

The AGE Inhibitor Pyridoxamine Inhibits Development of Retinopathy in Experimental Diabetes

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We examined the ability of pyridoxamine (PM), an inhibitor of formation of advanced glycation end products (AGEs) and lipoxidation end products (ALEs), to protect against diabetes-induced retinal vascular lesions. The effects of PM were compared with the antioxidants vitamin E (VE) and R- α -lipoic acid (LA) in streptozotocin-induced diabetic rats. Animals were given either PM (1 g/l drinking water), VE (2,000 IU/kg diet), or LA (0.05%/kg diet). After 29 weeks of diabetes, retinas were examined for pathogenic changes, alterations in extracellular matrix (ECM) gene expression, and accumulation of the immunoreactive AGE/ALE N^ε-(carboxymethyl)lysine (CML). Acellular capillaries were increased more than threefold, accompanied by significant upregulation of laminin immunoreactivity in the retinal microvasculature. Diabetes also increased mRNA expression for fibronectin (2-fold), collagen IV (1.6-fold), and laminin β chain (2.6-fold) in untreated diabetic rats compared with nondiabetic rats. PM treatment protected against capillary drop-out and limited laminin protein upregulation and ECM mRNA expression and the increase in CML in the retinal vasculature. VE and LA failed to protect against retinal capillary closure and had inconsistent effects on diabetes-related upregulation of ECM mRNAs. These results indicate that the AGE/ALE inhibitor PM protected against a range of pathological changes in the diabetic retina and may be useful for treating diabetic retinopathy. *Diabetes* 51:2826–2832, 2002

Retinopathy is a major complication of diabetes in both humans and animal models. The clinicopathology of microvascular lesions and the dysregulation of an array of biochemical pathways in the diabetic retina have been extensively studied, although the relative contribution of various biochemical

sequelae of hyperglycemia remains ill-defined (1). There is little doubt that the pathogenesis of this diabetic complication is highly complex, and there is a pressing need to establish new therapeutic regimens that can effectively prevent or retard the initiation and progression of the retinal microvascular cell dysfunction and death that is characteristic of the vaso-degenerative stages of diabetic retinopathy (1).

Among the several pathogenic mechanisms that may contribute to diabetic retinopathy is the formation and accumulation of advanced glycation end products (AGEs) (2). AGEs can form on the amino groups of proteins, lipids, and DNA through a number of complex pathways including nonenzymatic glycation by glucose and reaction with ascorbate, metabolic intermediates, and reactive dicarbonyl intermediates. These reactions not only modify the structure and function of proteins, but also cause intramolecular and intermolecular cross-link formation (3,4). Metal-catalyzed oxidative reactions give rise to a group of AGEs, classified as “glycoxidation” products [e.g., N^ε-(carboxymethyl)lysine (CML) or N^ε-(carboxyethyl)lysine (CEL) (5,6)], which also accumulate on protein and may chelate and activate redox-active metal ions (7,8). Nishikawa et al. (9) have proposed that production of reactive oxygen species by alterations in glucose homeostasis and increased AGE formation and polyol pathway activity during hyperglycemia may be the common pathway leading to activation of protein kinase C and downstream signaling events that lead to development of diabetic complications.

AGEs are known to have important effects on retinal vascular cells in vitro, effects that may be mediated through AGE receptors (10–12). In vivo systems, however, the role of AGE formation in diabetic retinopathy continues to remain uncertain. AGEs are known to accumulate in the neural retina and vascular cells of diabetic patients and rats (13,14) and are associated with pathophysiological changes in retinal function (15). AGE inhibitors such as aminoguanidine can attenuate retinal microvascular lesion formation in diabetic animal models (16–19). However, recent studies with aminoguanidine suggest this drug can significantly prevent diabetic retinopathic lesions independently without significantly affecting the formation of the AGE pentosidine in retinal basement membrane collagen (16,20), an effect that may be related to aminoguanidine’s antioxidative property rather than AGE inhibitory properties (21,22).

While intimately linked with AGE formation, oxidative imbalances in diabetic retinopathy have also been widely reported (23–26). Vitamin E (VE) can cause normalization

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Received for publication 18 February 2002 and accepted in revised form 3 June 2002.

A.S. has received stock options and consulting fees from BioStratum as a member of their Scientific Advisory Board, as well as research support from BioStratum.

AGE, advanced glycation end product; ALE, advanced lipoxidation end product; CEL, carboxyethyl lysine; CML, carboxymethyl lysine; CSLM, confocal scanning laser microscopy; ECM, extracellular matrix; LA, lipoic acid; NGS, normal goat serum; PM, pyridoxamine; STZ, streptozotocin; VE, vitamin E.

of diabetes-related retinal blood flow abnormalities (27), while this antioxidant, in combination with vitamin C, can inhibit capillary cell death (28). It has also been demonstrated that biochemical markers of retinal oxidative stress can be inhibited by α -lipoic acid (LA) (29). By contrast, some studies have reported no significant amelioration of retinal vascular lesions after treatment with antioxidants (19,30). Therefore, it is clear that the precise role played by oxidative stress in unison with advanced glycation reactions needs to be more closely examined.

In recent work, we have demonstrated that pyridoxamine (PM) is a potent inhibitor of the development of early renal disease (albuminuria, creatinemia) in streptozotocin (STZ)-induced diabetic rats (31). In the present study, we have investigated the effect of PM on the major microvascular lesions of diabetic retinopathy and compare/contrast this with the effects of the antioxidants VE and LA. PM has been shown to be an effective carbonyl scavenger (32,33,39,40) and, importantly, unlike aminoguanidine, can inhibit the formation of AGEs from Amadori compounds, the first stable product of glycation of proteins. PM can also prevent protein modification by products of lipid peroxidation reactions leading to the formation of advanced lipoxidation end products (ALEs) (3,33). This may be an important consideration for neural tissues such as the retina that possess high levels of polyunsaturated fatty acids, which are particularly susceptible to peroxidation reactions (34). One of the most frequently measured and abundant AGEs in tissues, N^{ϵ} -(carboxymethyl)lysine (CML) is also an ALE formed during lipid peroxidation (33). This is the first report of the potential of PM to prevent diabetic retinopathy.

RESEARCH DESIGN AND METHODS

All chemicals and kits were obtained from Sigma Chemical (St. Louis, MO or Poole Dorset, U.K.) unless otherwise noted. Standard rat chow [Rodent Diet (W) 8604] and chow supplemented with either VE (2,000 IU/kg) or LA (0.05%) was obtained from Teklad (Madison, WI); all diets were stored at 4°C. R- α -lipoic acid was a generous gift from Dr. Klaus Kraemer, BASF (Ludwigshafen, Germany).

Animal studies. Animal experiments were carried out in compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of South Carolina. Diabetes was induced in female Sprague-Dawley rats (~175 g) by a single intravenous injection of STZ (45 mg/kg in 0.1 mol/l citrate buffer); nondiabetic control rats (NonDb, $n = 12$) were sham injected with citrate buffer only. One week after induction of diabetes, animals with blood glucose levels >15 mmol/l were enrolled in the study. Rats were divided into one of the following treatment groups ($n = 16$ –17/group): Db, untreated diabetic; PM, 1 g/l in the drinking water; VE, 2,000 IU/kg diet; and LA, 0.05%/kg diet; the animals were followed for 29 weeks. Diabetic rats were treated three times a week with 3–4 IU ultralente insulin (Eli Lilly, Indianapolis, IN) to promote weight gain and limit hyperglycemia. Animal weight and food and water intake were measured every other week. Based on water and food intake during the last half of the study, daily intake of PM, VE, and LA were ~155 mg, 89 mg, and 20 mg per animal, respectively. Blood was sampled monthly for measurement of plasma glucose concentrations and percent total glycated hemoglobin (GHb) using commercial kits.

Necropsy and tissue sampling. Animals were anesthetized with isoflurane (Abbot Laboratories, Chicago, IL), blood was removed by cardiac puncture, and the animals were then killed by over-anesthetization. The eyes were removed immediately. One eye from each animal was enucleated and placed immediately in 10% buffered formalin for transport and later trypsin digestion or sectioning. Retinas from the companion eyes (minimum of eight per group) were dissected immediately and placed in RNA-later (Ambion, Austin, TX) for transport and subsequent mRNA extraction (see below). Eye samples were coded and all analyses of ocular tissue were conducted in a blinded manner in the Department of Ophthalmology, Queen's University, Belfast. Data were sent to South Carolina for unmasking. An ~2-cm² section of abdominal skin was also removed for isolation of insoluble collagen and measurement of

collagen glycation and AGE/ALEs by gas chromatography/mass spectrometry as described previously (35).

Retinal vascular digests. Before isolation of the retinal vasculature, the anterior segment and lens were removed and the neural retina separated from the posterior eye cup. The retina was digested in 3% trypsin (according to previously described protocols [14]) and the entire retinal vascular tree was then washed in distilled water and mounted onto a Silane-treated slide as described in detail previously (14). The digests were prepared for immunocytochemistry by washing in PBS containing 0.1% BSA and 0.01% Triton X-100, then incubated in 5% normal goat serum (NGS) (30 min) to block nonspecific binding before addition of polyclonal rabbit anti-laminin antibody for 18 h at 4°C. The digests were then washed with PBS (3 \times 10 min) blocked in 5% NGS for a further 30 min, and then exposed to an Alexa 488-conjugated anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 h. The slides were mounted in 50% aqueous glycerol and photographed using confocal scanning laser microscopy (CSLM) (Bio-Rad MicroRadiance). Retinas were then graded for laminin-immunoreactivity according to a four-part scale of varying fluorescence intensity (0 = no fluorescence; ++++ = strong fluorescence).

After immunocytochemistry and photography, the coverslips were gently removed from the slides (in PBS) and the digests washed to remove mountant and then histologically stained with hematoxylin and light green. The gross pathology was noted and the density of acellular capillaries was analyzed by an experienced investigator counting the number of acellular strands per field in the central retinal capillary beds (mean of 12 fields of view/specimen at 250 \times magnification). The fields were counted in a clockwise fashion at a fixed distance from the optic disc and expressed as acellular strands per square millimeter of retina.

Intraretinal AGEs. Before sectioning, the anterior segments and lens were removed and the posterior segment was then cryoprotected in solutions of increasing sucrose concentration (5–30%). Cryosections were cut, dried, and hydrated in PBS. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide, and the sections were washed in PBS and blocked with 5% NGS, 1% BSA, and 0.01% Triton-X100. A rabbit anti-CML-antisera (36) (or control rabbit IgG at a similar concentration) was added to the sections overnight at 4°C in a humidified chamber at dilution of 1:200. After extensive washing and a further blocking step, a 1:200 dilution of biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark) was added, followed by streptavidin in the form of the ABC complex (Vector Laboratories, Glostrup, Denmark). Detection was performed by addition of 3-amino-9-ethylcarbazol (AEC; Vector Laboratories) to yield a red reaction product. After stopping the development reaction by immersion in water at an appropriate stage, the sections were briefly washed, counter-stained with 0.02% methyl green, and mounted in Glycermount (Dako). CML immunoreactivity was then graded according to a four-part scale of varying reaction product intensity (0 = no product; ++++ = high color intensity) with a minimum of two retinal sections from at least six animals per group.

Expression of basement membrane component mRNA. Retinal RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, U.K.), and the quantity of RNA in each sample was determined spectrophotometrically. The purity and quality of each RNA sample was estimated by visualization of clear 18S and 28S ribosomal RNA bands after electrophoresing 1 μ g of each sample on a 1% agarose gel.

RNA samples were reverse transcribed into cDNA using the 1st Strand cDNA Synthesis Kit (Invitrogen, Paisley, U.K.) and the resulting single-stranded cDNA was amplified in a PCR using sequence-specific primers for rat fibronectin (forward primer: 5' CGA AAC CAT GAA CTT TCT GC 3'; reverse primer: 5' CCT CAG TGG GCA CAC ACT CC 3'), laminin (β chain) (forward primer: 5' ACA GAA GTC GAA GTG GTG 3'; reverse primer: 5' GGC TAA TAA ATA GAG GGT AGG 3') or collagen IV (forward primer: 5' CGA AAC CAT GAA CTT TCT GC 3'; reverse primer: 5' CCT CAG TGG GCA CAC ACT CC 3') to amplify 302, 888, and 503 bp fragments, respectively. The housekeeping gene, acidic ribosomal phosphoprotein (ARP) (37) (forward primer: 5' CGA CCT GGA AGT CCA ACT AC 3'; reverse primer: 5' ATC TGC TGC ATC TGC TTG 3'), was also examined using the same cDNAs.

Real-time PCR was performed using a LightCycler rapid thermal cycler system, (Roche, Hertfordshire, U.K.) according to protocols outlined by Simpson et al. (38). Briefly, PCR was performed in glass capillary reaction vessels (20 μ l volume with 0.5 μ mol/l primers and 4 mmol/l MgCl₂). Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix (Roche). Amplification of cDNAs involved a 30-s denaturation step followed by 40 cycles with a 95°C denaturation for 30 s, 55–58°C annealing for 5 s, and 72°C for an appropriate extension time (4–14 s). Detection of fluorescence from SYBR Green I bound to the PCR product was carried out at the end of each 72°C extension period. The specificity of the amplification reactions was confirmed by melting curve analysis and subsequently by agarose gel electrophoresis (38). The data were analyzed with the

TABLE 1
General characteristics of nondiabetic and diabetic rat groups*

Group	Plasma glucose† (mmol/l)	GHb‡ (%)	Collagen glycation§ (mmol FL per mol lysine)	Body weight§ (g)
Nondiabetic	5.2–5.5	6.6–7.0	4.4 ± 0.5	275 ± 10.5
All diabetic	24.5–25.5	12.1–12.9	22.6–23.9	222–238

Data are means ± SD or range of means. *Data for “all diabetic” groups show range of means for all diabetic groups; †throughout study; ‡weeks 17–29; §end of study. FL, fructose lysine.

LightCycler analysis software. The baseline for each reaction was equalized by calculating the mean value of the five lowest measured data points for each sample and subtracting this from each reading point. Background fluorescence was removed by setting a noise band. The number of cycles at which the best-fit line through the log-linear portion of each amplification curve intersects the noise band is proportional to the log of copy number (38). A dilution series of a reference cDNA sample was used to generate a standard curve against which the experimental samples were quantified. To enable direct comparison between separate experiments ($n = 3$), the level of expression at each time point was expressed as a fraction of the total expression for each specific gene over all the time periods.

Statistical analyses. All data sets were tested to verify that they fulfilled requirements for a normal distribution. One-way ANOVA was conducted to compare overall treatment differences, and a P value <0.05 was deemed significant. When a statistically significant difference was detected, posthoc multiple pairwise comparisons were performed using Duncan's multiple range test.

RESULTS

Characteristics of diabetic animals. A more detailed description of the biochemical and physiological characteristics of the animals in this study will be presented elsewhere (Alderson et al., in preparation). An overview of glycemic control and weight gain is presented in Table 1.

All measures of glycemic control were significantly elevated in the diabetic animals compared with the NonDb rats. However, there were no statistical differences at any time point among any of the diabetic groups for any of the parameters shown. Because nonfasting blood was used for measurement of plasma glucose measures, we also measured fructose-lysine, the initial glycation product on protein, in long-lived (insoluble) skin collagen, as an independent assessment of glycemic control. The approximate fivefold increase in skin collagen glycation in the diabetic animals was similar to the fold increase in blood glucose. Because overall glycemia was unaffected by any intervention, outcome differences are, therefore, not likely the result of differences in average blood glucose or rate of glycation of protein. As expected, weight gain in the diabetic animals was somewhat lower than in the NonDb group.

Acellular capillaries. Acellular capillaries, one of the earliest hallmarks of diabetic retinopathy, were identifiable by a lack of (hemotoxylin-positive) nuclei in a naked, collapsed basement membrane tube (Fig. 1). In comparison to NonDb controls, 29 weeks of STZ-diabetes resulted in an approximate threefold increase in acellular strands in the retinal capillaries (Table 2). Treatment of diabetic animals with PM resulted in reduction of acellular capillaries in the retina to a level not statistically different than that for NonDb animals, but neither VE nor LA had any apparent effect on this retinal microvascular lesion (Table 2), although there was a trend for VE to reduce the mean number of lesions. There was a small unexpected increase in the number of lesions in the LA group.

Laminin in capillary basement membranes. Laminin is

a major component of vascular basement membranes. Using the trypsin digest preparations, basement membrane-associated laminin-immunoreactivity was assessed in the retinal vascular tree. By both qualitative and quantitative assessment, it was evident that diabetes caused a marked increase in the levels of this protein in the vessel walls (compare Fig. 2A, B, and F) ($P = 0.0004$), especially on the precapillary arterioles and associated capillaries (Fig. 2B, D, and E). PM normalized laminin-immunoreactivity (Fig. 2C and F) with no significant difference between this group and NonDb controls (Fig. 2F). VE treatment showed a trend toward reduction in laminin fluorescence yielding an intermediate level of fluorescence that was not significantly different than that of either the NonDb or Db groups. LA treatment had no effect when compared with Db animals (Fig. 2F).

Expression of extracellular matrix mRNAs. Using mRNA of extracellular matrix (ECM) (and basement membrane)-related genes as parameters of diabetes-related cellular dysfunction, expression of three key genes were assessed by quantitative real-time PCR. There was a more than twofold increase in laminin and fibronectin expression ($P < 0.001$) and a 1.6-fold increase in collagen IV

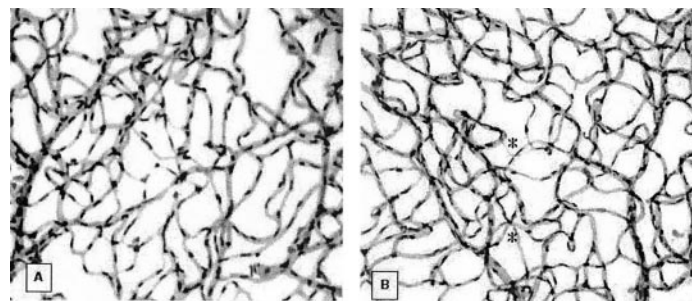


FIG. 1. STZ-induced diabetes causes measurable death of retinal vascular cells. The photographs show representative trypsin digest preparations from the retinal vascular tree of a NonDb (A) and Db (B) rat. Clearly evident are the retinal capillaries with darkly stained nuclei from endothelial cells and pericytes. In the diabetic retina, acellular strands, denoted by naked basement membrane tubes (*) can be observed.

TABLE 2
Effect of interventions on increase in acellular capillaries in diabetic rats

Group	Acellular capillaries/mm ²	P vs. NonDb	P vs. Db
NonDb	5.06 ± 1.34	—	<0.001
Db	14.87 ± 1.89	<0.001	—
PM	7.84 ± 1.83	NS	<0.001
VE	10.33 ± 3.73	0.0015	NS
LA	17.31 ± 2.59	<0.001	<0.001

Data are means ± SE.

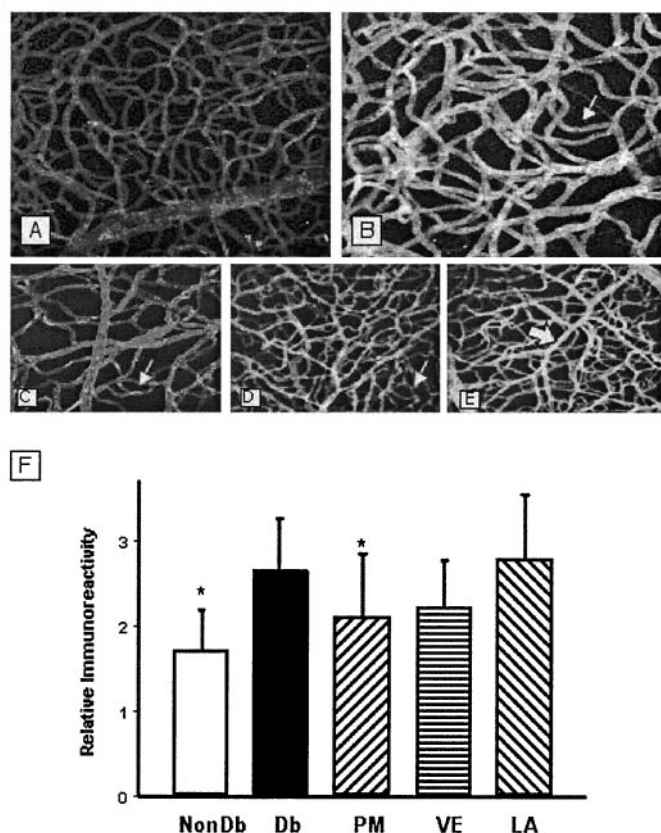


FIG. 2. Capillary basement membrane-associated laminin is upregulated in diabetes. Compared with NonDb retinal vasculature (A), there was a markedly increased laminin-immunoreactivity in the retinal capillaries of the diabetic retina (small arrows) (B). PM treatment prevented this diabetes-induced increase (C), while VE treatment had an intermediate benefit (D) and LA treatment failed to alter the intensity of immunofluorescence (E), as evident in the capillaries, veins, and arteries/arterioles (large arrow). F: Results of scoring for relative immunofluorescence intensity. Data are means \pm SD for eight to nine animals per group. *Significantly different from Db.

expression in diabetic versus control retinas (Fig. 3) ($P < 0.005$). PM treatment decreased the expression of laminin mRNA by $\sim 50\%$ ($P < 0.001$) and reduced fibronectin and collagen IV expression to levels not different from those in NonDb animals. VE failed to alter the diabetes-induced effect on mRNA for any of the extracellular proteins, whereas LA normalized collagen IV expression.

Intraretinal accumulation of CML. STZ diabetes induced a marked (more than twofold) increase in CML immunoreactivity in the neural retina of diabetic rats, compared with normal, age-matched controls (compare Fig. 4A and B). Qualitatively, this deposition was largely localized to the inner retina with less immunoreactivity at the level of the retinal pigment epithelium (RPE) and outer plexiform layer (Figs. 4A–E). CML immunoreactivity was also particularly evident around the retinal vasculature of diabetic animals (Fig. 4B). While PM caused a marked reduction in CML in this tissue (compare Fig. 4B with C), there was still some immunoreactivity detectable around the large retinal arteries (Fig. 4E); upon scoring, this was found to be significantly different from the diabetic controls (Fig. 4H). There was no significant difference in CML immunoreactivity in animals treated with VE (Fig. 4F). Upon quantification, it was evident that LA significantly reduced staining levels to an intermediate level (Fig. 4H).

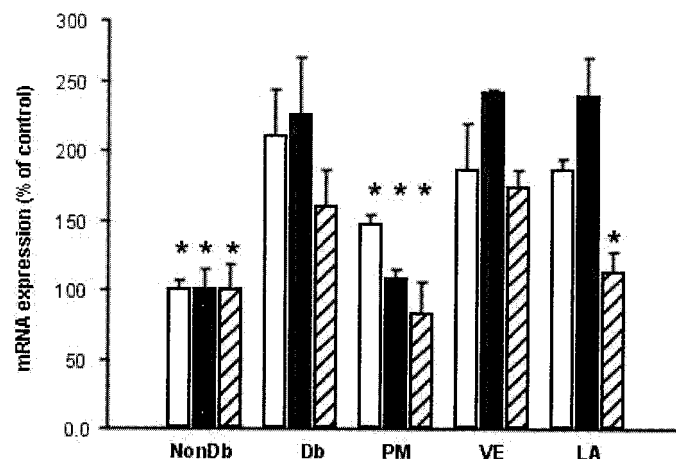


FIG. 3. Diabetes causes an upregulation of ECM mRNA expression. As measured by real-time PCR, there was a marked upregulation of the mRNAs for laminin (□), fibronectin (■), and collagen IV (▨) that was significantly normalized by PM. Retinal RNA was pooled from six animals per group and analyzed in triplicate. Results for each mRNA were normalized to mean value for mRNA levels in NonDb animals, set as 100%. Data are means \pm SD. *Significantly different from Db.

DISCUSSION

PM, a member of the B₆ vitamer family, is a potent scavenger of reactive carbonyls, inhibiting the late stages of glycation reactions that lead to AGE formation (32,39,40). In vivo, PM limited the formation of CML and CEL and cross-linking in skin collagen and, ultimately inhibited the development of nephropathy in STZ-diabetic rats (31). PM does not appear to function as an antioxidant since it does not prevent lipid peroxidation reactions (33). At the same time, it does prevent protein modification by products of lipid peroxidation, including inhibiting formation of malondialdehyde and 4-hydroxynonenal adducts on protein, in vitro (33), and in Zucker rats in vivo (Alderson et al., submitted). Based on immunohistochemical measurements, the current study has confirmed that PM also prevents CML formation in the diabetic retina during chronic hyperglycemia. We have demonstrated that this inhibition correlates with prevention of several key aspects of retinal microvascular pathology in STZ-diabetic rats.

The vaso-degenerative stage of diabetic retinopathy is characterized by progressive vascular dysfunction and loss of retinal capillary viability. Diabetic rodents do not generally show microaneurysm formation, which is characteristic of human diabetic retinopathy, but after 6 months of diabetes there is an increase in the number of acellular capillaries in the retinal microvasculature (14,16,17). The diabetic animals in the current study had a more than threefold increase in acellular strands in comparison to nondiabetic controls. This is similar to results of other studies on STZ-diabetic rats for a similar time period and provides an accepted morphological criterion to assess drug efficacy against diabetic retinopathy (14,16,28). Our results demonstrate that PM can inhibit the formation of acellular strands in the retinas of diabetic rats, maintaining microvascular cellularity at normal (non-diabetic) levels. Several previous studies have indicated that increases in AGEs are associated with injury to retinal microvascular endothelial cells and pericytes in vitro (11,12,41,42). Our data suggest that PM inhibition of AGE

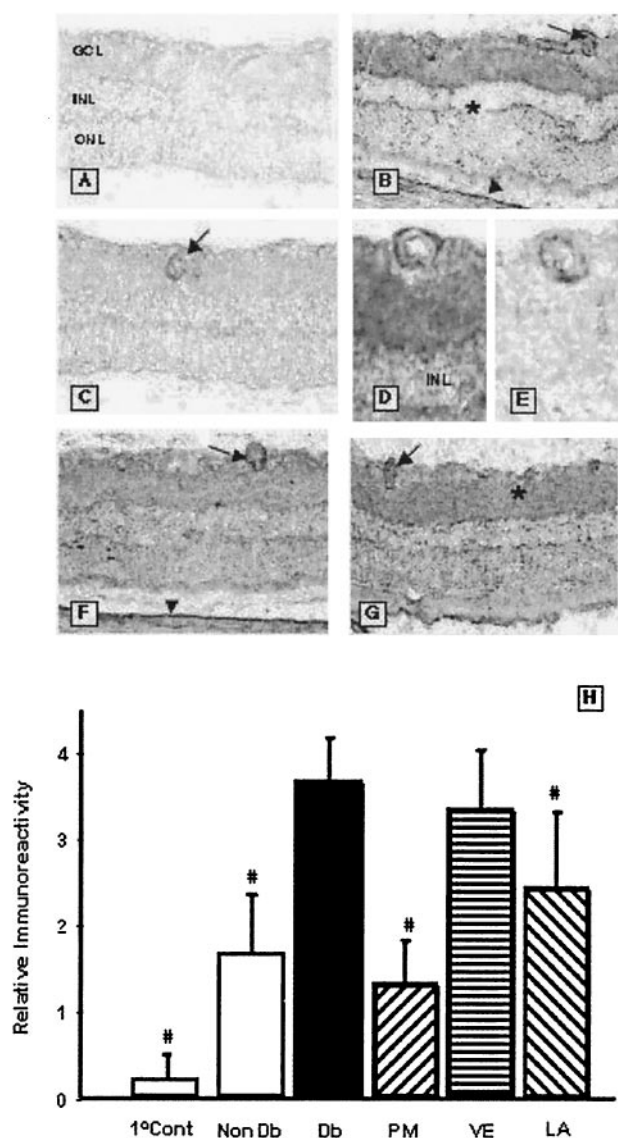


FIG. 4. CML accumulates in the neural retina during diabetes. While nondiabetic animals (A) had only sparse, diffuse CML immunoreactivity, diabetic rats showed CML accumulations at the level of the inner retina, especially around the plexiform layers (*) and the nerve fiber/ganglion cell layer (GCL) (B). By contrast, there was little staining in either the inner nuclear layer (INL) or outer nuclear layers (ONL). The retinal pigment epithelium also showed CML immunoreactivity (arrowhead) while there was intense reaction product around profiles of the retinal vasculature (arrow). PM prevented CML immunoreactivity in the neural retina (C). This was also evident around the retinal blood vessels at higher magnification [compare untreated diabetic control (D) with PM-treated diabetic rat (E)]. In contrast to PM, VE (F) did not affect the intensity of staining. LA (G) did not generally appear to alter CML immunoreactivity, and there was particularly intense staining at the inner retina (*). Panel H shows that scoring of the CML immunoreactivity demonstrated an approximate twofold increase in the intensity of CML immunostaining in the retina of Db animals ($P \leq 0.0001$). The increase in CML was normalized to NonDb levels by PM treatment and partially reduced by LA treatment ($P = 0.019$ and 0.00066 compared with NonDb and Db, respectively). First-degree control (1° Cont) indicates isotype control antibody. Data are means \pm SD for at least two retinal sections from at least six animals per group. #Significantly different from Db.

formation may protect against premature pericyte cell death and maintain capillary viability *in vivo*.

It is generally recognized that basement membrane thickening is a hallmark of diabetic microvasculopathy,

and inappropriate upregulation of the component mRNAs and proteins is thought to be a major factor contributing to this lesion (43–45). AGEs accumulate on diabetic retinal capillary basement membranes (14) where they are thought to contribute to structural and functional abnormalities on this specialized ECM, including rendering it more resistant to protease modification (46–48). It is significant that in the current study, PM limited CML accumulation in and around retinal vessels albeit to a lesser extent than in the neural retina. Treatment with this agent also prevented the diabetes-induced upregulation of laminin protein and of mRNA for three basement membrane components in the retinal microvasculature, although it remains unelucidated if the observed effects of PM of ECM gene expression are primary, secondary, or synergistically related to its anti-AGE properties. The link between basement membrane abnormalities and retinal vascular cell death remains uncertain, but it seems likely that both phenomena are intimately related. PM may act to limit AGE modifications that initiate inappropriate cross-linking of ECM proteins leading to chemical modification of key amino acids and conformational changes in the structure of the molecular lattice. This may serve to mask vital adhesion molecules including integrin and nonintegrin receptor-recognition motifs that are manifested by altered vascular integrity, increased cell death, and other pathophysiological responses (49). Vascular cells may upregulate their ECM gene expression to compensate for this loss of recognition, and further studies are underway to investigate if this phenomenon occurs during diabetes.

Treatment of diabetic animals with antioxidants is also known to prevent many biochemical abnormalities in the retina (29,50), although their effects on recognized pathogenic lesions in the retinal microvasculature are less well defined (17,30,51). In our study, treatment of diabetic rats with LA did not limit development of acellular strands or altered expression of ECM components. It is possible that a different route of administration and/or higher dose may be required to achieve significant effects on lesion formation. However, in the present study, the daily intake per rat of LA was ~ 20 mg of the pure R-isomer, while ~ 30 mg of the mixed isomer was effective in limiting the altered expression of vascular endothelial growth factor (VEGF) in STZ-diabetic rats after 6 weeks (34). Further, both LA and VE limited the development of renal disease (microalbuminuria decreased by 35–40%) in our animals (52), suggesting that the doses used were at least effective for treatment of nephropathy. Of interest, however, is that LA but not VE treatment limited immunoreactive CML formation (see Fig. 7). This result suggests that rather than acting as an antioxidant, LA may have served to trap reactive carbonyl precursors of CML, a chemical property not shared by VE. Animals receiving VE did show a trend toward decreased acellular capillaries, but the diabetes-induced upregulation of ECM components was not improved. Given the differences in their respective modes of action, it is possible that coadministration of VE and LA may have been more efficacious than administration of either antioxidant alone. In fact, a recent study of experimental retinopathy has reported a significant benefit from a long-term combined vitamin C and E or “multi-antioxidant diet” treatment of diabetic rats, albeit it used much

higher doses of VE than the present study (28). However, this therapy also failed to completely "normalize" the occurrence of acellular strands (28).

Hammes et al. (13) have previously demonstrated the accumulation of CML in the neural retina and its associated vasculature with marked increases during diabetes. Neural retinal dysfunction is now an acknowledged phenomenon during diabetes (1), and it is significant that the current investigation has confirmed similar CML localization patterns in the retina. This may suggest a putative role for AGEs in this aspect of diabetic retinopathy, although such an involvement requires a thorough evaluation. The precise protein substrate modified by CML was not characterized; likewise, the source of the CML is not clear since it may be derived from lipid peroxidation (53), given that the polyunsaturated fatty acid content of the retina is particularly high (34). In view of the known mode of action of PM (3,32,33), it remains possible that the beneficial effects of this drug lie in its combined AGE/ALE inhibitory capacity in the diabetic retina.

During the development of diabetic retinopathy, it is clear that enzymatic and nonenzymatic pathogenic mechanisms proceed simultaneously and perhaps synergistically. Recently proposed unifying concepts of diabetic complications suggest that altered mitochondrial function and resultant oxidative stress may exacerbate both tissue damage and AGE/ALE formation. In our studies, PM inhibited a range of pathological changes in the retinal microvasculature in diabetic rats when the treatment was initiated at diabetes induction. The data from the current study and related investigations suggest that inhibition of both AGE and ALE formation by PM may be key to its beneficial effects in the retina. Further work is underway to determine whether this drug can be effective in an intervention strategy after diabetes has been well established.

ACKNOWLEDGMENTS

This work was supported by Research Grant 1-1999-796 from the Juvenile Diabetes Research Foundation, and U.S. Public Health Service Grant DK-19971 from the National Institute of Diabetes and Digestive and Kidney Diseases. The authors also acknowledge the support of the Wellcome Trust, Fight for Sight (U.K.), and the Research and Development Office (N.I.).

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