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European Journal of Pharmacology 378 (1999) 283–289

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Inhibition of advanced protein glycation by a Schiff base between aminoguanidine and pyridoxal

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Received 21 April 1999; received in revised form 18 June 1999; accepted 22 June 1999

Abstract

Aminoguanidine is a well-known inhibitor of the formation of advanced glycation end products and is considered to be promising for the treatment of diabetic complications. We recently reported, however, that administration of aminoguanidine caused the formation of a Schiff base adduct between aminoguanidine and pyridoxal phosphate in the liver and kidney of mice and a concomitant decrease in the amount of liver pyridoxal phosphate. Our study led us to hypothesize that the Schiff base adduct and/or another Schiff base adduct formed from aminoguanidine and pyridoxal might be a better compound than aminoguanidine. In the present study, we examined the in vitro inhibitory potency of the latter adduct against advanced glycation end product formation and its effect on the tissue contents of pyridoxal and its phosphate. Aminoguanidine-pyridoxal phosphate adduct was not employed in this study because of its poor solubility in water. Aminoguanidine-pyridoxal adduct was hydrolyzed by only about 15% during 10 days at pH 7.4 and 37°C. The adduct at 1 mM did not inhibit Amadori product formation induced by incubation of albumin with 100 mM mannose for 10 days. The adduct, when tested at 1 and 2 mM, dose-dependently inhibited advanced glycation end product formation induced by incubation of albumin with mannose; and the inhibitory potency of the adduct was similar to or higher than that of aminoguanidine. The presence of an appreciable amount of aminoguanidine-pyridoxal adduct in the kidney of mice given the adduct suggested that at least part of the adduct administered was absorbed from the gastrointestinal duct. The amounts of pyridoxal and its phosphate in tissues were not at all decreased by administration of the aminoguanidine-pyridoxal Schiff base. We conclude that the Schiff base may be a more promising inhibitor of advanced protein glycation than aminoguanidine.

Keywords: Aminoguanidine; Aminoguanidine-pyridoxal adduct; Pyridoxal; Pyridoxal phosphate; Glycation; Diabetes

1. Introduction

Formation of advanced glycation end products of proteins plays a critical role in the pathogenesis of diabetic complications including neuropathy, retinopathy, nephropathy, and cataract (Brownlee et al., 1988). The initial step of glycation is a nucleophilic additive reaction of free amino groups of proteins with the aldehyde group of glucose. The intermediate products (i.e., Schiff bases) become converted to stable ketoamines (i.e., 1-amino-1-deoxyfructose derivatives) by an Amadori rearrangement. Further spontaneous reactions take place via the formation of degradation products such as 3-deoxyglucosone and lead to the appearance of stable and fluorescent advanced glycation end products. Attempts have been made to seek suitable glycation inhibitors for the treatment of diabetic complications. Among various inhibitors of advanced protein glycation, aminoguanidine (Fig. 1) is one of the most promising compounds (Bucala et al., 1995).

Aminoguanidine, a nucleophilic hydrazine, reacts with Amadori fragmentation products such as 3-deoxyglucosone (Edelstein and Brownlee, 1992). It is known that hydrazine compounds are capable of interacting with carbonyl groups of different biological constituents. Recently, we reported that the amount of pyridoxal phosphate in the liver of mice given aminoguanidine in their drinking water for 18 weeks was significantly decreased as compared with that in the liver of control mice and that a Schiff base adduct (Fig. 1) between aminoguanidine and pyridoxal phosphate was concomitantly formed in the liver of aminoguanidine-treated mice (Taguchi et al., 1998). This

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PII: S0014-2999(99)00471-9

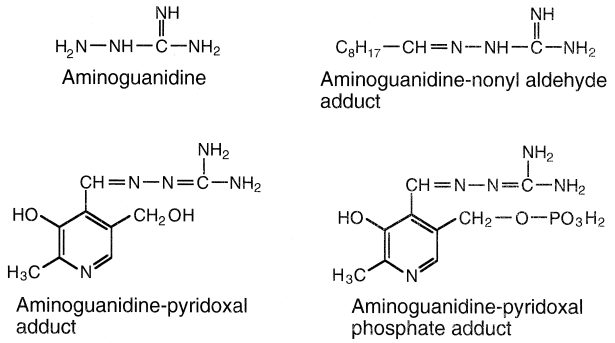

Fig. 1. Structures of aminoguanidine and its adducts.

finding suggested that the clinical use of aminoguanidine should be carefully considered, especially in view of the deficiency of tissue pyridoxal phosphate, and prompted us to consider the idea that an aminoguanidine-pyridoxal phosphate Schiff base adduct and/or another Schiff base adduct (Fig. 1) formed between aminoguanidine and pyridoxal would be a safer and more promising compound than aminoguanidine, provided that their inhibitory activity against advanced protein glycation was similar to or higher than that of aminoguanidine.

In the present study, we compared aminoguanidine and its adduct with pyridoxal for in vitro inhibitory potency against advanced protein glycation and examined whether the adduct affects the tissue pyridoxal phosphate and pyridoxal contents. Because aminoguanidine-pyridoxal phosphate adduct was hardly soluble in water, we did not use it in this study. In order to assess the efficacy of aminoguanidine-pyridoxal adduct, we also tested another Schiff base adduct (Fig. 1), one formed from aminoguanidine and nonyl aldehyde, for its ability to prevent advanced glycation end product formation.

2. Materials and methods

2.1. Materials

Aminoguanidine-pyridoxal adduct and aminoguanidine-pyridoxal phosphate adduct were synthesized as described previously (Taguchi et al., 1998). Bovine serum albumin (fraction V, A2153) was from Sigma (St. Louis, MO, USA). Monoclonal anti-advanced glycation end product antibody (clone No. 6D12) was purchased from Wako (Osaka, Japan). A kit (Vectastain Elite ABC kit) containing biotinylated horse anti-mouse immunoglobulin G antibody and avidin-biotin horseradish peroxidase complexes was obtained from Vector (Burlingame, CA, USA). Male 4-week old ddY mice, weighing 18-20 g, were purchased from Japan SLC (Hamamatsu, Japan) and given laboratory chow and water ad libitum. The experimental procedures employed were approved by our Animal Care and Use Committee.

2.2. Synthesis of aminoguanidine-nonyl aldehyde adduct

Nonyl aldehyde (30 mmol) was slowly added to a stirred solution of aminoguanidine · H_2CO_3 (30 mmol) in H_2O (50 ml), and the mixture was kept at room temperature for 24 h with stirring, followed by extraction with CH_2Cl_2. The CH_2Cl_2 layer was dried over $MgSO_4$, filtered, and concentrated. For purification, the residue was subjected to silica gel column chromatography with 1% CH_3OH in CH_2Cl_2. The eluate was concentrated to give a mixture of syn and anti isomers in the monoamine form (Fig. 1). The ratio of the syn to the anti was 3:1 as analyzed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrometry. The yield of aminoguanidine-nonyl aldehyde Schiff base adduct (yellow oil) was 83%. The spectral data for the adduct were as follows: mass spectrum (M^+) m/z: 198. IR cm^{-1}: $\nu_{C=N}$ 1629, 1670. ^{1}H-NMR ($CDCl_3$) δ: 11.44 and 10.98 (C=NH, 1H, bs), 7.37 and 7.92 (NH_2, 2H, bs), 6.26 and 6.47 (N–NH–C=$_N$H, 1H, bs), 7.51 and 6.73 (CH_2–CH=N–, 1H, t), 2.27 and 2.39 (CH_2–CH_2–CH=N–, 2H, m), 1.2–1.6 ($(CH_2)_7$, 14H, m), 0.88 (CH_3, 3H, t). ^{13}C-NMR ($CDCl_3$) δ: 152.59 and 151.71 (CH_2–CH=N–), 155.46 and 156.51 (C=NH), 32.29 and 28.43 (CH_2–CH_2–CH=N–), 13.91 (CH_3).

2.3. Measurement of Amadori product

Bovine serum albumin (40 mg/ml) was incubated with 100 mM mannose in the presence or absence of the desired drug for 10 days at 37°C in the dark under sterile conditions in 50 mM sodium phosphate buffer (pH 7.4) containing 15 mM NaN_3 (Miwa et al., 1996). We described the reason for the use of mannose instead of glucose in the same paper. Aminoguanidine-pyridoxal adduct was used after neutralization with HCl. Blank values were obtained by incubation of albumin in the absence of both mannose and drug. Incubations were performed in a final volume of 1 ml under sterile conditions. After incubation, the reaction mixture was dialyzed three times (8 h each) against H_2O. The Amadori product of albumin in the dialyzed solution was measured as reported previously (Ahmed and Furth, 1991). This method is based on the colorimetric determination of the formaldehyde released by periodate oxidation of C-1 hydroxyls in the Amadori product form of glycated proteins.

2.4. Measurement of advanced glycation end products by fluorometry

Incubation of bovine serum albumin with mannose and subsequent dialysis were performed as described above. The amount of glycation products in the dialyzed solution was assayed by measurement of fluorescence intensity with excitation and emission wavelengths set at 370 and 440 nm, respectively (Brownlee et al., 1986).

2.5. Measurement of advanced glycation end products by enzyme-linked immunosorbent assay (ELISA)

The same dialyzed solution as described above was analyzed for glycation products by a noncompetitive ELISA with a monoclonal anti-advanced glycation end product antibody and a Vectastain Elite ABC kit as reported previously (Horiuchi et al., 1991).

2.6. Administration of aminoguanidine and its pyridoxal adduct

Mice were divided into three groups of five each: group 1 for vehicle (H_2O), group 2 for aminoguanidine · HCl, and group 3 for aminoguanidine-pyridoxal adduct. The adduct was neutralized with HCl. Drugs were given by gavage once a day at a dosage of 0.9 mmol/kg/day for 6 weeks.

2.7. Preparation of tissue extracts

Mice were sacrificed under anesthesia with pentobarbital on the day after the final administration of the drug. Extracts of livers and kidneys of the mice were prepared as described previously (Taguchi et al., 1998). Briefly, tissues were homogenized in 50 mM sodium phosphate buffer (pH 7.4) and centrifuged, and then the supernatants were deproteinized by addition of perchloric acid to yield the tissue extracts.

2.8. Analysis of pyridoxal and its derivatives

Pyridoxal and its aminoguanidine-adduct as well as pyridoxal phosphate and its aminoguanidine-adduct in both incubation medium and tissue extracts were analyzed by a combination of two high-performance liquid chromatography (HPLC) methods as described previously (Taguchi et al., 1998).

Table 1
Concentrations of aminoguanidine-pyridoxal adduct and pyridoxal after incubation of the adduct with or without albumin and/or mannose
Aminoguanidine-pyridoxal adduct (1 mM) neutralized with HCl was incubated in the presence or absence of 40 mg/ml bovine serum albumin and/or 100 mM mannose for 10 days at 37°C in 50 mM sodium phosphate buffer (pH 7.4) containing 15 mM NaN_3. The incubation was performed in the dark under sterile conditions. After incubation, the reaction mixtures were deproteinized by addition of an equal volume of 0.6 M $HClO_4$, and then the resultant protein-free solutions were analyzed for aminoguanidine-pyridoxal adduct and pyridoxal by the HPLC methods. Values are the means ± S.D. of four experiments.

Condition	Adduct (mM)	Pyridoxal (mM)
Without albumin/mannose	0.85 ± 0.04	0.078 ± 0.005
With albumin only	0.90 ± 0.04	0.015 ± 0.003[a]
With albumin/mannose	0.69 ± 0.09[b]	0.065 ± 0.012

[a] $P < 0.001$ vs. the other two conditions.
[b] $P < 0.005$ vs. the other two conditions.

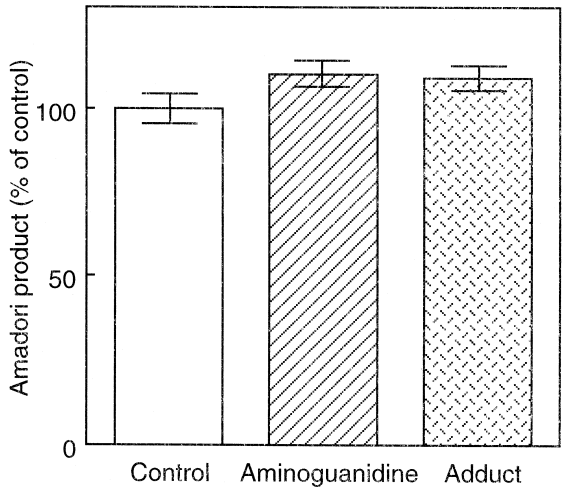

Fig. 2. Effects of aminoguanidine and its pyridoxal adduct on Amadori product formation. Bovine serum albumin (40 mg/ml) was incubated with 100 mM mannose for 10 days at 37°C in the presence or absence of 1 mM drug and then dialyzed thoroughly. The Amadori product was determined by the periodate method. Values are the means ± S.D. of five experiments.

2.9. Statistical analysis

The Bonferroni–Dunn test was used for all statistical analyses. The level of significance was set at 0.05.

3. Results

The aminoguanidine-pyridoxal Schiff base adduct was appreciably hydrolyzed to produce pyridoxal when incu-

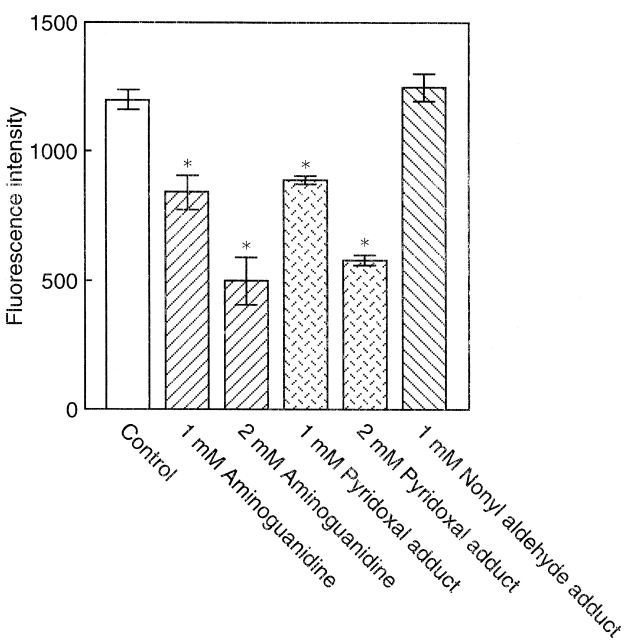

Fig. 3. Effects of aminoguanidine and its derivatives on advanced glycation end product formation assessed from fluorescence intensity. Bovine serum albumin was incubated and then dialyzed as described in the legend to Fig. 2. Advanced protein glycation was determined by measurement of fluorescence intensity. Values are the means ± S.D. of five experiments. * $P < 0.001$ vs. control.

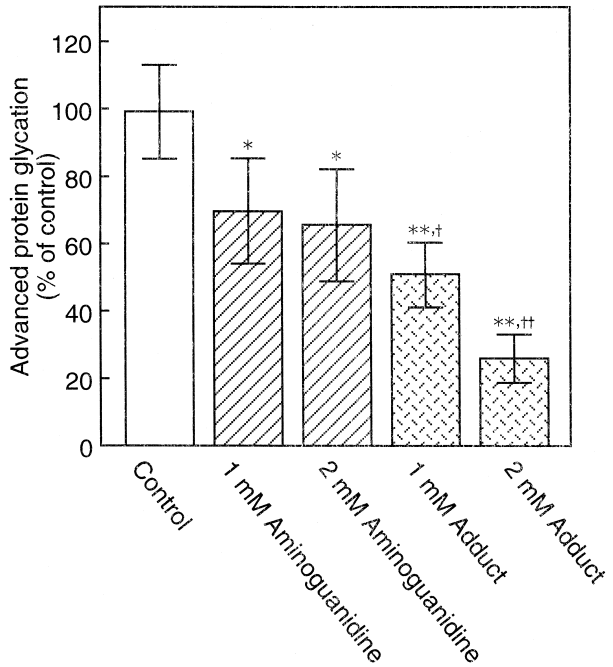

Fig. 4. Effects of aminoguanidine and its pyridoxal adduct on advanced glycation end product formation assessed by ELISA. Bovine serum albumin was incubated and then dialyzed as described in the legend to Fig. 2. Advanced protein glycation was determined by the ELISA method. Values are the means ± S.D. of 12 experiments. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. control, $^{+}P < 0.01$ vs. 1 mM aminoguanidine, $^{++}P < 0.005$ vs. 2 mM aminoguanidine.

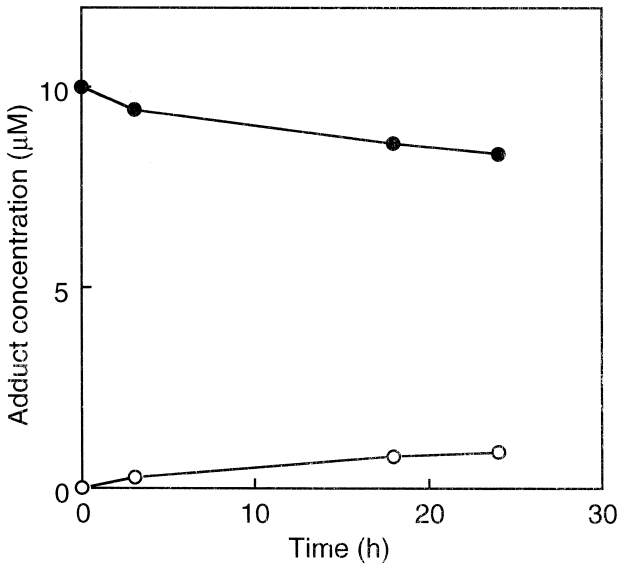

Fig. 5. Formation of aminoguanidine-pyridoxal phosphate adduct by incubation of aminoguanidine-pyridoxal adduct with pyridoxal phosphate. Pyridoxal adduct (10 μM) neutralized with HCl was incubated with 100 μM pyridoxal phosphate in 50 mM sodium phosphate buffer (pH 7.4) at 37°C. Pyridoxal adduct (●) and pyridoxal phosphate adduct (○) in the reaction mixture were assayed by HPLC. Values are the means of two experiments.

bated for 10 days at pH 7.4 and 37°C in the absence of both albumin and mannose (Table 1). The concentration (0.078 mM) of pyridoxal found after the incubation, however, was not the same as that (0.15 mM) of the adduct lost during the incubation. The pyridoxal concentration (0.065 mM) was far lower than that (0.31 mM) of the adduct lost especially when incubation was performed in the presence of both albumin and mannose. The aminoguanidine-pyridoxal adduct was thus found to decrease more rapidly in amount when incubated in the presence of both albumin and mannose, i.e., under the conditions in which glycation takes place, than when incubated either in the presence of albumin alone or in the absence of both albumin and mannose.

Neither aminoguanidine nor its pyridoxal adduct at 1 mM inhibited Amadori product formation induced by incubation of albumin with 100 mM mannose for 10 days (Fig. 2).

When advanced protein glycation was assessed in terms of fluorescence intensity, however, both aminoguanidine and its pyridoxal adduct dose-dependently attenuated advanced glycation end product formation with comparable potency (Fig. 3). The Schiff base formed between aminoguanidine and nonyl aldehyde did not affect advanced protein glycation, indicating that not all of the Schiff bases of aminoguanidine are necessarily effective in inhibiting advanced glycation end product formation. As-

Table 2
Amounts of aminoguanidine-pyridoxal phosphate adduct and aminoguanidine-pyridoxal adduct in liver and kidney of mice given aminoguanidine or the latter adduct
Values are the means ± S.D. for five animals.

Treatment	Liver		Kidney	
	Pyridoxal phosphate adduct	Pyridoxal adduct	Pyridoxal phosphate adduct	Pyridoxal adduct
	(nmol/g tissue)			
Vehicle (water)	ND[a]	ND	ND	ND
Aminoguanidine	3.5 ± 1.3	0.02 ± 0.03	0.61 ± 0.20	0.01 ± 0.05
Pyridoxal adduct	5.4 ± 2.1	0.10 ± 0.05	0.58 ± 0.15	0.47 ± 0.18[b]

[a] Not detected.
[b] $P < 0.005$ vs. aminoguanidine.

Table 3
Amounts of pyridoxal phosphate and pyridoxal in liver and kidney of mice given aminoguanidine or aminoguanidine-pyridoxal adduct
Values are the means ± S.D. for five animals.

Treatment	Liver		Kidney	
	Pyridoxal phosphate	Pyridoxal	Pyridoxal phosphate	Pyridoxal
	(nmol/g tissue)			
Vehicle (water)	19.6 ± 3.0	1.7 ± 0.44	7.1 ± 1.6	< 0.1
Aminoguanidine	13.7 ± 1.7[a]	1.8 ± 0.37	6.0 ± 1.1	< 0.1
Adduct	18.1 ± 3.0[b]	1.5 ± 0.30	7.2 ± 1.0	< 0.1

[a] $P < 0.01$ vs. vehicle.
[b] $P < 0.05$ vs. aminoguanidine.

sessment by ELISA also showed the ability of both aminoguanidine and its pyridoxal adduct to inhibit advanced protein glycation (Fig. 4). In the assay using ELISA, however, the aminoguanidine-pyridoxal adduct was more potent than aminoguanidine.

The amounts of aminoguanidine adduct with pyridoxal and with pyridoxal phosphate in the liver and kidney of mice given aminoguanidine or its pyridoxal adduct at 0.9 mmol/kg/day for 6 weeks were measured (Table 2). The amount of kidney aminoguanidine-pyridoxal adduct in mice given the adduct was significantly higher than that in mice given aminoguanidine. The amounts of pyridoxal adduct in the liver and pyridoxal phosphate adduct in both tissues were not significantly different between the two groups.

In order to address the possibility that the pyridoxal phosphate adduct found in the liver and kidney of mice given aminoguanidine-pyridoxal adduct is formed by reaction of the latter adduct with pyridoxal phosphate, we incubated 10 μM pyridoxal adduct with 100 μM pyridoxal phosphate. As shown in Fig. 5, pyridoxal phosphate adduct was time-dependently formed in accordance with the decrease in the concentration of the pyridoxal adduct.

The amount of pyridoxal phosphate in the liver of mice given aminoguanidine was significantly lower than that of mice given vehicle, whereas the amount of liver pyridoxal phosphate was not affected by administration of the aminoguanidine-pyridoxal adduct (Table 3). There was no significant difference in the amount of liver pyridoxal or kidney pyridoxal phosphate among vehicle, aminoguanidine, and adduct groups. The amount of kidney pyridoxal was too small to be measured precisely by our method.

4. Discussion

Our results clearly show that a Schiff base adduct between aminoguanidine and pyridoxal was capable of inhibiting advanced glycation end product formation, whereas Amadori product formation was not affected by the Schiff base adduct. Moreover, we found that the inhibitory potency of the adduct against advanced protein glycation was similar to or more than that of aminoguanidine when assessed by fluorometry and ELISA. The fluorometry can assess the amount of fluorescent glycation products, whereas non-fluorescent N^{ε}-(carboxymethyl)-lysine residues, one of the advanced glycation end product structures, are recognized by the monoclonal antibody used in our ELISA (Ikeda et al., 1996). This may be the cause for the difference in inhibitory potency of the adduct between the two assessment methods.

The structures of aminoguanidine derivatives, nonyl aldehyde adduct, and pyridoxal adduct, shown in Fig. 1 were mainly based on the data of ^{1}H-NMR and ^{13}C-NMR, with measurements made in organic solvent ($CDCl_3$ or $DMSO_{d-6}$). It is highly probable that both compounds tautomerize to give a mixture of $-N=C(-NH_2)_2$ structure and $-NH-C(=NH)-NH_2$ structure in aqueous media. Therefore, the nonyl aldehyde adduct does not necessarily resemble more closely aminoguanidine than pyridoxal adduct with respect to the functional amino groups. Further studies are needed to clarify the reason for the ineffectiveness of the nonyl aldehyde adduct in inhibition of advanced protein glycation.

The mechanism of inhibition of advanced protein glycation by the aminoguanidine-pyridoxal adduct remains to be elucidated. Aminoguanidine formed by spontaneous hydrolysis of the adduct may act, at least partly, as the inhibitor of advanced protein glycation. However, when the adduct (1 mM) was incubated for 10 days in the presence of albumin and mannose, only 0.31 mM aminoguanidine would be produced during the incubation provided that the adduct was lost by hydrolysis (Table 1). In view of the fact that the adduct (1 or 2 mM) was similar to or higher than aminoguanidine (1 or 2 mM) in inhibitory potency against advanced protein glycation (Figs. 3 and 4), aminoguanidine formed by hydrolysis of the adduct is unlikely to be the sole inhibitor of glycation. This notion inevitably suggests that not only aminoguanidine formed but also the adduct itself seems to have an inhibitory activity. The adduct may directly react with Amadori-derived products, e.g., 3-deoxyglucosone, as is thought to occur in the inhibition of advanced protein glycation by aminoguanidine (Edelstein and Brownlee, 1992). A more rapid decrease in adduct by incubation under the conditions in which glycation takes place than under the other two conditions (Table 1) supports this view.

The fact that the concentration (0.078 mM) of pyridoxal found after incubation of the aminoguanidine-pyridoxal adduct in the absence of both albumin and mannose was lower than that (0.15 mM) of the adduct decreased during the incubation (Table 1) implies that at least part of the pyridoxal produced by hydrolysis of the adduct is degraded during the incubation. A marked loss of pyridoxal during the incubation of the adduct in the presence of both albumin and mannose may suggest that pyridoxal, known as an inhibitor of advanced protein glycation (Khatami et

al., 1988), can also be implicated in inhibition of advanced glycation end product formation by the aminoguanidine-pyridoxal adduct.

The presence of an appreciable amount of aminoguanidine-pyridoxal adduct in the kidney of mice given the adduct strongly suggests that at least part of the adduct was absorbed with retention of its form, i.e., without its hydrolysis to pyridoxal and aminoguanidine, from the gastrointestinal duct; since only a marginally low amount of the adduct was observed in the kidney of mice given aminoguanidine (Table 2). It is conceivable that the aminoguanidine-pyridoxal phosphate adduct in the tissues of mice given the aminoguanidine-pyridoxal adduct was formed by reaction of the latter adduct with pyridoxal phosphate. In mice given the pyridoxal adduct, moreover, the higher pyridoxal phosphate adduct/pyridoxal adduct ratio in the liver than in the kidney might be due to the higher pyridoxal phosphate content in the former tissue than in the latter (Taguchi et al., 1998). The fact that aminoguanidine-pyridoxal phosphate adduct was actually formed by incubation of pyridoxal adduct with pyridoxal phosphate (Fig. 5) supports the above possibility. Incidentally, the prevention of loss of pyridoxal phosphate in the liver of adduct-treated mice (Table 3) may be explained by conversion of the pyridoxal, formed from the administered adduct, into pyridoxal phosphate.

The lack of any significant decrease in the kidney pyridoxal phosphate content in aminoguanidine-treated mice as compared with vehicle-treated mice (Table 3) is probably due to the short duration of the dosing, because the amount of kidney pyridoxal phosphate was significantly lower in mice given aminoguanidine than in mice given vehicle (1.5 ± 0.4 vs. 5.3 ± 0.4 nmol/g tissue; mean $\pm$ S.D. of six animals) when the drug was administered for 18 weeks at a dosage similar to that used in the present study (Taguchi et al., 1998). This view suggests that the deficiency of pyridoxal phosphate may take place in other tissues than liver and kidney in the case of long-term administration of aminoguanidine.

It has been reported that a deficiency of vitamin B_6 may be a plausible molecular basis of various diseases including carpal tunnel syndrome (Ellis et al., 1978), diabetic retinopathy (Ellis et al., 1991), arteriosclerosis (Kang et al., 1992), and myocardial infarction (Ellis and McCully, 1995). The present study, together with these reports, suggests that aminoguanidine-pyridoxal Schiff base adduct is a more promising inhibitor of advanced protein glycation than aminoguanidine especially because of no depletion of tissue pyridoxal phosphate. We are now planning to test whether the Schiff base adduct is able to inhibit both the in vivo formation of advanced glycation end products and the development of diabetic complications such as nephropathy, retinopathy, and neuropathy. Prior to the clinical testing of the aminoguanidine-pyridoxal adduct, the adduct should be carefully examined for certain likely properties, e.g., inhibition of pyridoxal phosphate-dependent enzymes (Okada and Ayabe, 1995) and inducible nitric oxide synthase (Misko et al., 1993) and the pro-oxidant and antioxidant activity toward low-density lipoprotein (Philis-Tsimikas et al., 1995), which actions have been reported to be intrinsic to the aminoguanidine structure.

Acknowledgements

This study was supported in part by the Grant-in-Aid (to I.M.) of High-Tech Research Center Project from the Ministry of Education, Science, Sports and Culture of Japan.

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