

Pyridoxamine Traps Intermediates in Lipid Peroxidation Reactions *in Vivo*

EVIDENCE ON THE ROLE OF LIPIDS IN CHEMICAL MODIFICATION OF PROTEIN AND DEVELOPMENT OF DIABETIC COMPLICATIONS*

Received for publication, April 24, 2003, and in revised form, July 31, 2003
Published, JBC Papers in Press, August 15, 2003, DOI 10.1074/jbc.M304292200

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Maillard or browning reactions between reducing sugars and protein lead to formation of advanced glycation end products (AGEs) and are thought to contribute to the pathogenesis of diabetic complications. AGE inhibitors such as aminoguanidine and pyridoxamine (PM) inhibit both the formation of AGEs and development of complications in animal models of diabetes. PM also inhibits the chemical modification of protein by advanced lipoxidation end products (ALEs) during lipid peroxidation reactions *in vitro*. We show here that several PM adducts, formed in incubations of PM with linoleate and arachidonate *in vitro*, are also excreted in the urine of PM-treated animals. The PM adducts *N*-nonanedioyl-PM (derived from linoleate), *N*-pentanedioyl-PM, *N*-pyrrolo-PM, and *N*-(2-formyl)-pyrrolo-PM (derived from arachidonate), and *N*-formyl-PM and *N*-hexanoyl-PM (derived from both fatty acids) were quantified by liquid chromatography-mass spectrometry analysis of rat urine. Levels of these adducts were increased 5–10-fold in the urine of PM-treated diabetic and hyperlipidemic rats, compared with control animals. We conclude that the PM functions, at least in part, by trapping intermediates in AGE/ALE formation and propose a mechanism for PM inhibition of AGE/ALE formation involving cleavage of α -dicarbonyl intermediates in glycoxidation and lipoxidation reactions. We also conclude that ALEs derived from polyunsaturated fatty acids are increased in diabetes and hyperlipidemia and may contribute to development of long term renal and vascular pathology in these diseases.

Non-enzymatic chemical modification of protein by reducing sugars, known as the Maillard reaction, is implicated in the development of pathology during aging and in chronic diseases such as diabetes, atherosclerosis, and Alzheimers disease (1–3). The Maillard reaction between sugar and protein proceeds through a labile Schiff base, which isomerizes to a ketoamine adduct, the Amadori compound. Oxidative decomposition and further reaction of the Amadori compound produce advanced

glycation end products (AGEs)¹, such as pentosidine and vesperlysines (2). Similarly, advanced lipoxidation end products (ALEs), such as the malondialdehyde and 4-hydroxy-2-nonenal adducts to lysine, are formed on protein during lipid peroxidation reactions (4, 5). *N*^ε-(Carboxymethyl)lysine and *N*^ε-(carboxyethyl)lysine, which are major products of both glycoxidation and lipoxidation reactions (4–6), are termed AGE/ALEs. Through effects on protein structure, function, and turnover, the accumulation of AGEs and ALEs in tissue proteins is thought to contribute to the development of diabetic complications.

AGE/ALE inhibitors are designed to limit the accumulation of AGE/ALEs in protein and thereby protect against the development of diabetic complications. Hudson and colleagues (7–9) reported that PM was a potent inhibitor of the formation of AGEs from Amadori adducts *in vitro*. Onorato *et al.* (10) later reported that PM traps intermediates in lipid peroxidation and protects proteins from chemical modification during lipid peroxidation reactions (lipoxidation) *in vitro*. Degenhardt *et al.* (11) and Alderson *et al.* (12) demonstrated that PM also inhibited the formation of AGE/ALEs *in vivo* and retarded the development of nephropathy in both streptozocin (STZ)-induced diabetic and Zucker (obese, hyperlipidemic) rats. The presence of severe hyperlipidemia in the STZ diabetic rats and the reno-protective effects of PM in both the diabetic and the Zucker rat suggested that lipids, rather than carbohydrates, might be the primary source of chemical modification of proteins in diabetes.

In the present study, we have extended earlier work on the reaction of PM with the polyunsaturated fatty acids (PUFAs), linoleate and arachidonate, to identify other intermediates trapped by PM during lipid peroxidation reactions and to determine whether PM functions as an inhibitor of advanced lipoxidation reactions *in vivo*. We show that several lipid-derived PM adducts formed in *in vitro* reactions are also detected in the urine of PM-treated control, diabetic, and hyperlipidemic rats and that these adducts are present at substantially higher

* This work was supported by research grants from the Juvenile Diabetes Research Foundation and the National Institutes of Health (DK-19971). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: AGE, advanced glycation end product; AA, arachidonic acid; AG, aminoguanidine; ALE, advanced lipoxidation end product; ESI⁺-LC/MS/MS, positive ion electrospray ionization liquid chromatography/mass spectrometry/mass spectrometry; GO, glyoxal; LA, linoleic acid; MGO, methylglyoxal; MRM-LC/MS/MS, multiple reaction monitoring-LC/MS/MS; PM, pyridoxamine; PUFA, polyunsaturated fatty acid; RP-HPLC, reversed phase high performance liquid chromatography; STZ, streptozocin; Db, diabetic; HAPM, *N*-hexanoyl-PM; NDAPM, *N*-nonanedioyl-PM; PDAPM, *N*-pentanedioyl-PM; FAPM, *N*-formyl-PM; PyPM, *N*-pyrrolo-PM; FPyPM, *N*-(2-formyl)-pyrrolo-PM; GOPM, glyoxal-PM; ZDF/Gmi-*fa*, male Zucker diabetic fatty rat; *+fa*, lean Zucker diabetic fatty rat; *fa/fa*, Zucker non-diabetic fatty rat.

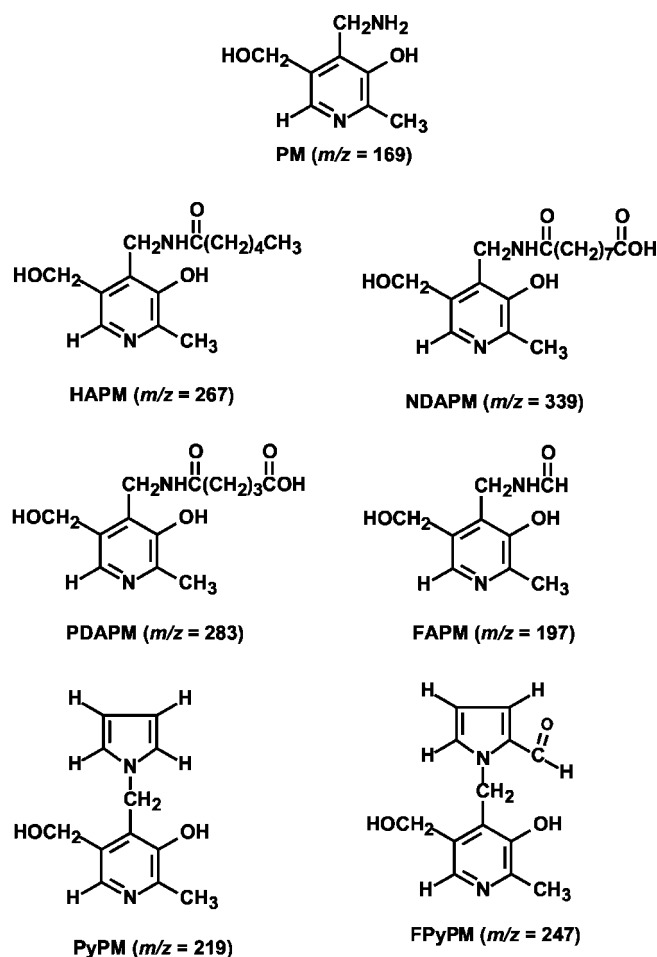


FIG. 1. PM adducts identified in reactions of PM with oxidizing PUFA and in the urine of animals treated with PM. HAPM and FAPM are found in reactions of PM with either LA or AA reactions. PDAPM, FPyPM, and PyPM are found only in the AA incubation, whereas NDAPM is unique to the LA system.

concentrations in the urine of diabetic and hyperlipidemic animals, compared with control rats. Our results support a role for lipoxidation reactions in the chemical modification of proteins and development of complications in diabetes and pre-diabetic hyperlipidemic states and demonstrate that the protective effects of PM are consistent with its role in reducing plasma lipids and trapping intermediates in lipoxidation reactions *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Except where indicated, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

Reaction of PM with Fatty Acids—PM (1 mM) and either linoleic acid (LA) (5 mM) or arachidonic acid (AA) (5 mM) were incubated in 200 mM phosphate buffer, pH 7.4, in a shaking water bath at 37 °C, and phosphate buffers were sterilized by ultrafiltration through 0.2- μ m syringe filters (Costar Corp., Acton, MA). Aliquots were taken at 0, 1, 3, and 6 days, quenched with a final concentration of 1 mM diethylenetriamine-pentaacetic acid, and frozen at -70 °C until analyzed. Samples were diluted 1:10 in 0.1% heptafluorobutyric acid (HFBA) (Acros Organics, Morris Plains, NJ) before analysis by reversed phase high performance liquid chromatography (RP-HPLC) and/or positive ion electrospray ionization liquid chromatography/mass spectrometry/mass spectrometry (ESI⁺-LC/MS/MS). Control incubations consisted of either PM or PUFA alone.

Synthesis of PM Adducts—All synthesized PM adduct standards were purified by RP-HPLC and characterized by ESI⁺-LC/MS/MS and/or by ¹H and ¹³C NMR, as described below. The structures of synthesized PM adducts that were also detected in urine are shown in Fig. 1.

N-Hexanoyl-PM (HAPM) and *N*-nonanedioyl-PM (NDAPM) were synthesized from PM and hexanoyl chloride or nonanedioic acid monomethyl ester, respectively, as described (10). For ESI⁺-MS of HAPM, m/z = 267 [M+H]⁺, and for NDAPM, m/z = 339 [M+H]⁺.

Heavy labeled *N*-hexanoyl-PM (*d*₁₁-HAPM) was synthesized from PM-(HCl)₂ and *d*₁₁-hexanoic acid according to the method of Kato *et al.* (13). Equimolar amounts (0.6 mmol) of *d*₁₁-hexanoic acid (Cambridge Isotope Laboratories, Inc., Andover, MA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and *N*-hydroxysulfosuccinimide (Pierce) were dissolved in 15 ml of dimethylformamide and incubated for 24 h at room temperature. PM-(HCl)₂ (0.6 mmol) in 1 ml of 200 mM borate buffer, pH 9, was added, and the reaction mixture was incubated for an additional 4 h. The reaction mixture was diluted to 30 ml with water, and the pH was adjusted to 8.1 with 1 M NaOH. The mixture was extracted three times with an equal volume of diethyl ether, and the pooled extracts were dried under nitrogen. The resulting white powder was reconstituted in 1% HFBA, and *d*₁₁-HAPM was purified in 20% yield by semi-preparative RP-HPLC. For ESI⁺-MS, m/z = 278 [M+H]⁺.

N-Pentanedioyl-PM (PDAPM) was synthesized from PM and glutaric anhydride. PM-(HCl)₂ (0.24 mg; 1 μ mol) was dissolved in 1 ml of 250 mM NaHCO₃, pH 10, and placed in a shaking water bath at 37 °C. A total of 3.4 mg (30 μ mol) of glutaric anhydride was added in three aliquots at 10-min intervals, and the reaction was continued for an additional 10 min after the last addition. The reaction mixture was then dried overnight *in vacuo* (Savant Speed Vac, Savant Instruments Inc., Farmingdale, NY). The residue was reconstituted in 1% HFBA, and PDAPM was purified (20% yield) by semi-preparative RP-HPLC. For ESI⁺-MS, m/z = 283 [M+H]⁺.

N-Formyl-PM (FAPM) was synthesized according to the method of Yamada and Okamoto (14). Briefly, pyridoxine-HCl (1.14 g, 10 mmol) was added to 4.8 ml of formamide (120 mmol) and incubated in a heating block at 100 °C for 24 h. The resulting red oil was diluted 1:100 in 1% HFBA, and a red precipitate formed immediately and was removed by centrifugation. The supernatant was fractionated by RP-HPLC, and a region containing FAPM was collected and dried overnight *in vacuo*. FAPM was subsequently purified in 50% yield upon re-injection of the collected fraction. For ESI⁺-MS, m/z = 197 [M+H]⁺. Structure conformation was provided by ¹H NMR on a Varian (Palo Alto, CA) Inova 500 MHz instrument using deuterated dimethyl sulfoxide (*d*₆-Me₂SO) as solvent. The chemical shifts were: δ 2.49 (s, 3H), 4.37 (d, 2H, J = 5.8 Hz), 4.72 (s, 2H), 5.20 (s, 1H), 5.75 (s, 1H), 8.01 (s, 1H), 8.92 (t, 1H, J = 5.8 Hz), and 11.2 (s, 1H).

N-Pyrrolo-PM (PyPM) was synthesized by the method of D'Silva and Walker (15). Briefly, PM-(HCl)₂ (0.27 g, 1 mmol) and 2,5-dimethoxytetrahydrofuran (0.16 g, 1 mmol) were dissolved in 4.25 ml of pyridine:acetic acid:water (1.9:1.35:1), and the mixture was reacted in a heating block at 100 °C for 2 h. The resulting brown liquid was diluted 1:100 in 1% HFBA, and PyPM was purified in 95% yield by semi-preparative RP-HPLC. For ESI⁺-MS, m/z = 219 [M+H]⁺. Structure conformation was provided by 500 MHz ¹H and ¹³C NMR using *d*₆-Me₂SO as solvent. The chemical shifts were: ¹H NMR δ 2.60 (s, 3H), 4.55 (s, 2H), 5.30 (s, 2H), 5.97 (s, 2H), 6.75 (s, 2H), and 8.20 (s, 1H). The chemical shifts were: ¹³C NMR δ 15.8 [-CH₃ (PM)], 42.3 [-CH₂- (PM)], 57.5 [-CH₂- (PM)], 107.9 [-CH= (pyrrole)], 121.2 [-CH= (pyrrole)], and 130.6–152.2 [5 pyridinyl (PM)].

N-(2-Formyl)-pyrrolo-PM (FPyPM) was synthesized from PM and xylose based on the method of Hayase and Kato (16). PM-(HCl)₂ (2.4 g, 10 mmol) and xylose (1.5 g, 10 mmol) were dissolved in 5 ml of water and heated at 95 °C for 2 h, after adjusting the pH to 4 with 6 N NaOH. The resulting brown liquid was extracted three times with equal volumes of diethyl ether, and the pooled ether extracts were evaporated under nitrogen. The residue was reconstituted in 1% HFBA, and FPyPM was purified in 0.1% yield by semi-preparative RP-HPLC. For ESI⁺-MS, m/z = 247 [M+H]⁺. Structure conformation was provided by 500 MHz ¹H NMR using *d*₆-Me₂SO as a solvent. The chemical shifts were: δ 2.35 (s, 3H), 4.38 (d, 2H, J = 4.7 Hz), 5.20 (t, 1H, J = 4.7 Hz), 5.60 (s, 2H), 6.15 (dd, 1H, J = 2.5, 3.9 Hz), 6.82 (unresolved dd, 1H), 7.00 (dd, 1H, J = 1.7, 3.9 Hz), 7.99 (s, 1H), and 9.61 (s, 1H).

Quantification of PM Adduct Standards—The concentrations of FAPM, HAPM, NDAPM, and PDAPM standards were determined by acid hydrolysis and subsequent quantification of released PM, as described (10). Briefly, aliquots of standard were hydrolyzed in 2 N HCl for 2 h at 95 °C, dried *in vacuo*, and then reconstituted in 1% HFBA. The concentrations of standards were determined, based on the yield of PM measured by RP-HPLC.

Because FPyPM and PyPM were stable to acid hydrolysis and did not exhibit the fluorescence characteristic of compounds containing PM, the concentrations of these standards were determined by RP-HPLC using

absorbance detection as described below. FPyPM and PyPM concentrations were estimated using the molar extinction coefficient of PM ($92,000 \text{ M}^{-1} \text{ cm}^{-1}$).

RP-HPLC—Samples were analyzed on a Waters (Milford, MA) 626/600 HPLC system, using an Aquasil (Thermo Hypersil-Keystone, Bellefonte, PA) C-18 column ($5 \mu\text{m}$; $250 \text{ mm} \times 4.6 \text{ mm}$) at a flow rate of 0.95 ml/min . Solvent A was 0.1% HFBA in water and Solvent B was 90% acetonitrile in water. The gradient was as follows: $0\text{--}2 \text{ min}$, 15% B; $2\text{--}37 \text{ min}$, linear ramp to 90% B; $37\text{--}40 \text{ min}$, hold at 90% B; $40\text{--}50 \text{ min}$, return to 15% B; $50\text{--}65 \text{ min}$, hold at 15% B. PM and PM adducts were detected by fluorescence ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 393 \text{ nm}$) and/or by absorbance at 294 nm .

Mass Spectrometry—ESI⁺-LC/MS/MS was performed on a Micro-mass (Beverly, MA) Quattro LC mass spectrometer equipped with an Agilent (Palo Alto, CA) 1100 series HPLC system and an Aquasil C-18 column ($5 \mu\text{m}$, $250 \times 2 \text{ mm}$) at a flow rate of 0.2 ml/min . Solvent A was 0.1% HFBA in water and Solvent B was 90% acetonitrile in water; all solvents including water were of HPLC grade. The gradient was as follows: $0\text{--}5 \text{ min}$, 15% B; $5\text{--}37 \text{ min}$, linear ramp to 90% B; $37\text{--}42 \text{ min}$, hold at 90% B; $42\text{--}52 \text{ min}$, return to 15% B; $52\text{--}60 \text{ min}$, hold at 15% B. The capillary was held at 3.11 kV , and the sampling cone and collision cell were held at 14 and 20 V , respectively. The source block and desolvation temperatures were maintained at 100 and 350°C , respectively.

The identity of all synthesized PM adducts detected in urine was unequivocally established by mixing experiments and by ESI⁺-LC/MS/MS, including precursor-ion and product-ion spectrum scans. PM and PM adducts generate $m/z = 152$ as the predominant product-ion, corresponding to the deaminated ion (PM), deaminated ion (FAPM, HAPM, NDAPM, and PDAPM), or loss of the pyrrole functional group (FPyPM and PyPM), when subjected to collision-induced dissociation. In addition to $m/z = 152$, unidentified intramolecular rearrangement ions of PM ($m/z = 140$ and 134) were consistently observed in product-ion analyses of all PM adducts, as well as the $[\text{M}+\text{H}]^+$ ion of PM, $m/z = 169$.

Animal Studies—All studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of South Carolina. Female Sprague-Dawley rats were purchased at 5 weeks of age from Harlan Industries (Indianapolis, IN). Male Zucker diabetic fatty (ZDF/Gmi-*fa*), lean Zucker diabetic fatty (*+fa*), and Zucker non-diabetic fatty (*fa/fa*) rats were purchased at 5 weeks of age from Genetic Models, Inc. (Charles River Laboratories, Wilmington, MA). Rats were housed in the Animal Resource Facility for 1 week before studies were started; all animals had free access to food and water throughout the study. Plasma glucose and plasma triglycerides were measured with Sigma Kit No. 315 and No. 339, respectively.

Sprague-Dawley rats were maintained on Harlan Teklad (Indianapolis, IN) rodent diet (W). Diabetes was induced by a single tail-vein injection of 45 mg/kg streptozocin in 0.1 M sodium citrate buffer, pH 4.5 , as described previously (11). Non-diabetic animals were sham-injected with buffer only. Animals with plasma glucose above 16 mM were classified as diabetic and assigned to either an untreated diabetic control group (STZ-Db, $n = 16$) or a diabetic group receiving PM (STZ-Db+PM, $n = 16$) in drinking water at 1 g/liter . Non-Db ($n = 12$) animals received PM (non-Db+PM) in drinking water at 2 g/liter , to compensate, in part, for the lower water consumption of these animals. Diabetic animals received insulin ($3\text{--}5 \text{ IU}$; Humulin; Eli Lilly, Indianapolis, IN) 3 times/week to maintain body weight and limit hyperglycemia. Plasma glucose in STZ-Db animals was $\sim 25 \text{ mM}$ throughout the study, whereas plasma glucose in non-Db animals remained at $\sim 5 \text{ mM}$. Further experimental details are provided elsewhere (11).

Zucker rats were maintained on Purina (St. Louis, MO) 5008 rat chow (16.7% of total calories from fat). The ZDF/Gmi-*fa* rat develops non-insulin-dependent diabetes on this diet. Plasma glucose increased to 20 mM by week 5 of the study and rose gradually to 40 mM during the course of the 23-week study. Animals were assigned to either untreated or treated groups ($n = 10/\text{group}$) designated as follows: *+fa* control (L), *+fa* receiving PM (L+PM) in drinking water at 2 g/liter , *fa/fa* control (F), *fa/fa* receiving PM (F+PM) in drinking water at 2 g/liter , ZDF/Gmi-*fa* control (ZDF-Db), and ZDF/Gmi-*fa* receiving PM (ZDF-Db+PM) in drinking water at 1 g/liter . Plasma glucose in *+fa* and *fa/fa* rats was ~ 5 and 8 mM , respectively.

Urine Preparation and Analysis—Rats were housed in metabolic cages for 24 h to collect urine; urine collection beakers contained several drops of toluene to prevent microbial growth. Urine samples were stored at -70°C , and aliquots of urine from each group of rats were pooled for analysis. For analyses of NDAPM and FPyPM, either 8 ml of diabetic or 1.5 ml of non-diabetic urine, containing $\sim 1 \text{ mg}$ of creatinine,

was adjusted to pH 1 with 6 M HCl and centrifuged (Marathon, Fisher Scientific) at 2000 rpm for 10 min at 4°C to remove particulate matter. The supernatant was then applied to a 60-mg OasisTM MCX column (Waters), which was washed sequentially with 4 ml each of 0.1 N HCl and methanol and then eluted with 4 ml of 10% concentrated NH_4OH in methanol. The eluted fractions were dried *in vacuo*, reconstituted in 1% HFBA, and ultrafiltered prior to analysis. For analyses of FAPM, HAPM, PDAPM, and PyPM, aliquots of urine were diluted $1:20$ in 1% HFBA, ultrafiltered as described above, and analyzed directly without further purification.

PM adducts were quantified using d_{11} -HAPM as the internal standard, which was added to urine samples prior to sample preparation. Standard curves were constructed with mixtures of serially diluted authentic standards and constant amounts of d_{11} -HAPM. Total daily excretion of PM adducts was calculated using average 24-h urine output volumes for each animal group: non-Db+PM and STZ-Db+PM animals, ~ 30 and 145 ml of urine, respectively; L+PM, F+PM, and ZDF-Db+PM animals, ~ 15 , 38 , and 158 ml of urine, respectively.

RESULTS

Reaction of PM with PUFAs—We have shown previously that PM traps intermediates formed during lipid peroxidation reactions *in vitro* (10). As an extension of these studies and to identify additional PM-lipid characteristic of each of these fatty acids, PM was incubated with either LA or AA in phosphate buffer at physiological pH for 6 days. Approximately 60% of PM was consumed in both of the PM-PUFA incubations after 6 days. As shown in Fig. 2, products containing PM were observed in both the LA and AA systems. Product characterization, described below, focused on those PM derivatives that were subsequently detected in the plasma and urine of PM-treated animals.

Eight PM adducts were observed in the incubation of PM with LA (Fig. 2A). Six of these ($m/z = 267, 305, 323, 339, 377$, and 479) had been observed previously, of which two had been identified, the hexanoate amide HAPM ($m/z = 267$) and the nonanedioate amide NDAPM ($m/z = 339$) (10). The PM derivative with $m/z = 197$ has now been identified as the formic acid amide FAPM, based on MRM-LC/MS/MS analysis of mixing experiments with authentic standard. The structure of the PM adducts having $m/z = 305, 321, 323, 377$, and 479 , which were not detected in urine, have not been determined. However, the product with $m/z = 479$ is consistent with a PM-LA dioxo derivative, and the products with $m/z = 323$ and 305 are consistent with a hydrated furan and a furan derivative of PM.

Although formed in different yields than in reactions with LA, four adducts (FAPM, HAPM, and unknowns with $m/z = 305$ and 377) were also detected in incubations of PM with AA (Fig. 2B). Four new compounds were also observed in the PM-AA incubation. Three of these were identified as pyrrole (PyPM), formylpyrrole (FPyPM), and pentanedioate (PDAPM) derivatives of PM, respectively, by MRM-LC/MS/MS analysis of mixing experiments with authentic standards. None of the other products in either the LA or AA reactions were detected in the urine of animals treated with PM, possibly because they are early or transient intermediates or were labile during urine collection or storage.

Detection of PM Adducts in Vivo—To determine whether PM also traps intermediates formed in lipid peroxidation reactions *in vivo*, we treated diabetic and obese animals with PM in drinking water. Urine samples (24 h) were collected at monthly intervals, and urine pools collected at the 7th month of the studies were analyzed for PM derivatives. Several compounds identified in incubations of PM with PUFA *in vitro* were detected in urine, and in each case, authentic standards were added to the urines to confirm the presence of the PM adduct in question by MRM-LC/MS/MS. A typical spiking experiment is shown in Fig. 3.

Six PM adducts observed in reactions of PM with LA (Fig.

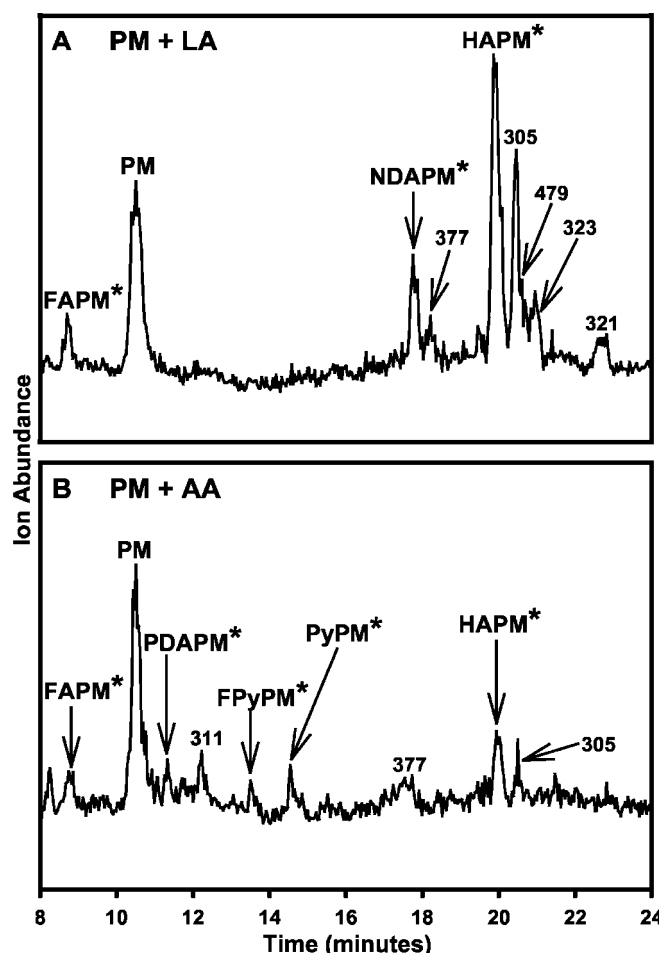


FIG. 2. PM traps intermediates of PUFA peroxidation. PM (1 mM) was incubated with either LA (5 mM) (A) or AA (5 mM) (B) in 200 mM phosphate buffer, pH 7.4, at 37 °C for 6 days. Aliquots of the 6-day incubations were analyzed by ESI⁺-LC/MS/MS using precursor-ion scans of $m/z = 152$. Products for which chemical identity has been established are indicated by *letter* abbreviations, and products identified with *numbers* are not yet characterized. PM adducts marked with an *asterisk* were subsequently identified in the urine of animals treated with PM (see Fig. 4).

4A) and AA (Fig. 4B) were also detected in the urine of animals treated with PM. NDAPM (derived from LA), PDAPM, PyPM, FPyPM (derived from AA), and FAPM and HAPM (derived from either PUFA) were readily detected in urine from STZ-Db+PM (Fig. 4C) and F+PM (Fig. 4D) animals by MRM-LC/MS/MS. None of these PM adducts was observed in the urine of untreated, control animals (data not shown). Other PM derivatives, which have not yet been characterized, were also detected in the urine of PM-treated rats (Fig. 4, C and D), and each of these products had fragment ions characteristic of PM when analyzed by daughter-ion LC/MS/MS. Most are more hydrophobic than PM, which elutes between FAPM and NDAPM, and may represent products from other lipid (or carbohydrate) precursors trapped during PM inhibition of oxidative modification of proteins *in vivo*.

Quantification of PM Adducts in Vivo—PM adducts in pooled urine of treated animals were quantified by MRM-LC/MS/MS, using d_{11} -HAPM as the internal standard. As shown in Fig. 5, the levels of these adducts in the urine of PM-treated diabetic animals were on average 5–10-fold higher than those in the urine of PM-treated non-diabetic animals. FAPM (Fig. 5A), which may be derived from both LA and AA, was the major adduct in the urine of control (non-Db) and STZ-Db animals (22.9 ± 0.8 and 132 ± 12 nmol/24 h, respectively), whereas

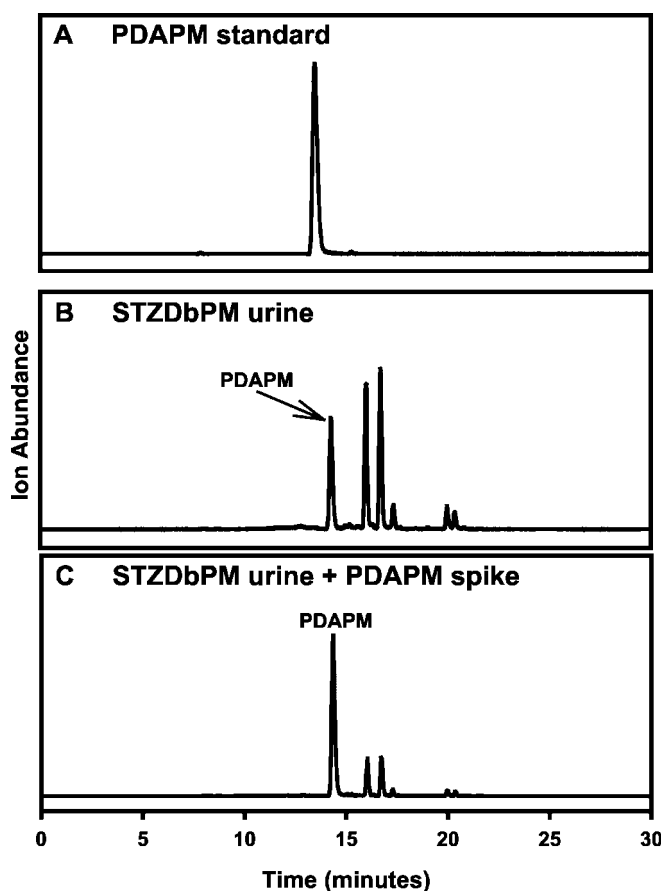


FIG. 3. The presence of PM adducts in urine was confirmed by spiking experiments. Synthetic PM adducts were spiked into urine samples from PM-treated animals and analyzed by ESI⁺-LC/MS/MS, using MRM. A representative spiking experiment for verifying the presence of PDAPM shows a PDAPM standard (A) monitored as 283 > 152 and a similar compound identified in the urine of STZ-Db+PM rats (B). A spiking experiment designed to double the peak height of PDAPM (C) confirmed the presence of PDAPM in the urine sample.

PDAPM (Fig. 5A) derived exclusively from AA was the major product in the urine of Zucker lean (L+PM), Zucker obese (F+PM), and Zucker type 2 diabetic (ZDF-Db) rats (9.1 ± 0.2 , 68.9 ± 7.0 , and 97.0 ± 4.5 nmol/24 h, respectively). Although FAPM and HAPM were produced in similar yield from both LA and AA *in vitro*, FAPM was detected in much larger relative amounts in urine (Fig. 5, A versus B), suggesting alternative sources of FAPM *in vivo*. HAPM excretion (Fig. 5B) was greatest in STZ-Db animals, about 800 pmol/24 h, and was 10-fold higher than in the urine of control (non-Db) animals. HAPM excretion in ZDF-Db animals was also 10-fold higher than in the urine of lean Zucker rats (L) and 7-fold higher than in the urine of obese (F) animals. The level of NDAPM (Fig. 5B), which is produced only from LA peroxidation (or other Δ^9 PUFAs) was typically the lowest of any of the measured adducts in the urine of animals, averaging about 7 pmol/24 h in non-diabetic animals and 50–100 pmol/24 h in diabetic animals. In general, PDAPM, FPyPM, and PyPM, products of AA oxidation, were more abundant than the LA-derived NDAPM (Fig. 5), suggesting that despite the higher concentration of LA in plasma and membrane lipids, AA contributes to the majority of PM adduct formation *in vivo*. This is consistent with the chemistry of these PUFAs, *i.e.* that AA is more readily oxidized than LA. Similar results were obtained during Cu²⁺-catalyzed oxidation of human low density lipoprotein in the presence of PM (data not shown), *i.e.* the yield of AA-derived products was greater than that of LA-derived products, despite the higher

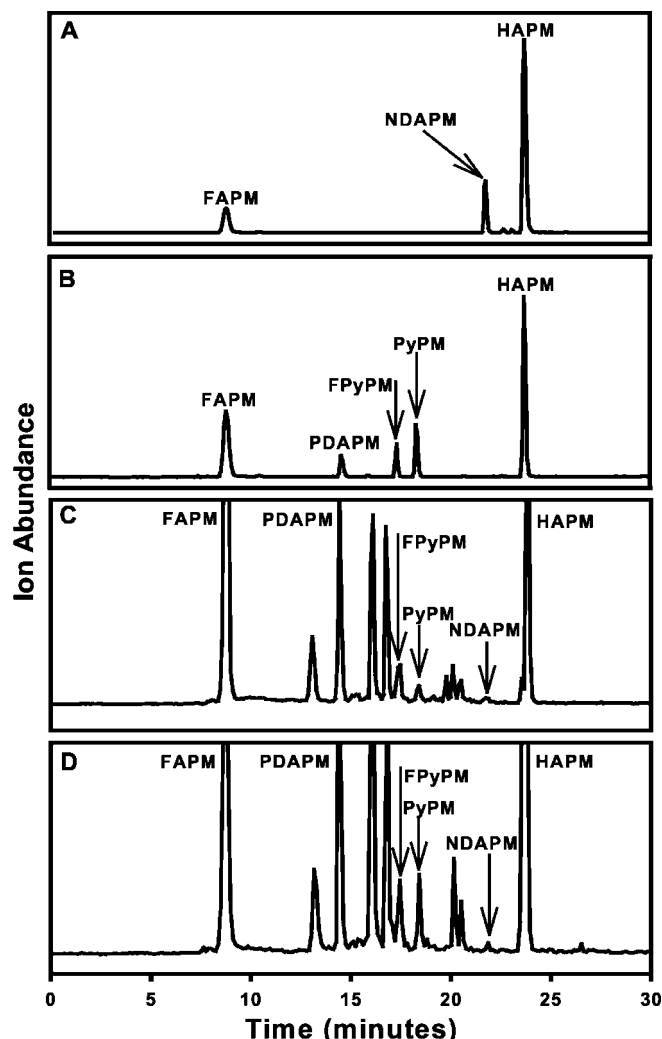


FIG. 4. PM adducts identified *in vitro* and *in vivo*. PM adducts were analyzed by MRM-LC/MS/MS in reactions of PM with PUFAs and in the urine of animals treated with PM. Shown are total ion currents of six MRM channels: FAPM (197 > 152), HAPM (267 > 152), NDAPM (339 > 152), PDAPM (283 > 152), PyPM (219 > 152), and FPyPM (247 > 152). FAPM and HAPM were found in reactions of PM with LA (A) and AA (B). NDAPM is unique to the LA reaction (A), whereas PDAPM, FPyPM, and PyPM are found only in the AA reaction (B). Analysis of STZ-Db+PM urine (C) and F+PM urine (D) revealed that all of these PM adducts were found in the urine of animals treated with PM.

concentration of LA compared with AA in low density lipoprotein.

Metabolic Analyses—Plasma glucose and triglyceride levels in various groups of rats are summarized in Fig. 6. As noted in several previous studies (11, 12, 17, 18), PM had no effect on plasma glucose concentrations in any group (Fig. 6A). However, PM significantly decreased levels of plasma triglycerides in both STZ-Db and ZDF animals (Fig. 6B) by ~50 and 35%, respectively, although the effects on hyperlipidemia in the obese rats, when fed a high fat diet (used to induce diabetes in the ZDF rat), were not statistically significant. Both groups of diabetic rats had dramatically lower levels of plasma triglycerides but higher levels of PM adducts (Fig. 5) compared with non-diabetic obese (F) rats, suggesting a role for diabetes in the enhancement of lipid peroxidation reactions (discussed below).

DISCUSSION

Summary—The goal of this study was to evaluate the role of PM as an inhibitor of lipoxidation reactions in obese and dia-

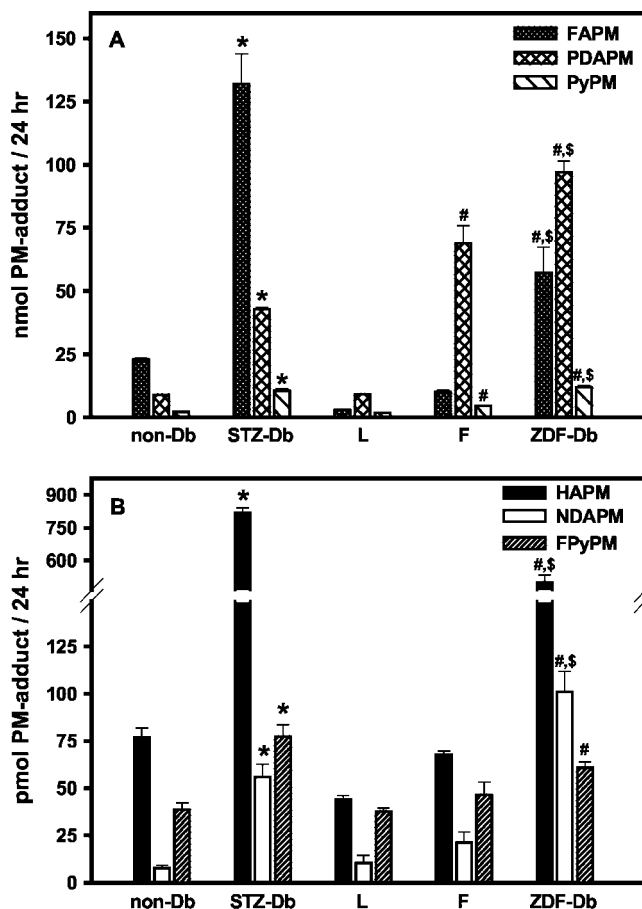


FIG. 5. Quantification of PM adducts in the urine of treated animals. PM adducts were quantified in urine pools by ESI⁺-LC/MS/MS, using MRM with *d*₁₁-HAPM as the internal standard. FAPM, PDAPM, and PyPM (A) were quantitatively the major adducts detected in the urine of PM-treated animals. Amounts of HAPM, NDAPM, and FPyPM (B) were generally <1 nmol/24 h. Data are mean ± S.D. *, *p* < 0.001 versus non-Db+PM; #, *p* < 0.001 versus L+PM; \$, *p* < 0.001 versus F+PM. One-way analysis of variance with Tukey-Kramer's post test was performed using GraphPad (San Diego, CA) InStat version 3.05 for Windows.

betic animals and thereby to gain a better understanding of the role of lipoxidation chemistry in the development of renal disease in diabetes. In preparatory studies, we identified 12 PM derivatives that were formed during incubation of PM with LA or AA under peroxidizing conditions *in vitro*. We then administered PM to type 1 and type 2 diabetic rats, to obese hyperlipidemic rats, and to lean controls, and chemically characterized the six PM derivatives that were detected in the plasma and urine of animals treated with PM. The elevated levels of PM adducts in the urine of diabetic and hyperlipidemic rats suggested that there was increased lipid peroxidation in these animals and that the protective effects of PM were consistent with its lipid-lowering activity and proposed role as an AGE/ALE inhibitor and chemical trap for intermediates in lipoxidation reactions.

Mechanism of Action of PM—Radical and hydroperoxide intermediates in lipid peroxidation reactions decompose to reactive carbonyl compounds, such as α -keto acids, α -ketoaldehydes, and α -hydroxyaldehydes (19, 20). The two most abundant adducts detected in incubations of PM with peroxidizing LA or AA were FAPM and HAPM. We had proposed previously (10) that HAPM was formed by reaction of PM with 13-oxo-9,11-octadecadienoic acid (13-keto-octadecadienoic acid (13-KODE)), an intermediate in peroxidation of LA (and also from the analogous product of peroxidation of AA and other ω^6

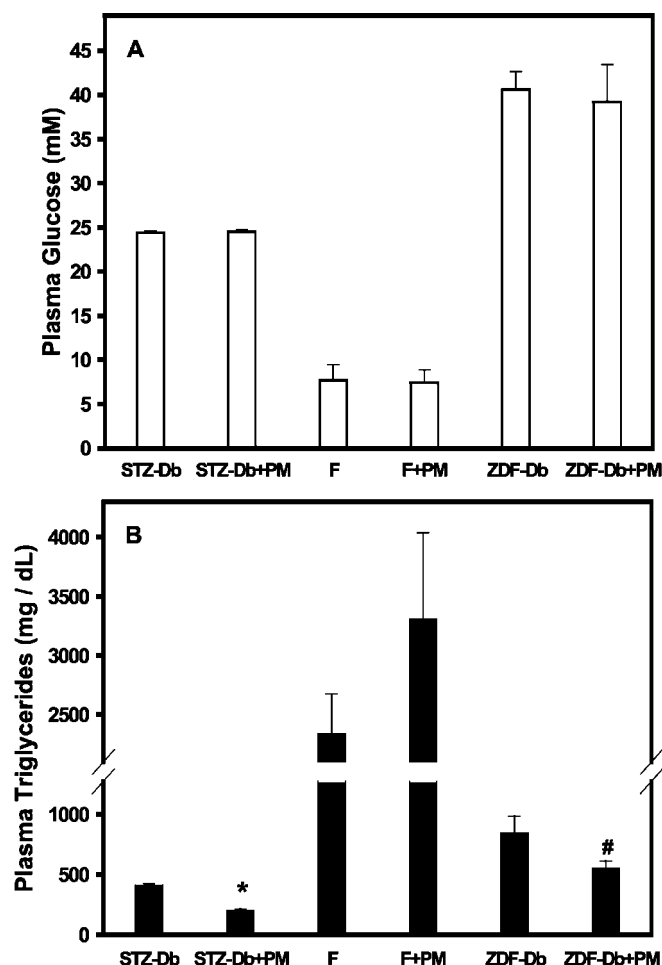


FIG. 6. Plasma glucose and plasma triglycerides. PM had no effect on plasma glucose concentrations (A) in treated animals, whereas it caused a 50 and 35% reduction in plasma triglycerides (B) of STZ-Db+PM and ZDF-Db+PM animals, respectively. PM had no significant effect on plasma triglycerides in F+PM animals. Data are means \pm S.E. *, $p > 0.05$ versus STZ-Db; #, $p > 0.05$ versus ZDF-Db. One-way analysis of variance with Tukey-Kramer's post test was performed using Graph-Pad InStat version 3.05 for Windows.

PUFAs). In that scheme, PM added to the 13-carbon of 13-keto-octadecadienoic acid, and HAPM was released on oxidative cleavage of the 12–13 carbon-carbon bond. NDAPM and PDAPM would be formed in an analogous manner by addition of PM to the 9- and 5-keto-octadecadienoic acids formed from LA and AA, respectively. However, although this scheme explained the formation of HAPM, NDAPM, and PDAPM, it did not account for the formation of one of the major products, the formyl adduct FAPM, nor have we been able to detect in *in vitro* reactions the putative oxidative cleavage products from the other ends of the lipid molecule. The present data, which identify FAPM as a major product formed during lipid peroxidation reactions in the presence of PM, suggest an alternative mechanism of action of PM (Scheme 1). In this scheme, the conjugated diene hydroperoxides decompose oxidatively to ketoaldehydes, which then may react with the primary amine and phenoxyl groups of PM to form a transient, seven-membered ring that undergoes a ring-opening rearrangement to form mixed amide and hemiacetal adducts to PM. The hemiacetals hydrolyze in aqueous solution to yield stable amide adducts, depending on the manner of addition of PM to the ketoaldehyde. This mechanism has been confirmed, in part, by detection of acetyl and benzoyl amide derivatives of PM in reactions of PM with the model dicarbonyl compound phenylpropanedi-

one.² The tertiary amine products FPyPM and PyPM are most likely formed by reaction of the primary amine of PM with 1,4-dioxo intermediates from the interior of AA.

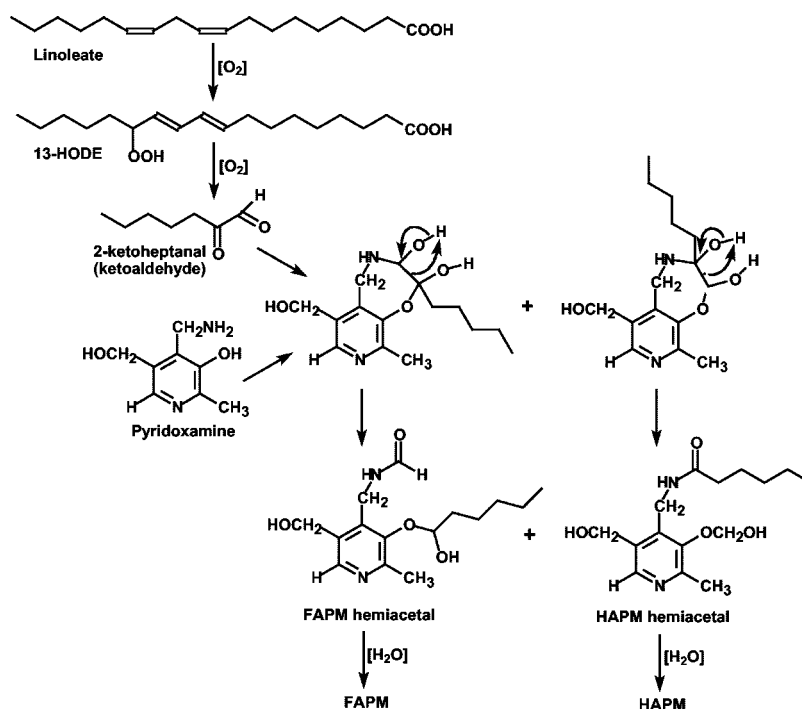
One of the unexpected observations from both the *in vitro* and *in vivo* studies was that no PM adducts analogous to classic AGE/ALEs were detected. Modification of protein during lipid peroxidation reactions produces characteristic lysine AGE/ALEs, such as *N*^ε-(carboxymethyl)lysine, *N*^ε-(carboxyethyl)lysine, and the malondialdehyde and 2-hydroxynonenal adducts, malondialdehyde-lysine and 4-hydroxy-2-nonenal-lysine (10, 21). However, we were unable to detect even traces of the analogous *N*-carboxymethyl-PM, *N*-carboxyethyl-PM, or the malondialdehyde or 4-hydroxy-2-nonenal adducts to PM, based on MRM-LC/MS/MS analyses, either *in vitro* or *in vivo*. Indeed, although PM is a powerful inhibitor of AGE/ALE formation, it actually exhibits low reactivity toward small dicarbonyl compounds such as glyoxal (GO), methylglyoxal (MGO), and malondialdehyde under physiological conditions (5, 22, 23). Voziyan *et al.* (23) isolated and characterized a stable 5-ring adduct between PM and GO, glyoxal-PM (GOPM), in incubations of PM with GO at equimolar and high concentration (10 mM each PM and GO). However, GOPM was not detectable *in vivo* nor in incubations of PM and GO at micromolar concentrations *in vitro* in the range of typical plasma concentrations of these compounds in PM-treated rats. Our *in vivo* data, combined with evidence that PM prevents the formation of characteristic ALEs on protein without forming the corresponding PM derivatives (10), suggest that the immediate precursors of these AGEs, such as GO, MGO, malondialdehyde, and 4-hydroxy-2-nonenal, are not formed. Rather, PM appears to trap earlier, as yet unidentified, diketone or ketoaldehyde intermediates in peroxidation of PUFAs before they decompose to the more reactive carbonyl and dicarbonyl compounds.

Formation of PM Adducts *ex Vivo*—In control experiments, to exclude the possibility that some adducts might be formed during the urine collection process, PM was added to the urine of untreated control, obese, and diabetic animals and allowed to incubate for 24 h at room temperature. Under these conditions, with the exception of FPyPM, <10% of the PM adducts recovered in urine was formed *ex vivo*. (The yield of FPyPM accounted for up to 50% of the amount recovered in the urine of PM-treated rats.) These experiments establish that some reactive lipoxidation intermediates are excreted in urine, probably in the form of polar lipid derivatives. However, all six of the PM adducts identified in urine were also detected in fresh frozen plasma samples from PM-treated animals in approximately the same ratio as they appeared in urine, indicating that the adducts were indeed formed *in vivo*. Newman *et al.* (24) have reported the urinary excretion of isomers of the dihydroxy and epoxy forms of AA, indicating that these more polar molecules are readily soluble in urine. Thus, early oxidation products of PUFA may be further oxidized during the standing of urine at room temperature forming reactive carbonyl intermediates, which would then react with PM. Incubation of PM with urine from diabetic and hyperlipidemic patients may prove useful as a diagnostic test to assess the oxidative state of plasma in diabetes, based on the amount of PM adducts formed during *ex vivo* incubation with urine. Uremia also enhances production of AGE/ALEs in plasma and tissues (25), possibly the result of carbonyl stress (26), and PM may also prove useful for identification of reactive carbonyl toxins and AGE/ALE precursors in uremic plasma.

The Interplay between Hyperglycemia and Hyperlipidemia—

² T. O. Metz, M. L. Stroman, S. R. Thorpe, and J. W. Baynes, manuscript in preparation.

SCHEME 1. Reaction of PM with 2-ketoheptanal. The 13-hydroperoxy-9,11-octadecadienoic acid (13-HODE) produced during oxidation of LA oxidatively decomposes to 2-ketoheptanal (ketoaldehyde), which may react with the primary amine and phenoxyl of PM to form a transient, seven-membered ring that undergoes a ring-opening rearrangement to form mixed amide and hemiacetal adducts to PM. The hemiacetals hydrolyze in aqueous solution to yield stable amide adducts.



Although the mechanism by which PM causes a reduction in triglycerides (and cholesterol) in STZ-Db and *fa/fa* rats (11, 12) is unknown, protection against kidney disease may result from both inhibition of hyperlipidemia and trapping of intermediates in formation of AGE/ALEs. Although both mechanisms may be operative, it is clear that PM acts at least in part as a trap for intermediates in the lipoxidative modification of proteins (Figs. 4 and 5), documenting for the first time the proposed mechanism of action of an AGE or ALE inhibitor *in vivo*.

At the conclusion of this study, the relative role of hyperglycemia *versus* hyperlipidemia in the chemical modification of proteins and the pathogenesis of diabetic complications still remains uncertain. The fact that several biomarkers of protein modification, such as *N*^ε-(carboxymethyl)lysine and *N*^ε-(carboxyethyl)lysine, can be derived from either sugar or lipid sources complicates the interpretation of analytical data. Our results suggest, however, that lipids may be the primary source of chemical modification of proteins in diabetes and obesity (11, 12), especially in the presence of hyperlipidemia or dyslipidemia. Thus, in all of the hyperlipidemic animals, including the non-diabetic *fa/fa* rat, there was a substantial increase in the urinary excretion of PM adducts derived from LA and AA, suggesting an increase in lipid peroxidation. There was also an indication that concurrent hyperglycemia exacerbated lipoxidative modification of protein. This argument is based on the comparison of levels of PM adducts recovered in the urine of the various animal models. The level of PM adducts were, in general, 2–4-fold higher in both STZ-Db+PM and ZDF-Db+PM animals than in non-diabetic obese F+PM animals (Fig. 5), despite dramatically higher levels of triglycerides and lipemia in the obese animals (Fig. 6). Thus, it appears that although severe hyperlipidemia may be sufficient to induce lipoxidative damage, hyperlipidemia, combined with hyperglycemia and possibly an increase in oxidative stress in diabetes (27), may exacerbate the chemical modification of proteins by lipids in diabetes.

In conclusion, although the focus of these studies has been on the mechanism of action of PM, the broader implications of the work suggest a major role for lipid peroxidation and lipoxidative modification of proteins in the development of chronic

complications in diabetes. These studies provide a mechanistic explanation for the results of recent clinical trials that have identified plasma triglyceride concentration as an independent risk factor for development of retinal (28), renal, and cardiovascular disease in both type 1 and type 2 diabetes (29–31) and in non-diabetic populations (32, 33). They also lend support to the current emphasis on more aggressive control of dyslipidemia in diabetes (34, 35).

Acknowledgments—We thank Dr. Perry J. Pellechia for performing and interpreting NMR experiments and Dr. William E. Cotham for assistance with LC/MS experiments.

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