

# Increased uptake of $\alpha$ -hydroxy aldehyde-modified low density lipoprotein by macrophage scavenger receptors

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**Abstract** Reactive aldehydes can be formed during the oxidation of lipids, glucose, and amino acids and during the nonenzymatic glycation of proteins. Low density lipoprotein (LDL) modified with malondialdehyde are taken up by scavenger receptors on macrophages. In the current studies we determined whether  $\alpha$ -hydroxy aldehydes also modify LDL to a form recognized by macrophage scavenger receptors. LDL modified by incubation with glycolaldehyde, glyceraldehyde, erythrose, arabinose, or glucose ( $\alpha$ -hydroxy aldehydes that possess two, three, four, five, and six carbon atoms, respectively) exhibited decreased free amino groups and increased mobility on agarose gel electrophoresis. The lower the molecular weight of the aldehyde used for LDL modification, the more rapid and extensive was the derivatization of free amino groups. Approximately 50–75% of free lysine groups in LDL were modified after incubation with glyceraldehyde, glycolaldehyde, or erythrose for 24–48 h. Less extensive reductions in free amino groups were observed when LDL was incubated with arabinose or glucose, even at high concentration for up to 5 days. LDL modified with glycolaldehyde and glyceraldehyde labeled with <sup>125</sup>I was degraded more extensively by human monocyte-derived macrophages than was <sup>125</sup>I-labeled native LDL. Conversely, LDL modified with <sup>125</sup>I-labeled erythrose, arabinose, or glucose was degraded less rapidly than <sup>125</sup>I-labeled native LDL. Competition for the degradation of LDL modified with <sup>125</sup>I-labeled glyceraldehyde was nearly complete with acetyl-, glycolaldehyde-, and glyceraldehyde-modified LDL, fucoidin, and advanced glycation end product-modified bovine serum albumin, and absent with unlabeled native LDL. These results suggest that short-chain  $\alpha$ -hydroxy aldehydes react with amino groups on LDL to yield moieties that are important determinants of recognition by macrophage scavenger receptors.—Kawamura, M., J. W. Heinecke, and A. Chait. **Increased uptake of  $\alpha$ -hydroxy aldehyde-modified low density lipoprotein by macrophage scavenger receptors.** *J. Lipid Res.* 2000. 41: 1054–1059.

**Supplementary key words** lipids • oxidation • aldehydes • macrophages • atherosclerosis • diabetes

The oxidative modification of low density lipoprotein (LDL) may play an important role in the pathogenesis of atherosclerosis (1–3). In vitro studies suggest multiple mechanisms whereby oxidized lipoproteins may be

come atherogenic. For example, reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are generated during the metal-catalyzed oxidation of polyunsaturated fatty acids in LDL (4, 5). Adducts form between these aldehydes and amino acid side chains of apolipoprotein B-100 (apoB) in oxidized LDL. Oxidized LDL is recognized by scavenger receptors on macrophages (4, 6, 7) and leads to the formation of foam cells in vitro (4, 6). Reactive aldehydes also are formed by amino acid oxidation (8). A physiologically relevant pathway may involve myeloperoxidase, a heme protein secreted by activated phagocytes. Myeloperoxidase converts tyrosine into *p*-hydroxyphenylacetaldehyde in near quantitative yield (9). It has been demonstrated that the enzyme similarly will convert serine into glycolaldehyde and threonine into 2-hydroxypropanal and acrolein (10). Because active myeloperoxidase is a component of human atherosclerotic lesions (11), LDL oxidation may involve aldehydes other than MDA or 4-HNE. Indeed, acrolein-modified LDL has been isolated from human atherosclerotic lesions (12).

Elevated levels of advanced glycation end products (AGEs) have been detected on LDL isolated from diabetic subjects (13). One important pathway for AGE formation involves glucose, which in its open-chain form is an aldehyde that covalently reacts with the amino groups of lysine residues on proteins (14). Highly reactive aldehydes also are formed by glucose autooxidation, during the nonenzymatic glycation of proteins, and by protein glycoxidation (14–16). Studies indicate that the conversion of protein-bound glucose into glycolaldehyde is likely to be a key intermediate in the formation of N<sup>ε</sup>-(carboxymethyl)lysine (17), an advanced glycation end-product (18). Glycolaldehyde and other aldehydes generated by myeloperoxidase may also play a role in the formation of advanced glycation end products (19).

Abbreviations: AGE, advanced glycation end product; apoB, apolipoprotein B; 4-HNE, 4-hydroxynonenal; LDL, low density lipoprotein; MDA, malondialdehyde; TNBS, trinitrobenzene sulfonic acid.

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These observations suggest that reactive aldehydes generated by oxidative stress and hyperglycemia might modify LDL to a form that plays a role in macrophage foam cell formation. To better understand the cell biology of aldehyde-modified lipoproteins, we have used a family of structurally related  $\alpha$ -hydroxy aldehydes ranging in size from two carbons to six carbons to modify LDL, and then determined the uptake and degradation of the modified LDL by macrophages.

## MATERIALS AND METHODS

### Materials

$\text{Na}^{125}\text{I}$  was obtained from DuPont-New England Nuclear (Boston, MA). Ficoll-Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). D-Glycolaldehyde, DL-glyceraldehyde, D-erythrose, D-arabinose, D-glucose, fucoidin, and sodium cyanoborohydride were obtained from Sigma (St. Louis, MO). RPMI 1640 culture medium, bovine calf serum, penicillin-streptomycin, and L-glutamine were from GIBCO (Grand Island, NY).

### Methods

**Lipoproteins.** LDL was prepared from human plasma (EDTA, 4 mM) that was treated immediately after separation from red blood cells with phenylmethylsulfonyl fluoride (1  $\mu\text{M}$  final concentration). The density of the plasma was increased to 1.30 g/mL by the addition of solid KBr, and LDL (d 1.019–1.063 g/mL) was isolated by discontinuous density gradient ultracentrifugation in a Beckman (Fullerton, CA) Vti-50 vertical rotor by the method of Chung et al. (20), as described (21). All solutions used for lipoprotein preparation contained 1 mM EDTA. After one wash at d 1.063 g/mL, the isolated LDL was dialyzed extensively under  $\text{N}_2$  at 4°C in the dark, and used within 2 weeks of preparation. LDL was radiolabeled by the iodine monochloride method as modified for lipoproteins by Bilheimer, Eisenberg, and Levy (22) using  $\text{Na}^{125}\text{I}$ .

**LDL modification.** LDL (1 mg of protein per mL) and  $^{125}\text{I}$ -labeled LDL (1 mg of protein per mL) were modified with different aldehydes by incubation with different concentrations of these compounds in phosphate-buffered saline (PBS; 150 mM NaCl, 50 mM  $\text{PO}_4$ , pH 7.4) supplemented with 1 mM EDTA for the indicated times at 37°C in a  $\text{CO}_2$  incubator. Aldehyde-modified LDL then was dialyzed extensively under  $\text{N}_2$  at 4°C in the dark. Significant aggregation of the modified lipoproteins was ruled out by lack of extensive turbidity, monitored as  $A_{450}$ ; no increase in precipitation of  $^{125}\text{I}$ -labeled lipoproteins pelleted by centrifugation at 10,000  $g$  for 30 min; and lack of detection of lipoprotein at the origin on agarose gel electrophoresis. Moreover, all lipoproteins were filtered through a 0.22- $\mu\text{m}$  pore size filter before their use in experiments. Also, there was no evidence of significant oxidation of those modified forms of LDL by measurement of thiobarbituric acid-reactive substance (23) or lipid hydroperoxides (24). Advanced glycation end product (AGE)-modified bovine serum albumin (BSA) was prepared by incubating BSA in PBS (pH 7.2) with 50 mM glucose at 37°C for 3–4 weeks in the presence of 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM EDTA, penicillin (100 units/mL), and gentamicin (40  $\mu\text{g}/\text{mL}$ ). LDL was acetylated by the method of Basu et al. (25).

**Cells.** Human monocyte-derived macrophages were isolated by density gradient centrifugation by the method of Böyum (26). The mononuclear cell band was washed twice at 4°C in RPMI 1640 medium and then plated at  $3 \times 10^5$  cells per 16-mm well.

After 2 h, nonadherent cells were removed, and RPMI 1640 medium containing 20% (v/v) autologous serum, penicillin (100 units/mL), streptomycin (100 mg/mL), and 2 mM glutamine was added. The cells were fed twice weekly with the same medium and used for experiments within 10 to 14 days of plating.

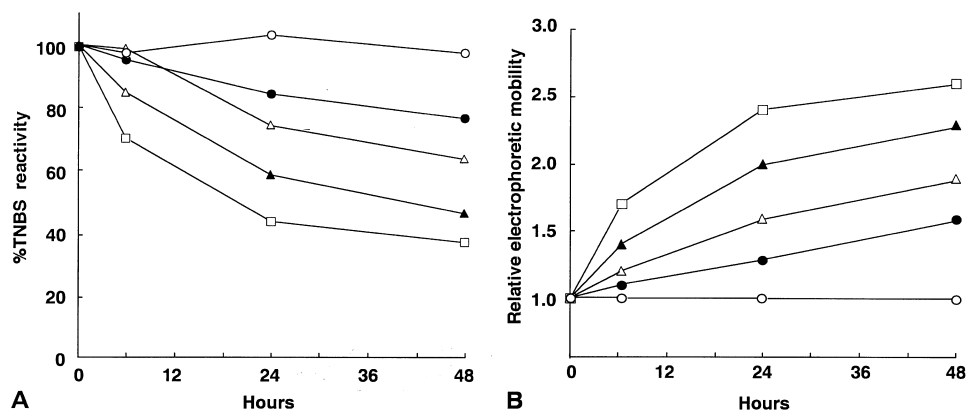
**Binding and cellular metabolism of radiolabeled lipoproteins.** Before incubation with macrophages,  $^{125}\text{I}$ -labeled lipoproteins were dialyzed extensively at 4°C against PBS with 1 mM EDTA (pH 7.4) to remove the aldehydes used for modification. Uptake and degradation of the  $^{125}\text{I}$ -labeled lipoprotein (150–250 cpm/ $\mu\text{g}$  of protein) in the presence and absence of unlabeled lipoproteins were assessed after a 5-h incubation with cells at 37°C. LDL degradation products in the incubation medium were assayed by measurement of trichloroacetic acid-soluble radioactivity (non-iodide). Cell-free LDL degradation, which represented less than 5% of total radioactivity, was subtracted from total degradation (27). Binding of  $^{125}\text{I}$ -labeled glyceraldehyde-modified LDL by macrophages was assessed after incubation of the cells with radiolabeled lipoprotein in the presence and absence of unlabeled lipoproteins for 2 h at 4°C. After extensive washing with PBS, the cell layer was washed in 0.5 N NaOH and the radioactivity was counted. For measurement of protein content, the cell layer was washed three times with PBS at the end of the incubation, and extracted by incubation at room temperature with 0.5 mL of 0.1 N NaOH for 1 h.

**Other assays.** Protein was measured by the method of Lowry et al. (28), using BSA as the standard. Free amino groups in LDL were estimated with trinitrobenzene sulfonic acid (TNBS; Pierce, Rockford, IL). LDL (50  $\mu\text{g}$  of protein) was mixed with 1 mL of 4%  $\text{NaHCO}_3$  (pH 8.4) and 50  $\mu\text{l}$  of 0.1% TNBS and heated for 1 h at 37°C, after which the absorbance at 340 nm was measured (29). LDL electrophoresis was carried out at pH 8.6 in barbital buffer on an 0.5% agarose gel as described. Electrophoretic mobility was expressed relative to native LDL (30).

## RESULTS

TNBS reactivity, a measure of free amino groups in LDL, decreased when LDL was incubated at 37°C with glyceraldehyde in a physiologic salt solution supplemented with EDTA at neutral pH. The change in TNBS reactivity was time- and concentration-dependent (Fig. 1A). Loss of TNBS reactivity presumably reflects the result of reaction of the  $\alpha$ -hydroxy aldehyde group of glyceraldehyde with free amino groups of lysine in the apoB of LDL (5, 12, 16). After 24 h, the reduction in TNBS reactivity began to plateau. Therefore, an incubation time of 48 h was used to obtain a relatively stable form of aldehyde-modified LDL for subsequent macrophage experiments. Relative electrophoretic mobility of LDL modified with glyceraldehyde increased in parallel with the loss of TNBS reactivity in a time- and concentration-dependent manner (Fig. 1B).

D-Glycolaldehyde, DL-glyceraldehyde, D-erythrose, and D-arabinose were used to investigate the effect of the molecular weight of  $\alpha$ -hydroxy aldehydes on their ability to modify LDL and to affect the interaction of the modified lipoprotein with macrophages. These compounds differ in their number of carbon atoms: glycolaldehyde, glyceraldehyde, erythrose, arabinose, and glucose have two, three, four, five, and six carbons, respectively (Fig. 2). When LDL was incubated with glycolaldehyde, glyceraldehyde, or erythrose, TNBS reactivity decreased by >50%, de-



**Fig. 1.** Time course and concentration dependence of LDL modification by glyceraldehyde. LDL (1 mg/mL) was incubated in PBS (pH 7.4) supplemented with 1 mM EDTA at 37°C with glyceraldehyde (open circles, 0 mM; solid circles, 5 mM; open triangles, 10 mM; solid triangles, 25 mM; open squares, 50 mM) for the indicated times. After incubation each mixture was dialyzed extensively at 4°C against PBS containing 1 mM EDTA. TNBS reactivity (A) and relative electrophoretic mobility (B) were measured as described in Materials and Methods and are expressed relative to native LDL.

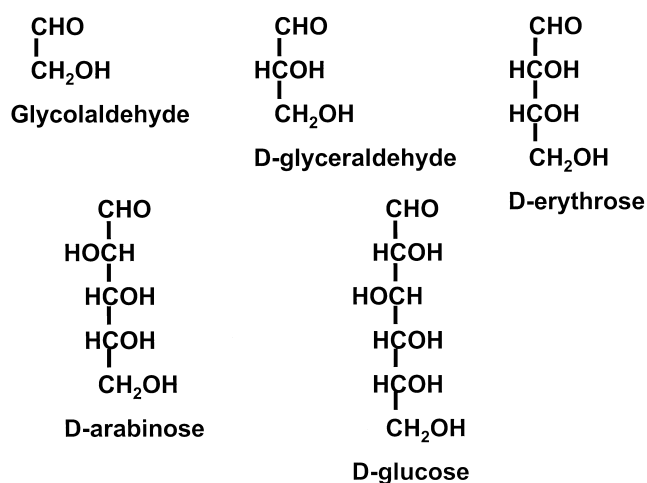
pending on the concentration of aldehyde used during the incubation. This effect appeared to saturate at high concentrations (20–100 mM) of these aldehydes (Fig. 3A). Arabinose and glucose were much less reactive. TNBS reactivity was reduced by only 27 and 25% after incubation for 5 days with 200 mM arabinose and 500 mM glucose, respectively (data not shown).

The relationship between relative mobility on agarose gel electrophoresis (an index of overall charge on the lipoprotein) and TNBS reactivity was examined for the more reactive of these  $\alpha$ -hydroxy aldehydes. Relative electrophoretic mobility increased in parallel with a decrease in TNBS reactivity (Fig. 3B). This relationship was the same independent of the aldehyde used for modification of LDL. The relative electrophoretic mobility and TNBS reactivity of acetyl LDL, used as a positive control, were 2.2 and 50%, respectively. Thus the degree of alteration of charge appeared to depend on the extent of modification

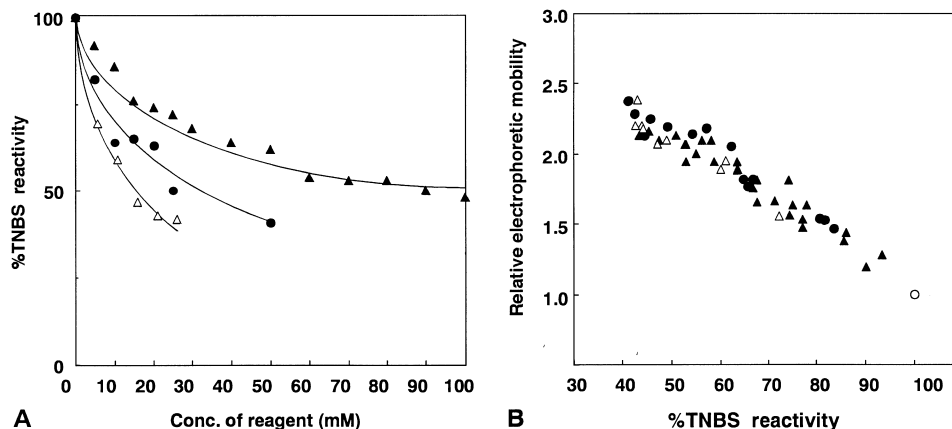
of lysine groups, irrespective of the nature of the reactive  $\alpha$ -hydroxy aldehyde. In contrast, there is little correlation between relative electrophoretic mobility and TNBS reactivity when other types of aldehydes have been used to modify LDL (7, 31).

To determine whether  $\alpha$ -hydroxy aldehydes might alter the cellular recognition of lipoproteins, we investigated the degradation by human monocyte-derived macrophages of  $^{125}\text{I}$ -labeled LDL modified by incubation with the various compounds.  $^{125}\text{I}$ -labeled acetyl LDL, and  $^{125}\text{I}$ -labeled glycolaldehyde- and glyceraldehyde-modified LDL, were all degraded to a greater extent than was  $^{125}\text{I}$ -labeled native LDL (Fig. 4A).  $^{125}\text{I}$ -labeled erythrose-modified LDL was degraded to a lesser extent than was  $^{125}\text{I}$ -labeled native LDL. The relationship between the concentration of aldehyde used and the degradation of the modified lipoprotein by macrophages also was evaluated. Higher concentrations of glycolaldehyde and glyceraldehyde resulted in increased uptake and degradation of the modified LDL by macrophages (Fig. 4B). In contrast to the short-chain hydroxy aldehydes,  $^{125}\text{I}$ -labeled LDL modified by arabinose or glucose was degraded more slowly by human monocyte macrophages relative to native  $^{125}\text{I}$ -labeled LDL (data not shown).

To determine the nature of the macrophage receptors to which these modified lipoprotein were binding, competition studies were performed with unlabeled ligands for the acetyl and LDL receptors (Fig. 5). A 40-fold m excess of either acetyl LDL or glycolaldehyde-modified LDL nearly completely inhibited the degradation of 5  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled glyceraldehyde-modified LDL by human macrophages. In contrast, 40-fold excess native, AGE-albumin had minor effects and erythrose-modified LDL had less effect on the degradation of radiolabeled glyceraldehyde-modified LDL. Fucoidin competed efficiently for degradation of glyceraldehyde-modified LDL (Fig. 5A). Glyceraldehyde-modified LDL also resulted in marked inhibition of degradation of  $^{125}\text{I}$ -labeled glyceraldehyde-modified LDL (Fig. 5B). Similar



**Fig. 2.** Structures of glycolaldehyde ( $\text{C}_2$ ), glyceraldehyde ( $\text{C}_3$ ), erythrose ( $\text{C}_4$ ), arabinose ( $\text{C}_5$ ), and glucose ( $\text{C}_6$ ).



**Fig. 3.** Effect of aldehydes on free amino groups in LDL. LDL (1 mg/mL) was incubated at 37°C in PBS with the indicated final concentrations of glycolaldehyde (open triangles), glyceraldehyde (solid circles), and erythrose (solid triangles) for 48 h, after which the LDL was dialyzed extensively. TNBS reactivity (A) and electrophoretic mobility relative to LDL (B) were then determined as described in the legend to Fig. 2. Open circle, unmodified LDL.

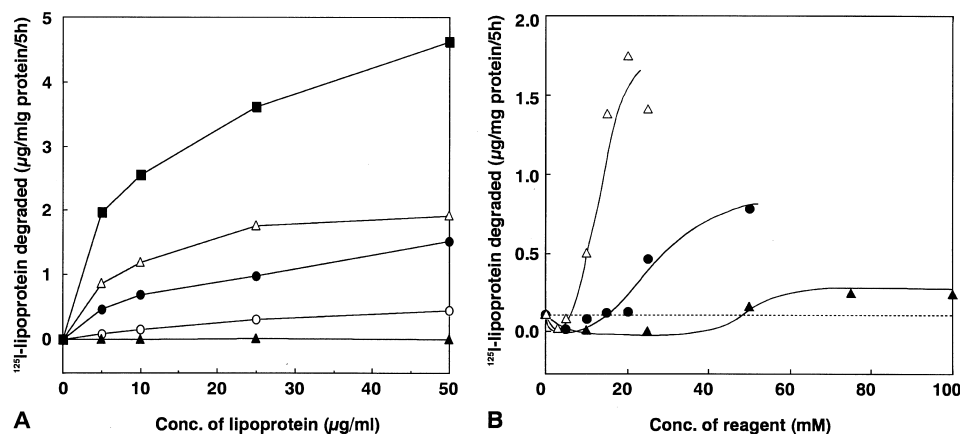
observations were made in binding studies performed at 4°C (data not shown). These observations are consistent with degradation of glyceraldehyde-modified LDL by the acetyl LDL receptor on human macrophages.

## DISCUSSION

This study demonstrates that incubation of LDL with  $\alpha$ -hydroxy aldehydes leads to reaction with free amino groups on LDL, which in turn affects their uptake and metabolism by cultured macrophages. The reactivity with amino groups on LDL apoB, as judged by loss of TNBS reactivity, was dependent on the concentration of the aldehyde, the duration of the incubation, and the size of the

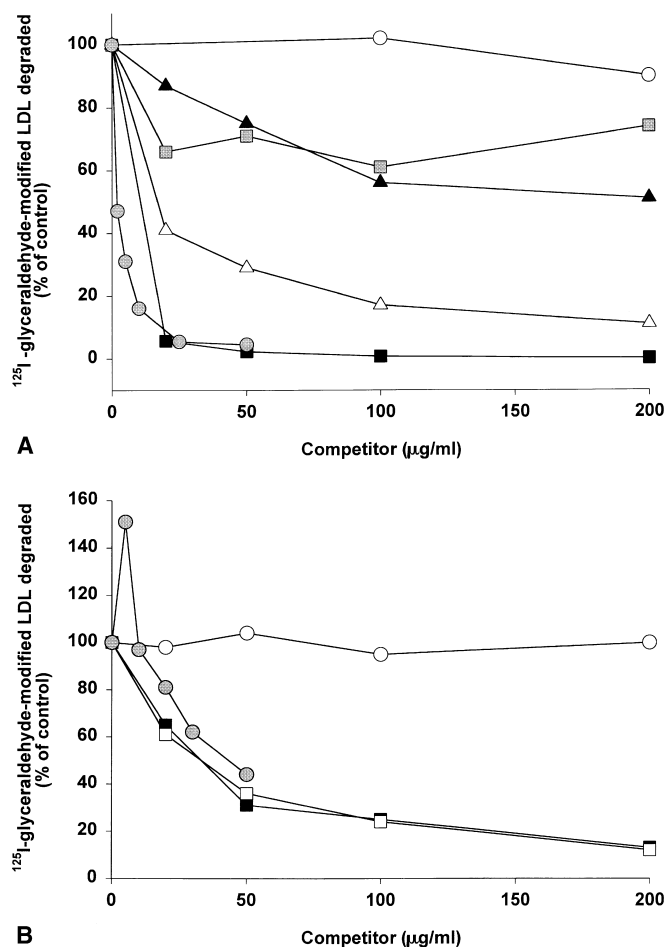
carbon skeleton of the reacting aldehyde. The smaller the aldehyde, the more reactive it was with free amino groups on LDL. A large proportion of the TNBS-reactive amino groups were modified after 24–48 h of incubation with glycolaldehyde, glyceraldehyde, and erythrose. Glucose and arabinose were less reactive and modified a lower percentage of protein amino groups on LDL.

Modification of LDL with the various aldehydes resulted in altered uptake and degradation by cultured human monocyte-derived macrophages. LDL modified by either glycolaldehyde or glyceraldehyde, the smallest and most reactive of the aldehydes (5, 16), was taken up and degraded by macrophages more rapidly than was native LDL. Competition studies suggest that glyceraldehyde-modified LDL is likely to be taken up by the acetyl LDL receptor,



**Fig. 4.** Degradation of aldehyde-modified LDL by cultured human monocyte-derived macrophages. <sup>125</sup>I-Labeled native LDL (open circles) was modified at 37°C either by incubation for 24 h with 25 mM glycolaldehyde (open triangles), glyceraldehyde (solid circles), erythrose (solid triangles), or by acetylation with acetic anhydride (solid squares) (A) or by incubating the lipoprotein with the indicated concentration of the glycolaldehyde, glyceraldehyde, or erythrose for 24 h (B). After dialysis against PBS supplemented with 1 mM EDTA, the aldehyde-modified lipoproteins were incubated at 37°C with human monocyte-derived macrophages at the indicated final concentrations of protein (A) or at 5 µg of protein per mL (B) for 5 h, and the degradation of radiolabeled lipoprotein was determined as described in Materials and Methods.





**Fig. 5.** Competition for the degradation of  $^{125}\text{I}$ -labeled glycer aldehyde-modified LDL by ligands for macrophage receptors.  $^{125}\text{I}$ -Labeled glycer aldehyde-modified LDL ( $5\ \mu\text{g}$  of protein per mL), prepared as described in the legend to Fig. 4A, was added to human monocyte-derived macrophages together with the indicated final concentration of various potential competitors of binding to macrophage receptors [(A) native LDL (open circles), AGE-albumin (shaded squares), glycolaldehyde-modified LDL (open triangles), erythrose-modified LDL (solid triangles), acetyl LDL (solid squares), and fucoidin (shaded circles); and (B) native LDL (open circles), acetyl LDL (solid squares), fucoidin (shaded circles), and glycer aldehyde-modified LDL (open squares)]. Unlabeled aldehyde-modified LDLs were prepared by incubating unlabeled LDL with a  $50\ \text{mM}$  concentration of the aldehyde for 48 h (A) or 24 h (B) at  $37^\circ\text{C}$ . LDL was acetylated as described in Materials and Methods. The cells were incubated with the mixtures of labeled lipoprotein and unlabeled competitor for 5 h at  $37^\circ\text{C}$  for measurement of the degradation of the lipoprotein by the method described in Materials and Methods.

the prototypic macrophage scavenger receptor described by Brown and Goldstein (32), because its degradation was almost completely inhibited by acetylated LDL and the polyanionic compound fucoidin, both of which bind to this family of receptors (32). Native LDL, which binds to the LDL receptor (32), did not compete. Further, the initial decrease in degradation of LDL modified with low concentrations of glycolaldehyde or glycer aldehyde, followed by increased uptake of LDL modified with higher concentrations of aldehyde, is consistent with an initial

loss of recognition by the LDL receptor, followed by gain of recognition by scavenger receptors, as has been observed previously for other modified forms of lipoproteins (30, 33). Jinnouchi et al. (34) reported that glycolaldehyde-modified LDL also was rapidly taken up and degraded by the macrophage scavenger receptor.

Macrophages also possess a cell surface scavenger receptor that binds and internalizes formaldehyde-modified proteins (35). This receptor recognizes proteins modified with a wide range of aliphatic, but not aromatic aldehydes. Importantly, this receptor for formaldehyde-modified proteins binds AGE-modified proteins with high affinity, and AGE-albumin is a potent competitive inhibitor of the cellular binding of formaldehyde-modified proteins (35). The failure of AGE-albumin to inhibit the uptake and degradation of  $^{125}\text{I}$ -labeled glycer aldehyde-modified LDL suggests that the receptor for formaldehyde-modified proteins does not mediate the uptake of glycolaldehyde- and glycer aldehyde-modified LDL.

LDL modified by long-chain  $\alpha$ -hydroxy aldehydes behaved differently from LDL modified by short-chain aldehydes. Erythrose-modified LDL bound less well to macrophages than did native LDL, similar to what has been described previously for LDL modified by incubation with glucose (36). The derivatization of lysines on apoB by glucose is believed to retard the ability of the lipoprotein to bind to the LDL receptor (33), but this modification is insufficient to lead to uptake by scavenger receptors. Uptake of modified lipoproteins by scavenger receptors is postulated to require a considerable increase in net negative charge of the lipoprotein, but also appears to require conformational specificity (6, 7, 32), which may occur with certain aldehydes, but not with others. The extent of uptake and degradation of the various modified forms of LDL was also related to the degree of derivatization of free amino groups on apoB, as assessed by changes in TNBS reactivity. The size of the  $\alpha$ -hydroxy aldehyde used for modification appeared to be the most important determinant of the extent of uptake of the modified lipoprotein by macrophages. This is likely to reflect in part the greater reactivity of short-chain  $\alpha$ -hydroxy aldehydes (5, 16). Our results support the hypothesis that short-chain aldehydes promote recognition of the modified lipoproteins by macrophage receptors.

Increased uptake of modified lipoproteins by scavenger receptors on cultured macrophages may lead to the accumulation of lipid and foam cell formation, because these receptors are not regulated by the cholesterol content of the cell (32). One important mechanism may involve the uptake of oxidized LDL, which has been postulated to play an important role in atherogenesis (1–3).

It is conceivable that other pathways for lipoprotein modifications, such as those described here, may take place in the artery wall. Reactive aldehydes are generated during the oxidation of lipids, amino acids, and reducing sugars (1–8, 10, 12–18). Aldehydes such as malondialdehyde and 4-HNE are formed during the oxidation of poly-

unsaturated fatty acids (4, 5), and myeloperoxidase generates *p*-hydroxyphenylacetaldehyde and glycolaldehyde from tyrosine and serine, respectively (9, 10). Glycolaldehyde has been postulated to be an intermediate in the formation of advanced glycation end-products (17), and more recently has been shown to be formed during lipid peroxidation (18) and amino acid oxidation by myeloperoxidase (10, 20).

These observations suggest that modified lipoproteins similar to those tested in this *in vitro* study might occur *in vivo* under conditions of increased oxidative stress or as a result of the nonenzymatic glycation of proteins. Altered uptake of aldehyde-modified LDL thus might contribute to atherogenesis by their ability to bind scavenger or other receptors on arterial wall macrophages. Similar reactions may play a role in altering the uptake and degradation of lipoproteins by macrophages at sites of inflammation. ■

Supported in part by NIH grants DK02456 and AG15013, and by the Monsanto-Searle/Washington University Biomedical Program. The authors thank Ms. Julie Kirk for assistance with preparation of this manuscript.

Manuscript received 21 December 1998 and in revised form 7 March 2000.

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