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Fructose Induced Deactivation of Glucose-6-Phosphate Dehydrogenase Activity and its Prevention by Pyruvate: Implications in Cataract Prevention

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Glucose-6-phosphate dehydrogenase (G6PDH) is an important lens enzyme diverting about 14% of the tissue glucose to the hexose monophosphate shunt pathway. The main function of such a pronounced activity of the enzyme is to support reductive biosyntheses, as well as to maintain a reducing environment in the tissue so as to prevent oxy-radical induced damage and consequent cataract formation. Sugars are one of the well-known cataractogenic agents. Several reports suggest that the cataractogenic effect of the sugars in diabetes as well as in normal aging is initiated by the glycation of the proteins including the enzymes and subsequent formation of more complex and biologically inactive or harmful structures. In a diabetic lens the concentration of fructose exceeds significantly the concentration of glucose, suggesting that the contribution of fructosylation may be greater than that of glucosylation. These studies were undertaken to examine further the possibility that in addition to glycation, generation of oxygen free radicals by fructose and consequent oxidative modifications in certain enzymes may be an important participant in the cataractogenic process. This hypothesis was tested by using G6PDH. The enzyme was incubated with various levels of fructose (0–20 mM) and its activity determined as a function of time. This led to a significant loss of its activity, which was prevented by superoxide dismutase, catalase, mannitol and myo-inositol. Most interestingly, pyruvate at levels between 0.2 and 1.0 mM also offered substantial protection. Hence, the results, while elucidating further the mechanism of enzyme deactivation by sugars such as fructose, also demonstrate the possibility of therapeutic prevention of cataracts by pyruvate and other such keto acids, in diabetes and other disabilities involving oxygen free radicals in the pathogenetic process.

Keywords: Glucose-6-phosphate dehydrogenase, deactivation, fructose, oxygen radicals, cataract, pyruvate

INTRODUCTION

Earlier studies have demonstrated that the ocular lens has a very active hexose mono-phosphate shunt activity. This is apparent by the high ratio of the 1-^{14}C-glucose derived $^{14}CO_2$ to 6-^{14}C-glucose derived $^{14}CO_2$.[1] The initial enzyme responsible for this high activity is glucose-6-phosphate dehydrogenase (G6PDH).[2] Functionally, one of

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its main roles is to maintain adequate levels of NADPH, required in reductive biosyntheses. In addition, it is important for preventing reactive oxygen species (ROS) dependent oxidative damage to the lens and other tissues through its integration with the glutathione (GSH) redox cycling process involving glutathione reductase and glutathione peroxidase.[3,4]

The activity of this enzyme is known to decrease with aging as well as cataract formation.[5,6] In a recent study, the enzyme has been reported to lose its activity by its glycation with fructose.[7] Earlier studies have demonstrated that the concentration of this sugar increases from <1 mM in a normal lens to ≈10–15 mM in a diabetic lens.[8,9]

Since fructose is more unstable in comparison to other sugars such as glucose and galactose, the deactivation of the enzyme by this sugar can also be due to the ROS generated during its auto-oxidation and their consequent deleterious effect on the enzyme structure and function. That fructose is more readily auto-oxidized in comparison to other sugars is apparent from the change of its color from white to pale yellow on exposing it to air and on long storage. The possibility that ROS generated from fructose by its auto-oxidation might also contribute to the enzyme deactivation was, therefore considered. The ROS generated by the Amadori product of the enzyme may also be destructive. Overall, ROS could be playing an important role in sugar induced deactivation of various enzymes including that of G6PDH. This hypothesis has been verified. Incubation of the enzyme with fructose at levels prevalent in diabetic lens led to a pronounced loss of its activity. This has been found to be preventable by superoxide dismutase (SOD), catalase and mannitol. In addition, pyruvic acid has also been found to be highly preventive. The latter findings are also considered important from the viewpoint of understanding the role of normal metabolism against the pathogenesis of cataracts and the development of future physiologically compatible anticataract agents.

MATERIALS AND METHODS

All the chemicals were purchased from Sigma Chemical Company, St. Louis, MO 63178.

Measurement of the Inhibition of G6PDH by Fructose

The effect of incubating G6PDH with fructose on its enzymatic activity was determined by measuring spectrophotometrically (OD_{340}) the generation of NADPH from $NADP^+$ in the presence of glucose-6-phosphate (G6P).[7] Briefly, 0.5 units of G6PDH (Sigma #Lot 47H8010) were incubated with various concentrations of fructose (0–20 mM) and 0.43 mM $NADP^+$ in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C for 0, 4 and 6 h. The volume of incubation mixture was 1 ml. Subsequently, magnesium chloride (6.7 mM) was added to the above mixture raising the volume to 2.9 ml. 0.1 ml of G6P (1 mM) was then added and the generation of NADPH followed for 5 min. The effect of fructose on the activity was expressed as the percentage of the basal activity obtained in the absence of fructose. Additional experiments were conducted to rule out the possibility of $NADP^+$ degradation by incubation with fructose and consequently a false appearance of the enzyme inactivation. Hence, in these studies $NADP^+$ was added after the enzyme was pre-incubated with fructose alone and then its activity determined. Also, experiments were conducted wherein $NADP^+$ was incubated with fructose (20 mM) prior to its use in the enzyme assay.

To determine the role of ROS in fructose-dependent loss of G6PDH activity, 0–5 units of catalase or 0–15 units of SOD were added to the incubation mixture containing 20 mM fructose. Mannitol (0–30 mM) was also used to study the effect of $OH^\bullet$. The protective effect of pyruvate (0–1 mM) was also similarly determined.

The production of $O_2^{\bullet -}$ by fructose as well as glucose and galactose as reference sugars was

ascertained by incubating these sugars with ferri-cytochrome c and measuring the $O_2^{\bullet-}$ dependent reduction spectrophotometrically at 550 nm. Ferri-cytochrome c (20 μM) in 0.1 M sodium phosphate buffer, pH 7.4 was incubated in 3 ml cuvettes at 37°C in the absence or presence of the above sugars and the reduction of ferri-cytochrome c monitored as a function of time. The concentration of fructose was varied between 0 and 20 mM. Glucose and galactose were used at a fixed concentration of 20 mM. That the reduction of ferri-cytochrome c in the presence of the sugars was $O_2^{\bullet-}$ dependent was affirmed by observing the inhibition of the process by SOD.

RESULTS

Data demonstrating the inhibitory effect of fructose on G6PDH activity are summarized in Figure 1. The activities were determined at 0, 4 and 6 h after incubation with fructose. The loss of activity was proportional to the time of incubation as well as to the fructose concentration. The activity decreased to about 50% of the controls in 6 h in the presence of 12 mM fructose. A similar inhibition took place only in 4 h if the sugar concentration was raised to ≈20 mM. However, in these experiments the enzyme was incubated with fructose and $NADP^+$ together. Hence, it was possible that the observed inhibition may be due to the degradation of $NADP^+$ by fructose, instead of the enzyme deactivation. Hence, further experiments were carried out in which the $NADP^+$ was added after the initial incubation of G6PDH with fructose. Inhibition of G6PDH was observed again, suggesting that the loss of enzyme activity is attributable to the effect of fructose on the enzyme *per se*. Additional experiments demonstrated that $NADP^+$ is not degraded to any significant extent by incubation with fructose.

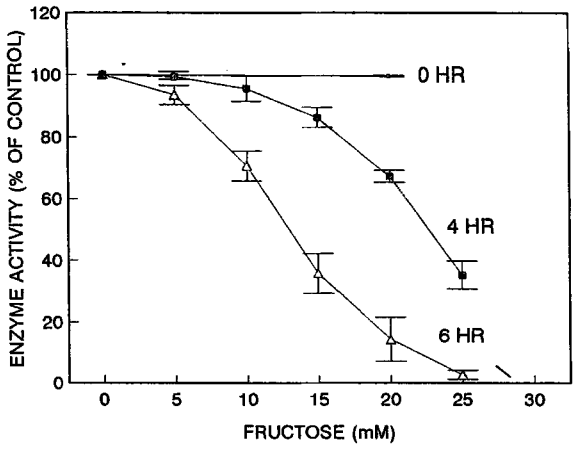

FIGURE 1 Loss of G6PDH activity on incubation with fructose: 0.5 units of G6PDH were incubated in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.43 mM $NADP^+$ and fructose (0–20 mM) at 37°C in 3 ml cuvettes. The final incubation volume was 1 ml. At indicated times, the enzyme activity was determined spectrophotometrically by following an increase in OD 340 nm due to NADPH generation, after the addition of magnesium chloride and G6P as described in the methods. The results (mean ± standard deviation) are expressed as the percent of controls wherein the enzyme was incubated without any fructose. $N = 4$ in each case.

The concentrations of fructose used in these experiments are also similar to those prevalent in a diabetic rat lens.[8,9] Human diabetic lens fructose level is about 10 mM.[10,11] The findings are hence considered physiologically relevant.

To determine if the loss of the enzyme activity is attributable to the production of $O_2^{\bullet-}$ and its derivatives generated by auto-oxidation of fructose, we determined the relative effectiveness of fructose in reducing ferri-cytochrome c. As summarized in Figure 2, such a reduction is quite measurable. The process is dose and time dependent. The range of fructose concentrations and the periods of incubation, were both similar to those used in Figure 1 showing the loss of enzyme activity. It was interesting to note, however, that the reduction of ferri-cytochrome c by glucose or galactose, even at 20 mM concentration, was very small if any, in comparison to fructose. That the reductive process induced by fructose is $O_2^{\bullet-}$ dependent was apparent by its inhibition with SOD (Figure 3). Catalase was not effective. Hence, further experiments were conducted to examine if the $O_2^{\bullet-}$ generated from fructose and its

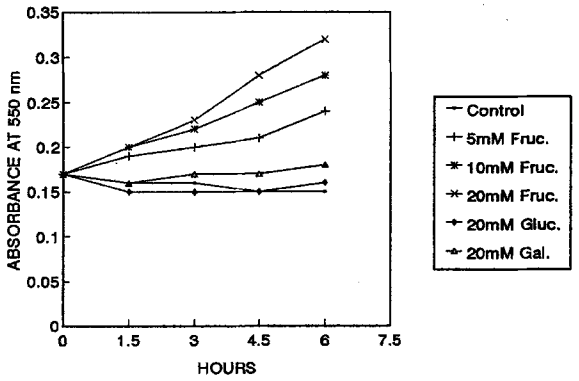

FIGURE 2 Superoxide generation by auto-oxidation of sugars: This was determined spectrophotometrically by following the reduction of ferri-cytochrome c. The sugars at indicated concentrations were incubated in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4 containing 20 μM ferri-cytochrome c at 37°C. The increase in OD 550 nm was measured at indicated times. A blank control was also run without any sugar. $N=3$.

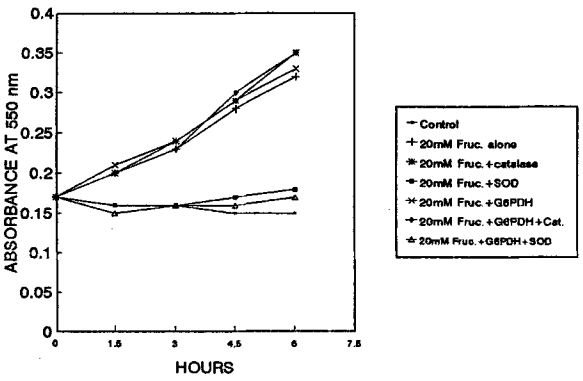

FIGURE 3 Inhibition of the fructose dependent reduction of ferri-cytochrome c by SOD: 0 or 5 units of SOD were added to the reaction mixtures as described in the methods and increase in OD at 550 nm measured. $N=3$.

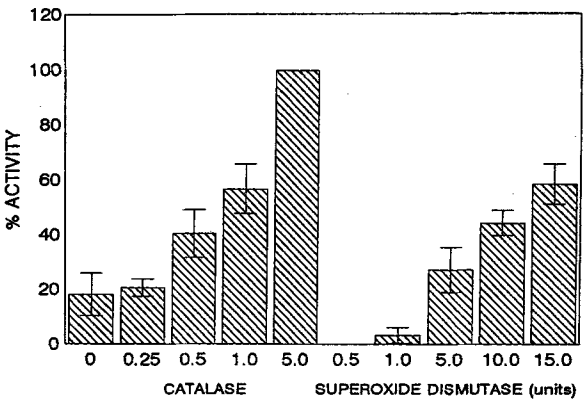

FIGURE 4 Protective effect of catalase and SOD against loss of G6PDH activity: Catalase or SOD was added to the reaction mixtures as described in the legend of Figure 1. $N=4$ in each case, except for the bar representing 5 units of catalase, where it is 2.

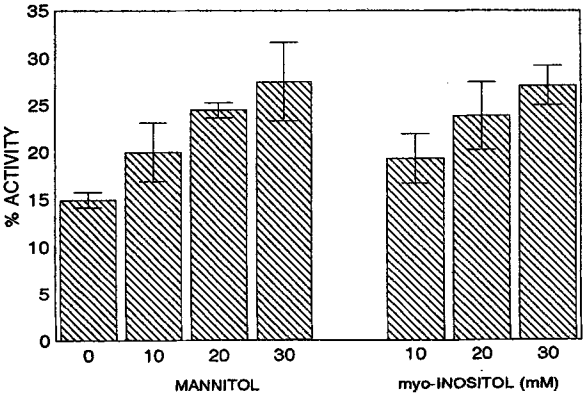

FIGURE 5 The effect of the $OH^{\bullet}$ scavengers, mannitol and myomositol on fructose-dependent loss of G6PDH activity: The enzyme was incubated as described in Figure 1, but in the absence or presence of the polyols at indicated levels. The activities are expressed as percent of the controls. $N=4$ in each case.

derivatives (ROS) were indeed responsible for inactivating the enzyme *per se*. This was done by monitoring the activity of G6PDH as influenced by fructose, in the presence of SOD, catalase, mannitol and myo-inositol. As shown in Figure 4, catalase was protective. The protection was proportional to the level of the enzyme used. The same was true with SOD, although in this case the protection observed was not complete. The protective effect of SOD however is not due to its possible contamination with catalase as sometimes occurs. This was evident from the inability of SOD to decompose H_2O_2. The effect of SOD coupled with the full protective effect of catalase is consistent with the hypothesis that the actual damaging agent is $OH^{\bullet}$ produced by the Haber–Weis reaction. In line with this thought, mannitol and myo-inositol, both were found to be partially protective (Figure 5). The partial effect of these agents can be explained on the basis of a

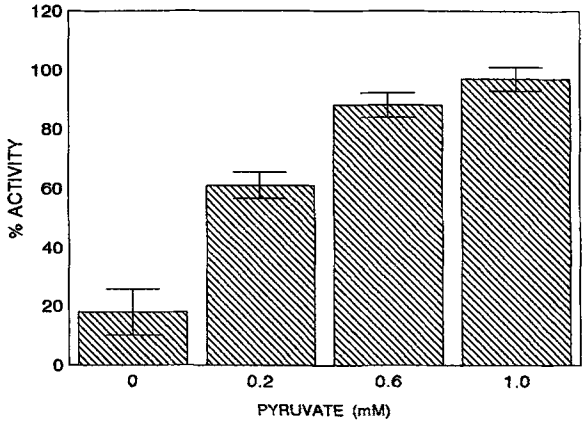

FIGURE 6 Protective effect of pyruvate against loss of G6PDH activity on incubation with fructose: This was determined by incubating the enzyme with fructose in the absence or presence of pyruvate (0–1 mM). The protocol was similar to that described in Figure 1. $N=4$ in each case.

more rapid reaction of the $OH^{\bullet}$ with the enzyme than with the sugar polyols that are devoid of any highly reactive site. The ROS scavengers used in this study do not have have any effect on the basal G6PDH activity.

Since the previous studies have demonstrated that pyruvate can also scavenge $O_2^{\bullet -}$ as well as H_2O_2,[12,13] we determined if it could also effectively prevent the fructose dependent enzyme deactivation. As shown in Figure 6, this metabolite was indeed very effective. It offered significant prevention at a low level of 200 μM. By increasing the concentration to 1 mM, the damaging effect of fructose was completely abolished.

DISCUSSION

Sugar-induced structural modifications in proteins have been suggested to participate in the genesis of several age dependent as well as diabetic manifestations including cataracts.[14–16] The possibility of such modifications increases with age, because of a decline in body metabolism and consequently a greater availability of unutilized sugars. While several studies exist on the modification of structural lens proteins (crystallins) by glycation, studies on the glycation of the enzymatic proteins are limited. The glycation of the proteins involves an initial reaction of their free $-NH_2$ groups with either phosphorylated or non-phosphorylated sugars forming a Schiff base. Among the non-phosphorylated sugars, glucose is the primary sugar available in the body, but its glycating activity is fairly low. On the other hand, fructose is much more potent.[14] This may therefore have a special significance in those tissues where its concentration is significantly high. Such high concentrations occur primarily in the accessory tissues of the reproductive system such as the seminal vesicles. Its occurrence in significant amounts in the lens however is a noticeable exception. For example, in the normal rat lens its level is approximately 100 mg/kg lens weight. In the older lenses it increases to about 340 mg/kg lens.[17] This increase may be due the age-related inhibition of glycolysis and consequent diversion of glucose into the sorbitol pathway whose activity in the lens is much more pronounced than in other tissues, with the exception of certain reproductive tissues. The concentration of glucose also rises by about 20% in older lenses. Hence, the significance of the phenomenon of the fructosylation of proteins in the lens should be much more than that of the glucosylation. Additionally, as previously known, fructose is a more potent glycating agent. It also undergoes much more rapid oxidation. This is apparent from a high rate of ferri-cytochrome c reduction by fructose than glucose. In line with these properties, the inhibition of the enzyme was also greater with fructose. That ROS generation is the primary factor in the loss of the enzyme activity on incubation with fructose is proven by the preventive effect of SOD and catalase as well as the effect of certain polyols acting as $OH^{\bullet}$ scavengers. Most interestingly, this inhibition is significantly prevented by pyruvate. We have previously demonstrated that pyruvate can also competitively inhibit fructosylation of the lens proteins.[18] Hence one may interpret that the prevention of the fructose

dependent loss of G6PDH activity may also be due to the inhibition of enzyme fructosylation alone. However, the effective concentration of pyruvate (1 mM) was much lower than the concentration of fructose (20 mM) required for maximal inactivation. Even at that high fructose concentration, pyruvate completely prevented the inhibition of the enzyme. Hence, the competitive inhibition of fructosylation by pyruvate appears to play a minor role if any in this case. The results hence clearly demonstrate that pyruvate is acting here as an ROS scavenger. The studies also provide further support to the hypothesis of cataract prevention by pyruvate or other ketoacids as suggested earlier.[19,20]

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