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Platelet Aggregation Increased by Advanced Glycated Hemoglobin

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Abstract

Introduction: Many studies have provided strong evidence for an association between diabetes complications and an increase in platelet (PLT) reactivity. Though some metabolic abnormalities have been reported as the major causes of this reactivity and malfunction, the precise mechanism has not been fully elucidated.

Aim: The aim of this study is to investigate the effect of advanced glycation end product of hemoglubin (AGE-Hb) on human PLT reactivity and malfunction.

Materials and Methods: A solution of bovine hemoglobin was prepared with fructose in phosphate buffer. The solution was then sterilized and incubated under sterile conditions at 37°C in the dark. The control solution was prepared in the same way, but without fructose. Human PLTs were isolated and prepared from 15 healthy volunteers, men and women. Blood was collected in the morning from fasting healthy subjects using a 21-gauge needle with 117 mM sodium citrate (1:9 v/v), as an anticoagulant. Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorometer. PLT aggregation was measured using the photometric system Packs-4 aggregometer.

Results: The relative fluorescence intensity increased in Hb samples but not in controls that incubated with fructose. PLTS aggregation did not change in controls while after incubation with 10, 22, 30-days fructose-glycated Hb decreased by 10, 12, 30%, respectively.

Taken together our data show that PLT secondary phase of ADP-induced aggregation gradually affected with advanced increase in Hb glycation.

Keywords: Human platelets; Diabetes; HbAc; Glycation; Blood transfusion; Hyperreactivity; *In-vitro*

Abbreviations:

PLTS: Human Blood Platelets; AGE: Advanced Glycation End Products; AGE-BSA: Advanced Glycation End Product-BSA; AGE-Hb: Advanced Glycation End Products-Hemoglobin

Introduction

Platelets (PLTs), the smallest enucleated cells in blood stream derived from bone marrow megakaryocytes, play some critical roles in haemostasis, thrombosis, and atherosclerosis [1]. It has been reported that PLTs from diabetic patients have different properties with those from normal individuals [2]. Many studies have also provided strong evidence for an association between diabetic complications and increase in PLT function defect and hyperactivity [3]. Though some metabolic abnormalities in proteins extracellular and/or interacelluarly have been reported as the major causes of this hyperactivity, the precise mechanism has not been fully elucidated [4,5].

However, it has been shown that free radicals increase the aggregation of PLTs5. Since these molecules are the major by-products of glycation reactions [6], they have been speculated as an important cause of PLT function inadequacy in diabetes.

Non-enzymatic glycosylation or glycation is a process in which reducing sugar(s) react spontaneously with amino groups in proteins to produce Advanced Glycation End products (AGEs) [7]. This phenomenon introduces considerable alteration on the structure of proteins, and consequently changes their function [8]. The chemistry of AGEs is not fully understood; in the meantime increased levels of circulating AGEs have been demonstrated in both animal and human diabetics [9]. AGE structures are associated with many pathophysiological abnormalities [10] as in diabetes and aging [11,12].

It has been assumed that PLT hyperactivity is influenced by protein glycation upon different mechanisms i.e. glycation of PLT membrane proteins [13], glycation of PLT cytosolic proteins [14], and interaction

between glycated proteins or AGEs and PLTs [15-**17].** Reports dealing with the interaction between AGEs and PLTs are limited to few cases in leterature. Hangaishi et al. have reported an increase in PLT aggregation upon treatment with AGE-BSA [16]. Furthermore, [17] Ferreti et al. have observed the same phenomenon during incubation of PLTs with glycated LDL, [17] along with some changes in PLT metabolism.

The most known form of the glycation product of hemoglobin is HbA1c, which has been attained significant prominence in the modern world of medical biology due to its use as a scale in the long-term control of diabetes mellitus [9,18,19] and aging-accelerated glycation diseases [11,18]. HbA1c is formed through a non enzymatic reaction between glucose and N-terminal valin residue of the β-chain of HbA0 [20].

In vivo, advancement of the glycation process, mostly involved by lysyl ε-amino groups, results in advanced glycated end products of Hb (AGE-Hb). These structures have been reported with vigorous altered structural features [21]. However, the presence and circulation of 0.42% and 0.75% AGE-Hb have been reported in non-diabetic and diabetic patients, respectively [19]. Regarding to the higher plasma concentration of AGE-Hb in diabetic patients [19,21], the interaction between this molecule and other blood elements such as PLTs in diabetics may changes the PLTs (ir)responsiveness and possible premature agglutionations, forming of non firmed revesible aggregates.

The aim of this study is to investigate the effect of AGE-Hb on human PLT aggregation, *invitro*.

Here we show that PLT secondary phase of ADP-induced aggregation affected by gradual increase in Hbglycation, as it is previously reported with increased glycated LDL17- and AGE-BSA [18].

Material

Bovine methemoglobin, and Griess reagent were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Thioflavin T was purchased from Fluka/*Sigma*-Aldrich (St.Louis, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

Blood sample preparation

This study was performed on 15 healthy volunteers, men and women, aged 22 to 40 years. Blood was collected in the morning from fasted healthy subjects using a 21-gauge needle with 117 mM sodium citrate (1:9 v/v), as an anticoagulant. None of the subjects had any signs or history of serious diseases, and they had abstained from taking medication for at least 10 days. Oral informed consent was obtained from all volunteers.

Whole blood was centrifuged at 150×g for 10 minutes at 25°C to prepare PLT-rich plasma (PRP), and the remaining supernatant was centrifuged at 2500 × g for 10 minutes at 25°C to obtain PLT-poor plasma (PPP). The PLTs count in PRP was measured and the PRP was adjusted to 3.8×10^5/ µl with PPP.

Invitro preparation of AGE-Hb

A solution of bovine hemoglobin (15 µM) was prepared with 30 mM fructose in a 50 mM phosphate buffer with pH 7.4, containing 0.02% (W/V) sodium azide. The solution was sterilized by filtering through low protein binding filter (Millex. ®.-GV 0.22. µm filter unit, Millipore, USA) and was then incubated under sterile conditions at 37°C in the dark.

The control solution was prepared in the same way, but without fructose. Samples were taken at various intervals and dialyzed extensively against 50 mM phosphate buffer with pH 7.4 at 4°C, to remove unbounded fructose molecules. After dialysis protein concentrations were estimated by the Bradford method [22] and the samples were immediately frozen and stored at -70°C until processed.

AGE-Hb associated fluorescence analysis

Fluorescence measurements were performed using a Hitachi F-4500 spectrofluorometer. AGE-related autofluorescence of the sample preparations was monitored by exciting at 370 nm and emission wavelength at 440 nm at 25°C which was determined through wavelength scanning in the range of 400-500 nm. The spectra were corrected with appropriate protein and buffer blanks. For all measurements, the samples contained 0.1 mg/ml protein.

Circular dichroism spectropolarimetry

Circular dichroism (CD) spectra were collected between 190 and 260 nm, at the far UV region with an Aviv CD spectropolarimeter model 215) at 25°C. The CD measurement was performed using a 1mm path length quartz cuvette, at the final protein concentration of 0.2 mg/ml. Deconvolution of the data was performed to estimate the (α)-helix and (β)-conformation content of AGE-Hbs using CDNN software [23].

Thioflavin T test

Thioflavin T (ThT) is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to diagnose amyloid fibrils, both *exvivo* and in vitro. Solutions of 1.5 µM fructated hemoglobin and control samples were incubated at 25°C with 5 µM thioflavin T for an hour. The free ThT remained in solution and did not affect signal-to-noise ratio. The sample fluorescence was measured through excitation at 435 nm and emission spectra were recorded in the wavelength range of 455-555 nm; band slit was adjusted at 10 nm.

PLT aggregation

PLT aggregation was measured using the photometric system Packs-4 aggregometer (Helena Laboratories, Beaumont, TX). In brief, 350 µl of PRP obtained from subjects and 100 µl of 3 µM hemoglobin at various levels of glycation were incubated with stirring (1000rpm) for 15 min at 37°C. PLTs count was adjusted to 300000 per microliter. Then 50 µl ADP (20 µM) was then added and the PLT aggregation was monitored for 15 minutes.

All aggregation phases were step-by-step analysed to unravel how glycated proteins affect agglutination and lag phase, shape change, primary phase, secondary phase, reversibility and maximal aggregation.

NO measurement

For NO assessment PLT aggregation was performed as described above. Fifteen minutes after PLT aggregation, 50 µl of fixative solution (36 mM EDTA, 1% formaldehyde) was added, and the samples were

centrifuged at 12000 rpm for 2 min at 4°C. The production of NO by PLTs was assayed through measuring nitrite in the supernatant of centrifuged samples.

In brief, 100 µl of supernatant of each sample was transferred to a 96-well flat-bottom microtiter plate and well mixed with equal volume of Griess reagent at room temperature for 10 min. The absorbance values at 540 nm were measured on an EIA Multiscan MS microplate reader. Moreover, $NaNO_2$ in a concentration range from 0 to 100 µM was used to construct standard curve.

Results

AGE-related fluorescence properties of Hb

The emitted fluorescence intensity values for fructated Hb and controls are plotted as a function of time in Figure 1. AGE-related fluorescence properties of the haemoglobin incubated with fructose were assessed through maximum emission peak at 440 nm after excitation at 370 nm.

Fluorescence studies over a 30-day incubation period revealed an increase in the fluorescence intensity for the samples incubated with fructose along with incubation time. However, AGE-related increase in the intensity of fluorescence was not observed for the controls.

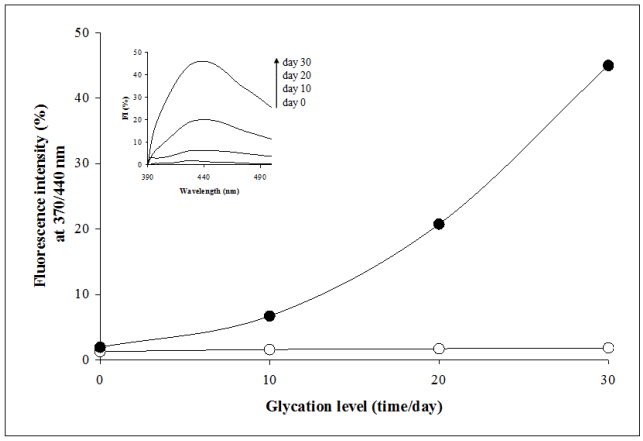

Figure 1: AGE-related fluorescence properties of Hb. The emitted fluorescence intensity values for fructated Hb and controls are plotted as a function of time. Filled dots are AGE-Hb treated samples and empty dots are controls. In inset are shown AGE-related fluorescence properties of the haemoglobin incubated with fructose which were assessed through maximum emission peak at 440 nm after excitation at 370 nm.

The CD spectra at the far-UV region of AGE-Hb

The CD spectra at the far-UV region of AGE-Hb are different from the controls and a significant change in the secondary structure of AGE-Hb was observed by a decrease in negative ellipticity in the wavelength rang of 205–235 nm (Figure 2).

Furthermore, in progressive glycation with fructose a decrease in α-helicity along with an increase in β-conformation content in AGE-Hb was observed (data not shown).

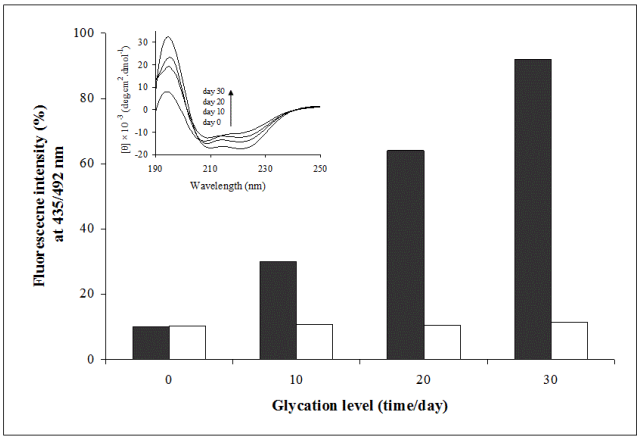

Figure 2: The CD spectra at the far-UV region of AGE-Hb. ThT-binding function against fructation time effect in which maximum fluorescence emission was measured through excitation at 435nm and spectra were recoded in the wavelength range of 455–555nm for glycated Hb (▮) and control samples (□). Inset to the figure presents resulting far-ultra-violet CD spectra for the samples of Hb treated with fructose during 30 days incubation time.

The thioflavin T-related fluorescence intensity increased with increasing incubation time

Fructated Hb was exposed to ThT, and fluorescence emission was measured in different days. The ThT-related fluorescence intensity increased with increasing the time of incubation, which shows the progressive formation of the amyloid structures (Figure 2).

In the samples, a gradual increase in the fluorescence emission of thioflavin T was observed at 492 nm upon binding to the glycated protein but not in control solutions.

AGE-Hb affects ADP-induced aggregation function

After validation of AGE-Hb quantity and quality under *in-vitro* condition the effect of AGE-Hb on human PLT aggregation function was investigated. Generally, glycated hemoglobin was revealed to have a significant effect on ADP-induced aggregation of human PLTs *invitro*.

As it is shown in Figure 3 with only ADP preincubated PLTs showed no remarkable decrease in niether primary nor secondary phase of ADP-induced aggregation (control group; PRP+ADP curve); while preincubation of PLTs with AGE-Hbday0 for 15 min, delayed and decreased the sceondary phase and maximal aggregation (PRP +ADP+AGEHb day0).

PLT aggregation induced by ADP was significantly blocked and decreased along with increase in hemoglobin glycation incubation time day 22 and 30 as a function of time. However, control samples prepared in different days caused no significant changes in ADP-induced aggregation.

In all samples that exposed to ADP immidiately their primary phase aggregation were not affected by AGEHbs irrespective of AGEHbs incubation time but the secondary phase were specially affected, which needs more investigation (Figure 3).

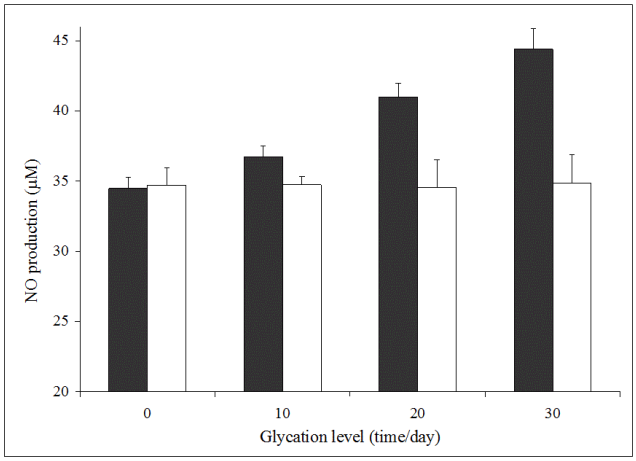

Figure 3: AGE-Hb caused remarkable increase in NO production. NO production in platelets pre-incubated with different level of glycated hemoglobin (■) and control samples (□). Each value represents the mean ± S.D. (n=5).

AGE-Hb caused significant increase in NO production

AGE-Hb caused significant increase in NO production in aggregated PLTs induced by ADP, which was monitored through incubation time (Figure 3). The NO production in those cells treated with control samples, however, was negligible. This increase in NO production seems to be a consequence of more platelet aggregation in samples treated with Hb-AGE.

Discussion

There are a lot of evidences which have shown a direct correlation between diabetic complications and PLTs reactivity [2]. This PLT malfunction has been attributed in part to hyperglycemia and glycation reactions that might aggravate with a higher rate in hyperglycemia conditions [24]. Aforementioned reactions affect PLT function by different mechanisms [15]. Several studies have revealed that an increase in PLT reactivity upon treatment with AGE proteins; though their influences vary remarkably [15-17].

In diabetics the AGE-Hb content raises extremely. Haemoglobin glycation results in an early and advanced glycation end-products, which are known as HbA1c and AGE-Hb, respectively. In our previous study we have shown that prolonged incubation of Hb with fructose increases consequential alteration in Hb structure [21]. In this study we show that pretreatment of PLTs with AGE-Hb affects ADP-induced PLT aggregation at different phases. These effects are absent in the controls and fresh proteins, which confirms previous studies dealing with the effects of AGE structures on these cells.

The general mechanism by which AGE proteins influence different cell types has been described to be involved particular types of receptors such as RAGE (receptor for AGE) and SR (scavenger receptor) [15,25].

Hasegawa et al. have suggested that AGE-BSA affects PLTs through serotonin receptor, the presence and even so the identity of receptor(s) for AGE proteins in PLTs has not been confirmed, yet. Herczenik et al. postulaNε-(carboxymethyl)lysine) ated that PLT activation by amyloid-containing proteins is mediated throught 2 independent routes. It has been also revealed that RAGE is the receptor for β-amyloid (Aβ) protein [26]. Since both AGE and Aβ are the ligands for RAGE, it seems that these structures have similar domains to interact with this receptor [27]. We observed that incubation time of AGEHb affects secondary phase of ADP-induced aggregation remarkably. Obviously AGEHb20 was able to mimic Aspirin-like and AGEHb30 as Clopidrogrel-like anticoagulating agents, however. How AGEHb20 and 30 inhibit ADP-induced secondary phase of aggregation needs more investigation.

AGE-related fluorescence properties of the AGE-Hb were assessed in this study through maximum emission peak at 440 nm. Fluorescence studies over a 30-day incubation period revealed an increase in the fluorescence intensity for the samples incubated with fructose *invitro*. ThT is used as a diagnostic dye for the amyloid fibrils due to its geometric fitness and therefore, binding to these structures [28,29]. Increase in fluorescence property of ThT upon interaction with AGE-Hb demonstrates the evolving of amyloid-like structure. Therefore, we monitored the amyloid fibrils formation as a result of alpha (α) to beta (β) transition during glycation process as a function of incubation time through increase of ThT-associated fluorescence [30]. These data were also quantified CD spectroscopy in which an increase in β content was resulted during the glycation process.

Since AGE-Hb affects PLT (ir-)responsiveness/ secretory phase, and it has amyloid-like structure, it seems that these domains are necessary for AGEs to affect PLTs (dys-)function. This assumption is consisted with the results from other groups who have already shown that AGE-BSA induces PLT reactivity [15,18] while some glycated structures like CML (Nε-(carboxymethyl)lysine) and pentosidine have no effect on these cells [16]. They have discussed their observation based on the presence of some essential epitopes in AGE-BSA. Moreover, Stanyer et al. [31] have shown an increase in PLT aggregation upon treatment with fibrilar form of Aβ-40, whereas aggregation is diminished when Aβ-peptide has been treated in the presences of plasma lipoprotein due to wrapping of the amyloid structures and reducing its effect on PLTs. Regarding to these facts, the presence of RAGE or SR-like receptors on PLT surface for conducting AGE signaling seems to be necessary.

It has been shown that AGE structures affect PLT reactivity through the production of free radicals [32]. Oxidative stress plays an important role in AGE pathogenesis [33]. The main intracellular signaling pathways introduced by AGE structures might involve these molecules [34]. Hangaishi et al. [16] have indicated that interaction between AGE-BSA and PLTs produces intracellular superoxide onion [35]. They have also shown that in presence of antioxidants, the effect of AGE-BSA on PLT reactivity is significantly reduced. It is well-known that O_2- triggers an increase in intracellular Ca^{+2} [36] which plays a central role in the regulation of PLT reactivity [37]. Therefore, it seems that AGE proteins cause a production in free radicals, and consequently an increase in inter- and intracellular calcium level in PLTs, which finally leads to PLT hyperactivity.

Effects of NOS and ROS endproducts

Nitric oxide acts as an essential molecule in the regulation of PLT function [38]. There are two NO synthase (NOS) insoforms in PLTs; iNOS and eNOS. The dominant form in normal individuals is eNOS, which is activated by Ca^{+2}-calmodulin complex [4]. Here we are reporting the increase in NO production in PLTs via AGE-Hb

treatment. Ferretti et al. have reported the same increase in NO level during incubation of PLT with glycated LDL. It seems that increase in intracellular Ca^{+2} during with the interaction between AGE and PLTs, probably through oxidative stress, activates eNOS, and consequently leads to production of NO [17]. It has been known that NO reduces Ca^{+2} concentration [39]; therefore, increase in NO production by PLT upon treatment with AGE may be a negative feedback mechanism for PLT hyperactivity. It has been revealed that O_2- reduces NO concentration by combining to it and form peroxynitrite ($ONOO^-$). This combination has been speculated as a mechanism by which O_2- diminishes the feedback effect of NO on Ca^{+2} and therefore, increases Ca^{+2} concentration [40,41]. Although, $ONOO^-$ is a pathogenic molecule in biological systems, its effect on PLT hyperactivity has not been clearly identified. In washed PLTs ONOO- induces aggregation; however, in PRP it acts as an inhibitor of PLT activity [42]. Therefore, it is not recognized that the formation of $ONOO^-$ is a mechanism for omitting NO by O_2- or diminishing O_2- effect by NO.

Collectively, the present work provided here, put together with the results obtained by other groups, suggest that interaction between AGE-Hb and PLTs might occur through special receptors and secretory granula leading to production of free radicals and thereby an increase in inter- and intracellular Ca^{+2} concentration. Calcium might act in dual mode function to induce eNOS activity and production of NO, which acts as a negative feedback for PLT function. Moreover, the combination between O_2- and NO produces $ONOO^-$, which has a dual effect on PLT (ir-)responsiveness and reactivity. How fructose affects Hb structure and function and PLTs hyperreactivity in diabetes patients is still need more investigation. However, more studies are required to evaluate the definite mechanisms underlying these events.

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Conflict of Interest

There is no conflict of interest.

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