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Aminoguanidine Pyridoxal Adduct is Superior to Aminoguanidine for Preventing Diabetic Nephropathy in Mice

Abstract

Aminoguanidine inhibits the formation of advanced glycation end-products, and has been extensively examined in animals. However, administration of aminoguanidine decreases the hepatic content of pyridoxal phosphate. In order to avoid this problem, we developed an aminoguanidine pyridoxal Schiff base adduct and examined its efficacy *in vitro* as well as in a model of diabetic nephropathy. Mice with streptozotocin-induced diabetes were treated with aminoguanidine or aminoguanidine pyridoxal adduct for 9 weeks. An *in vitro* study was also performed to assess the antioxidant activity of aminoguanidine and its pyridoxal adduct. Neither drug altered glycemic control. Aminoguanidine pyridoxal adduct significantly improved urinary albumin excretion by 78.1 % compared with the diabetic control, and also had a better preventive effect on the progression of renal pathology than aminoguanidine did. Inhibition of glycation by both drugs was similar, but the antioxidant activity of the pyridoxal adduct was far superior. These findings suggest that aminoguanidine pyridoxal adduct may be superior to aminoguanidine, as it not only prevents vitamin B_6 deficiency but is also better at controlling diabetic nephropathy, as this adduct inhibits oxidation as well as glycation.

Key words
Aminoguanidine · Pyridoxal · Vitamin B_6 · Glycation · Antioxidant · Nephropathy

Introduction

Various attempts at finding suitable glycation inhibitors for the treatment of diabetic complications have been undertaken. Brownlee et al. studied aminoguanidine (AG), a dicarbonyl trapping agent that inhibits AGE formation by the Maillard reaction *in vivo*, and reported that it could delay or prevent the onset of diabetic complications [1,2]. The beneficial effects of AG treatment have been confirmed in many studies on diabetic animals [3], and it is now planned for use in humans.

It is well-known that diabetic patients have low levels of vitamin B_6, folic acid, and cobalamin, although the mechanism involved remains to be elucidated [4]. Administration of AG would be expected to cause a further reduction in the levels of the vitamin B_6 derivatives pyridoxal (PL) and pyridoxal phosphate (PLP) [5,6]. In humans, vitamin B_6 deficiency causes symptoms such as numbness, tingling, pain, and swelling of the hands and feet, and may be a plausible molecular basis for various diseases including carpal tunnel syndrome [7], diabetic retinopathy [8], arteriosclerosis, and myocardial infarction [9]. Vitamin B_6, especially PLP, is important in the functioning of various PLP-dependent enzymes,

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including amino acid transaminases and amino acid decarboxylases [10].

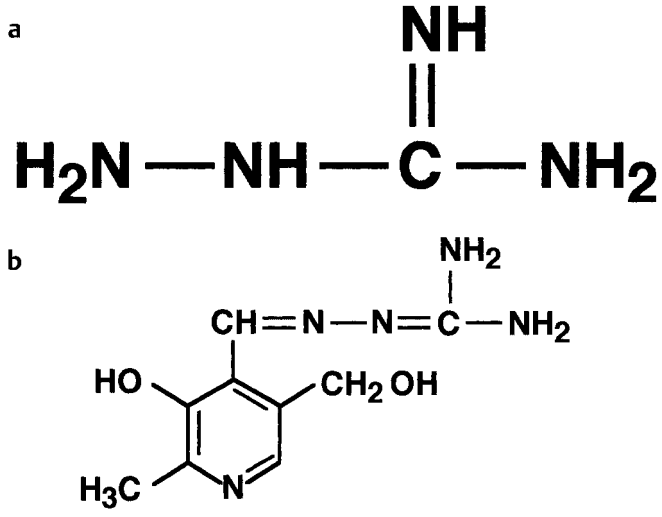
Recently, we demonstrated that the hepatic PLP content of mice that were given AG was significantly decreased compared with control mice, and that a Schiff base adduct consisting of AG and PLP (PLP-AG) was formed in AG-treated mice's livers. Administration of pyridoxine (PN) in addition to AG did not prevent a decrease in tissue PLP content, presumably because any PLP that was formed from PN subsequently reacted with AG to produce PLP-AG [11]. Accordingly, we synthesized an aminoguanidine pyridoxal Schiff base adduct (PL-AG) that we expected to be safer than AG (Fig. **1**). PL-AG showed a similar or stronger inhibitory effect than AG on AGE formation after incubation of bovine serum albumin with mannose, and its administration did not cause any decrease in tissue PLP in normal mice [12]. Therefore, PL-AG seems to be a more promising AGE inhibitor than AG itself, especially as it does not cause any depletion of tissue PLP.

In the present study, we examined the effects of PL-AG and AG in diabetic mice. We also compared the antioxidant activity of both drugs *in vitro* since AG is not only an AGE inhibitor, but also an antioxidant [13].

Materials and Methods

Animal models of diabetes

Aminoguanidine hydrochloride (AG-HCl) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA); aminoguanidine pyridoxal adduct (PL-AG) was synthesized as described previously [11] (Fig. **1**). Male ddY mice (Japan SLC, Hamamatsu, Japan) aged six weeks weighing around 30 g were randomized to a normal (n = 6) and diabetic group (n = 18). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ: Sigma S-0130, 200 mg/kg) in citrate buffer (pH 4.5). Mice with blood glucose levels above 22mmol/l at two weeks after STZ injection were randomized into three groups: group 1 was an untreated diabetic control group (n = 6), group 2 was treated with AG-HCl (n = 6), and group 3 was treated with PL-AG (n = 6). The adduct was neutralized with HCl before use. Drugs were added to the mice's drinking water at 27 µmol per day for 9 weeks. The experimental procedures were approved by our Animal Care and Use Committee.



Aminoguanidine pyridoxal adduct (PL-AG)

Fig. **1** Structure of aminoguanidine (**a**) and aminoguanidine pyridoxal adduct (**b**).

After 9 weeks, the mice were weighed and urine was collected for 24 h in metabolic cages. After that, each mouse was killed, and both kidneys were isolated and weighed. Blood was collected for the determination of serum glucose, serum creatinine, and serum AGE levels. Urinary albumin was measured by a specific to mouse albumin (Microalb, Bayer Sankyo, Tokyo, Japan). The serum creatinine concentration was estimated by Jaffe's method, and the serum glucose level was measured using the glucose oxidase method. We prepared polyclonal antibodies by immunizing rabbits with AGE-rabbit albumin as previously described [14]. After that, the circulating level of AGE was measured in the mice using a competitive ELISA as described elsewhere [15,16].

Glomerular stereology

Half of each right kidney was fixed in neutralized 10% formaldehyde, after which sections were cut and stained with periodic acid-Schiff reagent using the standard method. The sections were examined under a standard AX-80 Olympus light microscope connected to a Sony Trinitron television monitor via a CCD color video camera (Olympus Victor KY-F55MD, Tokyo, Japan). We selected 20 glomeruli per mouse (120 per group) and calculated the mesangial area and glomerular size using an automated image analyzer (Olympus Frovel VM-30, Tokyo, Japan), after which the fractional mesangial volume was determined. The mean glomerular volume was estimated using the method developed by Weibel and Gomez, as reported previously [17].

The remaining half of each right kidney was prepared for electron microscopy; glomerular basement membrane (GBM) thickness was measured at a magnification of 11500 × using the orthogonal intercept method developed by Jensen et al. [18].

The left kidney was immersed in embedding medium (Tissue-Tek O.C.T. compound) and frozen for AGE immunostaining using the immunoperoxidase technique and SAB method [19]. Frozen tissue was cut into 5 µm sections, fixed in acetone, and washed twice with phosphate-buffered saline. The sections were then dipped into 0.3% H_2O_2-100% methanol for 10 min to block endogenous peroxidase, after which nonspecific protein binding was blocked by incubating the sections with 10% goat serum in Tris-buffered saline for 20 min. Subsequently, the sections were incubated overnight at 4 °C with an AGE antibody [14] in a humidified chamber. A Histofine SAB-PO (R) kit (Nichirei, Tokyo, Japan) was used for color development. Briefly, the sections were first reacted with biotin-labeled rabbit anti-IgG antibody, then with peroxidase-labeled streptavidin, and finally with 3,3-diaminobenzidine (DAB). The reaction was stopped by placing the sections in water. Meyer hematoxylin was used as a counterstain. Microscopy was performed by a pathologist who was unaware of the animal groups. The amount of AGE in each kidney was scored from the intensity of the immunostaining.

Table **1** Characteristics of each group of mice

	Normal control	*Untreated diabetes*	*Diabetes plus aminoguanidine*	*Diabetes plus PL-AG*
Number	6	6	6	6
Body weight (g)	46 ± 1.2	26 ± 0.9**	29.5 ± 2.4**	26.5 ± 2.5**
Kidney weight/Body weight (mg/g BW)	12.8 ± 0.4	20.1 ± 0.7**	17.5 ± 1.0**	18.9 ± 1.0**
Glucose (nmol/l)	181.2 ± 29.5	392.7 ± 73.6**	406.4 ± 111.8**	437 ± 129.0**
Creatine (mg/dl)	0.68 ± 0.14	0.87 ± 0.09*	0.86 ± 0.15*	0.81 ± 0.24
AGE (U/ml)	4.76 ± 0.95	6.33 ± 0.62	6.88 ± 0.82	7.34 ± 0.52
Urine volume (ml/day)	1.55 ± 1.06	29.03 ± 3.95**	31.65 ± 5.74**	23.42 ± 2.99**†
Urinary albumin excretion (μg/day)	10 ± 10	330 ± 120**	330 ± 180**	80 ± 50*††

Data are the mean ± SE. *$p < 0.05$, **$p < 0.01$ vs. the normal control group; †$p < 0.01$ vs. the untreated diabetic group. PL-AG: aminoguanidine pyridoxal adduct.

Photo-oxidation of methyl orange in the presence of zinc oxide

The hydroxyl radical scavenging activity of PL-AG and AG was determined by the method developed by Russell et al. [20]. Briefly, a solution of methyl orange and a suspension of zinc oxide were prepared in 5 mM sodium borate buffer (pH 9.2) and mixed to give a final concentration of 40 μM and the equivalent of 6 mM, respectively. PL-AG or AG was then added at a final concentration range of 0 – 200 μM, and each mixture was exposed to a 500 W light from a distance of 20 cm for 30 min. Blank control mixtures prepared by the same procedure were incubated in the dark for 30 min. Subsequently, each mixture was centrifuged at 1500 × g for 5 min to remove suspended zinc oxide. Photo-oxidation of methyl orange by hydroxyl radicals generated by photolysis of zinc oxide was assessed by measuring the decrease in absorbance 465 nm, and the hydroxyl radical scavenging efficiency of each test compound was calculated as described elsewhere [20]. The experiment was performed three times.

H_2O_2-induced hydroxylation of benzoate

Hydroxylation of benzoate by H_2O_2 was measured by the method devised by Giardino et al. [13]. Briefly, 30 mM sodium benzoate in 100 mM sodium phosphate buffer (pH 7.4) was incubated with 10 mM H_2O_2 for 16 h at 37 °C in the presence or absence of various concentrations (0.1 – 100 μM) of PL-AG or AG. The formation of salicylate from the reaction was then determined using HPLC with a TSKgel ODS-80TM column (150 × 4.6 mm, 5 μm; Tosoh, Tokyo, Japan). The mobile phase was 10% (v/v) acetonitrile containing 20 mM potassium dihydrogen phosphate; chromatography was performed at a flow rate of 1.0 ml/min at room temperature. Salicylate was monitored by UV detection at 308 nm; this experiment was performed five times.

Statistical analysis

All results are expressed as the mean ± SD or SE. Statistical analysis was performed on a Macintosh computer using Stat View-J 4.5 software (Abacus Concepts, Berkeley, CA, USA). Statistical significance was tested by Student's *t*-test or one-way analysis of variance for multiple comparisons followed by Fisher's PLSD test. Probability values of less than 0.05 were considered to indicate significance.

Results

Body weight, serum glucose, and urine volume

At the time of final assessment, weight gain was significantly impaired by diabetes ($p < 0.01$). In the diabetic mice, serum glucose levels were increased by 2-fold to 3-fold ($p < 0.01$) relative to the control value, and urine volume was increased by approximately 15-fold ($p < 0.01$). None of these parameters were affected by either of the test drugs (Table **1**).

Kidney weight, serum creatinine, urinary albumin excretion, and serum AGE

We calculated the ratio of kidney weight to body weight in the various groups of mice to assess renal hypertrophy. The diabetic mice had a significantly increased ratio compared with the non-diabetic mice ($p < 0.01$). Serum creatinine levels were significantly increased in untreated and AG-treated mice compared with the levels in non-diabetic mice ($p < 0.05$). Urinary albumin excretion was increased by approximately 30-fold in untreated diabetic mice. Albuminuria was not reduced by treatment with AG, but was decreased by 78.1% in mice treated with PL-AG when compared with the untreated diabetic mice. The serum AGE level did not differ significantly among all the groups of mice (Table **1**).

Glomerular stereology

Expansion of the mesangial matrix and mesangial area was seen in diabetic mice, although there were no nodular lesions (Fig. **2**). The mean glomerular volume was significantly increased by 75.6% in untreated diabetic mice compared with control mice ($p < 0.01$). Both AG and PL-AG significantly reduced the increase of glomerular volume by 39.4% and 76.9%, respectively (Fig. **3A**). Untreated diabetic mice showed a significant increase in fractional mesangial volume (FMV) compared to control mice (22.2% *vs.* 16.9%, respectively, $p < 0.01$). Administration of AG partly prevented this increase (reducing it by 71.1%), while administration of PL-AG returned FMV to a value close to that in normal controls (reversing the increase by 96.6%, see Fig. **3B**). GBM thickness was significantly increased in untreated diabetic mice at 39.1% higher than in control mice ($p < 0.01$). Both AG and PL-AG significantly prevented an increase in GBM thickness ($p < 0.01$, see Fig. **3C**).

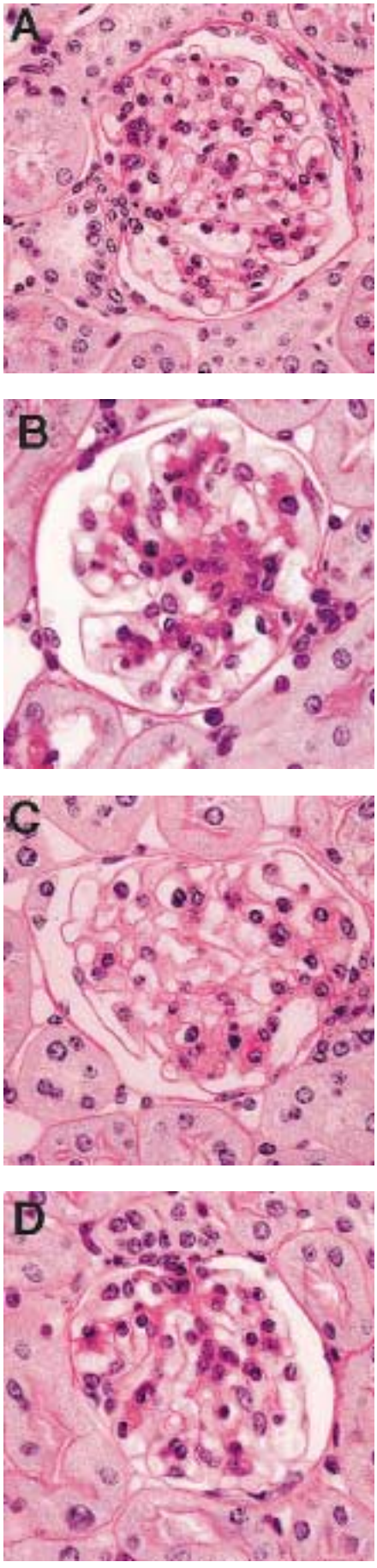


Fig. **2** Light micrographs of glomeruli from a normal control mouse (**A**), an untreated diabetic mouse (**B**), an aminoguanidine-treated diabetic mouse (**C**), and an aminoguanidine pyridoxal adduct-treated diabetic mouse (**D**) (PAS; Magnification × 400).

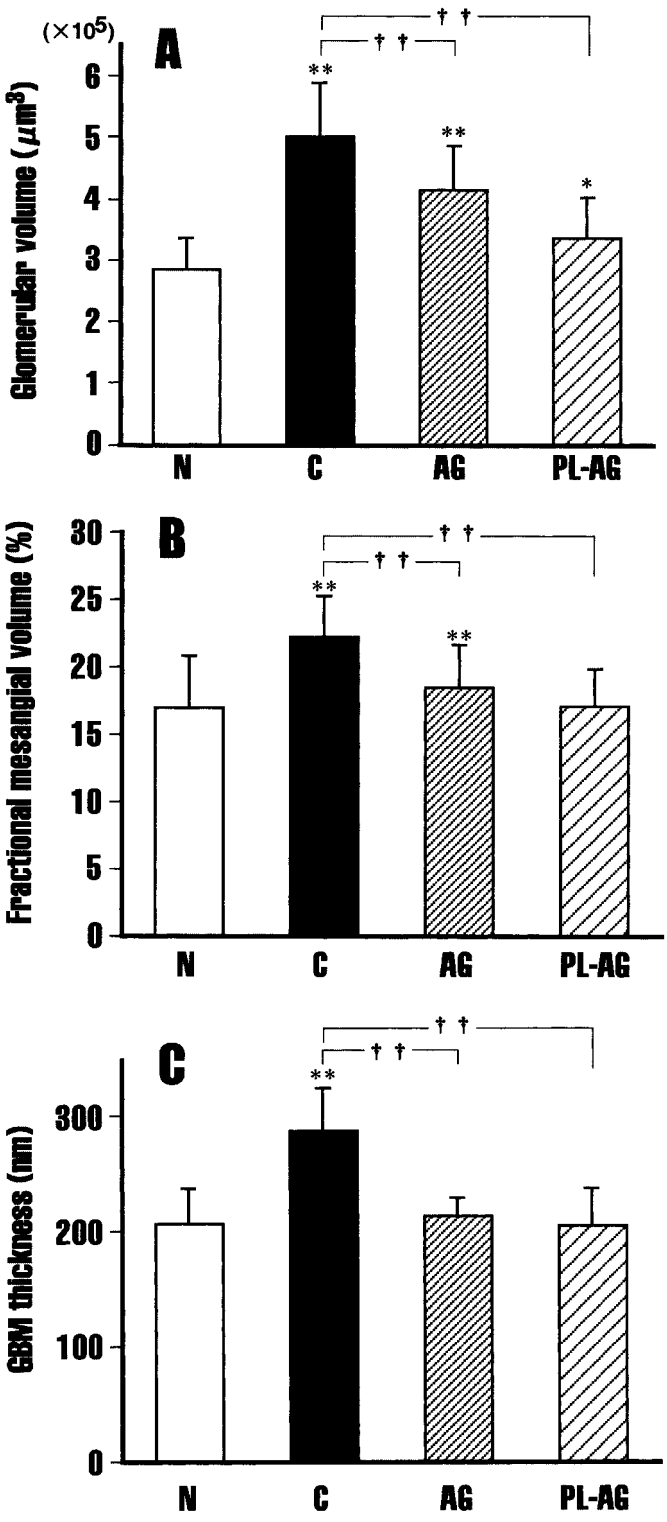


Fig. **3** Effect of diabetes and treatment for 9 wk with aminoguanidine or aminoguanidine pyridoxal adduct on glomerular volume (**A**), fractional mesangial volume (**B**), and glomerular basement membrane thickness (**C**) in mice with streptozotocin-induced diabetes. Data are the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* the normal control group; $^{\dagger}p < 0.01$ *vs.* the untreated diabetic group. N: Normal control mice, C: Untreated diabetic mice, AG: Diabetic mice treated with aminoguanidine, PL-AG: Diabetic mice treated with aminoguanidine pyridoxal adduct.

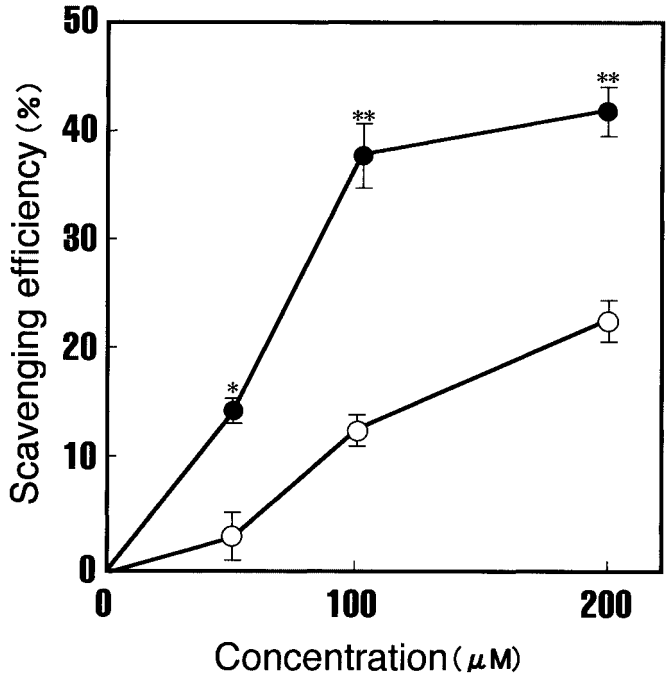


Fig. **4** Inhibitory effect of AG (○) and PL-AG (●) on photo-oxidation of methyl orange in the presence of zinc oxide. Values are the mean ± SD of three experiments. $^{*}p < 0.05, ^{**}p < 0.01$ *vs*. AG.

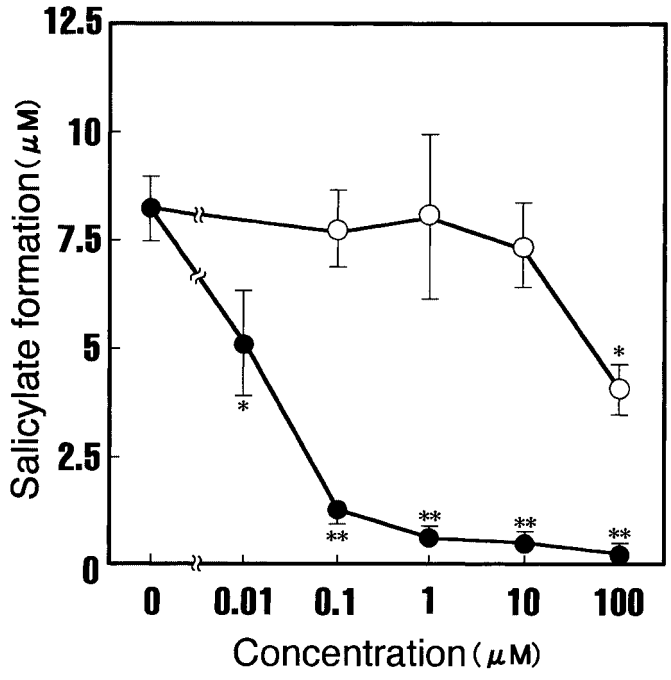


Fig. **5** Inhibitory effect of AG (○) and PL-AG (●) on H_2O_2-induced hydroxylation of benzoate. Values are the mean ± SD of five experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. no AG or PL-AG.

Immunostaining for AGE

Intense immunostaining for AGE was observed in the interstitial arteries of all mice, while varying levels of AGE staining were also seen in the glomerular mesangium and GBM. Untreated diabetic mice had slightly stronger glomerular mesangium and GBM staining compared to the other groups. However, no significant differences could be detected between any of the groups due to the wide variation in staining intensity (data not shown).

Photo-oxidation of methyl orange in the presence of zinc oxide

Hydroxyl radical-scavenging efficiency increased with concentration for both AG and PL-AG, although the increase was far less prominent when the concentration increased from 100 µM to 200 µM (Fig. **4**). The radical scavenging efficiency of PL-AG was significantly greater than that of AG ($p < 0.05$ at 50 µM and $p < 0.01$ at 100 µM or more).

H2O2-induced hydroxylation of benzoate

The concentration of salicylate formed by hydroxylation of benzoate decreased as the concentration of AG or PL-AG was increased (Fig. **5**). The salicylate concentration was reduced by half in the presence of 100 µM AG ($p < 0.05$ *vs*. the absence of AG), a finding that was consistent with the previous data of Giardino et al. [13]. In contrast to the effect of AG, significant inhibition of salicylate production was seen from a very low concentration (0.01 µM) of PL-AG ($p < 0.05$ *vs*. the absence of PL-AG), and the salicylate level was reduced by half even in the presence of 0.02 µM PL-AG. The inhibitory effect of PL-AG on hydroxylation was approximately 20 000 times that of AG.

Discussion

Many studies have suggested that the accumulation of AGEs may play an important role in the complications of diabetes mellitus, especially diabetic nephropathy [21,22]. Numerous drugs have been developed to inhibit AGE formation [23], including OPB-9195 [24] and N-phenacylthiazolium bromide [25]. Among them, AG is known to effectively inhibit AGE formation, and has been extensively examined in animals. In 1994, the first clinical trials to assess the effects of AG on diabetic nephropathy were started in the United States, but these trials have not yielded any definite result. He et al. [26] recently reported a potential protective effect of AG against the toxicity of excessive tissue AGE levels in diabetic patients with renal disease.

Administration of AG would be expected to cause a reduction of vitamin B_6 levels since this drug reacts with other biological compounds containing carbonyl group(s), such as the vitamin B_6 derivatives pyridoxal (PL) and pyridoxal phosphate (PLP) [5,6]. Okada et al. [10] have shown that adducts of AG and PLP are formed *in vitro*, and that AG inhibits cytosolic aspartate transaminase, which requires PLP as a coenzyme. In addition, we have previously demonstrated a concomitant decrease of PLP and PL in the livers and kidneys of non-diabetic mice administered with AG [11].

In the present study, diabetic mice showed a significant increase in relative kidney weight, glomerular volume, fractional mesangial volume, and GBM thickness when compared to control mice (Table **1**). Despite a similar severity of hyperglycemia in diabetic mice with and without AG or PL-AG therapy, there was a significant difference in the severity of renal changes between the treated and untreated groups (Fig. **3**). Although both drugs prevented an increase of glomerular volume, the relative kidney weight did not differ among all of the diabetic groups, so the increase of kidney weight might be explained by an increase in renal tubulointerstitial lesions caused by several factors related to hyperglycemia [27,28].

In the AG-group treated, there was a discrepancy between UAE and renal pathology. Many previous studies concerning the effects of AG have yielded conflicting data [3,29,30]. Differences in the species used, the dose or period of AG administration, the age at the time of study, and the severity of diabetes could explain the different findings obtained by previous animal studies. In general, AG is more effective against advanced diabetic nephropathy, while STZ mice are a model of early diabetic nephropathy. In the present study, there were no significant differences in serum AGE levels or tissue AGE staining between any of the groups. It is possible that UAE in diabetic mice treated with AG may have shown a significant difference from that of untreated diabetic mice after a longer follow-up period than 9 weeks.

We recently assessed the *in vitro* inhibition of glycation by PL-AG, and found that it had a similar activity profile to AG [12]. After oral administration, at least part of a dose of PL-AG is absorbed from the gastrointestinal tract without hydrolysis to PL and AG since appreciable levels of PL-AG have been detected in the livers and kidneys of mice [12]. Treatment with AG did not improve the UAE increase in this study, whereas PL-AG almost completely normalized albumin excretion. PL-AG not only prevented any increase in UAE, but also prevented the progression of pathological changes in the kidney. These results suggest that there is an additional effect of PL-AG on diabetic nephropathy besides compensating for the loss of vitamin B_6 caused by administration of AG. One possibility is that additional inhibition of diabetic renal changes occurred *via* the *in vivo* transformation of PL to other vitamin B_6 derivatives. Among several vitamin B_6 derivatives, pyridoxamine has recently been reported to act as an inhibitor of both glycoxidation and lipoxidation reactions, and its inhibitory effect is stronger than that of AG [31–33]. Another possibility is that PL-AG itself has a much stronger antioxidant activity than AG. Oxidative stress is defined as tissue injury induced by an increase in reactive oxygen species, such as the hydroxyl radical, superoxide anion, and hydrogen peroxide, and is one of the proposed mechanisms underlying diabetic complications. Our *in vitro* study used photo-oxidation of methyl orange to monitor hydroxyl radical production. In this system, the scavenging efficiency of PL-AG was far stronger than that of AG (Fig. **4**). Formation of salicylate was also monitored to estimate the protective effect of both drugs against H_2O_2-induced oxidant stress. The antioxidant effect of PL-AG was already significant at a very low concentration (0.01 µM), and it completely inhibited salicylate formation at 0.1 µM (Fig. **5**). These results suggest that there is a marked difference between AG and PL-AG regarding antioxidant activity.

Vitamin B_6 is an essential nutrient for humans, since it is necessary for the activity of several PLP-dependent enzymes, and vitamin B_6 deficiency may be related to various diseases including myocardial infarction. In this study, PL-AG, which does not decrease vitamin B_6 levels, was shown to be superior to AG for the prevention of diabetic nephropathy. Of course, we have to investigate further to determine the most effective and safe dose of PL-AG using other experimental animals treated over a longer term, and we must not forget the problems with extrapolating from experimental animals to patients with diabetes. The ultimate goal of our studies is to develop non-toxic and effective drugs that can completely prevent the development of diabetic complications. PL-AG seems to be a promising compound in diabetes treatment.

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Abbreviations

AG, Aminoguanidine; PL-AG, aminoguanidine pyridoxal adduct; AGE, advanced glycation end-products; STZ, streptozotocin; UAE, urinary albumin excretion; GBM, glomerular basement membrane thickness; FMV, fractional mesangial volume.

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