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The Advanced Glycation End Product, N^{ϵ}-(Carboxymethyl)lysine, Is a Product of both Lipid Peroxidation and Glycoxidation Reactions*

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N^{ϵ}-(Carboxymethyl)lysine (CML) is an advanced glycation end product formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) reactions. We now report that CML is also formed during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of protein. During copper-catalyzed oxidation *in vitro*, the CML content of low density lipoprotein increased in concert with conjugated dienes but was independent of the presence of the Amadori compound, fructoselysine, on the protein. CML was also formed in a time-dependent manner in RNase incubated under aerobic conditions in phosphate buffer containing arachidonate or linoleate; only trace amounts of CML were formed from oleate. After 6 days of incubation the yield of CML in RNase from arachidonate was ~0.7 mmol/mol lysine compared with only 0.03 mmol/mol lysine for protein incubated under the same conditions with glucose. Glyoxal, a known precursor of CML, was also formed during incubation of RNase with arachidonate. These results suggest that lipid peroxidation, as well as glycoxidation, may be an important source of CML in tissue proteins *in vivo* and that CML may be a general marker of oxidative stress and long term damage to protein in aging, atherosclerosis, and diabetes.

Oxidative stress, an imbalance resulting from increased prooxidant generation and/or decreased antioxidant defenses, is implicated in the pathogenesis of numerous diseases, including atherosclerosis, diabetes, hemochromatosis, ischemic reperfusion injury, and rheumatoid arthritis, and in the normal aging process (1). In atherosclerosis, the oxidation of circulating low density lipoproteins (LDLs)[1] and their increased uptake by the scavenger receptor is thought to promote the deposition of lipid-laden macrophages in the vascular wall, leading to fatty streaks that precede the development of plaque (2). In diabetes, oxidative stress, resulting from the metabolic sequelae of hyperglycemia and/or nonenzymatic glycation of protein, leads to increased formation of advanced glycation end products (AGEs) in tissue proteins (3, 4). Among these AGEs are the glycoxidation products, N^{ϵ}-(carboxymethyl)lysine (CML) and pentosidine, which are the only chemically characterized AGEs known to accumulate in protein with age and at an accelerated rate in diabetes (5). Glycation and glycoxidation products and AGEs affect the structure and function of proteins and cross-linking of extracellular proteins, processes that are thought to contribute to the pathogenesis of vascular disease in diabetes and atherosclerosis (3–5).

The observations that the extent of glycation of plasma proteins, including LDL, is increased in diabetes (6) and that glycated proteins are redox-active and may thus catalyze lipid peroxidation reactions have suggested that increased glycation of LDL in diabetes might accelerate its oxidation, providing a mechanism for the excess risk for atherosclerosis associated with diabetes (7). Other studies have shown that plasma antioxidant defenses, including ascorbic acid and vitamin E concentrations, and total radical trapping activity are also compromised in diabetes (8). Although the combination of increased glycation of LDL and decreased antioxidant defenses in diabetic plasma provides the potential for catalysis of lipid peroxidation in plasma lipoproteins, there is no direct evidence of a correlation between glycation and enhanced oxidation of LDL in diabetes.

In an effort to understand the potential interplay between glycation and lipid peroxidation reactions, we initiated studies on how changes in levels of glycation affect lipid peroxidation and formation of glycoxidation products during metal-catalyzed oxidation of LDL *in vitro*. We report here the unexpected observation that CML is formed during copper-mediated oxidation of LDL, independent of the presence of the putative carbohydrate precursor, the Amadori compound fructoselysine (FL). We also report that CML, heretofore described as a glycoxidation product, is formed during peroxidation of polyunsaturated fatty acids (PUFA) in the presence of ribonuclease A (RNase), a protein that contains neither enzymatically nor nonenzymatically attached carbohydrate.

MATERIALS AND METHODS

Reagents—The following reagents were purchased from Sigma: diethylenetriaminepentaacetic acid, arachidonic acid, linoleic acid, oleic acid, and bovine pancreatic ribonuclease A (RNase, type II-A). Girard T reagent ((carboxymethyl)trimethyl-ammonium chloride) and glyoxal (40% monomer solution) were from Aldrich. CML and FL standards were synthesized as described (9).

Preparation and Copper Oxidation of LDL—Blood was obtained from healthy volunteers after an overnight fast. Blood was collected in EDTA (1.5 mg/ml), and plasma was separated by centrifugation. LDL was isolated by single vertical spin centrifugation (10), stored at 4 °C under nitrogen, and used within 48 h of isolation. The purity of LDL preparations was confirmed by agarose gel electrophoresis followed by stain-

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[1] The abbreviations used are: LDL, low density lipoprotein; AGE, advanced glycation end product; CML, N^{ϵ}-(carboxymethyl)lysine; FL, N^{ϵ}-(fructose)lysine; RNase, bovine pancreatic ribonuclease A; GC/MS, gas chromatography-mass spectrometry; PBS, phosphate-buffered saline (12); PUFA, polyunsaturated fatty acid; HPLC, high pressure liquid chromatography.

ing for both lipid and protein using a Paragon LIPOEPG system (Beckman Instruments, Palo Alto, CA), which showed a single lipid and protein band; contamination with albumin was not detected. LDL protein concentration was estimated by the method of Lowry (11). LDL pools were prepared by combining LDL from three individuals.

To remove salts, EDTA, and water-soluble antioxidants prior to copper oxidation experiments, LDL was chromatographed on PD-10 columns (Pharmacia Biotech Inc.) equilibrated in phosphate-buffered saline (PBS; Ref. 12) that had been gassed with nitrogen. The LDL was sterilized by ultrafiltration (0.22-μ filters, CoStar, Cambridge, MA) and then diluted to ~100 μg of protein/ml using PBS that had been bubbled with oxygen for at least 10 min. The diluted samples were adjusted to 5 μM $CuCl_2$, placed in loosely capped plastic bottles, and incubated at 32 °C; copper was omitted in control samples. The progress of the oxidation reaction was monitored by measuring absorbance at 234 nm in aliquots removed at various times. For analysis of CML, aliquots (~1 mg of protein) were adjusted to 1 mM in diethylenetriaminepentaacetic acid (0.1 M) in sodium borate, pH 9.2, and reduced overnight at 4 °C with a final concentration of 25 mM $NaBH_4$. The samples were then dialyzed against water, dried by centrifugal evaporation (Savant SpeedVac, Farmingdale, NY), and delipidated using methanol:ether (3:10) (13). For analyses of FL, samples were treated with diethylenetriaminepentaacetic acid, dialyzed immediately, dried, and delipidated.

Modification of RNase with Fatty Acids or Glucose—Reaction mixtures, in triplicate, were prepared containing 1 mM RNase (equal to 10 mM lysine) and either 100 mM fatty acid or 100 mM glucose in PBS. The required amounts of fatty acids dissolved in $CHCl_3$ were placed in sterile 20-ml glass scintillation vials, and the solvent was removed under a stream of nitrogen passed through a 0.22-μ filter. All subsequent additions and the removal of sample aliquots at various times were conducted using sterile technique in a tissue culture hood. Solutions of RNase or glucose prepared in sterile PBS were sterilized by ultrafiltration (0.22-μ filter) and added as appropriate to the vials containing fatty acid. The vials were covered with sterile caps and placed in a shaking water bath at 37 °C. Aliquots (0.2–0.4 ml) were removed at desired times and processed immediately. Fatty acids were removed by Folch extraction (14). The aqueous layer and protein interface from the Folch extracts were back-extracted with theoretical Folch lower phases and dialyzed against water, and the protein was then processed for measurement of CML. To limit oxidation of FL to CML during sample hydrolysis, aliquots of protein incubated with glucose were first reduced with $NaBH_4$ prior to measurement of CML (9). Recovery of CML in protein incubated with fatty acids only was unaffected by prior reduction with $NaBH_4$. In some experiments a second aliquot (0.7 ml) was used for measurement of glyoxal. Following acidification with 25 μl of 3 M HCl and the addition of 100 μl of Girard T reagent (0.5 N) (16), samples were incubated at room temperature for 1 h and then stored at −20 °C; samples were analyzed by HPLC (16) in a single batch at the end of the experiment.

Protein Hydrolysis and GC/MS Analyses—Following the addition of d_8-lysine and d_8-CML or $^{13}C_6$-FL internal standards, proteins were hydrolyzed in 6 N HCl for 24 h at 110 °C. After drying by centrifugal evaporation, the dried hydrolysates were dissolved in 3 ml of 20% methanol containing 0.1% trifluoroacetic acid and applied to 3-ml Sep-Pak columns (Supelco, Bellefonte, PA) to remove brown and lipophilic materials prior to derivatization for GC/MS. The eluates were again dried and then derivatized for measurement of CML or FL by GC/MS as their trifluoroacetyl methyl ester derivatives (9).

RESULTS

Fig. 1 describes the kinetics of metal-catalyzed oxidation of normal LDL and concurrent changes in the protein's content of CML and FL. Comparison of the kinetics of conjugated diene (*panel A*) and CML (*panel B*) formation indicates that the rate of CML formation parallels the rate of fatty acid peroxidation. Neither conjugated dienes nor CML increased in the absence of copper. Because we assumed initially that the CML was derived from oxidation of the Amadori adduct on LDL, the FL content of the protein was also measured at various stages in the oxidation reaction. The data in *panel C* demonstrate unexpectedly that, unlike CML, the FL content of LDL remained constant during the course of the copper oxidation. We concluded tentatively that the CML was being formed from a product of oxidation of the lipid component of the LDL. To verify that CML could be formed independent of the presence of

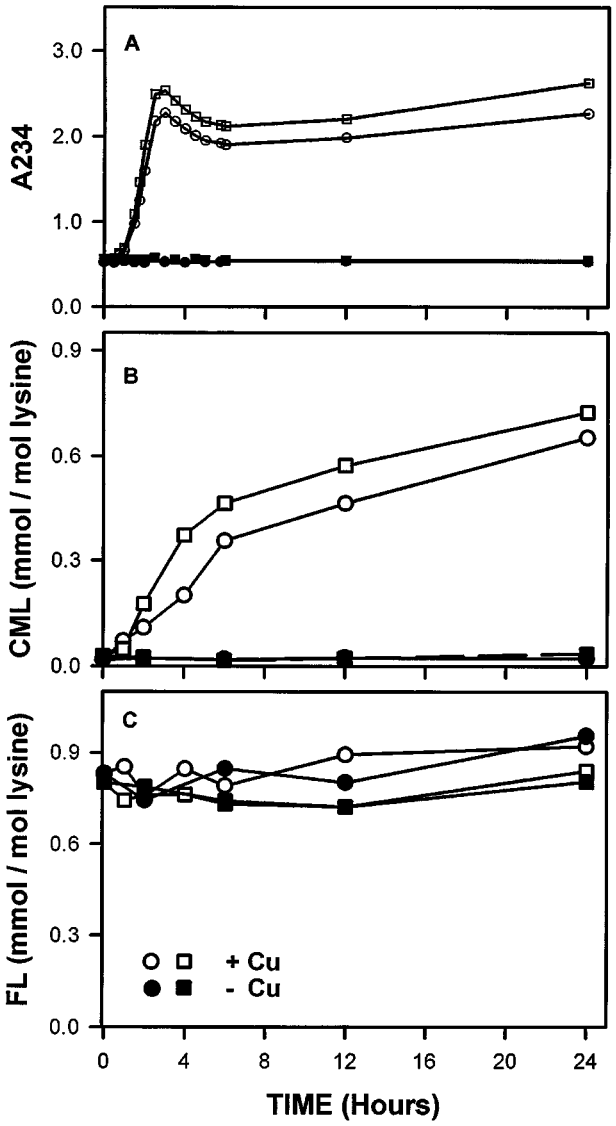

FIG. 1. **Copper-catalyzed oxidation of LDL results in an increase in CML without a change in the FL content of the protein.** LDL was incubated with (*open symbols*) or without (*closed symbols*) 5 μM copper at 32 °C. The progress of the oxidation reaction was monitored by following conjugated diene formation at 234 nm (*A*). The amounts of CML (*B*) and FL (*C*) were measured by GC/MS as described under "Materials and Methods." Data are shown for two different pools of LDL.

FL, the LDL was reduced with $NaBH_4$ to convert FL to the inert, redox-inactive hexitollysine adduct. Table I shows that ≤5% of the original FL remained on the reduced LDL, yet following oxidation of the reduced lipoprotein, the yield of CML was similar to that formed during oxidation of the native protein. The absorbance traces during metal-catalyzed oxidation of $NaBH_4$-reduced LDL were similar to those of the native protein (data not shown). These results indicate that CML can be formed on lipoprotein from product(s) of PUFA oxidation.

To further characterize the formation of CML during lipoxidation reactions, model experiments were carried out in which PUFAs were oxidized in the presence of RNase A, a protein devoid of carbohydrate. As shown in Fig. 2, oxidation of linoleate and arachidonate in the presence of RNase yielded a time-dependent increase in CML residues in the protein. In these experiments the fatty acids were progressively solubilized in buffer as they autoxidized, and the experiments were arbitrarily terminated at 6 days when the arachidonate reactions

TABLE I
Effect of $NaBH_4$ reduction on formation of FL and CML during copper oxidation of LDL

Sample	Time	FL	CML
	h	*mmol/mol lysine*	
Native LDL[a]	0	0.76–0.80	0.034–0.059
Native LDL[a]	24	0.76–0.84	0.67–1.09
Reduced LDL[b]	0	≤0.05	0.043–0.077
Reduced LDL[b]	24	≤0.05	0.98–1.1

[a] LDL was incubated at 32 °C with 5 μM copper, and samples were removed at indicated times for measurement of FL and CML as described under "Materials and Methods." Data are values for two separate experiments.

[b] LDL was reduced with $NaBH_4$ in the presence of 1 mM diethylenetriaminepentaacetic acid and then dialyzed against PBS prior to incubation with copper as described above for native LDL. Data are values for two separate experiments.

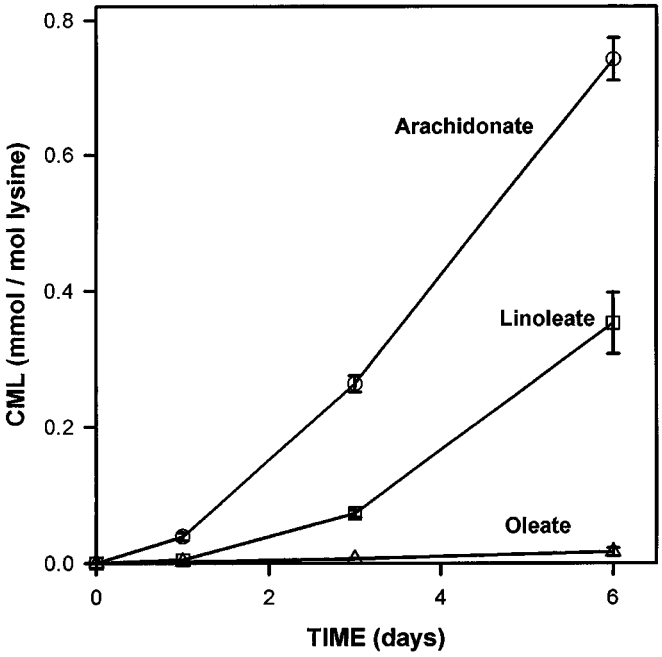

FIG. 2. **CML is formed during incubation of RNase with PUFA.** RNase (1 mM) was incubated with 100 mM arachidonate (○), linoleate (□), or oleate (△) in PBS at 37 °C, and aliquots were removed at indicated times. CML was measured by GC/MS as described under "Materials and Methods." There was no CML detected in RNase incubated in the absence of fatty acid. Data (mean ± S.D.) are for single measurements of individual aliquots from three separate incubations; absence of error bars indicates error was within size of *symbol*.

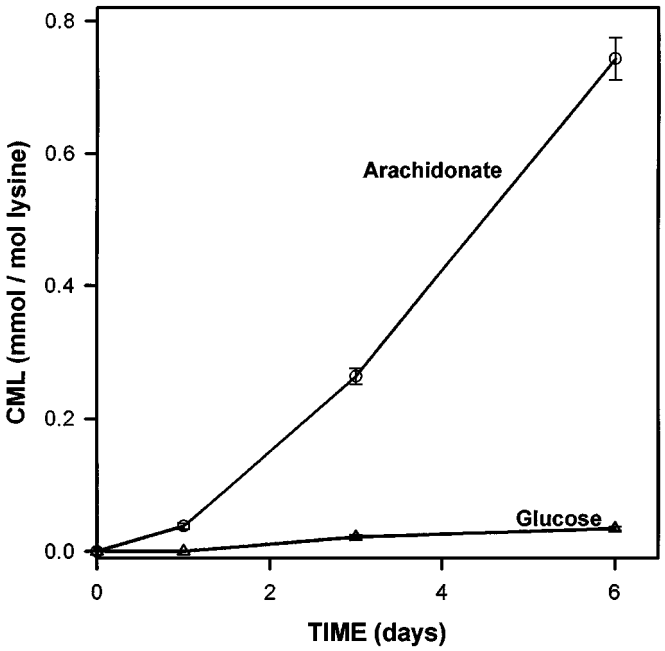

FIG. 3. **Comparison of CML formation in RNase from arachidonate or glucose.** RNase (1 mM) was incubated with 100 mM arachidonate (○, replotted from Fig. 2), or 100 mM glucose (△) in PBS. CML was measured by GC/MS as described under "Materials and Methods." Data are expressed as described in the legend to Fig. 2.

became a single phase. The overall amount of CML formed was dependent on the degree of unsaturation and oxidizability of the fatty acid, with the greatest yield of CML formed from arachidonate, an intermediate yield from linoleate, and only trace amounts formed from oleate. The difference in yields of CML from the various fatty acids probably reflects differences in their extent of oxidation at the end of the experiment.

Because CML was originally identified as a glycoxidation product (5, 17), *i.e.* the result of combined glycation and oxidation reactions, we also compared the relative yield of CML from glucose and arachidonate. Fig. 3 shows that at the end of 6 days' incubation the yield of CML from autoxidizing arachidonic acid, 0.74 ± 0.03 mmol/mol RNase, was significantly greater than that from glucose, 0.03 ± 0.003 mmol/mol RNase. These results indicate that both carbohydrates and lipids may contribute to formation of CML during autoxidation reactions in physiological buffer. However, oxidation of fatty acid is clearly a more efficient source of CML, despite the fact that the glucose is in solution throughout the course of the experiment, while the PUFA are only progressively solubilized. Further, after 6 days of incubation, a large fraction of the arachidonate was oxidized based on its solubilization in the aqueous phase, while ≤2% of the glucose is oxidized during this same time period (16). Finally, pentosidine, a second glycoxidation product known to form during glycoxidation reactions *in vitro* and *in vivo*, was detected in incubations of RNase and glucose, but no pentosidine was detected either in incubations of RNase with PUFA or in copper-oxidized LDL.

We have previously shown that glyoxal is both a product of glucose autoxidation and a source of CML in protein (16). Glyoxal formation has also been reported during UV irradiation of PUFA (18) and during oxidation of linolenic acid in an iron ascorbate model system (19, 20), although in the latter case formation of glyoxal from iron ascorbate itself was not excluded. In the present experiments the formation of glyoxal from PUFA was monitored by trapping it as the Girard T adduct (16). The data in Fig. 4 show that there was a progressive increase in the amount of glyoxal formed in autoxidizing arachidonic acid incubations, which was not significantly affected by the presence of protein. The data also indicate that the amount of glyoxal formed during arachidonate oxidation was more than sufficient to account for the amount of CML formed on protein, if glyoxal were the only source of all the CML formed.

DISCUSSION

The observations described above indicate that CML, previously described as a glycoxidation product or AGE, may, in fact, be derived from PUFA during lipid peroxidation reactions. These observations require a reassessment of previous work on (*a*) the biochemical origin of AGEs, (*b*) the significance of carbohydrate oxidation, autoxidative glycosylation, and glycoxidation in the chemical modification of proteins in diabetes, and, in general, (*c*) mechanisms of oxidative stress and pathways of oxidative damage to protein and other biomolecules in aging, atherosclerosis, and diabetes.

Biochemical Origin of CML—Our studies indicate that lipid

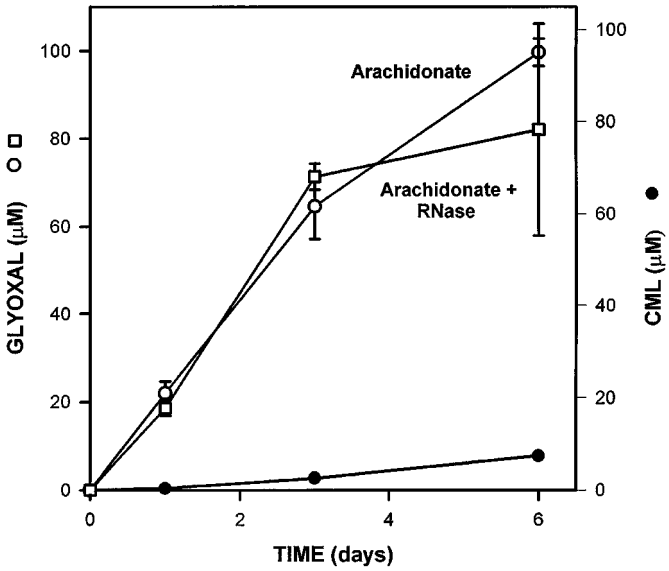

FIG. 4. **Glyoxal formation from arachidonic acid.** Aliquots of reaction mixtures containing either arachidonate alone (○) or arachidonate and RNase (□) were removed at indicated times, and glyoxal was measured by HPLC as described under "Materials and Methods." Data are the average ± range for two separate experiments. For comparison the data from Fig. 2 for CML formation from arachidonate (●), expressed as μM, are also shown; absence of error bars indicates error was within size of *symbol*.

peroxidation is a potential source of CML during lipoprotein oxidation and is the major source of CML formed during metal-catalyzed oxidation of LDL *in vitro*. Since PUFAs, both in biological systems and *in vitro* (Fig. 3) are, in general, more easily autoxidized in free radical reactions than are carbohydrates, it is quite possible that the majority of CML in tissue proteins is derived from lipid peroxidation reactions, even during hyperglycemia when concentrations of glucose and Amadori products on protein are increased. Indeed, the fact that the FL on LDL was not a significant source of CML formation during metal-catalyzed oxidation of LDL suggests that glucose and Amadori adducts may be less important sources of CML in protein, even in diabetes.

The mechanism of formation of CML and other AGEs during carbohydrate oxidation reactions is still uncertain. The pathway may involve oxidation of free glucose or protein-bound intermediates, including carbinolamine, Schiff base, and Amadori adducts (17, 21, 22). Wells-Knecht *et al.* (16) identified glyoxal as one intermediate in the formation of CML from glucose. Since glyoxal is also formed during peroxidation of PUFA (Refs. 18–20 and Fig. 4), it may be a common intermediate in the formation of CML during oxidation of both carbohydrates and lipids. However, other common intermediates may also be involved, such as glycolaldehyde (21), α-hydroxyaldehydes, or β,γ-unsaturated α-hydroxyaldehydes or dicarbonyls, which may be formed from both carbohydrate and PUFA oxidation. The intersection of carbohydrate and lipid autoxidation reactions in the formation of CML emphasizes the relationship between the fundamental chemistry and biochemistry of these molecules.

Significance of Lipid Peroxidation Reactions in the Maillard Reaction in Vivo—The importance of lipid peroxidation in the formation of AGEs and cross-linking of proteins is suggested by several experimental observations. The browning and cross-linking of extracellular proteins increase so rapidly in animal models of diabetes (23) that, in the absence of large endogenous pools of decompartmentalized transition metal ions, the origin of the browning products is more readily explained by lipid rather than carbohydrate oxidation reactions. AGEs also accumulate within a few days on intracellular proteins of cells grown in high glucose media (24). The rapidity of AGE formation and the effect of antioxidants as inhibitors of acute glucose toxicity (25) suggests that they may act by inhibiting glucose-induced lipid peroxidation reactions (26). Finally, it should be noted that aminoguanidine, which is a potent inhibitor of glycoxidation reactions *in vitro* (17, 21), the browning and cross-linking of collagen *in vivo* (23), and the development of complications in diabetic animals (3, 4), also inhibits lipid peroxidation reactions (27–29) Thus, this compound, which has been described primarily as an inhibitor of advanced glycation reactions, may in fact be exerting its effects *in vivo* by inhibition of lipid peroxidation reactions and/or by trapping of lipid peroxides and PUFA-derived carbonyl compounds, including glyoxal.

Mechanisms of Oxidative Stress and Damage in Vivo—Because similar intermediates may be derived from both carbohydrate and lipid peroxidation reactions, it has become difficult to discern the primary source of oxidative damage to protein in complex biological matrices. Palinski *et al.* (30) have recently reported, for example, that anti-AGE antibodies detect an increase in AGE epitopes in atherosclerotic plaque in *normo*glycemic animals. Our previous work identifying CML as a major antigenic determinant in AGE proteins (31) and the present work identifying CML as a product of lipid peroxidation suggest that the increase in AGE epitopes in atherosclerotic plaque could result from an increase in CML derived from lipid peroxidation reactions. In addition, lipid-derived CML may also be the major AGE epitope accumulating in intracellular protein in endothelial cells exposed to glucose-induced oxidative stress (24).

To distinguish carbohydrate- from lipid-mediated damage and assess the relative importance of oxidation of these two substrates in the development of diabetic complications, it will be necessary to identify unique products derived from each of these precursors. In the meantime, since CML has been identified as a major AGE antigen in tissue proteins and a product of both carbohydrate and lipid peroxidation reactions, our results suggest that CML may be more useful as a general biomarker of oxidative stress and damage in tissue proteins.

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Carbohydrates, Lipids, and Other Natural Products:

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