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Research Article

The role of enzymes of the glyoxalase system in relation to complications in type II diabetes mellitus

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ABSTRACT

Background: Metabolism of methylglyoxal by the glyoxalase system may be linked to the development of diabetic complications. It was considered worthwhile to find out whether changes observed in the levels of glyoxalase I, glyoxalase II, aldose reductase & D-lactate are prognostic indicators for the development of complications of diabetes or merely reflect the result of changes associated with complications.
Methods: The glyoxalase system was characterized in erythrocytes of blood samples from patients with type II diabetes mellitus (n=177), and normal healthy control subjects (n=40). Diabetics were divided into 3 main groups based on presence or absence of complications.
Results: The concentrations of RBC glyoxalase I, glyoxalase II, aldose reductase, and D-lactate were significantly increased in all groups of diabetic patients, ($P < 0.001$) relative to controls. Comparison between groups showed maximum rise of enzymes in group I and group III ($P < 0.001$); and maximum rise of D-lactate in group III ($P < 0.001$). Within the groups of patients with complications, enzyme levels were markedly increased in patients with IHD/PVD (ischaemic heart disease/peripheral vascular disease) and decreased in patients with nephropathy.
Conclusion: Results of this study suggests a positive relationship between increased activity of erythrocyte enzymes of glyoxalase system and poor or moderate glycemic control. The increased enzyme levels in patients without complications indicate their role as prognostic markers for development of complications. Molecular mechanisms for development of Nephropathy appear to be different from those of Neuropathy and Retinopathy.

Keywords: Glyoxalase I, Glyoxalase II, Lactate, Aldose reductase

INTRODUCTION

The last half century has seen a surge in the worldwide incidence of Diabetes Mellitus (DM) and cardiovascular diseases (CADs).[1] India is the unofficial "Diabetes capital of the world" with an estimated 40 million affected and the numbers expected to escalate and reach 80 million[2] by 2030. The prevailing risk factors in Indians and the so called "Asian Indian Phenotype" predisposes them to DM type II and its associated complications. Prolonged exposure to hyperglycemia, leads to accumulation of the triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in endothelial cells, and to the formation of the highly reactive dicarbonyl compound methylglyoxal (MG).

Recent research has demonstrated a significant role of MG in DM and in the development of diabetic complications.[3] Elevated MG levels are observed in DM. It has numerous metabolic origins, has effects on insulin secretion through signalling pathways, and is a major precursor of Advanced Glycation End products (AGEs).

AGEs cause irreversible modification of proteins, DNA and RNA and inhibition of cell growth leading to complications.[4-6] The glyoxalase system is the major route for the detoxification of MG and for prevention of its toxicity. In hyperglycemia associated with diabetes the increased formation of MG, by the down regulation of the glyoxalase system has been linked to the development of vascular complications of diabetes - nephropathy, retinopathy, neuropathy and cardiovascular disease.[7] The glyoxalase system comprising two separate metalloenzymes, glyoxalase I and glyoxalase II, bring about two consecutive reactions involving the thiol-containing tripeptide glutathione as a cofactor. Methylglyoxal is converted by glyoxalase I into S-D-lactoyl-glutathione in the first reaction. Subsequently, glyoxalase II catalyzes the hydrolysis of this thiolester into D-lactic acid and free glutathione. In addition to the glyoxalase system, aldose reductase plays a role in conversion of the toxic aldehydes into inactive alcohols (Figure 1).

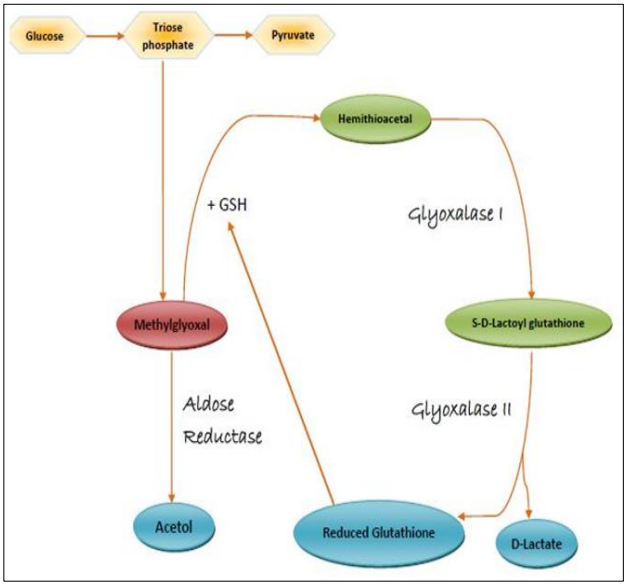

Figure 1: Detoxification of methylglyoxal by the glyoxalase system.

The purpose of this study was to investigate the possible modifications in the glyoxalase system, its relationship with degree of glycemic control, and its protective role, if any, against the deleterious reactions of MG associated with Diabetes and its complications. Limited attention has been given to the role of these detoxification enzyme systems. Hence, it was considered worthwhile to find out whether changes (if any) observed in the levels of glyoxalase I, glyoxalase II, aldose reductase are prognostic for the development of complications of DM or merely reflect the result of changes associated with complications. 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 177 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 3 major groups. The new patients who were diagnosed for the first time as diabetic and not under medication, and without any complications were considered as group I (n=30). Patients in group II (n=60) under medication, were not having any complications. The patients under various combinations of medications, with complications of diabetes were included in group III (n=87), and further subgrouped as: a) Retinopathy (n=22), b) Neuropathy (n=23), c) Nephropathy (n=16) and d) Ischemic heart disease/Peripheral Vascular Disease (IHD/PVD) (n=26), as per standard clinical criteria. These patients had diabetes for the duration of 6 to 11 years. Patients with major metabolic or organ disease were excluded from the study. The age and sex matched 40 normal subjects were selected randomly as normal controls, who had no history of diabetes and no other co-morbid illness (Table 1).

Analysis

Isolation of red blood cells and preparation of hemolysate:

Approximately 12 ml of blood was collected in heparinised container by venipuncture from all normal subjects and patients. Informed consent was obtained from all subjects. Part of the blood was used for estimation of glycosylated hemoglobin and D-lactate. Red cell hemolysate was prepared by the method of Thornalley.[6]

Estimation of biochemical variables

Samples were assayed for glyoxalase I, glyoxalase II, & aldose reductase activity. Glucose was estimated by GOD POD method of Trinder;[8] and glycosylated hemoglobin (HbA_{1C}) was measured using cation exchange resin.[9]

Activity of glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6) from hemolysate was determined spectrophotometrically by method of Ratliff et al.[10] Activity is expressed as units/ml of packed RBC, where 1 unit is the amount of enzyme required to catalyze the formation/hydrolysis of 1 µmol of S-D-lactoylglutathione per unit under the assay condition.

Aldose reductase activity in red cell hemolysate was determined spectrophotometrically using method of David Vander Jagt.[11]

Concentration of D-lactate, end product of glyoxalase system, was determined in blood by an end point enzymatic assay using D-lactate dehydrogenase and measuring the rate of formation of NADH at 340 nm.[12]

Statistics

Data are presented as mean values with Standard Error of Mean (SEM). 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

The results of biochemical parameters analysed are summarized in Table 2.

Table 1: Age sex distribution of control and diabetic patients.

Group	Total No.	Age (years)	Distribution of sex	
		Mean ± SEM	Males	Females
Control	40	53.8 ± 2.16	22	18
Group I*	30	52.7 ± 1.78	16	14
Group II**	60	54.1 ± 1.37	28	32
Group III#	87	55.6 ± 1.94	45	42

*Diabetics with no complications, not under medication,
**Diabetics with no complications, under medication,
#Diabetics with complications, under medication

Table 2: Biochemical parameters in control and three diabetic groups.

Parameter (Mean ± SEM)	Control (n=40)	Group I (n=30)	Group II (n=60)	Group III (n=87)
Glucose (mg% in plasma)	80.85 ± 9.96	203 ± 8.13	140 ± 7.85*	181 ± 8.41
HbA_{1C} (% in blood)	5.61 ± 0.32	8.2 ± 0.81	7.74 ± 0.66*	8.81 ± 0.53
Glyoxalase I (Units/ml of packed RBC)	28.1 ± 2.43	48.2 ± 3.61	39.3 ± 2.49*	44.5 ± 3.75
Glyoxalase II (Units/ml of packed RBC)	9.3 ± 1.46	25.7 ± 1.88	21.7 ± 2.91**	24.9 ± 3.33
Aldose reductase (Units/ml of packed RBC)	0.45 ± 0.15	1.47 ± 0.18	0.95 ± 0.13*	1.17 ± 0.14
D-lactate (µmoles/L in blood)	22 ± 1.2	67 ± 2.2	66 ± 2.4#	96 ± 3.3

All values in Diabetic groups, P <0.001 v/s control
*P <0.001 v/s I & v/s III
**P <0.01 v/s I & v/s III
#Non-significant v/s I & P <0.001 v/s III

The mean concentration of glucose, HbA_{1C}, glyoxalase I, glyoxalase II, aldose reductase, & D-lactate were found to be increased significantly (P <0.001) in all the three groups of diabetics when compared to the control group.

Comparison between the groups of patients revealed decreased levels of all the parameters in group II when compared with corresponding levels in group I and group III.

Levels of D-lactate in group I and group II were significantly lower (P <0.001) when compared with group III. The rise observed for all parameters (except D-lactate) was highest in group I, followed by group III and then group II.

Table 3 depicts biochemical parameters in four subgroups of diabetics with complications and control group.

The mean concentration of glucose, HbA_{1C}, glyoxalase I, glyoxalase II, aldose reductase & D-lactate were increased significantly (P <0.001) in all the 4 subgroups of diabetics with complications when compared to the control group.

When comparison was done between the subgroups of patients with complications, D-lactate levels decreased significantly, and all other parameters increased significantly in IHD/PVD, when compared with nephropathy group. In the group of patients with nephropathy, glyoxalase II and aldose reductase levels declined significantly when compared with all the other subgroups.

A highly significant, nearly 2 fold rise in D-lactate was observed in patients with nephropathy when compared with other subgroups.

Table 3: Biochemical parameters in control group and diabetics with complications.

Parameter (Mean ± SEM)	Control (n=40)	IHD/PVD (n=26)	Neuropathy (n=23)	Nephropathy (n=16)	Retinopathy (n=22)
Glucose (mg% in plasma)	80.85 ± 9.9	190.5 ± 8.51*	176.8 ± 4.52	167.2 ± 2.61	185.4 ± 3.83
HbA_{1C} (% in blood)	5.61 ± 0.32	9.9 ± 0.42*	9.7 ± 0.21	9.3 ± 0.15	9.8 ± 0.18
Glyoxalase I (Units/ml of packed RBC)	28.1 ± 2.43	46.9 ± 3.1*	44.2 ± 2.9	40.9 ± 2.3	43.1 ± 2.2
Glyoxalase II (Units/ml of packed RBC)	9.3 ± 1.46	25.6 ± 1.6*	25.2 ± 2.4	21.5 ± 1.9**	27.39 ± 0.91
Aldose reductase (Units/ml of packed RBC)	0.45 ± 0.15	1.27 ± 0.31*	1.35 ± 0.12	0.77 ± 0.03**	1.31 ± 0.14
D-lactate (µmoles/L in blood)	22 ± 1.2	87 ± 4.5*	85 ± 3.9	130 ± 5.1**	82 ± 4.8

All values in groups I, II, III, P <0.001 v/s control
*P <0.01 v/s nephropathy
**P <0.005 v/s neuropathy & v/s retinopathy

DISCUSSION

It has been documented that prolonged hyperglycemia leads to increased formation of methylglyoxal. And further, modification of proteins by MG leads to formation of AGEs which play a key role in development of complications. Hence the diabetic patients were subdivided into 3 major groups based on presence or absence of complications.

As shown in Table 2, in all the groups, the patients were hyperglycemic with inadequate glycemic control. However, group II patients who were under medication and without any complications showed relatively better glycemic control than Group I and Group III. This is clearly evinced from the levels of glucose and Hb_{A1c}. Severe hyperglycemia is expected in Group I patients because they were being diagnosed for the first time and hence were not under any medication. Similarly, in Group III, the poor glycemic control could be attributed to history of long standing diabetes (6 to 11 years), and consequent manifestations of complications.

Glyoxalase I, glyoxalase II and aldose reductase play an important role in detoxification of MG (Figure 1). Results from Table 2 shows significantly increased levels of these enzymes in all groups of diabetic patients compared to the 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 untreated persistent hyperglycemia in group I patients and long standing diabetes in group III. In group II, the diabetics did not show any 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 phenomenon of "hyperglycemic memory",[13] inducing changes that remain unaltered during subsequent periods of normal glucose homeostasis.

Aldose reductase (EC 1.1.1.21), a member of multigene family of NADPH dependent aldoketo reductase catalyzes the reduction of glucose to sorbitol, via polyol pathway under hyperglycemic conditions.[14,15] 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.[16] A similar rising trend in levels of aldose reductase has been reported by Hamada Y and Kitoh R.[17,18]

The glyoxalase system is present in cytosol of all cells and is the major route for metabolism of methylglyoxal. The action of these enzymes on MG finally culminates in the formation of D-lactate. D-lactate, being the endproduct of glyoxalase system, 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 clinical DM, D-Lactate levels may be elevated during episodes of hyperglycemia.

Blood D-Lactate levels were increased 2 to 3 fold in group I patients and 4 to 6 fold in group III, probably reflecting poor glycemic control in these patients.

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. Here, time duration is the risk factor for development of complications. As shown in Table 3, all the patients showed poor glycemic control, despite receiving various combinations of medications. Maximum induction of glyoxalase I, glyoxalase II and aldose reductase was observed in IHD/PVD group as

compared with other subgroups; but only when compared with nephropathy, the mean concentrations were statistically significant (P <0.01). Hotspot protein targets of methylglyoxal most likely play a key role in the mechanisms underlying the development of vascular complications in diabetes. In particular, modification of integrin binding sites in extracellular matrix proteins leads to endothelial cell shedding. And further, modification of mitochondrial proteins, increased formation of reactive oxygen species and modification of apolipoprotein B100 of low density lipoprotein lead to increased atherogenicity.[9]

The enzyme levels declined significantly in nephropathy as compared to neuropathy and retinopathy group. This implies that molecular mechanisms underlying development of nephropathy is not reflected in the altered enzyme levels to the same extent as in cases of neuropathy and retinopathy. Increased aldose reductase in neuropathy and retinopathy[25-28] as well as increased lactate in retinopathy has been documented. Maximum significant rise was observed in patients with nephropathy. In general, all group III patients were under mixed medication including metformin. Metformin therapy increases levels of reduced glutathione which is required for the continuing action of glyoxalase system leading to production of its end product i.e. D-lactate. Large prospective studies[29] show a strong relationship between hyperglycemia and macrovascular and microvascular complications. In all subgroups of diabetics with complications a significant correlation (r >0.9, P <0.01) was observed between the enzyme levels and degree of glycemic control. As shown in Table 2, the increased levels of enzymes in group II diabetics who did not present any complications, probably serve as signals for the impending development of complications. Secondly, a consistent overall increase in all enzyme levels in most of the patients with complications, indicate lack of specificity of a particular enzyme for a specific nature of complication.

Hence the increased enzyme activities cannot serve as diagnostic indicators for a specific complication, but they can serve as prognostic indicators, because the induction of enzyme activity probably occurs much earlier than the onset of complications.

In conclusion, result of this study suggest a positive relationship between increased activity of erythrocyte enzymes of glyoxalase system, and poor or moderate glycemic control in diabetes mellitus type II. Molecular mechanisms of altered glyoxalase system related to development of nephropathy appear to be different from neuropathy and retinopathy. Increased D-Lactate levels pose a risk factor for nephropathy. The increased enzyme levels in patients without complications indicate their role as prognostic indicators for development of complications in future.

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