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Reaction of Metformin with Dicarbonyl Compounds. Possible Implication in the Inhibition of Advanced Glycation End Product Formation

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ABSTRACT. Dicarbonyl compounds such as methylglyoxal and glyoxal are extremely reactive glycating agents involved in the formation of advanced glycation end products (AGEs), which in turn are associated with diabetic vascular complications. Guanidino compounds such as aminoguanidine appear to inhibit AGE formation by reacting with α-dicarbonyl compounds. The aim of this work was to study whether the antihyperglycemic agent metformin (a guanidine-like compound) might react with reactive α-dicarbonyls. Metformin was incubated at pH 7.4 and 37° in the presence of either methylglyoxal or glyoxal and reaction products analysed by HPLC coupled to mass tandem spectrometry. AGE formation on albumin by methylglyoxal and glyoxal in the presence or absence of metformin was also studied by measuring the fluorescence at 370/440 nm after albumin–AGE isolation by ultrafiltration. As a standard for mass spectra analysis, a metformin–methylglyoxal adduct was chemically synthesised and characterised as a triazepinone (2-amino-4-(dimethyl-amino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one). The results obtained showed that metformin strongly reacted with methylglyoxal and glyoxal, forming original guanidine–dicarbonyl adducts. Reaction kinetic studies as well as mass fragmentation spectra of the reaction products were compatible with the presence of triazepinone derivatives. In the presence of metformin, AGE-related fluorescence after albumin incubation with either glyoxal or methylglyoxal was decreased by 37% and 45%, respectively. These results suggest that besides its known antihyperglycemic effect, metformin could also decrease AGE formation by reacting with α-dicarbonyl compounds. This is relevant to a potential clinical use of metformin in the prevention of diabetic complications by inhibition of carbonyl stress. BIOCHEM PHARMACOL **58**;11:1765–1773, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. advanced glycation end products; carbonyl stress; dicarbonyl compounds; glycoxidation; metformin; triazepin

The non-enzymatic reaction between reducing sugars, such as glucose, and amino structures in proteins (also called Maillard reaction or glycoxidation) has been shown to play an important role in the development of chronic complications of diabetes mellitus [1, 2]. In living systems, the Maillard reaction proceeds through complex reaction pathways resulting in a large number of structures [3]. After the initial formation of a Schiff base adduct between the carbonyl and the amine moiety, the aldimine rearranges into a more stable ketoamine or Amadori product. The Amadori product further undergoes a series of reactions through dicarbonyl reactive intermediates that ultimately lead to stable end products called advanced glycation end products, which are associated with diabetic vascular complications. AGEs§ constitute a heterogenous class of structures that are yellow–brown pigments, fluoresce, tend to form cross-links, generate reactive oxygen intermediates, and recognise a class of receptors on cellular surfaces [4]. Some AGEs, such as pentosidine, N^{ϵ}-(carboxymethyl) lysine, pyrraline, and crosslines, have been successfully identified *in vivo* [5].

Substantial recent data indicate that glucose toxicity is mediated through increased production of highly chemically reactive α-dicarbonyl precursors of AGEs. Amadori compounds, such as fructoselysine, are considered to be precursors of dicarbonyl sugars, such as glyoxal and 3-deoxyglucosone, which are more reactive with proteins than glucose itself [3, 6]. Approximately 40–50% of the AGE N^{ϵ}-(carboxymethyl)lysine originates from a Schiff base adduct via glyoxal [3]. Glyoxal also seems to be the major α-dicarbonyl formed in glucose autoxidation, a process which could also contribute to sugar protein modification

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§ *Abbreviations:* AGE(s): advanced glycation end product(s); and NIDDM, non-insulin-dependent diabetes mellitus.

in diabetes [7, 8]. Moreover, glyoxal can be formed as a lipid peroxidation product [9].

Methylglyoxal is another reactive dicarbonyl compound whose level is increased in diabetes [10]. Methylglyoxal is a physiological metabolite of the glyoxalase system, formed by the non-enzymatic and enzymatic elimination of phosphate from glycolytic intermediates such as glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, and the oxidation of hydroxyacetone and aminoacetone [11]. Methylglyoxal can also react with amino groups of proteins generating AGEs, and it has been linked to the development of diabetic complications [12]. Recently, evidence for methylglyoxal-derived modifications in human tissues has been reported, arguing for dicarbonyl-mediated protein modification by Maillard reaction *in vivo* [13].

Because AGE formation *in vivo* has been hypothesised to play a role in the development of chronic diabetes complications, pharmacological agents were sought to inhibit this process by selectively blocking the reaction of amino groups with either reducing sugars or reactive dicarbonyls. Aminoguanidine has been the most extensively investigated compound of this type and effectively inhibits the formation of AGEs *in vitro* and *in vivo* [14]. Aminoguanidine acts by reacting with Amadori-derived fragmentation products such as 3-deoxyglucosone and other dicarbonyl sugars, thereby preventing subsequent AGE formation on susceptible proteins [14–16]. However, aminoguanidine has other biochemical side effects which might raise chronic toxicity problems in a long-term treatment [17]. The biguanide metformin (dimethylbiguanide) was introduced into clinical practice in 1957 as an oral antihyperglycemic agent for the management of non-insulin-dependent diabetes mellitus [18]. This drug is structurally related to guanidine as well as aminoguanidine (Fig. 1), although it has never been investigated whether it might react with dicarbonyl compounds. We report here the results concerning the reaction of metformin with glyoxal and methylglyoxal, as well as the effect of metformin on the formation of albumin-AGEs by dicarbonyl compounds.

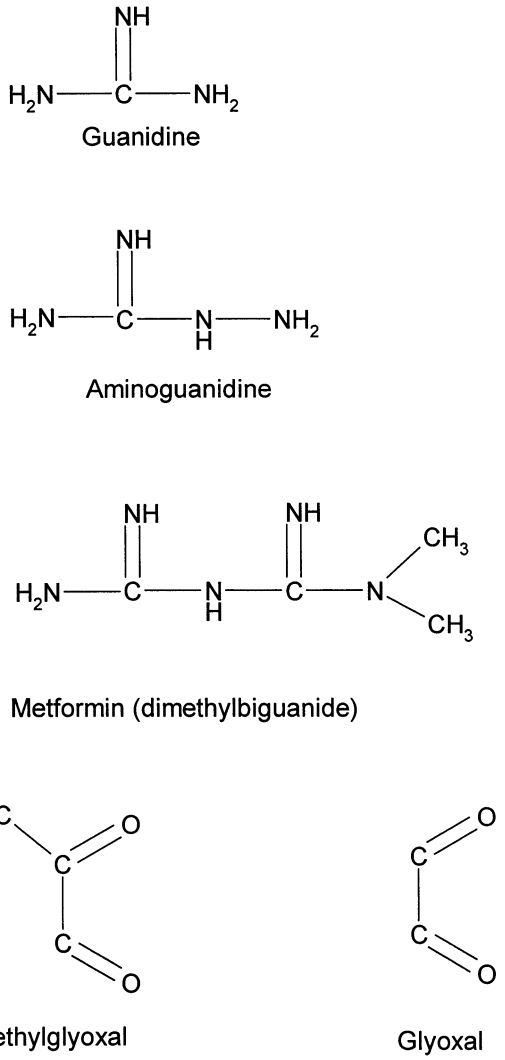

FIG. 1. Chemical structures of guanidine derivatives and dicarbonyl compounds.

MATERIALS AND METHODS

Materials

Methylglyoxal and globulin-free BSA were purchased from Sigma. Glyoxal and 3-amino-1,2,4-triazine were purchased from Aldrich. Metformin was obtained from Lipha S.A.

Chemical Synthesis of Triazepinone

Methylglyoxal (0.21 mol; 34 mL of a 40% aqueous solution) was added between 0° and +5° to a solution of 0.2 mol of *N*,*N*-dimethylbiguanide base in 100 mL of water. The reaction mixture was stirred for 1 hr at 5° and for a further 4 hr at 20°. A crystalline precipitate was obtained, washed with water, and evaporated in vacuum to dryness (white product, 18.2 g, 50% yield). The melting point of the product was determined on a Kofler instrument. Infrared spectrum was performed with an FITR Mattson 1020 Unicam instrument. Mass spectrum was obtained with a Nermag R10-10C using either electron impact or chemical ionisation. ^{1}H NMR (200 MHz), ^{13}C NMR (500 MHz), and ^{15}N NMR (500 MHz) spectra were recorded in DMSO on Bruker Avance DPX (200 MHz) and Bruker AM 500 MHz instruments.

Reaction of Metformin with Dicarbonyl Compounds at Physiological pH and Temperature

Metformin (25 mM) was incubated with either glyoxal or methylglyoxal (25 mM) in 0.2 M sodium phosphate buffer pH 7.4, under sterile conditions at 37° under air. In order to perform kinetic reaction studies, incubations were done over a period of time between 0 and 24 hr. Reactions were stopped after tubes were snap-frozen in liquid nitrogen. The reaction product analysis was performed by ion exchange

HPLC coupled to a diode array detector as well as by LC–MS.

HPLC Analysis

HPLC was performed with a Hewlett Packard 1100 series HPLC system equipped with a diode array detector. The column was a Supelcosil LC-SCX 5-μm (0.46 × 25 cm) stainless steel column fitted with a Supelcosil LC-SCX precolumn (0.46 × 2 cm). The mobile phase was 100 mM ammonium acetate pH 4.4 and the flow rate was 1.5 mL/min. The eluate was monitored by UV detection at 235 nm.

MS Analysis

MS analyses of the different reaction products resolved on HPLC were performed by HPLC coupled to mass tandem spectrometry (LC–MS–MS) using a Perkin Elmer SCIEX API 300 LC/MS/MS system. The same HPLC conditions described above were used. For secondary ion fragmentation (MS–MS) spectra, an ion spray source (5.7 kV) with an infusion flow rate of 5 μL/min was used, and 50–1000 amu scans were first realised and then reduced according to the mass observed. Spectra were obtained with a collision energy of 30 eV. External standards such as metformin, chemically synthesised triazepinone, or 3-amino-1,2,4-triazine were analysed in the same way.

Effect of Metformin on the Reaction of Albumin with Dicarbonyl Compounds

Globulin-free BSA (100 μM) was incubated with either methylglyoxal or glyoxal (1 mM) in the presence or absence of either metformin or aminoguanidine (1 mM) in 0.2 M sodium phosphate buffer, pH 7.4, under sterile conditions at 37° for 6 days. The serum albumin was then isolated by ultrafiltration using Microcon-10 devices from Amicon, according to the manufacturer's instructions. Albumin retained on the membrane was further washed three times with phosphate buffer and recovered in 200 μL of phosphate buffer. Protein concentration was measured by the bicinchoninic acid method using a Pierce kit, and adjusted to 1 mg/ml before the fluorescence was read at an excitation/emission wavelength of 370/440 nm, which is characteristic of AGEs [19].

RESULTS

Characterisation of Synthetic Triazepinone

As a standard for mass spectrometry analysis, a synthetic metformin–methylglyoxal adduct was chemically prepared and characterised. This product had the following characteristics: melting point 264–266°, elemental analysis (calculated/found): C 45.94/45.89%, H 7.29/7.15%, N 38.03/38.22%, O 8.65/8.73%. Mass spectrum showed a molecular mass of 183 compatible with the calculated molecular mass of 183.21 for $C_7H_{13}N_5O$. The infrared spectra of this compound exhibited the absorptions assignable to C = O, C = N, and vNH at 1672 cm^{-1}, 1541 cm^{-1}, and 3233 cm^{-1}, respectively. ^{1}H-NMR spectrum exhibited the following signals: δ_H (D_2O): 1.25 (d, 3H, CH_3), 3.13 (s, 6H, N-$(CH_3)_2$), 3.75 (q, 1H, CH), 8.06 (s, 2H, NH_2). ^{13}C-NMR spectrum showed the following signals: δ_C (D_2O): 17.519 (CH_3), 36.730 (($CH_3)_2$ − N), 52.963 (CH), 159.68 (>C = 4), 173.99 (>C = 2), 189.95 (>C = O). ^{15}N-NMR exhibited the following signals: δ_C (D_2O): −177.66 (s, N_1), −224,1 (s, N_2), −268.4 (d, NH), −293.6 (t, NH_2), −302.1 (s, N-$(CH_3)_2$). These results are compatible with the structure shown in Fig. 2, which corresponds to a 2-amino-4-(dimethylamino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one (triazepinone). Mass tandem spectrometry of the synthetic triazepinone (Fig. 2) showed 139 and 167 m/z fragment ions which are compatible with a triazepin ring with two different substitutions (NH_2 and N-$(CH_3)_2$). Ion-exchange HPLC analysis of the synthetic triazepinone standard showed a retention time of 16.5 min.

Reaction of Metformin with Glyoxal and Methylglyoxal

Metformin was incubated in the presence of either glyoxal or methylglyoxal in 0.2 M sodium phosphate buffer pH 7.4 at 37° for 0 to 24 hr. Reaction mixtures were analysed by ion-exchange HPLC coupled to a diode array detector as well as by LC–MS–MS without secondary ion fragmentation (LC–MS). Different reaction products were formed (Fig. 3). Their maximum UV absorbance was at 235 nm. In metformin–methylglyoxal reaction mixtures, five main peaks were detected (Fig. 3a). Their retention times were 6.3, 9.4, 11.2, 13.4, and 16.5 min and their m/z assessed by LC–MS were 202, 256, 256, 130, and 184, respectively. These m/z correspond to the mass of metformin (m/z 130), triazepinone (m/z 184), a linear condensation product of metformin with methylglyoxal (m/z 202), and an addition product of triazepinone with a second molecule of methylglyoxal (m/z 256). Concerning metformin–glyoxal reaction mixtures, three main HPLC peaks were observed, with retention times of 4.8, 5.9, and 13.0 min (Fig. 3b). Their m/z, assessed by LC–MS, were 188, 170, and 130, respectively. These m/z correspond to the mass of a linear condensation product of metformin with glyoxal (m/z 188), a non-methylated triazepinone (m/z 170), and metformin (m/z 130).

In order to study the formation of these products, reaction kinetics from 0 to 24 hr were performed. As shown in Fig. 4a, in the metformin–methylglyoxal reaction, the m/z 202 product formation increased quickly to reach a maximum after two hours of incubation and then decreased, whereas the m/z 184 product formation progressively increased. Concerning the m/z 130 product, its concentration quickly decreased at a logarithmic-like rate. The HPLC peak with a m/z of 256 and retention time of 9.4, showed a formation rate superimposable to the m/z 184 reaction rate, whereas the m/z 256 peak with a retention

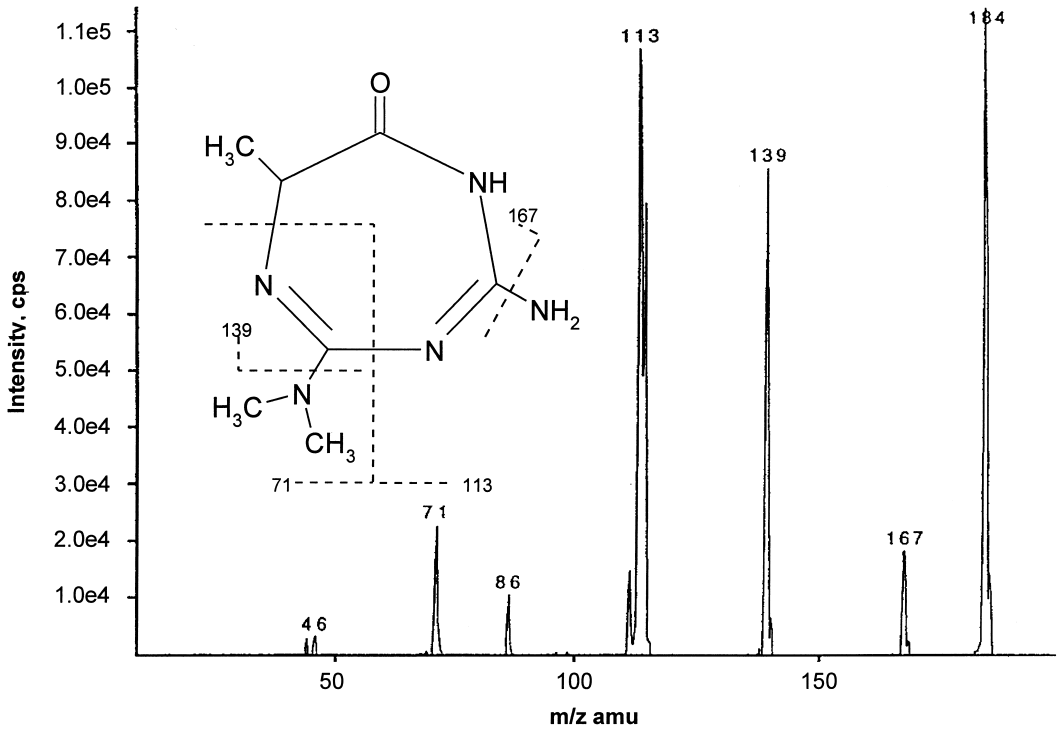

FIG. 2. Mass fragmentation spectrum of synthetic 2-amino-4-(dimethylamino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one. 2-Amino-4-(dimethylamino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one (triazepinone) was chemically synthesised and analysed by mass spectrometry as described in Materials and Methods.

time of 11.2 showed a different profile (Fig. 4a, inset). In the metformin–glyoxal reaction (Fig. 4b), m/z 188, 170, and 130 products exhibited similar formation kinetics as observed for m/z 202, 184, and 130 products of the metformin–methylglyoxal reaction, respectively.

Control incubations of either glyoxal or methylglyoxal without metformin up to 24 hr did not show any peak by HPLC analysis. HPLC analysis of control incubations of metformin alone showed only one peak with a retention time of 13.0 min (data not shown). Neither HPLC peaks nor mass spectra ions (m/z 97, for example) compatible with the presence of triazine derivatives (the main products of the aminoguanidine–methylglyoxal reaction [15]) were detected in the different reaction mixtures analysed.

LC–MS–MS Analysis of Metformin Reaction Products

Fragmentation mass spectra of the reaction products obtained after incubation of metformin with either methylglyoxal or glyoxal were obtained by LC–MS–MS and compared to a synthetic standard of triazepinone and metformin (Table 1). In metformin–methylglyoxal reaction mixtures, analysis of the fragmentation spectra of the m/z 130 and 184 protonated molecular ions $[M + H]^+$ showed strong similarities with mass fragmentation spectra of metformin and synthetic triazepinone, respectively, although some differences in relative abundances of some product ions were observed. The presence of m/z 139 and 167 product ions after fragmentation of the m/z 184 precursor ion was compatible with the existence of a triazepin ring such as in the synthetic triazepinone (see Fig. 2). A m/z 167 product ion was also detected after mass fragmentation of the m/z 256 reaction product. On the contrary, m/z 139 and 167 product ions were not observed after fragmentation of the m/z 202 reaction product (Table 1). Mass fragmentation of m/z 202 and m/z 256 also showed product ions of m/z 130 and 184 which correspond to the m/z of standard metformin and triazepinone, respectively. Concerning metformin–glyoxal reaction products, mass fragmentation spectra of m/z 170 showed product ions of m/z 125 and 153 which correspond to the mass of non-methylated m/z 139 and 167 product ions of the synthetic triazepinone fragmentation.

Comparison of Metformin and Aminoguanidine Effects on Serum Albumin Modification by Dicarbonyl Compounds In Vitro

In order to study the possible inhibitory effect of metformin on AGE formation, BSA was incubated with glyoxal or methylglyoxal in the presence or absence of metformin, and compared to aminoguanidine. After incubation, modified albumin was isolated by ultrafiltration in order to eliminate low-molecular-weight reaction products which could interfere with fluorescence measurement at 370/440 nm. Then, AGE formation was assessed by reading the fluorescence at 370/440 nm, which is characteristic of AGEs [19]. After 6 days of incubation of 100 μM BSA with 1 mM glyoxal or methylglyoxal at 37°, the fluorescence increased by 11.8-

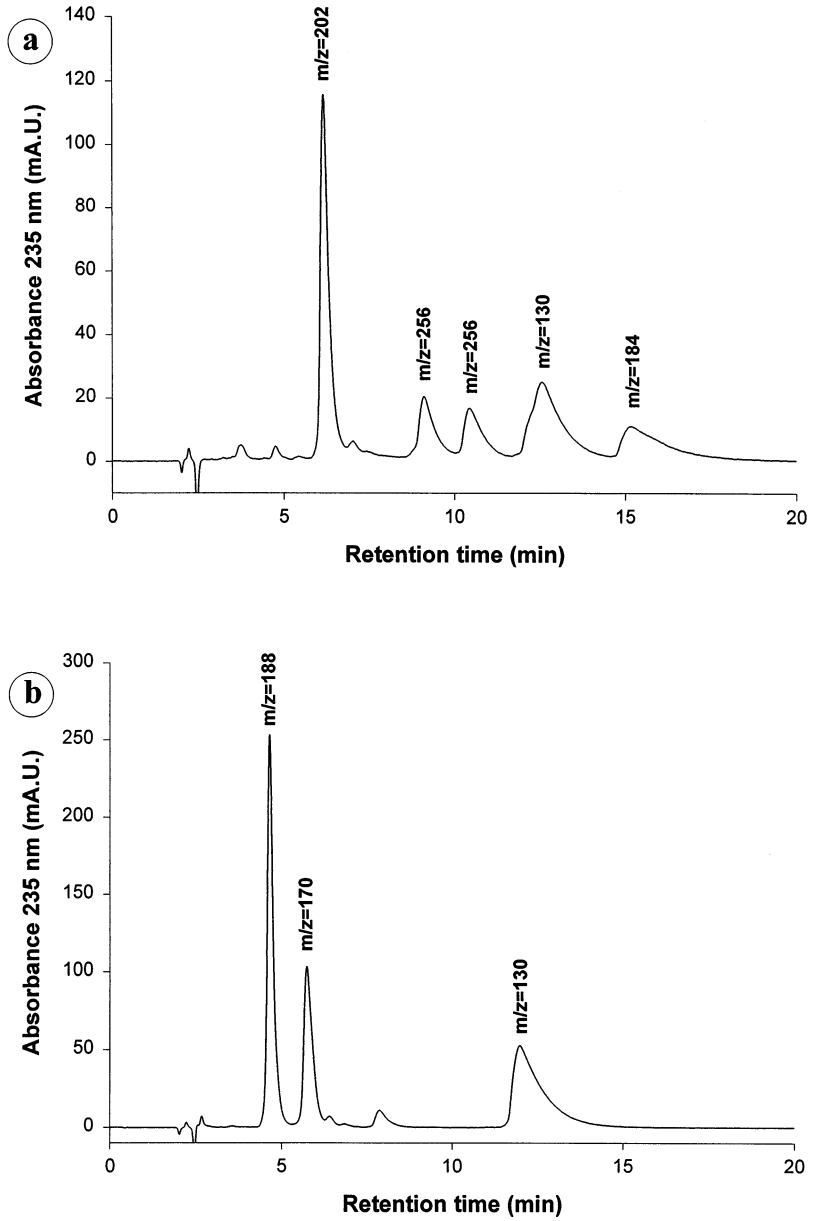

FIG. 3. HPLC–MS analysis of metformin/α-dicarbonyl reaction mixtures. Metformin (25 mM) was incubated with either methylglyoxal or glyoxal (25 mM) in 0.2 M sodium phosphate buffer, pH 7.4 at 37°. After 4 hr of incubation, reaction mixtures were diluted 100-fold and 10 μL injected into an HPLC–MS system equipped with Supelcosil LC-SCX column as described in Materials and Methods. m/z: mass/charge ratio of peaks determined by mass spectrometry. m.A.U., milliAbsorbance units. (a) metformin/methylglyoxal incubation mixture. (b) metformin/glyoxal incubation mixture.

and 37.7-fold, respectively, as compared to BSA alone. In contrast, the development of the fluorescence characteristics of AGEs was partially but markedly inhibited after incubation with 1 mM of aminoguanidine or metformin (Fig. 5). Indeed, metformin decreased the albumin fluorescence at 370/440 nm by 37% after incubation with glyoxal and by 45% after incubation with methylglyoxal. After incubation with aminoguanidine, the fluorescence reduction was 85% for glyoxal and 58% for methylglyoxal.

DISCUSSION

Metformin is an oral antihyperglycemic agent used for the management of non-insulin-dependent diabetes mellitus (NIDDM). It is structurally related to guanidines, which elicit a marked interest in glycation studies and in the prevention of diabetic complications. The most studied guanidine compound with a clear inhibitory effect on AGE formation is aminoguanidine [14]. Several groups have shown that this compound was quite effective in inhibiting the formation of AGEs *in vitro* and *in vivo* [2]. Clinical trials are currently underway to evaluate the effect of aminoguanidine therapy on the progression of diabetic complications. The presence of a guanidino group in the metformin structure confers a potential use of this drug for the inhibition of the Maillard reaction by reacting with carbonyl groups of reducing sugars and dicarbonyl compounds. It has been shown previously that biguanides react with α-diketones in strongly alkaline ethanolic media [20]. However, the metformin reaction with α-dicarbonyl compounds involved in AGE formation has never been studied under more physiological conditions. Therefore, we studied

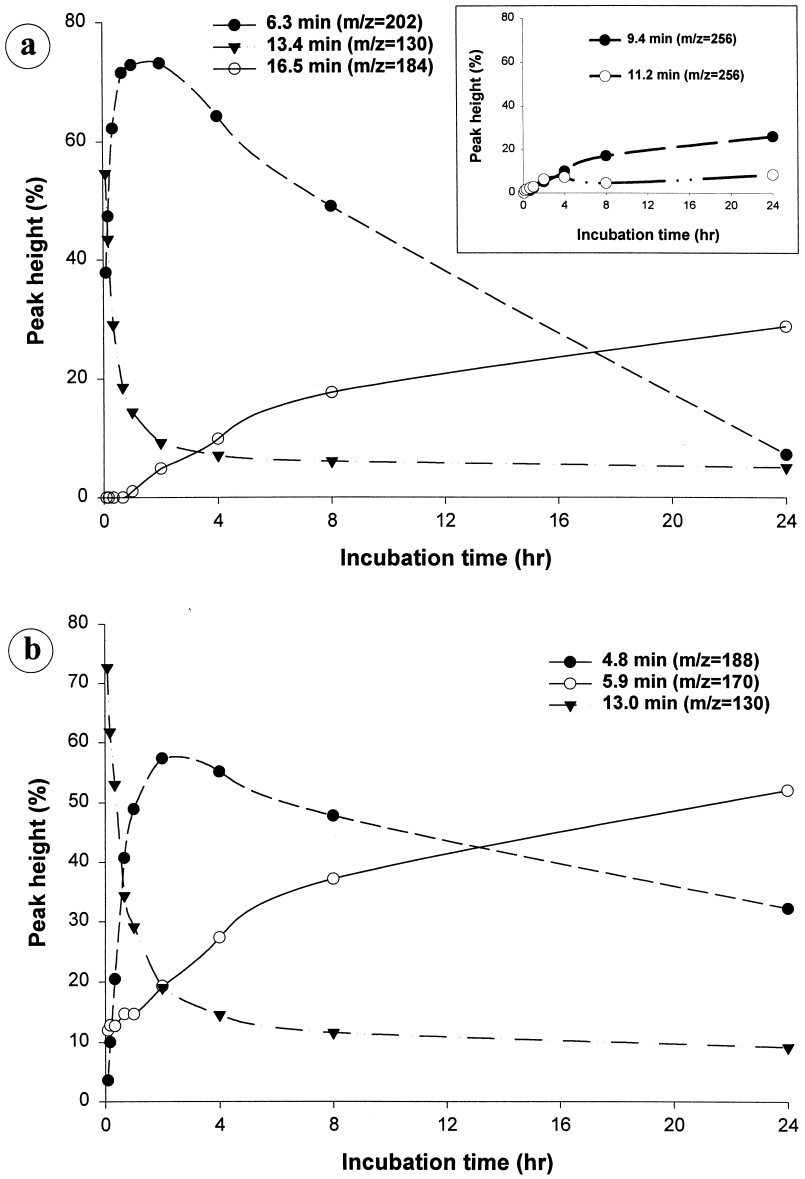

FIG. 4. Reaction kinetics of metformin with methylglyoxal and glyoxal. Metformin (25 mM) was incubated with either methylglyoxal or glyoxal (25 mM) in 0.2 M sodium phosphate buffer, pH 7.4 at 37°. After 5, 10, 20, 40, 60, 120, 240, 480, and 1440 min of incubation, fractions were analysed by HPLC as described in Materials and Methods. After integration, peaks were identified by their HPLC retention times (min), and height of selected peaks (expressed as percentage) was plotted against incubation times. In brackets: m/z of HPLC peaks. (a) metformin/methylglyoxal incubations; inset: reaction kinetics of 9.4- and 11.2-min HPLC peaks. (b) metformin/glyoxal incubations.

whether metformin could react with either methylglyoxal or glyoxal at pH 7.4 and 37°. HPLC analysis of the reaction mixtures after incubation showed a strong reaction of metformin with methylglyoxal and glyoxal and the formation of several products with different kinetics. In order to characterise these products, mass tandem spectrometry analysis was performed. In parallel, a metformin–methylglyoxal adduct was chemically synthesised, characterised as a triazepinone (2-amino-4-(dimethylamino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one) and used as standard for MS–MS studies.

Kinetic data, as well as comparison of product mass fragmentation spectra to standards, suggest the metformin–α-dicarbonyl reaction steps shown in Fig. 6. In the metformin–methylglyoxal reaction, a condensation product would be quickly formed by the reaction of methylglyoxal with the primary amino group of metformin (m/z 202 product). Thereafter, the triazepinone (m/z 184 product) would be obtained by dehydration. MS–MS and kinetic results also suggest that a second molecule of methylglyoxal could react with free amino groups of triazepinone, leading to a m/z 256 condensation product. The existence of two m/z 256 products (Figs. 3a and 4a, inset) with the same fragmentation spectra could correspond to isomers of the same product. Concerning the metformin–glyoxal reaction, the same reaction mechanism could be proposed, leading to similar products without the methyl group. However, a condensation product obtained by reaction of non-methylated triazepinone with a second molecule of glyoxal was not detected in the incubation mixtures.

The reaction of aminoguanidine with methylglyoxal or other dicarbonyl compounds leads to the formation of substituted 3-amino-1,2,4-triazine derivatives as major reaction products [15, 16]. In the LC–MS–MS system used,

TABLE 1. Mass tandem spectrometry analysis of metformin–α-dicarbonyl reaction products

	Precursor ions (m/z)	Product ions (m/z) / relative abundance (%)	Suggested identification
Metformin	130	71 (100), 60 (68.6), 85 (59.8), 88 (37.9), 113 (25.3)	
Synthetic triazepinone*	184	113 (100), 71 (20.9), 86 (9.9), 139 (80.5), 167 (17.0)	
Metformin/Methylglyoxal reaction products	130	71 (100), 60 (33.0), 85 (65.9), 88 (66.5), 113 (66.5)	metformin
	184	139 (100), 71 (22.0), 86 (22.0), 99 (22.0), 113 (66.2), 125 (21.4), 167 (21.4)	triazepinone
	202	113 (100), 90 (11.4), 130 (33.3), 184 (44.8)	metformin + methylglyoxal condensation
	256	184 (100), 137 (50), 167 (50)	triazepinone + methylglyoxal condensation
Metformin/Glyoxal reaction products	130	71 (100), 60 (58.0), 85 (66.5), 88 (25), 113 (24.4)	metformin
	170	100 (100), 71 (32.7), 110 (65.4), 113 (15.4), 125 (65.4), 140 (50), 153 (15.8)	non-methylated triazepinone
	188	118 (100), 72 (14.3), 100 (14.3), 113 (22.9), 143 (85.7), 144 (14.6), 170 (22.9)	metformin + glyoxal condensation

Product ions listed by increasing order of m/z, base peak (100% relative abundance) listed first. Numbers in brackets give relative abundances. Suggested identification based on mass fragmentation similarities.

*: 2-amino-4-(dimethylamino)-7-methyl-5,7-dihydro-6*H*-[1,3,5]triazepin-6-one.

we never detected the presence of triazine derivatives. Moreover, fragmentation spectra obtained were not compatible with the presence of argpyrimidine [21], a reaction product of methylglyoxal with arginine which has a guanidino group. We can conclude that the metformin reaction with α-dicarbonyls at physiological pH and temperature leads to the formation of original guanidine–α-dicarbonyl adducts such as triazepin-like structures.

Dicarbonyl compounds are more reactive in the glycation reaction than reducing sugars and are an important step for cross-linking proteins in the Maillard reaction [22]. It has been suggested that increased chemical modification of proteins by carbohydrates and lipids in diabetes is the result of overload on metabolic pathways involved in detoxification of reactive carbonyl species leading to an increased carbonyl stress [23]. If metformin reacts with α-dicarbonyl compounds, it could thereby prevent subsequent AGE formation on susceptible proteins acting as a carbonyl scavenger. Therefore, we studied the effect of metformin on albumin modification by either methylglyoxal or glyoxal. The results obtained showed that albumin AGE fluorescence induced by these two dicarbonyl

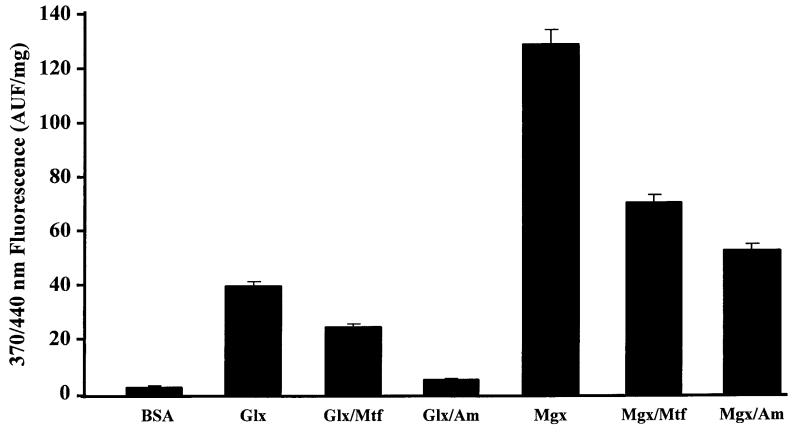

FIG. 5. Comparative effects of metformin and aminoguanidine on the glycoxidation of albumin by α-dicarbonyl compounds. BSA (100 μM) was incubated alone or with dicarbonyl compounds (1 mM) in the presence or absence of metformin or aminoguanidine (1 mM) in 0.2 M sodium phosphate buffer, pH 7.4, under aseptic conditions at 37° for 6 days. After isolation of albumin by ultrafiltration, protein concentration was adjusted to 1 mg/mL and fluorescence measured at 370/440 nm. Results are expressed as fluorescence arbitrary units per mg of protein (AUF/mg) and represented the means ± SD of three independent determinations. BSA: albumin alone; Glx: albumin + glyoxal; Glx/Mtf: albumin + glyoxal + metformin; Glx/Am: albumin + glyoxal + aminoguanidine; Mgx: albumin + methylglyoxal; Mgx/Mtf: albumin + methylglyoxal + metformin; Mgx/Am: albumin + methylglyoxal + aminoguanidine.

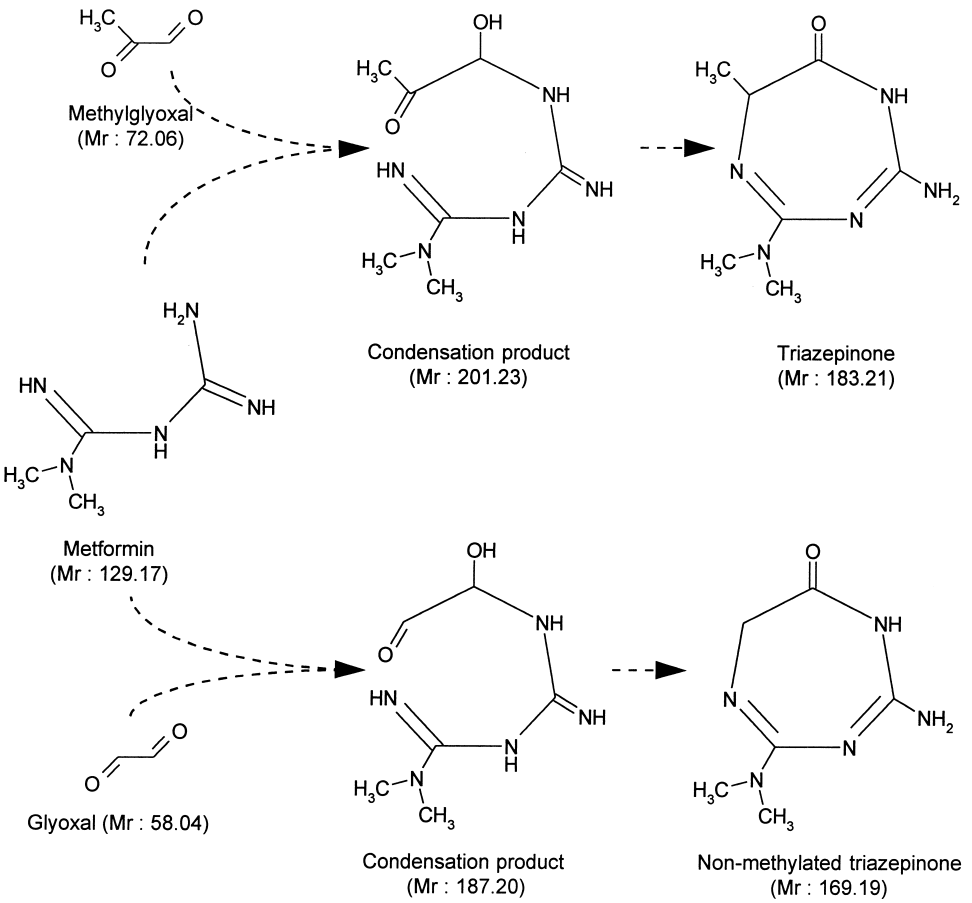

FIG. 6. Possible reaction steps of metformin with α-dicarbonyl compounds (methylglyoxal and glyoxal). Mr, molecular weight.

compounds was markedly reduced by metformin, supporting our hypothesis of an inhibitory effect of metformin on AGE formation. The metformin effect was stronger with methylglyoxal than with glyoxal, but not as pronounced as that of aminoguanidine, probably because of structural differences between the guanidines (Fig. 1). It is noteworthy that albumin AGE fluorescence was 3-fold higher after incubation with methylglyoxal than with glyoxal, suggesting that methylglyoxal is a more potent glycoxidation agent than glyoxal.

As suggested by the results presented above, the mechanism by which metformin inhibits glycoxidation of albumin by glyoxal and methylglyoxal most likely relates to a reaction between the metformin guanidino groups and the carbonyl groups of methylglyoxal and glyoxal. However, an alternative or complementary mechanism by metal-chelating action could not be excluded. It is known that biguanides can chelate metals [24] and therefore could interfere with glycoxidation reactions. It has recently been reported that metformin exhibits an inhibitory effect on glycoxidation of albumin by glucose under aerobic conditions [25] where glyoxal is formed. In preliminary experiments, we observed that metformin also reacts with glucose itself, although the reaction was 300-fold stronger with glyoxal (unpublished data). Reaction with dicarbonyl compounds (glyoxal and 3-deoxyglucosone) derived from the oxidative fragmentation of glucose has been proposed as a mechanism for aminoguanidine inhibition of AGE formation by glucose [14]. It could be that metformin inhibition of albumin glycoxidation by glucose was the consequence of several non-mutually-exclusive mechanisms: metformin reaction with glucose itself and with carbonyl compounds derived from glucose oxidative fragmentation, as well as by metal-chelating action.

The pharmacological inhibition of AGE formation seems to be a good approach in the prevention of diabetic complications. The results presented here suggest that metformin acting as a scavenger of dicarbonyl compounds could decrease carbonyl stress and inhibit AGE formation in proteins. Even though the effect of metformin on preventing AGE formation was weaker than that of aminoguanidine in the present experiments, metformin has the advantage of being a drug with a long history of therapeutic use. Since the normalisation of the glycemic level reduces the development of diabetic complications by itself [26], it is very difficult to determine, in NIDDM patients, a preventive effect of metformin on the diabetic complication development independently of the metformin antihyperglycemic effect. It has previously been shown that metformin was effective in preventing microvascular alter-

ations such as enhanced permeability in diabetic animals, independently of its antihyperglycemic effect [27]. Recent results from the UKPDS trial have shown that metformin significantly reduced the mortality risk in NIDDM patients, suggesting additional effects of metformin besides its antihyperglycemic effect [28]. Very recently, Beisswenger *et al.* reported that metformin reduces systemic methylglyoxal levels in type 2 diabetic patients with similar glycemic levels [29]. As suggested by these authors and supported by our results, methylglyoxal plasma levels could be lowered by a metformin-binding effect. However, only the precise identification of metformin–dicarbonyl reaction products such as the triazepinone in diabetic patients currently treated with metformin could make it possible to determine to which extent the *in vitro* reaction of metformin reported here occurs *in vivo*.

In conclusion, in the present study we have shown that metformin reacted with methylglyoxal and glyoxal forming original adducts such as triazepin-like structures and inhibited AGE related fluorescence when albumin was incubated in the presence of these α-dicarbonyl compounds. These results are relevant to a potential clinical use of metformin in the prevention of diabetic complications by inhibition of carbonyl stress.

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References

1. Vlassara H, Bucala R and Striker L, Pathogenic effects of advanced glycosylation: Biochemical, biologic, and clinical implications for diabetes and aging. *Lab Invest* **70:** 138–151, 1994.
2. Bucala R, Cerami A and Vlassara H, Advanced glycosylation end products in diabetic complications. *Diabetes Rev* **3:** 258–268, 1995.
3. Glomb MA and Monnier VM, Mechanism of protein modification by glyoxal and glycoaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem* **270:** 10017–10026, 1995.
4. Schmidt AM, Hori O, Brett J, Yan SD, Wautier JL and Stern D, Cellular receptors for advanced glycation end products. *Arterioscler Thromb* **14:** 1521–1528, 1994.
5. Wells-Knecht KJ, Brinkman E, Wells-Knecht MC, Litchfield JE, Ahmed MU, Reddy S, Zyzak DV, Thorpe SR and Baynes JW, New biomarkers of Maillard reaction damage to proteins. *Nephrol Dial Transplant* **11** (Suppl 5): 41–47, 1996.
6. Zyzak DV, Richardson JM, Thorpe SR and Baynes JW, Formation of reactive intermediates from Amadori compounds under physiological conditions. *Arch Biochem Biophys* **316:** 547–554, 1995.
7. Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR and Baynes JW, Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* **34:** 3702–3709, 1995.
8. Hunt JV, Dean RT and Wolff SP, Hydroxyl radical production and autoxidative glycosylation. *Biochem J* **256:** 205–212, 1988.
9. Fu M, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW and Thorpe SR, The advanced glycation end product, N^{ϵ}-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem* **271:** 9982–9986, 1996.
10. McLellan AC, Thornalley PJ, Benn J and Sonksen PH, The glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci* **87:** 21–29, 1994.
11. Thornalley PJ, Methylglyoxal, glyoxalases and the development of diabetic complications. *Amino Acids* **6:** 15–23, 1994.
12. Lo TWC, Westwood ME, McLellan AC, Selwood T and Thornalley PJ, Binding and modification of proteins by methylglyoxal under physiological conditions. *J Biol Chem* **269:** 32299–32305, 1994.
13. Shamsi FA, Partal A, Sady C, Glomb MA and Nagaraj RH, Immunological evidence for methylglyoxal-derived modifications *in vivo*. *J Biol Chem* **273:** 6928–6936, 1998.
14. Edelstein D and Brownlee M, Mechanistic studies of advanced glycosylation end product inhibition by aminoguanidine. *Diabetes* **41:** 26–29, 1992.
15. Lo TWC, Selwood T and Thornalley PJ, The reaction of methylglyoxal with aminoguanidine under physiological conditions and prevention of methylglyoxal binding to plasma proteins. *Biochem Pharmacol* **48:** 1865–1870, 1994.
16. Hirsch J, Petrakova E and Feather MS, The reaction of some dicarbonyl sugars with aminoguanidine. *Carbohydr Res* **232:** 125–130, 1992.
17. Ou P and Wolff SP, Aminoguanidine: A drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide *in vitro*. *Biochem Pharmacol* **46:** 1139–1144, 1993.
18. Wiernsperger N and Rapin JR, Metformin–insulin interactions: From organ to cell. *Diabetes Metab Rev* **11:** S3–S12, 1995.
19. Njoroge FG, Sayre LM and Monnier VM, Detection of D-glucose-derived pyrrole compounds during Maillard reaction under physiological conditions. *Carbohydr Res* **167:** 211–220, 1987.
20. Tanabe S and Sakaguchi T, Reaction of guanidines with α-diketones. VL. Structures of fluorescent products of biguanides with 9,10-phenanthraquinone. *Chem Pharm Bull* (Tokyo) **26:** 423–428, 1978.
21. Shipanova IN, Glomb MA and Nagaraj RH, Protein modification by methylglyoxal: Chemical nature and synthetic mechanism of a major fluorescent adduct. *Arch Biochem Biophys* **344:** 29–36, 1997.
22. Yim H, Kang S, Hah Y, Chock B and Yim M, Free radicals generated during the glycation reaction of amino acids by methylglyoxal. *J Biol Chem* **270:** 28228–28233, 1995.
23. Baynes JW and Thorpe SR, Role of oxidative stress in diabetic complications. *Diabetes* **48:** 1–9, 1999.
24. Ray P, Complex compounds of biguanides and guanylureas with metallic elements. *Chem Rev* **61:** 313–359, 1961.
25. Tanaka Y, Iwamoto H, Onuma T and Kawamori R, Inhibitory effect of metformin on formation of advanced glycation end products. *Curr Ther Res Clin Exp* **58:** 693–697, 1997.
26. The Diabetes Control Complications Trial Research Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* **329:** 977–986, 1993.
27. Colantuoni A, Bertuglia S and Donato L, Effects of metformin on microvascular permeability in diabetic syrian hamsters. *Diabetes Metab* **14:** 549–553, 1988.
28. UK Prospective Diabetes Study Group, Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes. *Lancet* **352:** 854–865, 1998.
29. Beisswenger PJ, Howell SK, Touchette AD, Lal S and Szwergold BS, Metformin reduces systemic methylglyoxal levels in type 2 diabetes. *Diabetes* **48:** 198–202, 1999.