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Free alanine, aspartic acid, or glutamic acid reduce the glycation of human lens proteins

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The amino acids lysine and glycine are reported to react with glucose at physiological pH and temperature and undergo non-enzymic glycation. Three other amino acids present in relatively larger amounts in the lens i.e. alanine, aspartic acid and glutamic acid were also found to undergo non-enzymic glycation as found by incorporation of uniformly labelled (U-[^{14}C]) glucose into the amino acids. The glucose incorporation was 1.6 to 2.5% for alanine, 35 to 50% for aspartic acid and 2.3 to 3.3% for glutamic acid. Each amino acid of varying concentrations lowered the extent of *in vitro* glycation of lens proteins significantly in glucose-treated homogenates of normal lens from humans. The decrease in glycation for alanine was between 32 and 69%, that for aspartate was between 18 and 74%, and for glutamate was between 52 to 74%. Decreased glycation was greater for higher concentrations of glucose. Scavenging of intracellular glucose and decreasing the extent of glycation of lens proteins could be the mechanism of action by which the amino acids alanine, aspartic acid and glutamic acid could exercise a beneficial effect on cataract and diabetic retinopathy.

Keywords: alanine, aspartic acid, glutamic acid, glycation, lens proteins, cataract, diabetic retinopathy

Introduction

The amino acid lysine has been reported to delay cataractogenesis by an unknown mechanism [1]. Ramakrishnan [2] and Ramakrishnan and Sulochana [3] have shown that lysine as well as glycine decreased glycation of lens proteins and thereby they could mitigate cataractogenesis. This is because, one of the biochemical causes for the formation or progression of cataract is the glycation of lens proteins, formation of Schiff's bases and Amadori and Advanced Glycation End (AGE) products which denature the proteins. Consequently, the proteins become insoluble and cause opacity to the lens [4, 5]. The free amino acids lysine and glycine formed adducts with glucose at physiological temperature and pH [6]. They could, therefore, scavenge intracellular glucose and protect the proteins from glycation. In essence, there would be competition between free amino acids and protein-lysine for glucose. As the basic reaction in the glycation of proteins is the interaction of the epsilon amino group of lysine with the aldehydic group of glucose, it was thought that other amino acids present in relatively larger amounts [7–9] in the lens like alanine, aspartic and glutamic acids might have antiglycating properties and could therefore mitigate cataractogenesis.

In this study the glycation of alanine, aspartic acid and glutamic acid was studied *in vitro*. The amino acids were treated with glucose to which U-[^{14}C] glucose was added. The reaction was conducted at physiological pH and temperature. The reaction mixture was separated by paper chromatography and the ^{14}C label in the adduct was measured by counting the radioactivity in the fractions.

In the second study, homogenate of the eye lens from normal human donors was treated with glucose and U-[^{14}C] glucose with and without the above amino acids. The incorporation of ^{14}C glucose into the proteins was then investigated.

Materials and methods

Amino acids (alanine, aspartic acid, glutamic acid) were obtained from Sigma, and Analar glucose was used. Different concentrations of the amino acids, namely 5 and 10 mM, were added to sodium phosphate buffer pH 6.9

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(probably the pH inside the lens), and to each tube, different concentrations of glucose were added together with 2 μ Ci of U-[^{14}C] glucose and sodium azide (0.1%) as a preservative. The tubes were kept at 37 °C with periodic shaking. At the end of 72 h the mixture was separated by paper chromatography as previously described [3]. The labelled glycated amino acids and unreacted U-[^{14}C] glucose were applied as a discrete spot to Whatman No. 3 chromatographic paper, the mobile phase being n-butanol, glacial acetic acid and water (4:1:1), and run for 16 h. After drying in air, the paper was cut at 90° into 2 cm wide strips and each piece was put into a tube containing liquid scintillant (Beckman with Tritonic surfactant). The activity in each strip was recorded as dpm in a Liquid Scintillation Counter (Beckman, automatic). A total of 12 strips was analysed for each run.

For studies with homogenates, normal clear lenses from donors to the eye bank of Sankara Nethralaya were used immediately. They were weighed and a homogenate was prepared in 20 mM phosphate buffer (pH 6.9), such that the concentration was 100 mg ml^{-1} protein. Lenses removed carefully from human eyeballs were washed with deionized water and weighed after removing water with filter paper. They were then homogenized in Teflon homogenizer. To each tube was added 2 μCi U-[^{14}C] glucose, 5 ml phosphate buffer, 1 ml lens homogenate, 0.1% sodium azide, cold glucose (5 or 10 mM) with or without amino acids (5 or 10 mM). The whole was incubated for 72 h at 37 °C. At the end of the incubation period, the contents of all the tubes were dialysed separately against millipore water in order to remove free unreacted radioactive glucose. Dialysis was continued until the water was free from radioactivity. Proteins were precipitated using 10% TCA and dissolved in 1 ml of 1 N NaOH. To 0.5 ml of this solution 4 ml of Beckman aqueous liquid scintillant was added and the radioactivity was measured in dpm using a liquid scintillation counter.

Results

It was found that all the amino acids used formed adducts with glucose at physiological pH and temperature as shown by the incorporation of U-[^{14}C] glucose. The radioactivity in terms of dpm measured for different zones of the chromatogram are given in Tables 1–3. Out of the 12 strips analysed in each experiment, a major peak and a moderate peak were obtained. For all the other strips the dpm was much less (see a typical example in Fig. 1). The

Table 1. A summary of the chromatography results after the incubation of glucose with alanine for 72 h at 37 °C

		Concentration of alanine	
		5 mM	10 mM
Zone	*Concentration of glucose (mM)*	*Radioactivity in each zone (dpm)*	
No free or adduct glucose	5	26 ± 4	16 ± 4
	10	19 ± 5	17 ± 2
Glycated alanine	5	442 ± 32 (2.4%)	474 ± 54 (2.5%)
	10	316 ± 28 (1.6%)	394 ± 35 (2.2%)
Free [^{14}C] glucose	5	18432 ± 4247	18986 ± 5118
	10	19765 ± 5200	17917 ± 4451

In Tables 1–3, values given as mean ± SE of six experiments; values in the parenthesis are % glucose bound to amino acid; and 2 μCi U-[^{14}C] glucose was used in each experiment.

Table 2. A summary of the chromatography results after the incubation of glucose with aspartic acid for 72 h at 37 °C

		Concentration of aspartic acid	
		5 mM	10 mM
Zone	*Concentration of glucose (mM)*	*Radioactivity in each zone (dpm)*	
No free or adduct glucose	5	22 ± 6	23 ± 5
	10	20 ± 7	24 ± 4
Glycated aspartic acid	5	8042 ± 405 (46%)	9775 ± 534 (54%)
	10	6559 ± 324 (35.2%)	9320 ± 578 (50.3%)
Free glucose	5	17484 ± 3124	18102 ± 3815
	10	18635 ± 4194	18529 ± 4274

Table 3. A summary of the chromatography results after the incubation of glucose with glutamic acid for 72 h at 37 °C

		Concentration of glutamic acid	
		5 mM	10 mM
Zone	*Concentration of glucose (mM)*	*Radioactivity in each zone (dpm)*	
No free or adduct glucose	5	16 ± 2	17 ± 2
	10	21 ± 5	19 ± 4
Glycated glutamic acid	5	410 ± 58 (2.3%)	492 ± 64 (2.9%)
	10	618 ± 49 (3.3%)	475 ± 65 (2.6%)
Free glucose	5	17846 ± 4114	16966 ± 4237
	10	18737 ± 5235	18267 ± 4674

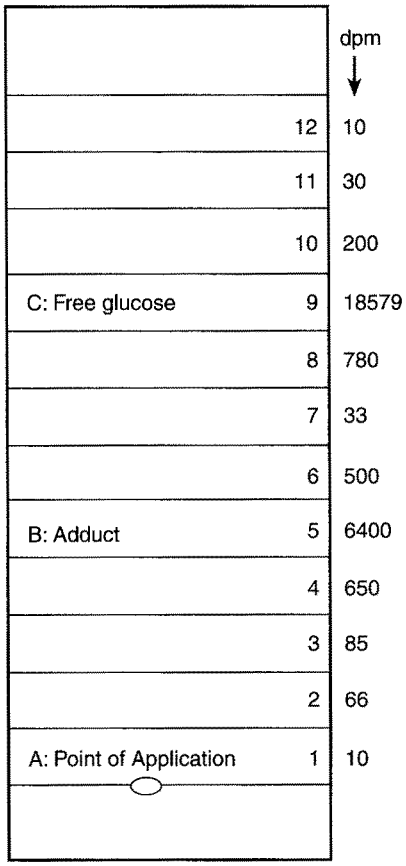

Figure 1. A typical paper chromatogram showing the separation of free glucose from the glucose/amino acid adduct. Radioactivity was measured by liquid scintillation counting of 12 × 2 cm wide strips. (A) point of application; (B) glucose/amino acid adduct; and (C) free glucose.

major peak was taken as free U-[^{14}C] glucose while the moderate one was taken as that of the adduct of glucose with the amino acid. The presence of free and bound glucose was also established in a separate chromatogram by spraying with thiobarbituric acid [10]. The results showed that the glucose reacting with alanine was between 1.6 and 2.5%; reacting with aspartate was between 35 and 50%; and reacting with glutamate was between 2.3 and 3.3%.

It was found that an homogenate of normal human lens could be glycated by incubation with glucose (Tables 4–6).

This glycation decreased when free amino acid was present. Addition of alanine in concentrations of 5 or 10 mM to equimolar concentrations of glucose decreased the glycation of lens proteins between 32 and 69% respectively (Table 4), while addition of aspartate decreased glycation by between 18 and 74% (Table 5). Glutamate decreased the glycation between 52 and 74% (Table 6). In all cases, the change was greater when a higher concentration (10 mM) of glucose was used.

Discussion

Glucose, fructose and derivatives of sugars react with the amino group of lysine of the lens-proteins, ie crystallins. When the amino group reacts with the aldehydic or ketonic group, Schiff's bases are formed. In the case of the aldehydic group of glucose, an aldimine is formed. This undergoes Amadori rearrangement to form a ketamine. As the reaction is non-enzymic, it is not subject to any regulation. Further reactions result in cross-links between molecules, and the production of Advanced Glycation End products (AGE). Due to the denaturation of proteins, the transparency of lens proteins is lost, which results in the formation of cataracts.

The free amino acids, lysine and glycine, can react with the aldehydic group of glucose through their amino groups and form glycosyl amino acid adducts. Being small molecules, these adducts can diffuse out of the lens. Thus, glucose can be scavenged from the lens

Table 4. The effect of alanine on the glycation of human lens proteins

Concentration of glucose/amino acid	*Radioactive incorporation (dpm)*	*% reduction in incorporation*	*p value*
Glucose 5 mM	23680 ± 2938		
Glucose 5 mM Alanine 5 mM	16233 ± 2003	31.5	<0.05
Glucose 5 mM Alanine 10 mM	14434 ± 2013	39.0	<0.05
Glucose 10 mM	28754 ± 4710		
Glucose 10 mM Alanine 5 mM	12002 ± 1378	58.3	<0.05
Glucose 10 mM Alanine 10 mM	9035 ± 2018	68.5	<0.05

In Tables 4–6, radioactive incorporation is given as mean ± SE from six experiments; the statistical effect of the added amino acid on the incorporation was determined using the paired Mann-Whitney test; and radioactive measurements were made after exhaustive dialysis and TCA precipitation of the proteins

Table 5. The effect of aspartic acid on the glycation of human lens proteins

Concentration of glucose/amino acid	*Radioactive incorporation (dpm)*	*% reduction in incorporation*	*p value*
Glucose 5 mM	23680 ± 2938		
Glucose 5 mM Aspartic acid 5 mM	19516 ± 2373	17.5	0.075
Glucose 5 mM Aspartic acid 10 mM	14817 ± 2107	37.5	<0.05
Glucose 10 mM	28754 ± 4710		
Glucose 10 mM Aspartic acid 5 mM	7648 ± 848	73.5	<0.05
Glucose 10 mM Aspartic acid 10 mM	10613 ± 1481	63.0	<0.05

Table 6. The effect of glutamic acid on the glycation of human lens proteins

Concentration of glucose/amino acid	*Radioactive incorporation (dpm)*	*% reduction in incorporation*	*p value*
Glucose 5 mM	23680 ± 2938		
Glucose 5 mM Glutamic acid 5 mM	9951 ± 1092	58.0	<0.05
Glucose 5 mM Glutamic acid 10 mM	11579 ± 3583	51.8	<0.05
Glucose 10 mM	28754 ± 4710		
Glucose 10 mM Glutamic acid 5 mM	8321 ± 2066	71.1	<0.05
Glucose 10 mM Glutamic acid 10 mM	7465 ± 2129	74.1	<0.05

fibres. This reaction with glucose could protect lens protein from glycation, denaturation and cross-linking of proteins and reduce cataractogenesis [2, 3].

The free amino acids alanine, aspartic acid and glutamic acid, which are also reported [7–9] to be present in relatively larger amounts in the lens, undergo non-enzymic glycation at physiological pH and temperature with glucose. Therefore, these amino acids may remove intracellular glucose and decrease the glycation of lens proteins. Although the amount of glycated alanine and glutamic acid formed in the presence of glucose was low, the effect of all these amino acids in decreasing glycation in the presence of lens homogenate was quite high. This could be because, in the presence of lens homogenate, some biotransformation of glucose or the amino acids might have taken place which augmented the glucose scavenging effect.

Swamy and Abraham [11] have demonstrated the antiglycating effects of aspirin and its beneficial effect on cataract, while Blakytny and Harding [12] have shown the prevention of cataract in diabetic rats by aspirin, paracetamol (acetaminophen) and ibuprofen. Blakytny and Harding [13] have also shown that glycation inactivates even enzymes like glutathione reductase. In this paper it is shown that amino acids other than lysine and glycine, can also decrease the extent of glycation [3, 14]. It is suggested therefore that a mixture of free amino acids may be useful in the future in reducing cataracts especially in diabetic individuals.

In addition to the protection of lens proteins, the free amino acids might even protect the retina from diabetic retinopathy by the same mechanism [15]. A desirable aspect of using amino acids in these ways is that they are available at all times, non-toxic and are normal physiological constituents of all living cells.

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