Aminoguanidine Inhibits Advanced Glycation End Products Formation on β2-Microglobulin

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Abstract. Because advanced glycation end products (AGE)-modified β2-microglobulin (AGE-β2M) is a dominant constituent of amyloid in dialysis-related amyloidosis (DRA), AGE-β2M may be directly involved in the pathobiology of DRA. In experimental diabetes mellitus, blocking the formation of AGE prevents AGE-mediated tissue damage. In this study, it is postulated that similar pharmacologic intervention may be beneficial in DRA. Aminoguanidine, a nucleophilic hydrazine compound that prevents AGE formation on collagen, may have a similar effect on the advanced glycation of β2M. To test this hypothesis, β2M was incubated in vitro with 50 or 100 mM D-glucose for 3 wk in the presence and absence of incremental concentrations of aminoguanidine. On the basis of enzyme-linked immunosorbent assay and immunoblots using anti-AGE-keyhole limpet hemocyanin antibody, aminoguanidine inhibited glucose-induced Nε-(carboxymethyl)lysine formation on β2M. At aminoguanidine-glucose molar ratios of 1:8 to 1:1, 26 to 53% inhibition occurred. Fluorospectrometry examination showed that aminoguanidine also inhibited the formation of fluorescent AGE on β2M in a dose-dependent manner. At aminoguanidine-glucose molar ratios of 1:8 to 1:1, fluorescent product generation was inhibited by 30 to 70%. Furthermore, aminoguanidine suppressed the AGE formation on β2M bound to AGE-modified collagen. If aminoguanidine is similarly active in vivo, this compound may be of clinical utility for treating DRA in patients on maintenance dialysis. (J Am Soc Nephrol 9: 277–283, 1998)

Dialysis-related amyloidosis (DRA) is a progressive and often incapacitating complication for patients undergoing maintenance dialysis (1,2). Amyloid deposits composed of fibrils of β2-microglobulin (β2M) are localized mainly in osteoarticular tissue and may result in shoulder periarthritis, carpal tunnel syndrome, hand flexor tenosynovitis, destructive spondyloarthropathy, and subchondral bone cysts (1,2). The pathogenesis of DRA is incompletely understood; therefore, preventive measures can be proposed only on the basis of retrospective studies and hypothetical considerations. Two principal solutions have been recommended: enhancing the intradialytic removal of β2M and minimizing β2M synthesis associated with the use of bioincompatible dialysis membranes (3,4). The longitudinal clinical sequelae of these interventions are unknown.

Recent work has demonstrated that β2M isolated from dialysis-related amyloid tissue is modified to advanced glycation end products (AGE), and AGE-modified β2M (AGE-β2M) is a major component of amyloid fibrils (5). AGE-β2M may directly participate in the pathobiology of DRA by stimulating: (1) chemotaxis of monocytes; (2) synthesis of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6 from macrophages; and (3) production of collagenase in synovial cells (6,7). On the basis of studies of diabetic complications, the pathobiologic consequences of AGE can be prevented if the reactive intermediates of early glycosylation products are pharmacologically blocked (8). Because aminoguanidine, a nucleophilic hydrazine compound, inhibits AGE formation on collagens in vitro by suppressing the cross-linking of soluble plasma proteins to collagen and the cross-linking of collagen to itself (8,9), it has been advocated for in vivo use. In animal models of diabetes mellitus, aminoguanidine minimizes the development of glomerulopathy, vasculopathy, and neuropathy (10). Based on the suggested efficacy of aminoguanidine in diabetes mellitus, a disorder associated with AGE, it may be beneficial in other AGE-associated diseases, such as DRA. As an appropriate and necessary preliminary investigation before the administration of this compound as a therapeutic agent for DRA, we examined the in vitro effect of aminoguanidine on nonenzymatic glycosylation of β2M.

Materials and Methods
Preparation of β2M-AGE in Solution
AGE-modified β2M was prepared in vitro as described previously (5,11,12). Briefly, 1.75 mg/ml purified normal human β2M (Corter Biochem, San Leandro, CA) was incubated at 37°C for 3 wk with 50 or 100 mM D-glucose (Sigma, St. Louis, MO) in 100 mM phosphate buffer (PB) containing 200 U/ml penicillin, 70 μg/ml gentamicin, and...
1.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). Samples were also incubated under identical conditions in the presence of various concentrations of aminoguanidine hydrochloride (a gift of Mr. Zee M. Look, Alteon, Ramsey, NJ). Samples incubated in the absence of glucose with and without aminoguanidine were used as controls. After incubation, all samples were dialyzed against phosphate-buffered solution (PBS), pH 7.4, and lyophilized. The protein concentration was determined by the Bradford method (13) using bovine serum albumin (BSA) as a standard.

**Characterization of β2M-AGE in Solution**

**Enzyme-Linked Immunosorbent Assay.** An enzyme-linked immunosorbent assay (ELISA) was performed using a polyclonal anti-AGE-KLH antibody raised in rabbits immunized with keyhole limpet hemocyanin (KLH) (a gift of Dr. John W. Baynes, University of South Carolina, Columbia, SC) (14). This antibody specifically reacted with AGE but not with the early products of Maillard reaction. The major epitope structure recognized by the antibody has been characterized as N'- (carboxymethyl)lysine (CML) (14). This anti-AGE-KLH antibody has been used in ELISA assays to measure β2M and collagen AGE formed in vitro (12) and in human lens protein (15). The reactivity of each prepared sample to the anti-AGE-KLH antibody was examined using the ELISA Starter Kit (Pierce, Rockford, IL), as described previously (12,15). Each well of a 96-well microtiter plate was incubated for 12 h at 4°C with 0.1 ml of sample (concentration of β2M, 0.01 to 20 μg/ml) in coating buffer. The wells were washed three times with washing buffer containing 0.05% Tween 20 (buffer A) and blocked with 1% bovine serum albumin. After washing with buffer A, each well was reacted with 0.1 ml of anti-AGE-KLH at 1:2500 dilution for 60 min at room temperature. The wells were then washed with buffer A, incubated for 60 min at room temperature with 0.1 ml of goat anti-rabbit IgG peroxidase at 1:5000 dilution (Pierce), and developed by the addition of the substrate 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid. The absorbance at 405 nm was measured on a micro-ELISA plate reader (Titertek Multiskan, Mcc/340). Control studies were performed using rabbit antihuman IgG (Boehringer Mannheim, Indianapolis, IN) instead of rabbit anti-AGE-KLH antibody and coating the plates with unmodified β2M instead of AGE-β2M.

In separate experiments, to evaluate the reactivity of reduced samples to the anti-AGE-KLH, 200 μg of AGE-β2M was incubated with 5 mM sodium borohydride (NaBH₄) in 1 ml of 0.2 M PB, pH 8.5, at room temperature for 4 h (16). The samples were dialyzed against 50 mM acetic acid to destroy excess NaBH₄ followed by dialysis against PBS, pH 7.4. ELISA was performed as described.

**Immunoblotting.** Immunoblots were performed as described (5). After incubation as described above, the samples were run on a 16% Tris-glycine gel (Novex, San Diego, CA). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was electrophoretically transblotted to a nitrocellulose membrane. The membranes were soaked in blocking solution and reacted with rabbit anti-AGE-KLH antibody at 1:3000 dilution for 60 min. After washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG at 1:3000 dilution (blotting grade, Bio-Rad, Hercules, CA), followed by the addition of enhanced chemiluminescence solution (Amersham Life Science, Arlington Heights, IL). The membranes were exposed to Kodak X-Omat films, which were developed by an automatic X-Omat processor (Kodak M35A).

**Fluorospectrometry.** The fluorescence spectra of the samples were measured at a protein concentration of 0.26 mg/ml in a fluorescence spectrometer (Aminco Bowman Series 2), as described previously (5). Fluorescence excitation and emission maxima were 360 and 430 nm, respectively, which is characteristic of AGE proteins (5).

**Preparation of AGE-Modified Collagen**

AGE-modified type I collagen (collagen-AGE) was prepared in vitro as described (11,12). Briefly, 0.45 mg/ml purified type I collagen (calf skin, Sigma) was incubated at 37°C for 30 d with 100 mM β-glucose in 100 mM PB containing 200 U/ml penicillin, 70 μg/ml gentamicin, and 1.5 mM PMSF. After incubation, the sample was dialyzed against PBS, pH 7.4. The prepared collagen-AGE was characterized by fluorospectrometry and ELISA using anti-AGE-KLH (12). AGE content quantified by fluorospectrometry was 36.9 U/mg of protein.

**Characterization of AGE Formation of β2M Bound to AGE-Collagen**

The buffers and substrate used in these experiments were obtained from the ELISA Starter Kit as described above. Under sterile conditions, all solutions were filtered through a 0.22-μm pore filter (Sarstedt, Newton, NC). Type I collagen-AGE was solubilized in 0.05N acetic acid and adjusted to 10 μg/ml using a coating buffer. The AGE-collagen solution (1 μg of protein/well) was aliquotted into 96-well polystyrene microplates and incubated for 12 h at 4°C. The solution was discarded, and the wells were rinsed three times with 100 μl of wash buffer. One hundred microliters of β-glucose solution (final concentration of glucose, 100 mM in 100 mM PB) with or without β2M (75 μg/ml) was then added and incubated at 37°C for 3 wk in the presence or absence of 100 mM aminoguanidine. The incubation solution also contained 200 U/ml penicillin, 70 μg/ml gentamicin, and 1.5 mM PMSF. Every 7 d, the incubation solution was changed by aspiration of the original incubation solution and its replacement with fresh solution containing the original concentration of β2M, glucose, and aminoguanidine. After this, the wells were rinsed three times with wash buffer. As a control, immobilized AGE-collagen (1 μg/well) was incubated with the same buffer without β-glucose, both in the presence and absence of β2M (75 μg/ml) and aminoguanidine (100 mM).

To quantify AGE formation, 100 μl of rabbit anti-AGE-KLH (dilution 1:2500) was added and incubated at room temperature for 1 h. Each well was rinsed three times, and 100 μl of goat-anti rabbit IgG peroxidase at 1:5000 dilution (Pierce) was added. After incubation at room temperature for 60 min, the wells were washed and reacted with 100 μl of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid at room temperature for 30 min. The absorbance at 405 nm was then measured using an ELISA reader as described above.

To confirm the binding of β2M to immobilized AGE-collagen, the microplates were incubated with affinity-purified sheep antihuman β2M antibody at 1:2500 dilution (BiosPacific, Emeryville, CA) and developed by the addition of rabbit anti-sheep IgG peroxidase at 1:5000 dilution (Pierce) (12). Control studies were performed using rabbit antihuman IgG (dilution 1:2500) instead of rabbit anti-AGE-KLH or sheep antihuman β2M antibody.

**Statistical Analyses**

All experiments were performed in triplicate. Continuous variables, expressed as mean ± SD, were compared using ANOVA. Multiplicative terms were included to evaluate for interaction among experimental variables. The Student–Newman–Keuls procedure was used to evaluate pairwise comparisons. Two-tailed P values <0.05 were considered statistically significant. Statistical analyses were conducted with SAS (The SAS Institute, Cary, NC).
Results

Effect of Aminoguanidine on AGE Modification of β2M in Solution

On the basis of ELISA, β2M modified by incubation with d-glucose reacted with anti-AGE-KLH (Figure 1), suggesting the formation of AGE-β2M. When β2M was incubated with equimolar concentrations of d-glucose and aminoguanidine, immunoreactivity of the product was significantly less. β2M incubated in the absence of glucose with or without aminoguanidine was not recognized by the anti-AGE-KLH (data not shown). The interaction of β2M incubated with glucose to the anti-AGE-KLH did not reflect nonspecific binding, because unmodified β2M did not react with anti-AGE-KLH. An irrelevant polyclonal antibody (antihuman IgG) did not bind to AGE-β2M (data not shown).

To determine whether early products of the Maillard reaction were recognized in the ELISA, β2M incubated with d-glucose was treated with NaBH4. Early products of the Maillard reaction are reduced by >90% by this treatment (17), but CML and AGE are not (14,17,18). As shown in Figure 1, the immunoreactivity to the anti-AGE-KLH antibody was not altered significantly by treatment with NaBH4, indicating that early products of the Maillard reaction are not recognized by the antibody. Because CML is the principal AGE antigen recognized by anti-AGE-KLH (14), these data suggest that glucose incubation of β2M resulted in its modification to CML-containing AGE, and this process was attenuated by aminoguanidine.

To further support the inhibitory effect of aminoguanidine on formation of AGE on β2M, a dose–response relationship of aminoguanidine on AGE modification was established by ELISA, using anti-AGE-KLH. The quantity of immunoreactive AGE-β2M formed was dependent on the concentration of aminoguanidine in the incubation solution (Figure 2A). The percentage inhibition induced by aminoguanidine was a func-
tion of the molar ratio of aminoguanidine and glucose (Figure 2B). Glucose-derived AGE formation on β2M was inhibited from 26 to 53% at aminoguanidine-glucose molar ratios of 1:8 to 1:1.

Figure 3 is the photograph of a representative immunoblot, using anti-AGE-KLH antibody. The incubation of glucose and β2M resulted in the formation of an immunoreactive product of the appropriate size for β2M monomer (M<sub>r</sub> 11,700) and dimer (M<sub>r</sub> 23,000) (lane 2). The formation of AGE-β2M product was suppressed by aminoguanidine (lane 1). Treating the samples with NaBH₄ did not alter the immunoblot results (data not shown).

MW

29,000 - 18,400 - 14,300 -

β2M + glucose + aminoguanidine

β2M + glucose

β2M alone

Because of the presence of fluorescent AGE in β2M amyloid in DRA (5), and the observation that CML does not fluoresce (14), we used fluorescence spectrometry to determine whether aminoguanidine also affects the formation of fluorescent AGE on β2M. β2M incubated with D-glucose alone showed typical excitation and emission fluorescence spectra, with a maximum emission intensity at 430 nm upon excitation at 360 nm (data not shown). Incubation of β2M and glucose with aminoguanidine inhibited the emission intensity in a dose-dependent manner (Figure 4). Glucose-derived fluorescence was inhibited from 30 to 70% at aminoguanidine-glucose molar ratios of 1:8 to 1:1, respectively. β2M incubated in the absence of glucose with or without aminoguanidine did not fluoresce.

Effect of Aminoguanidine on AGE Modification of β2M Bound to AGE-Collagen

Previously, we demonstrated that β2M preferentially binds to immobilized type I collagen-AGE in a dose-dependent manner (12). The binding of β2M to AGE-collagen-coated microplates was unaffected by glucose and/or aminoguanidine at 100 mM each (data not shown). The wells incubated without β2M were not recognized by the anti-β2M antibody (data not shown). Incubation of AGE-collagen-coated wells with 100 mM D-glucose increased their immunoreactivity to anti-AGE compared with wells incubated without glucose (Figure 5). This finding suggests that immobilized AGE-collagen was further AGE-modified. The addition of β2M to the wells incubated with glucose resulted in even more AGE formation, indicating that β2M bound to AGE-collagen subsequently underwent AGE formation. The inclusion of 100 mM aminoguanidine: Glucose (Molar Ratio)

Figure 4. The effect of aminoguanidine on the formation of fluorescent AGE-β2M. β2M was incubated for 3 wk with 100 mM (open bars) or 50 mM (filled bars) D-glucose in the presence of incremental concentrations of aminoguanidine. Formation of AGE-β2M was quantified by fluorospectrometry. Data from three independent experiments were expressed as mean ± SD. ANOVA, P < 0.0001; aminoguanidine and glucose molar ratio, P < 0.0001.
Aminoguanidine inhibited AGE modification of β2M bound to AGE-collagen. Therefore, aminoguanidine to the glucose-containing incubation solution reduced AGE formation to near baseline (Figure 5). Therefore, aminoguanidine to the glucose-containing incubation solution reduced AGE formation to near baseline (Figure 5). Therefore, aminoguanidine inhibited AGE modification of β2M bound to collagen. Because the immunoreactivity to anti-β2M in wells incubated with β2M was unchanged in the presence of aminoguanidine, the inhibitory effect of aminoguanidine was not a consequence of a nonspecific effect that decreased binding of β2M to collagen. When antihuman IgG was used instead of anti-AGE-KLH as the primary antibody, no immunoreactivity was seen.

Discussion

The end products of nonenzymatic protein glycosylation may contribute to the development of chronic diabetic complications, as well as certain physical stigmata of aging (8,10). Protein amino groups add to glucose by nucleophilic addition to form a chemically reversible Schiff base product, which subsequently rearranges to the more stable but still chemically reversible Amadori compound. Amadori products slowly undergo a series of reactions with free amino groups, leading to cross-linking, browning, and fluorescence of the proteins via the formation of AGE in the late stage of the Maillard reaction (19–21). The chemical progression from the Amadori product to stable AGE requires the formation of “reactive intermediates” capable of attaching to a second amino group present on either the same or a neighboring polypeptide chain (10). Because aminoguanidine is chemically more reactive than the epsilon-amino group of lysine in proteins, it has been hypothesized to be able to compete to form unreactive substituted early glycosylation products rather than AGE (8). Alternatively, aminoguanidine may act by trapping protein-bound or free dicarbonyl intermediates, which are involved in the formation of AGE (22). A number of studies have demonstrated that aminoguanidine markedly reduces the glucose-induced AGE formation of proteins such as collagen and albumin (9), and prevents AGE-mediated tissue damage in vivo in experimental diabetes mellitus (23–26).

Because AGE products on β2M have been hypothesized to be of pathobiologic significance in DRA, we investigated the capacity of aminoguanidine to block AGE-β2M formation in vitro. Immunochemical analysis and fluorescence examination demonstrated that aminoguanidine attenuates AGE formation on β2M in solution. These may seem trivial findings based on the demonstration of aminoguanidine’s effects on other AGE-modified proteins. Because AGE are heterogeneous in structure and form under distinct conditions, it is necessary to determine the efficacy of inhibitors of AGE formation in the individual situation of the affected macromolecules. This study examined the effect of aminoguanidine on the formation of AGE-β2M, a major constituent in amyloid fibrils and present both in diabetic and nondiabetic dialysis patients.

It was recently demonstrated that the amyloid β2M extracted from DRA tissue is modified with CML, indicating that CML is one of the native structures in AGE-β2M (27). Because the major epitope recognized by the anti-AGE antibody used for these experiments is CML, our results suggest that the appropriate AGE epitope was formed in vitro. Therefore, aminoguanidine inhibits CML formation on β2M. CML was originally detected in vitro as a product of oxidative cleavage of glucose-derived Amadori compounds (28). However, CML has also been detected in vivo in lens proteins (29) and skin collagen (30) as the result of aging. Age-adjusted levels of CML in skin collagen are increased in diabetic patients, particularly those with diabetic complications (31,32). Therefore, the descriptor AGE is now applied to a wide range of advanced products of the Maillard reaction, which have been detected in tissue proteins, including CML and pyrroline. These compounds are colorless, do not fluoresce, and are not protein cross-linked (14,27).

AGE-β2M isolated from amyloid fibrils of DRA is a mixture of heterogeneous molecular adducts that includes fluorescent cross-linked molecules (16). Among the glycated β2M products, pigmented and fluorescent AGE-β2M exhibit biological activity with monocytes/macrophages (16). Thus, inhibition of the formation of the fluorescent β2M-AGE may be clinically important. Our results demonstrated that aminoguanidine also inhibits the formation of fluorescent AGE on β2M in a dose-dependent manner. Glucose-derived fluorescence was diminished by 30% to 70% at aminoguanidine-glucose molar ratios of 1:8 to 1:1, respectively.

The implications of β2M binding to AGE-collagen may be relevant to the recruitment of β2M amyloid in DRA (12). Recently, we reported that β2M binds to AGE-modified collagen to a much greater extent than to unmodified collagen, and this incorporated β2M is more readily modified to AGE-β2M. Immunohistological study also demonstrated that β2M skin deposits are associated with collagen fibers (33). There-
fore, we postulate that AGE-β2M in amyloid may be formed in situ as a subsequent event of binding to AGE-collagen (12). This finding may account for the predisposition of older dialysis patients to this disorder (1). In the present study, we demonstrated that aminoguanidine also suppressed the AGE formation on β2M bound to AGE-collagen. These data suggest that there may be two potential points in the development of β2M amyloidosis in which aminoguanidine could interfere. The first would be to attenuate AGE modification of collagen, which then predisposes to β2M deposition. The second would be to interfere with the subsequent AGE modification on β2M after it has bound to AGE-modified collagen. This would prevent its cross-linking to itself and other proteins. Because of the important role of β2M in the pathogenesis of DRA, preventing AGE modification on β2M may have a potential therapeutic role for DRA.

Other than renal transplantation, strategies to prevent and/or treat DRA have been only modestly successful. The administration of nonsteroidal anti-inflammatory drugs may result in short-term relief of arthralgias and other symptoms, but their long-term efficacy is poor (2). The use of low-dose oral steroid treatment has been recommended, but data on their long-term effectiveness and steroid-related side effects are lacking (2). Renal transplantation has been shown to arrest the progression of DRA (34–36). However, because transplantation is not feasible for most patients with end-stage renal disease, especially those likely to get DRA, alternative strategies to prevent and treat DRA must be explored. The data presented here suggest that the in vivo use of aminoguanidine may be one of these strategies, and should be the basis of an intervention trial.

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