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Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients

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Nonenzymatic glycation, the reaction of glucose and other reducing sugars with protein, reversibly produces Amadori products and over a long period irreversible advanced glycation end products. In diabetes, these reactions are greatly accelerated and are important in the pathogenesis of diabetic complications.

In vitro glycation was studied with bovine albumin as the model protein. A mixture of 25 mM glucose/fructose was used as the glycating agent. The Amadori product was quantitated by thiobarbituric acid colorimetry after hydrolysis. Advanced glycation end products were measured by their intrinsic fluorescence. A number of vitamins and nutrients were found to be potent inhibitors of both the glycation reaction and the subsequent end products. The nutrients were effective at physiological concentrations and exhibited dose-response relationships. The inhibitors included ascorbic acid, tocopherol, pyridoxal, niacinamide, sodium selenite, selenium yeast, and carnosine. A significant correlation was found between the inhibition of glycation and the inhibition of AGE formation ($P < 0.001$). One of the nutrients, ascorbic acid, was used in a pilot study. Eighteen normal subjects, 7 college age and 10 middle age, were supplemented with 1,000 mg of ascorbic acid in the form of Re-Natured Vitamin C® for a period of 4 weeks. Serum protein glycation was decreased an average of 46.8% ($P < 0.01$). These results underline the importance of nutrition in diabetes and indicate the possibility of therapeutic use of these nutrients for the prevention of diabetic complications. © Elsevier Science Inc. 1996 (J. Nutr. Biochem. 7:659–663, 1996.)

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Introduction

Although there have been important advances in the control of the hyperglycemia of diabetes by means of diet, hypoglycemic drugs, insulin, the insulin pump and islet transplantation, the long-term complications of diabetes are still leading causes of death.[1] These complications are a direct result of protein alterations which result in irreversible tissue damage. One of the consequences of hyperglycemia is the excessive nonenzymatic glycation of proteins known as the Maillard reaction, which is shown schematically below.

$$\text{Protein} + \text{sugar} \rightleftarrows \underset{\text{Base}}{\text{Schiff}} \rightleftarrows \underset{\text{Products (AP)}}{\text{Amadori}} \rightarrow \rightarrow \underset{\text{End Products (AGE)}}{\text{Advanced Glycation}}$$

Equilibrium levels of Schiff base and Amadori products (AP) are reached quickly, in hours and weeks, respectively.[2] The levels of these early glycation products change in response to blood glucose and are reflected in the analysis of glycated hemoglobin (GHb) and glycated albumin to monitor average blood glucose control over several months and several weeks, respectively, in diabetic patients. However the irreversibly formed advanced glycation end products (AGE) do not return to normal when hyperglycemia is corrected and continue to accumulate over the lifetime of the protein. These substances can form covalent bonds with amino groups on other proteins and thus cause protein cross-linking.[3] Glycation and AGE modifications result in pathological changes to the protein such as enzyme activa-

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tion of aldose reductase,[4] deactivation of superoxide dismutase,[5] increased atherogenicity of LDL,[6] increased basement membrane permeability[7] and decreased insulin binding to insulin receptors.[8] All of these modifications contribute to diabetic complications such as cataracts, nephropathy, vasculopathy, proliferative retinopathy, and atherosclerosis.

A major effort has been launched to find a therapeutic means of mediating protein glycation. The most studied and successful agent has been aminoguanidine which reacts with AP and early glycation products and limits the formation of AGE.[9] Aspirin, acetaminophen, and ibuprofen have been shown to decrease glycation of lens proteins and prevent diabetic cataracts in rats.[10] In this report we investigate vitamins and nutrients as possible inhibitors of protein glycation and AGE formation.

Methods and materials

Chemicals

All biochemicals were obtained from Sigma Chemical Company (St. Louis, MO USA). Other chemicals were reagent grade. Selenium yeast (containing 1% selenium) and Re-Natured Vitamin C® (25.1% ascorbic acid, 10.0% bioflavonoids, and a minimum 30% carbohydrates and 15% protein) were gifts from Grow Company, Inc. (Hackensack, NJ USA). Dubelco's Formula Buffered Saline, pH 7.4, was obtained from Flow Laboratories (McLean, VA USA).

Incubations

Bovine serum albumin (BSA) solutions were made in the buffer with 0.02% sodium azide to prevent degradation. Fructose and glucose were made together in 0.02% sodium azide. Inhibitors were freshly prepared in water or, if lipid soluble, in Tween 80. Aliquots of BSA, sugar, inhibitor, and distilled water or Tween 80 as a control, were added to 5-cm screw-capped test tubes to give final solutions of 7 mg/mL BSA, 25 mM glucose, 25 mM fructose, and the inhibitors at concentrations in the physiological range. All incubations were done in quadruplicate and the tubes were degassed with nitrogen before being placed in a constant temperature bath at 37°C for 3 to 30 days.

Glycation analysis

Glycated proteins were measured in the form of the AP from BSA reacting with both glucose and fructose. Sugars were removed from the solution by extensive dialysis in the cold and the proteins precipitated with trichloroacetic acid and hydrolyzed with oxalic acid. The product was reacted with thiobarbituric acid and the chromogen measured using hydroxymethylfurfural as a standard. Serum samples were treated similarly, except that sugars were removed by precipitating with trichloroacetic acid and washing the protein precipitate with water.

AGE analysis

Fluorescence of the samples was measured at the excitation and emission maxima of 350 and 450 nM, respectively versus an unincubated blank containing the protein, sugar and inhibitor.

Supplementation study

Seven healthy college students, three females and four males, aged 18 to 22, and 11 healthy normoglycemic subjects, five females and six males, aged 39 to 76, participated with informed consent. Fasting blood samples were drawn before and after 3 weeks supplementation with 1000 mg of ascorbic acid/day as Re-Natured Vitamin C® (4 g/day). The blood was converted to serum and analyzed for glycated proteins.

Results

Incubations

Albumin was chosen as the protein for the in vitro reactions because it is the most abundant protein in serum. Fructose was included in the incubation because it is present in the tissues at comparable concentrations to that of glucose and, as Suarez has pointed out, reacts with proteins at faster rates than glucose and produces about 10 times more protein-bound fluorescence than glucose.[11] Fructose is also elevated in those tissues where the polyol pathway is active. The AGE product in the present study was a mixture of both protein-glucose and protein-fructose reaction products. Ascorbic acid, one of the inhibitors tested, was incubated at 20 μM with BSA, glucose, and fructose over a period of 30 days. The inhibition of glycation relative to the control remained constant; 3 day—70.4%, 15 days—75% and 30 days—73%. The AGE inhibition gradually increased; 3 days—56.6%, 15 days—67.7%, and 30 days—80.4%.

The results of 15-day incubations of various vitamins and nutrients and the inhibition of glycation and AGE are shown in *Table 1*. All substances showed a dose-response inhibition of both glycation and AGE at concentrations in the physiological range. An example of the inhibition at different concentrations is shown for ascorbic acid in *Figure 1*. In comparison to the control with no inhibitor, all nutrients at the physiological concentration except selenite produced a significant inhibition of glycation and AGE by the ANOVA test, $P < 0.05$. Niacinamide and pyridoxal almost completely inhibited glycation and AGE at physiological concentrations. For example, niacinamide at 70 μm inhibited glycation 100% and AGE 99.7%; pyridoxal at 1.1 μM inhibited glycation 100% and AGE 87.2%. The order of effectiveness for both glycation and AGE inhibition is selenium yeast > ascorbic acid > niacinamide > carnosine > tocopherol > pyridoxal > sodium selenite. The combination of Vitamins C and E were examined as inhibitors at physiological concentrations (20 μM) and the inhibition was 95.2% for glycation and 85.2% for AGE formation. The

Table 1 Concentration of nutrients for 50% inhibition (IC_{50}) of *in vitro* protein glycation and AGE formation

Nutrient	IC_{50} Albumin		Physiological Concentration of Nutrient
	Glycation	AGE	
Ascorbic acid	11.8 μM	13.3 μM	20 μM
Tocopherol	195 μM	219 μM	20 μM
Niacinamide	16.0 μM	17.8 μM	41 μM
Pyridoxal	417 μM	458 μM	649 nM
Sodium selenite	652 μM	791 μM	380 nM
Selenium yeast	5.3 μM	6.2 μM	380 nM
Carnosine	20.6 μM	16.3 μM	10 mM

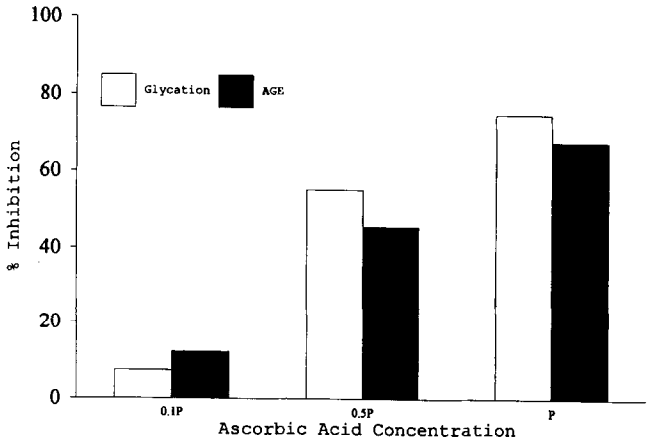

Figure 1 Dose response of % inhibition of protein glycation and AGE after 15 days of incubation with ascorbic acid at concentrations near physiological (P), 0.1 P = 2 μM, 0.5 P = 10 μM, P = 20 μM relative to control.

combination was a significantly greater inhibitor than either vitamin alone at that concentration, ANOVA $P < 0.001$.

The 15 day data for all the nutrients was pooled and the inhibition of glycation and AGE formation was found to be highly correlated ($P < 0.001$). The regression line was % AGE inhibition = 0.852% glycation inhibition + 2.96 with Pearson's correlation coefficient of 0.988 (*Figure 2*).

Supplementation

Vitamin C was chosen for a supplementation study since it is a very inexpensive and non-toxic nutrient. Re-Natured Vitamin C® was used for supplementation because it has been found to be more bioavailable to animals and humans than ascorbic acid alone.[12,13] The supplementation produced a significant decrease in serum protein glycation of both the college age and middle age groups, $P < 0.05$ by a paired *t* test (*Table 2*). Five of the 7 college students and all 11 of the middle-age subjects experienced a decline in glycation as a result of the Vitamin C regimen.

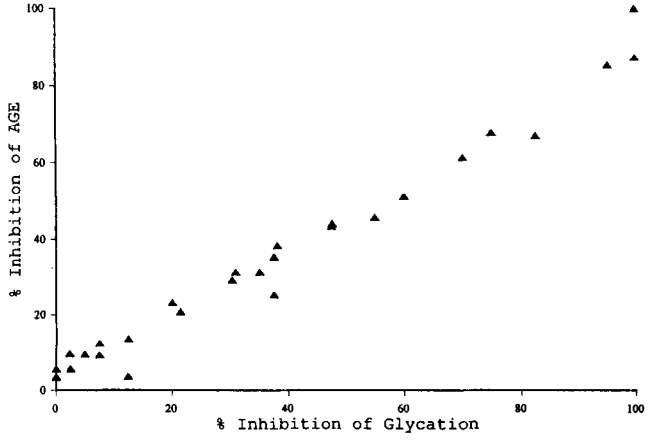

Figure 2 Correlation between 15 day % inhibition of glycation and AGE formation by vitamins and nutrients relative to control.

Table 2 Effect of ascorbate supplementation (1 g/day) on serum protein glycation of college-age and middle-age subjects

Subjects	Serum Protein Glycation (μM)	
	Before Supplementation	After Supplementation
Normal college age (n = 7)	8.57 ± 6.65	3.38 ± 2.50*
Middle age (n = 11)	15.70 ± 7.53	7.89 ± 6.25*
All subjects	12.76 ± 7.85	6.04 ± 5.44**

*$P < 0.05$ by a paired *t* test.
**$P < 0.01$.

Discussion

Although the mechanism of inhibition is at present unknown, all of the substances can bind to sugars or proteins and/or are proven antioxidants. Binding to sugar or protein would inhibit AP production and subsequent AGE formation. The antioxidant function would decrease the concentration of free radicals. Several reports indicate the production of radicals and highly reactive oxidants from glycated proteins under physiological conditions.[14–16] Free radicals are known to stimulate AGE production by autoxidation of sugars.[16] Oxidative stress has been linked to diabetic complications[17] and diabetic atherogenesis.[18] Recently in vitro AGE formation from glucose and albumin was found to be due in part to oxidants and free radicals acting on the lipid moiety of the albumin.[19] Bucala and co-workers[20] found a linear relationship between low density lipoprotein oxidation and AGE in diabetic subjects. A linear correlation between plasma glycation and AGE fluorescence has recently been reported,[21] which our results confirm.

Carnosine is a natural dipeptide and a major brain and muscle antioxidant[22] which can also compete with proteins for binding with the sugars. Pyridoxal can bind to proteins via the aldehyde group and thus be a competitive inhibitor as found by Khatami.[23] This vitamin has been used at high doses to decrease glycosylated hemoglobin in human diabetics.[24] Nicotinamide decreases the severity of streptozotocin-induced diabetes in animals due to its antioxidant effect.[25] Niacinamide supplementation was also found to prevent the onset on diabetes in children with first-degree relative with type I diabetes.[26] The greater oxygen radical scavenging ability of organic forms of selenium[27] may explain the much larger inhibition of glycation and AGE of the selenium yeast compared with selenite. Also, the yeast proteins contain lysine, which has been shown to inhibit in vitro protein glycation.[28]

Ascorbate and its free radical, semihydroascorbate, form ionic bonds with biological molecules such as proteins.[29] The carbonyl group of ascorbate and its oxidation product may also compete with glucose for protein as seen with in vitro erythrocyte glycation.[30] Vitamin C has been shown to inhibit in vitro oxidation of low density lipoprotein by a dual action; scavenging aqueous oxidants and by stable modification of the protein by its oxidation product dehydroascorbate.[31]

Vitamin E, α-tocopherol, is a potent antioxidant. It previously has been shown by Ceriello to be an anti-glycating

substance in vitro[32] and in vivo as a supplement to diabetics.[33] Jain recently demonstrated Vitamin E blocks glycation of erythrocyte hemoglobin by inhibiting in vitro lipid peroxidation.[34] The present study confirms these results that vitamin E is both a glycation and AGE inhibitor.

The fact that the combination of Vitamin C and E more effectively inhibited glycation and AGE than the single vitamins is evidence for the antioxidant mechanism of inhibition. These vitamins have been shown to synergistically inhibit free radical mediated lipid oxidation.[35]

The present Vitamin C supplementation study (in a bioflavonoid mixture) demonstrated an average decrease of 46.8% in protein glycation in normoglycemic subjects in good agreement with Davie.[30] He showed a 33% decrease in glycated albumin after 3 months with 1 g/day of ascorbate and an 18% reduction in GHb. Stolba also found a significant decrease in fructosamine in insulin-dependent diabetics given 1.5 g of ascorbate/day.[36] The glycation-lowering effect in the supplement could be due to both the ascorbate and flavonoids in the mixture. Odetti found that the flavonoid rutin decreased collagen-linked AGE fluorescence in diabetic rats.[37]

Epidemiological studies have recently found a negative correlation between vitamin C intake and GHb in normal subjects.[38] Ascorbate either alone or in the form of a citrus extract has also been found to be an effective agent in lowering red blood cell sorbitol in human diabetic subjects at 2 g/day,[39] 1 g/day,[40] and 100 mg/day.[41] Vitamin C thus beneficially influences the two mechanisms of diabetic complications; glycation and the sorbitol pathway.

An important therapeutic factor to consider is that it would be necessary to administer the inhibitor as soon as possible after diagnosis of diabetes and regularly throughout the lifetime of the patient. This is essential, because it has been proposed that once the progress of excessive glycation has begun, subsequent remediation of hyperglycemia would not prevent diabetic complications.[2] Important attributes of an ideal inhibitor would be easy absorption and excretion, little or no toxicity, and the absence of serious side effects.[28] Vitamin C should, therefore, be an ideal candidate for diabetes supplementation.

All of the substances studied are naturally occurring vitamins and nutrients in the body and several of them are known to be lowered in the tissues of subjects with diabetes, such as ascorbate,[42] pyridoxal,[43] and nicotinamide.[25] This fact and the results of the present study point to the necessity of good nutrition in diabetes and to the possibility of inexpensive, relatively non-toxic therapies for the prevention and treatment of diabetic complications. Because AGE and atherogenic oxidized LDL are correlated in vivo,[21] inhibition of glycation and oxidation by nutrients may also form the basis of future antiatherogenic strategies in both diabetic and non-diabetic individuals.

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