Oxidation of Heparin-Isolated LDL by Hemin
The Effect of Serum Components

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Abstract

Oxidation of low-density lipoprotein (LDL) in the artery wall is probably determined by several factors, some of which may include physiological oxidants such as heme and hydrogen peroxide, blood serum components, and the interaction of the lipoprotein with glycosaminoglycans. Glycosaminoglycans form complexes with LDL that increase its susceptibility to oxidation in vitro. To examine the effect of these factors on oxidation of LDL in vitro, we isolated LDL from serum by using heparin and oxidized the resolubilized lipoprotein (Hep-LDL) with heme and hydrogen peroxide in the presence of apolipoprotein B lipoprotein-deficient serum (BLPDS). Low levels (2.1%) of BLPDS stimulated the oxidation of Hep-LDL by approximately fivefold, and increasing concentrations reduced oxidation to baseline rates. By comparison, the oxidation of native LDL was stimulated to a similar extent at lower concentrations of BLPDS (0.83%) and returned to baseline more rapidly with increasing levels of the serum fraction. Oxidation rates did not change significantly with increasing concentrations of BLPDS alone. Human serum albumin (HSA) at comparable levels produced changes in the oxidation of Hep-LDL similar to those seen with BLPDS. Degradation of heme was accelerated by low levels of BLPDS or HSA in the presence of hydrogen peroxide but not by higher levels, and maximal degradation rates were inhibited by comparably low levels of butylated hydroxytoluene (35 μmol/L). This antioxidant also effectively inhibited oxidation of Hep-LDL maximally stimulated by BLPDS. The data suggest that serum components, particularly HSA, modulate the peroxidation of both glycosaminoglycan-treated LDL and native LDL by heme and hydrogen peroxide via mechanisms that may involve oxidative interactions between heme and HSA. This phenomenon may influence oxidation of LDL in vivo, where levels of HSA in regions of the artery wall are comparable with levels that stimulate the oxidation of Hep-LDL in vitro. (Arterioscler Thromb. 1994;14:1966-1975.)

Key Words • LDL • oxidation • heme • serum albumin • heparin

There is increasing evidence that oxidation of low-density lipoprotein (LDL) in the artery wall may be implicated in the development of atherosclerosis.1 Oxidized LDL has been identified in atherosclerotic lesions by immunocytochemistry,2,3 and partially oxidized LDL has been gently extracted from lesions.4 Treatment of hyperlipidemic animals with antioxidants reduces lesion formation independent of lipid lowering.5-7 Oxidized LDL has several potentially atherogenic properties, including the ability to attract and immobilize macrophages and to promote accumulation of cholesteryl esters in these cells in vitro.9,10

The presence of potent antioxidant protection in blood has led to the concept that oxidative modification of LDL occurs in microdomains of the artery wall, where conditions favoring oxidation may exist.9 Immobilization or delayed clearance of LDL from the artery wall as a result of lipoprotein interaction with proteoglycans and glycosaminoglycans is thought to facilitate the oxidation of LDL11 and the formation of atherosclerotic plaque.12-14 Apolipoprotein (apo) B lipoprotein–proteoglycan complexes, which have been isolated from fatty streaks and fibrous plaques of human aortas, promote cholesteryl ester accumulation in cultured human monocyte-macrophages.14,15 These complexes contain increased levels of lipid peroxides (thiobarbituric acid–reacting substances [TBARS]) that may potentiate oxidative modification of the lipoprotein and subsequent cellular lipid accumulation.12 There is further evidence that the interaction of LDL with arterial proteoglycans and glycosaminoglycans renders LDL more susceptible to oxidation. Formation of complexes between LDL and arterial proteoglycans, heparin, or chondroitin sulfate in vitro alter the structure of the lipoprotein, which is then more readily oxidized by copper ions.11,16

The factors determining oxidation of LDL in vivo are largely unknown. Hemin, a biologically important iron-containing compound that can enter hydrophobic domains, can oxidize LDL in vitro, particularly in the presence of hydrogen peroxide, and could be a physiological mediator of LDL oxidation.17 Furthermore, LDL that has interacted with heme is cytotoxic,18 a property that may encourage the development of arterial lesions. Modification of LDL in the artery wall presumably occurs in the presence of several components of blood and extracellular fluid that are capable of modulating the oxidation of LDL. For example, vitamin C and vitamin E are well-known plasma antioxidants that can reduce oxidation of LDL by copper ions19,20 and heme.17 However, there is little information on the effect of serum components on hemin-catalyzed oxidation of LDL, particularly LDL that has interacted with heparin (Hep-LDL). Heparin was chosen as a
model glycosaminoglycan for several reasons. First, complexes containing apoB lipoprotein and heparin proteoglycans have been isolated from human atherosclerotic plaques. Second, heparin or glycosaminoglycans that are more common in the arterial intima produce similar modifications to LDL in vitro. Third, LDL can be rapidly isolated from serum by using heparin.

**Methods**

**Materials**

Hemin, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, vitamin E, butylated hydroxytoluene (BHT), HEPES, chondroitin sulfate C (from shark cartilage), and human serum albumin (HSA) were obtained from Sigma Chemical Co. (Multipar, from porcine mucosa, 5000 IU/mL) was purchased from Fisons Pty, Ltd, hydrogen peroxide (Analar, 30% wt/vol) was obtained from BDH, and normal human γ-globulin was obtained from the Commonwealth Serum Laboratories. Ascorbic acid was obtained from J.T. Baker Chemical Co.

**Preparation of Lipoprotein Fractions**

Blood from healthy volunteers was collected into tubes containing disodium EDTA (1.5 mg/mL) and tubes containing no anticoagulant. Serum and EDTA plasma were isolated by low-speed centrifugation at 4°C. Hep-LDL was isolated by precipitation with heparin. Briefly, 5 mL sodium citrate buffer (64 mmol/L, pH 5.12) containing heparin (50 000 IU/L) was added to serum (0.5 mL). The tubes were left at room temperature for 10 minutes and then centrifuged for 15 minutes at 2800 g at room temperature. The precipitate (Hep-LDL) was washed three times with HEPES (5 mmol/L), sodium chloride (20 mmol/L), magnesium chloride (2 mmol/L), and magnesium chloride (2 mmol/L), pH 7.2, then redissolved in 0.5 mL phosphate-buffered saline (PBS), pH 7.4, containing 4% sodium chloride (4% NaCl/PBS) or in a solution of 0.5 mL of sodium citrate (64 mmol/L, pH 7.7). This preparation, which was contaminated with small quantities of serum proteins, was used for most of the experiments. These contaminating proteins were removed essentially by the method of Burstein and coworkers. The precipitated Hep-LDL from 0.5 mL serum was washed twice and then redissolved by adding 100 μL 4% NaCl/PBS. To this solution was added 5 mL Tris (0.02 mol/L, pH 7.7), followed by 125 μL magnesium chloride (2 mol/L), which reprocentrifuged the Hep-LDL. The precipitate was pelleted by centrifuging the mixture for 15 minutes at 2800 g. The pelleted Hep-LDL was washed three times with PBS and redissolved in 4% NaCl/PBS. Native LDL (d = 1.019 to 1.063 g/mL) was isolated by sequentially ultracentrifuging EDTA plasma at the appropriate densities for 20 hours and 24 hours, respectively, at 40 000 rpm in a Beckman 50.3 Ti rotor at 4°C. The isolated LDL was dialyzed twice against PBS (2 L) overnight at 4°C in the dark. The dialyzed LDL was stored under argon in the dark at 4°C and was used within 2 days. Serum fractions d > 1.063 g/mL and d = 1.019 to 1.21 g/mL and high-density lipoprotein (HDL) (d = 1.063 to 1.21 g/mL) were similarly isolated by 24-hour sequential ultracentrifugation of serum at the appropriate densities. These lipoprotein fractions were also dialyzed against PBS (2 L) with one change and were stored similar to LDL. LDL that had interacted with chondroitin sulfate C (CS-LDL) was prepared from native LDL (2.6 mg protein in 3 mL PBS) by adding 200 μL of a solution of chondroitin sulfate C (13 mg/mL in PBS) and adjusting the pH of the final solution to 4.6. The precipitate formed was pelleted by centrifugation (30 minutes at 2800g and 4°C). After decanting the supernatant, the pellet (CS-LDL) was washed with PBS and was redissolved in 4% NaCl/PBS. No precipitate was formed when LDL solution was adjusted to pH 4.6 in the absence of chondroitin sulfate. ApoB lipoprotein-deficient serum (BLPDS) was prepared by the addition of dextran sulfate/magnesium chloride to serum according to the method of Warnick and coworkers. The apoB-containing lipoproteins were pelleted by centrifugation in a Beckman Microfuge for 5 minutes, yielding a BLPDS supernatant. Free fatty acid-depleted HSA was prepared as described.

**Analytical Methods**

Cholesterol, triglycerides, free cholesterol, and phospholipids were measured by using enzymatic kits and calibrators supplied by Boehringer Mannheim. Protein was measured by the method of Lowry et al by using bovine serum albumin as the standard. Vitamin E was measured by high-performance liquid chromatography, and organic lipid peroxides in native LDL and Hep-LDL preparations were measured. Two sets of duplicate aliquots (100 μL) of LDL were taken, and 50 μL EDTA solution (3.7 mmol/L) and 10 μL BHT (0.4 mmol/L) in methanol were added. Next, 7 μL aqueous iodine (1 mmol/L) and 86 μL acetic acid solution (3%) were added to reduce iodine binding to LDL. Water (7 μL) and acetic acid solution were added to blank and hydrogen peroxide standards. A volume (1 mL) of a commercial cholesterol iodide reagent (Merk) was added to each set of tubes to determine iodine binding to LDL. These were incubated for 30 minutes in the dark at room temperature. Iodide reagent was added to the remaining set of LDL aliquots immediately prior to reading all tubes at 365 nm. Absorbances for these LDL blanks were subtracted from the readings in LDL solutions incubated with reagent.

**Electrophoresis**

Agarose gel electrophoresis on Hep-LDL preparations was performed by using the method of Noble. Cellulose acetate electrophoresis was performed by using a Beckman Microzone system according to methods supplied with the equipment.

**Oxidation of LDL**

The formation of TBARS was used to monitor the oxidation of lipoproteins. The use of TBARS is justified by the close correlation between TBARS and other measures of lipid peroxidation during the oxidation of LDL by hemin. To 800 μL HEPES-saline buffer (pH 7.4) was added a serum fraction (0 to 200 μL), Hep-LDL or native LDL (100 to 150 μL), or 4% NaCl/PBS (100 μL), hemin (20 μL of 1.20 mmol/L in 20 mmol/L sodium hydroxide; final concentration, 20 μmol/L), hydrogen peroxide (20 μL of 17 mmol/L; final concentration, 286 μmol/L) and HEPES-saline buffer, pH 7.4, to give a final volume of 1.20 mL. The solution in duplicate was incubated at 37°C for 2 hours. At the end of the incubation, 4 mmol/L disodium EDTA (50 μL) and 4 mmol/L BHT (10 μL) were added to the tubes. TBARS were then measured essentially by the method of Beuge and Aust. The standards and blank contained 1.15 mL HEPES-saline buffer, 50 μL EDTA (4 mmol/L), 10 μL BHT (4 mmol/L), 50 μL 1,1,3,3-tetraethoxypropane in isopropanol (standards), and 50 μL isopropanol (blank). Thiobarbituric acid solution (1 mL) containing 0.37% thiobarbituric acid in 15% trichloroacetic acid and 0.25 mol/L hydrochloric acid was added to all tubes, which were then heated for 15 minutes on a boiling water bath. After cooling, the tubes were centrifuged at 150g for 30 minutes. The optical density of the clear supernatant was read at 532 nm against the blank, and the concentration of TBARS was calculated by using the standards. Solutions of BLPDS, HSA, γ-globulin, d = 1.21 g/mL and d > 1.063 g/mL plasma fractions, and one set of tubes containing LDL oxidation assay were at concentrations that approached those in human serum.

**Statistical Analysis**

Student's t test was used to compare mean values. A two-sided test of significance was used, and a P value of less
reduced in 4% NaCl/PBS and in a sodium citrate solution (64 mmol/L, pH 7.7). By contrast, the precipitate obtained after similar treatments of plasma did not completely redissolve. Cellulose acetate electrophoresis of Hep-LDL isolated from plasma showed a band consistent with the presence of fibrinogen. Thus, Hep-LDL from serum was used in all experiments. Measurement of the protein content in Hep-LDL using the Lowry method or a dye-binding method gave values that were approximately twice those in native LDL. Consequently, total cholesterol in Hep-LDL was used to determine the quantity of the lipoprotein (0.2 μmol lipoprotein cholesterol) added to oxidation assays. Measurement of cholesterol in serum diluted 1:2 (vol/vol) with PBS, 4% NaCl/PBS, or 4% NaCl/PBS containing heparin (75 IU/mL) gave similar values, indicating that the determination was not influenced by the small quantities of heparin present in the Hep-LDL preparations. Agarose gel electrophoresis of Hep-LDL with Coomassie Blue staining showed the presence of contaminating proteins, mainly globulins. The data in Table 1 show that purification of Hep-LDL by precipitation gave a protein content that was comparable with that in native LDL. The content of triglycerides was significantly higher and the content of cholesteryl esters lower at a marginal level of significance (P = 0.07) in purified Hep-LDL compared with native LDL due to the presence of intermediate-density lipoproteins and remnants of very-low-density lipoprotein (VLDL) catabolism. To a lesser extent, the free cholesterol content was lower and the phospholipid content higher in Hep-LDL. The vitamin E content was not significantly different, and lipid peroxide levels were significantly and twofold higher in purified Hep-LDL compared with native LDL. The lipid composition was similar in purified Hep-LDL compared with the protein-contaminated preparation, and both showed higher triglyceride and lower cholesteryl ester contents than the corresponding composition in native LDL. When heparin–sodium citrate solution was added to serum, the amount of triglycerides in the supernatant after pelleting Hep-LDL was 46% of the corresponding amount in serum diluted similarly with PBS. This indicates that little if any VLDL was precipitated. Ultracentrifugation of serum Hep-LDL dissolved in sodium citrate (64 mmol/L, pH 7.7) and adjusted to d = 1.019 g/mL at 40 000 rpm for 20 hours in a Beckman type 50.3 Ti rotor showed that 88.3% of total lipoprotein cholesterol was in the d > 1.019 g/mL fraction. Thus, the proportion of lipoprotein in the LDL density range is similar to reported values.

The effect of BLPS on hemin-mediated oxidation of Hep-LDL, purified Hep-LDL, or native LDL is shown in Fig 1. Hep-LDL gave maximal production of TBARS in the presence of 2.1% BLPS, and increasing volumes of BLPSD progressively reduced TBARS formation to baseline rates. The profile of TBARS production with increasing BLPS was essentially similar to purified Hep-LDL. When native LDL was tested in the assay, a maximum TBARS production rate was attained at a lower proportion of BLPSD (0.8%). This maximum rate was not significantly different from the maximum rate attained when serum Hep-LDL was oxidized in the presence of 2.1% BLPS. At proportions of BLPSD between 2.1% and 8.3%, rates of TBARS formation from Hep-LDL were significantly (P < .001) higher than rates from LDL. When 4% NaCl/PBS instead of Hep-LDL was added, the TBARS production rate did not change significantly with increasing amounts of BLPS. Thus TBARS produced during oxidation of Hep-LDL and stimulated by low levels of BLPSD were derived from Hep-LDL and not from BLPSD. Redissolving the Hep-LDL precipitate in a solution of sodium citrate (64 mmol/L, pH 7.7) did not significantly alter the TBARS production with increasing amounts of BLPS. This means that the solution used to redissolve Hep-LDL did not affect the oxidation of the lipoprotein.

### Table 1. Composition of Heparin-Isolated LDL Preparations

<table>
<thead>
<tr>
<th>Component</th>
<th>Purified Hep-LDL</th>
<th>Native LDL</th>
<th>Purified Hep-LDL</th>
<th>Crude Hep-LDL</th>
<th>Native LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, %</td>
<td>24.3±1.8</td>
<td>25.1±1.9</td>
<td>9.8±2.6</td>
<td>10.8±1.1</td>
<td>13.6±2.2</td>
</tr>
<tr>
<td>Free cholesterol, %</td>
<td>7.5±1.9</td>
<td>10.2±1.8</td>
<td>46.8±4.4*</td>
<td>45.5±3.4</td>
<td>54.7±2.1</td>
</tr>
<tr>
<td>Cholesterol ester, %</td>
<td>35.5±3.2</td>
<td>41.0±2.2</td>
<td>17.1±3.8*</td>
<td>17.9±5.5</td>
<td>7.9±1.4</td>
</tr>
<tr>
<td>Triglycerides, %</td>
<td>13.0±3.1*</td>
<td>5.9±0.9</td>
<td>26.0±1.6</td>
<td>25.6±2.2</td>
<td>23.6±0.5</td>
</tr>
<tr>
<td>Phospholipids, %</td>
<td>19.7±1.2</td>
<td>17.7±1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E, μmol/g protein</td>
<td>10.5±3.7</td>
<td>8.5±2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxides, μmol/g protein</td>
<td>75±8*</td>
<td>37±6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; Hep-LDL, heparin-isolated LDL. Native LDL, purified Hep-LDL, and crude Hep-LDL were obtained from the same three subjects. Crude Hep-LDL was contaminated with small quantities of serum proteins and was used in virtually all experiments in the present study. Values are mean±SD. Each experiment was performed three times.

*P < .05, tP < .01 vs native LDL.
The effect of BLPDS on the oxidation of CS-LDL by hemin–hydrogen peroxide is shown in Fig 2. The production of TBARS was stimulated by 2.1% BLPDS, and the increase was approximately four times less than the corresponding increase with Hep-LDL (Fig 1). Increasing amounts of BLPDS reduced TBARS formation to baseline levels.

The time course of the hemin-mediated oxidation of Hep-LDL or native LDL in the presence of 2.1% BLPDS is shown in Fig 3. With both LDL preparations the formation of TBARS reached a plateau after about 100 minutes and with no evidence of an initial lag phase. When Hep-LDL was oxidized, the bulk of the TBARS production occurred in the first 20 minutes. With native LDL a lesser proportion of maximal TBARS formation was seen during this period, and thereafter there was an approximately linear increase in TBARS until the plateau was reached. The lower maximum TBARS production from native LDL compared with Hep-LDL in the presence of 2.1% BLPDS is in accord with the data in Fig 1.

The dose-response curves for hemin-mediated oxidation of Hep-LDL in the presence of hydrogen peroxide (286 μmol/L) and 2.1% BLPDS. The rate of TBARS formation increased steeply with increasing hemin concentrations of up to 20 μmol/L and then slowed. Levels of hemin as low as 1 μmol/L significantly increased TBARS production in the presence of 286 μmol/L hydrogen peroxide. The concentrations of hemin (20 μmol/L) and hydrogen peroxide (286 μmol/L) routinely used gave virtually maximal oxidation of Hep-LDL in the presence of 2.1% BLPDS. When hemin (0.64±0.14 nmol·2 h⁻¹·0.2 μmol cholesterol⁻¹; n=3 experiments) or hydrogen peroxide (0.32±0.14 nmol·2 h⁻¹·0.2 μmol cholesterol⁻¹; n=3) was added singly, TBARS production was significantly (P<.001) lower than when both were added together (2.80±0.38...
of three experiments performed in duplicate. The end of the incubation, thiobarbituric acid-reacting substances (TBARS) were determined and are expressed as nanomoles per 2 hours (multiply by 5 to convert to nanomoles per micromole LDL cholesterol per 2 hours).

Fig 4. Line graph showing dose-response curve of the oxidation of heparin-isolated low-density lipoprotein (Hep-LDL) by hemin and hydrogen peroxide. Increasing quantities of Hep-LDL were added to HEPES-saline buffer containing apolipoprotein B lipoprotein-deficient serum (BLPDS), and hemin and hydrogen peroxide were added. The mixture in duplicate was incubated for 2 hours at 37°C, and thiobarbituric acid-reacting substances (TBARS) were measured and are expressed as nanomoles per 2 hours (multiply by 5 to convert to nanomoles per micromole LDL cholesterol per 2 hours).

nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹; n=3). Reducing the hydrogen peroxide concentration to 25 μmol/L and maintaining the hemin concentration at 20 μmol/L did not significantly alter TBARS formation (2.49±1.10 nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹; undialyzed, 3.52±0.65 nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹; n=3). Thus, small dialyzable molecules were not responsible for the stimulated TBARS formation at low levels of BLPDS.

The effects of serum fractions d>1.063 g/mL and d>1.21 g/mL on hemin-mediated oxidation of Hep-LDL are shown in Fig 6. The serum fractions were at concentrations that were comparable to those usually found in serum. Highest TBARS production rates were observed when 2.1% of the d>1.063 g/mL or the d>1.21 g/mL plasma fractions were present, and rates were reduced with increasing amounts of the fractions. These results are similar to those obtained when Hep-LDL was oxidized in the presence of BLPDS and indicate that the stimulatory component of BLPDS is in the d>1.063 g/mL and d>1.21 g/mL serum fractions.

Stimulation of hemin-catalyzed oxidation of Hep-LDL by the d>1.063 g/mL fraction also indicates that dextran sulfate and magnesium chloride in BLPDS are not responsible for its stimulatory effect.

Fig 7 shows the effects of HSA, free fatty acid–free HSA, and γ-globulin on hemin-catalyzed oxidation of serum Hep-LDL. The amounts of proteins added were typical of those in the aliquots of BLPDS added to incubations. The TBARS production rate in the presence of 15 μmol/L HSA was 2.75 times the rate with Hep-LDL alone and approximately two times the rates at higher concentrations of HSA. When Hep-LDL was oxidized in the presence of HSA the rates of TBARS formation were not significantly different from the corresponding rates in the presence of BLPDS shown in Fig 1. Increasing concentrations of γ-globulin (amounts similar to those in aliquots of BLPDS added to incubations) did not significantly alter rates of TBARS production from values observed with Hep-LDL alone.

The effects of BLPDS and HSA on the degradation of hemin (decrease, OD 412 nm) in the presence of hydrogen peroxide are shown in Fig 8. There was a marked decrease in OD₄₁₂ in the presence of 2.1% BLPDS that became progressively less with increasing volumes of the serum fraction. Similar findings were obtained with the addition of HSA at concentrations comparable with those obtained by addition of BLPDS. Addition of BHT reduced the rapid fall in OD₄₁₂ observed with hemin in the presence of 2.1% BLPDS or 15 μmol/L HSA.

Formation of TBARS was not stimulated when Hep-LDL was incubated with ferrous sulfate (20 μmol/L) alone (0.21±0.01 nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹) or in the presence of 2.1% (vol/vol) BLPDS (0.23±0.03 nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹) or 15 μmol/L HSA (0.19±0.04 nmol · 2 h⁻¹ · 0.2 μmol cholesterol⁻¹). These results are mean±SD of two experiments. Similar results were obtained when ferric chloride (20 μmol/L) was used instead of ferrous sulfate (data not shown).

Increasing concentrations of HDL with and without added Hep-LDL were treated with hemin and hydrogen peroxide, and TBARS production was measured (Fig 9). The amounts of HDL added were typical of those present in the aliquots of BLPDS added to incubations. TBARS formation increased linearly with increasing HDL concentration and was not significantly affected by the addition of Hep-LDL.
Sutherland Serum Components and Heparin-LDL Oxidation by Hemin 1971

FIG 6. Line graphs showing effect of d>1.063 g/mL and d>1.21 g/mL serum fractions on oxidation of heparin-lisolated low-density lipoprotein (Hep-LDL) by hemin and hydrogen peroxide. The serum fractions were isolated by ultracentrifuging serum at the appropriate densities as described in "Methods." The concentrations of these fractions were typical of concentrations in apolipoprotein B lipoprotein-deficient serum. A, Hep-LDL was added to HEPES-saline buffer and incubated for 2 hours at 37°C in the presence of hemin, hydrogen peroxide, and varying amounts (0% to 16.7%) of d>1.063 g/mL serum fraction. A control incubation contained 4% NaCl-phosphate-buffered saline (NaCl/PBS). Thiobarbituric acid-reacting substances (TBARS) were measured at the end of the incubation and are expressed as nanomoles per 2 hours (multiply by 5 to convert to nanomoles per micromole LDL cholesterol). Results are mean±SD of two experiments performed in duplicate. B, Oxidation of Hep-LDL in the presence of d>1.21 g/mL serum fraction. Conditions were the same as described in panel A except that 0% to 16.7% of d>1.21 g/mL serum fraction replaced the d>1.063 g/mL fraction. Results are mean±SD of three experiments performed in duplicate.

Table 2 shows the effect of EDTA and antioxidants on hemin/hydrogen peroxide-induced formation of TBARS from serum Hep-LDL in the presence of 2.1% BLPDS. BHT (35 μmol/L) completely inhibited the increase in TBARS produced by BLPDS, and vitamin E (605 μmol/L) reduced TBARS production significantly by 51%. EDTA (180 μmol/L) and ascorbic acid (102 μmol/L) did not reduce TBARS production significantly under the same conditions.

FIG 7. Line graph showing effect of human serum albumin (HSA), free fatty acid-HSA (F-HSA), and γ-globulin on oxidation of heparin-isolated low-density lipoprotein (Hep-LDL) by hemin and hydrogen peroxide. Hep-LDL was added to HEPES-saline buffer and oxidized by hemin and hydrogen peroxide in the presence of varying concentrations of HSA, F-HSA, and γ-globulin at 37°C for 2 hours. The concentrations of the proteins in the incubations were typical of concentrations present when apolipoprotein B lipoprotein-deficient serum was added in the other experiments. Control incubations contained varying concentrations of HSA and 4% NaCl-phosphate-buffered saline (NaCl/PBS). At the end of the incubation period thiobarbituric acid-reacting substances (TBARS) were measured and are expressed as nanomoles per 2 hours (multiply by 5 to convert to nanomoles per micromole LDL cholesterol). Results are mean±SD of three experiments performed in duplicate.

Discussion

This study demonstrates that hemin/hydrogen peroxide-induced oxidation of LDL isolated by interaction with heparin is stimulated by low levels of BLPDS and in particular by HSA, which is present in the serum fraction. Furthermore, oxidation of CS-LDL is also stimulated by low levels of BLPDS. Chondroitin sulfate is a major component of arterial wall glycosaminoglycans,33 and together with heparin it accounts for the bulk of these compounds in the apoB lipoprotein-proteoglycan complexes that have been isolated from human atherosclerotic plaques.14,15 The present findings may thus be relevant to oxidation of LDL in the artery wall.
The composition of Hep-LDL, after purification to remove small quantities of serum proteins, was similar in many respects to that of native LDL except that the contents of triglycerides and lipid peroxides were higher in Hep-LDL. This increased triglyceride content was undoubtedly due to comparatively low levels of VLDL remnants, which have been identified in Hep-LDL. The raised lipid peroxide content suggests that Hep-LDL may have undergone minimal oxidative modification during isolation from serum. Interaction with glycosaminoglycans is reported to alter the structure and oxidizability of LDL\(^1\) and therefore may also increase LDL lipid peroxide levels. In the present study, the vitamin E contents in Hep-LDL and native LDL were comparable, which is in line with the finding of similar levels of vitamin E in native LDL and LDL treated with glycosaminoglycans.\(^1\) Thus, the increased lipid peroxide levels do not appear to be due to any reduction in vitamin E–mediated antioxidant protection of Hep-LDL. Purification did not influence the lipid composition or the oxidation of Hep-LDL in this study.

### TABLE 2. Effect of Antioxidants and EDTA on the Maximal Oxidation Rate of Heparitin-Isolated Low-Density Lipoprotein by Hemin and Hydrogen Peroxide in the Presence of Apolipoprotein B Lipoprotein–Deficient Serum

<table>
<thead>
<tr>
<th>Additions</th>
<th>Fraction of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid, 102 (\mu)mol/L</td>
<td>0.81±0.11</td>
</tr>
<tr>
<td>Vitamin E, 605 (\mu)mol/L</td>
<td>0.49±0.07*</td>
</tr>
<tr>
<td>BHT, 35 (\mu)mol/L</td>
<td>0.11±0.05†</td>
</tr>
<tr>
<td>EDTA, 180 (\mu)mol/L</td>
<td>1.08±0.10</td>
</tr>
</tbody>
</table>

BHT indicates butylated hydroxytoluene. Hemin (20 \(\mu\)mol/L), hydrogen peroxide (286 \(\mu\)mol/L), and apolipoprotein B lipoprotein–deficient serum (2.1%) were present in all incubations. Vitamin E and BHT were dissolved in ethanol, and 10-\(\mu\)L aliquots of the solutions were added to incubations. This quantity of ethanol did not influence the formation of thiobarbituric acid–reacting substances. Values are mean±SD from three experiments performed in duplicate.  

\(*P<.05, \dagger P<.01\) vs control.
hemin and hydrogen peroxide lower than current levels (HSA, 6 to 120 μmol/L). Other serum components may also influence the oxidation of Hep-LDL by hemin. For example, hemopexin, a serum protein that binds heme avidly and thereby inhibits lipid peroxidation, could conceivably reduce the oxidation of Hep-LDL, particularly at higher levels of BLPDS. The BLPDS fraction also contains HDL, which has inhibited the peroxidation of LDL in previous studies. However, in the present study, lipid peroxidation was accelerated when Hep-LDL and high levels of HDL were oxidized, suggesting that increasing levels of HDL were not responsible for the return of oxidation rates to baseline at high levels of BLPDS. Although lipids in isolated HDL were readily oxidized by hemin (Fig 9), they were not the source of increased levels of lipid oxidation products during BLPDS-stimulated oxidation of Hep-LDL because oxidation rates remained at baseline when BLPDS alone was treated with hemin and hydrogen peroxide. This finding also suggests that there is an inhibitor of hemin-catalyzed HDL oxidation in BLPDS.

Vincent and coworkers report that low levels of HSA stimulate and high levels inhibit peroxidation of rat liver microsomes by hemin and hydrogen peroxide, an effect of HSA on hemin-catalyzed lipid peroxidation that was comparable with the present findings. They postulate that nonspecific protein binding of heme increases its availability for lipid oxidation and that high levels of HSA reduce peroxidation by effectively competing with membranes for heme. Thus, binding of hemin to comparatively low concentrations of HSA may facilitate peroxidation of LDL by intercalation of the hydrophobic hemin molecule within the lipids of the LDL particle. Lipid peroxidation may then occur as a result of oxidative destruction of the hemin ring with the release of catalytic free iron in the lipid domain. The present finding that low levels of the lipid-soluble antioxidant BHT inhibited but higher levels of the lipid-insoluble chelator EDTA did not influence hemin-catalyzed and BLPDS-stimulated oxidation of Hep-LDL is consistent with this theory.

The mechanism underlying this stimulated oxidation of Hep-LDL (or native LDL) may also involve oxidative interactions between heme and HSA. The effect of HSA or BLPDS on hemin degradation in the presence of hydrogen peroxide essentially paralleled the effect of these serum fractions on hemin-mediated oxidation of Hep-LDL, suggesting a possible link between the two processes. Oxidative mechanisms were evidently involved in the destruction of hemin at low levels of HSA because hemin degradation was inhibited by BHT. Free iron is released when the heme ring is destroyed, but our data show that neither ferrous ions nor ferric ions in the aqueous medium influence the peroxidation of Hep-LDL in the presence or absence of low levels of HSA or BLPDS. Hemin in the presence of hydrogen peroxide modifies HSA to a number of hemin-containing products, but whether these are more potent catalysts of lipid peroxidation than hemin itself remains to be determined.

The molar ratio of HSA/hemin appears to influence the effect of the protein on both the oxidative degradation of hemin and the hemin-catalyzed peroxidation of Hep-LDL. At low levels of HSA, which maximally stimulated peroxidation of Hep-LDL, the ratio was approximately unity; at higher levels of HSA, which were ineffective, the ratio was ~8. An increase in the HSA/hemin molar ratio from unity may lead to binding of more than one molecule of HSA to hemin. Increased binding of HSA to hemin might prevent subsequent oxidative degradation of the compound by hydrogen peroxide, possibly by interacting with further coordination sites on heme iron. These sites are apparently
important for the oxidative degradation of protein-heme complexes by hydrogen peroxide.26

When native LDL is oxidized by hemin and hydrogen peroxide there is a lag time in lipid peroxidation, measured by hemin degradation, that is prolonged twofold by physiological concentrations of ascorbate and higher concentrations of vitamin E.17 In the present study, there was no appreciable lag time in hemin degradation during the oxidation of Hep-LDL stimulated by low levels of BLPSD except when unphysiologically high levels of ascorbate were added (Fig 10). Several factors might contribute to this finding, including raised levels of lipid peroxides in Hep-LDL, reported structural changes in LDL that has interacted with heparin,11 comparatively high levels of hemin and hydrogen peroxide, and effects of BLPSD. An important influence of raised lipid peroxide levels on the lag time is suggested by the finding that there is no delay in the onset of oxidation when minimally oxidized LDL is treated with hemin/hydrogen peroxide.15 It is doubtful that high levels of oxidants were solely responsible for the insensitivity of stimulated Hep-LDL oxidation to inhibition by ascorbate. Ascorbate at levels (50 μmol/L) similar to those used here markedly inhibited (70%) the peroxidation of rat liver microsomes by concentrations of hemin and hydrogen peroxide that were similar and higher, respectively, than current levels.36

Balla and coworkers36 have suggested that hemin derived from trivial hemolysis in areas of turbulent arterial flow coupled with hydrogen peroxide released from activated macrophages might peroxidize LDL in the vessel wall and thereby promote atherogenesis. We extend this hypothesis by suggesting that low levels of albumin in regions of the artery wall37-39 may accelerate hemin-induced peroxidation of LDL that has complexed with glycosaminoglycans. However, in areas of the vessel wall where levels of albumin are higher,37-39 oxidation of LDL may not be stimulated in this manner. It can be calculated from published data37-38 that albumin levels in the arterial media region may be sufficiently low (approximately 4% of plasma albumin levels) to stimulate hemin-catalyzed peroxidation of LDL that has interacted with glycosaminoglycans (but not native LDL), particularly if plasma albumin levels are low. Interestingly, low plasma albumin levels have been associated with an increased incidence of coronary heart disease in an epidemiological study.40 The low levels of hydrogen peroxide that could catalyze the rapid oxidation of Hep-LDL stimulated by HSA and BLPDS were within the range calculated to occur near activated neutrophils41 and therefore are likely to occur in vivo. The levels of hemin that catalyzed this oxidation were also low, but whether levels in the artery wall become sufficiently high to oxidize LDL remains to be seen.

In conclusion, the present study showed that serum components and particularly HSA modulate the oxidation of glycosaminoglycan-treated LDL by hemin and hydrogen peroxide in vitro, indicating that certain physiological factors can influence the formation of oxidized LDL, which is Potentially atherogenic.

Acknowledgments
This work was supported by the Nye Research Fund. The author is grateful to Vicky Phillips and Kevin Sanderson for the measurement of vitamin E.

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doi: 10.1161/01.ATV.14.12.1966

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