Iranian Biomedical Journal 10 (3): 139-143 (July 2006)

Cytotoxic Effect of "Glycated Albumin-Transition Metal Ion" on Rat Hepatocyte Suspension

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Received 16 August 2005; revised 28 January 2006; accepted 6 March 2006

ABSTRACT

Background: Combination of glycation and oxidation is associated with diabetes mellitus. The aim of this study was to clarify the effect of glycated proteins in presence of transition metal ions on production of reactive oxygen species (ROS) in rat hepatocyte suspension. **Methods**: Glycated albumin was prepared by incubation of bovine serum albumin with 100 mM glucose in 0.3 M phosphate buffer at 37°C for 2, 4 , and 6 weeks. The prepared rat liver cell suspension was treated with glycated albumin in presence of either Fe^{+++} or Cu^{++} ions. Produced malone-dialdehyde was measured as an indicator of ROS and of cell injury. **Results**: The results showed Fe^{+++} and Cu^{++} ions increase the ROS production in presence of glycated albumin ($p<0.01$). All prepared glycated albumin showed cytotoxicity in rat hepatocytes suspension in the presence of cupric and ferric ions, and this injury was dependent to metal ion concentration. Higher degree of glycation showed higher effect on ROS production ($p<0.01$). Comparing the effect of Fe^{+++} and Cu^{++}, cupric ion had higher cytotoxic effect ($p<0.01$). **Conclusion**: These results indicated that hepatocytes may be damaged by ROS which are produced by the interaction of the glycated albumin and transition metal ion. *Iran. Biomed. J. 10 (3): 139-143, 2006*

Keywords: Albumin, Glycation, Reactive oxygen species (ROS), Transition metal ion

INTRODUCTION

Reducing sugars react non-enzymatically with proteins or nucleic acids to initiate post-translational modification known as non-enzymatic glycosylation or glycation. This reaction leads to alteration in protein structure and subsequently altering their functions. Most of the late complications of the diabetes are the results of this modification. The early stage of the non-enzymatic glycation of proteins leads to the formation of early glycation products [1]. In this reaction, sugars condense with amino groups of macromolecules to form an adduct known as Amadori product [2]. The Amadori products very slowly undergoes a series of still unknown rearrangement reactions to form yellow-brown, fluorescent and cross-linking substances, called advanced glycation end products (AGE) [2].

Elevated level of blood glucose ignites a vicious cycle of metabolic disturbances within the intra- and extra-cellular environment that if left unchecked, lead to a broad array of complications in macro-vessel and micro-vessel structures [3]. Several proteins have known to undergo glycation including hemoglobin [4, 5], albumin [6], and lipoproteins [7]. The glycation products such as AGE have been shown to accumulate in various human tissues that contain long-half-lives proteins such as collagen [8], lens crystalline [9] and myelin [10].

It has been reported that glycation may influences in the generation of oxygen free radicals [11]. On the other hand, the early and intermediate products of glycated proteins have also been shown to induce the oxidative cleavage of DNA and proteins and lipid peroxidation in the presence of copper and iron [12, 13]. Glycated or oxidized LDL has been shown to induce atherogenesis by its cytotoxicity for some of tissues such as endothelial cells and macrophages in a manner which correlated with oxidation [14].

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[DOR: 20.1001.1.1028852.2006.10.3.4.7]

Glycated albumin has been shown to account for 6-15% of the total serum albumin in normal persons; little is yet known about its pathologic significance in diabetes [15]. The source of reactive oxygen species (ROS) associated with atherosclerosis and diabetes also remains to be clarified. In this study, we examined the oxidative injury of hepatocytes suspension induced by the non-enzymatic glycation of albumin in presence of transition metal ions.

MATERIALS AND METHODS

All chemicals (Analytical grade) were obtained from Sigma (USA), unless stated otherwise. All solutions were prepared using deionized water. All prepared solution was sterilized by Millipore filtration (pore size 0.45 um).

Preparation of glycated albumin. The glycated albumin was prepared according to the method of Monnier [16] with minor modification. Briefly, aliquots of BSA (0.10 g/ml) in 0.3 M phosphate buffer (pH 7.4) containing 0.04% sodium azide in dialysis bag was incubated with 100 mM D-glucose at 37°C for either 2, 4 or 6 weeks (g-BSA-2, g-BSA-4, and g-BSA-6, respectively). Controls were treated under the same condition without glucose. On days 10, 20, and 30 from the beginning of incubation, microbiological testing of the samples was carried out to confirm the absence of microbiological contamination. To avoid the interference by glucose, the samples were dialyzed overnight against one liter of 0.01 M PBS (pH 7.4) (dialysis bag from Biogen cut off 12000-14000). To remove any toxin produced by probable microbial contamination, the samples were subsequently applied to an endotoxin-absorption column (Pyrosep, Daicel Chemical Japan). The column was equilibrated with 0.01 M PBS and the samples in 5 mg/ml concentration were applied to column. The fraction was monitored at 280 nm in a UV-Vis spectrophotometer (Spectronic Genesis 2).

Measurement of the level of glycation. Glycation of albumin was measured using thiobarbituric acid (TBA) colorimetric reaction [17]. The colorimetric method with TBA is based on the hydrolysis of the glycated proteins using oxalic acid at 100°C yielding 5-hydroxymethyl furfural (5-HMF) which react with TBA. The absorbance was measured at 443 nm. 5-HMF was used as standard and glycation of albumin was calculated and expressed as μmol HMF per mg protein.

Preparation of rat hepatocyte suspension. Wistar rats weighing 250-300 g were sacrificed and their liver were removed, minced and homogenized (Heidolph homogenizer, Diax900 , 20,000 rpm) at 4°C in 3 ml per g liver in 20 mM Tris buffer (pH 7.4) containing 0.15 mM NaCl, 1 mM $CaCl_2$ and 1 mM Phenyl Methyl Sulfonyl Fluoride. The samples were centrifuged at 800 ×g for 10 min and the supernatant was removed and filtered through Miracloth. The resulting filtrate was quickly frozen in liquid nitrogen and stored at -20°C for performing the other experiments.

Treatment of rat hepatocyte suspension with glycated albumin-transition metal ion. Glycated and non-glycated albumin were treated with rat hepatocyte suspension in presence of different concentration (0, 20, and 40 μmol/l) of either $FeCl_3$ or $CuCl_2$. This cocktail was incubated at 37°C for 30 min, and then the samples were centrifuged at 4000 ×g for 20 min and supernatants were isolated.

Measurement of cytotoxic effects of glycated albumin-transition metal-ion on suspension of rat hepatocytes. The cytotoxic effect of glycated albumin-metal ion was estimated by measuring produced malone-dialdehyde (MDA) using TBA colorimetric reaction [18]. The above prepared cocktail (0.1 ml) was mixed with 1 ml of 0.67% TBA and 0.5 ml of trichloroacetic acid and incubated at 100°C for 20 min. After cooling, the reaction mixture was centrifuged at 2000 ×g for 5 min and the absorbance of supernatant was read at 532 nm. The concentration of produced ROS was calculated and expressed as nmol of MDA per mg protein using a freshly diluted 1,1,3,3-tetraethoxypropane for preparing standard curve.

Statistical analysis. The obtained data were analyzed using descriptive statistics and Wilcoxon matched pairs test. The *p* values less than 0.05 were considered significant.

RESULTS

The level of glycation of albumin was measured and the results are summarized in Table 1. As this Table shows the albumin was glycated in presence of glucose and the level of glycation was dependent on time of exposure with glucose ($p<0.05$).

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Table 1. Glycation of albumin (μmol hydroxymethyl furfural /mg protein) in presence of 100 mM glucose and in different incubation time at 37°C.

Glucose (mM)	Time of incubation (week)		
	2	4	6
0 (control)	5.02 ± 0.46	21.9 ± 1.88	23.29 ± 1.92
100	31.13 ± 1.77*	53.23 ± 2.37*	123.25 ± 7.6*

The data are mean ± SD of two separate triplicate experiments. *p<0.05 compared to the relevant control.

Table 2 shows the effects of glycated albumin in presence of Fe^{+++} ion on the formation of ROS on rat hepatocytes. Although the basal concentration of ROS on hepatocyte suspension was extremely low, glycated albumin-Fe^{+++} ions system increased the formation of ROS (4-6 fold, p<0.01). The formation of ROS on rat hepatocytes suspension under the influence of Fe^{+++} ions and glycated albumin increased with increasing the Fe^{+++} ions concentration. Moreover glycated albumin in combination with Fe^{+++} ions at concentration of 40 μM/l promoted the greatest increase (6.4 fold, p<0.01) in formation of ROS.

Table 2. The level of produced reactive oxygen species (nmol MDA/mg protein) in the presence of glycated albumin and the various concentrations of Fe^{+++} ions on rat hepatocytes.

Albumin	Fe^{+++} (μM)		
	0	20	40
BSA-2	8.12 ± 0.75	13.4 ± 0.770	29.92 ± 1.94
g-BSA-2	12.70 ± 1.05	19.3 ± 1.128*	52.90 ± 2.68**
BSA-4	11.50 ± 0.98	21.8 ± 1.080	34.90 ± 1.96
g-BSA-4	16.80 ± 1.45	33.3 ± 1.420*	53.90 ± 2.38**
BSA-6	13.50 ± 1.08	37.4 ± 1.310	43.70 ± 2.54
g-BSA-6	24.80 ± 1.88	51.3 ± 1.820**	85.90 ± 3.68**

In Tables 2 and 3: g-BSA-2, 4, and 6 are albumin treaded with glucose at 2, 4, and 6 weeks, respectively. BSA are non-glycated albumin (control). *p<0.05 and **p<0.01 compared to the relevant control. The data are mean ± SD of two separate triplicate experiments.

We also measured the effects of the glycated albumin and the various concentrations of Cu^{++} ions on the formation of ROS on rat hepatocytes. The results of this test are shown on Table 3. According to this Table, glycated albumin-Cu^{++} ions system proportionately increased the formation of ROS (p<0.01). These effects were dose-dependent and Cu^{++} ions at the concentration of 40 μM/l promoted the greatest increase (7.6 fold, p<0.01) in formation of ROS on rat hepatocytes compared to the basal stage. Therefore, the formation of ROS on rat hepatocytes significantly increased in the presence of glycated albumin and Cu^{++} or Fe^{+++} ions (p<0.01). Comparing the effect of iron and cupric ions indicated the stronger effect of Cu^{++} (p<0.01) (Fig. 1).

Table 3. The level of produced reactive oxygen species (nmol MDA/mg protein) in the presence of glycated albumin and the various concentrations of Cu^{++} ions on rat hepatocyte suspension.

Albumin	Cu^{++}(μM)		
	0	20	40
BSA-2	8.12 ± 0.75	18.4 ± 0.87	27.6 ± 1.84
g-BSA-2	12.7 ± 1.05	23.3 ± 1.42*	58.90 ± 2.28**
BSA-4	11.5 ± 0.98	24.3 ± 1.86	34.7 ± 1.88
g-BSA-4	16.8 ± 1.45	35.1 ± 2.12**	57.9 ± 2.62**
BSA-6	13.5 ± 1.08	29.1 ± 1.51	46.1 ± 2.81
g-BSA-6	24.8 ± 1.88	57.3 ± 1.92*	102.9 ± 4.28**

* and **, see the footnote on Table 2.

DISCUSSION

Glycation, which is the non-enzymatic binding of glucose to protein molecules, occurs both *in vitro* and *in vivo*. *In vitro* early glycation products can be prepared by the incubation of proteins with glucose for about 1-6 weeks, while AGE require more than 6 weeks [19]. Therefore we selected this time range for albumin glycation reaction.

Recently, the glycation and increased generation of ROS have been suggested as playing a role in tissue damage in several human diseases associated with both diabetes and aging [20]. Nakayama *et al.* [21] showed that glucosone which is a typical enediol product in Maillard reaction, had a cytotoxic effect on cultured fibroblasts in the presence of cupric ion. In our study, g-BSA-2, g-BSA-4 and g-BSA-6 showed dose-dependent cytotoxicity in rat

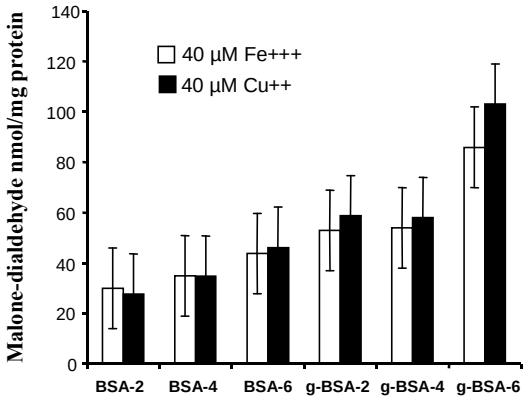

Fig. 1. Comparison of the effect of Fe^{+++} with Cu^{++} on ROS production in presence of glycated and non-glycated albumin.

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hepatocytes in the presence of cupric or ferric ions. The degree of cell injury increased with an increase in the concentration of ferric or cupric ions. ROS are thought to play a role in the development of some of diseases such as diabetes and arthrosclerosis. The auto-oxidation of glucose, the intermediate products of cyclooxygenase, and intracellular production from mitochondria have been proposed to be the source of oxygen free radical [22]. Mullarkey *et al.* [23] reported that Amadori products generated free radicals at physiological pH levels *in vitro* and caused lipid peroxidation. Moreover, the oxidative depolymerization of proteins has also been shown to be caused by oxygen free radical generation from Amadori compounds by the addition of cupric or ferric ions [24].

The chemical mechanism by which oxidant species damage the cells and tissues is not clearly understood yet. Glucose itself has been reported to undergo autoxidation in the presence of transitional metal ions, and hydrogen peroxide and hydroxyl radical have been proposed to play a role in the pathogenesis of diabetic complications [25]. In the recent study, the ROS may be a candidate for damage to hepatocytes by glycated albumin-transition metal ions system. Our results indicated that cupric ion participated in the oxidative damage of hepatocytes by glycated albumin more potent than ferric ion. Both metal ions are known to exist in advanced atherosclerosis and cataract [26, 27]. Indirect evidence has been demonstrated the participation of transition metals in oxidative damage of tissues during aging and diabetes [28]. Evidences show that protein-bound copper ceruloplasmin and protein bound iron; transferring is in part responsible for the oxidative modification in lipids, lipoproteins and other biomolecules [29]. Roy *et al.* [30] also showed that myoglobin undergoes glycation *in vitro*, and they found the amount of iron released from the heme pocket of myoglobin was directly related with the extent of glycation.

We concluded that transition metal such as copper and iron, may participate in the oxidative damage of cells and tissues. The cell damage correlates with the extent of lipid peroxidation of the cell membrane which is catalyzed by these two ions. The hepatocyte membrane contains high levels of polyunsaturated lipids and thus it is susceptible to the free radical chain reaction of lipid peroxidation. However, there is also a possibility that cupric and ferric ions directly cause lipid peroxidation of the hepatocyte membrane.

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