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In Vivo Formation of a Schiff Base of Aminoguanidine with Pyridoxal Phosphate

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ABSTRACT. Aminoguanidine (AG) is considered to be a promising compound for the treatment of diabetic complications. We examined the *in vitro* and *in vivo* formation of Schiff bases of AG with pyridoxal 5′-phosphate (PLP) and pyridoxal (PL). AG reacted *in vitro* far more rapidly with PLP to form a Schiff base (PLP–AG) than with PL to form another Schiff base (PL–AG). Administration of AG at 7 mM in drinking water for 18 weeks caused the formation of PLP–AG in the liver and kidney of mice (12.1 ± 1.6 and 3.8 ± 0.64 nmol/g of tissue, respectively, mean ± SD, $N = 6$). The amount of PLP in the liver of mice AG administered was significantly lower than that of control mice (4.0 ± 1.4 vs 17.4 ± 1.3 nmol/g of wet tissue, mean ± SD, $N = 6$). Simultaneous administration of pyridoxine (1 mM in drinking water) with AG (7 mM in drinking water) did not ameliorate the decrease in tissue PLP and caused the excess formation of PLP–AG. The results suggest that attention should be paid to the deficiency of tissue PLP in the clinical use of AG. BIOCHEM PHARMACOL **55**;10:1667–1671, 1998.

KEY WORDS. aminoguanidine; pyridoxal; pyridoxal 5′-phosphate; pyridoxine; diabetes; glycation

Hyperglycemia-induced formation of AGEs§ plays a central role in the pathogenesis of such diabetic complications as neuropathy, retinopathy, and nephropathy [1]. Glycation is initiated with attachment of the aldehyde group of glucose to a protein amino group via nucleophilic addition, forming a Schiff base. This intermediate product undergoes an Amadori rearrangement to form a 1-amino-1-deoxyfructose derivative in stable ketoamine linkage. The Amadori compound is then degraded to highly reactive carbonyl compounds, such as 3-deoxyglucosone, that react again with free amino groups to form various intermediates and AGEs. Among various pharmacological agents that have been developed to prevent AGE formation, AG is known to be one of the most promising inhibitors [2–4]. AG is thought to inhibit AGE formation primarily by reacting with Amadori-derived products including 3-deoxyglucosone, rather than forming adducts with peptide-bound early glycation products [5]. AG, due to this property, reacts also with other biological compounds having a carbonyl group(s). Khatami [6] has reported that PLP and PL easily react *in vitro* with AG. Okada and Ayabe [7] also demonstrated that an adduct of AG with PLP is formed *in vitro* and that AG inhibits cytosolic aspartate aminotransferase, requiring PLP as a coenzyme. Therefore, it is necessary to examine whether AG administration affects the amounts of PLP and PL in tissues.

In this paper, we report the *in vitro* and *in vivo* formation of Schiff bases (PLP–AG and PL–AG) of AG with PLP and PL, respectively, and the concomitant decrease in the amounts of PLP and PL in the liver and kidney of mice given AG.

MATERIALS AND METHODS

Materials

AG·HCl was obtained from the Aldrich Chemical Co. AG·H_2CO_3, PLP·H_2O, PL·HCl, PN, and all other reagents were purchased from Nacalai Tesque.

Male 4-week-old ddY mice, weighing 18–20 g, were obtained from Charles River and given laboratory chow and water *ad lib*.

Synthesis of PLP–AG and PL–AG

AG · H_2CO_3 (10 nmol) was added slowly with stirring to a solution (200 mL) of PLP · H_2O (10 mmol) in 50% CH_3OH, and the mixture was kept at room temperature for 30 min. The precipitate was collected by filtration and then recrystallized from CH_3OH-CH_2Cl_2 (1:1, v/v) to give 8.5 mmol of PLP–AG as yellow crystals, m.p. 270° (dec.).

AG·H_2CO_3 (30 mmol) was added slowly with stirring to a solution (20 mL) of PL·HCl (30 mmol) in H_2O, and the mixture was kept at room temperature for 30 min. The precipitate was collected by filtration on paper and dis-

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§ *Abbreviations:* AG, aminoguanidine; AGEs, advanced glycation end products, PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PL–AG, Schiff base of AG with PL; PLP–AG, Schiff base of AG with PLP; and PN, pyridoxine.

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solved in 2 L of H_2O. Addition of $NaHCO_3$ (10 g) yielded a yellow precipitate, which was recrystallized from CH_3OH-CH_2Cl_2 (1:1, v/v) to give 28 mmol of PL–AG as yellow crystals, m.p. 170° (dec.).

The structures of PL–AG and PLP–AG were analyzed by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry, IR spectrometry, and elementary analysis. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on JNM A-400 (400 MHz) and JNM A-600 (600 MHz) spectrometers (JEOL), respectively, with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ) and signals are expressed as s (singlet), d (doublet), m (multiplet), and br (broad). Mass spectra (MS) were taken with an M-80B-GC-MS spectrometer (Hitachi).

The data for PL–AG (a Schiff base of AG with PL) were as follows: MS (EI) *m/z*: 223 (M^+). IR cm^{-1}: $\nu_{C=N}$ 1628, 1695. ^{1}H-NMR ($DMSO_{d-6}$) δ: 2.36 (2 − C$\underline{H}_3$, 3H, s), 4.54 (5-C$\underline{H}_2$OH, 2H, d, J = 3.6 Hz), 5.19 (5 − CH$_2$O$\underline{H}$, 1H, br), 5.79 (N$\underline{H}_2$, 2H, br), 5.92 (N$\underline{H}_2$, 2H, br), 7.83 (6 − $\underline{H}$, 1H, s), 8.42 (4 − C$\underline{H}$═N—, 1H, s), 12.19 (3 − O$\underline{H}$, 1H, s). ^{13}C-NMR ($DMSO_{d-6}$) δ: 18.74 (2 − $\underline{C}H_3$), 58.78 (5 − $\underline{C}H_2$), 122.02 (4 − $\underline{C}$), 131.06 (5 − $\underline{C}$), 138.48 (6 − $\underline{C}$H), 143.46 (4 − $\underline{C}$H═N), 145.77 (2 − $\underline{C}$), 150.17 (3 − $\underline{C}$), 159.95 (4 − C═NH—N═$\underline{C}$<).

The data for PLP–AG (a Schiff base of AG with PLP) were as follows: MS (FAB+) m/z: 329 (2Na salt of $MH^+ - H_2O$). IR cm^{-1}: $\nu_{c=N}$ 1616, 1695. ^{1}H-NMR (1% $NaHCO_3$ in D_2O) δ: 2.32 (2 −C$\underline{H}_3$, 3H, s), 4.8 (5C$\underline{H}_2$O, 2H, d, J = 5.6 Hz), 7.73 (6 −C$\underline{H}$, 1H, s), 8.40 (4 −C$\underline{H}$—N—, 1H, s).

In Vitro *Formation of PLP–AG and PL–AG*

PLP and PL solutions (100 μM), in 50 mM of sodium phosphate buffer (pH 7.0), were incubated in the presence of AG (1 mM) over 5 hr at 37° in the dark. The amounts of PLP, PL, PLP–AG, and PL–AG were determined by the HPLC methods described below.

Administration of AG or AG + PN

Mice were randomized into three groups of six mice each: group 1 for vehicle (drinking water), group 2 for AG, and group 3 for AG + PN. AG · HCl was dissolved in drinking water to be 7 mM and given to group 2 for 18 weeks. AG · HCl and PN were dissolved in drinking water to be 7 and 1 mM, respectively, and given to group 3 for 18 weeks.

The change in body weight and the consumption of drinking water were assessed weekly and every 3 days, respectively.

Preparation of HPLC Samples

Animals were killed under anesthesia by pentobarbital after an 18-week administration of AG or AG + PN. Liver and kidneys were removed immediately, frozen in liquid nitrogen, and stored at −70° in light-tight containers until used. Pieces of the tissues were homogenized with 2 vol. of 50 mM of sodium phosphate buffer (pH 7.4) and centrifuged (8500 *g*) for 10 min at 4°. The supernatants were deproteinized by the addition of an equal volume of 0.8 M perchloric acid. The protein-free solutions obtained after centrifugation were used for the analysis of PLP, PL, PLP–AG, and PL–AG by HPLC methods.

Analysis of PLP and PL by HPLC

PLP and PL contents were assayed by a modification of the HPLC method of Kimura *et al.* [8]. We used a liquid chromatograph 880-PU (JASCO) equipped with a column of TSKgel ODS-120A (250 × 4.6 mm, 5 μm; Tosoh). The mobile phase, 0.1 M of potassium phosphate buffer (pH 4.3) containing 0.1 M of sodium perchlorate and 0.5 g/L of sodium bisulfite, was degassed by sonication under reduced pressure. The flow rate was 0.5 mL/min, and the column temperature was maintained at 50°. PLP and PL were detected with a fluorescence detector (model FP-920, JASCO) at an emission wavelength of 400 nm (λ_{ex} 300 nm). Peak areas were calculated with a Chromato-integrator D-2500 (Hitachi).

Analysis of PLP–AG and PL–AG by HPLC

PLP–AG and PL–AG were determined by HPLC with a TSKgel ODS-80TM column (150 × 4.6 mm, 5 μm; Tosoh). The mobile phase was 50 mM of sodium phosphate buffer (pH 4.3) containing 2% (v/v) acetonitrile. The chromatography was performed at a flow rate of 0.5 mL/min at ambient temperature (*ca.* 25°). PLP–AG and PL–AG were detected with a fluorescence detector at an emission wavelength of 460 nm (λ_{ex} 380 nm).

Statistical Analysis

Student's *t*-test was used for all statistical analyses. The level of significance was 0.05.

RESULTS

Determination of PLP–AG and PL–AG

Structures of PLP–AG and PL–AG are shown in Fig. 1. Figure 2 illustrates the typical chromatographic behavior of PLP–AG and PL–AG. Linear calibration curves were obtained for synthesized PLP–AG and PL–AG at least over a range between 2 and 200 pmol in 20-μL samples.

In Vitro *Formation of PLP–AG and PL–AG*

As shown in Fig. 3, PLP and PL reacted with AG to form PLP–AG and PL–AG, respectively, when incubated in 50 mM sodium phosphate buffer (pH 7.0) at 37°. The amounts of AG adducts formed were the mirror images of those of PLP and PL that disappeared. The velocity of PLP–AG formation was far more rapid than that of PL–AG formation, indicating that AG has a higher reactivity toward PLP

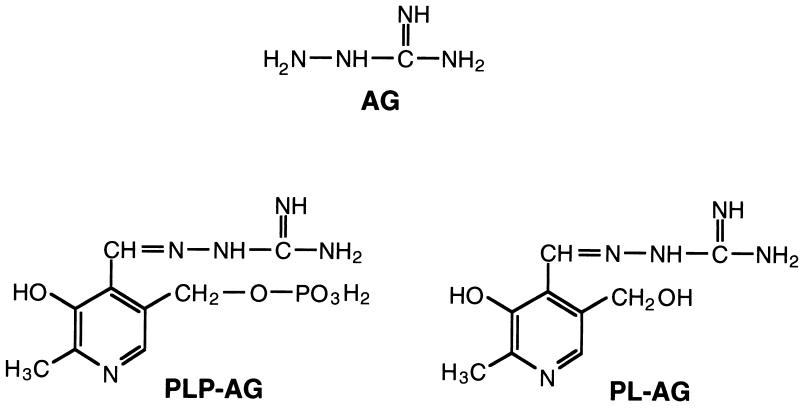

FIG. 1. Structures of AG, PLP–AG, and PL–AG.

than toward PL under the physiological conditions employed.

In Vivo *Formation of PLP–AG*

There was no significant differences in body weight or in the consumption of drinking water among the three groups (i.e. vehicle group, AG group, and AG + PN group) throughout the study. After administration for 18 weeks, the body weight (mean ± SD, $N = 18$) of mice of the three groups was 39.2 ± 1.8.

Chromatograms of liver extracts indicated that PLP–AG was formed in the liver of mice given AG (Fig. 2). Table 1 shows the amount of PLP–AG in the liver and kidney of mice administered AG or AG + PN. The amount of PLP–AG in the liver of mice given AG was three times higher than that in the kidney. Simultaneous administration of PN and AG increased the amount of PLP–AG in both the liver and the kidney. PL–AG was formed in both tissues, but the amounts of it were too small to allow its quantitative determination.

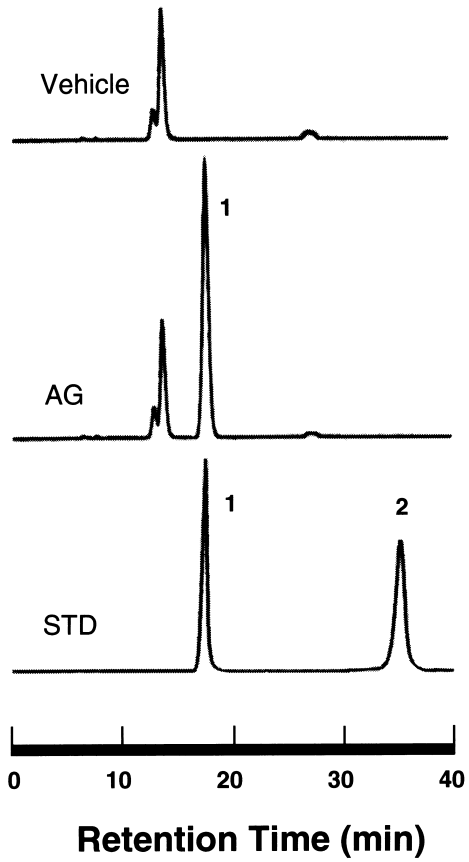

FIG. 2. Typical chromatograms of liver extracts of mice given vehicle or AG and of standard PLP–AG and PL–AG. PLP–AG and PL–AG were dissolved in 0.4 M of perchloric acid to be 1 μM. Aliquots (20 μL) of a standard solution and deproteinized extracts were injected into the HPLC system described under Materials and Methods. Peak identification: 1, PLP–AG; and 2, PL–AG.

Effect of AG on Tissue PLP and PL

The amount of PLP in the liver of mice given AG was markedly lower than that of control mice (4.0 ± 1.4 vs

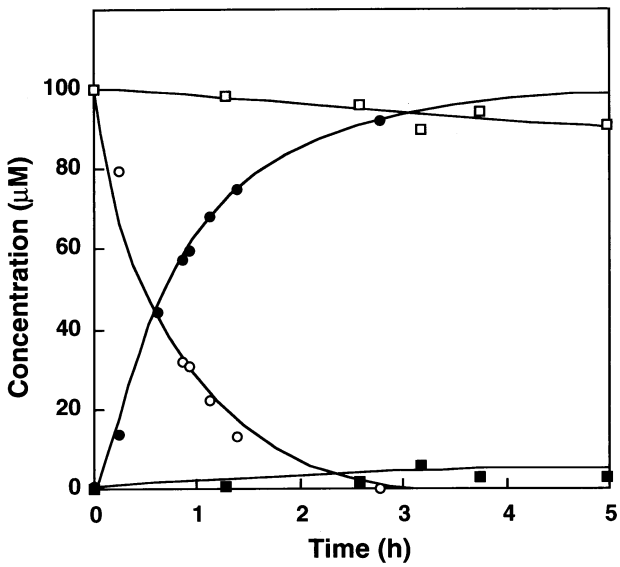

FIG. 3. *In vitro* formation of PLP–AG and PL–AG. PLP or PL at 100 μM in 50 mM of sodium phosphate buffer (pH 7.0) was incubated in the presence of 1 mM of AG at 37° for the indicated periods. PLP (○), PL (□), PLP–AG (●), and PL–AG (■) were determined by HPLC methods. Values are the means of two experiments.

TABLE 1. Amounts of PLP–AG in liver and kidney of mice given AG or AG + PN

Drug	PLP–AG (nmol/g tissue)	
	Liver	Kidney
AG	12.1 ± 1.6	3.8 ± 0.64
AG + PN	26.8 ± 3.0	5.5 ± 0.44

Values are means ± SD for 6 animals.

17.4 ± 1.3 nmol/g of wet tissue), as shown in Fig. 4. The amount of kidney PLP was also decreased significantly by administration of AG. There was, however, no significant difference in the amount of liver PL between AG and control groups. The amount of PL in the kidney was too small to be measured precisely by our method. Administration of PN in addition to AG did not ameliorate the decrease in tissue PLP induced by the administration of AG alone.

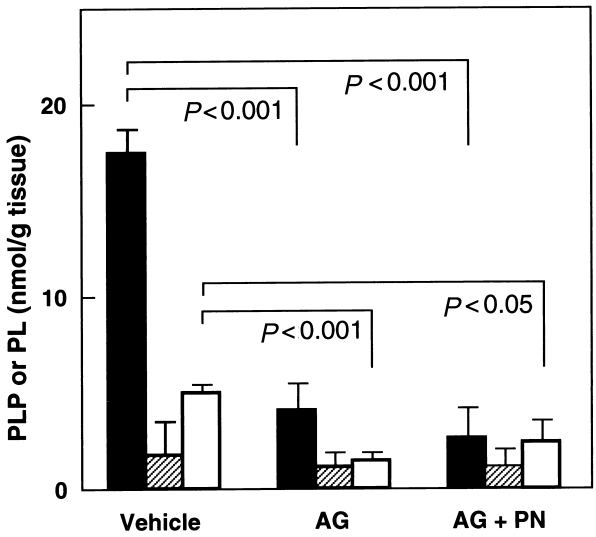

FIG. 4. Amounts of PLP and PL in liver and kidney of mice given vehicle, AG, or AG + PN. Closed columns, PLP in liver; striped columns, PL in liver; open columns, PLP in kidney. Values are means ± SD for 6 animals.

DISCUSSION

In the present study, mice were given water containing 7 mM of AG · HCl. This concentration of AG in drinking water was similar to those reported in some papers [9–11] dealing with the *in vivo* effect of AG on diabetic complications and/or AGE formation: 7.35 mM [9], 8.13 mM [10], and 7.35 mM [11].

The present study demonstrated that PLP–AG was formed in the liver and kidney of mice administered AG. This is the first demonstration of PLP–AG formation *in vivo*. The pharmacological and toxicological effects of the adduct remain to be elucidated.

PL, in solution, exists to a great extent in a hemiacetal form [12]. Thus, much of its free aldehyde group is unavailable to form a Schiff base with the amino group of AG. This should be the reason that PLP reacted *in vitro* with AG much faster than PL and that PLP–AG was formed *in vivo* far more than PL–AG.

The amounts of PLP in the liver and kidney of control mice (17.4 ± 1.3 and 5.0 ± 0.4 nmol/g of tissue, respectively) were in good agreement with those (21.7 ± 3.3 and 5.3 ± 1.1 nmol/g of tissue) reported by Waymire *et al.* [13]. Marked decreases in tissue PLP by the administration of AG may cause the impairment of metabolism related to a variety of PLP-dependent enzymes including amino acid aminotransferases and amino acid decarboxylases. In addition, in light of the fact that PLP is potent as an inhibitor of protein glycation [14–16] and AGE formation [17], the decrease in tissue PLP would possibly have an adverse effect. Attention should also be paid to a report [18] that a deficiency of vitamin B_6 may be a plausible cause of diabetic retinopathy.

We administered PN in addition to AG in an attempt to increase PL and PLP in tissues. However, no amelioration of the decrease in tissue PL and PLP was observed; in fact, the amount of tissue PLP–AG was increased. This suggests that the PLP formed from PN reacts with AG to produce PLP–AG without being accumulated in tissues.

The results suggest that the use of AG for the treatment of diabetic complications should be considered carefully especially in view of the deficiency of tissue PLP.

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