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Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal

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Abstract

Advanced glycation end products (AGEs) from the Maillard reaction contribute to protein aging and the pathogenesis of age- and diabetes-associated complications. The α-dicarbonyl compound methylglyoxal (MG) is an important intermediate in AGE synthesis. Recent studies suggest that pyridoxamine inhibits formation of advanced glycation and lipoxidation products. We wanted to determine if pyridoxamine could inhibit MG-mediated Maillard reactions and thereby prevent AGE formation. When lens proteins were incubated with MG at 37 °C, pH 7.4, we found that pyridoxamine inhibits formation of methylglyoxal-derived AGEs concentration dependently. Pyridoxamine reduces MG levels in red blood cells and plasma and blocks formation of methylglyoxal–lysine dimer in plasma proteins from diabetic rats and it prevents pentosidine (an AGE derived from sugars) from forming in plasma proteins. Pyridoxamine also decreases formation of protein carbonyls and thiobarbituric-acid-reactive substances in plasma proteins from diabetic rats. Pyridoxamine treatment did not restore erythrocyte glutathione (which was reduced by almost half) in diabetic animals, but it enhanced erythrocyte glyoxalase I activity. We isolated a major product of the reaction between MG and pyridoxamine and identified it as methylglyoxal–pyridoxamine dimer. Our studies show that pyridoxamine reduces oxidative stress and AGE formation. We suspect that a direct interaction of pyridoxamine with MG partly accounts for AGE inhibition.

Keywords: α-Dicarbonyls; Maillard reaction; AGEs; Imidazolium crosslinks; Pyridoxamine

Protein amino groups combine with aldehydes and ketones, in what is known as the Maillard reaction, to produce advanced glycation end products or AGEs[2] [1,2]. The reaction damages proteins by covalent cross-linking and altering their conformation. AGEs accumulate in tissues affected by diabetes and aging, including the lens of the eye (cataract) [3], the aorta (atherosclerosis) [4], and the brain (Alzheimer's disease) [5].

Although aldoses and ketoses were originally thought to be the sole precursors of AGEs (thus providing the name advanced glycation end products), recent studies indicate that carbonyl compounds other than sugars are even more reactive and that they produce AGE-like products. Potential reactants include the α-dicarbonyls, methylglyoxal (MG), and glyoxal. MG is produced primarily by nonenzymatic pathways from glycolytic

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[2] *Abbreviations used:* AGE, advanced glycation end product; MG, methylglyoxal; MOLD, methylglyoxal–lysine dimer; GOLD, glyoxal–lysine dimer; BLP, bovine lens protein; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; HFBA, heptafluorobutyric acid; DQFCOSY, double-quantum-filtered COSY; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; GSH, glutathione; TBARS, thiobarbituric-acid-reactive substances; PITC, phenyl isothiocyanate.

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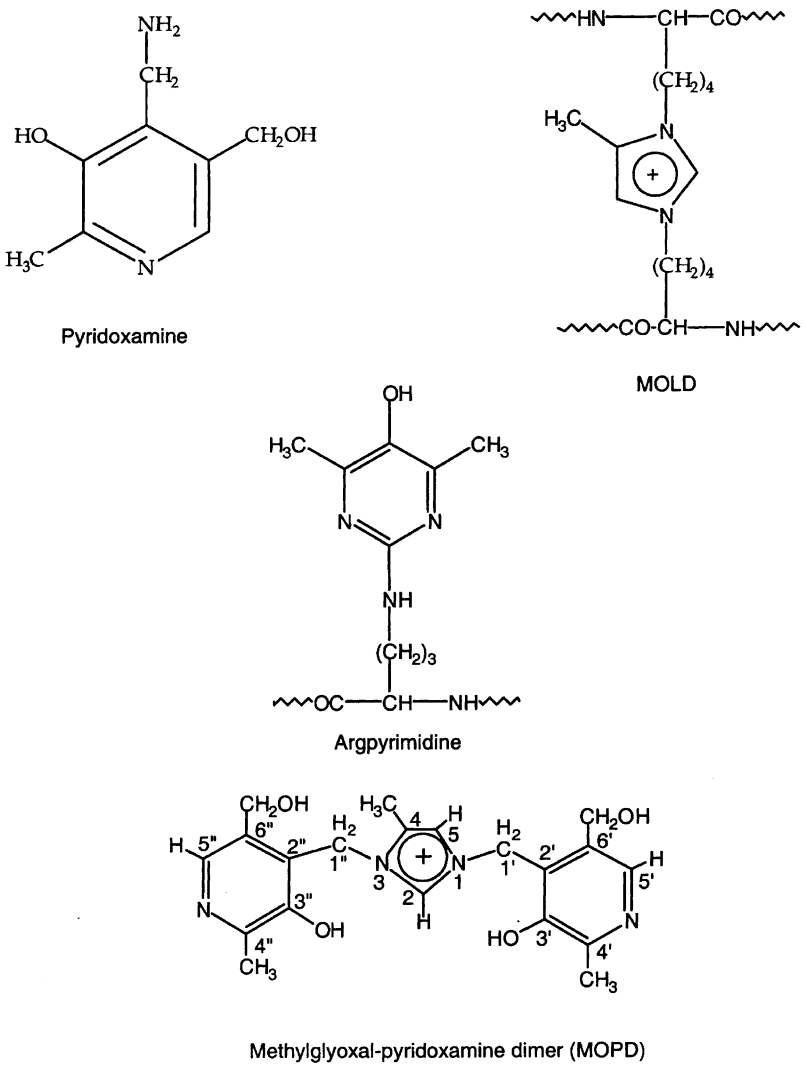

Fig. 1. Structure of pyridoxamine, methylglyoxal–lysine dimer (MOLD) argpyrimidine and methylglyoxal–pyridoxamine dimer (MOPD).

intermediates, such as glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and also by several minor metabolic pathways, including the Maillard reaction [6]. Glyoxal is formed in the course of lipid peroxidation, autoxidation of sugars, and by the Maillard reaction [7]. These two α-dicarbonyl compounds are extremely reactive and readily modify lysine, arginine, and cysteine residues on proteins.

Diabetic individuals have elevated levels of MG in plasma and tissues [6], and Odani et al. reported that uremic patients have significant increases in plasma glyoxal [8]. Several studies identified dicarbonyl-derived products in proteins from diabetic individuals; these include imidazolium crosslinks, MOLD (imidazolysine) (Fig. 1) [9–12], GOLD (glyoxal–lysine dimer) [13,14], carboxyethyllysine [15], and argpyrimidine (Fig. 1) [16,17].

In vitro incubation experiments showed that pyridoxamine (Fig. 1) can inhibit formation of AGEs [18,19] and pyridoxamine also inhibits protein modifications by lipid oxidation products [20]. The mechanism by which pyridoxamine prevents AGE formation is still unconfirmed, although it is thought that pyridoxamine reacts with dicarbonyl intermediates of AGE synthesis. Our experiments show that pyridoxamine inhibits AGE formation from MG both in vitro and in diabetic rats. We also find that the production of methylglyoxal–AGEs depends, in part, on formation of methylglyoxal–pyridoxamine dimer (MOPD) (Fig. 1), an adduct of two molecules of pyridoxamine and two molecules of MG.

Materials and methods

Bovine lenses were obtained from Pel-Freeze Biologicals (Roger, AR). Pyridoxamine, methylglyoxal (40% aqueous solution), and glyoxal (40% aqueous solution) were from Sigma Chemical (St. Louis, MO). MG

was purified by vacuum distillation. All other chemicals were of the highest high-performance liquid chromatography (HPLC) grade.

Extraction of bovine lens proteins. Bovine lenses were decapsulated and homogenized in 50 mM Tris buffer (pH 7.4) (7.46 g/liter KCl, 0.37 g/liter EDTA, 0.35 g/liter mercaptoethanol, 0.7 g/liter NaN_3) using a glass homogenizer. The homogenates were centrifuged for 30 min at 10,000*g*, and the water-soluble supernatant fraction was dialyzed against water for 24 h, concentrated, lyophilized, and stored at −80 °C.

Incubation of bovine lens proteins with MG and pyridoxamine. We incubated bovine lens proteins (BLP) (30 mg/ml) with 5 or 50 mM MG in the presence or absence of pyridoxamine. Incubations with BLP alone were done simultaneously as controls. Pyridoxamine was added to both sets at three different concentrations (5, 15, or 50 mM) to make a total volume of 3.0 ml. All incubations were done in 0.2 M phosphate buffer (pH 7.4) at 37 °C. Aliquots were drawn after 0, 15, and 30 days of incubation and dialyzed against 2.0 l of PBS for 48 h. Samples containing 50 mM pyridoxamine and 50 mM MG developed precipitate after 15 days of incubation. These reaction mixtures were centrifuged, and protein concentration in the supernatant was measured and used for assays.

Animal experiments. All experimental procedures involving animals were designed to conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Resources Center of the Case Western Reserve University (Cleveland, OH). Sprague–Dawley rats (3 months old, approximately 150 g) were made diabetic by a single intraperitoneal injection of streptozotocin (65 mg/kg body wt in citrate buffer). Development of diabetes was confirmed by the appearance of glycosuria and hyperglycemia. All animals used in this study had <250 mg/dl plasma glucose. Nondiabetic animals of comparable age and weight were maintained as controls (C). Each group had at least 12 animals. Pyridoxamine was administered by dissolving it in the drinking water. Diabetic animals received 400 mg/dl (D + PM) and nondiabetic animals received 800 mg/dl (C + PM). The dosage was lower in diabetic animals to compensate for higher water intake when compared to nondiabetic control animals (D). The animals had access to water throughout the day and were maintained for 21 weeks, after which they were killed with a high dose of pentobarbital. Blood was drawn from heart immediately after death; plasma and blood cells were then separated by centrifugation.

Preparation of erythrocyte and plasma proteins. Erythrocytes were lysed in distilled water and centrifuged at 4000 rpm in an Eppendorf microcentrifuge for 10 min at 4 °C to remove the membranes. The supernatant fraction was mixed with an equal volume of 10% trichloroacetic acid (TCA) to precipitate the proteins. Plasma samples were similarly treated with 10% TCA. The precipitated proteins were washed twice with diethylether, dried under vacuum, and then suspended in 1.0 ml of water and lyophilized to a powder. All protein preparations were stored at 20 °C until use.

HPLC assays for AGEs. We identified and measured the imidazolium crosslink, MOLD, by a two-step HPLC assay [21]. Argpyrimidine and pentosidine were measured by HPLC as described by Wilker et al. [22].

Characterization of major reaction product of pyridoxamine and methylglyoxal. MG and pyridoxamine (0.1 M each) were mixed together in 100 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 24 h. HPLC analysis of the incubation (HPLC conditions below) indicated the presence of unreacted pyridoxamine. When two more equivalents of MG were added, and the incubation was extended for an additional 48 h, nearly all of the pyridoxamine was reacted; the products, obtained under these conditions, were then applied to purification. HPLC of the reactants from the pyridoxamine + MG incubation was performed on a semipreparative reverse-phase column (C18, 10 μm, Separations Group, Vydac, Hesperia, CA). The mobile phase consisted of solvent A (0.01 M HFBA in water) and solvent B (40% water in acetonitrile with 0.01 M HFBA). The gradient was as follows: 0–3 min, 0% B; 3–15 min, 50% B; 15–25 min, 50% B; 25–30 min, 100% B; 30–33 min, 100% B; 33–45 min 0% B. The flow rate was 2.0 ml/min. The column effluent was monitored for absorption at 325 nm, and we found that pyridoxamine eluted at approximately 18 min. We noted a major peak at 31 min; this was collected from repeated injections, pooled, and lyophilized.

We then subjected the lyophilized product to NMR and mass spectrometry. ^1H (300 MHz), ^{13}C (75.4 MHz), double-quantum-filtered COSY (DQFCOSY) [23] heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) NMR spectra were recorded on a Varian Unity Inova 300 spectrometer (Darmstadt, Germany) in D_2O. Chemical shifts (δ) are given in ppm relative to external sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propanoate and coupling constants (J) in Hz.

We used a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an ESI interface for accurate mass determination [24]. Mass spectrometry parameters were ESI+, source temperature 80 °C, capillary 3.0 kV, HV lens 0.5 kV, cone voltage 35 V. The data were collected in the multichannel acquisition mode with 128 channels per m/z unit using nine scans (6 s) with 0.1-s reset time. The resolution was 960 (10% valley definition). The sample was dissolved for analysis in water/acetonitrile (1/1) containing polyethylene glycol 400 (0.1 μg/μl) as reference material, ammonium formate (0.2%), and formic acid (1%); the sample concentration was similar to that of polyethylene glycol. The solution was introduced into the ESI source at a flow

rate of 5 μl/min. We calibrated six reference peaks with *m/z* 325–435 scan range: *m/z* 327.2019, 344.2284, 371.2281, 388.2547, 415.2543, 432.2809. From these data we assigned the structure to be 1,3-bis{[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]methyl}-4-methylimidazolium, methylglyoxal pyridoxamine dimer.

Detection of methylglyoxal pyridoxamine dimer (MOPD) in protein incubation. Bovine serum albumin (10 mg/ml) was incubated with 25 mM each of MG and pyridoxamine at 37 °C in 3.0 ml of 50 mM phosphate buffer (pH 7.4). Samples (0.05 ml) were drawn after 0, 1, 3, 5, and 7 days of incubation. The protein was precipitated by addition of 0.5 ml of 10% trichloroacetic acid. Pyridoxamine and MOPD were measured in the trichloroacetic acid supernatant by HPLC using a C-18 reverse-phase analytical column (Vydac). HPLC conditions were the same as described for purification of methylglyoxal pyridoxamine dimer, except that the flow rate was 1.0 ml/min. Under these conditions, pyridoxamine eluted at 18.6 min and MOPD at 33.6 min.

Other methods. Glycated hemoglobin was measured by a kit (Sigma total glycated hemoglobin kit, Cat. No. 442-B) according to the manufacturer's instructions. MG was measured as described by Shamsi et al. [25]. Glyoxalase I activity was measured in erythrocyte lysates as described by Haik et al. [26]. Plasma protein carbonyl content was measured by the method of Oliver et al. [27]. GSH levels in erythrocytes were measured by the method of D'Aquino et al. [28]. Thiobarbituric-acid-reactive substances (TBARS) in plasma proteins were measured by the method of Kakkar et al. [29]. Protein was quantified with a Bio-Rad kit using bovine serum albumin as the standard. Amino acid content of acid hydrolyzates was determined by the ninhydrin reaction method [30].

Results

To determine how pyridoxamine affects methylglyoxal-mediated AGE synthesis, we used HPLC to measure MOLD, the imidazolium lysine–lysine crosslinking structure, in samples of bovine lens proteins that were incubated for 30 days. Incubation for 30 days were performed to determine if intermolecular crosslinking, which occurs as a result of advanced glycation end product formation, is affected in the presence of pyridoxamine. We were unable to measure MOLD at the earlier time periods (0 and 15 days) because of the low levels of product in those incubation samples. Fig. 2A shows that 50 mM pyridoxamine inhibited MOLD formation by nearly 80% when bovine lens proteins were incubated with 5 mM MG. However, inhibition was less impressive in samples exposed to 50 mM MG; here the

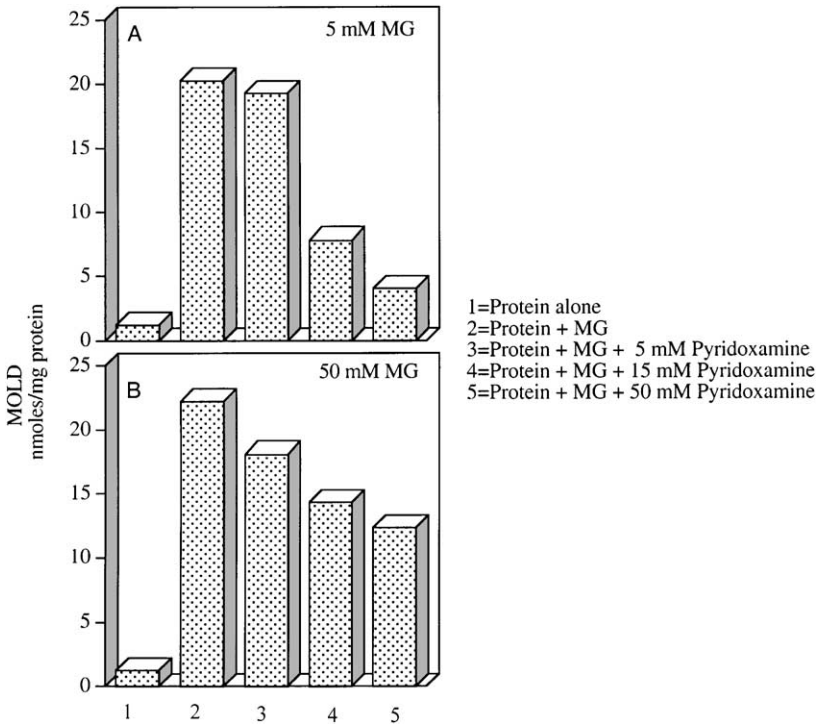

Fig. 2. (A) Effect of pyridoxamine on MOLD formation. Bovine lens proteins were incubated in vitro with MG (5 mM) in the presence of 0, 5, 15, or 50 mM pyridoxamine for 30 days and MOLD was measured by HPLC of the acid-hydrolyzed protein. Values indicated by bars are the average of two determinations. (B) Bovine lens proteins were incubated under the same conditions with 50 mM MG and varying concentrations of pyridoxamine.

Table 1
Physical and biochemical parameters of animals in treatment groups

Group	C ($n = 12$)	D ($n = 13$)	C + pyridoxamine ($n = 12$)	D + pyridoxamine ($n = 12$)
Body weight (g)	628 ± 60	299 ± 59	552 ± 52	340 ± 81
Blood glucose (mg/dl)	76 ± 9	413 ± 39	69 ± 5	375 ± 62
Glycated hemoglobin (%)	5.23 ± 1.07	14.39 ± 1.43	5.46 ± 0.74	17.85 ± 3.78

Note. C, control nondiabetic rats; D, diabetic; C + pyridoxamine, nondiabetic rats treated with 0.8 g/liter pyridoxamine; D + pyridoxamine, diabetic rats treated with 0.4 g/liter pyridoxamine.

same concentration of pyridoxamine (50 mM) inhibited MOLD formation by approximately 45% (Fig. 2B). We concluded that under these conditions, pyridoxamine inhibits MOLD formation more effectively at the lower MG concentration. SDS–polyacrylamide gel electrophoresis of the incubated samples revealed that pyridoxamine (at 50 mM concentration) partly inhibited MG-induced protein crosslinking (data not shown).

Table 1 shows body weights and biochemical parameters in control and diabetic animals at the end of the study. Mean blood glucose was five to six times higher in diabetic animals (D) than in control animals (C). Treatment with pyridoxamine slightly improved glycemia in diabetic animals (D + PM) and slightly inhibited the loss in body weight from untreated diabetes. Glycated hemoglobin levels were three to four times higher in diabetic animals, and there was a slight trend toward further elevations in pyridoxamine-treated diabetic animals.

Fig. 3 shows that MG levels were approximately 10–15 times higher in plasma (Fig. 3A) than in erythrocytes (Fig. 3B). In diabetic animals, MG levels increased in both erythrocytes and plasma, but the increase was significant ($P < 0.05$) only in plasma. Pyridoxamine treatment did not alter MG levels in nondiabetic rats (C + PM), but in both fractions from diabetic rats, it significantly reduced MG levels ($P < 0.05$). Surprisingly, MG levels were lower in pyridoxamine-treated diabetic rats (D + PM) when compared to control rats.

To determine if pyridoxamine affected glyoxalase I activity, we measured the enzyme activity in erythrocytes from diabetic and control animals. Cell lysates were dialyzed against PBS before assay, thereby excluding the effect of endogenous MG and glutathione.

Glutathione is essential for the metabolism of MG through glyoxalase. Glutathione reacts nonenzymatically with MG to form a hemithioacetal complex, which is a substrate for glyoxalase I. The product of the glyoxalase I reaction, *S*-D-lactoylglutathione, is then converted to D-lactate by glyoxalase II. We found significant reduction of glutathione levels in lysed erythrocyte preparations from diabetic animals, but pyridoxamine treatment in vivo did not alter this phenomenon (Fig. 4A). As shown in Fig. 4B, glyoxalase I activity was unchanged in diabetes, but we found a significant increase in activity in erythrocytes from pyridoxamine-treated diabetic rats (D + PM). These data suggest that pyridoxamine increases the glyoxalase I activity under the hyperglycemic conditions.

To determine if pyridoxamine affects methylglyoxal-mediated Maillard reactions in diabetes, we measured MOLD in plasma proteins. There was a trend towards increased MOLD levels in diabetes; mean levels were approximately 20% higher in plasma proteins from

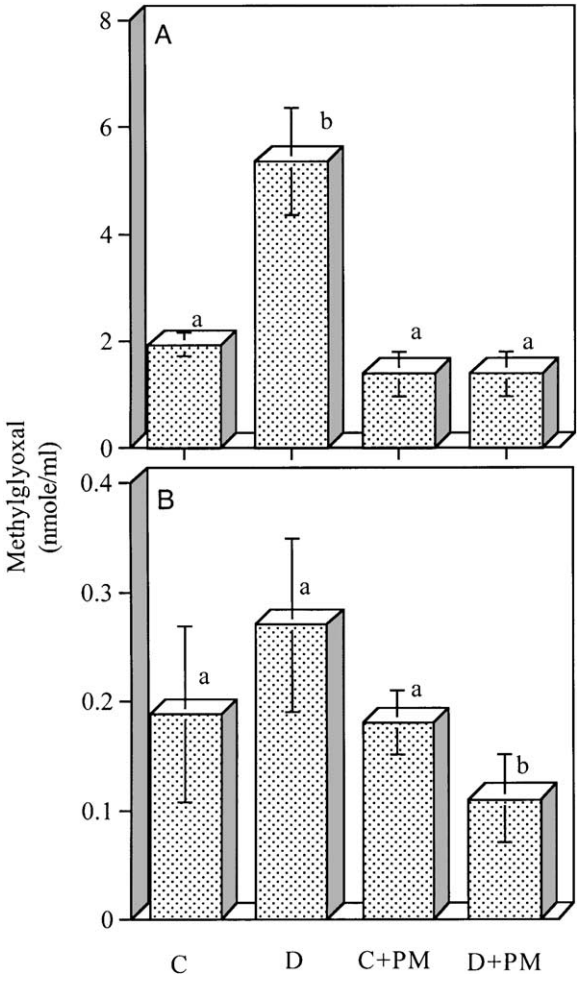

Fig. 3. MG in plasma (A) and erythrocytes (B) from control (C) and diabetic (D) rats and those treated with pyridoxamine. MG was measured by HPLC. The heights of the bars indicate MG concentrations (nmol/ml). Bars that do not share a common superscript are statistically significant ($P < 0.05$).

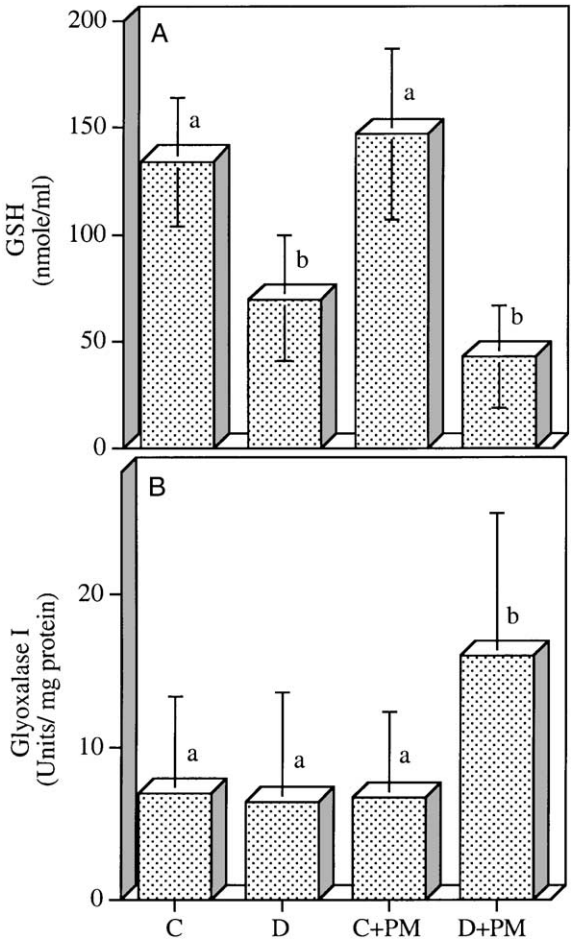

Fig. 4. Effect of pyridoxamine on glutathione and glyoxalase I in erythrocytes. Rats were treated with pyridoxamine in drinking water for 5 months. (A) Glutathione concentrations (nmol/ml) in control (C) and diabetic (D) animals. Pyridoxamine was administered to both groups in drinking water (0.8 g/liter (C + PM) or 0.4 g/liter (D + PM). (B) Glyoxalase I activity for the same treatment groups. Statistical determination as in Fig. 3.

diabetic animals than in those from controls, but the differences were not significant (Fig. 5). Pyridoxamine treatment caused a trend toward increased MOLD in nondiabetic animals, but in diabetic animals it reduced MOLD levels by nearly 40% ($P < 0.05$).

We also used HPLC to measure argpyrimidine, another methylglyoxal-derived AGE in plasma proteins. Our HPLC chromatogram showed a peak that had identical retention time to that of synthetic argpyrimidine (data not shown). The material in this peak was quantified based on a standard curve generated by injecting several concentrations of synthetic argpyrimidine. Mass spectrometric analysis is necessary to further confirm argpyrimidine in this peak. Our results showed that mean "argpyrimidine" levels were higher in diabetic rats (5.9 ± 0.2 pmol/mmol of amino acids) compared to nondiabetic controls (4.2 ± 0.2), but the increase was not statistically significant. Pyridoxamine treatment of

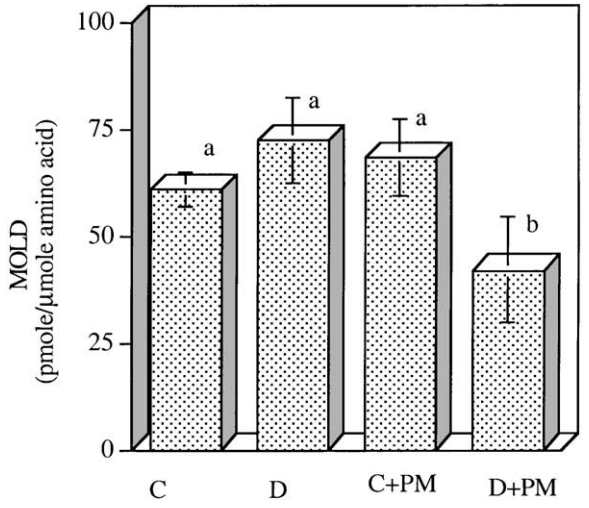

Fig. 5. Effect of pyridoxamine treatment on plasma protein MOLD. Samples were acid hydrolyzed and separated on a C-18 column, and fractions corresponding to MOLD were collected, derivatized with PITC, and separated by C-18 HPLC.

diabetic rats reduced the levels to control levels (4.3 ± 0.3). Pyridoxamine treatment in nondiabetic rats had no effect on argpyrimidine levels (4.0 ± 0.1).

Fig. 6 shows accumulation of pentosidine in plasma proteins from diabetic rats. The samples from untreated diabetic rats had nearly 30% higher concentrations of pentosidine ($P < 0.05$) than those from controls. Pyridoxamine suppressed this increase, reducing the mean value to nearly 17% less than that for the pyridoxamine-treated control group.

We reasoned that the beneficial effects of pyridoxamine on AGE formation might be due to a reduction of oxidative stress. To test this possibility we measured TBARS and protein carbonyl content, since both of these parameters increase with oxidative stress. As can

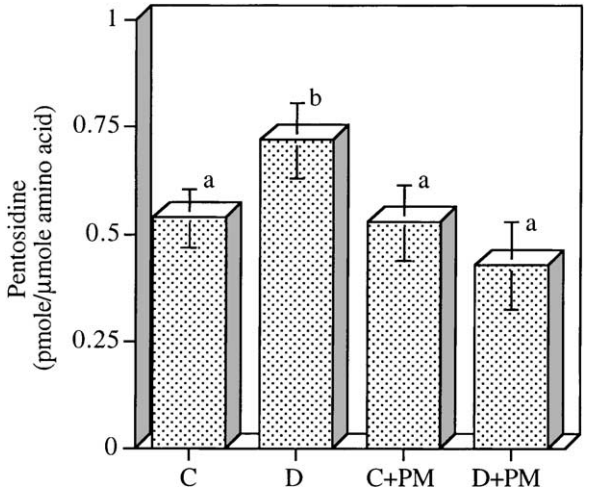

Fig. 6. Effect of pyridoxamine on plasma protein pentosidine. Control and diabetic rats were treated with pyridoxamine in vivo as described in the legend to Fig. 4. Statistical determination as in Fig. 3.

be seen in Fig. 7A and B, both TBARS and protein carbonyl content increased in samples from diabetic animals relative to controls. The increase in TBARS was significant ($P < 0.05$), and pyridoxamine treatment suppressed this increase by nearly 22% (Fig. 7A). Plasma proteins from diabetic rats had more protein carbonyls (by approximately 30%) than those from nondiabetic controls (Fig. 7B). Surprisingly, pyridoxamine treatment decreased protein carbonyls in the diabetic group well below the comparable controls; this did not occur in nondiabetic animals.

To determine if the inhibition of MOLD formation by pyridoxamine was due to a direct reaction of MG with pyridoxamine, we incubated MG with pyridoxamine in vitro using conditions that closely approximate those in vivo. We injected this sample into the HPLC column and monitored the effluent at 325 nm. We found one major peak and several minor peaks. We purified the major peak by preparative HPLC and reexamined the product by spectroscopy. When this product was reinjected into the same column using the same gradient system, we obtained a single peak (Fig. 8A) that was more hydrophobic than pyridoxamine (retention time 18.6 min) (Fig. 8B). The spectrum of the purified compound was nearly identical to that of pyridoxamine; both show absorption maxima at approximately 325 nm (data not shown).

Table 2 shows chemical shifts from ^{1}H NMR and ^{13}C NMR spectra. Our isolated product contains two pyridoxamine residues, as indicated by the signal for two methyl groups of the pyridoxamine moieties at δ 2.55 showing a 6H integral. The methyl group on the imidazolium ring shows a chemical shift of δ 2.16. ^{13}C

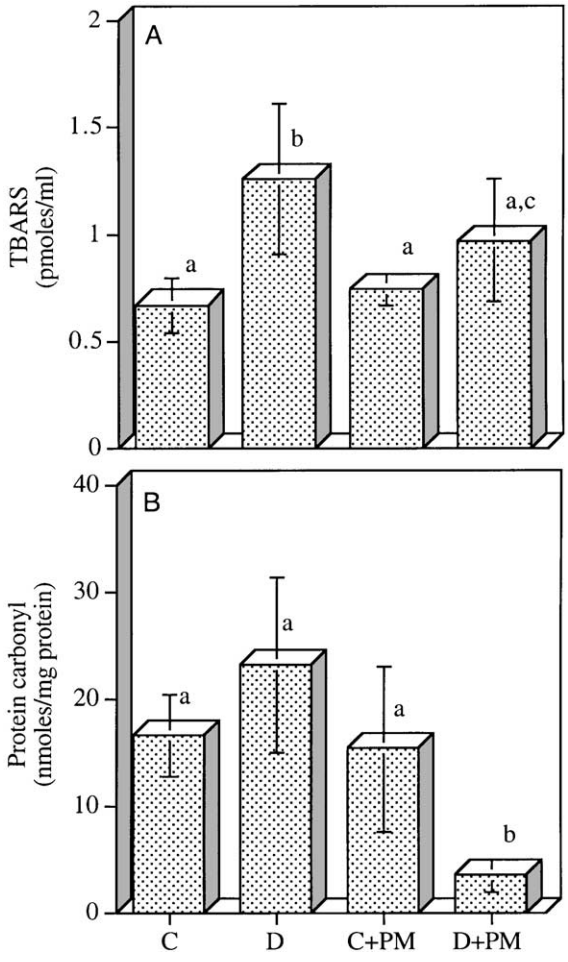

Fig. 7. Pyridoxamine effect on TBARS and protein carbonyl in plasma proteins. (A) TBARS (pmol/ml) in plasma proteins from control and diabetic rats following in vivo treatment with pyridoxamine. (B) Pyridoxamine effect on protein carbonyl content (nmol/mg protein). Statistical determination as in Fig. 3.

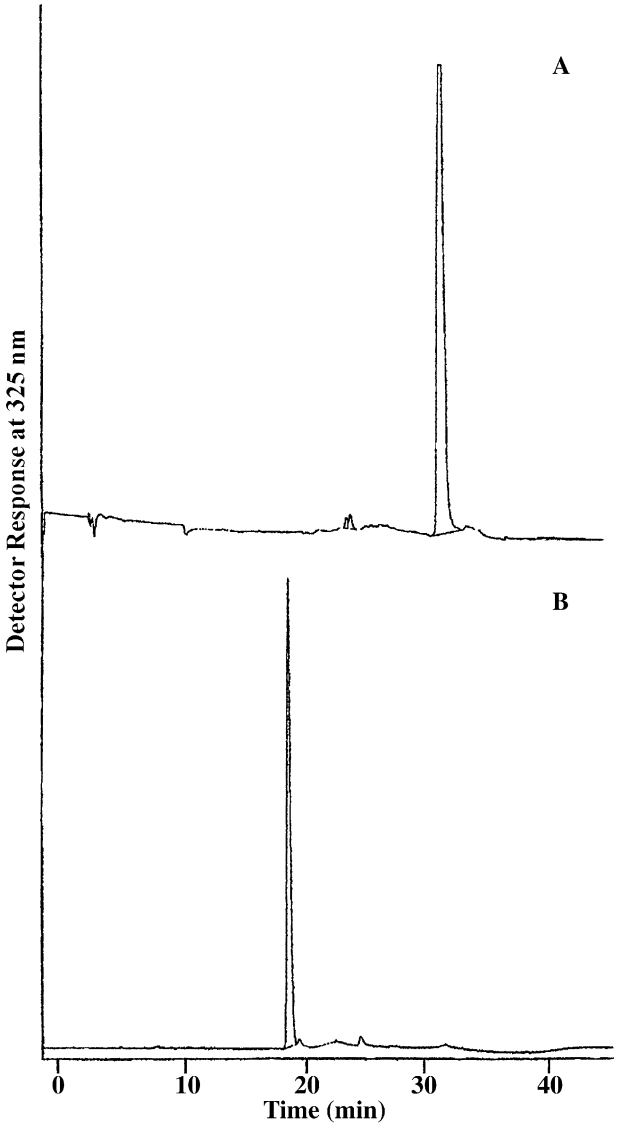

Fig. 8. HPLC of pyridoxamine + MG incubation. Reverse-phase HPLC showed a major absorbance product at 325 nm. This product was collected from several injections and reinjected onto the same column. (A) The chromatogram for the purified product and (B) the chromatogram for pyridoxamine.

Table 2
Chemical shifts for the purified product

No.	^{1}H	^{13}C
2	8.70 (lH, d, $J = 1.7$ Hz)	140.07
4		135.66
4-CH_3	2.16 (3H, d, $J = 0.8$ Hz)	11.16
5	7.19 (1H, m)	122.28
1′	5.43 (2H)	46.68
2′		139.41
3′		156.50
4′		146.28
4′-CH_3	2.55 (3H)	17.86
5′	8.14 (1H)	134.44
6′		140.84
6′-CH_2OH	4.73 (2H)	61.26
1″	5.42 (2H)	44.92
2″		138.99
3″		156.34
4″		146.43
4″-CH_3	2.55 (3H)	17.86
5″	8.19 (1H)	134.36
6″		140.98
6″-CH_2OH	4.72 (2H)	61.31

Note. Chemical shift (δ) is expressed in ppm relative to external standard, sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate. Coupling constant (J) is expressed in Hz. s, singlet; d, doublet; m, multiplet. See Fig. 1 for structure.

NMR displays 20 carbon atoms. Hydrogen and carbon assignments were validated by DQFCOSY, HSQC, and HMBC. The accurate mass (mean ± SD from 10 measurements) was determined to be *m/z* 385. 1882 ± 0.0006 $[M + H]^+$. The *m/z* of 385.1876 corresponded to the empirical formula $C_{20}H_{25}N_4O_4$. From these data we assigned the structure of the compound as 1,3-bis{[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]methyl}-4-methylimidazolium (Table 2). We designated MOPD as the abbreviation for this structure.

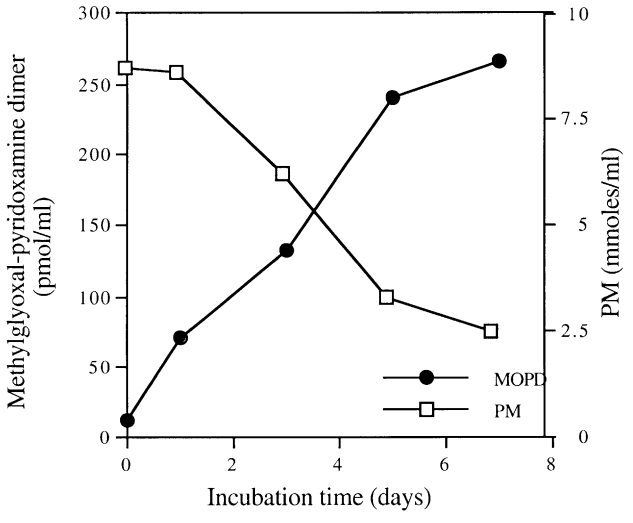

Fig. 9. MOPD formation and MG utilization of pyridoxamine. Bovine serum albumin was incubated in vitro with MG and pyridoxamine, and MOPD and pyridoxamine were measured by HPLC in supernatant samples. The time-dependent increase in methylglyoxal–pyridoxamine dimer mirrors the decrease in pyridoxamine in the incubation medium.

Finally, when we incubated BSA with MG and pyridoxamine, we found that the time-dependent increase in methylglyoxal–pyridoxamine dimer coincided with a decrease in pyridoxamine (Fig. 9). This suggests that inhibition of the MG-mediated reaction by pyridoxamine is related to formation of methylglyoxal–pyridoxamine dimer. Since we now believe that methylglyoxal–pyridoxamine dimer is an important product of MG and pyridoxamine, our data indicate that the inhibitory effects of pyridoxamine are due to its interaction with MG.

Discussion

Booth et al. were the first investigators to report that pyridoxamine could block post-Amadori reactions that lead to AGEs [31]. They found that pyridoxamine inhibited AGE formation in samples of bovine serum albumin incubated with glucose and noted that it was a more effective inhibitor than aminoguanidine. Baynes and colleagues reported that pyridoxamine inhibited advanced glycation and lipoxidation end products and suggested that some of the beneficial effects of pyridoxamine were due to this mechanism [20]. The same authors recently showed that pyridoxamine treatment prevents development of pathological changes associated with renal functions in diabetic rats [32].

Although it is not clear exactly how pyridoxamine inhibits AGE formation, one possibility is that it acts through nucleophilic reactions with carbonyl intermediates of the Maillard reaction. A recent study by Voziyan et al. [33] showed that pyridoxamine reacts directly with Maillard reaction intermediates glyoxal and glycoaldehyde and inhibits formation of carboxylmethyllysine. Because we wanted to determine if the beneficial effects of pyridoxamine were due, in part, to inhibition of MG-mediated reactions, we used diabetic rats. These animals provided a metabolic model of enhanced AGE formation in vivo, in which, over 5 months of treatment, we were able to determine how pyridoxamine affects specific products of the reaction of proteins with MG.

Our in vitro experiments with plasma proteins showed that pyridoxamine was an ineffective inhibitor of MOLD formation at a pyridoxamine:MG ratio of 1:1 (Fig. 2). However, when the ratio of pyridoxamine:MG was 10:1, MOLD formation was inhibited by 80%. This finding suggests that MG reacts to a greater extent with proteins than with pyridoxamine. In this regard, Degenhardt et al. [34] described steady-state levels of pyridoxamine that can reach 100 μM in diabetic rats

treated with 1 g/liter of pyridoxamine. We used a lower dose (0.4 g/liter) to treat our diabetic animals, but assuming that a proportionately lower steady-state level of pyridoxamine occurs, our animals should have plasma concentrations of pyridoxamine around 40 μM. Since we found plasma MG levels to be approximately 5 μM, 40 μM pyridoxamine would be sufficient to react with all the plasma MG.

We were surprised to find that MOLD levels in pyridoxamine-treated diabetic rats were 30–35% lower than in nondiabetic controls and nondiabetic + pyridoxamine rats (Fig. 5). One possible explanation is that, because of increased cellular permeability in diabetes, pyridoxamine binds to MG within the cells, thus preventing leakage of MG into plasma. Our data on erythrocyte MG levels support this (Fig. 3). We find that MG concentrations are significantly lower in pyridoxamine-treated diabetic rats than in untreated diabetic or nondiabetic, pyridoxamine-treated animals. MG levels in plasma proteins did not correlate with the levels of plasma protein–MOLD. MOLD was not significantly elevated in samples from diabetic rats, but pyridoxamine treatment did reduce it in the diabetic group relative to controls.

It should be noted that MG reacts with proteins to produce a variety of other compounds in addition to MOLD and argpyrimidine. For example, other products of MG-protein reactions include N^5-(4-methyl-5-oxo-4,5-dihydro-1*H*-imidazol-2-yl)ornithine [35] and N^6-carboxyethyllsyine [36]. In order to understand how pyridoxamine influences all the various methylglyoxal-mediated modifications, we would need methods to measure all of the diverse AGEs that result from MG–protein interactions. Pending availability of such methods, we can only speculate that pyridoxamine inhibits other modifications by MG in addition to the ones we observed.

The glyoxalase enzyme system is important for the detoxification of MG in cells [6]. Glyoxalase I, the first of two enzymes, converts the hemithioacetal formed from the nonenzymatic reaction of MG and GSH to D-*S*-lactoylglutathione. Glyoxalase II then converts this product to D-lactate. We measured glyoxalase I activity in the dialyzed proteins from erythrocytes and found that glyoxalase I activity was unaltered in diabetic rats (Fig. 4B). This result confirmed observations from a previous study, but we were surprised to find that diabetic animals treated with pyridoxamine had higher enzyme activity than other treatment groups. This suggests that pyridoxamine either induces synthesis of glyoxalase I or activates native erythrocyte glyoxalase I.

Increased erythrocyte glyoxalase I activity may account for decreased MG concentrations in diabetic animals treated with pyridoxamine. Glutathione levels were not significantly altered by pyridoxamine treatment, but glutathione was significantly lower in both pyridoxamine-treated and untreated diabetic animals compared to nondiabetic controls (Fig. 4A). Whether the diabetic animals had sufficient glutathione to support the observed increase in glyoxalase I activity remains to be determined.

We recorded significantly elevated levels of pentosidine in diabetic rats and found that pyridoxamine treatment suppressed formation of this product (Fig. 6). Like MOLD, the pentosidine levels were less in pyridoxamine-treated diabetic rats than either untreated or nondiabetic controls. This finding supports our idea that pyridoxamine probably prevents pentosidine precursors from forming at their sites of origin. This might occur at selected sites in cell metabolism., e.g., the formation of ribose or oxidation of ascorbate. In fact, a recent study indicates that pyridoxamine inhibits degradation of ascorbate [37] by binding to metal ions. Another recent study suggests that pyridoxamine inhibits formation of oxygen free radicals and lipid oxidation [38]. These effects may reduce synthesis of AGEs.

Inhibition of MG-mediated Maillard reactions by pyridoxamine, at least in part, appears to be due to formation of MOPD a compound that we identified by spectroscopic methods. Methylglyoxal pyridoxamine dimer is similar in structure to MOLD [9,11], the difference being that there are two pyridoxamine molecules in place of two lysines. The ability of pyridoxamine to sequester MG before it reacts with lysines and arginines of target proteins depends upon several factors, including the relative reactivity of MG with pyridoxamine, the reactivity with lysine and arginine and the concentration of reactants. The inhibition of MOLD formation observed in our incubation experiments suggests that pyridoxamine prevents protein crosslinking by MG. Further experiments with our animal models should clarify the mechanism. Incubation of bovine serum albumin with MG in the presence of pyridoxamine showed a time-dependent increase in the formation of methylglyoxal pyridoxamine dimer that correlated with the disappearance of pyridoxamine (Fig. 9). Thus, MOPD formation appears to be a mechanism by which pyridoxamine protects against MG-mediated reactions.

In summary, our study establishes that pyridoxamine inhibits formation of AGEs by preventing dicarbonyl-mediated reactions. Whether this inhibition occurs through direct reaction of α-dicarbonyls with pyridoxamine or by inhibition of pathways that enable α-dicarbonyls to react with proteins remains to be established.

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