Reducing Sugars Trigger δ-Aminolevulinic Dehydratase Inactivation: Evidence of In Vitro Aspirin Prevention*

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INTRODUCTION

Nonenzymatic glycation of proteins, mainly hemoglobin, and the relation between this process and diabetes mellitus has been extensively reviewed (Strowig and Raskin, 1995). The aldehyde group of glucose is known to react with amino groups of proteins, resulting in a Schiff-base conjugate formation, which subsequently undergoes an Amadori rearrangement leading to a stable ketoamine adduct (Nagaraj et al., 1996).

Glycation is the first of a series of reactions known as “Maillard browning” (Nagaraj et al., 1996). This process can promote in vitro cross-linking of proteins through ε-NH2 groups of lysine or hydroxylysine. Maillard browning is enhanced in vivo in the diabetic condition, especially in tissues in which protein turnover is slow and physiological effects are stronger than in blood, such as in lens crystallin (Thorpe and Baynes, 1996) and tendons (Kochakian et al., 1996). There is also some evidence suggesting that protein glycation may be implicated in the long-term complications of diabetes mellitus (Brownlee, 1992; Cerami et al., 1991), and in aging diseases (Thorpe and Baynes, 1996), and it is the primary causal factor for the development of diabetic microvascular complications (Brownlee, 1995). It is known that protein cross-linking may either alter protein functions or change their susceptibility to enzymatic degradation (Fu et al., 1994); moreover, in vitro and in vivo modifications in enzymatic activities have been well documented (Brownlee, 1994). On the other hand, it has been demonstrated that aspirin can prevent in vitro and in vivo glycation of albumin and hemoglobin by acetylation of the potential glycation sites (Rendell et al., 1986).

Studies carried out in a diabetic population showed that the activities of several heme enzymes such as δ-aminolevulinic dehydratase (ALA-D), porphobilinogen deaminase and uroporphyrinogen de-carboxylase are diminished in blood (Caballero et al., 1995). Alterations in the heme pathway were also observed in streptozotocin-induced diabetic mice (Polo et al., 1995).

The aim of this study was to investigate the effect of in vitro glycation on ALA-D under different experimental conditions; the evolution of this process was correlated with the amount of glycated hemoglobin produced. To determine if lipid peroxidation products can promote protein glycation, the peroxidation index was estimated. Furthermore, the potential role of acetyl salicylic acid (ASA) (aspirin) in preventing the nonenzymatic glycation was examined.

MATERIALS AND METHODS

Materials

All chemicals used were reagent degree and obtained from Sigma (St. Louis, MO, USA).

Enzymatic source

Blood from healthy volunteers was obtained by venipuncture, withdrawn in a heparin- or EDTA-containing flask and kept at 4°C.

Glycation procedure

Red blood cells (RBCs) were separated, washed with saline and preincubated at 37°C in the dark and aerobiosis, for as long as 20 hr in humid atmosphere, with shaking, after adding the sugar at a final concentration of 500 mM in a balanced salt solution (glucose free), unless otherwise specified (RBC:balanced salt solution, 1:10 v/v).

ABSTRACT. 1. The effect of in vitro glycation on δ-aminolevulinic dehydratase (ALA-D) under several experimental conditions was investigated. When preincubated with 500 mM glucose at 37°C for 20 hr, ALA-D was 80% inactivated and glycated hemoglobin levels were increased more than fourfold.

2. Thiobarbituric acid species were not modified during glycation; therefore ALA-D inactivation cannot be attributed to glucose autoxidation.

3. Acetyl salicylic acid was effective in preventing both hemoglobin glycation and ALA-D inactivation by glucose.

4. A method has been developed for measuring protein glycation in vitro, in a crude preparation of red blood cells, which can also be applied to sugars other than glucose.

KEY WORDS. Glycation, carbohydrates, acetyl salicylic acid, lipid peroxidation, δ-aminolevulinic dehydratase, enzyme inactivation, glycated hemoglobin

To the memory of our beloved César.

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Received 5 September 1997; accepted 15 December 1997.
Controls, without sugar but preincubated in the same conditions, were included in all experiments. The addition of any carbohydrate to the enzyme assay mixture during incubation, at a final concentration of 500 mM, had no effect on enzyme activity. Samples of RBCs were preincubated with or without glucose in the presence of ASA (10 mM) for as long as 20 hr.

**Enzyme assays**

ALA-D activity was determined by the method of Batlle et al. (1967). Because preincubation periods could be as long as 20 hr, zinc acetate was added to the medium at a final concentration of 1 mM to prevent oxidation of thiol groups. Activity was expressed as units per milligram of hemoglobin (Hb). An enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product under the standard incubation conditions.

Glycosylated hemoglobin (GHb) was measured by using the method described by Parker et al. (1981) and was expressed as nanomoles of hydroxymethylfurfural (HMF) per milligram of Hb.

The peroxidation index was estimated by the formation of malondialdehyde (MDA) and determined as thiobarbituric acid reactive species (TBARS) by the method of Niehaus and Samuelson (1968).

**Statistics**

Data represent mean values ± SD of at least four separate experiments conducted in triplicate and were analyzed statistically by using nonpaired Student’s t-test with the Sigma Plot 2.0 statistical software for IBM.

**RESULTS**

**Effect of glucose concentration**

ALA-D activity was determined after preincubating RBCs with different glucose concentrations (range, 50–500 mM). Enzyme activity was diminished by about 10% at the lowest concentration of sugar assayed; the inhibition profile was sharper for higher glucose concentrations and reached an inhibition of 80% at 500 mM of glucose, with respect to controls under the same conditions but in the absence of glucose (Fig. 1). The progression of glycation was followed by the detection of GHb, which augmented more than fourfold at 500 mM glucose (Fig. 1).

**Effect of temperature**

When preincubation was carried out at 4°C with or without 500 mM glucose for 20 hr, ALA-D activity was not altered. A slight variation (15%) between the enzyme activity evaluated without and with glucose was detected when glycation was at 22°C. Instead, at 37°C, a great difference between ALA-D activity preincubated with and without glucose was detected, reaching an inactivation of about 80% in the presence of the sugar. Hemoglobin glycation occurred only at 37°C under the assayed conditions (Fig. 2).

**Effect of time**

Preincubation with 500 mM glucose for different intervals showed a sharp diminution of ALA-D activity already observed at the shortest time tested, reaching its lowest value after 20 hr of preincubation. Controls without sugar showed a diminution of enzyme activity, which was significantly lower than that detected in the presence of the sugar (Fig. 3A).

GHb was determined in samples run in parallel, as a control of the glycation process. GHb levels augmented along the period of assay; a correlation between ALA-D inactivation and GHb formation was found (Fig. 3B).

The peroxidation index was determined to evaluate if the enzyme inactivation was produced as a consequence of glucose oxidation. No

**FIGURE 1.** Effect of preincubation in the presence of different glucose concentrations on ALA-D activity (●) and GHb formation (■). Red blood cells were preincubated for 20 hr at 37°C with glucose (50–500 mM final concentration) or without the sugar. Other experimental details were as indicated in the text. *P<0.05. HMF=hydroxymethylfurfural.

**FIGURE 2.** Effect of preincubation temperature in the presence of glucose on ALA-D activity (open bars) and GHb formation (hatched bars). Red blood cells were preincubated for 20 hr at 4, 22 and 37°C in the presence of 500 mM glucose. Results are expressed as percentage of the controls preincubated in the same conditions without sugar at the indicated temperatures. Other experimental details were as indicated in the text. Control values at zero time: ALA-D=11.77±0.70 U/mg Hb (n=9); GHb=0.67±0.03 nmol HMF/mg Hb (n=9). *P<0.05.
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TABLE 1. Effect of different carbohydrates on ALA-D activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Enzymatic activity (%)</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>20.8 ± 1.0*</td>
</tr>
<tr>
<td>Galactose</td>
<td>81.5 ± 3.3</td>
</tr>
<tr>
<td>Mannose</td>
<td>44.8 ± 2.1*</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>48.1 ± 1.9*</td>
</tr>
<tr>
<td>Fructose</td>
<td>77.6 ± 3.9</td>
</tr>
<tr>
<td>Xylose</td>
<td>15.1 ± 0.9*</td>
</tr>
<tr>
<td>Arabinose</td>
<td>22.4 ± 1.2*</td>
</tr>
<tr>
<td>Lactose</td>
<td>108.2 ± 5.4</td>
</tr>
<tr>
<td>Maltose</td>
<td>43.9 ± 2.6*</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>91.2 ± 4.6</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>34.1 ± 2.4*</td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>47.0 ± 2.8*</td>
</tr>
</tbody>
</table>

Red blood cells were preincubated 20 hr at 37°C in the presence of the indicated carbohydrates at a final concentration of 500 mM. Results are expressed as percentage of the controls preincubated without carbohydrate. Other experimental details were as indicated in the legend to Figure 2 or in the text.

*P < 0.05.

the glycation of hemoglobin (Fig. 4B), reaching the GHb basal levels after 20 hr of preincubation in the presence of both agents.

DISCUSSION

Several studies in past decade have highlighted the importance of hexose sugars, especially glucose, as being responsible for alterations in the presence (C) of glucose on (A) ALA-D activity, (B) GHb formation (C) and TBARS formation. Red blood cells were preincubated at 37°C for 20 hr in all cases. Glucose (500 mM) was added at different times during the whole period of preincubation, being in contact with the enzymes at the indicated times (2, 4, 6, 14, 16 or 20 hr). Other experimental details were as indicated in the text. *P < 0.05.

Effect of other carbohydrates

With the aim of determining whether glycation was specific for glucose or whether it could also occur with other sugars, different hexoses, pentoses and disaccharides were tested. All the hexoses assayed inactivated ALA-D between 50 and 80%, except galactose and fructose, which reduced the enzyme activity by only 20%. Pentoses (xylose and arabinose) strongly inactivated ALA-D (80%). In glycation by disaccharides, lactose (α-β-galactoside) did not alter the activity of ALA-D; however, maltose (glucose homogeneous disaccharide) inhibited it by 60%. Sorbitol slightly reduced ALA-D activity. Glucosamine reduced the enzyme activity by more than 50% and 2-deoxyglucose produced a 50% inhibition (Table 1).

Effect of ASA

We relied on our in vitro glycation system to study the effects of ASA on ALA-D inactivation and GHb formation. Preincubation with glucose (500 mM) and ASA (10 mM) prevented the inactivation of ALA-D during the whole period studied (Fig. 4A) and even prevented changes in TBARS levels were detected along the whole period studied, either in the controls or in the glucose-treated RBCs (Fig. 3C).

FIGURE 3. Effect of preincubation time in the absence (□) or presence (○) of glucose on (A) ALA-D activity, (B) GHb formation (C) and TBARS formation. Red blood cells were preincubated at 37°C for 20 hr in all cases. Glucose (500 mM) was added at different times during the whole period of preincubation, being in contact with the enzymes at the indicated times (2, 4, 6, 14, 16 or 20 hr). Other experimental details were as indicated in the text. *P < 0.05.

FIGURE 4. Effect of ASA on (A) ALA-D activity and (B) GHb formation. Red blood cells were preincubated at 37°C in the presence of glucose (500 mM) and ASA (10 mM) (●) or glucose (500 mM) (■) for different periods of time. Other experimental details were as indicated in the text and in Figure 3. *P < 0.05.
procedures, the activity of an enzyme of the heme pathway also can be modified. The dose–response curve for the inhibitory effect of glucose on this enzyme was correlated with the glycation process, which was evaluated through the formation of GHb. The level of GHb in blood has been considered to indicate the degree of glycation of other proteins that are freely exposed to circulating glucose (Schwartz, 1995).

Considering the effect of the temperature during glycation, we assume that this phenomenon would occur only at 37°C, at least under our experimental protocol.

Although glycation appeared to be effective even at the shortest periods assayed, its greater effects on ALA-D activity and hemoglobin glycation were detected at longer times. Furthermore, the glycation procedure used here also proved to be useful for investigating nonenzymatic glycation by sugars other than glucose. Each hexose assayed inactivated ALA-D at a different degree. It was demonstrated that 11 different sugars were able to produce glycation of myofibrillar proteins and inactivation of ALA-D activity (Syvory, 1994). Similar differences in the effects of pentoses and disaccharides also were found.

From these findings, it appears that varying effects on the inactivation of the enzymes by different sugars could not be ascribed to steric reasons but to differential affinity of the sugar for lysine groups at or near the active site; the hydrophobicity of the active site environment also might play a significant role in the sugar–enzyme interaction (Brownlee, 1994).

Sorbitol did not inactivate ALA-D. It is interesting to recall that Trüeb et al. (1980) reported that sorbitol, which does not bear a reactive aldehyde or keto group, only weakly binds collagen.

Beswick and Harding (1985) reported that the rate of binding of 2-deoxyglucose (a nonautoxidizable sugar) to lens α-crystallin is similar to that of glucose (an autoxidizable sugar). These authors concluded that it is the open-chain aldehyde of glucose that binds initially to the protein amino groups and that there is no participation of dicarbonyl autoxidation products in the initial nonenzymatic protein glycation reaction. In contrast, Wolff and Dean (1987) suggested that a component of protein glycation depends upon glucose autoxidation and subsequent covalent attachment of ketoaldehydes.

It is noticeable that enzyme inactivation could result not only from glycation, but also from the side effects of glucose autoxidation involving the formation of free radicals, which might in turn directly act on enzyme activity (Jain and Palmer, 1997). In our system, we could not attribute ALA-D inactivation to free-radical formation, because we did not detect any lipid peroxidation; however, accumulated effects occurring in vitro, cannot be discarded, because glycation itself could lead to formation of reactive oxygen species (Yim et al., 1996).

Glycation of lens proteins might be a contributory factor in the development of diabetic and senile cataracts. In vitro studies with rat lens crystallins demonstrated that ASA inhibits cataratogenesis (Cherian and Abraham, 1993).

Acetylation by aspirin would prevent glycation inhibition by blocking the potential glycation residues (α-NH2 groups). Aspirin inhibited in vitro and in vivo lens crystallin glycation, thiol oxidation and the formation of high-molecular-weight aggregates (Swamy and Abraham, 1989).

In our experimental model ASA was effective in inhibiting glycation of Hb as well as preventing ALA-D inactivation. These results provide additional support for the potential use of aspirin in the late stage of the Maillard reaction.

At present, investigations on the isolated enzyme are being performed to identify the amino acid(s) taking part in the carbohydrate linkage.

**SUMMARY**

The interaction between proteins and reducing sugars, known as the Maillard reaction, proceeds through the formation of early products, such as Schiff-base conjugates, to protein cross-linking, leading to the alteration of their functions. A frequent coexistence of diabetes and porphyria has been reported. 

The effect of in vitro glycation on one of the heme pathway enzymes, (ALA-D), under several experimental conditions was investigated. In this paper, we provide evidence showing that the activity of ALA-D can be modified, depending on the conditions of the glycation procedure. When preincubated with 500 mM glucose at 37°C for 20 hr, ALA-D was 80% inactivated and GHb levels were increased more than fourfold. ALA-D inactivation could not be attributed to glucose autoxidation, because TBARS were not modified during glycation. It has also been shown that ASA (aspirin) was effective in preventing both Hb glycation and ALA-D inactivation by glucose. These findings are providing further support for the potential use of aspirin during the late stages of the Maillard reaction. A method has been developed for measuring protein glycation in vitro, in a crude preparation of RBCs, that can also be applied to sugars other than glucose.

**References**


Niehaus W. and Samuelson B. (1968) Formation of malondialdehyde from...
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