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A Comparison of the Kinetics of Low-Density Lipoprotein Oxidation Induced by AGEs-Modified Hemoglobin or by Glycohemoglobin*

Porównanie kinetyki reakcji utleniania hemoglobiny zmodyfikowanej AGEs lub glikowanej hemoglobiny w obecności LDL

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Abstract

Background. Hb-mediated reactions can be implicated in oxidative stress during diabetes, characterized by inflammatory reactions induced by hemolytic complications. Diabetes is associated with a marked increase in the glycation of numerous proteins, including Hb.
Objectives. The aim of this study was to compare the kinetics of low-density lipoprotein oxidation induced by advanced glycation end product-modified hemoglobin (AGEs-Hb) or by glycohemoglobin (the glycated fraction of hemoglobin, GHb). The effects of pH on LDL peroxidation kinetics were also studied.
Material and Methods. Experiments were performed using human hemoglobin obtained commercially or fresh blood obtained from a blood bank. LDL was selectively precipitated from the plasma by addition of BioMerieux precipitating reagent of an LDL-cholesterol kit and separation by centrifugation. The oxidation of LDL was determined by the formation of conjugated dienes after oxidation with AGEs-Hb or GHb. The end product of lipid peroxidation was estimated from the spectrophotometric measurement of thiobarbituric acid-reactive substance (TBARS) using 1, 1, 3,3-tetra-ethoxypropane as the standard.
Results. Hemoglobin can continue to modify proteins such as LDL oxidatively within the vessel wall. It was shown that at physiological pH, AGEs-Hb is more effective than GHb in promoting LDL oxidation. A comparative study was also carried out of the reactivity of antioxidants in AGEs-Hb equimolar concentrations as inhibitors of LDL peroxidation. The end products of lipid peroxidation were decreased by 10–14% in samples supplemented with the haptoglobin 2-2 and 1-1. The addition of radical scavengers such as butylated hydroxytoluene inhibited oxidation of LDL (99%), indicating that the reaction is mediated by radicals.
Conclusions. These results support the hypothesis that heme released from a damaged AGEs-Hb molecule as well as iron released from heme are plausibly secondary factors creating oxygen radicals and contributing to increased lipid oxidation. In the development of artery atherosclerosis as a complication of diabetes, the cooperation of GHb and AGEs-Hb probably plays an essential role by retaining the oxidative activity of the glycohemoglobin and the AGEs--modified hemoglobin, both bound in a complex with haptoglobin (**Adv Clin Exp Med. 2008, 17, 5, 531–537**).

Key words: AGEs-modified hemoglobin, low-density lipoprotein, oxidative stress, antioxidants, haptoglobin.

Streszczenie

Wprowadzenie. Istnieje współzależność między stresem oksydacyjnym oraz rozwojem zmian miażdżycowych związanych z hemolizą wewnątrznaczyniową w przebiegu cukrzycy. Peroksydacja lipidów lipoprotein o małej gęstości (LDL) jest zwiększona w cukrzycy z powodu hiperglikemii, wzmożonego stresu oksydacyjnego oraz zmniejszenia aktywności mechanizmów przeciwutleniających. We krwi stężenie glikowanej frakcji hemoglobiny (glikohemoglobiny; GHb) lub hemoglobiny zmodyfikowanej późnymi produktami glikacji (AGEs-Hb) zależy od uśrednionego stężenia glukozy w czasie jej trwania w krążeniu oraz od samego stężenia hemoglobiny. Tematem klinicznych poszukiwań jest pytanie, czy krążąca AGEs-Hb może mieć wpływ na ryzyko rozwoju miażdżycy jako powikłania cukrzycy.

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Cel pracy. Porównanie charakterystyk aktywności utleniającej GHb i AGEs-Hb w obecności LDL w środowisku kwaśnym i fizjologicznym, w warunkach stresu oksydacyjnego.
Materiał i metody. Do badań użyto komercyjnych preparatów Hb oraz krwi pełnej osób zdrowych lub chorych na cukrzycę. Oceniono kinetykę peroksydacji lipidów LDL w obecności GHb i AGEs-Hb, przyjmując za kryterium oceny stężenie substancji reagujących z kwasem tiobarbiturowym (TBARS) oraz stężenie sprzężonych dienów (metoda fotometryczna) w warunkach zastosowanego pH środowiska.
Wyniki. Reakcje związane z powstawaniem TBARS prowadzone przez 5 godz. w środowisku kwaśnym nie wykazały istotnych różnic w mechanizmie peroksydacji lipidów w obecności AGEs-Hb i GHb. W przypadku pH fizjologicznego AGEs-Hb okazała się bardziej aktywna od GHb w reakcjach utleniania LDL. Hamowanie peroksydacji lipidów LDL w obecności stałego stężenia AGEs-Hb po inkubacji z haptoglobiną 1-1 i 2-2 przebiegało na niskim poziomie, odpowiednio 14 i 10%.
Wnioski. Zmodyfikowana późnymi produktami glikacji hemoglobina jest równie aktywna jak glikohemoglobina jako czynnik inicjujący peroksydację lipidów LDL w środowisku kwaśnym, bardziej aktywna natomiast jest w środowisku fizjologicznym. Zaobserwowano zachowanie aktywności utleniającej GHb i AGEs-Hb związanych w kompleks z haptoglobiną. Uzyskane wyniki pogłębiają wiedzę odnośnie do molekularnego mechanizmu działania GHb i AGEs-Hb w przypadku wczesnych zmian naczyniowych jako powikłania cukrzycy (**Adv Clin Exp Med 2008, 17, 5, 531–537**).

Słowa kluczowe: hemoglobina zmodyfikowana późnymi produktami glikacji, lipoproteiny o małej gęstości, stres oksydacyjny, przeciwutleniacze, haptoglobina.

Atherosclerotic vascular disease develops at an accelerated rate among persons with diabetes mellitus. Therefore there could be a more direct link between diabetes and atherosclerosis. A possible link between glucose and vascular disease is the production of advanced glycation end products (AGEs). The augmented presence of glucose inside cells results in reactive dicarbonyl molecules such as glyoxal, methylglyoxal, and 3-deoxyglucosone, which react with the amine groups of proteins to form AGEs. The modification process does not require the presence of an enzyme and the two-step reaction is not reversible [1]. AGEs have been thought to form only on long-lived extracellular macromolecules since the rate of AGEs formation from glucose is so slow that more rapidly turned over intracellular proteins would not exist long enough to accumulate them. However, it has been demonstrated that AGEs do in fact form on proteins *in vivo*. In erythrocytes, AGEs-modified hemoglobin (AGEs-Hb) accounts for 0.42% of circulating hemoglobin in normal subjects and 0.75% in diabetics [1, 2].

Low-density lipoprotein (LDL) oxidation appears to be a critical step in the pathogenesis of atherosclerotic vascular disease [3]. This oxidation is associated with free radical-mediated peroxidation of polyunsaturated lipids within the LDL. The amplification of LDL peroxidation *in vitro* by hemoglobin is a well-studied biochemical approach for investigating the oxidative modification of LDL and its role in the pathogenesis of atherosclerosis [4–7]. Hb-mediated reactions can be implicated in oxidative stress during diabetes, characterized by inflammatory reactions induced by hemolytic complications [8]. Haptoglobin (Hp) is thought to inhibit hemoglobin-induced oxidation by preventing the release of redox active iron from hemoglobin. However, the Hb-binding protein Hp is rapidly consumed during intravascular hemolysis [9]. It has also been shown that the antioxidant function of Hp is impaired against glycohemoglobin (the glycated fraction of human hemoglobin, GHb), an early product of glycation which is markedly increased in the diabetic state. The GHb-Hp complex can continue to modify LDL oxidatively [10]. Likewise, a decreased uptake of GHb-Hp complex through the macrophage scavenger receptor CD163 contributes to its slower catabolism [11]. It is therefore imperative to continue the search for other fractions of Hb (for example, AGEs-modified hemoglobin) that may display an increased oxidant activity in relation to LDL.

The aim of this study was to compare the kinetics of low-density lipoprotein peroxidation induced by AGEs-Hb or by glycohemoglobin. The effects of pH on LDL peroxidation kinetics were also studied.

Material and Methods

Preparation of Glycohemoglobin and AGEs-Modified Hemoglobin

Glycohemoglobin was prepared from lysed human blood cells by boronate affinity chromatography [13]. Fresh human blood from persons with diabetes and HbA_{1c} values of 10% or greater as obtained by cation exchange (Bio-Rad) was collected in EDTA-containing tubes, pooled, and frozen. The affinity protocol used a Pierce Glycogel II boronate affinity (Pierce) column with ammonium acetate (250 mM), magnesium chloride (50 mM), and phenoxyethanol (4.5 ml/L) in equilibration buffer

(pH 8.5) and sorbitol (200 mM), Tris (100 mM), and phenoxyethanol (4.5 ml/L) in the elution buffer. Hemolysate was applied to the column. Both the unbound and bound fractions were eluted and collected in glass bottles and concentrated using ultrafiltration (Amicon) before storage at –70°C. The cation exchange protocol used Bio-Rex 70–400 mesh cation exchange resin ~40 mm in size and a column 15 cm in length and 2 cm in diameter. Buffer A consisted of sodium phosphate (monobasic, 33 mM), sodium phosphate (dibasic; 80 mM), and potassium cyanide (10 mM) adjusted to pH 6.75. Buffer B consisted of sodium phosphate (monobasic; 104 mM) and sodium phosphate (dibasic; 45 mM) adjusted to pH 6.4 and with a conductivity between 7.5 and 8.2 g/L total dissolved solids. The column was eluted by using a linear gradient at a flow rate of 1.4 ml/min after an initial equilibration for 45 min using Buffer A. Approximately 500 mg of hemoglobin was applied. After affinity chromatography, the unbound and bound effluents were collected, concentrated (10,000 M_r cutoff, Amicon), and chromatographed on Bio-Rex 70 cation exchange resin.

AGEs-modified hemoglobin was prepared as described previously [14]. Briefly, Hb (0.25 g) was dissolved with 3.0 g of D-glucose in 10 ml of 500 mM sodium phosphate buffer (pH 7.4). Each solution was deoxygenated with nitrogen and sterilized by ultrafiltration (0.45-µm filter). Each sample was incubated at 37ºC for 90 days and dialyzed against 20 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaC1. One preparation of control hemoglobin was incubated under the same conditions but without sugar. All incubations were performed under sterile conditions, in the dark, and at 37°C. After incubation, unbound material was removed by extensive dialysis against 20 mM sodium phosphate buffer (pH 7.4) or by gel filtration over Sephadex G-10 (Pharmacia, Uppsala, Sweden). AGEs-Hb showed absorption and fluorescent spectra identical to those reported previously [14].

Purification of LDL

LDL was selectively precipitated in duplicate from 60 ml of serum by adding 1 ml of BioMerieux precipitating reagent (BioMerieux) and separation by centrifugation (3000 × g for 10 min). The BioMerieux reagent does not contain interfering substances such as antioxidants, prooxidants, a high concentration of potassium bromide, or ion chelator and was discarded after centrifugation. The LDL pellet was dissolved in 600 ml of 15 mM NaOH, which does not contain interfering substances; therefore, dialysis was unnecessary. Only the LDL fraction was precipitated by the BioMerieux reagent. The purity of the LDL was not checked by agarose/sodium dodecyl sulfate-polyacrylamide gel electrophoresis because all the LDL samples were apo-A free. Just before use, the LDL solution was sterilized by passing through a 0.2-µm filter (Millipore) and the concentration of LDL is expressed in milligrams of protein as determined by the Lowry assay [12].

Assay of Thiobarbituric Acid-Reactive Substances

Lipid peroxidation end product was determined as thiobarbituric acid-reactive substances (TBARS) according to the method of Rice-Evans et al. [15]. Briefly, LDL (200 µg protein/ml) was incubated with either 10 µM GHb or 10 µM AGEs-Hb in 150 mM NaCl, 10 mM phosphate buffer solution (PBS) at 37ºC and at pH 5, 6, and 7.4. After incubation, 0.1 ml of 25% TCA and 0.5 ml 1% thiobarbituric acid (in 50% acetic acid) were added and heated at 95°C for 45 min. The solution was centrifuged at 13,000 rpm for 10 min and the supernatant was read at 532 nm. TBARS were expressed as nanomoles of malondialdehyde (MDA) per milligram of LDL protein.

Assay of Conjugated Dienes Formation During GHb- and AGEs-Hb-Mediated Oxidation of LDL

Conjugated diene formation during GHb- and AGE-Hb-mediated oxidation of LDL was measured by monitoring the formation of conjugated dienes [16]. Briefly, LDL (200 µg/ml) was oxidized using either 10 µM GHb or 10 µM AGE-Hb at room temperature in a quartz cuvette. Absorbance values at 234 nm were measured as a function of time as a marker of the rate of conjugated diene formation.

Data Analysis

All data were expressed as the mean ± *SEM*. Statistical comparisons were performed using Student's *t*-test (P values < 0.05 were considered statistically significant).

Results

The Effect of pH on the LDL--Conjugated Diene Formation

The kinetics of the oxidation of human low-density lipoprotein can be measured continuously

by monitoring the change in the 234 nm diene absorption. The time-course shows three consecutive phases: a lag-phase during which the diene absorption increases only weakly, a propagation phase with a rapid increase in diene absorption, and finally a decomposition phase. When the extent of LDL oxidation by glycohemoglobin was assessed by conjugated diene formation, a progressive increase in oxidation and an enhancement in the rate of the propagation phase were observed on reduction of the pH from 7.4 to 5 (Fig. 1). In contrast, when applying AGEs-Hb as the pro-oxidant there was a progressive augmentation in the lag phase and a decrease in the rate of the propagation phase as pH fell.

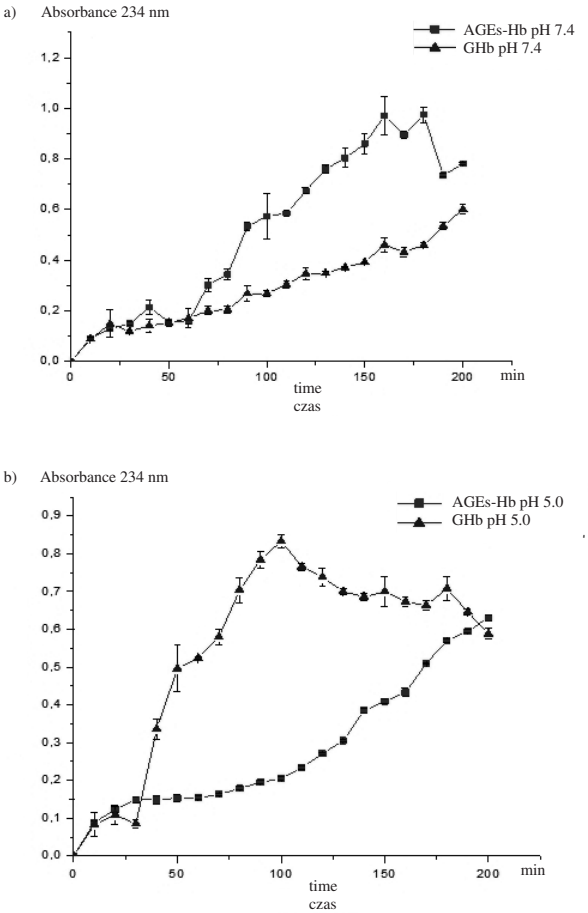

Fig. 1. The effect of pH on LDL-conjugated diene formation. LDL (200 μg protein/ml) was incubated in the presence of either 10 μM GHb or 10 μM AGEs-Hb for 200 minutes at 37ºC at pH 7.4 (a) and 5 (b). The data represent the mean ± *SD* (n = 4)

Ryc. 1. Wpływ pH 7,4 (a) i pH 5 (b) na powstawanie sprzężonych dienów w obecności LDL (200 μg białka/ml) oraz GHb i AGEs-Hb (odpowiednio 10 μM) w czasie 200 min w temperaturze 37ºC. Wyniki przedstawiono jako wartości średnie ± SD (n = 4)

The Time-Course of TBARS Formation at pH 5, 6, and 7.4

Comparison of the levels of the decomposition products of lipid hydroperoxides produced on interaction of AGE-Hb with LDL to those with GHb is shown in Fig. 2. Reactions conducted in an acidic environment (pH 5 and 6) for 300 minutes did not show any significant differences in the lipid peroxidation mechanism in the presence of AGE-Hb or GHb. The oxidation rate was enhanced progressively when GHb was the pro-oxidant, attaining 65 and 140 nmol/mg LDL protein levels at pH 7.4 and pH 5, respectively. The rate in the AGE-Hb oxidation system, however, was more rapid at 7.4, and by 300 minutes 80% of

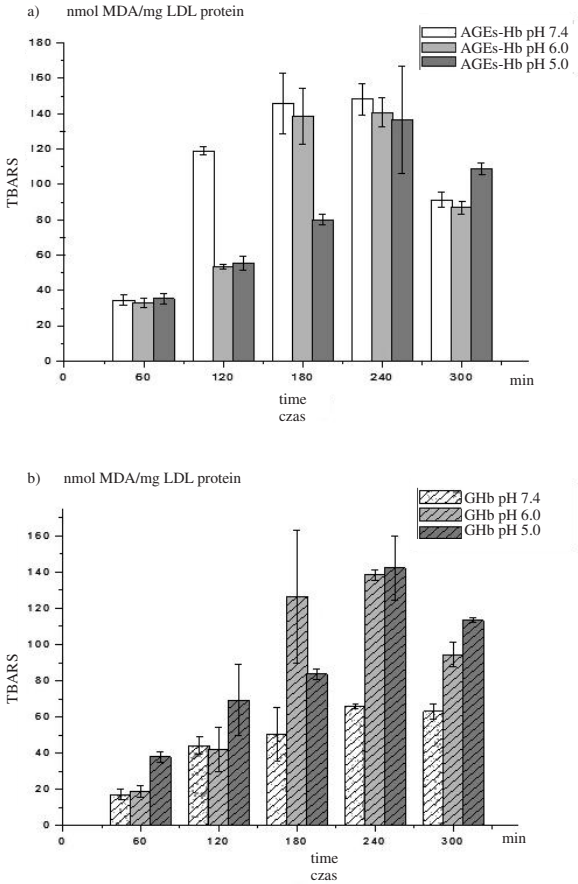

Fig. 2. The time-course of thiobarbituric acid-reactive substances (TBARS) formation at pH 5, 6, and 7.4. LDL (200 μg protein/ml) was incubated in the presence of either 10 μM GHb (a) or 10 μM AGEs-Hb (b) at 37ºC. The data represent the mean ± *SD* (n = 4)

Ryc. 2. Przebieg tworzenia substancji reagujących z kwasem tiobarbiturowym (TBARS) jako markerem peroksydacji LDL (200 μg protein/ml) w środowisku o pH 5, 6 i 7,4 z udziałem AGEs-Hb (a) i GHb (b) (odpowiednio 10 μM) w temperaturze 37ºC w czasie 300 min. Wyniki przedstawiono jako wartości średnie ± SD (n = 4)

the peroxides had decomposed. Thus although the level appeared to be enhanced by AGEs-Hb-induced oxidation, this was due to less breakdown of the lipid hydroperoxide at acidic pH.

AGEs-Hb was much more active than GHb in LDL lipid oxidation and the difference in reactivity in favor of AGEs-Hb was even more pronounced at a concentration of 10 μM than at 17 μM (data not shown). Although at pH 7.4 GHb generated fewer decomposition products of peroxides than AGEs-Hb, the levels produced at pH 5 as a consequence of GHb-LDL interaction were increased, implying an enhancement in the rate of formation and decomposition of peroxides under these conditions.

Effect of Antioxidants on AGE-Hb-Induced LDL Lipid Peroxidation

In this study the effect of antioxidants on GHb- and AGEs-Hb-induced LDL lipid oxidation was examined (Table 1). BHT (butylated hydroxytoluene) almost completely inhibited LDL peroxidation (99%), while EDTA provided limited antioxidative activity (25%) under the same experimental conditions. As seen, the relative inhibition of LDL peroxidation by the antioxidants was different. For example, haptoglobin 2-2 as an inhibitor of LDL peroxidation in the presence of AGEs-Hb was more potent than albumin (10 vs. 5%, respectively), but Hp 1-1 was more reactive than Hp 2-2 (14 vs. 10%, respectively).

Discussion

In the present study it was shown that, at physiological pH, AGE-Hb is more effective than GHb in promoting LDL peroxidation. However, under acidic conditions, GHb becomes more effective in catalyzing the oxidative modification of LDL and AGE-Hb becomes less effective (at least in the early stage of LDL oxidation). It has been shown that glucose is capable of destroying the heme cofactor in hemoglobin, even at diabetic glucose concentrations [17]. The precise chemical mechanisms of this destruction are probably very complex. The peroxide complex of ferryl heme readily auto-oxidizes to form the highly reactive superoxide radical. There is strong evidence emerging from studies by Cussimanio et al. [17] suggesting that hydrogen peroxide produced during early glycation is the initiating agent in the degradative mechanism. By analogy to other inflammatory and ischemic sites, atherosclerotic lesions may well have an acidic extracellular pH. The superoxide radical in acidic pH most frequently takes in a hydrogen ion and forms the hydroperoxide radical because its dissociation constant into the superoxide radical is 4.8. Furthermore, Bedwell et al.

Table 1. Effect of antioxidants on AGEs-modified hemoglobin-induced LDL lipid peroxidation

Tabela 1. Wpływ wybranych przeciwutleniaczy na utlenianie LDL w obecności AGE-Hb

Antioxidant (Antyoksydant)	Thiobarbituric acid-reactive substances; TBARS (Związki reagujące z kwasem tiobarbiturowym)	
	nmol MDA/mg LDL protein	% inhibition (% hamowania)
None (Bez obecności przeciwutleniacza)	130.3 ± 16.2	—
Butylated hydroxytoluene; BHT (3, 5-diizobutylo-4-hydroksytoluen)	1.3 ± 0.15	99
Ethylene diamine tetraacetic acid; EDTA (Kwas etylenodiaminotetraoctowy)	97.7 ± 12.1	25
Haptoglobin type 1-1 (Haptoglobina typ 1-1)	112.1 ± 13.9	14
Haptoglobin type 2-2 (Haptoglobina typ 2-2)	117.3 ± 14.5	10
Albumin (Albumina)	123.8 ± 15.4	5

LDL (200 μg/ml) was oxidized by AGEs-modified hemoglobin (10 μM) + H_2O_2 (10 μM) at 37°C for 300 minutes in the absence and presence (10 μM) of each of the antioxidants listed. Lipid peroxidation measured as thiobarbituric acid-reactive substances (TBARS).

Do mieszaniny zawierającej LDL (200 μg/ml) oraz AGEs-hemoglobinę (10 μM) dodano równomolarną ilość (10 μM) wybranego przeciwutleniacza i następnie inkubowano w obecności H_2O_2 (10 μM) w temperaturze 37°C przez 300 min. Peroksydację lipidów mierzono jako stężenie związków reagujących z kwasem tiobarbiturowym (TBARS)

[18] also observed that hydroperoxide radicals are more active in LDL peroxidation than superoxide radicals.

Yim et al. [19] suggested that glycated proteins, including AGEs-Hb, accumulated *in vivo* provide stable active sites for catalyzing the formation of free radicals. The glycation of protein generates active centers for catalyzing one-electron oxidation-reduction reactions. This active center, which exhibits an enzyme-like character, is suggested to be the cross-linked Schiff-based radical cation of the protein. It mimics the characteristics of the metal-catalyzed oxidation system. Similar types of reactive centers, if formed *in vivo*, may exert significant effects on their biological environment by generating free radicals for a long duration. The addition of radical scavengers such as butylated hydroxytoluene inhibit peroxide oxidation of lipids (Table 1), indicating that the reaction is mediated by radicals that escape the hem pocket. The oxidation by AGEs-Hb is retarded at pH 5 compared with pH 7.4, as shown by the time-dependency of conjugated diene and TBARS formation. The observation of different levels of TBARS at 300 minutes of incubation may be due to differences in the time-courses for oxidation in which the conjugated dienes reach a maximum and then remain stable or only slowly decrease, whereas TBARS reach a peak and gradually decline. With GHb, however, the formation of conjugated dienes and TBARS are all increased at acidic pH.

It has been shown that Hb is a strong lipid oxidant, especially in an acidic environment (pH 6.5), and its binding by Hp inhibits this pro-oxidative activity [20]. The haptoglobin contains four binding sites, two for each hemoglobin dimer ($\alpha\beta$). Hp binds an $\alpha\beta$ dimer in its α-specific binding site, decreasing the formation of globin radicals as a result of oxidation by heme [21]. In transgenic knock-out mice (whose Hp gene has been damaged), the release of hemoglobin during intravascular hemolysis is the cause of the oxidation of the guanine base in renal tubules' DNA. It is believed that enhanced guanine oxidation in transgenic mice is caused by singlet oxygen or the superoxide radical [20]. Identifying the types of radicals that participate in the oxidation-induced damage during intravascular hemolysis might allow us to explain the molecular mechanism of Hp's role as an antioxidant. The results of the present study indicate that haptoglobin 2-2 was as active as haptoglobin 1-1 and much more active than albumin as an inhibitor of AGE-Hb-mediated oxidation (Table 1). It has been observed in an experimental animal model [20] that under the conditions of increased hemolysis, the greater heme oxygenase induction in Hp knockout mice than in control mice (with the presence of Hp) might be caused by the release of heme from hemoglobin. On the other hand, it is a well-known fact that haptoglobin, unlike hemopexin, does not prevent lipid oxidation that is catalyzed by heme [22]. It is believed that heme's binding by albumin prevents lipid peroxidation that is catalyzed by heme. Albumin displays antioxidative properties which also react directly with oxygen radicals [23], so its low (5%) protection influence is not necessarily evidence in favor of the role that heme plays in initiating LDL lipid oxidation. This suggests that heme released from a damaged AGEs-Hb molecule as well as iron released from heme are plausibly secondary factors formatting oxygen radicals and contributing to increased lipid oxidation. As seen from the results presented in Table 1, EDTA-inhibited AGEs-Hb promoted TBARS formation (25%). This inhibition does not relate to the metal chelation, but rather to the antioxidative properties of EDTA [24].

In summary, the results presented above show that in the development of artery atherosclerosis as a complication of diabetes, the cooperation of GHb and AGEs-Hb probably plays an essential role not only through direct oxidative activity of both hemoglobins, but also through retaining the oxidant activity of glycohemoglobin and AGEs--modified hemoglobin, both bound in a complex with haptoglobin.

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