Metformin inhibition of glycation processes

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SUMMARY
A number of studies have shown that metformin is beneficial in reducing diabetes associated vascular risk beyond the benefits expected from its anti-hyperglycaemic effect. One of the main pathogenic mechanisms leading to chronic complications of diabetes is non-enzymatic glycation where damage is mediated through increased production of highly chemically reactive glucose and β-dicarbonyl compounds which lead to production of advanced glycation products (AGEs). We present laboratory and clinical data supporting the hypothesis that one important explanation of metformin’s effect on diabetic complications could be its ability to reduce toxic dicarbonyls and AGEs. This effect could be related either to the binding of the β-dicarbonyls, methylglyoxal (MG) or 3-deoxyglucosone, or to an increase in enzymatic detoxification. Our studies presented in this manuscript document extracellular binding of MG by metformin to form a specific product (triazepinone) in vivo. This condensation product appears to be only one of several inactive end products resulting from this chemical reaction and we discuss the possibility that these or other condensation products (hydroimidazolones) could be indicative of inactivation of MG by metformin. Additional studies of other possible condensation products, as well as other potential cellular effects of metformin on MG production, will help to clarify this potentially important effect of metformin and provide a further rationale for using metformin to prevent long-term complications.

Key-words: Metformin · Glycation · Methylglyoxal · Diabetic complications · Triazepinone.

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Glycation and diabetic complications

There is substantial data indicating that glucose toxicity leads to dramatically accelerated atherosclerosis, retinal-renal disease, and neuropathy in diabetes. It is widely recognized that one of the main pathogenic mechanisms leading to the chronic complications of diabetes is non-enzymatic glycation where damage is mediated through increased production of highly chemically reactive glucose and α-dicarbonyl compounds [1] which lead to production of advanced glycation products (AGEs) [2, 3] through distinct chemical pathways [4]. The non-enzymatic reaction between sugars, such as glucose, and free amino groups of proteins is also called the Maillard reaction, glycation or glycoxidation. The Maillard reaction proceeds through complex non-enzymatic reaction pathways resulting in early glycation products and a large number of complex, chemically irreversible structures called AGEs. In addition to the damage caused by AGEs, substantial recent data indicate that glucose toxicity is also mediated through increased production of very reactive carbonyl species such as glyoxal, methylglyoxal and 3-deoxyglucosone (3DG) [5] (Fig 1). The cumulative results of damage caused to protein, lipid and DNA by these compounds leads to a state of increased "carbonyl stress".

The production of methylglyoxal (MG) occurs primarily from the triosephosphate intermediates in the glycolytic pathway (Fig 2), which include dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) [6, 7]. MG

![Diagram of Guanidine derivatives and α-dicarbonyl compound chemical structures.](image)

is generated by two processes: A) A spontaneous non-enzymatic elimination of the phosphate group, or B) Decomposition of an enediol triose phosphate intermediate that “leaks” from the active site of triose phosphate isomerase [8]. Other possible sources of MG include cytochrome P4502E1 catalysed oxidation of acetone from ketone bodies [9], cleavage of Amadori products to 3DG and fragmentation to MG by a reverse aldol reaction [10], and catabolism of threonine via aminoacetone [11].

Carbonyl species (α-oxoaldehydes) are extremely reactive as glycating agents for collagen, enzymes and other important cellular components [12-15] and have been shown to be toxic to cultured cells [15-17]. They react with lysine and arginine residues in proteins under anaerobic conditions to form specific AGEs, including pyrraline, imidazolones, arg-pyrimidine, Nε(carboxyethyl)lysine and various cross-linking structures (MOLD and others) [18]. Although oxidation is not required for modification of protein by carbohydrates, oxidation reactions accelerate the chemical modification of proteins by hexoses. Metal catalysed and non-metal catalysed oxidation and peroxidation of lipids can also lead to a multitude of reactive carbonyl compounds [18].

Our studies and other investigations have shown that MG is significantly elevated in plasma from diabetics [19-23] and shows a strong correlation with glycaemic control [19, 24, 26]. MG can also lead to increased AGE formation which in turn has been shown to be associated with diabetic vascular and neuropathic complications [26-28]. For example MG may play a role in the development of a number of diabetic complications including diabetic nephropathy. This is suggested by several lines of evidence, including studies of the early stages of diabetic renal dysfunction which have shown that glomerular hyperfiltration (GFR) is associated with elevated MG levels, [29] and that elevated levels of MG and its metabolites may be associated with early albuminuria [30].

Pharmacological inhibition of glycation

Several glycation inhibitors have been proposed based on their mechanism of action and include, glycating agent scavengers, amadorins, chelators and antioxidants as proposed by Khalifa et al. [31]. Indeed, some inhibitors of glycation interfere with the reaction by trapping glycating agents such as α-dicarbonyl compounds, whereas other inhibitory compounds act in a non-specific way as antioxidants and transition metal chelators thereby inhibiting the glycoxidative process but not glycation. In vitro screening of glycation inhibitors is complicated due to the fact that most of the assays that are utilised measure the suppression of oxygen-dependent AGE formation (glycoxidation) which can also be inhibited by compounds with antioxidant and metal-chelating activity. To identify specific glycation inhibitors one should optimally use assays that avoid oxidative conditions [32] and lead to reaction products between the potential inhibitor and the glycating agent as the major end points to determine efficacy.

Aminoguanidine is one of the best characterised glycation inhibitors. The primary mechanism of action of aminoguanidine appears to involve reactions which inactivate α-carbonyls such as 3DG and MG [33, 34], and it has been shown to react with MG at near physiological conditions to form triazine derivatives [35]. This trapping mechanism could explain its positive effects preventing AGE formation and the development of diabetic complications in animal models of diabetes. A number of groups have shown that this compound is quite effective in inhibiting the formation of AGEs and alleviating diabetic complications [36-38], although toxic side effects of aminoguanidine have limited its therapeutic use in humans [39, 40].

Several others inhibitors of glycooxidation processes have been described in the literature [31, 41-43]. Among them pyridoxamine, a compound belonging to family of post-Amadori reaction inhibitors called Amadorins, has shown a
powerful effect on inhibition of advanced glycation as well as advanced lipoxidation end products formation [18]. Because of its potential as an anti-complication agent, this compound is currently undergoing clinical trials in humans.

The biguanide metformin (dimethylbiguanide) was introduced into clinical practice in 1957 as an oral antihyperglycaemic agent for the management of non-insulin-dependent diabetes mellitus [44]. This drug is a guanidino compound that is structurally related to guanidine as well as aminoguanidine (Fig 1) suggesting that it may also have a potential effect on the inhibition of glycation reactions.

**Metformin and diabetic complications: UKPDS**

A number of studies have shown that metformin is beneficial in reducing diabetes associated vascular risk [45] beyond the benefits expected from its antihyperglycaemic effect. The recently completed UKPDS is perhaps the most dramatic illustration of this phenomenon where a 40-50% reduction in coronary events was observed in the metformin group, even though the mean glycaemic control in this group was slightly higher than the group treated intensively with sulfonylureas or insulin [46]. This difference could not be accounted for by reduction in traditional cardiovascular risk factors such as hypertension or hyperlipidaemia, and other possible explanations for this observation have been proposed. Recently we have shown that metformin reduces tissue levels of MG in diabetic patients [45] and that it reacts chemically in vitro with MG to form specific condensation products, one of which has been identified as triazepinone (TZP) [47]. Other recent studies have confirmed that metformin can reduce AGE levels in lens, kidney, and nerves in diabetic animals [48].

These results have led us to postulate that some of the beneficial effect of metformin may be due to scavenging of MG through the formation of TZP and related products.

**AGE formation inhibition by metformin**

Due to its beneficial effect on diabetic complications, and our lack of information on the mechanism(s) by which this occurs, we and others have performed studies to investigate whether metformin might inhibit glycation processes independently from its antihyperglycaemic effect.

**In vitro studies**

Regan et al. [49] have shown that chronic metformin treatment prevented functional and structural alterations of diabetic myocardium associated to glycation. In diabetic alloxan-induced diabetic dogs a significant increase of left ventricular end-diastolic pressure and ventricular stiffness is observed after 4 months of diabetes. These functional alterations were associated with a significant increase of collagen-linked AGEs in left ventricule myocardium. Metformin was able to completely prevent the functional myocardial alterations as well as myocardial AGE formation, and were independent of its antihyperglycaemic effect. The authors concluded that improvement of diastolic function in these diabetic animals occurred through a reduction of protein cross-links attributable to prevention of glycation by metformin.

In another in vivo study, Tanaka et al. [48] showed that metformin inhibited AGE formation in peripheral nerves and improved their function in diabetic animals. In streptozotocin-induced diabetic rats a significant increase of Nε-(carboxymethyl)lysine and pentosidine, two well known AGEs, was observed in lens, sciatic nerve, renal cortex and plasma. In the same animals, sciatic nerve conduction velocity was significantly decreased by diabetes. When a parallel group of diabetic animals was treated for 10 weeks with metformin, tissue deposition of AGEs was significantly reduced in association with a significant improvement of sciatic nerve conduction velocity. In this study, a slight decrease in glycemia and HbA1c was observed in metformin-treated animals compared to non-treated animals but it was not sufficient to explain the decreased deposition of AGEs in tissue.

**Mechanisms by which metformin inhibits glycation**

The antiglycation effects of metformin reported in animals are supported by in vitro results showing that metformin inhibits protein glycation. When bovine serum albumin (BSA) is incubated in 0.2 M phosphate buffer, pH 7.4 at 37°C in the presence of either glucose or dicarbonyl compounds such as glyoxal or MG, metformin is able to significantly inhibit albumin AGE-related fluorescence [47, 50]. The metformin inhibitory effect on albumin glycation by glucose was confirmed by Tanaka et al. [51] using specific polyclonal antibodies against Nε-(carboxymethyl)lysine. Metformin seems to be more effective in inhibiting late glycation and AGE formation (post-Amadori) than early stage glycation as reported by Rahbar et al. [52]. Indeed, metformin had only moderate inhibitory effects on glycation of hemoglobin by the α-glucosonolactone, reaction which leads to the formation of HbA1c, a well known early glycation product. However, metformin showed a significant stronger inhibitory effect on N-acetylglycyl-lysine methyl ester dipeptide cross-linking by ribose and on glycated BSA crosslinking to rat tail tendon collagen, assays which evaluate AGE formation and AGE cross-linking.
Glycation inhibition by metformin

**Metformin reaction with sugars and dicarbonyl compounds**

One possible explanation for the glycation inhibiting properties of metformin can be explained by its ability to trap reactive carbonyl species. This mechanism has been demonstrated in vitro where reaction products of metformin with either glyoxal or MG have been identified. In near physiological conditions, metformin directly reacts in vitro with glyoxal and methylglyoxal leading to the formation of stable TZP derivatives (Fig 3) [47]. Several research groups also confirmed the formation of TZP products from metformin – MG reaction [53, 54]. Aminoguanidine has been shown to react with MG to form two isomeric triazines, while TZP-like products have not been identified following this reaction [35]. This suggests that different chemical reactions leading to dicarbonyl trapping occur with metformin and aminoguanidine in spite of their structural similarities. TZP derivatives are not the only reaction products of metformin with MG. Substantial levels of less stable metformin-MG products were identified, and it is possible that these also could in-part account for the observed reduction in MG associated with metformin therapy [47]. Recently, Thornalley’s group has identified hydroimidazolone structural isomers and tautomers, as early stage adducts of metformin reaction with MG [54]. These early stage adducts have a formation rate faster than TZP derivatives.

In addition to this carbonyl trapping effect of metformin as potential mechanism for inhibiting glyoxidation reactions, a metal-chelating action could be also involved. It is very well known that biguanides chelate metal ions [55] which are key co-factors of oxidation processes in Maillard reaction [56]. Moreover, an antioxidative effect of metformin has been also reported [57]. It could not be excluded that both these additional mechanisms contribute to the glycation inhibitory effect of metformin.

**In vivo evidence of metformin reaction with reactive carbonyl species**

TZP derivatives have been also detected in vivo indicating that the dicarbonyl trapping effect of metformin also occurs in animal models. Chou et al. have performed an interesting in vivo study looking for metformin-MG reaction products in different rat tissues by means of mass spectrometry [53]. Two hours after a single bolus intravenous administration of metformin (50 mg/kg) in non-diabetic Sprague-Dawley rats, a TZP precursor of molecular weight 201.23 resulting from metformin-MG linear condensation (Fig 3), was found in kidneys, liver, pancreas, intestinal tract, heart and eye. This precursor was also found in rat plasma and urine. On the contrary, it was not detected in brain where metformin levels were extremely low. These results show that metformin is able to react in vivo with MG even at the MG levels present in non-diabetic animals. The TZP end product was not detected in the Chou et al. in vivo study most probably because of the short time between metformin administration and sample analysis. Indeed, in in vitro experiments high TZP levels are found after 24-hour incubation whereas after 2 hours levels are very low [47].

**Metformin effects on carbonyl compound detoxification**

An alternative way to inhibit glycation process is decreasing glycating agent levels either by inhibiting their synthesis or increasing their elimination. For example, MG formation can be suppressed by decreasing accumulation of triosephosphates that occurs under hyperglycaemic conditions [58]. Very recently Brownlee’s group [59] have shown that a lipid-soluble thiamine derivative benfotiamine prevents experimental retinopathy by activating the...
pentose phosphate pathway enzyme transketolase, which converts glyceraldehyde-3-phosphate and fructose-6-phosphate into pentose-5-phosphate and other sugars. By decreasing glyceraldehyde-3-phosphate levels less MG is formed.

A complementary approach is to increase MG detoxification through activation of the glyoxalase system. Our studies have also suggested that an alternative explanation for the ability of metformin to reduce MG levels is enhancement of detoxification through the glyoxalase pathway [45]. This pathway has been shown to be the major factor responsible for oxidative detoxification of MG and leads to the production of D-lactate [60]. Red blood cell (RBC) incubations performed in 30 mM glucose have shown that MG levels increase progressively over 24 and 48 hours followed by elevation of D-lactate levels, indicating that hyperglycaemia can produce a consistent increase in flux through this pathway. In shorter incubations of RBCs with MG we have also quantified the Km and Vmax of glyoxalase, as well as the half life of MG, in control and diabetic subjects (Fig 4).

A possible role for enhanced glyoxalase activity in reduction of MG levels is supported by the observation that levels of D-lactate are significantly increased in the subjects on metformin therapy. The mechanism by which metformin might increase the activity of this pathway is not clear. Since reduced glutathione (GSH) is required for optimal glyoxalase activity [61], and the oxidative stress associated with the diabetic state frequently leads to decreased levels of reduced glutathione, one possibility is that metformin therapy may lead to increased GSH levels and enhance MG detoxification. The observation that metformin therapy can reduce oxidative stress and improved levels of reduced glutathione in vivo further supports this possibility [62].

Numerous past publications have suggested that metformin may be beneficial in reducing diabetes associated vascular risk [44, 57] and that its effect may exceed the benefits expected from its hypoglycaemic effect. Our initial clinical data suggest that one important explanation for this effect could be metformin-associated reduction in toxic dicarbonyls related either to the binding of α-carbonyls or to an increase in enzymatic detoxification. To further document this potentially important effect of metformin and to determine the mechanisms responsible for dicarbonyl reduction, we have performed additional studies.

**Evidence for glycation inhibition by metformin in diabetic patients**

Studies that we have performed in two separate populations with type 2 diabetes have demonstrated significant decreases in plasma MG levels in subjects treated with metformin. We have published results documenting this effect [45] and have subsequently confirmed these findings by comparing MG levels in an additional 25 type 2 diabetic subjects treated with > 1.5 g/day of metformin with 25 diabetic sub-
jects not on metformin with equivalent levels of glycaemic control. In agreement with previous results, we found that plasma MG was significantly reduced in metformin (+) vs metformin (−) patients (173.5 ± 29.8 vs 216.1 ± 51.0 nM, p = 0.03).

In subsequent studies we were able to identify the MG-metformin end product, TZP, in 52 type 2 diabetic patients treated with metformin [63]. Using also an accurate mass spectrometry technique TZP was identified in plasma and urine from type 2 diabetic patients treated with metformin. In metformin-treated patients, 24 h urine TZP levels were 528.3 ± 340.1 nmoles/g creatinine while plasma levels were 1.22 ± 0.94 nM. No TZP was detected in diabetic patients not treated with metformin. Renal clearance of TZP was ten times higher than that of MG (532.4 ± 802.4 vs 49.9 ± 18.9 ml/min) suggesting active renal excretion. Plasma and urinary levels of TZP were highly correlated, while urinary levels were significantly higher in subjects treated with more than 1,500 mg/day metformin compared to those treated with less than 1,500 mg/day (677.7 ± 392.6 vs 368.2 ± 224.0 nmoles). A significant inverse correlation was seen between MG and TZP supporting a possible role for metformin in lowering reactive carbonyl species and glycation processes in diabetic patients treated with metformin (Fig 5).

Although extracellular binding of MG by metformin is one possible mechanism accounting for the observed reduction of MG levels, TZP appears to be only one of several inactive end products resulting from this chemical reaction. The reduction in MG levels resulting from metformin therapy in our prior study was 30.9 nM relative to subjects not taking metformin [45], while the plasma concentrations of TZP have now been found to be 1.22 nM. Although a purely stoichiometric relationship between MG and TZP is difficult to determine, due to the rapid renal clearance of TZP relative to MG, it is unlikely that the observed decrease in MG is solely due to the formation of TZP. In the work of Ruggiero-Lopez et al. substantial levels of less stable metformin-MG products were identified, and it is possible that these could in-part account for the observed reduction in MG associated with metformin therapy [47]. Another short study of MG-metformin reactions in cell-free systems has suggested that the formation of other products, such as hydroimidazolones, could also account for binding and inactivation of MG [54]. Considering the significant relationship observed between in vivo TZP levels and reduction in MG, TZP does appear to serve as a useful marker of metformin-MG condensation.

An alternate hypothesis for the effect of metformin on MG levels in vivo would involve the glucose-lowering effect of metformin. This is unlikely to be playing an important role in our studies since the glycaemic control in the subjects treated or not treated with metformin were similar. In these studies we also investigated levels of another α-dicarbonyl compound, 3-deoxyglucosone (3DG), and found that they are not significantly affected by metformin treatment [45]. Since we have previously shown that 3DG levels correlate with glycaemic control [64], this finding would not support the lowering of glucose levels as the primary mechanism for the decrease in MG levels. Metformin has also been shown to reduce hepatic triglyceride production [44, 65, 66], which could subsequently result in less MG production by lipoxidation. This is unlikely to be a mechanism in our study population, however, since triglyceride levels were actually higher in the metformin-treated subjects relative to those on other agents.

As discussed above, metformin induced reduction in oxidative stress and increased GSH could have beneficial effects on MG detoxification by the glyoxylase pathway. Another potential benefit of reducing oxidative stress could relate to less oxidative modification and enhanced activity of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) [67]. Since GAPDH activity is important in regulating cellular levels of dihydroxyacetone and glyceraldehyde phosphate [68], this perturbation could also result in reduced MG production.
Conclusion

Metformin’s capacity to inhibit glycation is a recent discovery although this antihyperglycaemic agent has been used for the treatment of diabetes for four decades. Although relatively little data are available on this unique property of metformin, the studies that have been performed to date have shown consistent in vitro, and in vivo, evidence supporting metformin’s ability to inhibit glycation by reducing MG and AGE levels. One possible explanation for this effect is trapping and inactivation of reactive carbonyl species, thus leading to lower carbonyl stress and decreased formation of deleterious AGEs.

Our studies presented in this manuscript document extracellular binding of MG by metformin to form TZP. This condensation product appears to be only one of several inactive end products resulting from this chemical reaction and it is possible that these or other condensation products (hydroimidazolones) could be more indicative of inactivation of MG by metformin. Even though it is unlikely that the observed decrease in MG is solely due to the formation of TZP this compound does appear to serve as a useful marker for metformin-MG condensation.

In conclusion, inactivation of glycation products provides one possible mechanism to explain the beneficial effects of metformin on diabetic complications by mechanisms which are independent of its antihyperglycaemic effect. They also confirm that one metformin-MG condensation product, TZP, can serve as a clinical marker for a chemical reaction leading to the lowering of the important α-oxoaldehyde, MG. Additional studies of other possible condensation products, as well as other potential cellular effects of metformin on MG production, will help to clarify this potentially important effect of metformin and provide further rationale for using metformin to prevent long-term diabetic complications.

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Effect of intensive

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Glycation inhibition by metformin