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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Science 8 (9): 1203-1206, 2005
ISSN 1028-8880

Effect of *in vitro* Glycation of Human Placental Collagen (Type IV) on Platelet Aggregation

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Abstract: The aim of this study was to analyze the *in vitro* glyation of human placental collagen (type IV) and to test the effect of glycation on its platelet aggregation activity. After isolation of collagen IV from a normal human placenta, it was glycated using different glucose concentration and different time of incubation. Glycation was measured using thiobarbituric acid colorimetric reaction. Using a turbidimetric method effect of glycated collagen on platelet aggregation was studied and compared with those of non-glycated collagen. The data obtained from this study indicated that collagen underwent *in vitro* glycation reaction and the level of glycation was dependent on glucose concentration and time of treatment with glucose ($p<0.01$). Glycated collagen increased platelet aggregation compared with native collagen ($p<0.01$). These results suggest that hyper-aggregability of platelet in interaction with glycated collagen can be the result of modification in collagen structure. The observed carbohydrate-induced modification apparently have a major impact on molecular conformation of the collagen IV, which might be the molecular basis for pathophysiological changes observed in diabetic microvascular complications.

Key words: Glycation, collagen, platelet aggregation, human placenta

INTRODUCTION

Hyperglycemic conditions of diabetes accelerate protein glycation, leading to formation of Advanced Glycation End products (AGEs)[1,2]. This protein modification is believed to play an important role in the development of long-term disorders associated with diabetes[3,4]. Proteins of extra cellular matrix are long-lived, so that accumulated non-enzymatic modifications may alter protein conformation and properties, hence leading to those functional deficiencies, which are observed in a variety of tissues, exposed to reducing carbohydrate and reactive oxygen radicals[5]. Human basal membrane collagen (type IV) is a protein that undergoes glycation reaction. Formations of inter-and intra-molecular cross-link in collagen in diabetics and stroptozocin-induced rats were shown by Raabe *et al.*[6]. First evidence for effects of glycation on basement-membrane collagen (type IV) was obtained by a series of *in vitro* experiments. For the NC1 domain of collagen IV a loss in the ability to interact with the triple-helical domain of collagen IV was reported by Tsilibary *et al.*[7]. The decrease interaction with heparin *in vitro* was shown for non-enzymatically glycated collagen IV[8].

Increase glycation of human fetal collagen in hyperglycemic mothers has been reported by Pape *et al.*[9]. Brownlee *et al.*[10] showed that glycated collagen can binds to IgG and albumin four times higher than non-glycated collagen. They also concluded that both albumin and IgG bound to glycated collagen retained their ability to form immune complex *in situ* with free antibody and antigen respectively[10].

Altered platelet function such as hyper-sensitivity of platelets to collagen, is prevalent in diabetes and may participate in the pathogenesis of diabetic vascular complications by promoting microthrombus formation[11]. Alteration of platelet function contributes to microthrombus formation and may play an important role in the pathogenesis of diabetic micro-and macroangiopathies[12]. Accerelated atherosclerosis and diabetic microvascular disease make diabetes a leading cause of coronary artery disease and a major cause of blindness and renal failure. However the molecular mechanism underlying diabetic platelet dysfunction has not been fully elucidated[11].

The aim of this study was to obtain further details about the *in vitro* glycation of human placental collagen (IV) and also to test the hypothesis that glycation of collagen alters its platelet aggregation activity.

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MATERIALS AND METHODS

Collagen IV preparation: Normal human placental tissue was frozen immediately following delivery and stored at –20°C until preparation. Collagen IV was isolated from placenta of a healthy subject using pepsin digestion and differential salt precipitation and subsequent ion-exchange chromatography[13,14]. The purity of prepared collagen was checked using SDS-PAGE[15] and protein concentration was determined using Bradford method[16].

Glycation of collagen IV: Prepared collagen was glycated by treatment with different concentrations of glucose and in different incubation time[17]. Aliquots of collagen IV (19.5 mg mL^{-1}) in dialysis bag was incubated with 100 and 150 mM glucose in 0.2 M phosphate buffer of, pH 7.4 containing 0.04% sodium azide, at 27 and 37°C for 20 and 30 days. Controls were treated under the same conditions but without glucose. On days 10, 20 and 30 from the beginning of incubation, microbiological testing of samples was carried out to confirm the absence of microbiological contamination. To avoid the interference by glucose we dialyzed the samples overnight against physiological saline at 4°C with three times changes of buffer.

Measurement of glycation: Glycation was measured using the thiobarbituric acid colorimetric reaction[17]. The colorimetric method with 2-thiobarbituric acid is based on the hydrolysis of the glycated proteins using oxalic acid at 100°C yielding 5-hydroxymethyl furfural (5-HMF) which react with thiobarbituric acid. The absorbance was measured at 443 nm. 5-HMF was used as a standard and glycation of collagen was calculated and expressed as μmol HMF per g protein.

Preparation of human platelets: Whole blood was drawn from a healthy individual and collected in test tube containing 3.8% sodium citrate. Platelet Rich Plasma (PRP) was harvested from this anti-coagulated whole blood after centrifugation at 2,500 rpm for 1 min at room temperature[11]. Platelet poor plasma was obtained by spinning the rest of the blood at 5,000 rpm for 10 min at room temperature. The PRP sample was adjusted using platelet poor plasma to a platelet concentration of $2x10^8$ cells mL^{-1} before use.

Measurement of platelet aggregation: Platelet aggregation was monitored based on a turbidimetric method[11] using a Pye Unicam UV-Vis spectrophotometer. Cuvetts containing 480 μL PRP were stirred at 1000 rpm for 1 min at 37°C before addition of 20 μL of 5 mg mL^{-1} collagen. The change in absorbance (ΔA) was recorded at 620 nm for 5 min. The maximum aggregation was determined as the peak of light absorbance after the addition of collagen.

Statistical analysis: Comparison of the data was carried out using ANOVA (p_1 value) and for more details post hoc test (Tukey) was used (p_2 value).

RESULTS

Glycation of collagen IV after treatment with different concentrations of glucose and different incubation times at 27 and 37°C was measured (Table 1 and 2). Data obtained from these experiments in two different temperatures showed that higher glucose concentration and longer period of incubation led to higher degree of glycation and analysis the results using ANOVA showed that these factors had statistical significance ($p_1<0.01$). To find out more details post hoc test (Tukey) was used to compare the level of glycation with those of control and the observed differences were significant ($p_2<0.01$). As these data showed the glycation was higher when glucose concentration and incubation time increased (Table 1, $p< 0.01$). Similar data was obtained for glycation of collagen at 37°C (Table 2). However the differences between data obtained in two different temperatures were not statistically significant.

Effect of glycation on platelet aggregation was studied based on changes in absorbance after adding collagen on platelet suspension. Absorbance and aggregation of platelet is dependent on glycation level (Table 3). At higher degree of glycation, changes in absorbance were higher and the difference was statistically significant (p_1 and p_2 <0.01). A similar result was obtained for glycation of collagen at 37°C (Table 4).

Table 1: Glycation of collagen IV (μmol HMF g^{-1} protein) in different incubation time and different concentrations of glucose at 27°C. (p_1 and p_2 <0.01)

Incubation time (days)	Glucose concentration (mM) 0 (Control) Mean±SD	100 Mean±SD	150 Mean±SD
20	4.22±0.19	21.6±0.75	27.5±1.05
30	4.55±0.14	23.36±0.85	31.56±1.4

Table 2: Glycation of collagen IV (μmol HMF g^{-1} protein) in different time and concentration of glucose at 37°C. (p_1 and p_2 <0.01)

Incubation time (days)	Glucose concentration (mM) 0 (Control) Mean±SD	100 Mean±SD	150 Mean±SD
20	4.48±0.19	22.63±0.85	29.26±1.30
30	5.19±0.29	25.00±1.1	32.18±1.38

Table 3: Effect of glycated collagen IV on platelet aggregation. ΔA is the difference in absorbance after adding collagen on platelet suspension (p_1 and p_2 <0.01)

	20 days		30 days	
Glucose concentration (mM)	Glycation (μmol HMF g^{-1} protein) Mean±SD	ΔA Mean±SD	Glycation (μmolHMF g^{-1} protein) Mean±SD	ΔA Mean±SD
0	4.22±0.19	0.04±0.01	4.55±0.14	0.04±0.01
100	21.6±0.75	0.18±0.013	20.36±0.85	0.20±0.012
150	27.5±1.05	0.23±0.010	31.56±1.4	0.26±0.014

Collagen IV was glycated at 27°C in presence of 100 and 150 mM Glc for 20 and 30 days

Table 4: Effect of glycated collagen IV on platelet aggregation. ΔA is difference in absorbance after adding collagen on platelet suspension (p_1 and p_2 <0.01)

	20 days		30 days	
Glucose concentration (mM)	Glycation (μmol HMF g^{-1} protein) Mean±SD	ΔA Mean±SD	Glycation (μmolHMF g^{-1} protein) Mean±SD	ΔA Mean±SD
0	4.48±0.19	0.04±0.012	5.19±0.29	0.04±0.014
100	22.63±0.85	0.19±0.015	25.00±1.1	0.21±0.016
150	29.26±1.30	0.24±0.013	32.18±1.38	0.27±0.014

Collagen IV was glycated at 37°C in presence of 100 and 150 mM Glc for 20 and 30 days

DISCUSSION

Collagen undergoes continuous non-enzymatic glycation during its long life period. The products resulting from the glycation reaction, so called Advanced Glyction End products (AGEs), were regarded as potential pathogens of various diseases such as diabetes complications. The main functional property of collagen is to provide a supporting framework to almost all tissues, the effect of non-enzymatic glycation on this protein are deleterious and in diabetes mellitus contribute to the mechanism of late complications[18]. Hyperaggregability of platelets in patients with diabetes has been reported by Leocini *et al.*[19]. Several factors have been recognized to play roles in this process. Hyperglycemia-induced mitochondrial superoxide generation may play an important role in platelet disfunction in patients with diabetes[11]. In this study we examined another factor i.e. collagen that is involved in platelet function and undergoes glycation in diabetes and aging. The results obtained from this study showed that glycation of collagen IV are dependent on glucose concentration and time of treatment with glucose. Hyper-aggregability of platelet was observed when these cells were treated with glycated collagen compared to non-glycated collagen. It can be concluded from these data that disfunction of platelet in diabetes can be at least to some part due to glycation of collagen. Yamagishi *et al.*[11] have shown that hyperglycemia increased ROS generation in human platelets and this effect was additive with that of collagen[11]. In the same study they also showed that marked irreversible aggregation was induced by collagen when PRP was preincubated with high glucose.

Increased platelet activity and an increased tendency for thrombus formation occur in atherosclerosis, heart disease, hypertension and diabetes[12]. An evolving concept is that enhanced platelet activity may not only derive from procoagulant activity but from unbridling platelet hyperfunction secondary to loss of the restraining action of the antiaggregatory mechanisms. Abnormalities may occur in potentially all of the mechanisms regulating platelet function, involving platlet-agonist interaction, platelet-vessel wall interaction, platelet-platelet interaction, platelet secretion and platelet-coagulant protein interaction[12]. Present results also can explain another mechanism for hyperfunction of platelet and support this concept. However still it is not clear at this time whether the platelet abnormalities are intrinsic to the platelet or are a consequence of circulation factors that affect platelet function.

Present results revealed changes in another activity of collagen due to glycation. Some investigators previously reported that glycated collagen could covalently trap low-density lipoprotein[20]. Altered cellular interaction between endothelial cells and non-enzymatically glycosylated collagen was also reported[21].

If the post translational modifications of proteins play a leading role in the pathogenesis of complications, it is possible to conclude that strict glycaemic control, obtained by accurate insulin therapy can prevent them by inhibiting the non-enzymatic modification of proteins and delaying their accumulation in collagen.

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