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Original article

Study of Nonenzymatic Glycation of Transferrin and its Effect on Iron –Binding Antioxidant Capacity

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Abstract

Objective(s)
Nonenzymatic glycosylation (glycation) occurs in many macromolecules in aging and diabetes due to exposure of biomolecules to high level of glucose. Glycation can changes function, activities and structure of many biomolecules. Considering this important role of transferrin (Trf) in iron transport and antioxidant activity in plasma this study was carried out to investigate the effect of glycation in these processes.
Materials and Methods
In this study, human apo-Trf (5 mg/ml in sodium phosphate buffer pH= 7.4) was treated with different concentrations of glucose in different period of times (10 days and 20 days). Rate of glycation was measured using thiobarbituric acid method. The effect of glycation on iron binding antioxidant capacity of apo-Trf was investigated using two methods (RBC hemolysis and fluorescent).
Results
Result showed that rate of glycation of apo-Trf was increased with increase in glucose concentration and time of incubation ($P< 0.05$). Lower iron binding antioxidant capacity was observed for glycted Trf as compared to native Trf ($P< 0.05$).
Conclusion
Impairment of antioxidant capacity of glycated Trf can suggest a relationship between glycation of Trf and oxidative stress that occurs due to hyperglycemia in diabetic patients.

Keywords: Apotransferrin, Diabetes mellitus, Glycation, Oxidative stress

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Introduction

Glycation, is a common spontaneous posttranslational modification of proteins that reducing sugars bind covalently to the free amino groups. The most common *in vivo* reducing sugar is glucose, which reacts with N-terminal amino acids or with free Lys and Arg amino groups. This process occurs in aging and diabetes due to exposure of biomolecules to high level of glucose. Many biomolecules, such as lens crystallins, collagen, hemoglobin (Hb), albumin, Igs, LDL and DNA, are modified by glycation (1). This structural alteration may lead to impaired protein function, and perhaps contribute to the long-term complications of diabetes. For example glycation had comparable increased effects on high-affinity binding of warfarin (site I), but decreased effects on high-affinity binding of dansyl sarcosine (site II) and esterase like activity of albumin (2). It has been shown that *in vitro* glycation can significantly decrease the affinity of an antibody for its antigen, and significantly increases the rate of dissociation of the antigen-antibody complex and impaired Fc function such as complement binding and binding to protein A (3). It has been reported that glycation of collagen affects platelet aggregation (4). Glycation of IgG induces structural alteration leading to changes in its interaction with anti IgG (5, 6).

Transferrin (Trf) is one of the most important plasma proteins which involves in several functions such as iron transport (7), proliferation and differentiation (8), antimicrobial (9) and antioxidant activity (10). Diabetes mellitus is invariably accompanied by oxidative stress (11), which has been postulated to be the unifying pathogenic mechanism mediating the appearance and progression of chronic diabetic complications (12). The increased levels of lipid and DNA peroxidation products (13, 14) found in diabetic patients are the result of an imbalance between pro-oxidants (free radicals) and antioxidants which is directly caused by hyperglycaemia. Main sources of free radicals in diabetes are auto-oxidation of glucose (15), non-enzymatic glycation of proteins (16), monocyte dysfunction (17) and ischemia–reperfusion (18). Due to important role of Trf in antioxidant activity in diabetes and few studies in this field, present study was carried out to investigate whether glycation of apo-Trf could affect the neutralization of free radicals (antioxidant activity).

Materials and Methods

Preparation of in vitro glycated apo- Tf

In this study, human apo-Trf (Sigma–Aldrich), at a concentration of 5 mg/ml, was dissolved in sodium phosphate buffer (PBS) (0.1 mol/l, pH 7.4, containing 0.1% NaN_3), 2 ml aliquots of this solution in dialysis bag were incubated with different concentrations (50, 100, 150, 200 mM) of D-glucose (Merck) in PBS and for two different period of times (10 days and 20 days) at room temperature. Thereafter remaining free glucose was removed by dialysis of the tubes against PBS in 24 hr with twice buffer exchange.

Measurment of glycated apo-Tf

Glycation was measured using the thiobarbituric acid colorimetric reaction (19). The colorimetric method with 2-thiobarbituric acid is based on the hydrolysis of the glycated proteins using oxalic acid at 100 ºC yielding 5-hydroxymethyl furfural (5-HMF) which reacts with thiobarbituric acid. The absorbance was measured at 443 nm. 5-hydroxy methyl furfural (5-HMF) was used as a standard and glycation of Trf was calculated and expressed as µmol 5- HMF per mg protein.

Measurement of antioxidant capacity by RBC hemolysis method

Antioxidant capacity of glycated Trf was measured according to the method of Koga *et al* (20) and was compared to those of non-glycated Trf. In this method human erythrocytes were oxidized by a water soluble radical generator, and the protective effect of Trf (or glycated Trf) on lipid peroxidation was assayed and compared with those of Vit C. Briefly 1 ml of washed erythrocytes were suspended in PBS and incubated at 37 ˚C water bath for 30 min. After adding PBS the mixture was centrifuged in 2000 g for 10 min.

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one ml of 150 mM 2, 2´- AZO bis- dihydro chloride (AAPH) was added to the suspension and oxidation was carried out at 37 ˚C with continuous shaking. After the incubation, the tubes were centrifuged at 2000 g for 10 min. The optical density of all samples were read at 540 nm using a spectrophotometer (Spectronic Genesys 2).

Measurement of antioxidant capacity using fluorescent method

Thermal decomposition of AAPH in H_2O produces free radicals that subsequently can converts 2′,7′ dicholoro fluorescein-diacetate (DCFH-DA) to fluorescent DCF (21) and produced DCF can be measured using a fluorometer. We used this system to measure the antioxidant capacity of Trf. Briefly, in a test tube containing 100 µl of a serum sample, 28 µl of DCFH-DA and 1622 µl of PBS and 50 µl of apo-Trf and 200 µl of AAPH were added and mixed. The intensity of fluorescence was measured by spectrofluorometer FP-6200 using excitation at 480 nm and emission at 526 nm. This experiment was repeated at the same condition using glycted Tf, and the obtained results were compared to those of apo-Tf. Vit C was used as a standard.

Statistical analysis

Results were expressed as mean±SD. The results were analyzed using statistical software SPSS 13. Mann-Whitney U-test and ANOVA with Dunnett-Tukey HDS as *post hoc* tests were used. *P*-values< 0.05 were considered as statistically significant.

Results

Glycation of apo-Trf

Figure 1 shows the calibration curve for measuring glycation of apo-Trf using 5-HMF as standard. The results of glyacation assay are summarized in Table 1. This data showed that glycation of apo-Trf was increased by increasing glucose concentration and time of incubation. On the other hand in the series of 10 days, glycation was increased parallel with increasing glucose concentration (*P*< 0.05). This phenomenon also was observed for series of 20 days (*P*< 0.05). Samples (apo-Trf) incubated for 20 days showed higher glycation as compared to 10 days samples (*P*< 0.05).

Antioxidant capacity assay using RBC hemolysis method

In this method we used AAPH as a source of free radical production and RBC as a detector of neutralization of free radical. We assumed that Trf similar to other antioxidant such as Vit C, was able to protect RBC hemolysis through neutralization of free radicals. Obtained results showed that increasing glycation of Trf decreases antioxidant capacity of Trf and this impairment of antioxidant capacity of Trf increased with increasing the rate of glycation (Table 2) (*P*< 0.05). The standard curve for this method plotted on the base of antioxidant capacity of Vit C is shown in Figure 2.

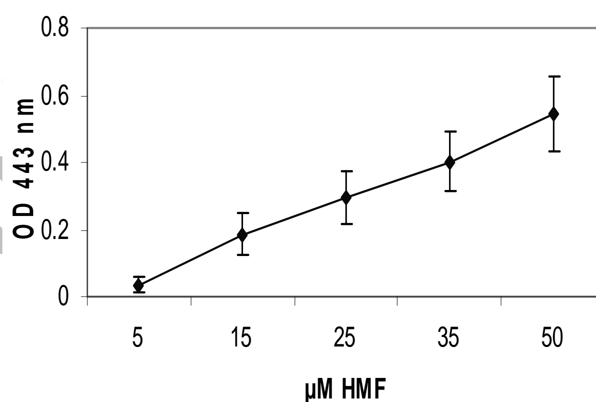

Figure 1. Standard curve of glycation assay.
Glycation was measured using the thiobarbituric acid colorimetric reaction. 5-hydroxy methyl furfural (5-HMF) was used as a standard. Data are results of 2 different triplicate experiments.

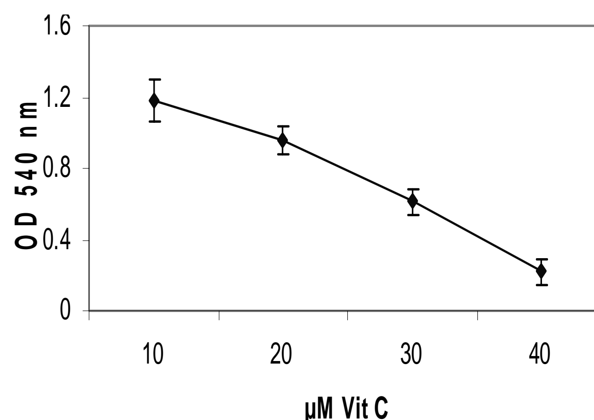

Figure 2. Standard curve of andioxidant assay.
In this method human erythrocytes were oxidized by a water soluble radical generator, and the protective effect of Vit C on lipid peroxidation was assayed. Data are results of 2 different triplicate experiments.

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Table 1. Rate of glycation of apotransferrin according to μ mole HMF/mg apoTransferrin.

	Glucose concentration (mM)				
Time (day)	0 (control)	50	100	150	200
10	5.06±1.10	20.95±1.17	28.64±1.54	39.04±1.10	51.04±1.32
20	6.08±1.09	25.02±1.26*	34.04±1.16*	45.10±1.21*	58.02±1.17*

*P= 0.029 compared to 10 days treatment with glucose.

Table 2. Antioxidant capacity of glycated and nonglycated (control) Trf by RBC hemolysis method according to μM Vit C.

	Glucose concentration (mM)				
Time (day)	0 (control)	50	100	150	200
10	29.74±1.21	26.78±1.18	22.93±1.15	17.97±1.09	13.01±1.09
20	28.95±1.12	23.70±1.59*	18.83±1.15*	12.90±1.2*	7.47±0.81*

*P= 0.03 compared to 10 days incubation. Data are results of 2 different triplicate experiments.

Table 3. Antioxidant capacity of glycated and nonglycated (control) Trf by fluorescent method according to μM Vit C.

	Glucose concentration (mM)				
Time (day)	0 (control)	50	100	150	200
10	43.85±1.2	39.90 ± 1.21	34.76±1.23	28.81±1.19	21.66±1.12
20	41.79±1.19	36.90±1.13*	30.72±1.17*	23.79±1.25*	15.83±1.18*

*P= 0.029 compared to 10 days incubation. Data are results of 2 different triplicate experiments.

Antioxidant capacity assay using fluorescent method

In this method we used AAPH as a source of production free radical and DCFH-DA as a detector of neutralization of free radical. The capacity of Trf to protect DCFH-DA from oxidation through neutralization of free radicals was measured and compared to that of glycated Trf. Obtained result showed that increasing glycation of Trf results in a decrease of its antioxidant capacity (Table 3). The results also showed that impairment of antioxidant capacity of Trf increased with increasing glycation rate (Table 3) (P< 0.05). The standard curve of this method was plotted on the base of antioxidant capacity of Vit C.

Discussion

In the present study, we used HMF method for investigation of glycation of apo-Trf. This method is economic, rapid, accurate and easy to perform. We illustrated that in *in vitro* condition, human apo-Trf can be glycated in treatment with different concentrations of glucose and the period of incubation with glucose. In 2006 Van Campenhout *et al* showed that in *in vitro* condition human apo-Trf can be glycated in different concentration of glucose, and rate of this glycation was dependent on glucose concentration (10). Our findings in this report were in agreement with the result of Van Campenhout (10). Diabetes is associated with oxidative stress that may result in damaging antioxidant function of Trf. In regard to iron binding antioxidant capacity of Trf we can suggest that Trf can scavenge the free radicals and subsequently can prevent oxidative stress in diabetes. Furthermore we investigated this subject with two methods for more confirmation of the results. We found a correlation between the obtained results from these two methods. Our findings showed that antioxidant capacity of Trf is impaired by glycation, and rate of this impairment increases by raising concentration of glucose and time of incubation that leads to higher glycation. Impairment of protein function resulting from glycation was shown for different proteins (4-6). Glycation of hemoglobin that forms HbA1c is studied vastly in diabetic patients and it is used as an index of

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glycemic control. It is shown that glycation induces structural alterations in Hb that leads to alteration of its function (22). This study also demonstrated that in the presence of hydrogen peroxide glycated Hb degrades DNA and protein more efficiently than non-glycated Hb (22). There is limited number of studies showing the alteration of Trf function due to glycation. Fujimoto *et al* showed that iron ions bound to glycated Trf loosely and glycated Trf produces more oxygen radicals (23). In an *in vitro* study, Van Campenhout *et al* showed that glycation impairs Fe^{3+} binding of Trf (24). They also demonstrated that glycation induces alteration in the distribution of different isoforms of Fe^{3+}-Trf (24). Lower iron binding antioxidant capacity is reported in plasma of diabetic patients (10). Van Campenhout developing a new method to quantify *in vivo* Trf glyaction indicated that total iron binding capacity of Trf is lower in diabetic patients that are induced by glycation (25).

Conclusion

Our *in vitro* findings supporting the previous reports indicating that structural alterations induced by glycation can leads to impairment of Trf function. According to this result, it can be suggested that an alteration in function of Trf which may occur in diabetes could be a result of alteration in Trf structure. Effect of glycation in other function of Trf is under investigation.

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