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Inhibition of hemoglobin-induced low density lipoprotein oxidation by haptoglobin

In-Sook Kim[1]

1 Department of Biochemistry, Catholic University Medical College
505 Banpo-Dong, Socho-Gu, Seoul 137-701, Korea

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Abbreviations: Hp, haptoglobin; Hb, hemoglobin; LDL, low density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substance

Abstract

The effects of hemoglobin (Hb) and haptoglobin-hemoglobin complex (Hp-Hb complex) on oxidation of low density lipoprotein (LDL) were investigated. Treatment of LDL (100 μg protein) with 60 μg (20 μM) of Hb for 6 h in phosphate-buffered saline resulted in an apparent LDL oxidation as determined by measuring lipid peroxidation, increasing electronegativity, aggregate formation, and decreasing lysine content of lipoprotein. Such effects of Hb on LDL oxidation showed in a dose-dependent manner at concentrations up to 60 μg. But $FeCl_3$ could not oxidize LDL even at 100 μM. Hb-induced LDL oxidation was blocked by addition of free Hp, and its inhibitory effect was a maximum when Hp:Hb molar ratio was 1:1. In addition, LDL incubated with purified Hp-Hb complex showed almost the same electronegativity and lysine content as native LDL. These findings indicate that Hb mediates LDL oxidation strongly and Hp inhibits Hb-stimulated LDL oxidation by forming Hp-Hb complex.

Keywords: haptoglobin-hemoglobin complex, hemoglobin, low density lipoprotein, oxidation

Introduction

One of the important and early events in atherosclerosis is the recruitment of circulating monocytes and T lymphocytes into the subendothelial space, and the formation of a fatty streak which is an aggregation of lipid-laden macrophages and T lymphocytes. As the process continues, various kinds of growth factors and cytokines are released from monocytes/macrophages, endothelial cells, smooth muscle cells and platelets, and the autocrine or paracrine stimulation of these cells is induced. As a result, the fatty streaks become to fibrous plaques (Ross, 1993).

It is now well established that an elevated level of low density lipoprotein (LDL) is a risk factor for atherosclerosis (Brown and Goldstein, 1983). Especially, some recent reports have shown that oxidatively modified LDL contribute to the pathogenesis of early and advanced atherosclerotic lesions (Haberland *et al.*, 1988; Steinberg *et al.*, 1989), and also play an important role in inflammatory reactions (Hartung *et al.*, 1985; Yokode *et al.*, 1988; Hamilton *et al.*, 1990).

LDL could be oxidized *in vitro* by incubation in the presence of 5-10 μM of copper(II) ion (Tomas, 1992). Treatment of LDL with cultured endothelial cells, smooth muscle cells or macrophages for 12 to 18 h *in vitro* also generates an oxidatively modified LDL that is scavenged by oxidized LDL receptors of macrophages (Morel *et al.*, 1984; Cathcart *et al.*, 1985; Sparrow *et al.*, 1989). Moreover, there are some accumulated evidences that LDL is oxidatively modified *in vivo* (Palinski *et al.*, 1989; Yla-Herttuala *et al.*, 1990). However, the exact mechanism of LDL oxidation *in vivo* remains unknown.

On the other hand, hemoglobin (Hb) can stimulate lipid peroxidation and act as a Fenton reagent which is able to promote hydroxyl radical generation in areas of inflammation (Sadrzadeh *et al.*, 1984). And Hb-stimulated lipid peroxidation can be inhibited by haptoglobin (Hp) that forms a stable complex with Hb (Hp-Hb complex) (Gutteridge, 1987; Halliwell and Gutteridge, 1990).

To study a source of the oxidation of LDL *in vivo*, we examined the effect of Hb and Hp-Hb complex on LDL oxidation. This study shows that free Hb stimulates the oxidation of LDL and serum Hp inhibits Hb-induced LDL oxidation.

Materials and Methods

Materials

Normal human plasma was obtained from Korean National Red Cross. DEAE-cellulose was obtained from Whatman (Clifton, NJ). Sephadex G-200 and Bio-Gel (A-5m) were purchased from Pharmacia (Uppsala, Sweden). Anti-rabbit serum against human LDL,

human albumin, agarose, $CuSO_4$, $FeCl_3$ and thiobarbituric acid were purchased from Sigma (St. Louis, MO). Malondialdehyde (MDA) and Coomassie Plus protein assay reagent were obtained from Aldrich (St. Louis, MO) and Pierce (Rockford, IL), respectively. All other chemicals used were of reagent grade.

Purification of human LDL

Lipoproteins (density < 1.1) were isolated from fresh normal human plasma by ultracentrifugation in a SW 28 rotor (Beckman) at 24,000 r.p.m. for 40 h. And then LDL was separated from the above isolated lipoproteins mixture by agarose-column chromatography (Bio-Gel A-5m) using buffer A (0.15 M NaCl, 1 mM EDTA, 0.01% NaN_3 (pH 7.4)) (Rudel *et al.*, 1974). After chromatography, LDL fraction was concentrated using Diaflo PM 10 membrane (Amicon, Danvers, MA.), dialyzed against PBS, filter sterilized (0.22 μm) and stored at 4°C. To prevent oxidation of LDL during purification, 1 mM EDTA was added in the all purification steps. Purified LDL was used within 2 weeks of preparation. Protein concentration was measured with Coomassie Plus protein assay reagent using bovine serum albumin as a standard.

Preparation of Hp, Hb, and Hp-Hb complex

Human Hp was purified from 2-1 type serum by $(NH_4)_2SO_4$ precipitation, DEAE-cellulose ion-exchange chromatography and Sephadex G-200 gel filtration as described by Shim (1973). Hb was isolated from human blood as described previously (Shim *et al.*, 1985). To prepare Hp-Hb complex, the purified Hp was mixed with the purified Hb in 1:2 molar ratio, and the mixture was subjected to a Sephadex G-200 gel filtration column (3 x 97 cm) preequilibrated with 0.1 M Tris buffer, pH 8.0, containing 0.5 M NaCl (Shim *et al.*, 1985).

LDL oxidation and assay for lipid peroxides

For oxidation of LDL by Hb or Hp-Hb complex, 100 μg protein of LDL dialyzed against PBS was mixed with 60 μg (20 μM) of Hb or Hp-Hb complex (60 μg Hb) in 50 μl of final volume, and then incubated for 6 h at 37°C in 5% CO_2/95% air incubator. For inhibition of Hb-induced LDL oxidation, Hp, EDTA or human albumin was added in the LDL preparation before addition of Hb. LDL oxidation was also carried out with 10 μM of $CuSO_4$ or 100 μM of $FeCl_3$ at 37°C for 6 h.

LDL oxidation was estimated by measuring lipid peroxidation in terms of thiobarbituric acid-reactive substance (TBARS) using modified method described previously by Schuh *et al.* (1978). Briefly, 50 μl of LDL incubated with Hb or Hp-Hb complex for 6 h was mixed with 0.2 ml of PBS and 0.5 ml of MDA reagent (0.755% thiobarbituric acid, 30% trichloroacetic acid, 0.45 M HCl). The mixture was boiled at 95°C for 30 min. After centrifugation at 12,000 *g* for 10 min, absorbance of the resultant supernatant was read at 532 nm. The results were presented as nmol of MDA equivalents.

Immunoelectrophoresis

For assessment of LDL oxidation, the LDL samples oxidized by Hb or Hp-Hb complex were analyzed immunoelectrophoresis on 1% agarose gel in barbital buffer, pH 8.5, and identified with anti-rabbit serum against human LDL.

Determination of lysine content

The lysine content in a native LDL and an oxidized LDL were determined by amino acid analysis (UNKN amino acid analyzer).

Results

Hb-induced LDL oxidation

To study the effect of Hb on LDL oxidation, we incubated LDL (100 μg protein) with various amount of Hb (15-150 μg) at 37°C for 6 h, and then measured TBARS for estimating of the LDL oxidation. As shown

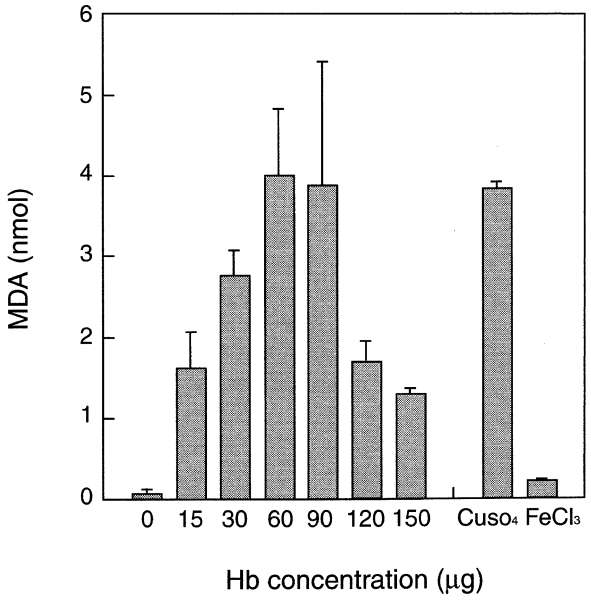

Figure 1. Effect of Hb on LDL oxidation. LDL (100 μg/50 μl) was incubated with the indicated amounts of Hb (15-150 μg) for 6 h at 37°C in a CO_2-incubator. To compare oxidizing activity, 10 μM $CuSO_4$ or 100 μM $FeCl_3$ was also used for incubations with LDL (100 μg) for the same time. After incubation, the level of LDL oxidation were estimated in terms of TBARS as outlined under Materials and Methods. Values represent the means ± S.D. of four separate determinations.

in Figure 1, Hb could oxidize LDL effectively in a dose-dependent fashion at concentrations up to 60 μg (20 μM). LDL treated with 60 μg Hb generated 4.1 nmol of MDA which was similar amount to 4.0 nmol of MDA produced by LDL oxidized with 10 μM $CuSO_4$, that is well known as the system for LDL oxidation *in vitro*. But $FeCl_3$ could not oxidize LDL even at 100 μM. Hb of concentrations greater than 60 μg decreased TBARS formation progressively (Figure 1). Therefore, 60 μg (20 μM) of Hb was used for LDL (100 μg protein) oxidation in all other experiments.

Oxidation of LDL with $CuSO_4$ or hypochlorite results in modification of lysine residues of apolipoprotein B-100 which is a single protein associated with LDL. As a result, oxidatively modified LDL has an increased negative charge and an aggregative form (Thomas, 1992; Hasell *et al.*, 1994). For assessment of apparent LDL oxidation by Hb, we examined a charge change, aggregates formation and lysine content in Hb-treated LDL. Figure 2 shows that LDL incubated with Hb moved faster toward anode than native LDL on an agarose gel, and also the results in Figure 3 indicate that Hb-exposed LDL eluted before native LDL from agarose-gel filtration column, which resulted from LDL aggregates having higher molecular weight. Indeed, lysine content of Hb-treated LDL was 85.4% of native LDL according to amino acid analysis (Table 1). Taken together, these results suggest that LDL oxidation could be mediated by Hb effectively.

Table 1. Lysine contents in native and oxidized LDLs. As described in Figure 3, after incubation of LDL with Hb or Hp-Hb complex for 6 h, oxidized LDLs were purified by agarose-gel chromatography. The lysine contents in native and purified oxidized LDLs (Hb- or Hp-Hb complex treated LDLs) were determined by amino acid analysis.

LDLs	Lysine content (% of total amino acid)	Lysine content (% of native LDL)
Native LDL	9.20	100
Hb-treated LDL	7.86	85.4
Hp-Hb treated LDL	8.49	92.3

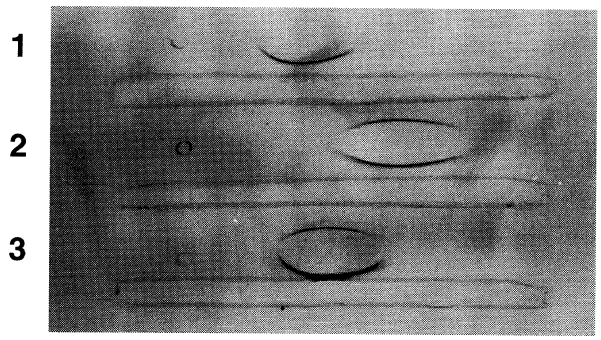

Figure 2. Inhibitory effect of Hp on Hb-stimulated LDL oxidation. Native LDL and LDL treated with Hb or Hp-Hb complex for 6 h were immunoelectrophoresed on 1% agarose-gel in barbital buffer, pH 8.5, and identified with anti-rabbit serum against human LDL. Well 1, native LDL, 10 μg; 2, LDL incubated with Hb, 10 μg; 3, LDL incubated with Hp-Hb complex, 10 μg. Troughs, antiserum against human LDL.

Inhibition of Hb-induced LDL oxidation by Hp

It was suggested that the Hp that forms stable complex with Hb (Hp-Hb complex; one mol of Hp bind one mol of Hb) might act as an antioxidant *in vivo*, protecting against Hb-stimulated lipid peroxidation (Gutterigdge, 1987). To test whether Hp could inhibit Hb-stimulated LDL oxidation, purified Hp was preincubated with LDL before addition of Hb.

Table 2 shows that Hb-induced LDL oxidation was inhibited by addition of free Hp. When 50 μg, 100 μg or 200 μg of Hp was added to the each LDL reaction mixture including of 60 μg Hb (Hp:Hb molar ratio = 1:2, 1:1, 2:1, respectively), Hb-induced LDL oxidation decreased to 57.5%, 27.6% or 24.8% of the control level, respectively. These data indicate that inhibition of LDL oxidation by Hp is maximum when Hp:Hb molar ratio is 1:1, and excess Hp doesn't participate in further

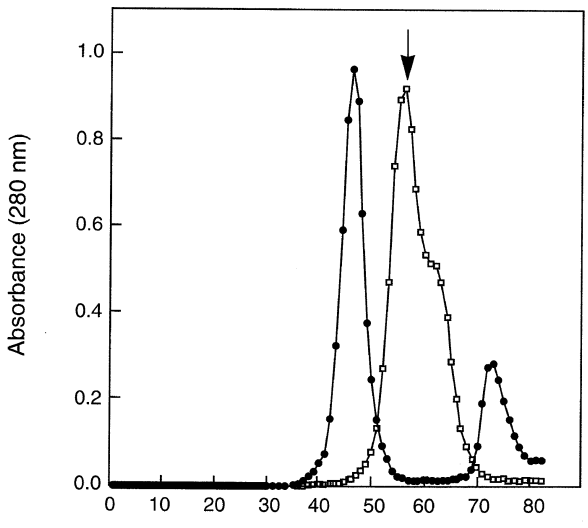

Figure 3. Oxidation of LDL with Hb causes aggregation of the lipoprotein. Four mg of LDL was incubated with 2.4 mg of Hb (closed circle) or Hp-Hb complex (2.4 mg Hb, open square) in 1 ml final volume for 6 h at 37°C and then the incubated samples were chromatographed using agarose-gel column (Bio-Gel A-5m) with buffer A. 3 ml fractions were collected and monitored at 280 nm. The arrow indicates elution position of native LDL.

Table 2. Effects of Hp, EDTA and human albumin on Hb-induced LDL oxidation. To inhibit Hb-induced LDL oxidation, Hp (50 μg, 100 μg or 200 μg), EDTA (20 μM, 40 μM or 100 μM) and human albumin (50 μg, 100 μg or 200 μg) were mixed with each LDL preparation (100 μg protein) and preincubated for 5 min. And then oxidation reactions were carried out by addition of 60 μg of Hb. Control reaction is for Hb-induced LDL oxidation without inhibitor. Level of LDL oxidation was estimated by the same method as that in Figure 1. Experiments were done in triplicates and the values are mean ± S.D.

Treatment		MDA (nmole)	Inhibition (% of control)
Hb		4.28 ± 0.683	100
Hb + Hp	50 μg	2.46 ± 0.537	57.5
	100 μg	1.18 ± 0.286	27.6
	200 μg	1.06 ± 0.167	24.8
Hb + EDTA	20 μM	1.23 ± 0.153	28.7
	40 μM	1.13 ± 0.153	26.4
	100 μM	1.17 ± 0.153	27.3
Hb + Albumin	50 μg	3.38 ± 0.811	79.0
	100 μg	2.54 ± 0.73	59.3
	200 μg	1.98 ± 0.589	46.3

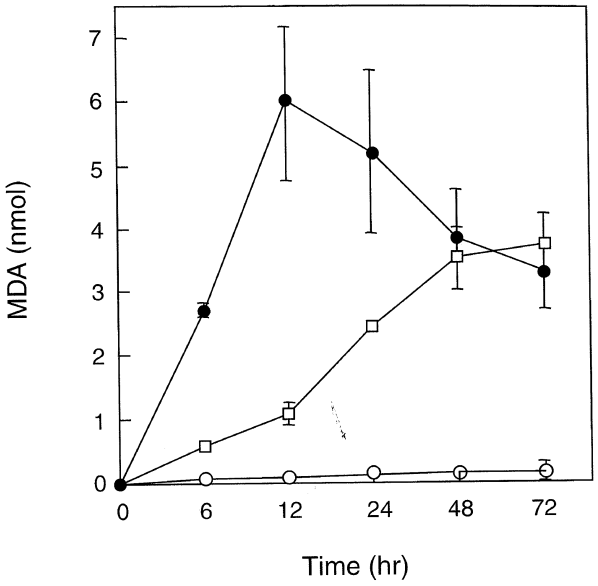

Figure 4. Time-dependent LDL oxidations by Hb and Hp-Hb complex. LDL samples (100 μg protein each) were mixed without (open circle), with Hb (60 μg, closed circle) or with Hp-Hb complex (60 μg Hb, open square), and incubated for up to 72 h using the same method as that in Figure1. The results shown represent means ± S.D. of three separate experiments.

inhibition, suggesting that Hp can block effect of Hb on LDL oxidation by formation of Hp-Hb complex. Inhibition of Hb-induced LDL oxidation with 100 μg Hp corresponded to inhibition by 20 μM EDTA. But human albumin inhibited LDL oxidation by 46.3% even at 200 μg concentration (Table 2). Therefore, it is likely that albumin is a less potent antioxidant toward Hb-stimulated LDL oxidation.

Because Hp acted as Hp-Hb complex, its effect on LDL oxidation was examined using purified Hp-Hb complex. Electronegativity of LDL (100 μg protein) treated with Hp-Hb complex (60 μg Hb) was almost the same as that of native LDL, in contrast to highly increased electronegativity of Hb-treated LDL (Figure 2). The lysine content of Hp-Hb complex-treated LDL was 92.3% of native LDL value (Table 1). In addition, LDL exposed to Hp-Hb complex was eluted in approximately the same elution volume as native LDL through agarose-gel filtration column (Figure 3). These results suggest again that Hp can inhibit Hb-induced LDL oxidation by formation of Hp-Hb complex.

The levels of LDL oxidation by Hb and Hp-Hb complex as a function of incubation time are shown in Figure 4. LDL oxidation in the presence of 60 μg Hb reached a peak at 12 h of incubation, thereafter declined gradually, while LDL oxidation with Hp-Hb complex (60 μg Hb) increased slowly until 72 h. These data indicate that LDL oxidation by Hp-Hb complex is weak and slow, in contrast to strong and rapid effect of Hb on the LDL oxidation.

Discussion

There are interesting evidences that oxidatively modified LDLs have an important role in atherosclerosis (Steinberg *et al.*, 1989). For example, oxidized LDLs could act as a chemoattractant in monocyte recruitment (Quinn *et al.*, 1987), and a ligand for receptor-mediated lipid loading of macrophages (Sparrow *et al.*, 1989). Moreover, it have been detected in atherosclerotic lesions (Haberland *et al.*, 1988; Palinski *et al.*, 1988). However, the mechanism by which LDL is oxidized *in vivo* is unclear yet. We described here that Hb released from damaged red blood cell could participate in LDL oxidation, which might contribute to early atherogenesis, and serum Hp blocked the Hb-induced LDL oxidation.

Hb stimulated LDL oxidation at ranges up to 60 μg (20 μM) and Hb of concentrations greater than 60 μg decreased TBARS formation progressively. And $FeCl_3$ could not oxidize LDL (Figure 1). These findings are consistent with the other report in which lipid peroxidation by heme in the presence of H_2O_2

decreased upon increasing the heme concentration above 30 μM and little TBARS could be detected in the incubation of lipids with ferric chloride (Vincent *et al.*, 1988).

It is well known that oxidatively modified LDLs have an increased electronegativity, an aggregates and decreased lysine content, which result from modification of apolipoprotein B-100 (Thomas, 1992; Hasell *et al.*, 1994). Likewise, Hb-treated LDL also showed markedly increased negative charge and an aggregative form as a result of the modification at lysine residues in apolipoprotein B-100 (Figure 3 and Table 1).

Addition of free Hp blocked Hb-stimulated LDL oxidation and its blocking effect was maximum when Hp:Hb molar ratio was 1:1 (Table 2). Moreover electronegativity and lysine content of LDL treated with purified Hp-Hb complex was almost the same as that of native LDL (Figure 2 and Table 1), suggesting that Hp inhibits Hb-induced LDL oxidation by formation Hp-Hb complex.

Several studies have demonstrated that chemically or oxidatively modified LDLs participate in inflammatory reactions, i.e., releasing some enzymes from macrophages, stimulating arachidonate metabolism and regulation of tumor necrosis factor-α expression (Hartung, *et al.*, 1985; Yokode *et al.*, 1988; Hamilton *et al.*, 1990). It is possible that circulating monocytes are recruited to the inflammation site and release various enzymes and arachidonic acid metabolites necessary for repair of tissue damage through action of LDL oxidized by Hb released from damaged site. But it doesn't seem likely that free Hb interacts with LDL directly for inflammatory response as described above, because Hb liberated by intravascular hemolysis is bond to serum Hp and the binding is strong and irreversible (Shim, 1973; Hershko, 1975). In addition, the level of serum Hp, which is one of the acute phase proteins, increases during inflammation (Shim, 1973) and Jue *et al.* reported that Hp-Hb complex could not be removed by cultured macrophages while free Hb uptake by macrophages occurs rapidly (Jue *et al.*, 1994). Therefore, it is likely that most released Hb exists as Hp-Hb complex at inflammation sites. In this study, we observed that free Hp diminished Hb-stimulated LDL oxidation, but its inhibitory effect was not complete although enough Hp to saturate Hb was used (24.8% of control, Table 2). Also, LDL oxidation could be mediated weakly and slowly by Hp-Hb complex (Figure 4). Recently, Cushing et al. showed that not only fully oxidized LDL but also minimally oxidized LDL induced monocyte chemotaxsis (Cushing *et al.*, 1990). In some studies, cytotoxic effects of fully oxidized LDLs were also observed (Steinberg, *et al.*, 1989; Fossel *et al.*, 1994). Therefore, it could not be excluded that weakly oxidized LDL by Hp-Hb complexes might act as a inflammatory regulator without cytotoxic effect in a localized environment such as injured tissue.

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