Advanced glycosylation end products (AGEs) have been identified to be present on both the apolipoprotein and lipid components of low density lipoprotein (LDL) and to act to prevent its recognition and uptake by high affinity, tissue LDL receptors. Lipid-linked AGEs form readily in vitro by the covalent addition of glucose to the amine-containing head groups of phospholipids. This process is accompanied by oxidation of the unsaturated fatty acid side chains and occurs in the absence of exogenously added transition metals or free radical generating systems, suggesting that AGE formation may contribute significantly to lipid oxidation in vivo. To assess more precisely the chemical basis of AGE-induced oxidative modification, we performed gas chromatography-mass spectrometry analysis of the lipid products which form over time during LDL-advanced glycosylation in vitro. Negative ion chemical ionization mass spectroscopy of two major compounds that were identified were consistent with the structures of the fatty acid oxidation products 4-hydroxyhexenal and 4-hydroxynonenal. These data support the concept that AGE formation in close proximity to unsaturated fatty acyl groups leads to lipid oxidation and provide additional evidence that advanced glycosylation is an important pathogenic modification of the LDL particle in vivo.

The progressive, non-enzymatic modification of various tissue constituents by advanced glycosylation end products (AGEs) has been linked to the development of many of the long term complications of diabetes, renal insufficiency, and normal aging (1, 2). This modification process follows the principles of the Maillard reaction and begins with the covalent addition of reducing sugars, such as glucose, to the primary amino groups of macromolecules (1–3). The "early" Schiff base and Amadori adducts which form then slowly undergo a succession of intramolecular rearrangement, dehydrogenation, and oxidation-reduction reactions to produce the "late" products, termed AGEs, which are chemically irreversible and persist for the life of the affected macromolecule. Although the structures of the most abundant AGEs which occur in vivo remain unknown, recent immunochromatographic studies have established that a common, AGE-derived epitope(s) accumulates with age on many proteins in vivo (4–6). Immunoreactive AGEs have been identified to form on basement membrane collagen, red cell hemoglobin, the serum protein β2-microglobulin, and the Alzheimer's disease β-amyloid peptide (4–9). In many cases, the presence of the AGE modification has been shown to critically affect the functional properties of the affected substrate (9–12).

Recent investigations have led to the identification of an AGE-modified form of LDL, termed AGE-LDL, which circulates in high amounts in patients with diabetes or end-stage renal disease (13, 14). Patients with diabetes or renal insufficiency suffer from a rapidly progressive vasculopathy, which results in part from an elevation in the circulating level of the apoprotein-B (apoB) containing lipoproteins VLDL, LDL, and LDL (15–18). AGE-modified LDL exhibits impaired plasma clearance kinetics and the treatment of diabetic patients with the advanced glycosylation inhibitor aminoguanidine decreases both circulating AGE levels and total serum LDL (14). These observations thus have led to the hypothesis that the advanced glycosylation of LDL may play an important role not only in the dyslipidemia and accelerated vasculopathy of diabetes or renal insufficiency, but also in the more generalized, age-related atherosclerosis that affects much of the population (13, 14).

The AGE modifications within LDL have been shown to be present on both the apoB and lipid components (13). In the case of apoB, the predominant site of advanced glycosylation has been identified to lie within a single 67-amino acid domain, and modification at this site prevents the uptake of LDL by high affinity, fibroblast LDL receptors (12). Immunochromatographic studies indicate that the lipid-associated AGEs are attached covalently to the amine-containing head groups of phospholipids such as phosphatidylethanolamine (PE) (13). These AGE-modified phospholipids have the same absorbance, fluorescence, and immunochromatographic properties as the AGEs which form on proteins. It is also noteworthy that lipid AGEs form at a more rapid rate within LDL than apoB-AGEs, evidently because nonpolar, lipid micro-environments facilitate the dehydration and subsequent rearrangement reactions which occur as part of the advanced glycosylation pathway (3, 13, 19). Of particular importance, however, is the observation that both phospholipid- and LDL-advanced glycosylation are accompanied by the progressive formation of lipid oxidation products. In model studies performed either with purified LDL or phospholipid, AGE-mediated oxidative modification was shown to occur in the absence of exogenously added transition metals, to be dependent on the presence of primary amino groups within the phospholipid, and to be inhibited by aminoguanidine, which blocks the late stages of the advanced glycosylation reaction (13). Because advanced glycosylation can involve a succession of both intra- and intermolecular oxidation-reductions (1–3), the AGEs that form directly within a lipid phase might act to

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readily oxidize the unsaturated bonds contained in fatty acid side chains. Furthermore, the observation that these AGE-induced oxidative reactions occur in the absence of exogenously added free metals, which are often utilized to initiate lipid oxidation in vitro (20, 21), has led to the proposal that lipid-advanced glycosylation may represent an important and previously unrecognized mechanism for oxidative modification in vivo (13).

To gain additional insight into the chemical mechanisms by which advanced glycosylation reactions promote lipid oxidation, we performed gas chromatography-mass spectrometry (GC-MS) analysis of lipid products that form during LDL-advanced glycosylation in vitro. We report herein the identification of two previously characterized hydroxyalkenals, 4-hydroxyhexenal and 4-hydroxy2-nonenal, as major products of LDL-advanced glycosylation.

MATERIALS AND METHODS

Reagents—Glucose, EDTA, butylated hydroxytoluene (2,6-di-t-butyl-p-cresol), PE, and phosphatidylcholine (PC) (each containing a variety of polyunsaturated fatty acyl residues) were obtained from Sigma. O-Pentafluorobenzyl hydroxylamine and N,O-bis(trimethylsilyl) trifluoroacetamide were purchased from Aldrich. Authentic 9-hydroxyhexenal and 9-hydroxy2-nonenal was synthesized and provided by Prof. Hermann Esterbauer (University of Graz, Graz, Austria).

Lipid- and Lipoprotein-advanced Glycosylation—Human LDL was isolated from the plasma of control, non-diabetic, and non-renal dialysis patients by ultracentrifugation (d = 1.025–1.063) (22) and modified by advanced glycosylation as described previously (13). Briefly, 5 mg of purified LDL was aliquoted into 10 ml of de-aerated buffer consisting of 0.5 M PIPES (pH 7.4), 1 mM EDTA, and 200 mM glucose. The mixture was de-aerated under a stream of nitrogen for 30 min, sealed, and incubated in the dark at 37 °C for various lengths of time. AGE-modified phospholipids were prepared by incubating 5 mg of PE or PC suspended in 10 ml of the above buffer as described (13). Control incubations were performed in the absence of glucose and were shown previously to produce only low levels of spontaneously arising lipid oxidation products, as assessed by thiobarbituric acid-reactive substances (13).

The progress of lipid-advanced glycosylation was measured by a direct enzyme-linked immunosorbent assay, which relies on the recognition of the AGE-phospholipid epitope by an AGE-specific polyclonal antibody (4). This antibody does not show cross-reactivity with Cu2+, acetyl-, or malondialdehyde-modified LDL (4, 23). AGE values are expressed in units calculated relative to a synthetic AGE-BSA standard as described previously (4).

Advanced glycosylation reactions were terminated at various intervals by extracting the lipid-soluble products into 5 ml of chloroform/methanol (2:1). The aqueous layers were then re-extracted once and the organic layers combined and evaporated under N2. The lipid-soluble products were re-dissolved in 1.5 ml of methanol, and the oxime derivatives prepared by adding 2 ml of 0.1 M PIPES buffer (pH 6.5) containing 50 mM O-pentafluorobenzyl hydroxylamine, vortexing, and incubating at room temperature for 30 min (24). The derivatives were extracted with n-hexane (GC quality), the solvent evaporated, and the residue treated by adding 0.2 ml of N,O-bis(trimethylsilyl) trifluoroacetamide and heating for 10 min at 70 °C.

Gas Chromatography-Mass Spectrometry Analyses—GC-MS were performed with a Finnigan MAT TSQ 70 GC/MS system (Finnigan MAT, Munich, Germany). The following conditions were employed: a 25 m × 0.25 mm inner diameter fused-silica column coated with DB-5, a temperature gradient consisting of 60°C (2 min), 60–175°C (over 4.6 min), 175°C (1 min), 175–250°C (over 7.5 min), 250°C (10 min); carrier gas: helium at a split ratio of 1:10; injection volume, 10 μl; ionization, negative chemical ionization with methane, 70 eV; mass range, 40–400 amu. Ion abundance was quantified by area integration and comparison with spectra obtained from authentic 4-hydroxyalkenal species.

RESULTS AND DISCUSSION

Oxidation product formation during lipid-advanced glycosylation was first studied by measuring the time- and glucose-dependent appearance of thiobarbituric acid-reactive substances in buffer suspensions of phospholipids or LDL (13). Although widely employed, the thiobarbituric acid-reactive substances test is relatively nonspecific and relies on the detection of chromophores that can arise from a variety of reactive aldehyde species (25, 26). To investigate more precisely the formation of lipid oxidation products during LDL-advanced glycosylation, we analyzed by GC-MS the profile of lipid-soluble compounds that form after incubating freshly isolated LDL with glucose in vitro. These reactions were performed in the presence of EDTA and without added transition metals, conditions which in the absence of glucose have been shown previously to result in only minimal levels of spontaneous oxidation (13). Although the glucose concentrations that were used to prepare AGE-LDL were supraphysiological, it is important to note that in vivo AGEs arise not only from glucose, but also from more reactive AGE intermediates that circulate as a result of the catabolism of AGE-modified proteins (27). Of note, the level of AGE modification of LDL produced by these reactions (400–4600 units of AGE/mg of lipid) is comparable with the level which has been identified to be present in the circulation of patients with end-stage renal insufficiency (230–3260 units of AGE/mg of lipid) (13, 14).

Aliquots of LDL were removed at intervals and the lipid-soluble products extracted with chloroform/methanol, derivitized with O-PFB hydroxylamine, and analyzed by negative chemical ionization GC-MS. Fig. 1 shows the total ion curve for the O-PFB oxime, tetramethyliisilane ether derivatives of products isolated from control, native LDL, and AGE-modified LDL. It is apparent that AGE modification results in the formation of several new molecular ions that are absent in control, native

2 Y. Al-Abed and R. Bucala, unpublished observations.
LDL that had been incubated over the same period of time, but in the absence of glucose (Fig. 1).

Lipid decomposition products of the 4-hydroxy-2-alkenal series form readily as a consequence of lipid peroxidation and represent an important class of reactive, aldehyde products in vivo (26). 4-Hydroxyhexenal forms by oxidation of docosahexanoic acid (22:6), an abundant member of the n-3 fatty acid series, and 4-hydroxynonenal forms by oxidation of fatty acids of the n-6 series, such as linoleic (18:2), γ-linolenic (18:3), and arachidonic acids (20:4) (24–26). Fig. 2, A and B, show the ion-reconstructed chromatograms for mass ions of 110 and 152 atomic mass units, which represent the parent carbons chains of 4-hydroxyhexenal and 4-hydroxynonenal, respectively, after loss of the PFB and hydroxytrimethylsilane groups (24, 25). Fig. 3 shows these chromatograms on an expanded scale. As reported previously, the two peaks in each chromatogram (scans 413 and 423 at m/z = 110, and scans 606 and 631 at m/z = 152) correspond to the anti and syn stereoisomers of the 4-hydroxyhexenal and 4-hydroxynonenal derivatives that form by conversion of the aldehydes into oximes (24, 25). This was confirmed by examining the full negative ion chemical ionization mass spectra of the indicated ion current peaks in Fig. 2. These spectra, displayed in Fig. 4, showed mass peaks that were consistent with previously published chemical ionization analyses (24, 28). Fragments 110 and 241 are specific for 4-hydroxyhexenal (Fig. 4A), and fragments 152 and 283 are specific for 4-hydroxynonenal (Fig. 4B) (24, 25, 28). These spectra were identical to those obtained for authentic 4-hydroxyhexenal and 4-hydroxynonenal, which also showed the expected syn and anti isomers present in the same scan numbers and intensity ratio as in Fig. 3. In separate studies, mass spectra consistent with the formation of these two hydroxylkenal species were obtained by analyzing AGE-modified preparations of PE esterified with mixed polyunsaturated fatty acid residues. By contrast, 4-hydroxyhexenal and 4-hydroxynonenal were detected in only trace amounts in control incubations of PE alone, PC alone, or PC plus glucose (data not shown). These results confirm prior thioisobarbituric acid-reactive substance-based studies, indicating that reaction between glucose and the primary amino group of the phospholipid is a necessary condition both for the formation of lipid AGEs and lipid oxidation products (13).

We next performed a time course analysis for 4-hydroxyalkenal formation during LDL-advanced glycosylation. Signals corresponding to mass ions at 110 and 152 atomic mass units were identified by selective ion monitoring and their relative abundance estimated by peak area integration. These values then were compared with lipid AGE content, as quantified by AGE-specific enzyme-linked immunosorbent assay. LDL-advanced glycosylation produced a significant, time-dependent increase in hydroxylkenal formation that peaked at 5 days of incubation and then decreased to trace levels after 15 days (Fig. 5). 4-Hydroxynonenal was detected in higher yield than 4-hydroxyhexenal, as expected by the greater abundance within LDL of the precursor lipids for hydroxynonenal than for hydroxyhexenal (30). The rapid formation of these two hydroxyalkenal species compared with the more slow, time-dependent production of lipid AGEs suggests that oxidative modification occurs early in the time course of AGE formation. Once formed, hydroxylkenals are evidently consumed by various secondary reactions involving the nucleophilic constituents of the apoB amino acid side chains (26, 28, 29). As lipid precursors become depleted from the LDL particle, the formation of 4-hydroxynonenal and 4-hydroxyhexenal is no longer detectable by GC-MS.

The present study provides important chemical confirmation of the role of advanced glycosylation reactions in the oxidation of the unsaturated fatty acid groups present in LDL. Because this pathway of oxidative modification proceeds in the appar-
ent absence of exogenously added transition metals or free radical-generating systems, lipid- and lipoprotein-advanced glycosylation reactions may represent an important mechanism for lipid oxidation in vivo. Both AGEs (31) and oxidized LDL (32, 33) have been shown to be present in situ in areas of lipid and lipoprotein sequestration within the arterial wall. Reactive AGE intermediates thus may form within these regions and readily promote, or possibly initiate, lipid oxidation. Whether AGE intermediates in fact possess sufficient reactivity to abstract a hydrogen atom from the bis-allylic position of unsaturated bonds, the event initiating lipid oxidation, is of considerable interest. Such a role for advanced glycosylation reactions would provide an important link in the relationship between AGE formation and oxidative modification and offer new insight into the origin of atherogenic forms of LDL in vivo.

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FIG. 4. Full negative ion chemical ionization spectra of the m/z = 110 (A) and m/z = 152 (B) products identified by selective ion monitoring.

FIG. 5. Time course analysis for the formation of lipid AGEs (●), 4-hydroxynonenal (▲), and 4-hydroxyhexenal (▼) during LDL-advanced glycosylation. LDL was incubated with glucose as described under "Materials and Methods" and the lipid-linked-AGEs measured by AGE-specific enzyme-linked immunosorbent assay. Hydroxylalkenals were quantified by peak area integrations for their respective selected ions (syn + anti isomers) and are expressed as relative integration units. Values shown are the mean of two to three determinations.
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