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LIPIDS AND LIPOPROTEINS: Pyridoxamine, an Inhibitor of Advanced Glycation Reactions, Also Inhibits Advanced Lipoxidation Reactions: MECHANISM OF ACTION OF PYRIDOXAMINE

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Maillard or browning reactions lead to formation of advanced glycation end products (AGEs) on protein and contribute to the increase in chemical modification of proteins during aging and in diabetes. AGE inhibitors such as aminoguanidine and pyridoxamine (PM) have proven effective in animal model and clinical studies as inhibitors of AGE formation and development of diabetic complications. We report here that PM also inhibits the chemical modification of proteins during lipid peroxidation (lipoxidation) reactions in vitro, and we show that it traps reactive intermediates formed during lipid peroxidation. In reactions of arachidonate with the model protein RNase, PM prevented modification of lysine residues and formation of the advanced lipoxidation end products (ALEs) N\textsuperscript{ε}-carboxymethyllysine, N\textsuperscript{ε}-carboxyethyllysine, malondialdehyde-lysine, and 4-hydroxynonenal-lysine. PM also inhibited lysine modification and formation of ALEs during copper-catalyzed oxidation of low density lipoprotein. Hexanoic acid amide and nonanedioic acid monoamide derivatives of PM were identified as major products formed during oxidation of linoleic acid in the presence of PM. We propose a mechanism for formation of these products from the 9- and 13-oxo-decadienoic acid intermediates formed during peroxidization of linoleic acid. PM, as a potent inhibitor of both AGE and ALE formation, may prove useful for limiting the increased chemical modification of tissue proteins and associated pathology in aging and chronic diseases, including both diabetes and atherosclerosis.

Aging and age-related diseases, such as diabetes, atherosclerosis, and neurodegenerative diseases, are characterized by increases in oxidative chemical modification of tissue proteins (1, 2). Some oxidation products, e.g. methionine sulfoxide and \( \alpha \)-tyrosine, are formed by direct oxidation of amino acids in proteins. Glycoxidation products or advanced glycoxidation end products (AGEs), such as the cross-links pentosidine and vesperlysines (3, 4), are produced by secondary modification of proteins by products of carbohydrate oxidation. Advanced lipoxidation end products (ALEs), including malondialdehyde (MDA) and hydroxynonenal (HNE) adducts to lysine (MDA-Lys, HNE-Lys) (5) and pyroles (6), are formed during lipid peroxidation reactions, whereas other compounds, such as N\textsuperscript{ε}-carboxymethyllysine (CML) and N\textsuperscript{ε}-carboxyethyllysine (CEL), are formed during both glycoxidation and lipoxidation reactions (7). Age-adjusted levels of pentosidine and CML are increased during chronic hyperglycemia and correlate with the severity of renal, retinal, and vascular complications in diabetes (8). Inhibitors of AGE formation, such as aminoguanidine (9), tenilstatem (10), OPB-9195 (11), and pyridoxamine (PM) (12, 13), also retard the development of diabetic complications in animal models, and aminoguanidine and PM are being evaluated in clinical trials for treatment of diabetic nephropathy. Although AGE inhibitors vary widely in structure, they are all nucleophilic compounds and are thought to function by trapping reactive carbonyl intermediates in AGE formation. Because they are relatively nonspecific nucleophiles, AGE inhibitors also affect other oxidative chemistry, e.g. the formation of ALEs. Aminoguanidine, for example, inhibits chemical modification of proteins during lipid peroxidation reactions (14) and inhibits metal-catalyzed oxidation of low density lipoprotein (LDL) and uptake of oxidized LDL into macrophages via the scavenger receptor (15).

The AGE inhibitor PM was introduced by Hudson and colleagues (12, 13) as an inhibitor of AGE formation from Amadori compounds, the first stable sugar-protein adducts formed during glycation of protein. In animal model studies, PM also inhibited AGE formation and retarded the development of nephropathy in streptozotocin-induced diabetic rats.\(^\text{\textsuperscript{2,3}}\) However, the renal protection was accompanied by a significant reduction in hyperglycemia, and there were strong correlations between plasma triglyceride concentration and the levels of CML and CEL, fluorescence, and cross-links in skin collagen. These observations suggested that, in addition to its AGE-inhibitory activity, PM might also affect lipid homeostasis and...
lipid-dependent chemical modification of protein in vivo. In this paper we describe experiments in vitro on the effects of PM on the chemical modification of proteins during lipid peroxidation reactions, and we show that PM is a potent inhibitor of modification of lysine by peroxidizing lipids, both in a model protein-lipid system and during copper-catalyzed oxidation of LDL. We also identify the major products formed on reaction of linoleic acid (LA) with PM under autioxidative conditions, the hexanoic acid amide and nonanedic acid monoamide derivatives of PM, and we propose a mechanism for PM inhibition of chemical modification of proteins during lipoxidation reactions. Our results provide insight into the mechanism of action of PM as an AGE and ALE inhibitor, and we suggest that PM may be useful for inhibiting the increased chemical modification of tissue proteins, whether derived from carbohydrate or lipids, in diabetes, atherosclerosis, and other chronic diseases.

MATERIALS AND METHODS

Chemicals—Arachidonic acid (AA), LA, oleic acid, palmitic acid, PM(HCl), pyridoxal(HCl), pyridoxine(HCl), ribonucleas A (type II-A from bovine pancreas; RNase), diethylenetriaminpentaaetic acid (DTPA), ptthylene glycol butyldiyethylhexyl amine, NaBH 4, hexanoyl (caproyl) chloride, methyl linoleate, and nonanedioic (azelaic) acid mono- methyl ester were purchased from Sigma. Boron trichloride-methanol was purchased from Pierce and heptfluorobutyric acid from Acros Chemicals (Atlanta, GA). [ 1 H]- and [ 2 H]-lysine were from Isotec (Miamisburg, OH). Deuterated and natural abundance internal standards (CML and [ 1 H]-CML, CEL and [ 1 H]-CEL, MDA-Lys and [ 1 H]-MDA-Lys, and HNE-Lys and [ 1 H]-HNE-Lys) were prepared as described previously (5, 18, 19).

Recom of PM with Fatty Acids—PM (1 mM) was incubated with AA, LA, or oleic acid (5 mM) in 5 ml of filter-sterilized 200 mM sodium phosphate buffer, pH 7.4, in 20-nl scintillation vials for 6 days at 37 °C in a shaking water bath. Reactions were initially biphasic but, in the case of the polyunsaturated fatty acids (PUFA) AA and LA, became monophasic by day 3. Aliquots were removed from the aqueous phase and stored at −20 °C until analyzed. Modification of the free amino group of PM was measured by the trinitrobenzenesulfonic acid (TNBS) assay (20), using PM as standard. Reversed-phase high performance liquid chromatographic analysis of the samples was carried out on a Waters (Milford, MA) HPLC system, using a Supelco (Bellefonte, PA) C-18 column (4.6 mm × 25 cm). The gradient (flow rate 1 ml/min; initial temperature 75 °C, 6 °C/min ramp to 110 °C, 10 °C/min ramp to 270 °C, hold 5 min) was collected, and the column was washed with 3 ml of 0.1% heptafluorobutyric acid in deionized water:acetonitrile (1:1, v/v). The eluate was pooled from the flow-through fraction, dried in vacuo, and the resulting free carboxylic acids (hexanoic and nonanedioic acid amide and nonanedioic acid monoamide derivatives of PM, acid amide and nonanedioic acid monoamide derivatives of PM, acid amide and nonanedioic acid monoamide derivatives of PM, acid amide and nonanedioic acid monoamide derivatives of PM, acid amide and nonanedioic acid monoamide derivatives of PM) were identified by TMS derivatization and by comparison with the authentic samples. The eluate was dissolved in CHCl 3:CH 3 OH, according to Folch (22), and the lower organic phase was removed. The upper aqueous phase and interfacial protein were reduced by addition of a 5-fold excess of NaBH 4 over lipid in 0.1 M borate buffer, pH 9, for 4 h at room temperature. The samples were then transferred to dialysis tubing (6000–8000 molecular weight cut-off) and dialyzed against 1000 ml of distilled water at 4 °C with several water changes. An aliquot of the dialysate, equivalent to ~100 µg of protein, was removed, diluted with an equal volume of concentrated HCl, and hydrolyzed (110 °C for 24 h) for amino acid analysis. Modification of lysine residues was measured by amino acid analysis, conducted by cation-exchange chromatography, using post-column derivatization with o-phthalaldehyde and fluorescence detection. Analysis of lipoxidation products, the reaction products, was carried out in vacuo, deuterated internal standards ([ 1 H]-Lys, [ 1 H]-CML, [ 1 H]-CEL, [ 1 H]-MDA-Lys, and [ 1 H]-HNE-Lys) were added, and the sample was hydrolyzed in 6 N HCl, as above. The hydrolysate was dried in vacuo, reconstituted in 1 ml of 0.1% trifluoroacetic acid, and applied to a 1 ml C-18 Sep-Pak (Waters Associates, Milford, MA) equilibrated with 0.1% trifluoroacetic acid. The flow-through fraction from the column was collected, and the conjugated dienes, an index of lipid peroxidation, was monitored at 234 nm. Aliquots corresponding to ~1 mg of LDL protein were removed at various times, reduced with NaBH 4, and hydrolyzed for amino acid analysis and measurement of ALEs, as described previously (18).

Other Methods—N-Hexanoyl-pyridoxamine was synthesized from PM(HCl), and hexanoyl chloride. PM (20 mg) was dissolved in 50 ml of 2 mM NaOH in a 250-ml round bottom flask. Hexanoyl chloride (22 mg in 30 ml of dry ether) was added droppewise over an hour while the reaction was kept on ice and stirred vigorously. The reaction was stirred for an additional 2 h and then the ether layer was removed and dried under nitrogen, yielding a white powder. N-Hexanoyl-PM was identified and isolated by RP-HPLC with detection by absorbance and electrospray ionization/mass spectrometry (ESI-LC-MS), as described below.

N-Nonanediyl-pyridoxamine was synthesized according to D’Alelio and Reid (23). Briefly, 0.3 g of PM in 0.3 ml of deionized water was added to 0.3 g of azelaic acid monomethyl ester and heated at 140 °C for 1 h. The brown reaction mixture was dried in vacuo and reconstituted in deionized water:acetonitrile (1:1, v/v), N-Nonanediyl-PM was isolated and identified by RP-HPLC with detection by absorbance and ESI-LC-MS, as described below.

N-Acyl-PD derivatives were hydrolyzed in 2 N HCl for 4 h at 95 °C, releasing free PM and carboxylic acids. The hydrolysate was dried in vacuo, and the resulting free carboxylic acids (hexanoic and nonanedioic acids) were analyzed by GC/MS as their propyl and dimethyl esters, respectively. Esterification was performed in 1 ml of 1 M HCl in n-propyl alcohol or methanol at 65 °C for 1 h. After drying in vacuo, the esters were extracted into 2 ml of hexane:water (2:1, v/v). Following vortexing and centrifugation, the hexane phase was analyzed by GC/MS, as described below. The aqueous phase, containing PM, was dried in vacuo, and the PM was acetylated by reaction with acetic anhydride: pyridine (1:1, v/v) for 2 h at room temperature. Following evaporation in vacuo, the product was reconstituted in methylene chloride for GC/MS analysis.

Mass Spectrometry—ESI-LC-MS was carried out in the positive ion mode on a VG (Manchester, UK) Trio-3 triple quadrupole mass spectrometer equipped with a Hewlett-Packard (Palo Alto, CA) series 1100 HPLC system and a Keystone (Bellefonte, PA) Aquasil C-18 microbore column. The solvent system consisted of 0.1% heptafluorobutyric acid (solvent A) and acetonitrile (solvent B), flow rate 0.05 ml/min. The program for analyses of PM and carboxylic acid esters was as follows: initial temperature 75 °C, 6 °C/min ramp to 110 °C, 10 °C/min ramp to 180 °C hold 5 min, 12 °C/min ramp to 270 °C/min, hold 5 min. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a VG (Manchester, UK) Septima instrument. A solution of PM in methanol (1:1, v/v) was spotted onto a glass slide, and the eluent was allowed to evaporate. The sample was then introduced into the FAB source by a vacuum-driven syringe. The mass spectrometric conditions and the instrument settings are summarized below. The aqueous phase, containing PM, was dried in vacuo, and the PM was acetylated by reaction with acetic anhydride: pyridine (1:1, v/v) for 2 h at room temperature. Following evaporation in vacuo, the product was reconstituted in methylene chloride for GC/MS analysis.
bombardment-MS was performed on a VG (Manchester, UK) 70 SQ high resolution, magnetic sector mass spectrometer.

RESULTS

Reaction of PM with PUFAs—To study the interaction of PM with products of lipid peroxidation, PM was incubated with oleate or PUFA in phosphate buffer at physiological pH, relying on endogenous metal ions in the phosphate buffer to catalyze PUFA oxidation. As shown in Fig. 1A, PM incubated alone or in the presence of oleate was recovered unchanged over the 6-day incubation period. However, in the presence of LA and AA, PM was gradually consumed, with ~60% PM loss at 6 days, based on HPLC analysis (see inset to Fig. 4). When the same samples were analyzed by the TNBS assay (Fig. 1B), ~60% of the free amino groups of PM was also modified during peroxidation of PUFA. The close agreement between the loss of PM by HPLC and the loss of amino groups by the TNBS reaction suggested that PM was reacting with products of lipid peroxidation via its benzylic amino group. PUFA were also solubilized at similar rates in the presence and absence of PM, and as shown in Fig. 2, the kinetics of formation and yield of TBARs and the rate of loss of LA, the primary PUFA in LDL (see below), were only slightly affected by the presence of PM in reaction mixtures at 1 mM concentration. These experiments indicate that PM, at the concentration used (1 mM) in the above experiment, did not prevent lipid peroxidation by chelation or antioxidant activity.

PM Inhibition of Lipoxidative Modification of RNase by AA—The activity of PM as an inhibitor of lipoxidative modification of proteins was studied in reactions of AA with RNase. As shown in Fig. 3, CML, CEL, MDA-Lys, and HNE-Lys were formed continuously on RNase during the incubation with AA. These studies confirm previous reports on the formation of CML during lipid peroxidation reactions (18) and also identify CEL as a product of arachidonate oxidation. The decline in MDA-Lys recovered after 1 day resembles that observed during copper oxidation of LDL and is thought to result from further reaction of MDA-Lys to form other products, such as Lys-MDA-Lys (5). Addition of 1 mM PM to the AA plus RNase mixture resulted in almost complete inhibition of formation of all the ALEs. Notably, PM provided potent protection against lipoxidative modification of RNase, although it was present at only 1% the concentration of AA, and 10% the concentration of lysine residues in RNase. The small increase in formation of lipoxidation products by day 6 likely reflects the fact that all of the PM had been consumed by that time, as shown in Fig. 4. Amino acid analysis indicated that there was a progressive loss of lysine during the reaction, with about 58% of lysine residues lost at the end of 6 days in the absence of PM (Fig. 5). In contrast, inclusion of PM in the incubation resulted in loss of only about 5% of lysine residues by day 6.

Inhibition of LDL Oxidation by PM—Because PM inhibited lipoxidation product formation in the AA plus RNase system, we also evaluated its effectiveness as an inhibitor of lipoxidation during copper-catalyzed oxidation of LDL, a common in vitro model for studies on lipoxidative modification of proteins. PM was used at concentrations of 100 and 250 μM, comparable to the ~120 μM concentration achieved in rats treated with 1 g/liter in drinking water (2, 3). At these concentrations, PM caused a 2–3-fold increase in the lag phase of LDL oxidation and a ~20% decrease in absorbance at 234 nm during the later plateau phase of the reaction (Fig. 6A). Confirming the results of experiments with AA and RNase, PM was an effective inhibitor of formation of CML, CEL, MDA-Lys, and...
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Fig. 3. Pyridoxamine inhibits formation of ALEs during peroxidation of arachidonate in the presence of RNase. RNase (1 mM) was incubated with arachidonate alone (■) (100 mM) or in the presence of PM (△) (1 mM) in 200 mM phosphate buffer, pH 7.4, for 6 days at 37 °C. By 3 days of incubation, all of the arachidonate had been solubilized, yielding a monophasic system. At various times, aliquots of the reaction were reduced with NaBH₄, then dialyzed and prepared for GC/MS as described under “Materials and Methods.” Approximately 1 mg of protein was analyzed by selected ion monitoring (SIM)-GC/MS for oxidation of arachidonate in the presence of RNase. Aliquots of the reaction mixture described in the legend to Fig. 3 were analyzed by amino acid analysis for their lysine content; data are expressed relative to lysine content of native RNase. Data are means ± S.D. of three independent experiments.

Fig. 4. Pyridoxamine is consumed during inhibition of chemical modification of RNase by arachidonate. RNase was incubated with arachidonate and PM (1 mM) as described in the legend to Fig. 3. Aliquots were removed at various times and assayed for residual PM by HPLC (inset). Pyridoxal (PL) was added as an internal standard. Data are means ± S.D. of three independent experiments.

HNE-Lys (Fig. 6B) during metal-catalyzed oxidation of LDL. The relative yields of the ALEs during oxidation of LDL differed significantly from yields obtained in reactions of AA with RNase, probably reflecting the heterogeneous fatty acid composition of LDL. PM was more effective in limiting formation of CML and CEL, compared with MDA-Lys and HNE-Lys (Fig. 6B), but was a potent inhibitor of the overall modification of lysine residues in oxidized LDL (Fig. 6C), with only 6% loss in the presence of PM compared with 29% in its absence.

Identification of PM Adducts—To identify products trapped by PM during PUFA oxidation, we studied the reaction of PM (1 mM) with LA, the primary PUFA in LDL. HPLC analyses of the LA + PM reaction, described in Fig. 7, indicated formation of products with fluorescence (Fig. 7, B versus A) and absorbance (Fig. 7C) maxima characteristic of PM. The consumption of PM over a period of 6 days of reaction is evident, as well as the time-dependent formation of new products eluting later than PM, indicating that these adducts were more hydrophobic than PM itself. Two major fluorescent products were consistently observed, eluting at ~24 min. Based on their fluorescence intensity, these two products accounted for ~10% of the PM in the starting reaction, whereas ~65% of the starting PM was consumed by 6 days (Fig. 1). The fluorescence of the total reaction mixture was also measured at 6 days, to establish that fluorescent reaction products were not binding irreversibly to the C-18 column. These measurements confirmed a significant ~50% decrease in fluorescence during the reaction, consistent with the overall loss of fluorescence observed in the HPLC analyses in Fig. 7C. This observation suggested that the PM derivatives were less fluorescent than PM itself.

Semi-preparative RP-HPLC analysis (Fig. 7C) showed that a number of additional products with absorbance maxima at 294 nm were also detected after 6 days of reaction. Those eluting after about 23 min (indicated by an asterisk) had absorbance maxima below 260 nm and were also formed from LA, in the absence of PM. However, products eluting between 19 and 22 min had extracted absorbance maxima at 294 nm (photodiode array detector), consistent with the presence of the intact pyridine ring. The peaks eluting in this region of the chromatogram were pooled and subjected to direct injection ESI-MS. Major products identified had m/z values of 267, 305, 339, 377, and 479 (Fig. 8A); none of these were present in incubations of LA or PM alone. Assignment of the various species to particular chromatographic peaks was accomplished by collecting individual peaks by semi-preparative RP-HPLC (Fig. 8B) and analysis by direct injection ESI-MS. Once peak assignments were made, the kinetics of formation of the three most abundant products (267, 305, and 339) were determined by RP-HPLC (Fig. 8C). The yields of all three compounds increased with time, with products 267 and 339 consistently forming in the highest yields. These products were chosen for further characterization.

Product Characterization—Products 267 and 339 were identified as the hexanoic acid amide and nonanedioic (azelaic) acid monoamide derivatives of PM (24). The hexanoic acid amide derivative of lysine has been identified previously by Kato et al. (25, 26) in reactions of linoleic acid hydroperoxides with N°-
blocked lysine, was shown to be formed during copper-catalyzed oxidation of LDL, and was identified in the vascular wall by immunohistochemical methods. Data on product 339 are therefore presented in more detail here. Products 267 and 339 were isolated by semi-preparative RP-HPLC. Based on absorbance area units on HPLC, both products yielded PM (90%) on acid hydrolysis (2 N HCl, 2 h at 95 °C) (shown for product 339 in Fig. 9, A versus B). The fluorescence response of these products on HPLC analysis was ~30% that of PM, consistent with the loss of PM fluorescence during reaction of LA with PM (Fig. 7, B versus A). Neither product contained a free amino group, based on lack of reactivity with TNBS, consistent with the loss of TNBS reactivity during lipid peroxidation reactions (Fig. 1B).

The hexanoic acid amide and nonanedioic acid monoamide derivative of PM were prepared synthetically, as described under "Materials and Methods." Analysis of the synthetic products by RP-HPLC-ESI-MS confirmed that they co-eluted with products 267 and 339 obtained from the reaction mixture and lysine loss (C) by amino acid analysis. Results are mean and range for analysis of two independent pools of LDL and are representative of two separate experiments.

**Fig. 6.** Pyridoxamine does not prevent copper-catalyzed oxidation of LDL but does inhibit ALE formation and lysine loss. LDL (50 μg/ml) was incubated at 37 °C in phosphate-buffered saline alone (□) or in the presence of 5 μM Cu²⁺ (○) or with 5 μM Cu²⁺ and 100 μM (△) or 250 μM (▲) PM. A, conjugated diene formation was monitored at 234 nm. At the end of the incubation ~1 mg of protein was removed, reduced with NaBH₄, dialyzed, delipidated, and then analyzed for CML, CEL, MDA-Lys, and HNE-Lys content by selected ion monitoring (SIM)-GC/MS (B) and lysine loss (C) by amino acid analysis. Results are mean and range for analysis of two independent pools of LDL and are representative of two separate experiments.

**Fig. 7.** Pyridoxamine is consumed, and fluorescent and absorbant products are formed during oxidation of linoleic acid. PM (1 mM) was incubated with LA (5 mM) in 0.2 M sodium phosphate buffer, pH 7.4, for 6 days at 37 °C. A and B, aliquots of the aqueous phase were analyzed by analytical RP-HPLC at various times using fluorescence detection with pyridoxal added as an internal standard, just prior to chromatography. Products indicated by brackets were consistently formed in reaction mixtures in the presence of PM. C, semi-preparative chromatographic analysis of a sample at 6 days, monitoring absorbance at 294 nm. The brackets in C indicate the region pooled from several semi-preparative column runs and were used for mass spectrometry analysis. The * indicates products produced during LA oxidation in the absence of PM.

**Fig. 8.** Identification and kinetics of formation of pyridoxamine adducts in reactions of pyridoxamine with linoleic acid. A, products formed after 6 days of reaction and eluting between 19 and 22 min (Fig. 7D) were analyzed by direct injection ESI-MS. Five major products were identified, with m/z = 267, 305, 339, 339, and 479. B, semi-preparative RP-HPLC (absorbance detection), identifying major products with m/z = 339, 267, and 305, in order of elution. C, kinetics of formation of 267 (●), 339 (■), and 305 (▲), based on their area ratios to pyridoxal, added as internal standard. The data shown are the mean and S.D. from three independent experiments.
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The inhibition of lipid oxidation in the RNase model system suggested that PM would exert similar effects during copper-catalyzed oxidation of LDL. As shown in Fig. 6, despite the high rate of lipoprotein oxidation catalyzed by $\text{Cu}^{2+}$, the gradual solubilization of the PUFAs and chemical modification of PM in reactions in either the absence or presence of protein, the formation of TBARS and decomposition of LA (Fig. 2), as well as the formation of conjugated dienes during oxidation of LDL (Fig. 6) document that lipid peroxidation occurred, even in the presence of PM. The overall observations were consistent with the hypothesis that PM acts as a sacrificial nucleophile, trapping reactive intermediates in lipid oxidation (and glycoxidation) reactions. The involvement of the amino group of PM in inhibition of ALE formation was confirmed by the loss of TNBS reactivity in reactions of PM with oxidizing PUFAs (Fig. 1B).

The fact that PM protects against modification of lysine residues and formation of lipid oxidation products in RNase, despite the 100-fold excess of PUFAs and the 10-fold higher concentration of lysine residues in RNase (Figs. 3 and 4), illustrates its efficiency in trapping reactive carbonyl intermediates. The efficiency of PM in protecting lysine amino groups suggests either that only a fraction of lipid peroxidation products participate in chemical modification of protein or that PM intercepts critical, early lipid peroxidation products, preventing their decomposition to a broader range of shorter-chain reactive carbonyl compounds. Consistent with the latter hypothesis, none of the anticipated ALE derivatives of PM, e.g. carboxymethyl-PM, carboxyethyl-PM, MDA-PM, or HNE-PM, were identifiable in the PM reaction mixture, based on $\text{LC-ESI-MS}$. Thus, it appears that, by trapping early intermediates in glycoxidation and lipid oxidation reactions, PM protects against the formation of a range of later precursors of ALEs on proteins.

DISCUSSION

PM Inhibition of Advanced Lipid Oxidation Reactions—PM was originally described as an AGE inhibitor and, like other AGE inhibitors, is thought to act as a nucleophilic trap for reactive carbonyl intermediates in AGE formation. Reactive carbonyl compounds are also involved in the formation of ALEs, so it seemed likely that this compound would also inhibit chemical modification of proteins during lipid peroxidation reactions. Indeed, PM was a potent inhibitor of chemical modification of both RNase and LDL during lipid peroxidation reactions, inhibiting both the modification of lysine residues and the formation of specific lipid oxidation products, CML, CEL, MDA-Lys, and HNE-Lys. Some weak antioxidant activity, a general characteristic of phenolic compounds, was also observed, as judged by a marginal effect of PM on the kinetics of oxidation of linoleate and LDL. However, the gradual solubilization of the PUFAs and chemical modification of PM in reactions in either the absence or presence of protein, the formation of TBARS and decomposition of LA (Fig. 2), as well as the formation of conjugated dienes during oxidation of LDL (Fig. 6) document that lipid peroxidation occurred, even in the presence of PM. The overall observations were consistent with the hypothesis that PM acts as a sacrificial nucleophile, trapping reactive intermediates in lipid oxidation (and glycoxidation) reactions. The involvement of the amino group of PM in inhibition of ALE formation was confirmed by the loss of TNBS reactivity in reactions of PM with oxidizing PUFAs (Fig. 1B).

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The inhibition of lipid oxidation in the RNase model system suggested that PM would exert similar effects during copper-catalyzed oxidation of LDL. As shown in Fig. 6, despite the high rate of lipoprotein oxidation catalyzed by $5 \mu\text{M} \text{Cu}^{2+}$ in this...
reaction system, PM, at a pharmacologically relevant concentration, was an effective inhibitor of chemical modification of LDL, including both the formation of specific ALEs and the modification of lysine residues. These inhibitory effects, coupled with the lipid lowering activity of PM in diabetic rats, suggest that PM may be effective in protecting against lipid-dependent, oxidative chemical modification of proteins in vivo and may, therefore, have anti-atherogenic activity. Experiments to assess the effects of PM on formation of lipoxidation products in vivo and development of diabetic vascular disease are currently under way.

**Mechanism of Action of PM**—PM is unique among current inhibitors of advanced glycation reactions because of its characteristic fluorescence spectrum. Thus, the carbonyl intermediates trapped by PM are not only amenable to RP-HPLC analysis but are also readily identifiable by fluorescence detection (Fig. 7). These features of PM, combined with previous work of Kato et al. (25, 26), facilitated the identification of the hexanoic acid amide and nonanedioic acid monoamide derivatives of PM. These compounds are clearly formed by addition of PM to the 9- and 13-carbons of LA, followed by oxidative cleavage of the 9–10 or 12–13 carbon-carbon bonds (Scheme I).

In support of this mechanism, KODEs have been identified as products of both non-enzymatic (27, 28) and enzymatic (29, 30) peroxidation of LA and have also been detected by chemical methods in atherosclerotic plaque (31). Thiol compounds are known to react directly with KODEs, whereas their reaction with hydroperoxyoctadecadienoic acids requires autoxidizing conditions (Fe3+ and O2) (16, 17). PM also fails to react with lipid peroxides (prepared from LA using soybean lipoygenase) under anti-oxidative conditions (anaerobic incubation in the presence of DTPA; data not shown.). Thus, PM may function as a surrogate, non-thiol nucleophile, trapping and inactivating KODE intermediates and thereby inhibiting further chemical modification of proteins by peroxidizing lipids.

**Concluding Remarks**—Based on the proposed mechanism of formation of PM adducts, the hexamido derivative of lysine is likely to be formed from any ω-6 PUFA. A number of other mono- and dicarboxylic acid amide derivatives should also be formed on oxidation of other families of PUFA. Analysis of the amide content of tissue proteins should therefore provide insight into both the origin of lipid-derived chemical modifications of proteins and their contribution to the overall chemical modification of proteins in vivo. These non-enzymatic lipid adducts to protein promote protein-membrane interactions, affecting the endocytosis, subcellular transport, compartmentalization, and catabolism of lipoxidized proteins. These and other lipid adducts may also contribute to the accumulation of lipofuscin in postmitotic tissues during aging and to the accumulation of lipids and lipoproteins in foam cells in the vascular wall in atherosclerosis. We are encouraged by the possibility that inhibition of lipoxidative modification of proteins, using compounds such as PM, may provide a route for limiting age-dependent and disease-related damage to proteins by both glycoxidation and lipoxidation reactions and may be useful for treatment of diseases involving hyperlipidemia and/or oxidative stress.

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