. Because of its mutagenicity and toxic, irritating properties, it would be useful to have a sensitive and simple biomarker of its exposure. Recently acrolein has been shown by our laboratory to form adducts with α_1-proteinase inhibitor and serum albumin [3,4].

MATERIALS AND METHODS

Chemicals

D-Glucose 6-phosphate (G6P); nicotinamide adenine dinucleotide phosphate (NADP); ethylenediaminetetraacetate, sodium salt (EDTA); sodium chloride (NaCl); mercaptoethanol; Tris buffer (Tris); DEAE-cellulose; G6PD type XII, from Torula yeast crystallized and lyophilized; were all obtained from Sigma Chemical Company, St. Louis, MO and were all reagent grade. Acrolein (99%) was obtained from Aldrich Chemical Co., St. Louis, MO.

Partial purification of glucose-6-phosphate dehydrogenase from human erythrocytes

The procedure used for partial purification of G6PD is a modification of Mills et al. [6]. Blood samples (10–20 ml) were obtained from anonymous adult volunteers using informed consent forms. Samples were collected in heparinized tubes and immediately centrifuged at room temperature in a Nanofuge ($2000 \times g$; 10 min). Two volumes of 1 mM EDTA (pH 7.0) were added to the erythrocytes and allowed to stand for 10 min for lysis. The hemolysate was transferred to a dialysis bag and dialyzed for 2–4 h at 20–40°C against 300 ml of the following solution: 0.01 M NaCl, 1 mM EDTA (pH 7.0), 20 μM NADP, with mercaptoethanol (1:2000). The dialyzed hemolysate was filtered through glass wool to remove small clots and debris prior to applying to the chromatographic column.

DEAE-cellulose was washed three to four times by suspending in a graduated cylinder and pouring off the fines after 30 min–1 h settling time. A slurry of the washed suspension was poured onto the column to give a 3 cm (length) $\times$ 2.4 cm (diameter) column. The column was washed with 50 ml of a 0.300 M phosphate buffer (pH 6.4) and then with 100 ml of a 1:100 dilution of the same buffer (using pressure if necessary). The dialyzed hemolysate (10–25 ml) was added to the prepared column without disturbing the DEAE cellulose. The column was washed with 0.02 M phosphate buffer, using pressure as necessary to flow drop-wise through the column. A total volume of 125 ml was obtained in 25-ml fractions, each fraction collected separately. All of the hemoglobin and 6-phosphogluconate dehydrogenase (6PGD) should be removed in these washes.

The G6PD was eluted from the column with two 15-ml portions (eluates 1 and 2, respectively) of 0.50 M KCl containing 1 mM EDTA, 20 μM NADP and mercaptoethanol (1:2000). Nearly all of the G6PD activity was eluted in eluate 1 and little in

eluate 2. The KCl was removed from eluate 1 by dialysis at 2–4°C against two 300-ml portions of a Tris buffer dialyzing medium (0.050 M Tris, pH 8.0; 10 μM NADP; 2.7 mM EDTA and mercaptoethanol, 1:2000). After 16–20 h of dialysis, the G6PD preparation was removed from the dialysis bag and stored in a refrigerator at 2–4°C until ready for use. Each preparation was regularly assayed for G6PD activity.

Assay of glucose-6-phosphate dehydrogenase

The spectrophotometric assay utilized was that of Alperin and Mills [5] and utilized a Beckman Model 25 Spectrophotometer, permitting kinetic determinations. The method depends on the increase in absorbance at 340 nm with time due to NADPH formation. The reference cuvette contains 2.7 ml 0.115 M Tris buffer (pH 8.0), 100 μl 20 mM glucose 6-phosphate, 100 μl 20 mM NADP, 100 μl 0.3 M $MgCl_2$. The sample cuvette contains the same as the reference cuvette except for containing 2.6 ml of buffer plus 100 μl of G6PD enzyme fraction. Immediately after the addition of 100 μl of G6PD fractions to cuvettes 2–4 the absorbance was measured at 340 nm at 20-s intervals and plotted on a chart recorder. The slope of the best fitted line was calculated using a linear regression. The greater the slope the higher the G6PD activity.

Inactivation of glucose-6-phosphate dehydrogenase activity with acrolein

Acrolein was freshly diluted with 0.115 M Tris buffer (pH 8.0) under a fume hood to a 1:1000 dilution and an appropriate volume added to the G6PD fraction to be assayed to give a final concentration of acrolein between 0.05–0.8 mM. For most experiments, a 30-min incubation time was used. After incubation assays were performed by taking appropriate aliquots directly from the incubation mixture.

Protein assays

Protein assays were performed using the Bio-Rad protein assay kit.

Treatment of human erythrocytes with acrolein

Heparinized human blood (10 ml) was processed to obtain erythrocytes, and the erythrocytes were resuspended in 5 ml physiological (0.9%) saline. This erythrocyte suspension was separated into 1.0-ml samples; the samples were incubated for 1 h at 25°C with varying concentrations of acrolein (0–10 mM). The samples were recentrifuged at 2000 × *g* and the pellets resuspended in 1 ml physiological saline, recentrifuged and the erythrocytes lysed as above and G6PD isolated by a micro-modification of the procedure described above and assayed.

Amino acid analysis

Three 2-ml samples of standard G6PD (1 μg/ml) from Torula yeast were incubated for 1 h with varying concentrations of acrolein (1, 5 and 10 mM), while a fourth sample was left as a control. One hundred μl of each sample was assayed for G6PD activity to ensure that inactivation had occurred from acrolein treatment. The remainder of the samples were used for amino acid analysis.

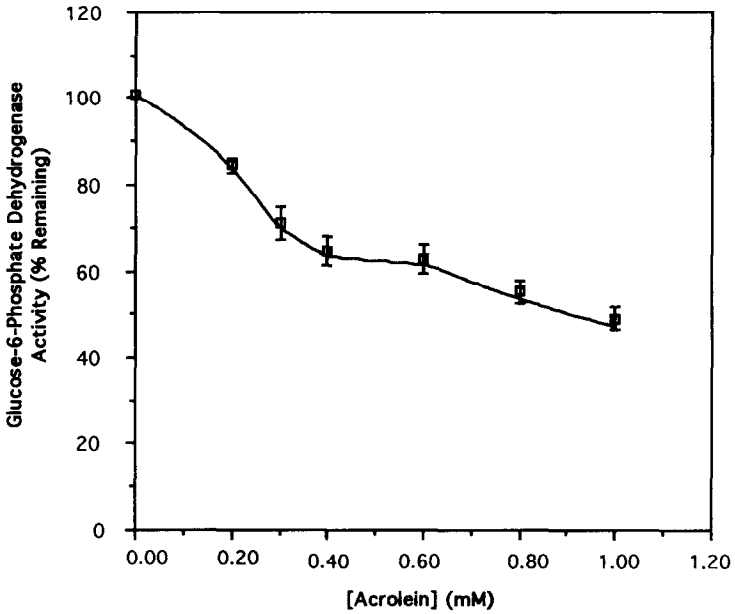

Fig. 1. Inactivation by acrolein of G6PD isolated and purified from human blood. G6PD activity (% remaining) vs. [acrolein] (mM). I = standard error.

RESULTS AND DISCUSSION

Figure 1 contains data on G6PD activity remaining after acrolein treatment for 30 min at room temperature. The G6PD was isolated and partially purified from human erythrocytes according to the method of Mills et al. [6]. With 0.6 mM acrolein (final concentration) about 40% of the initial activity of G6PD was lost, while at 1.0 mM acrolein nearly 55% of the activity was removed. The activity vs. [acrolein] curve appears to pass through a plateau, although it is unclear whether this is real or an artifact.

In Figure 2 the data for G6PD from Torula yeast are plotted. The two figures are quite similar except for Figure 2 being somewhat smoother and showing slightly higher inactivations for the same acrolein concentration compared to Figure 1, both being consequences of the erythrocyte-isolated G6PD containing other proteins.

The reaction of acrolein appears to follow a first-order reaction since linear plots were obtained (data not shown) when log C_0 was plotted vs. time (min) where C is the activity of G6PD at time t, while C_0 is the activity at zero time. Only slight differences in slope were obtained between 0.1 mM acrolein and 0.05 mM acrolein, with 0.1 mM acrolein having a slightly more negative slope (1.33:1).

Amino acid analysis shows that as a consequence of acrolein incubation there is both a reduction in the lysine peak and a dose-dependent increase of a new peak. We

believe that this latter peak is an adduct due to the reaction of acrolein with the ε-amino group of lysine, as found by our earlier investigations [3].

When acrolein was incubated directly with human erythrocytes and the G6PD isolated subsequently, about 10-times the concentration of acrolein was required for comparable inactivation (Fig. 3) compared with the case of isolated and partially purified G6PD (Fig. 1). The higher concentrations required are likely due to binding of acrolein to other blood proteins and erythrocyte glutathione.

Gan and Ansari [3] have previously shown that four new peaks were observed upon amino acid analysis of α_1-proteinase inhibitor (α-PI) which was inactivated by acrolein under in vitro conditions. One peak was identified as 3-oxopropyl lysine and the other three due to adducts of acrolein with histidine.

Acrolein is found in cigarette smoke [7] as well as in the environment [8]. The fact that it can inactivate G6PD in vitro suggests that it may also inactivate G6PD in vivo, although this has yet to be established. An inactivation of G6PD in vivo such as in the case of smokers or those exposed occupationally might well chronically diminish these individual's protection against oxidative hemolytic agents (nitro-compounds, aniline, sulfonamides, primaquine, chloroquine) that could induce a hemolytic crisis, as they do in those individuals with G6PD deficiency [9]. These individuals, even if they possessed normal levels of G6PD, after chronic exposure to acrolein and other G6PD-depleting agents could be at increased risk of red blood cell rupture. Further,

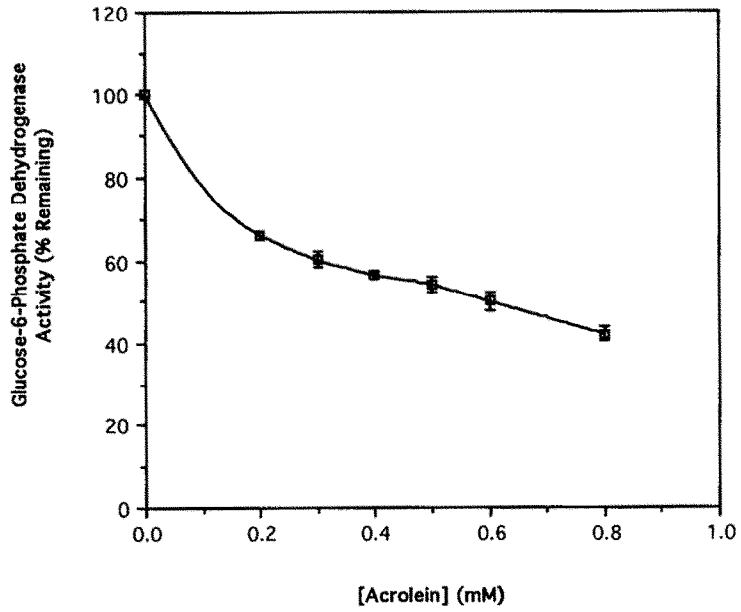

Fig. 2. Inactivation by acrolein of purified G6PD from Torula yeast. G6PD activity (% remaining) vs. [acrolein] (mM).

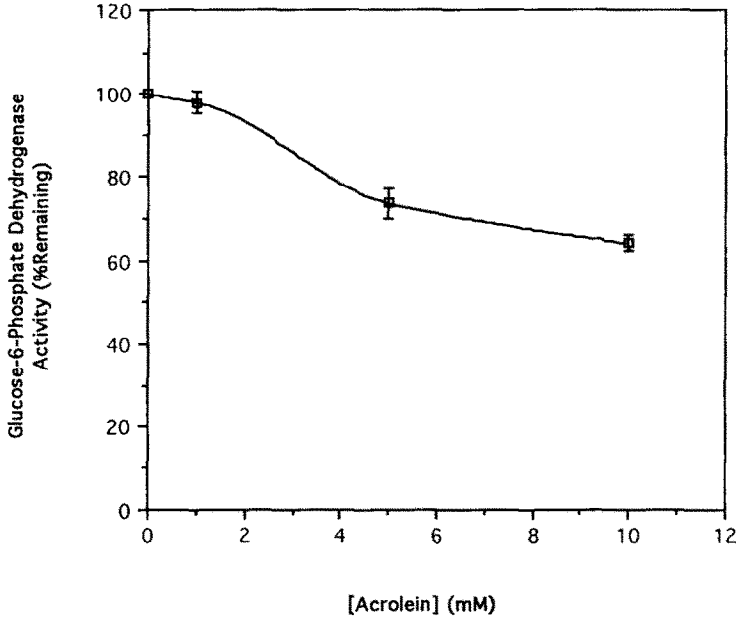

Fig. 3. Inactivation of G6PD in human blood by treatment with acrolein. G6PD activity (% remaining) vs. [acrolein] (mM).

acrolein exposure might also place individuals with genetic G6PD deficiency at increased risk of experiencing a hemolytic crisis.

Our results in the present study show that acrolein inactivates G6PD in vitro. These findings occur in purified enzyme (Torula yeast), enzyme partially purified from human erythrocytes and in human erythrocytes with subsequent isolation of G6PD. The inactivation is nearly complete (60–80% inactivation) between 30 min–1 h at room temperature. The assay may be a useful biomonitor because it provides a direct measure of enzymatic activity, it is a simple, accurate assay and furthermore, the human red blood cell, with a lifetime of about 120 days, would provide G6PD with 120 days of exposure to low doses of acrolein and essentially perform as an integrated dose monitor. Therefore, it offers an advantage over serum proteins which have shorter half-lives.

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