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Thiamine Pyrophosphate and Pyridoxamine Inhibit the Formation of Antigenic Advanced Glycation End-Products: Comparison with Aminoguanidine[1]

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Nonenzymatic glycation of proteins by glucose leading to the formation of toxic and immunogenic advanced glycation end products (AGEs) may be a major contributor to the pathological manifestations of diabetes mellitus, aging, and, possibly, neurodegenerative diseases such as Alzheimer's. We tested the *in vitro* inhibition of antigenic AGE formation on bovine serum albumin, ribonuclease A, and human hemoglobin by various vitamin B_1 and B_6 derivatives. Among the inhibitors, *pyridoxamine and thiamine pyrophosphate potently inhibited AGE formation and were more effective than aminoguanidine,* suggesting that these two compounds may have novel therapeutic potential in preventing vascular complications of diabetes. An unexpected finding was that aminoguanidine inhibited the late kinetic stages of glycation much more weakly than the early phase.

The chronic hyperglycemia observed in diabetes leads to microvascular and macrovascular complications underlying nephropathy, neuropathy, retinopathy and atherosclerotic disease (1,2). The recent Diabetes Control and Complication Trial (3) confirmed that patients with higher levels of mean blood glucose have a higher prevalence of diabetic complications. This supports the hypothesis of many investigators (1,2) that significant pathogenic alterations arise from irreversible *nonenzymatic* chemical modifications of plasma and tissue components that are initiated by glucose. Furthermore, nonenzymatically glycated serum albumin is elevated in diabetic sera (4), and diabetic nephropathy can be induced by administration of glycated albumin (5–7). It is preferentially transported into the renal glomerulus, where it has been shown to contribute to basement membrane thickening and mesangial expansion (8,9). The inhibition of glycation ("glucosylation") reactions of reducing sugars is thus potentially of much medical and pharmacological importance (1,2). We report here that pyridoxamine and thiamine pyrophosphate are at least as potent as aminoguanidine (currently in clinical trials for the prevention of diabetic nephropathy) in inhibiting the *in vitro* formation of *antigenic* advanced glycation end products.

Glucose initially reacts with proteins, amino acids and nucleic acids through its minor, open-chain aldehydic form (10) via Schiff base condensation with amino groups **(Scheme I).** It then undergoes a nearly irreversible rearrangement to form an Amadori product. Further Maillard reactions ensue to slowly produce reactive and toxic covalent adducts of largely unknown structure that are often termed advanced glycation end products or "AGEs." AGE products can also arise from reactive dicarbonyl fragments generated from free glucose or from the "early" glycation products (Schiff base and Amadori intermediates) through "glycoxidation" reactions (11). The discovery of AGE inhibitors and the elucidation of their mechanisms of inhibition will be clearly challenging in view of the complexity of the glycation reaction cascade. Aminoguanidine, the most prominent AGE inhibitor, prevents a variety of diabetic complications in experimental animal

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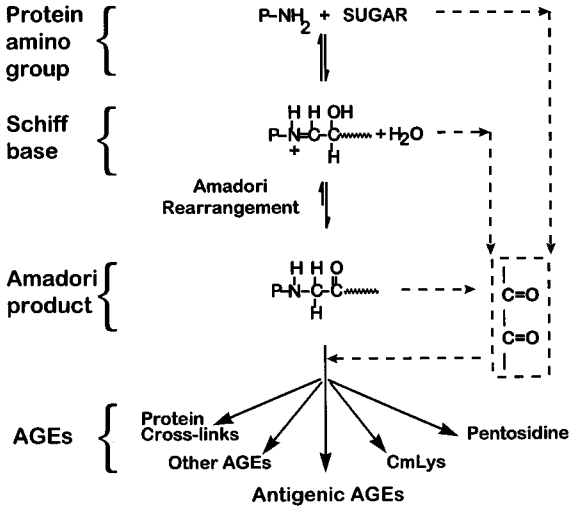

SCHEME 1.

models (1,2,12) and is currently being investigated in diabetic clinical trials (1). This compound combines hydrazine and guanidinium moieties, making it difficult to ascertain its precise mechanism of inhibition (13–15).

We have examined potential inhibition of late AGE formation by members of vitamin B_1 and B_6 families, participants in a myriad of enzymatic reactions that involve carbonyl metabolism (16). We are unaware of previous studies on possible inhibition by thiamine or its vitamers. Some of the B_6 vitamers, especially pyridoxal phosphate (PLP), have been previously proposed to act as "competitive inhibitors" of early glycation, since as aldehydes they themselves can form Schiff bases adducts with protein amino groups (17) and thus limit the amount of amines available for glucose attachment. However, effectiveness in limiting initial sugar attachment is not a predictor of inhibition of the conversion of any Amadori products formed to AGEs. We have searched, in contrast, for potential inhibitors of *late* glycation reactions as indicated by their effects on the *in vitro* formation of *antigenic* AGEs.

MATERIALS AND METHODS

Chemicals. Bovine pancreatic ribonuclease A (RNase) was a chromatographically pure, aggregate-free grade from Worthington Biochemicals. Bovine serum albumin (BSA) (fraction V, fatty acid-free), human methemoglobin (Hb), D-glucose, pyridoxine, pyridoxal, pyridoxal 5′phosphate, pyridoxamine, thiamine, thiamine monophosphate, thiamine pyrophosphate, and goat alkaline phosphatase-conjugated anti-rabbit IgG were all from Sigma Chemicals. Aminoguanidine hydrochloride was purchased from Aldrich Chemicals.

Glycation. Bovine serum albumin, ribonuclease A, and human hemoglobin were incubated with glucose at 37°C in 0.4 M sodium phosphate buffer of pH 7.5 containing 0.02% sodium azide. The protein, glucose (at 1.0 M), and prospective inhibitors (at 0.5, 3, 15 and 50 mM) were introduced into the incubation mixture simultaneously. Solutions were kept in the dark in capped tubes. Aliquots taken were immediately frozen until analyzed by ELISA at the conclusion of the reaction. The incubations were for 3 weeks (Hb) or 6 weeks (RNase, BSA).

Preparation of polyclonal antibodies to AGE Proteins. Immunogen preparation followed earlier protocols (18–20). Immunogen was prepared by glycation of BSA (R479 antibodies) or RNase (R618 antibodies) at 1.6 g protein in 15 ml for 60–90 days using 1.5 M glucose in 0.4 M phosphate containing 0.05 % sodium azide at pH 7.4 and 37°C. New Zealand white rabbit males of 8–12 weeks were immunized by subcutaneous administration of a 1 ml solution containing 1 mg/ml of glycated protein in Freund's adjuvant. The primary injection used the complete adjuvant and three boosters were made at three week intervals with Freund's incomplete adjuvant. The rabbits were bled three weeks after the last booster. The serum was collected by centrifugation of clotted whole blood. The antibodies are AGE-specific,[3] being unreactive with either native proteins (except for the carrier) or with Amadori intermediates.

[3] The polyclonal anti-AGE antibodies have proven to be a sensitive and valuable analytical tool for the study of "late"

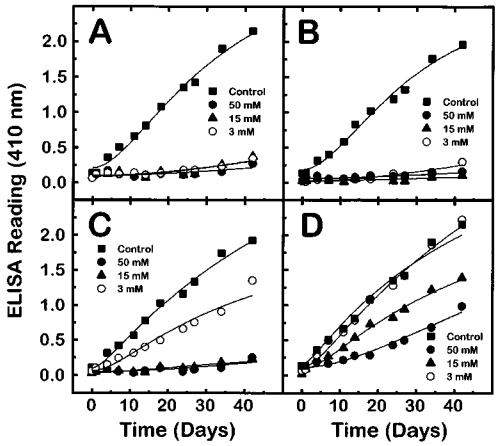

FIG. 1. Effect of vitamin B_6 derivatives on AGE formation in bovine serum albumin. BSA (10 mg/ml) was incubated with 1.0 M glc in the presence and absence of the various indicated derivatives in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 6 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 3, 15 and 50 mM. (A) Pyridoxamine (PM); (B) pyridoxal phosphate (PLP); (C) pyridoxal (PL); (D) pyridoxine (PN).

ELISA detection of AGE products. The general method of Engvall (21) was used to perform the ELISA. Glycated protein samples were diluted to approximately 1.5 μg/ml in 0.1 M sodium carbonate buffer of pH 9.5 to 9.7. The protein was coated overnight at room temperature onto a 96-well polystyrene plates by pippetting 200 μl of the protein solution in each well (0.3 μg/well). After coating, the protein was washed from the wells with a saline solution containing 0.05% Tween-20. The wells were then blocked with 200 μl of 1% casein in carbonate buffer for 2 h at 37°C followed by washing. Rabbit anti-AGE antibodies were diluted at a titer of 1:350 in incubation buffer and incubated for 1 h at 37°C, followed by washing. In order to minimize background readings, antibodies R479 used to detect glycated RNase were raised against glycated BSA, and antibodies R618 used to detect glycated BSA and glycated Hb were raised against glycated RNase. An alkaline phosphatase-conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of 1:2000 and incubated for 1 h at 37°C, followed by washing. The *p*-nitrophenylphosphate substrate solution was then added (200 μl/well) to the plates, with the absorbance of the released *p*-nitrophenolate being monitored at 410 nm with a Dynatech MR4000 microplate reader.

RESULTS

Figure 1 (control curves) demonstrates that reaction of BSA with 1.0 M glucose is slow and incomplete after 40 days, even at this high sugar concentration used to accelerate the reaction. The simultaneous inclusion of different concentrations of various B_6 vitamers markedly affects the formation of antigenic AGEs **(Fig. 1A–D).** Pyridoxamine and pyridoxal phosphate strongly suppressed antigenic AGE formation at even the lowest concentrations tested, while pyridoxal was effective above 15 mM. Pyridoxine was slightly effective at the highest concentrations tested. Of the various B_1 vitamers similarly tested **(Fig. 2A–D),** thiamine pyrophosphate was effective at all concentrations investigated **(Fig. 2C),** whereas thiamine and thiamine monophosphate were not inhibitory. Note the remarkable decrease in the *final levels* of AGEs formed that is observed with thiamine pyrophosphate, pyridoxal phosphate and pyridoxamine. Aminoguanidine **(Fig. 2D)** produced some inhibition of AGE formation in BSA but less so than the above compounds.

Similar studies were carried out with human methemoglobin and bovine ribonuclease A. Makita et al. (22) had previously detected Hb in diabetic patients that binds to anti-AGE antibodies, and

AGE formation *in vitro* and *in vivo*. The nature of the dominant antigenic AGE epitope or hapten remains in doubt, although recently Baynes and coworkers (25) proposed that the protein glycoxidation product carboxymethyl lysine (CmL) may be a dominant antigen of their antibodies. Earlier studies, however, failed to reveal ELISA reactivity with model carboxymethyl lysine compounds (20).

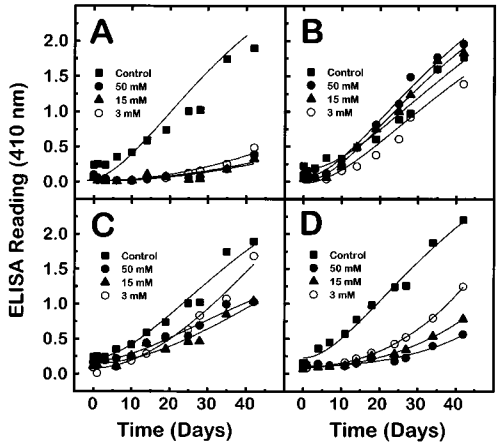

FIG. 2. Effects of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation in bovine serum albumin. BSA (10 mg/ml) was incubated with 1.0 M glc in the presence and absence of the various indicated derivatives in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 6 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 3, 15 and 50 mM. (A) Thiamine pyrophosphate (TPP); (B) thiamine monophosphate (TP); (C) thiamine (T); (D) aminoguanidine (AG).

proposed that this glycated Hb (termed Hb-AGE, not to be confused with Hb_{A1c}) could be useful in measuring long-term exposure to glucose. We find that the *in vitro* incubation of Hb with glucose produces antigenic AGEs at an apparently faster rates than observed with BSA. Nevertheless, the different B_6 **(Fig. 3A–D)** and B_1 **(Fig. 4A–C)** vitamers exhibited the same inhibition trends in Hb, with pyridoxamine and thiamine pyrophosphate being the most effective inhibitors in each of their respective families. *Aminoguanidine appeared to only decrease the rate of AGE formation in Hb and not the final levels formed* ***(Fig. 4D),*** *thus being without effect after 21 days of glycation.* With RNase the rate of antigenic AGE formation by glucose was intermediate between that of Hb and BSA, but the extent of inhibition within each vitamer series was maintained. Again, pyridoxamine and thiamine pyrophosphate appeared as effective or more effective than aminoguanidine **(Fig. 5).**

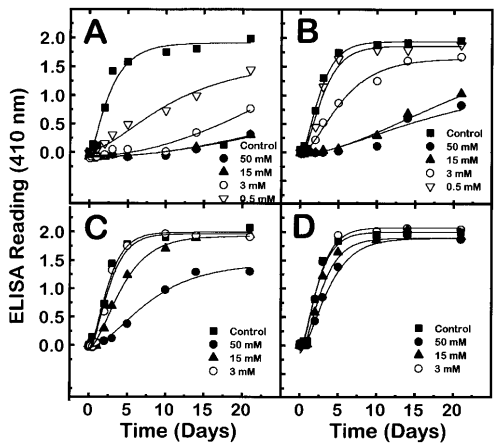

FIG. 3. Effect of vitamin B_6 derivatives on AGE formation in human methemoglobin. Hb (1 mg/ml) was incubated with 1.0 M glc in the presence and absence of the various indicated derivatives in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 3 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 0.5, 3, 15 and 50 mM. (A) Pyridoxamine (PM); (B) pyridoxal phosphate (PLP); (C) pyridoxal (PL); (D) pyridoxine (PN).

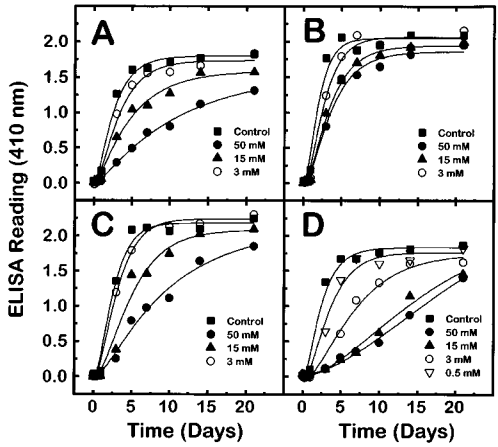

FIG. 4. Effects of vitamin B_1 derivatives and aminoguanidine (AG) on AGE formation in human methemoglobin. Hb (1 mg/ml) was incubated with 1.0 M glc in the presence and absence of the various indicated derivatives in 0.4 M sodium phosphate buffer of pH 7.5 at 37°C for 3 weeks. Aliquots were assayed by ELISA using R618 anti-AGE antibodies. Concentrations of the inhibitors were 0.5, 3, 15 and 50 mM. (A) Thiamine pyrophosphate (TPP); (B) thiamine monophosphate (TP); (C) thiamine (T); (D) aminoguanidine (AG).

DISCUSSION

Our results demonstrate that certain derivatives of B_1 and B_6 vitamins are capable of inhibiting "late" AGE formation. Some of these vitamers successfully inhibited the final levels of AGE produced, in contrast to aminoguanidine, suggesting they have greater interactions with Amadori or post-Amadori precursors to antigenic AGEs. The Amadori and post-Amadori intermediates represent a crucial juncture where the "classical" pathway of nonenzymatic glycation begins to become essentially irreversible (Scheme I). In earlier inhibition studies "glycation" was usually measured either as Schiff base formed (after reduction with labeled cyanoborohydride) or as

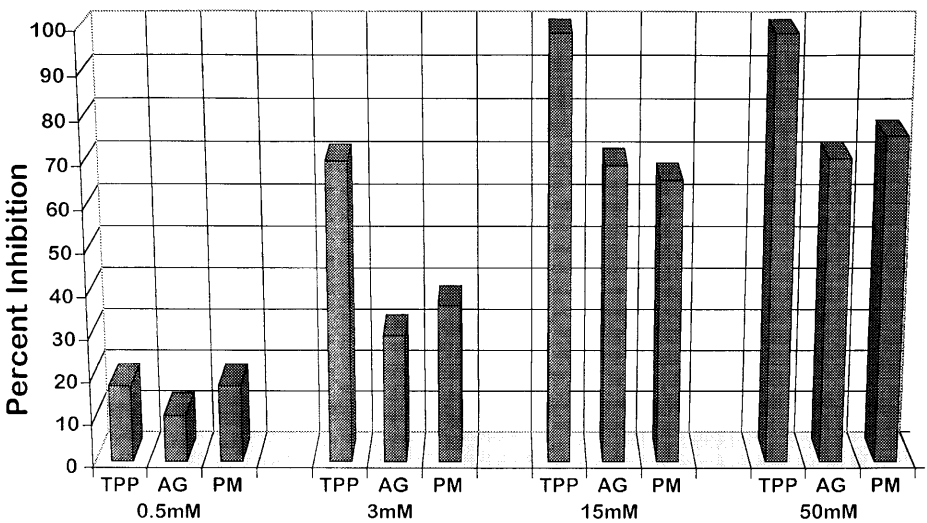

FIG. 5. Comparison of the inhibition of the glycation of ribonuclease A by thiamine pyrophosphate (TPP), pyridoxamine (PM) and aminoguanidine (AG). RNase (1 mg/ml) was incubated with 1.0 M glucose (glc) in the presence and absence of the indicated inhibitors for 6 weeks in 0.4 M phosphate buffer of pH 7.5 at 37°C. Aliquots were assayed by ELISA using R479 anti-AGE antibodies. The indicated percent inhibition (ordinate values) was computed from ELISA readings in the absence and presence of the inhibitors at the 6 weeks time point.

Amadori product formed (after acid precipitation using labeled sugar). Such assays do not yield information on inhibition of post-Amadori conversion steps to "late" AGE products, since there is no change in the amount of labeled sugar that is attached. Other "glycation" assays have relied on the sugar-induced increase of non-specific protein fluorescence, but this can also be induced by dicarbonyl oxidative fragments of free sugar, such as glycoaldehyde or glyoxal (23), independently of Amadori product formation.

In the case of pyridoxal (PL) and pyridoxal phosphate (PLP), our data support the simple mechanism of inhibition involving competitive Schiff-base condensation of these aldehydes with protein amino groups at glycation sites (17). Due to internal hemiacetal formation in pyridoxal but not pyridoxal phosphate (24), stronger inhibition of AGE formation by PLP is expected by this competitive mechanism. This indeed is observed **(Fig. 1B,1C, Fig. 3B,3C).** *The inhibition by pyridoxamine is necessarily different, as it lacks an aldehyde group.* It is, however, a candidate amine potentially capable of forming a Schiff-base linkage with the carbonyls of open-chain sugars, with dicarbonyl fragments, with Amadori products, or with post-Amadori intermediates. The mechanism of inhibition of B_1 compounds is not obvious. All the forms contain an amino functionality, so that the marked efficacy of only the pyrophosphate form suggests an important requirement for strong negative charge.

A significant unexpected observation is that the extent of inhibition by aminoguanidine, and some of the other compounds, is *considerably less at late stages of the reaction than during the initial phase.* One possible explanation is a steady-state Schiff-base sequestration by aminoguanidine of the open-chain aldehyde form of glucose (normally 0.002%), a possibility first suggested by Khatami et al. (17) and recently verified by Hirsch et al. (15). Our results indicate that *glycation assays that emphasize initial rates, even over two weeks under these conditions, may greatly overestimate the efficacy of inhibition.* While further studies are clearly required to elucidate the mechanisms of AGE inhibition by pyridoxamine and thiamine pyrophosphate *in vitro*, the possible differences in their mechanism of inhibition from that of aminoguanidine raises the intriguing possibility that the therapeutic potential of these inhibitors may be significantly enhanced by co-administration together with aminoguanidine.

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