

Research Article

Erythrocyte enzymes of Glyoxalase system as indicators of beneficial effects of antihyperglycemic agents in Type 2 Diabetes

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ABSTRACT

Background: Methylglyoxal (MG), a product of sustained hyperglycemia, is a reactive carbonyl toxin responsible for development of complications in diabetes. Glyoxalase system detoxify MG to prevent complications. Some antihyperglycemic agents, may inhibit deleterious effects of MG by independent mechanisms. It was considered worthwhile to identify such agents and to find out whether changes observed in the erythrocyte levels of Glyoxalase I, Glyoxalase II, Aldose Reductase & D-Lactate are indicators of the beneficial effects through their direct action on MG, or merely a result of good glycemic control in response to treatment.

Methods: The glyoxalase system was characterized in erythrocytes of blood samples from patients with Type 2 Diabetes (n = 147), and normal healthy control subjects (n = 40). Diabetics were divided into groups based on presence or absence of complications; & further divided into subgroups based on medication with sulphonylurea, metformin, insulin and combination therapy.

Results: Erythrocyte Glyoxalase I, Glyoxalase II, Aldose Reductase, and D-Lactate levels significantly increased in all diabetics, (p<0.001) relative to controls. A maximum rise of enzymes in T2D with complications was observed as compared to patients without complications (p<0.001). Inadequate glycemic control was observed in all diabetics, and enzyme levels significantly declined in groups treated with metformin, either as monotherapy or in combination with insulin.

Conclusions: Enzymes of Glyoxalase system indicate beneficial effects of metformin. Metformin reduces MG and minimizes worsening glycemic control leading to complications. Metformin renders protection through mechanism independent of its antihyperglycemic action.

Keywords: Glyoxalase, Lactate, Aldose Reductase, Metformin

INTRODUCTION

In Type 2 Diabetes (T2D), chronic hyperglycemia plays an important role in the development of complications.¹ Prolonged exposure to hyperglycaemia leads to accumulation of Methyl glyoxal (MG), a highly reactive dicarbonyl compound. MG is a major precursor of Advanced Glycation End products (AGEs) which are responsible for glycation of collagen, irreversible modifications of proteins and inhibition of cell growth leading to complications like Retinopathy, Neuropathy, Nephropathy and Ischemic Heart Disease (IHD)

/Peripheral Vascular Disease (PVD).²⁻⁴ MG is oxidized by Glyoxalase I & Glyoxalase II to D-Lactate, and also reduced by Aldose Reductase to acetol.⁵ (Fig. 1). Glutathione acts as a cofactor.

In terms of pathophysiology, T2D is characterized by decreased insulin secretion from pancreatic β cells and decreased tissue responsiveness to the normal action of insulin (i.e., insulin resistance), leading to hyperglycemia.⁶ The tiered drug treatment approach serves as a useful guidance for the pharmacotherapeutic management of T2D. Tier 1 therapies include lifestyle

interventions to decrease weight and increase physical activity, in addition to medication with metformin, sulfonylureas and insulin. Sulfonylureas stimulate insulin release from pancreatic β cells by binding to ATP-sensitive potassium channels on pancreatic β cells in a glucose-independent manner.⁷ Although Sulfonylureas are effective antihyperglycemic agents, interindividual variability exists in drug response. Sulfonylureas and insulin are known to improve hypertriglyceridemia, secondary to the glucose-lowering effect.

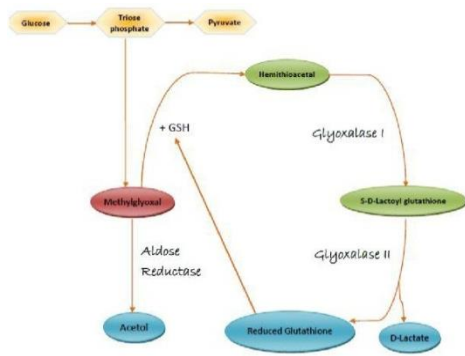


Figure 1: Detoxification of Methylglyoxal by glyoxalase system.

Management of diabetes is aimed at effective glycemic control & prevention of complications, through life style modification and medications. Some antihyperglycemic agents not only lower plasma glucose levels but may also decrease MG levels through its direct action on MG,⁸⁻¹⁰ thereby inhibiting formation of AGEs and thus alleviate diabetic complications.

The purpose of this study was to assess the effect of antihyperglycemic agents on Methylglyoxal metabolizing

enzymes in T2D. Limited attention has been given to the role of these detoxification enzyme systems. Hence, by observing the modifications in the glyoxalase system, it is worthwhile to find out whether complications can be prevented by glycemic control and/or by action of antihyperglycemics that lower the total MG load through mechanisms other than their glucose lowering action. This would presumably help in development of strategies, whereby the sequence from hyperglycemia to complications can be interrupted therapeutically.

METHODS

Study Subjects

The study included randomly selected 147 Type II diabetic patients from the outpatient Department of Endocrinology, BYL Nair Charitable Hospital, Mumbai Central, Mumbai. The patients were selected using a full standardized clinical review. Complications of diabetes like Retinopathy, Nephropathy, Neuropathy and Ischemic Heart Disease/Peripheral Vascular Disease were determined and graded according to standard clinical criteria. The patients were first divided into 2 major groups. Patients in Group I (n=60) under medication for 3 to 6 months, were not having any complications. The patients with complications, who were either receiving monotherapy or multitherapy, were included in Group II (n=87). Age and sex matched distribution of control and diabetic patients is depicted in Table I. Those who had no history of diabetes or no other co-morbid illness were selected as control. Group I and Group II patients were further grouped based on antihyperglycemic medication given to the patients. (Table 1). Group II patients had diabetes for the duration of 6 to 11 years. Patients with major metabolic or organ disease were excluded from the study.

Table 1: Age Sex distribution of control and Type 2 diabetics under medication.

Group	Medication	Total No	Age (Yrs.) (Mean±SEM)	Distribution of Sex Males Females	
Control	No Treatment	40	53.8 ± 2.16	22	18
	Sulphonylurea	19	55.1 ± 1.37	8	11
Group I*	Metformin	20	52.3 ± 1.67	9	11
	Insulin	21	54.8 ± 1.92	11	10
Group II#	Metformin	19	55.1 ± 1.37	9	10
	Insulin	23	53.3 ± 1.67	11	12
	Insulin + Metformin	20	55.8 ± 1.92	8	12
	Insulin + Sulphonylurea	25	56.1 ± 1.73	14	11

*Diabetics with no complications, receiving only monotherapy.

#Diabetics with complications, under monotherapy/combination therapy

Analysis

Isolation of red blood cells and preparation of hemolysate

Approximately 12 ml of blood was collected in heparinised container by venepuncture from all normal subjects and patients. Informed consent was obtained from all subjects. Part of the blood was used for estimation of Glycosylated Haemoglobin and D- Lactate. Red cell hemolysate was prepared by the method of Thornalley.¹¹

Estimation of biochemical variables

Glucose was estimated by GOD POD method of Trinder,¹² Glycosylated hemoglobin (HbA_{1c}) was measured using cation exchange resin.¹³ Activity of Glyoxalase I, Glyoxalase II,¹⁴ and Aldose Reductase¹⁵ were determined from hemolysate. D-Lactate, end product of Glyoxalase system, was determined in blood using D-lactate Dehydrogenase.¹⁶

Statistics

Data are presented as mean values with standard error of mean. The continuous variables were compared using the Student’s unpaired t-test. All the calculations were done using Microsoft Office Excel 2007. A two tailed P-value of less than 0.05 (P < 0.05) was considered to be statistically significant.

The study protocol was approved by the Institutional Ethics Committee of Topiwala National Medical College and B.Y.L. Nair Charitable Hospital, Mumbai.

RESULTS

Table 2 depicts biochemical parameters in three treatment groups of diabetics without complications and control

group. In the T2D groups without complications and under monotherapy, the levels of Glucose, HbA_{1c}, Glyoxalase I, Glyoxalase II, Aldose Reductase, & D-Lactate increased significantly (p < 0.001) in all the three subgroups of diabetics as compared to the control group. Glucose levels increased nearly 1.8 fold equally in metformin & insulin groups, and 1.6 fold in sulphonylurea group; whereas HbA_{1c} levels increased by 20 % in all the three treatment groups as compared to control.

Comparison between the three treatment groups, showed no significant difference in glucose and HbA_{1c} values. On comparing metformin treated group with insulin group and with sulphonylurea group, highly significant (P<0.001) increased levels of D-Lactate and highly significant (P<0.001) decreased levels of Glyoxalase I, Glyoxalase II & Aldose Reductase were observed in the patients treated with metformin as monotherapy.

Biochemical parameters in diabetics with complications and control are shown in Table 4. The levels of Glucose, HbA_{1c}, Glyoxalase I, Glyoxalase II, Aldose Reductase & D-Lactate increased significantly (p < 0.001) in all the four subgroups of diabetics as compared to the control group. Glucose levels increased more than 2 fold; whereas HbA_{1c} levels increased by 55% as compared to control. Comparison within the four subgroups of T2D with complications revealed no significant change in glycemic control (nearly equal HbA_{1c} values) in all the subgroups. Significantly decreased levels of Glyoxalase I, Glyoxalase II & Aldose Reductase along with significantly increased D- Lactate values were observed in metformin group & in (insulin + metformin) group when individually compared with insulin & with (insulin + sulphonylurea) groups. In general, all biochemical parameters were increased in T2D with complications (Table 3) as compared to T2D without complications (Table 2).

Table 2: Biochemical Parameters in control group and Diabetics without complications.

Parameter (Mean ± S.E.M.)	Control (n=40)	Sulphonylurea (n = 19)	Metformin (n = 20)	Insulin (n = 21)
Glucose (mg % in plasma)	80.85 ± 9.9	135.5 ± 4.32	141.75 ± 9.14 [#]	145.66 ± 3.31
HbA_{1c} (% in Blood)	5.61 ± 0.32	7.83 ± 0.72	7.57 ± 0.56 [#]	7.83 ± 0.70
Glyoxalase I (Units/ml of packed RBC)	28.11 ± 2.43	40.79 ± 7.88	33.85 ± 6.12 [*]	42.38 ± 5.47
Glyoxalase II Units/ml of packed RB(C)	9.3 ± 1.46	22.72 ± 7.15	16.35 ± 2.23 ^{**}	22.14 ± 5.33
Aldose Reductase (Units/ml of packed RBC)	0.45 ± 0.15	1.17 ± 0.86	0.76 ± 0.64 [*]	0.94 ± 0.40
D-Lactate (µmoles/L in blood)	22 ± 1.2	58 ± 2.1	74 ± 3.2 [*]	67 ± 3.4

All values for Sulphonylurea, Insulin & Metformin subgroups p < 0.001 v/s Control.

* p < 0.001 v/s Sulphonylurea & v/s Insulin

** p < 0.05 v/s Sulphonylurea & v/s Insulin

Nonsignificant v/s Sulphonylurea & v/s Insulin

Table 3: Biochemical Parameters in control group and Diabetics with complications.

Parameter (Mean ± S.E.M.)	Control (n=40)	Metformin (n = 19)	Insulin (n = 23)	Insulin + Metformin (n = 20)	Insulin + Sulphonylurea (n = 25)
Glucose (mg % in plasma)	80.85 ± 9.9	183.57 ± 8.5 [#]	177 ± 2.6	175 ± 3.8 [#]	176.78 ± 4.5
HbA1c (% in Blood)	5.61 ± 0.32	8.7 ± 0.21 [#]	8.4 ± 0.42	8.3 ± 0.18 [#]	8.4 ± 15
Glyoxalase I (Units/ml of packed RBC)	28.1 ± 2.43	37.15 ± 3.12*	53.19 ± 2.32	36.14 ± 2.16*	52.72 ± 3.87
Glyoxalase II (Units/ml of packed RBC)	9.3 ± 1.46	19.72 ± 7.15*	29.35 ± 2.23	17.14 ± 5.33*	28.17 ± 2.17
Aldose Reductase (Units/ml of packed RBC)	0.45 ± 0.15	0.77 ± 0.26*	1.38 ± 0.64	0.71 ± 0.23*	1.35 ± 0.22
D-Lactate (μmoles/L in blood)	22 ± 1.2	116 ± 4.1*	85 ± 4.2	120 ± 3.4*	87 ± 5.27

All values for Insulin, Metformin, (Insulin + Metformin), (Insulin + Sulphonylurea) subgroups $p < 0.001$ v/s Control.

* $p < 0.001$ v/s (Insulin + Sulphonylurea) & v/s Insulin

Nonsignificant v/s (insulin + sulphonylurea) & v/s insulin

DISCUSSION

In diabetics, prolonged hyperglycemia leads to increased formation of Methylglyoxal, leading to formation of AGEs which play a key role in development of complications. Hence the diabetic patients were divided into groups based on presence or absence of complications.

As shown in Table 2, glycemic control in diabetics without complications was inadequate. Patients did not achieve the expected normoglycemic state, despite receiving medications for 3 to 6 months. The extent of hyperglycemia indicated by HbA1c levels was nearly equal in all the treatment groups. Thus any relative modification of glyoxalase system in a treatment group is probably not a direct result of hyperglycemia, but reflects involvement of molecular mechanisms independent of the antihyperglycemic effect of medication.

Glyoxalase I, Glyoxalase II and Aldose Reductase play an important role in detoxification of MG (Fig. 1). Results from Table 2 shows significantly increased levels of these enzymes in all treatment groups under monotherapy compared to control. A similar increase has also been reported by Antony McLellan et al.⁴ The elevated levels of Glyoxalase I, Glyoxalase II and Aldose Reductase in erythrocytes can be possibly attributed to the induction of erythrocyte Glyoxalase system, due to the effect of hyperglycemia. The decline in Glyoxalase I, Glyoxalase II and Aldose Reductase and increase in D-Lactate was most significant ($P < 0.001$) in patients treated with metformin as compared to diabetics under sulphonylurea or insulin treatment. In the present study, though MG levels were not determined, it appears that decreased activity of glyoxalase enzymes is probably due to decrease in MG levels, through action of metformin. Here the decreased MG levels are not sufficient enough to induce enzyme synthesis. This implies that patients

taking dosage of metformin (1 gm. daily) get protected against the deleterious effects of increased levels of MG. This beneficial effect of metformin appears to be independent of its antihyperglycemic action¹⁷ wherein MG is inactivated by the direct binding of guadino group of metformin with the alpha dicarbonyl group of MG. Metformin also leads to increased production of glutathione which is required for MG detoxification via glyoxalase pathway¹⁸. Aldose Reductase catalyses the reduction of glucose to sorbitol, under hyperglycemia.^{19,20} However MG appears to be the preferred substrate for Aldose Reductase where it converts MG to form acetol. Hence it is possible that protection from MG toxicity is the normal function of Aldose Reductase.²¹ Increased levels of D-Lactate, the end product of glyoxalase pathway, indicated increased flux in glyoxalase pathway in diabetics showing periodic hyperglycaemic episodes. Sulphonylureas and insulin are known to improve hypertriglyceridemia, secondary to the glucose-lowering effect.²² However the present study revealed that Sulphonylurea and insulin had no role in detoxifying MG. In treatment groups of T2D patients without complications (Table 2), there was no significant correlation between the enzyme levels and degree of glycemic control (Coefficient of correlation $r < 0.4$, $p > 0.5$). Probably in these patients glucose control was not the decisive factor at the level of expression of any of these enzymes. Here, nonenzymic glycation may increase Glyoxalase activities due to prolonged chronic exposure to low or moderate glucose levels, leading to increased MG concentration. The persistent level of hyperglycemia (mild to moderate) leads to the phenomenon of "Hyperglycemia Memory",²³ inducing changes that remain unaltered during subsequent periods of normal glucose homeostasis. Since MG is mainly implicated in complications of DM, prevention of its formation or promotion of its detoxification might provide a means to limit the diabetic complications.

T2D patients with complications (Table 3) showed poor glycemic control for all treatment groups (monotherapy and combination therapy). The poor glycemic control could be attributed to history of long standing diabetes (6 to 11 years) and consequent manifestations of complications. The increased HbA1c levels (Mean 8.5 %) were nearly equal in all the groups, indicating unbiased effectiveness of antihyperglycemic agents observed in the four drug regimen groups. The beneficial effect of metformin is also observed in patients.

Table 3 treated with either metformin as monotherapy or in combination with insulin; as evinced by marked decline in Glyoxalase I, Glyoxalase II, & Aldose Reductase along with significant increase in D-Lactate. These patients had complications of diabetes like Retinopathy, Nephropathy, Neuropathy and Ischemic Heart Disease/Peripheral Vascular Disease. Maximum induction of Glyoxalase I, Glyoxalase II and Aldose Reductase in patients without complications (Table 2) and with complications (Table 3) was observed for treatment groups not under metformin medication. Here enzyme induction is caused by relative increase in MG levels, due to lack of protection rendered by metformin. Here, molecular mechanisms underlying development of complications is directly reflected by the increased enzyme levels.

In diabetics with complications (Table 3), the enzyme levels were greater as compared to diabetics without complications (Table 2); more so in groups not treated with metformin.

In the present study, though MG levels were not determined, it appears that decreased activity of glyoxalase enzymes is probably due to decrease in MG levels through action of metformin. With the exception of metformin group, in all other diabetics with complications a significant correlation ($r > 0.9$, $p < 0.01$) was observed between the enzyme levels and degree of glycemic control. Thus, in the absence of metformin, there is increased MG production or decreased detoxification leading to greater induction of enzymes associated with increasing degrees of hyperglycemia. In presence of metformin, the enzyme induction is decreased because MG levels are decreased; and there was no positive relationship between enzyme levels and HbA1c levels ($r = 0.2$, $p > 0.5$).

Action of glyoxalase enzymes on MG finally culminates in the formation of D-Lactate, which serves as an important index of the activity of Glyoxalase enzymes and has been indicated in diabetic complications.¹⁹⁻²³ It has been reported that in human red cell cultures,²⁴ D-lactate levels were increased during hyperglycemia. Thus in diabetics, D-Lactate levels may be elevated during episodes of hyperglycemia. Blood D-Lactate levels were increased 4 to 6 fold in patients with complications, reflecting poor glycemic control in these patients (Table 3). The management of T2D requires aggressive

treatment to achieve glycemic control and prevent complications. When lifestyle interventions fail or are not feasible, pharmacological therapy may be an important resource to prevent T2D. Several different drug classes have been studied for this purpose. Metformin lowers plasma glucose levels by suppressing hepatic gluconeogenesis and glycogenolysis, while increasing peripheral sensitivity to insulin. Metformin's negligible risk of hypoglycemia in monotherapy, its efficacy, and security profile, beneficial cardiovascular and metabolic effects makes this drug the first glucose lowering agent of choice. A large amount of evidence in literature supports its use even in cases where it would be contra-indicated mainly due to the fear of lactic acidosis; which has been over-emphasized as the available data suggest that lactate levels and risk of lactic acidosis do not differ appreciably in patients taking this drug versus other glucose-lowering agents.³⁰

Metformin has been proposed to cause a mild and transient inhibition of mitochondrial complex I which decreases ATP levels and activates AMPK-dependent catabolic pathways,²⁶ increasing lipolysis and β -oxidation in white adipose tissue²⁸ and reducing neoglucogenesis. The resultant reduction in triglycerides and glucose levels could decrease Methylglyoxal (MG) production through lipoxidation and glycooxidation, respectively.^{25,27} Despite all its benefits, metformin is contraindicated in patients with heart failure due to the potential risk of developing lactic acidosis, a rare but potentially fatal metabolic condition resulting from severe tissue hypoperfusion.²⁹

In conclusion, all parameters were increased in diabetics, maximum rise being observed in patients with complications. Degree of inadequate glycemic control was nearly equal within T2D subgroups without complications and within T2D subgroups with complications. Decreased levels of enzymes & increased values of D-Lactate in all diabetics treated with either metformin as monotherapy or metformin in combination with insulin, indicates tendency of metformin to render protection against development of complications. Positive significant correlation, between the enzyme levels and degree of glycemic control, for non-metformin groups and lack of positive correlation for the same parameters in metformin groups is suggestive of protective role of metformin through mechanism independent of its antihyperglycemic action. Prevention or delay in development of complications in diabetes can be achieved by sulphonylurea and insulin only through glycemic control, and by metformin through glycemic control and by antihyperglycemics lowering MG levels. Thus Glyoxalase I, Glyoxalase II, Aldose Reductase & D-Lactate can serve as indicators of the beneficial effects of metformin through its direct action on MG.

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