

Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat

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Background. Nonenzymatic reactions between sugars or lipids and protein and formation of advanced glycation and lipoxidation end products (AGE/ALEs) contribute to the chemical modification and cross-linking of tissue proteins with age. Accelerated formation of AGE/ALEs during hyperglycemia is implicated in the development of diabetic complications. In this study, we examined the effect of the AGE/ALE inhibitor pyridoxamine on chemical modification and cross-linking of collagen and development of renal disease in the streptozotocin-diabetic rat.

Methods. Diabetic rats were treated with pyridoxamine; parallel experiments were conducted with aminoguanidine, the prototype AGE inhibitor. Progression of renal disease was evaluated by measurements of albuminuria and plasma creatinine concentration. Plasma triglycerides, cholesterol, lactate and pyruvate were measured by enzymatic assays, and AGE/ALEs in skin collagen by HPLC and GC-MS assays.

Results. Pyridoxamine significantly inhibited the increase in albuminuria, plasma creatinine, hyperlipidemia and plasma lactate/pyruvate ratio in diabetic rats, without an effect on blood glucose or glycated hemoglobin. AGE/ALEs, fluorescence and cross-linking of skin collagen increased approximately twofold in diabetic versus control rats after seven months of diabetes. Pyridoxamine caused a significant (25 to 50%) decrease the AGE/ALEs, carboxymethyllysine and carboxyethyllysine, cross-linking and fluorescence in skin collagen of diabetic rats, but did not affect pentosidine.

Conclusions. Pyridoxamine inhibits the progression of renal disease, and decreases hyperlipidemia and apparent redox imbalances in diabetic rats. Pyridoxamine and aminoguanidine had similar effects on parameters measured, supporting a mechanism of action involving AGE/ALE inhibition.

Advanced glycation end products (AGE) are formed by chemical reaction of carbohydrates with protein in a

process known as the Maillard or browning reaction. This reaction begins with adduction of a reducing sugar to an amino group in protein, typically the ϵ -amino group of a lysine residue, to form a Schiff base (imine), which then rearranges to an Amadori compound (ketoamine), such as fructoselysine. The Amadori product is a precursor to AGEs, which are a more permanent, irreversible modification of proteins. According to the AGE hypothesis [1, 2], the increased rate of chemical modification and cross-linking of tissue proteins during hyperglycemia compromises the structure and function of proteins and contributes to the development of chronic complications in diabetes. The accumulation of AGEs in collagen, a long-lived protein in the extracellular matrix, is thought to effect changes in elasticity, ionic charge, thickness and turnover of basement membrane components, setting the stage for development of diabetic renal, retinal and vascular disease.

Reactive carbonyl and dicarbonyl compounds derived from sugars are intermediates in the formation of AGEs, and oxygen, redox active transition metal ions and reactive oxygen species are recognized as catalysts of AGE formation [3]. The precise chemical origin of specific AGEs is not always clear. Some AGEs, such as pentosidine, are clearly derived from carbohydrates, but may be formed from glucose, ascorbate, 3-deoxyglucosone or other sugars in vivo [4]. The major AGEs, including the lysine adducts N ϵ -(carboxymethyl)lysine (CML) and N ϵ -(carboxyethyl)lysine (CEL), and the imidazolium crosslinks glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD), may be formed from early, protein-bound intermediates in the Maillard reaction, such as the Schiff base or Amadori compound. However, they also may be formed directly from glyoxal or methylglyoxal, which in turn may be derived by either chemical or enzymatic reactions from both carbohydrates and lipids [4, 5]. Thus, the increase in CML and CEL in atherosclerotic plaque from non-diabetic individuals is most likely the result of peroxidation of lipids in lipopro-

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teins in the vascular wall. In this case, CML and CEL are more properly described as advanced lipoxidation end-products (ALE) [5]. In the absence of firm evidence on their origin, even during hyperglycemia in diabetes, these compounds should be referred to as AGE/ALEs, that is, products that may be derived from reactions of carbohydrate and/or lipids with proteins.

A number of compounds have been described as AGE inhibitors, based on their inhibition of AGE formation during incubation of proteins with glucose *in vitro*. These inhibitors vary widely in structure, the common theme being their nucleophilicity or reactivity with reactive carbonyl intermediates in AGE formation. Aminoguanidine (AG), the prototype AGE inhibitor, inhibited the increase in fluorescence and cross-linking of aortic collagen in diabetic rats [6]. In subsequent studies, AG and the related compounds, OPB-9195 (2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yl-acetanilide) and 2,3-diaminophenazine retarded the development of retinal, vascular and renal pathology in diabetic animals [7–9]. Protective effects of these inhibitors were associated with a decrease in AGEs in serum proteins measured by enzyme-linked immunosorbent assay (ELISA) [10], or in lens proteins [11] or tissues collagens [8, 9, 12] by immunohistochemistry. In no instance have the immunohistochemical observations been confirmed by direct chemical analysis for specific AGEs in tissue proteins.

Hudson and colleagues recently described a new class of post-Amadori AGE inhibitors, termed Amadorins, which block the formation of AGEs from Amadori adducts on proteins [13–15]. Although both AG and the Amadorin pyridoxamine (PM) inhibited the chemical modification of protein by ribose and glucose *in vitro*, only PM was effective in inhibiting the formation of AGEs from Amadori adducts on glycated proteins, prepared by anaerobic incubation with ribose or glucose. Thus, there is overlap in the AGE-inhibitory activity of these compounds, with PM having a broader range of inhibitory activity, that is, inhibition of post-Amadori reactions. In a recent report [16] we showed that PM was also an effective inhibitor of advanced lipoxidation reactions, inhibiting the modification of lysine residues in RNase and low-density lipoprotein (LDL) during metal-catalyzed oxidation reactions, as well as the formation of CML and CEL, malondialdehyde-lysine (MDA-Lys) and hydroxynonenal-lysine (HNE-Lys). We report here that AGE/ALE inhibitor PM inhibits the development of early renal dysfunction, as measured by the increase in urinary albumin and plasma creatinine concentrations in the streptozotocin (STZ)-induced diabetic rat. We also made the unexpected observation that PM reduced the increase in hyperlipidemia and the alterations in redox balance in the STZ-diabetic rat. Considering the growing recognition of the importance of hyperlipidemia and dyslipidemia in the development of both diabetic

and non-diabetic renal disease [17–21], our results suggest that the renoprotective effects of PM may derive from both its AGE/ALE inhibitory and its lipid-lowering activities. The relationship between these two activities of PM is also discussed.

METHODS

Materials

Female Sprague-Dawley rats (8 weeks old, ~150 g) were obtained from Harlan Corp. (Indianapolis, IN, USA). PM · (HCl)₂, AG hemisulfate, STZ and clinical assay kits were obtained from Sigma Chemicals Inc. (St. Louis, MO, USA).

Animal studies

Rats were treated according to the guidelines of the Institutional Animal Care and Use Committee of the University of South Carolina. Diabetes was induced by a single tail vein injection of 45 mg/kg of STZ in 0.1 mol/L sodium citrate buffer, pH 4.5. Non-diabetic animals were sham injected with buffer only. Diabetes was confirmed by measuring blood glucose levels at two and three days after the STZ-injection. Animals with plasma glucose higher than 16 mmol/L were classified as diabetic. The diabetic rats were divided randomly into an untreated diabetic group (diabetic control; *N* = 12) and two diabetic treatment groups, receiving either PM (diabetic + PM; *N* = 13) or AG (diabetic + AG; *N* = 12) at 1 g/L in drinking water. Two non-diabetic groups were studied, one receiving no treatment (non-diabetic control; *N* = 13), the other receiving PM at 2 g/L in drinking water (non-diabetic + PM; *N* = 12). The higher dose of PM in the PM-treated control group was designed to compensate, in part, for the lower water intake of non-diabetic, compared to diabetic animals. All animals were housed individually with a light dark cycle of 12 hours each, and had free access to food and water. To maintain body weight and to limit hyperglycemia, all diabetic animals were treated with 3 IU of ultralente insulin (Humulin U; Eli Lilly) three times per week in the afternoon (3 to 5 PM); the dose was increased to 5 IU after week 15 to maintain glycemic control as the rats gained weight.

Glycemic control

Glycemia was monitored monthly by measurement of plasma glucose and glycated hemoglobin. Non-fasting blood was obtained in the morning from the tail vein, using heparinized microhematocrit tubes, and plasma recovered by centrifugation. Plasma glucose was measured using the Trinder assay Kit: (Sigma # 315). Total glycated hemoglobin (GlcHb) was measured on whole blood by boronate affinity chromatography (Sigma kit # 442-B).

Measurement of drug levels in plasma and urine

Pyridoxamine and AG concentrations in plasma were measured by reverse phase–high performance liquid chromatography (RP-HPLC). PM was assayed as described by Sampson and O'Connor [22], with excitation and emission wavelengths of 293 nm 393 nm, respectively. AG was quantified at 380 nm as the 4-nitrobenzaldehyde conjugate [23].

Renal function

Progression of renal disease was assessed by monthly measurements of urinary albumin and plasma creatinine concentrations. For urinary measurements, rats were housed in metabolic rat cages (Nalgene; Nalge Company, Rochester, NY, USA) for 24 hours. Several drops of toluene were added to the urine collection beaker to inhibit microbial growth. Urinary albumin was quantified by a competitive ELISA assay. Rabbit antiserum to rat albumin and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from ICN Biomedical Research Products (Costa Mesa, CA, USA). Briefly, wells were coated with 50 ng of rat albumin (Sigma) in 0.1 mol/L sodium carbonate buffer, pH 10.4, overnight at 4°C. In the competition step 100 µL of standard or urine sample was incubated with 100 µL of rabbit anti-rat albumin antiserum for three hours at room temperature on a microplate shaker. After washing, horseradish peroxidase-conjugated goat anti-rabbit IgG (200 µL) was applied for one hour at 25°C with shaking, and the plate was developed with 200 µL of ABTS-reagent (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) for 45 minutes at room temperature. Absorbance was measured at 405 nm.

Plasma creatinine concentration was measured by the Jaffé picric acid procedure, using Sigma kit # 555-A. Absorbance at 490 nm was measured before and after addition of the acid reagent. In these and other clinical assays, samples were diluted so that measurements were within the range of the standard curve. None of the assays was subject to interference by aminoguanidine or pyridoxamine.

Plasma lipids and other metabolites

Total cholesterol and triglycerides were measured by enzymatic, colorimetric, end-point assays using Sigma kits for total cholesterol (#352) and total triglycerides (#37, GPO Trinder). Free fatty acids were measured as copper complexes in organic solvent, as described by Noma et al [24]. Glycerol was measured by a spectrometric enzymatic assay using glycerol kinase and glycerol 3-phosphate dehydrogenase, as described by Bergman [25]. Lactate and pyruvate were measured spectrophotometrically, using NADH/NAD⁺ linked assays (Sigma Kits #826 and 726, respectively).

Necropsy and tissue sampling

Blood was drawn from anaesthetized (isoflurane) rats by heart puncture and transferred into heparinized vacuum tubes on ice. Rats were killed by over-anesthetization, and liver and kidneys were removed, the kidney decapsulated, and both organs rinsed in phosphate buffered saline, and weighed; sections were dissected and fixed for electron and light microscopy. Abdominal skin (2 cm²) also was removed, rinsed in phosphate buffered saline (PBS) and stored at -70°C. Blood was stored on ice and separated into plasma and red blood cells (RBC) within 30 minutes by centrifugation in a refrigerated benchtop centrifuge. Aliquots of plasma were stored at -70°C until time of analysis.

Isolation and analysis of collagen

The insoluble fraction of skin collagen was prepared by scraping the skin with a single-edged razor blade to remove hair and adventitious tissue, followed by a series of extractions with 0.5 mol/L NaCl, 0.5 mol/L acetic acid and chloroform/methanol (2:1 vol/vol), as described previously [26]. Fructoselysine (FL), a measure of the extent of glycation of collagen, and the AGE/ALEs, CML and CEL, were assayed by isotope dilution, selected ion monitoring gas chromatography–mass spectrometry (SIM-GC/MS), and pentosidine by RP-HPLC [26]. Levels of these AGE/ALEs were normalized to the lysine content of the collagen since lysine is a common component of all of the compounds.

The relative extent of cross-linking of collagen was estimated from the kinetics of digestion of the collagen by pepsin. Digestion of collagen (3 mg suspended in 7.5 mL 0.5 mol/L acetic acid) was initiated by addition of pepsin [3 µL of 1% (wt/vol) solution, yielding 20 µg pepsin/mL final concentration or 50 µg pepsin/mg collagen], and samples were incubated at 37°C in a water bath with shaking at 60 Hz. Aliquots (250 µL) of the supernatant were removed at various times and mixed with an equal volume of concentrated HCl and hydrolyzed for 24 hours at 110°C. Percent digestion of collagen at each time point was calculated as percent hydroxyproline released into supernatant, compared to hydroxyproline in the supernatant at 24 hours when complete digestion of all samples was achieved, that is, <1% residual collagen was present in the pellet fraction. Hydroxyproline was measured in acid hydrolysates of the protein by the method of Stegemann and Stalder [27]. The final hydrolysate diluted 10-fold in 0.5 mol/L acetic acid was analyzed directly for total Maillard-type fluorescence (Ex = 370 nm, Em = 440 nm), and normalized to the Hyp content of the solution.

Renal morphometry

A sample from the right kidney of each rat was fixed in 10% buffered formalin and embedded in JB-4TM. Five-

Table 1. Body weights and metabolic control in various groups of animals^a

	<i>N</i>	Final weight ^b <i>g</i>	Plasma glucose <i>mmol/L</i>	GlcHb <i>%</i>	Collagen glycation <i>mol FL/mol Lys</i>
Non-diabetic control	13	285 ± 10.7	5.1 ± 0.4	7.3 ± 0.7	4.8 ± 1.6
PM-treated control	12	261 ± 11.9 ^c	5.0 ± 0.3	7.3 ± 0.4	5.0 ± 1.7
Untreated diabetic	12	248 ± 24.9	26.7 ± 2.1	15.8 ± 0.6	23.8 ± 3.4
PM-treated diabetic	12	213 ± 23.0 ^d	26.0 ± 2.9	15.4 ± 1.2	21.1 ± 2.8
AG-treated diabetic	13	225 ± 23.0 ^d	25.3 ± 4.0	14.4 ± 1.6	21.5 ± 4.0

^a Data are means ± SD. Measurements are shown for week 28 and are representative of results obtained throughout the study. Abbreviations are: GlcHb, glycated hemoglobin; FL, fructoselysine; Lys, lysine; PM, pyridoxamine; AG, aminoguanidine.

^b Initial weights for all groups were 178 ± 8.4 g

^c *P* < 0.05 vs. untreated control rats

^d *P* < 0.05 vs. untreated diabetic rats

micrometer thick sections were cut and stained with Toluidine Blue. These sections were processed, as described elsewhere, to determine glomerular volume [28]. A second sample of kidney was cut into one-millimeter cubes, fixed in 2.5% glutaraldehyde and embedded in PolyBed 812®. Images were obtained with a JEOL 100CX electron microscope (JEOL, Tokyo, Japan) and used to determine fractional volume of mesangium per glomerulus [29] and glomerular basement membrane (GBM) width [30]. Because of the costs involved and the focus on PM, morphological studies were limited to diabetic and PM-treated diabetic rats.

Statistical analysis

Statistical analyses were performed using SigmaStat for Windows V1.00 (SPSS, Inc., Chicago, IL, USA). *P* values were calculated by non-parametric Mann-Whitney Rank Sum analysis. Correlation analyses were performed by the Pearson Product Moment method. Unless indicated otherwise, all data are presented as mean ± SD.

RESULTS

Animal weights

The mean weight of diabetic animals was significantly lower than that of non-diabetic animals (Table 1), however, the insulin regimen was sufficient both to support weight gain in the diabetic animals during the early stages and to maintain their weight at the later stages of the study. Mortality was less than 5% among diabetic animals during the study. Control animals treated with PM weighed ~10% less than untreated controls (*P* < 0.05). With the exception of the weight difference, all other parameters, including apparent redox imbalances, lipemia, AGEs, etc., measured in PM-treated, non-diabetic animals were statistically identical to those in untreated, non-diabetic controls. Diabetic animals consuming PM or AG also weighed ~10% less than the untreated diabetic group (*P* < 0.05); the weights of the PM- and AG-treated diabetic animals were statistically identical (Table 1). There was no evidence of any nutritional de-

ficiency associated with the decrease in body weight of rats treated with AGE inhibitors. Mean water intake for the untreated diabetic rats, measured at monthly intervals between 14 and 28 weeks, was 235 mL/day; water intake was decreased to 185 and 145 mL/day in PM- and AG-treated animals, respectively. Mean 24-hour urine volumes were 200, 155 and 125 mL/day in these groups, respectively. Effects of drugs on food and water intake are being followed more closely in subsequent studies.

Metabolic control

Acute, intermediate and long term measures of glycemic control were obtained by monthly measurements of plasma glucose and glycated hemoglobin, and by measurement of glycated skin collagen at the end of the experiment (Table 1). Mean plasma glucose levels (morning, non-fasting) in both groups of non-diabetic animals were ~5 mmol/L for the duration of the study. In the diabetic groups, mean plasma glucose levels were ~21 mmol/L at one week and stayed at that level during the first eight weeks of the study. During the next several weeks, plasma glucose levels rose gradually to nearly 30 mmol/L in the diabetic groups. At week 15, the insulin dose was adjusted from 3 to 5 IU every other day in all diabetic groups, such that plasma glucose levels were ~26 mmol/L or fivefold higher than the non-diabetic animals throughout the remainder of the study. There were no statistically significant differences in plasma glucose among any of the diabetic groups at any time point.

The increase in plasma glucose levels in the diabetic animals was accompanied by an increase in GlcHb. By eight weeks, GlcHb reached a mean value of ~16% in all diabetic groups, compared to 7.3% in the non-diabetic animals (Table 1). Neither PM nor AG had an effect on glycemic control in diabetic animals, as measured by either plasma glucose or GlcHb. Assuming slow turnover of insoluble skin collagen, glycation of this collagen should reflect very long term, steady-state glycemic control. As shown in Table 1, the mean concentration of FL in diabetic animals was 4.9-fold higher than FL in

non-diabetic animals, compared to a 5.2-fold increase in mean plasma glucose and 2.2-fold increase in GlcHb in the diabetic animals. Thus, measurement of FL in collagen provided a more sensitive and accurate index of the increase in glycemia, than did GlcHb.

Drug concentrations in plasma and urine

Concentrations of PM and AG were measured in morning plasma specimens during the 28th week of the study. In non-diabetic animals receiving 2 g PM/L of drinking water, the plasma PM concentration was $6.3 \pm 2.1 \mu\text{mol/L}$. In polydipsic and polyuric diabetic animals receiving 1 g PM/L in drinking water, the plasma PM concentration was $106 \pm 17 \mu\text{mol/L}$, compared to approximately 5 mmol/L concentration of PM in their drinking water and urine. The intent of the treatment of the non-diabetic animal with a twofold higher concentration of PM in their drinking water was to achieve comparable levels of PM in plasma of both diabetic animals and non-diabetic controls, however the concentration of PM in plasma of control animals reached only ~6% of that in diabetic animals. Urinary PM excretion measured in 24-hour urine specimens from diabetic rats at 23 weeks was $824 \pm 44 \mu\text{mol/day}$, while the PM intake estimated from water consumption was $801 \pm 22 \mu\text{mol/day}$ ($P > 0.05$), indicating that most of the PM was excreted unchanged in urine. Although the molar concentration of PM was approximately half that of AG in the drinking water of the diabetic rats (both were administered at 1 g of the salt form/L), the mean concentration of PM in diabetic rat plasma was approximately 20% greater than that of AG ($87 \pm 10 \mu\text{mol/L}$; $P < 0.01$ vs. PM). This is consistent with pharmacokinetic studies indicating that the half-times for clearance of AG and PM, administered by gavage, were approximately 30 minutes and 1.5 hours, respectively (unpublished observations).

Progression of renal disease

Urinary albumin and plasma creatinine concentrations were used as indicators of renal function (Fig. 1). In contrast to control or PM-treated non-diabetic animals, albuminuria increased with time in all diabetic groups, with untreated diabetic animals showing the greatest increase. At 23 weeks and thereafter, all three diabetic groups were significantly different from one another, and from non-diabetic controls, with respect to albuminuria (Fig. 1A). Plasma creatinine values also began to rise above normal after the 17th week (Fig. 1B). Both PM and AG inhibited the rise in albuminuria and plasma creatinine, with PM being significantly more effective. Urinary glucose concentration in diabetic rats was unaffected by drug treatment (not shown).

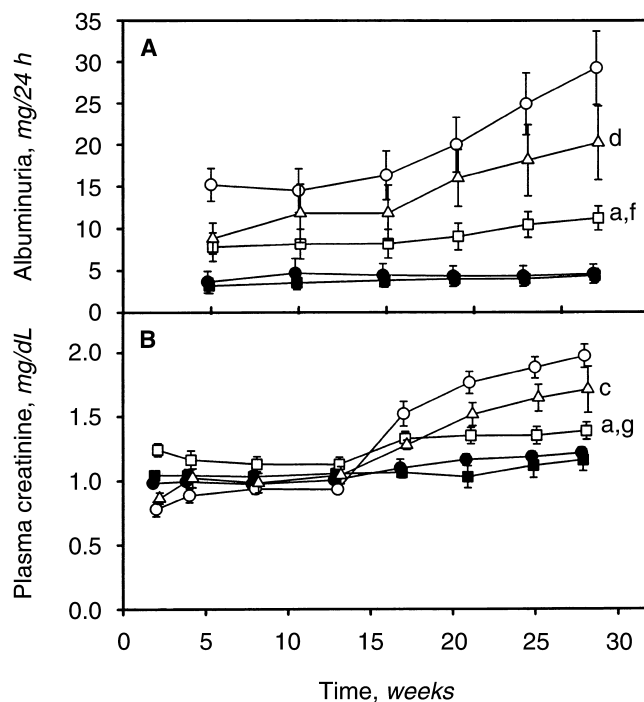


Fig. 1. Effect of pyridoxamine (PM) and aminoguanidine (AG) on development of nephropathy in streptozotocin (STZ)-diabetic rats. Urine (24-hour samples) and blood were collected at 4-week intervals for measurement of urinary albumin (A) and plasma creatinine (B) concentrations. Symbols are: (●) untreated, non-diabetic control; (■) PM-treated, non-diabetic control; (○) diabetic control; (△) AG-treated diabetic; and (□) PM-treated diabetic rats. All diabetic groups vs. non-diabetic controls in A and B, $P \leq 0.001$. For this and subsequent graphs: ^{a,b,c,d,e} $P < 0.0001$, < 0.001 , < 0.01 , < 0.05 , and NS vs. D; ^{f,g} $P < 0.01$, $P < 0.05$ vs. D + AG; ^h $P > 0.05$ vs. C.

Dyslipidemia

At necropsy we observed that the plasma of untreated diabetic animals was visibly more lipemic than that from control and PM- or AG-treated animals. Assays of plasma lipids (Fig. 2) documented a substantial increase in lipemia in untreated STZ-diabetic rats, and significant correction of hyperlipidemia by both PM and AG. Triglycerides (Fig. 2A) increased to a mean of approximately 700 mg/dL in diabetic rats, compared to 100 mg/dL in non-diabetic controls, and were reduced 80 to 60%, to means of 220 and 350 mg/dL by PM and AG, respectively. Plasma cholesterol (Fig. 2B) was increased by approximately 50%, and was normalized by PM and partially corrected by AG.

Redox imbalances

Alterations in intermediary metabolism in diabetes may result from glucose-induced redox imbalances (pseudohypoxia) or decreased tissue oxygenation (true hypoxia) [31–33]. These changes are reflected by a change in the cytosolic ratio of NADH/NAD⁺, which can be assessed indirectly by measuring the ratio of lactate to pyruvate

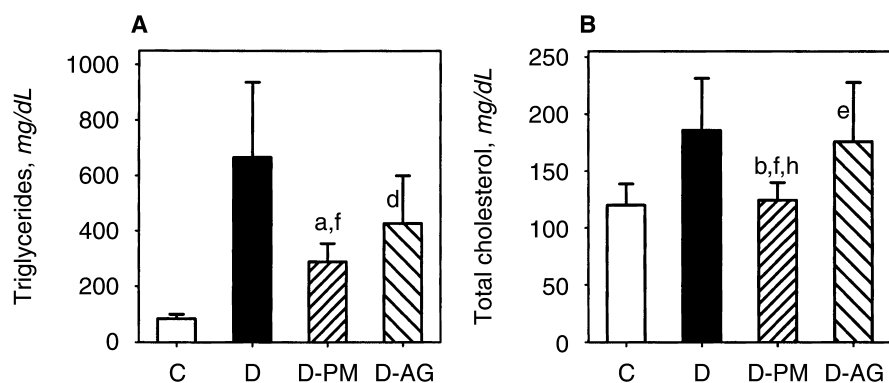


Fig. 2. Effect of AG and PM on dyslipidemia in STZ-diabetic rats. Plasma (28 weeks) was analyzed for triglyceride (A), total cholesterol (B). All diabetic groups vs. untreated, non-diabetic controls: $P < 0.001$. See footnote key in legend to Figure 1.

in plasma. As shown in Figure 3, diabetic animals had significantly higher levels of both lactate and pyruvate in plasma, as well as an increased ratio of lactate to pyruvate. Both PM and AG significantly corrected this apparent redox imbalance in the diabetic animals.

Chemical modification and cross-linking of collagen

The effects of various drug treatments on AGE/ALE concentrations in skin collagen at the end of the study are shown in Figure 4. AGE/ALEs and fluorescence in PM-treated non-diabetic animals were identical to those in the untreated non-diabetic group (not shown). CML concentrations (Fig. 4A) increased approximately 100%, from 0.057 ± 0.01 to 0.124 ± 0.01 mmol/mol lysine in non-diabetic controls versus untreated diabetic animals. PM and AG treatment of diabetic animals limited the increase in CML to 0.092 ± 0.02 and 0.10 ± 0.02 mmol/mol lysine, respectively, a 35 to 45% reduction toward the level in non-diabetic animals. CEL (Fig. 4B) increased approximately 50%, from 0.018 ± 0.004 mmol/mol lysine in control to 0.028 ± 0.004 mmol/mol lysine in diabetic animals. As with CML, both drugs limited the accumulation of CEL, reducing it up to 50% toward levels in non-diabetic control animals. The fluorescent AGE cross-link, pentosidine also increased approximately twofold, from 0.58 ± 0.10 μ mol/mol Lys in control to 1.1 ± 0.16 μ mol/mol Lys in diabetic rats (Fig. 4C). However, in contrast to CML and CEL, pentosidine concentrations were similar in all diabetic groups, that is, neither drug had a significant effect on the pentosidine content of skin collagen. At the same time, both drugs caused approximately a 40% decrease in Maillard-type fluorescence in skin collagen of diabetic rats (Fig. 4D). The unexpected results obtained for pentosidine are discussed later in this article.

In addition to the increase in chemical modification, collagen from diabetic animals typically shows a decrease in susceptibility to enzymatic digestion [34]. The kinetics of pepsin digestion of skin collagen from the various experimental groups are shown in Figure 5A. In non-

diabetic animals these kinetics were complex, with a burst of hydroxyproline-containing peptides released during the first hour, followed by somewhat slower rates of digestion thereafter. In contrast, collagen from diabetic animals was digested in a more linear fashion, and at a slower rate throughout the 24-hour experiment. The half time for digestion of skin collagen (50% release of Hyp-peptides) increased approximately fivefold, from 90 minutes in control animals to 450 minutes in diabetic animals (Fig. 5B). PM and AG reduced the half time to mean values of 350 and 300 minutes, respectively, that is, approximately 25 to 40% toward the control values. Differences in kinetics of digestion of control and diabetic collagen were more impressive at shorter times, such as at 20%, rather than 50% solubilization.

Anatomical observations

As shown in Table 2, mean kidney and liver weights were increased in diabetic rats, both in absolute terms and as a fraction of total body mass. These changes in tissue weight were partially reversed by both PM and AG. Occasional cysts were observed in kidneys of diabetic animals, but were not increased in frequency in drug-treated groups. There was no evidence of an increase in tumors in other major organs (heart, liver, intestines), nor of other unusual pathologies associated with either PM or AG therapy. Characteristic changes in morphology were observed in the diabetic kidney (Table 3). PM caused a significant, partial correction of the increase in glomerular volume, but the effects of PM on GBM thickness and mesangial volume were not statistically significant.

DISCUSSION

These studies were designed to evaluate the efficacy of PM as an inhibitor of AGE formation and development of renal disease in the STZ-diabetic rat. The STZ-rat was chosen because of the reproducible development of renal disease and several reports on the protective

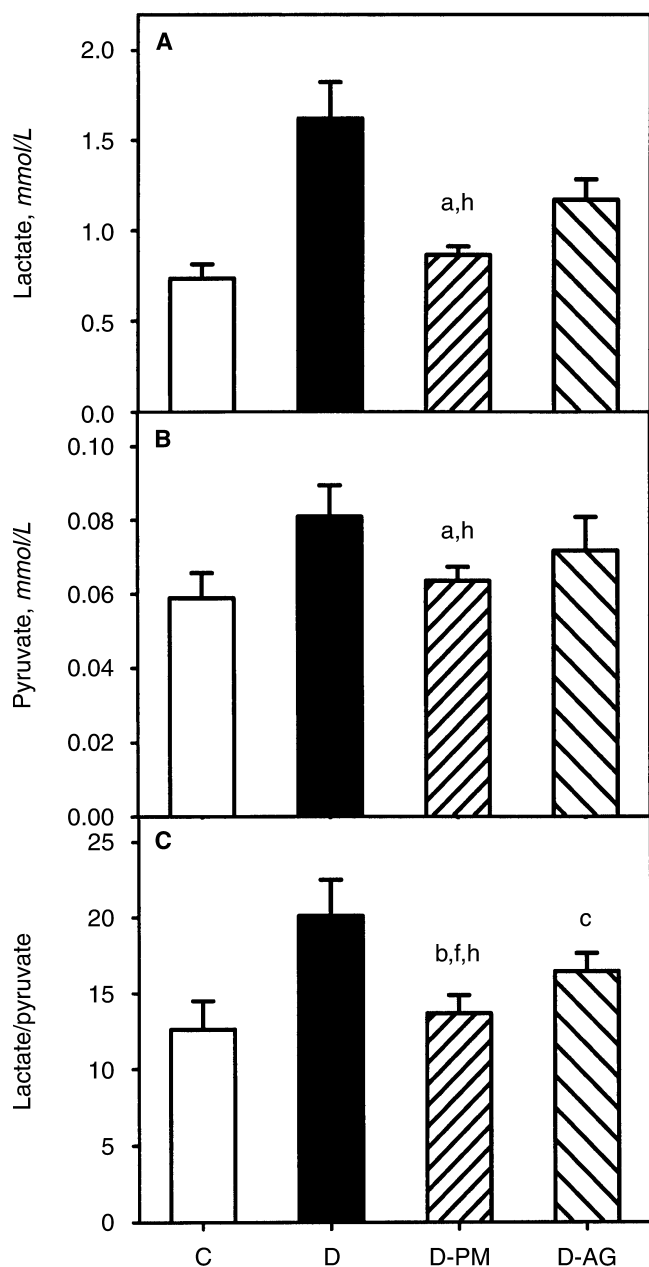


Fig. 3. Effect of AG and PM on redox state in STZ-diabetic rats. Plasma (28 weeks) was analyzed for lactate (A) and pyruvate (B) concentrations. The ratio of lactate/pyruvate, an index of intracellular redox status, is shown in (C). See footnote key in legend to Figure 1.

effects of the AGE inhibitor AG against both AGE formation and nephropathy in this model [10, 12; earlier work reviewed in 7]. While these studies were in progress, we learned that PM was also an effective inhibitor of formation of ALEs during lipid peroxidation reactions [16]. As well, we learned at the end of the study that, like AG, PM exerted profound effects on hyperlipidemia and redox imbalances in the STZ-diabetic rat. The activity of PM as an ALE inhibitor and the complicating effects of hyperlipidemia and its partial correction by

PM (and AG) were not anticipated in the original experimental design. These factors have both complicated the interpretation of the experimental results and raised some interesting questions regarding the origin of renal dysfunction in the STZ-diabetic rat.

Effect of PM on progression of renal disease in the STZ-diabetic rat

Diabetic rats exhibited characteristic changes in renal function and structure, including increasing urinary albumin and plasma creatinine concentrations, accompanied by renal hypertrophy (Table 2), increased thickening of GBM, and glomerular and mesangial volume expansion (Table 3). Both PM and AG limited renal hypertrophy, as measured by trends toward a reduction in absolute kidney weight and significant effects on relative kidney weight (Table 2). Although morphological changes in the kidney were limited during the seven-month period of the study, PM significantly inhibited the increase in glomerular volume. Effects on the increase in mesangial volume were encouraging, but not statistically significant. There was only a 14%, but statistically significant increase in basement membrane thickening in the diabetic rats; this small increase was not significantly affected by treatment with PM. Although effects of AG on renal morphology were not measured in this study, studies on AG treatment of the STZ-diabetic rat have also yielded mixed results. One study reported inhibition of GBM thickening in the Lewis rat [35], while there are two reports that AG had no effect on GBM thickening in Sprague-Dawley rats [36, 37]. The discordance between the magnitudes of the structural and functional changes in the diabetic kidney is puzzling; however, in a recent study, anti-transforming growth factor- β (anti-TGF- β) antibody inhibited renal morphological changes in the db/db diabetic mouse, but had no effect on albuminuria [38]. Because the AGE inhibitors do not correct all of the structural and functional changes in the diabetic kidney, they may be useful in combination with other renoprotective therapies, such as ACE inhibitors.

Metabolic effects of AGE/ALE inhibitors

Although lipid lowering effects of AG in diabetic humans have been reported previously [39], the magnitude of the effects of both PM and AG on plasma cholesterol and triglycerides in the STZ-diabetic rats has not been noted previously. These changes could result from either primary effects of the drugs on lipid metabolism or secondary effects related AGE/ALE-inhibition. It is unlikely, however, that the metabolic effects are related to the vitamin B₆ activity of PM. PM and AG are structurally unrelated compounds, and the fact that they produce similar effects in diabetic animals is most consistent with a mechanism of action based on their common nucleophilic character.

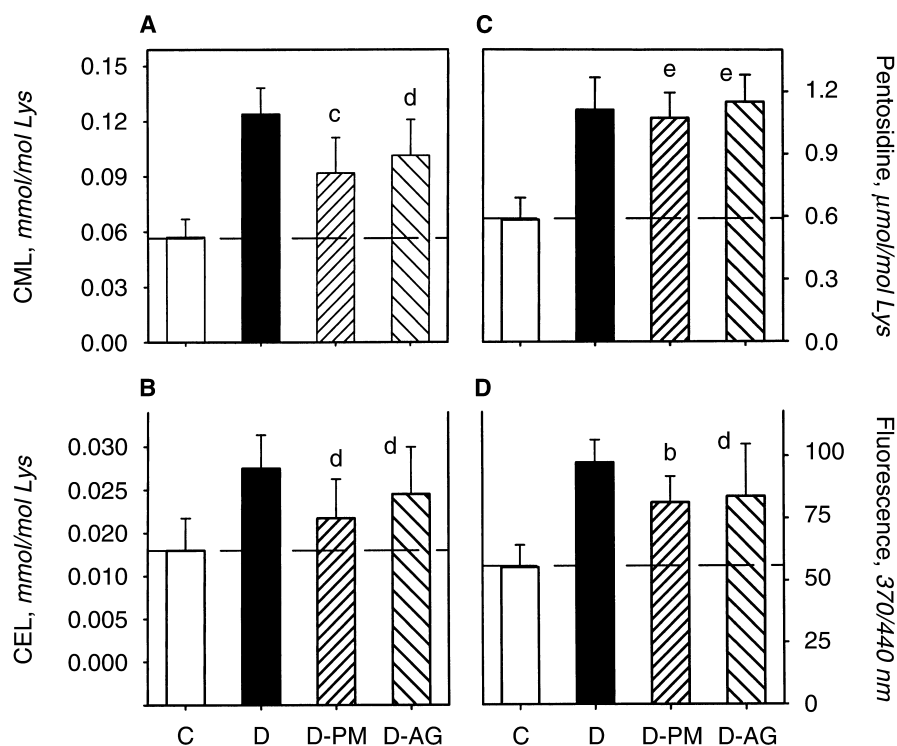


Fig. 4. Effect of diabetes and drug treatment on levels of advanced glycation end-products (AGEs) and fluorescence in skin collagen. Skin collagen was analyzed for concentrations of CML (A), CEL (B) pentosidine (C), and fluorescence (D). All diabetic groups vs. untreated, non-diabetic control in A, B, C & D, $P < 0.0001$. See footnote key in legend to Figure 1.

Hyperglycemia is known to induce shifts in intracellular ratios of redox coenzymes, both in vitro and in vivo, leading to a state of pseudohypoxia [31]. However, the significant increase in lactate and the lactate to pyruvate ratio in plasma of diabetic rats suggests true hypoxia in major tissues in the body. Decreased perfusion and oxygenation of muscle and nerve in both humans and animal models of diabetes [32, 33, 40–42] could be caused by AGE/ALE-dependent thickening and stiffening of basement membranes or loss of autoregulation in the peripheral vasculature. The resultant increase in anaerobic metabolism of carbohydrates in the diabetic animals suggests that aerobic metabolism of lipids might also be impaired, contributing, in part, to the hyperlipidemia in the diabetic rat. Studies on the kinetics of response to intervention therapy with AGE/ALE inhibitors should provide insight into whether the drugs acutely and directly affect metabolic pathways of lipid metabolism or exert their effects secondarily through inhibition of long-term structural changes in the vascular wall.

Effect of PM on AGE/ALEs in collagen

As in previous studies with AG and other AGE inhibitors [6–12], PM had no effect on the increase in glycemia or glycation of hemoglobin or collagen in the diabetic rats. Like AG [43, 44], PM also did not appear to affect polyol pathway activity or osmotically-induced oxidative stress in the lens of diabetic rats, since all diabetic animals developed bilateral sugar cataracts after 8 to 12 weeks

of diabetes. The AGE/ALEs, CML, CEL and pentosidine, as well as Maillard-type fluorescence, were increased approximately twofold in both skin collagen of diabetic animals, compared to controls, after seven months of diabetes. At the doses of PM and AG tested, we observed an approximately 50% inhibition of the increase in CML, CEL and fluorescence in skin collagen of diabetic animals, supporting the activity of these compounds as AGE/ALE inhibitors in vivo. Although our HPLC and GC/MS methods lacked the sensitivity for measurement of AGEs in isolated renal glomerular or tubular collagen, immunohistochemical studies indicate that there is a significant decrease in CML or other AGE/ALEs in the kidney [12] and mesenteric vasculature [45] of AG-treated versus untreated STZ-diabetic rats. AG also is known to inhibit the increase in cross-linking of collagen in diabetic rats, as measured by changes in tail tendon stability [46] or protease digestibility of aortic [6], renal [47], or tail tendon [48] collagen. In the present studies, the half-time for solubilization of skin collagen by pepsin was increased about fourfold in diabetic, compared to control rats, and was decreased by 25 to 40% toward normal in diabetic rats treated with PM or AG. The lack of an effect of either PM or AG on pentosidine, despite significant effects on other AGE/ALEs, fluorescence and pepsin digestibility, suggests that other fluorescent cross-links (such as vesperlysines, crosslines and fluorolink) or non-fluorescent cross-links (such as dityrosine imidazolium salts formed from glyoxal and meth-

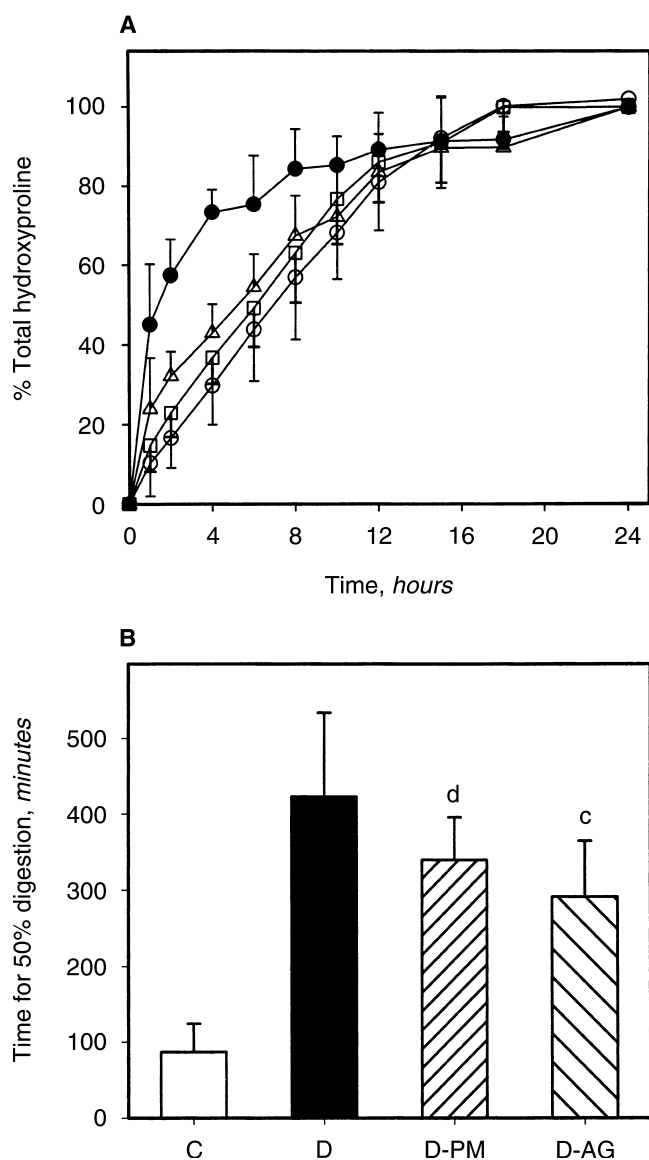


Fig. 5. Influence of diabetes and drug-treatment on cross-linking (proteolytic digestibility) of rat skin collagen. Collagen was digested with pepsin and Hyp release in the supernatant was measured as a function of time of digestion. (A) Kinetics of digestion: (●) untreated, non-diabetic control; (○) diabetic control; (△) AG-treated diabetic; and (□) PM-treated diabetic rats. (B) Time for 50% digestion. See footnote key in legend to Figure 1.

ylglyoxal (GOLD and MOLD) [1, 2]) may be more sensitive to inhibition by PM and AG. In general, despite differences in their mechanism of action as AGE inhibitors in vitro, that is, PM, but not AG, inhibits post-Amadori reactions, the two drugs had comparable effects on AGE/ALE formation, fluorescence and cross-linking of skin collagen of diabetic rats, including their failure to inhibit the increase in pentosidine formation.

Origin of AGE/ALEs: Carbohydrates or lipids?

Carbohydrates. Although the exact source and route of formation of pentosidine in vivo is uncertain, pentosi-

Table 2. Effects of diabetes and drug treatment on liver and kidney weight

	Kidney g	Kidney g/100 g	Liver g	Liver g/100 g
Control	0.84 ± 0.04	0.32 ± 0.02	7.96 ± 0.5	3.37 ± 0.22
Untreated diabetic	1.53 ± 0.21	0.73 ± 0.23	16.7 ± 3.4	7.03 ± 0.94
Diabetic + PM	1.25 ± 0.30	0.59 ± 0.05	12.8 ± 2.1	6.00 ± 0.59
Diabetic + AG	1.31 ± 0.18	0.59 ± 0.07	14.0 ± 2.6	6.20 ± 0.99
P value:				
D + PM vs. D	>0.05	<0.001	=0.0025	=0.002
P value:				
D + AG vs. D	>0.05	=0.002	<0.05	>0.05

Abbreviations are: PM, pyridoxamine; AG, aminoguanidine; D, diabetic rats.

Table 3. Effect of diabetes and drug treatment on renal morphometry

	Glomerular volume	Mesangial volume	GBM width
	× 10 ⁶ μm ³		nm
Non-diabetic control	1.30 ± 0.10 ^a	0.25 ± 0.05 ^a	201 ± 14.9 ^a
Diabetic control	2.08 ± 0.27	0.52 ± 0.22	229 ± 19.4
Diabetic + PM	1.68 ± 0.08 ^b	0.46 ± 0.12	225 ± 25.1

^a *P* < 0.001 vs. untreated diabetic animals

^b *P* < 0.025 vs. untreated diabetic animals

dine appears to be derived exclusively from carbohydrates [49]. The failure of PM to inhibit the increase in pentosidine in diabetic skin collagen was unexpected, but is consistent with the observation that PM is also less effective in inhibiting formation of pentosidine, compared to CML, from glycated protein in vitro (personal communication; Voziyan PA and Hudson BG, University of Kansas Medical Center). Higher concentrations of PM may be required for efficient trapping of intermediates in pentosidine formation, while the failure of AG to inhibit the increase in pentosidine may be attributed, at least in part, to the fact that AG is not an effective in vitro inhibitor of the formation of AGEs from Amadori-modified proteins [13, 14]. AG inhibition of pentosidine formation in numerous in vitro studies may be attributed to its chelating activity at the high concentrations of AG commonly used in in vitro experiments [50] [Price et al, *J Biol Chem* (in press)]. Although CML and CEL may be derived from lipids, the relative increases in CML, CEL and pentosidine are similar in diabetic compared to control rats, consistent with their origin from a common precursor and pathway. The relative levels of CML and pentosidine in diabetic skin collagen are also comparable to their relative yields during modification of collagen by glucose or ribose in vitro, that is, 50- to 100-fold higher concentration of CML, compared to pentosidine [51–53]. Thus, it is reasonable to propose that all three of these products, as well as the increase in fluorescence and cross-linking of diabetic collagen, are derived from gly-

cooxidation reactions. It is possible that ascorbate, which is a common precursor of CML, CEL and pentosidine [51, 54], rather than glucose, may be the common carbohydrate source of these compounds in the diabetic animals. Notably, ascorbate is the more readily oxidized, compared to glucose and has an important supporting role as an inhibitor of lipid peroxidation chain reactions by maintaining vitamin E in the reduced state [55]. Its oxidation product, dehydroascorbate, is also a potent source of both CML and pentosidine [51, 56]. Significant alterations in ascorbate homeostasis occur commonly in diabetes [57], including the STZ-diabetic rat model [58]. Overall, despite the lack of an effect of either PM or AG on the increase in pentosidine in skin collagen, our results are reasonably consistent with a role for carbohydrate, probably ascorbate, as the primary source of the increased chemical modification of proteins in the STZ-diabetic rat.

Lipids. Dyslipidemia and hyperlipidemia are recognized as independent risk factors for development of diabetic nephropathy [59, 60], and also are associated with nephrotic syndromes, independent of diabetes [61, 62]. We have observed recently that CML, CEL and, unexpectedly, pentosidine are increased in skin collagen of the non-diabetic (normoglycemic) Zucker *fa/fa* rat, and that PM also inhibits the development of nephropathy, reduces the hyperlipidemia and inhibits the increase in CML and CEL, as well as pentosidine, in skin collagen of these animals (manuscript in preparation). Since *fa/fa* rats are normoglycemic, glucose is an unlikely source of the increase in these compounds. Thus, the increase in chemical modification of proteins could be entirely attributable to ascorbate and/or lipids. A sequence of events might be considered in which AGE/ALE-dependent changes in collagen and basement membranes in the microvasculature may limit tissue perfusion, causing hypoxia, limiting oxidative metabolism of lipids, and leading to secondary hyperlipidemia. Intervention in this sequence of events by AGE/ALE inhibitors might correct the vascular changes, leading eventually to increased oxidative metabolism of lipids and a decrease in hyperlipidemia. At this time, however, we cannot exclude the possibility that the hyperlipidemia is entirely secondary to alterations in renal function (proteinuria), and that protection of renal function by unknown mechanisms may lead to the decrease in lipemia in PM-treated rats.

Although we did not address the temporal relationship between dyslipidemia, renal dysfunction and AGE/ALE formation in the present study, the fractional decrease in AGE/ALEs in skin collagen was comparable to, and correlated with, the fractional decrease in lipemia in the PM-treated diabetic rats (Fig. 2 vs. Fig. 4). In other work in progress, we have observed that the dyslipidemia develops slowly in the STZ-diabetic rat, with a half-time of approximately ten weeks, in contrast to the immediate

onset of hyperglycemia. This time-course for development of hyperlipidemia is consistent with an effect resulting from slow, AGE/ALE-mediated changes in vascular architecture. It also is possible that the decrease in AGE/ALEs in the STZ-diabetic rat might be attributed to direct effects of PM (and AG) on lipid metabolism by some mechanism independent of their ALE inhibitory activity. Although we cannot exclude metabolic effects, independent of AGE/ALE inhibition, this seems less likely because the two compounds, PM and AG, are significantly different in structure and therefore less likely to have identical regulatory effects on metabolic pathways.

CONCLUSION

We have demonstrated that the AGE/ALE inhibitors, PM and AG, retard the decline in renal function, substantially reverse dyslipidemia and apparent redox imbalances, and inhibit the chemical modification and cross-linking of tissue proteins in the STZ-diabetic rat. Our results indicate a complex interplay between the altered chemistry and biochemistry of carbohydrates, lipids and ascorbate in the development of diabetic complications, but are consistent with the proposed mechanism of action of PM and AG as AGE/ALE inhibitors. PM achieved slightly higher plasma concentrations than AG although it was administered at 50% the AG dose: both drugs were administered at 1 g/L, equivalent to 4 mmol/L PM and 8 mmol/L AG, while mean plasma concentrations were 106 mmol/L PM and 87 mmol/L AG. The higher concentration of PM at lower dosage may be attributed to differences in bioavailability and/or pharmacokinetics. Although PM was present at 20% higher concentration in plasma, it was substantially more effective than AG in retarding the development of renal disease, hyperlipidemia and redox changes in the diabetic rat. Despite these differences, the two drugs had similar effects on inhibition of AGE/ALE formation and collagen cross-linking, including their failure to inhibit pentosidine formation, so that their mechanism(s) of action must involve more than inhibition of AGE/ALE formation alone. The better bioavailability of PM and its low toxicity (5 to 7.5 g/kg oral LD₅₀ in rodents) [63], combined with its more pronounced effects on renal disease, hyperlipidemia and metabolic changes, compared to AG, suggest that PM may be useful for clinical treatment of a wide range of diabetic complications, including vascular disease and neuropathy. Future studies on progression of disease in normolipidemic, diabetic animals and in nephrotic, non-diabetic animals, on lipid-lowering agents without AGE/ALE inhibitory activity, and quantification of PM adducts in urine of hyperglycemic and hyperlipidemic animals should lead to a better understanding of the major chemical and biochemical

targets of AGE/ALE inhibitors and clarify the mechanism of action these drugs in protection against nephropathy and possibly other diabetic complications.

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APPENDIX

Abbreviations are: AG, aminoguanidine; AGE, advanced glycation end product; ALE, advanced lipoxidation end product; AGE/ALE, compounds such as CML and CEL that may be either AGEs or ALEs; CEL, N^ε-(carboxyethyl)lysine; CML, N^ε-(carboxymethyl)lysine; FL, fructoselysine; GBM, glomerular basement membrane; GlcHb, glycosylated hemoglobin; PM, pyridoxamine; RP-HPLC, reverse phase-high performance liquid chromatography; SIM-GC/MS, selected ion monitoring gas chromatography-mass spectrometry; STZ, streptozotocin.

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