Pergamon

Biochemical Pharmacology, Vol. 48, No. 10, pp. 1865–1870, 1994.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/94 $7.00 + 0.00

0006-2952(94)00379-3

THE REACTION OF METHYLGLYOXAL WITH AMINOGUANIDINE UNDER PHYSIOLOGICAL CONDITIONS AND PREVENTION OF METHYLGLYOXAL BINDING TO PLASMA PROTEINS

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(*Received* 18 *February* 1994; *accepted* 17 *August* 1994)

Abstract—Increased formation of methylglyoxal in clinical diabetes mellitus and metabolism by the glyoxalase system has been linked to the development of clinical complications of diabetes: retinopathy, neuropathy and nephropathy. Aminoguanidine has been proposed as a prophylactic agent for preventive therapy of diabetic complications. Methylglyoxal reacted with aminoguanidine under physiological conditions to form two isomeric triazines, 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine. The mean second order rate constant for the reaction of methylglyoxal with aminoguanidine, $k_{MG,AG} = 0.39 \pm 0.06\ M^{-1}\,sec^{-1}$ at pH 7.4 and 37°. Under these conditions, no methylglyoxal bisguanylhydrazone was detected. Aminoguanidine prevented the irreversible modification of human plasma protein by a physiological concentration of methylglyoxal (1 μM); the median inhibitory concentration IC_{50} value of aminoguanidine was 203 ± 16 μM (N = 28). The scavenging of methylglyoxal by aminoguanidine may contribute to the beneficial effects of aminoguanidine in the prevention of vascular pathogenesis in diabetes.

Key words: methylglyoxal; aminoguanidine; triazine; diabetic complications; glyoxalase; L-arginine

The metabolism of methylglyoxal by the glyoxalase system [1] has been linked to the development of clinical complications (retinopathy, neuropathy, nephropathy) associated with diabetes mellitus [2, 3]. In both patient groups with IDDM† and NIDDM, the activity of glyoxalase I in red blood cells was significantly higher in patients with complications (retinopathy, neuropathy and/or nephropathy), relative to the duration of disease-matched uncomplicated patients [3]. The concentration of methylglyoxal in blood samples was increased 6-fold from IDDM patients and 2–3-fold in NIDDM patients, relative to normal controls [3]. The concentration of methylglyoxal was also increased in the lens, kidney and blood samples from streptozotocin-induced diabetic rats, relative to normal controls [4]. Diabetic patients are chronically exposed to increased systemic concentrations of methylglyoxal and this may induce increased glyoxalase activities in red blood cells; a precedent for this is the induction of glyoxalase I activity in red blood cells of rats exposed to the non-physiological glyoxalase I substrate, glyoxal [5]. A causal link between methylglyoxal, glyoxalase I and the development of diabetic complications may involve the binding and modification of protein by methylglyoxal [3].

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† Abbreviations: IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.

The modification of protein by glucose in diabetes mellitus to form advanced glycation end products and prevention by aminoguanidine has been well-considered with respect to the development and therapy of diabetic complications [6]. In contrast, the involvement of the α-oxoaldehyde metabolite methylglyoxal and scavenging of methylglyoxal by aminoguanidine has not. Methylglyoxal reacts with cysteine, lysine and arginine residues in proteins [7, 8]. Initially, reversible hemithioacetal and glycosylamine adducts are formed but thereafter with arginine residues there is slow irreversible reaction which forms an imidazolone derivative, N_δ-(3,4-dihydro-4-methylimidazol-5-on-2-yl)-2,5-diaminopentanoic acid [9]. Derivatization of proteins with methylglyoxal produced a characteristic fluorescence [10] and protein crosslinking [11]. Binding and modification of protein by methylglyoxal is normally associated with protein turnover and age-associated changes in extracellular matrix proteins. However, chronic exposure to abnormally high concentrations of methylglyoxal may induce pathological changes in protein structure and cell function. Compounds that scavenge methylglyoxal under physiological conditions may find therapeutic application, providing they or their methylglyoxal adducts are not toxic. One such scavenger is aminoguanidine.

The reaction of methylglyoxal with excess aminoguanidine under acidic conditions has been used to produce methylglyoxal bisguanylhydrazone [12], a clinical anti-tumour agent [13]. However, studies of the reaction of glyoxal derivatives

Table 1. NMR spectra of 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine

3-Amino-5-methyl-1,2,4-triazine		3-Amino-6-methyl-1,2,4-triazine	
Assignment	δ_H (ppm)	Assignment	δ_H (ppm)
	Proton NMR spectroscopy		
5-Methyl	2.25 (3H)	5-H	8.02 (1H)
6-H	8.42 (1H)	6-Methyl	2.39 (3H)
3-Amino	5.71 (2H)	3-Amino	5.31 (2H)
	Carbon-13 NMR spectroscopy		
	δ_C (ppm)		δ_C (ppm)
3-C	162	3-C	163
5-C	136	5-C	150
6-C	152	6-C	143
5-Methyl	22	6-Methyl	18

with aminoguanidine at neutral pH and ambient temperature suggest that 5- and 6-substituted 3-amino-1,2,4-triazine derivatives are formed [14, 15]. In this report, we describe the reaction of methylglyoxal with aminoguanidine under physiological conditions produces two isomeric triazines, 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine. Aminoguanidine prevented the irreversible modification of human plasma protein by methylglyoxal under physiological conditions.

MATERIALS AND METHODS

Materials. Reduced glutathione, glyoxalase I (from yeast), glyoxalase II (from bovine liver), ethylenediaminetetra-acetic acid (tetra sodium salt), and methylglyoxal bisguanylhydrazone were purchased from the Sigma Chemical Co. Ltd (Poole, U.K.). Aminoguanidine bicarbonate and 3-amino-1,4,5-triazine were purchased from the Aldrich Chemical Co. Ltd (Poole, U.K.). Methylglyoxal was synthesized from methylglyoxal dimethylacetal as described [16], and assayed by endpoint enzymatic assay with reduced glutathione, glyoxalase I and glyoxalase II [17]. [^{14}C]Methylglyoxal was synthesized from [^{14}C]acetone and had a specific activity of 1 mCi/mmol [18].

3-Amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine were synthesized by reacting methylglyoxal with aminoguanidine [14]. The triazines were characterized by ^{1}H and ^{13}C NMR spectroscopy (Table 1), mass spectrometry, UV spectrophotometry and TLC. Mass spectrometric analysis gave molecular ions of m/z values = 111, consistent with the triazine products. The isomeric triazines had similar absorption spectra [14] with absorbance peaks λ_{max} at 320 nm and extinction coefficients which were not significantly different. The mean extinction coefficient for 5-methyl and 6-methyl- derivatives of 3-amino-1,2,4-triazine ε_{320} in 10 mM sodium phosphate, pH 7.4 and 37°, was 2411 ± 9 M/cm (N = 11; band width 1 nm). TLC was performed on silica gel F_{254} with a mobile phase of ethanol:acetic acid:water, 10:5:5 (6 g vol.). 5-Methyl-3-amino-1,2,4-triazine and 6-Methyl-3-amino-1,2,4-triazine had R_f value of 0.58.

Human plasma was isolated from blood samples from normal healthy human subjects with consent. Blood was drawn by venepuncture into tubes containing sodium EDTA anticoagulant. Blood cells were sedimented by centrifugation (2000 *g*, 10 min), the plasma removed and used in experiments to study the binding of [^{14}C]methylglyoxal to plasma protein under physiological conditions. Ultrafiltration was performed with CF25 Centriflo membrane cones (nominal molecular mass cut-off 25,000 Da; Amicon Ltd, Stonehouse, U.K.). Scintillation cocktail (Ultima gold) was purchased from Packard (Pangbourne, U.K.).

The rate of reaction of aminoguanidine with methylglyoxal. The reaction of methylglyoxal with aminoguanidine was investigated by UV spectrophotometry and product analysis by ion-pair reversed-phase HPLC [19]. HPLC was performed with a Waters HPLC system (2 × 510 pumps, a Lambda Max 481 LC spectrophotometer with a 680 automated gradient controller). The column was a Nova-Pak octadecylsilica (ODS) 4 μm (0.8 × 10 cm) cartridge fitted with a precolumn in a 8 × 10 radial compression unit. The mobile phase was 50 mM sodium phosphate, pH 7.4, containing 1 mM sodium hexylsulphate and 15% methanol and the flow rate 2 mL/min. The eluate was monitored by flow spectrophotometry at 280 nm to detect the internal standard (3-amino-1,2,4-triazine), 3-amino-5-methyl-1,2,4-triazine, 3-amino-6-methyl-1,2,4-triazine and methylglyoxal bisguanylhydrazone; detection at 210 nm was used to investigate the formation of other intermediates and products. Under these conditions, methylglyoxal bisguanylhydrazone was resolved from the triazine products but the triazine isomers were not resolved (if the methanol was omitted from the mobile phase, retention times were increased but the triazine isomers were only partially resolved). HPLC analysis therefore gave measurements of 5-methyl- and 6-methyl derivatives of 3-amino-1,2,4-triazine (both isomers) and methylglyoxal bisguanylhydrazone; the proportion of each triazine isomer formed was determined from integration of ^{1}H NMR spectra of the reaction mixtures. The concentration of methylglyoxal bisguanylhydrazone and triazine products were determined from calibrated analyte/internal standard integral ratios. The limits of detection of isomeric triazines and methylglyoxal bisguanyl-hydrazone were 6.94 and 19.4 pmol, respectively.

Methylglyoxal (0.89 mM) was reacted with aminoguanidine (1.0 mM) in 50 mM sodium phosphate buffer, pH 7.4 and 37°. Aliquots (100 μL) were withdrawn at the times indicated (Fig. 1), and further reaction progress slowed by 10-fold dilution in mobile phase. The internal standard, 3-amino-1,2,4-triazine (0.3 mM in mobile phase; 100 μL) was added. This was analysed immediately by HPLC, as described above. The injection volume was 100 μL.

The rate of formation of the isomeric triazines from the reaction of aminoguanidine with methylglyoxal in sodium phosphate, pH 7.4 and 37°, was determined

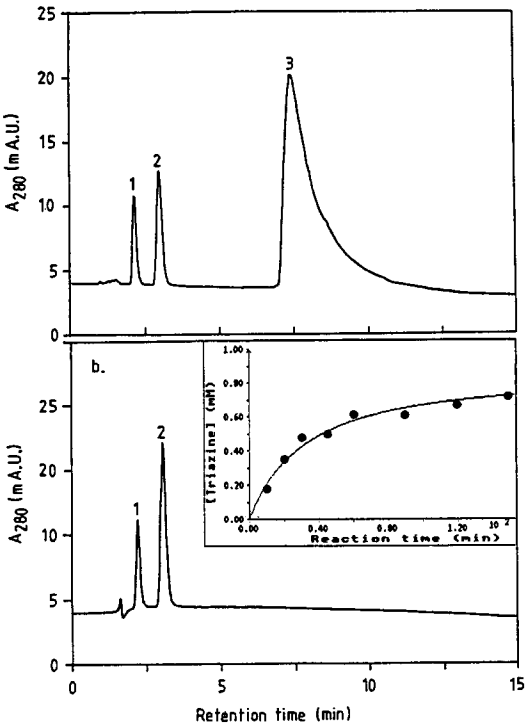

Fig. 1. Reaction of methylglyoxal with aminoguanidine studied by HPLC. (Top panel) Standard chromatogram: 30 μM 3-amino-1,2,4-triazine—internal standard (peak 1), 50 μM methyl triazines—3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine (peak 2), and 25 μM methylglyoxal bisguanylhydrazone (peak 3); injection volume 100 μL. (Bottom panel) Reaction mixture: methylglyoxal (0.89 mM) and aminoguanidine (1.00 mM) in 50 mM sodium phosphate buffer, pH 7.4 and 37°. Aliquots (100 μL) were withdrawn at the times indicated and analysed by HPLC as described in the Materials and Methods. (Inset) Kinetic analysis (see Materials and Methods) gave $k_{MG,AG} = 0.39 \pm 0.06\ M^{-1}\ sec^{-1}$ (N = 9) at pH 7.4 and 37°.

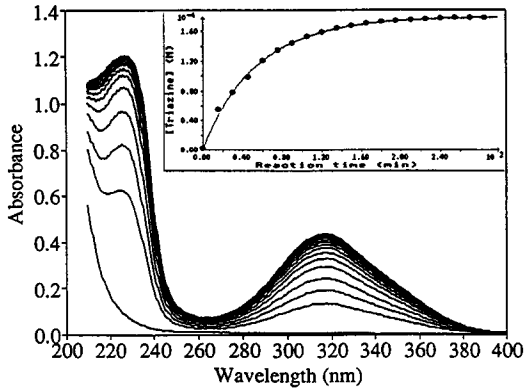

Fig. 2. Reaction of methylglyoxal with aminoguanidine studied by UV spectrophotometry. Reaction mixture: methylglyoxal (180 μM) and aminoguanidine (1.00 mM) in 50 mM sodium phosphate buffer, pH 7.4 and 37°. Spectra were recorded at 15 min intervals. Inset: kinetic analysis (see Materials and Methods) gave $k_{MG,AG} = 0.34 \pm 0.01\ M^{-1}\ sec^{-1}$ (N = 20) pH 7.4 and 37°.

by measuring the increase in absorbance at 320 nm. The rate of reaction was investigated for reaction mixtures containing initial concentrations of 45–890 μM methylglyoxal and 21–1045 μM aminoguanidine.

The reaction of methylglyoxal with plasma protein and the effect of aminoguanidine. Human blood plasma (500 μL) was incubated with [^{14}C]methylglyoxal (101 μM, 5 μL; final concentration 1 μM), in the absence and presence of aminoguanidine (1 μM–1 mM) in sodium phosphate buffer (10 mM, pH 7.4 at 37°, 5 μL), under aseptic conditions at 37° for 21 days. The sample was then diluted with 3 mL of phosphate buffer and the plasma proteins isolated by ultrafiltration. Ultrafiltration was performed by centrifugation (1600 *g*, 30 min, room temperature) of samples through membrane cones. The membrane was washed with phosphate buffer (10 mM, pH 7.4; 3 mL) by ultrafiltration a further four times. The protein was then washed from the membrane with 1 mL of 0.3 M sodium hydroxide solution containing 1% (w/v) sodium dodecyl sulphate. The membrane and wash were mixed with scintillation cocktail (15 mL) in a scintillation vial and counted (10 min).

Data analysis. Data of the formation of triazine products from methylglyoxal and aminoguanidine, determined by spectrophotometric and HPLC analysis, were fitted to a second order integrated rate equation to determine the rate constant for the reaction of methylglyoxal with aminoguanidine $k_{MG,AG}$. Data from the binding of methylglyoxal to plasma protein were fitted to the logistical equation

$$[\text{MG-Prot}] = [\text{MG-Prot}]_{max} \cdot IC_{50}{}^{n} / (IC_{50}{}^{n} + [\text{Scavenger}]^{n})$$

where [MG-Prot] and $[\text{MG-Prot}]_{max}$ are the binding of methylglyoxal to protein in the presence and absence of scavenger (aminoguanidine or L-arginine), respectively, IC_{50} is the median inhibitory concentration of aminoguanidine and n the logistical regression coefficient. Limits of detection of isomeric 5/6-methyl-3-amino-1,2,4-triazines and methylglyoxal bisguanylhydrazone by HPLC analysis were determined as the concentration of analyte equivalent to three standard deviations on the zero control on the peak integral ratio-analyte concentration calibration curves. Linear and non-linear regression was performed using the ENZFITTER programme (BIOSOFT, Cambridge, U.K.).

RESULTS

The reaction of methylglyoxal with aminoguanidine

When methylglyoxal (0.89 mM) reacted with aminoguanidine (1 mM) in 50 mM sodium phosphate buffer, pH 7.4 and 37°, the products formed were two isomeric triazines, 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine—as evidenced by ^{1}H and ^{13}C NMR spectroscopy (Table 1);

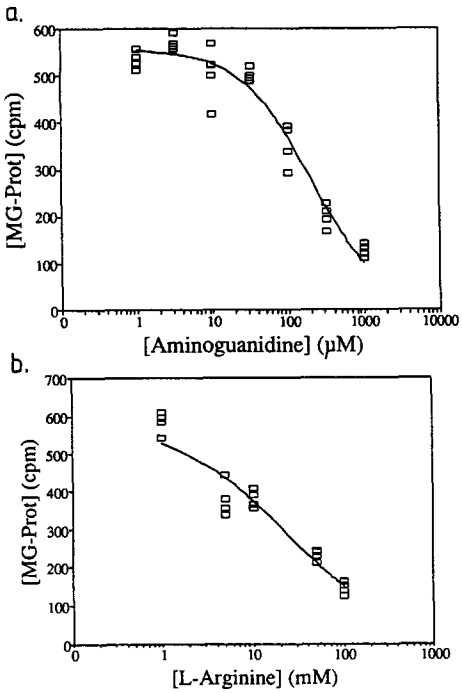

Fig. 3. Binding of methylglyoxal to plasma proteins. (Panel a) Inhibition of methylglyoxal modification of protein by aminoguanidine: the IC_{50} value was 203 ± 16 μM and the logistical regression coefficient value was 0.95 ± 0.07 (N = 28). (Panel b) Inhibition of methylglyoxal modification of protein by L-arginine: the IC_{50} value was 23 ± 3 μM and the logistical regression coefficient value was 0.72 ± 0.07 (N = 20). The total count value for [^{14}C]methylglyoxal 894 ± 4 cpm (N = 4) and the maximum count value of MG-Prot (when [scavenger] = 0) was 557 ± 20 (N = 3).

^{13}C chemical shift values were similar to those previously reported [20]. HPLC product analysis confirmed the formation of isomeric triazines and the absence of methylglyoxal bisguanylhydrazone (<190 nM or <0.019% of products) and other products (Fig. 1). This was confirmed by the change in the absorption spectrum during the reaction progress (Fig. 2) showing the characteristic development of the spectrum of the isomeric triazines. The product distribution was 56% 3-amino-5-methyl-1,2,4-triazine and 44% 3-amino-6-methyl-1,2,4-triazine at pH 7.4 and 37°.

When data from HPLC product analysis and spectrophotometric analysis were fitted to a second order integrated rate equation, the rate constant $k_{MG,AG}$ values for the reaction of methylglyoxal with aminoguanidine were 0.39 ± 0.06 M^{-1} sec^{-1} (HPLC) and 0.34 ± 0.01 M^{-1} sec^{-1} (spectrophotometric) at pH 7.4 and 37°. The $k_{MG,AG}$ value was independent of phosphate buffer concentration and ionic strength.

The effect of aminoguanidine on the modification of plasma proteins by methylglyoxal in vitro

When [^{14}C]methylglyoxal (1 μM) was incubated with blood plasma for 3 weeks under aseptic conditions at 37°, approximately 62% of the methylglyoxal added was irreversibly bound to the plasma protein (Fig. 3, panel a). Incubation with aminoguanidine (1 μM–1 mM) prevented the modification of the plasma proteins by methylglyoxal. The median inhibitory concentration IC_{50} value was 203 ± 16 μM and the logistical regression coefficient value was 0.95 ± 0.07 (N = 28). 3-Amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine were not bound to the protein after washing by ultrafiltration under these conditions, as evidenced by the lack of binding of 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine (preformed from [^{14}C]methylglyoxal and aminoguanidine) to protein.

A similar study with L-arginine (1–100 mM) gave a median inhibitory concentration IC_{50} value of 23 ± 3 mM and a logistical regression coefficient value of 0.72 ± 0.07 (N = 20) (Fig. 3, panel b).

DISCUSSION

The initial stage of the reaction of methylglyoxal with aminoguanidine involves the reaction of the aldehyde group of the reactive unhydrated form of methylglyoxal with aminoguanidine, with subsequent cyclization to the triazine. The formation of approximately equal amounts of the 5-methyl and 6-methyl 3-amino-1,2,4-triazine and the logistical regression coefficient value for inhibition of methylglyoxal binding to protein of approximately 1.0 are consistent with reaction of methylglyoxal occurring at the hydrazino and guanidino moieties of aminoguanidine with similar rates. The logistical regression coefficient value for the inhibition of methylglyoxal binding to plasma protein by L-arginine significantly less than 1.0 is consistent with methylglyoxal reacting with two sites on arginine of different reactivity i.e. the α-amino group and the guanidino group. Aminoguanidine was approximately 100-fold more effective than L-arginine in the prevention of irreversible modification of plasma proteins by methylglyoxal. Aminoguanidine is expected to reverse the increase in the concentration of methylglyoxal in cells and tissues in diabetic patients during hyperglycaemia. High dose arginine therapy may have a similar effect.

Aminoguanidine (50 mg/kg/day) inhibited the development of fluorescence associated with advanced glycation end products in renal basement membrane [21] and prevented diabetes-induced increase in mesangial volume of renal glomeruli in streptozotocin-induced diabetic rats [22]. It also reversed ischaemia [23], improved electrophysiology of caudal and sciatic nerves [24], prevented the development of acellular capillaries in the retina of diabetic rats [25], and prevented protein cross-linking in the aortal wall of diabetic rats [26] and stabilization of rat tail collagen [27, 28]. These effects of aminoguanidine have been attributed to the prevention of the formation of advanced glycation end products, particularly by reaction with 3-deoxyglucosone [29] which forms the triazine derivatives 3-amino-5-(2,3,4-trihydroxybutan-1-yl)-1,2,4-triazine and 3-amino-6-(2,3,4-trihydroxybutan-1-yl)-1,2,4-triazine [30, 31]. However, the prevention of protein modification by methyl-

glyoxal should also be considered since the reaction of methylglyoxal with proteins forms compounds with similar fluorescence characteristics to those of advanced glycation end products [9, 10], and the reaction of methylglyoxal with aminoguanidine forms analogous triazines, 3-amino-5-methyl-1,2,4-triazine and 3-amino-6-methyl-1,2,4-triazine. Scavenging of methylglyoxal by high dose L-arginine may also contribute to the inhibition of collagen crosslinking in diabetes rats and diabetic patients by L-arginine [32, 33].

The maximum concentration of aminoguanidine expected to be achieved in plasma *in vivo* at doses producing pharmacologic effects associated with the prevention of diabetic complications (50 mg/kg of aminoguanidine bicarbonate) [21–28] is *ca.* 669 μM, given distribution throughout the total body water volume. This exceeds the IC_{50} value 203 μM for the prevention of methylglyoxal modification of plasma proteins and, therefore, aminoguanidine is expected to be a competent scavenger of methylglyoxal at the therapeutic dose *in vivo*. In contrast, L-arginine is expected to be a less competent scavenger of methylglyoxal *in vivo*.

The recently reported [3] increased concentration of methylglyoxal in blood samples of diabetic patients (in the range 60 nM–2.4 μM) and correlation of methylglyoxal metabolism with the development of diabetic complications suggest that pharmacological intervention to prevent increased methylglyoxal concentration, and thereby prevent irreversible protein modification by methylglyoxal, may be an effective strategy for prophylactic therapy of diabetic complications. Moreover, in IDDM patients there was a positive correlation of the concentration of methylglyoxal in blood samples with duration of diabetes; therefore, without such intervention, the methylglyoxal concentration progressively increases further and the risk of irreversible modification of protein becomes greater.

Acknowledgements—The authors thank the Medical Research Council for support for our research programme. This is a contribution from the Glyoxalase Research Group at the University of Essex.

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