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Glyceraldehyde-3-phosphate dehydrogenase activity as an independent modifier of methylglyoxal levels in diabetes

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Abstract

Methylglyoxal (MG) may be an important cause of diabetic complications. Its primary source is dihydroxyacetone phosphate (DHAP) whose levels are partially controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Using a human red blood cell (RBC) culture, we examined the effect of modifying GAPDH activity on MG production. With the inhibitor koningic acid (KA), we showed a linear, concentration-dependent GAPDH inhibition, with 5 μM KA leading to a 79% reduction of GAPDH activity and a sixfold increase in MG. Changes in redox state produced by elevated pH also resulted in a 2.4-fold increase in MG production at pH 7.5 and a 13.4-fold increase at pH 7.8. We found substantial inter-individual variation in DHAP and MG levels and an inverse relationship between GAPDH activity and MG production ($R=0.57$, $P=0.005$) in type 2 diabetes. A similar relationship between GAPDH activity and MG was observed in vivo in type 1 diabetes ($R=0.29$, $P=0.0018$).

Widely varying rates of progression of diabetic complications are seen among individuals. We postulate that modification of GAPDH by environmental factors or genetic dysregulation and the resultant differences in MG production could at least partially account for this observation.

Keywords: Methylglyoxal; Glyceraldehyde-3-phosphate dehydrogenase; Diabetes; Red blood cell; Carbohydrate metabolism

1. Introduction

Nonenzymatic glycation has been shown to play an important role in the development of diabetic complications [1]. One of the most powerful glycating agents is methylglyoxal (MG), an alpha ketoaldehyde that is found at elevated concentrations in diabetes. This compound is extremely reactive as a glycating agent of proteins and other important cellular components and has been shown to be toxic to cultured cells [2–6]. In vivo, chronic oral administration of MG in mice induces renal damage [7]. Additional support for the role of MG in the development of complications comes from studies demonstrating a protective effect for the guanidino compounds (aminoguanidine and metformin) which have the ability to scavenge this dicarbonyl [8–10]. Because of the toxic potential of MG and its role as an important source of advanced glycation end-products (AGEs), it is important to understand the mechanisms that regulate its production in the disordered metabolism associated with diabetes.

The production of MG occurs primarily from the triose-phosphate (TP) intermediates in the glycolytic pathway, which include dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) [11,12]. MG is produced by spontaneous nonenzymatic elimination of the phosphate group or decomposition of the enediol intermediate at the active site of triose phosphate isomerase [13]. Other minor sources of MG include cytochrome P4502E1 catalyzed oxidation of acetone from ketone bodies, [2] cleavage of Amadori products, [14] and catabolism of threonine via aminoacetone [15].

Accumulation of TPs and the subsequent production of MG can occur as a consequence of increased glycolytic flux and/or a reduced ability of the glycolytic enzymes downstream from TP to handle the increased flux. Of particular importance is the enzyme responsible for the metabolism of GAP, glyceraldehyde-3-phosphate dehydro-

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genase (GAPDH) [16], which catalyzes the reaction of GAP to 1,3 diphosphoglycerate.

Recent evidence has suggested a variety of functions for GAPDH, including a role in endocytosis, translational control of gene expression, nuclear tRNA export, DNA replication and repair as well as cell apoptosis [17]. Several other seemingly unrelated functions have now been attributed to this enzyme, including involvement in membrane fusion and microtubule bundling [18]. In light of its many apparent functions, perturbations in GAPDH have recently been connected to cellular dysfunction in several neurodegenerative diseases [19–24].

The GAPDH gene has been sequenced and its structure appears to be highly conserved throughout nature [25]. Only one copy of the gene appears to be expressed, although recent work in brain cells questions this assumption and suggests the presence of heterogeneity in GAPDH [26]. Multiple isoforms of GAPDH, probably due to posttranslational modification, are found in nature although their significance is not clear.

The activity of GAPDH is exquisitely sensitive to modification by cellular processes that have been shown to be significantly perturbed in diabetes. Since the enzyme contains a highly reactive thiol at its active site (cysteine149) [27], it is sensitive to modification by a variety of compounds with subsequent loss of enzyme activity [28–30]. These compounds include reactive aldehydes [31–34], glycating agents [35,36] and oxidative radicals [37]. Pseudohypoxia secondary to excessive accumulation of L-lactate from pyruvate in diabetes can result in a decreased ability to generate NAD+, which in turn leads to impaired GAPDH activity. Since GAPDH activity is dependent on regeneration of the oxidized form of this nucleotide, any factor that produces elevated NADH/NAD+ ratios could have similar effects [16,38].

Structural mutation of the GAPDH gene and altered gene expression could lead to significantly altered function and increased TP and MG production in response to hyperglycemia. GAPDH gene expression can be regulated by hormonal, nutritional and metabolic factors, including insulin, which can increase GAPDH mRNA and activity up to 10-fold in liver and fat cells. Mutations induced in the promoter region of GAPDH can lead to altered regulation of gene expression by nuclear transcription factors [39]. Circadian controller genes have also been shown to produce cyclic regulation of GAPDH activity [40].

To our knowledge, there is currently very little information concerning the activity of GAPDH as it relates to its effects on MG production and potential complication outcomes in diabetes. The work presented here will explore some basic questions concerning these issues.

These studies examine the effect of the specific GAPDH inhibitor, koningic acid (KA), as well as changes in redox state and reduced glutathione (GSH) levels on the production of triose phosphates and MG. We will also determine the relationship of these same parameters to GAPDH activity and MG production in red blood cells (RBCs) from patients with type 2 diabetes with varying degrees of glycemic control. Finally, we examined the relationship between GAPDH activity and plasma MG levels in a large group of patients with type 1 diabetes.

2. Materials and methods

2.1. Materials

All materials are from Sigma/Aldrich with the exception of the following: 1,2-diamino-4,5-dimethoxybenzene (DDB) (Toronto Research Chemicals), C18 solid phase extraction cartridges (Phenomenex), diaphorase (ICN Biomedicals), Eagles Essential Vitamin Mix (Gibco/BRL). KA was a gift from K Hasumi of Tokyo Noko University, Tokyo, Japan.

2.1.1. Determination of reduced glutathione

A colorimetric DTNB-based GSH assay from Beutler et al. [41] is used in the determination of RBC glutathione levels.

2.1.2. Determination of GAPDH

Red cell GAPDH was determined spectrophotometrically using the method of Byers [42].

2.1.3. Determination of DHAP

Red cell DHAP was determined spectrophotometrically using the method of Michal and Beutler [43].

2.1.4. Determination of MG by HPLC

MG determinations by HPLC utilized a modification of the procedure by Ohmori et al. [44]. The assay is based on derivatization of MG and the internal standard 2-oxopentanoic acid with *o*-phenylenediamine (*o*-PDA), extraction and quantitation by UV monitoring. In brief, the sample of media or plasma is prepared for derivatization by perchloric acid (PCA) precipitation with 0.075 ml of 60% PCA per milliliter of sample. The PCA supernatant is treated with PDA and 2-oxopentanoic acid internal standard to a final concentration of 3.5 mM and 80 μM, respectively. Samples are incubated at 50 C for 1 h and subsequently brought to a pH of 2.5 with 1 N KOH and extracted twice with ethyl ether. Due to the volatility of the MG derivative, an aqueous phase is added to the sample using 20 μl of 10 mM potassium phosphate pH 2.8 and mixed well. Samples are dried in a centrifugal evaporator until only the aqueous portion remains. The sample is then brought to a volume of 50 μl with acetonitrile, and 20 μl is injected for HPLC analysis.

Chromatography was performed using gradient elution on a Prodigy 5 μM, ODS-3, 250 × 4.5-mm column. Solvent A was 10 mM potassium phosphate, pH 2.8, and solvent B was acetonitrile. Ultraviolet detection is at 316 nm. Quantitation is performed against an internal standard based

calibration curve. The lower limit of quantitation for a 1.5-ml sample is 71 nM. In day coefficient of variation for 20 determinations was 6.4%.

2.1.5. Determination of MG by gas chromatography-mass spectrometry (GC-MS)

The GC-MS assay was based on the method of McClellan et al. [45] using DDB as the derivatizing agent. We have modified the assay for GC-MS and have enhanced the sensitivity and reliability of quantitation by using a C13 labeled MG internal standard. Following addition of 45 μl of 60% PCA on ice the sample is centrifuged at 18,000 × g for 20 min; 225 μl of the supernatant is then mixed with 25 μl of 10 mM DDB in duplicate, samples are incubated at 50 C for 90 min and immediately adjusted to pH 2.3 with 0.5 M dibasic sodium phosphate. This step is followed by solid phase extraction utilizing 100 mg, C-18 cartridges that were pretreated with 1-ml methanol and 1-ml water, followed by 1 ml of 20 mM ammonium phosphate pH 2.3. After loading, the cartridge is washed twice with 1 ml of 20 mM ammonium phosphate pH 2.3, and eluted with 1 ml of methanol. The eluent was dried in a centrifugal evaporator until approximately 50 μl of total volume remains. Ethyl acetate, 100 μl, was added to each tube and the samples were centrifuged at 1000 × *g*. The ethyl acetate supernatant was used for GC-MS analysis.

Analysis was performed with a Shimadzu QP5050A GC/MS equipped with an HP-1701, 0.25-μm film, 0.25-mm ID, 3-m capillary column. Carrier gas is helium with a split-less injection mode, injector at 280 °C, and interface at 290 °C. Sampling time is 1.5 min, followed by a temperature program where column temperature was increased from 40 to 280 °C at 40°/min and held at 280 °C for 4 min. The advanced flow control is used to maintain a constant carrier gas flow. One-microliter samples are analyzed by selective ion monitoring of the C12 and C13 MG derivative 6,7-dimethoxy-2-methylquinoxaline with target ions of 204.1, 189.1 and 161.1 for the C12 derivative and 207.1, 192.1 and 164.1 for the C13 derivative. Quantities were determined by comparing to a known quantity of C13 MG internal standard. For 0.6-ml plasma samples between day coefficient of variation was 4% while the lower limit of quantitation was 25 nM.

2.1.6. Determination of D-lactate by HPLC

We modified the assay of Ohmori and Iwamoto [46] and improved its reproducibility while shortening the analysis time by pre-treating the sample to remove endogenous pyruvate by derivatization with PDA and removal with a C18 resin. With endogenous pyruvate removed, the D-lactate concentration is equivalent to the enzymatically derived pyruvate.

Briefly, 1 ml of plasma or media samples is treated with 75 μl of 60% PCA, centrifuged, and the supernatant is treated with 35 mM PDA for 2 h at 50 C to react with endogenous pyruvate. Sample pH is adjusted to 6.0 with KOH, centrifuged and applied to a pre-equilibrated C-18 cartridge. The eluent from the cartridge is used in the rest of the analysis. It is added to a reaction mixture containing thioctamide, diaphorase, NAD, hydrazine sulfate and D-lactate dehydrogenase (D-LDH), and incubated at 37 C for 2 h. The 2-ketobutyric acid internal standard is added at this point along with PDA, and acidification with HCl. Samples are incubated 1 h at 50 C. After the pH is adjusted to 3.0 with sodium phosphate dibasic they are extracted with 1 ml of ethyl acetate. Samples are taken to dryness in a centrifugal evaporator and reconstituted with 125 μl of HPLC buffer. Twenty microliters of sample was used in the analysis.

Chromatography was performed on a Phenomenex Prodigy, 5 μM, C-18, 4.6 × 250-mm column. Samples were eluted isocratically with 75% 10 mM potassium phosphate pH 2.9 and 25% acetonitrile. Ultraviolet detection was at 334 nM. Between day coefficient of variation for media was 9% with a lower limit of detection of 4.0 μM.

2.1.7. RBC culture conditions

RBCs were cultured according to the culture system developed by Freedman [47]. Media consisted of Earle's salts with 18 mM HEPES and 25 mM sodium bicarbonate supplemented with Eagle's Essential Vitamin Mix, 1% BSA, L-cysteine, L-glutamine, L-glutamic acid, glycine and gentamycin sulfate. Cells were incubated at 37 °C and 5% CO_2 in 25-cm^2 tissue culture flasks. In our experiments strict control of media pH was essential for reproducible results in the system.

2.1.8. RBC culture

Blood was collected in the fasting state into EDTA vacutainer tubes and immediately placed on ice. Samples were centrifuged and RBCs were separated from the plasma and buffy coat. RBCs were cultured for 2.5–3 days with a 5% hematocrit. In order to stabilize pH, media was pre-incubated overnight at 5% CO_2 prior to the addition of cells. Cells were then added and incubated overnight with 5 mM glucose before increasing the glucose concentration to 30 mM. At time zero, 25 mM glucose was added to the cells and one half of the media and cell suspension were removed as a zero-time sample. After 32 or 48 h of incubation the remaining cells were harvested and processed. Cells were pelleted and snap-frozen and media was frozen for later analysis. Typically, red cells were analyzed for MG, GAPDH activity and GSH. Media was analyzed for MG and DL.

When the above analyses were performed in six separate cell cultures utilizing RBCs from the same subject, the following coefficients of variation were achieved for each parameter at the 48-h time point: Media MG = 12%, media DL = 9%, GSH = 10% and GAPDH = 7%.

2.1.9. Effect of pH on media MG

RBCs from normal donors were isolated and cultured for 24 h in EBSS with 30 mM glucose and varying sodium bicarbonate concentrations designed to produce a range of

pH from 7.1 to 7.8 in a 5% CO_2 environment. Media MG levels were determined by HPLC.

2.1.10. Incubations with KA

Normal RBCs were isolated, washed with EBSS and treated for 1 h with KA. The cells were washed twice and placed into the usual culture conditions. The effect of KA on the activity of GAPDH, the concentration of GSH and the production of MG and DL were evaluated after 48-h exposure to 30 mM glucose.

2.2. Diabetic study populations

The first population was made up of 20 subjects with type 2 diabetes being treated with varying combinations of oral agents and insulin. All subjects had normal renal function with a mean creatinine clearance of 110 ml/min. The mean HbA1c was 7.9% and the mean age was 64 years. There were equal numbers of males and females and the mean duration of diabetes was 15 years.

The second population consisted of 110 subjects with type 1 diabetes participating in "The Natural History of Diabetic Nephropathy Study" [48]. The mean age was 22.3 ± 7 years and the mean duration of diabetes was 12.3 ± 4.1 years. All subjects had normal renal function with mean GFR (iothalamate clearance) of 131 ± 26 ml/min.

We also studied 29 non-diabetic controls with a mean age of 41.1 ± 13.8 years.

3. Results

3.1. Inhibition of GAPDH by KA

Since KA is a potent and specific inhibitor of GAPDH [49] we took advantage of this effect to determine the relationship between the level of GAPDH inhibition and production of MG in our cell culture system. As shown in Fig. 1A, there is an inverse linear relationship between GAPDH activity and KA concentration with a detectable decrease of GAPDH activity at 2 μM and a decrease to 21% of control at 5 μM KA. Production of both MG and D-lactate were significantly enhanced at 48 h in a nonlinear dose response (Fig. 1B) with a sixfold increase in MG and a fourfold increase in DL over control levels. This effect did not appear to be secondary to changes in oxidative stress since GSH levels were minimally effected by 5 μM KA (Fig. 1B). An increase in the abundance of MG relative to DL was observed after exposure to KA, with the MG/DL ratio (pmol/nmol) increasing from 0.278 in control cells to 0.454 in cells treated with 5 μM KA.

3.2. Effect of pH on GAPDH activity and MG production

The NADH/NAD ratio of the cell is an important modulator of GAPDH activity [16]. Since alterations in pH can influence the NADH/NAD ratio and inhibit GAPDH, we cultured normal RBCs with modified sodium bicarbonate levels resulting in precise changes in pH. As shown in Fig. 2, increasing pH dramatically increased media MG concentrations, with a sharp 2.4-fold increase occurring as pH rises just above the physiological range (pH = 7.5), and 13.4-fold increase at pH 7.8. DL levels were also measured and followed a nearly identical pattern. Concurrently we observed a decrease in media pyruvate from 80 μM at pH 7.4 to 15 μM at pH 7.8, indicating an altered L-lactate-to-pyruvate ratio.

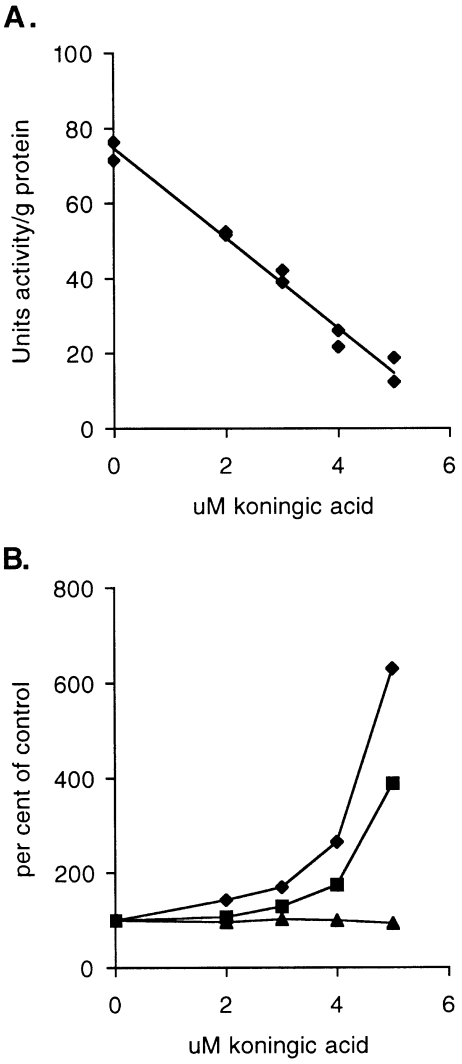

Fig. 1. (A) Inhibition of GAPDH enzyme activity by koningic acid. RBCs from a normal donor were isolated, washed with Earle's Balanced Salts and treated for 1 h with the indicated concentrations of KA. The cells were again washed twice and placed into the usual culture conditions described in Materials and methods and incubated for 48 h. Each point represents duplicate analysis of GAPDH activity for individual flasks. Line statistics: $y = -11.922x + 74.797$, $R^2 = 0.9828$. (B) Effect of koningic acid on MG, DL, and GSH levels. Using RBCs treated as in 1A, media MG and DL levels and intracellular GSH levels were determined and expressed as percent of control levels. Each point represents the mean of two flasks. Chart symbols: (♦) methylglyoxal, (■) D-lactate, (▲) GSH.

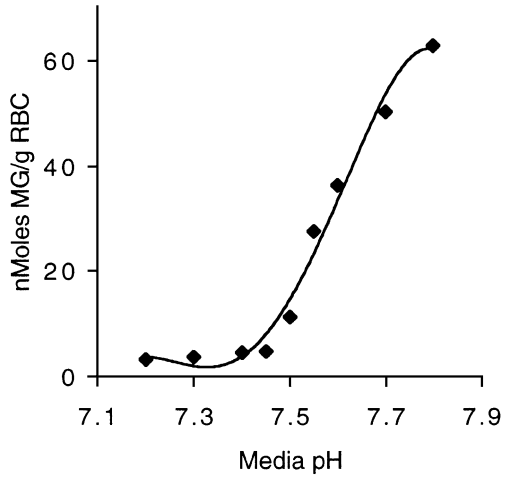

Fig. 2. Effect of pH on media MG. RBCs from a normal donor were isolated and cultured for 24 h in EBSS with 30 mM glucose and varying sodium bicarbonate concentrations designed to produce a range of pHs from 7.1 to 7.8 in a 5% CO_2 environment. Media MG levels were determined. Each point represents the mean of three individual flasks.

3.3. GAPDH activity and MG levels in type 2 diabetes

To study the effect of variation in GAPDH activity on MG levels in diabetic patients we utilized freshly harvested RBCs in our culture system. Cells obtained from type 2 diabetic subjects exhibited wide variation in intracellular DHAP and MG levels when exposed to 30 mM glucose for 32 h. DHAP levels ranged from 28 to 195 nM/g protein and MG levels from 900 to 2100 pM/g protein (Fig. 3). However, we observed no correlation between the preceding level of glycemic control as measured by HbA_{1c} and the production of MG and DL in culture. GAPDH activity in these cells showed a significant negative correlation ($P=0.005$) with MG concentration at 32 h (Fig. 4A). GAPDH activity at zero hour (prior to the 32-h incubation with 30 mM glucose) also showed a significant inverse correlation with MG ($P=0.016$, $R=0.5$, data not shown). As expected, intracellular DHAP levels showed a significant positive correlation with production of MG, (Fig. 4B).

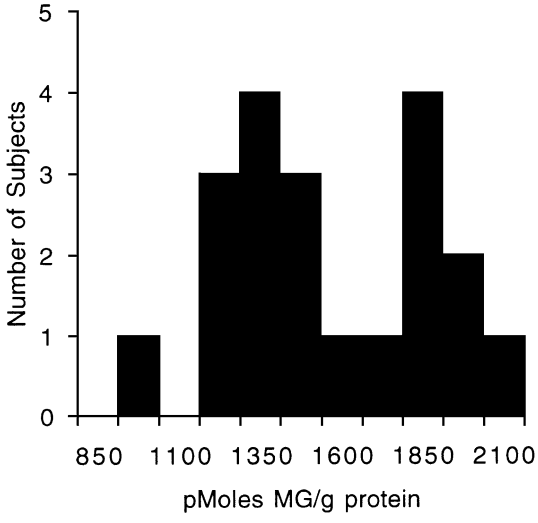

Fig. 3. Variable generations of MG from hyperglycemia. RBCs from patients with type 2 diabetes were cultured for 32 h under conditions described in Materials and methods. Subjects showed widely divergent increases in intracellular levels of MG from 900 to 2100 pmol of MG per gram of protein.

3.4. MG production and GAPDH activity and diabetic complications

We observed a significant increase in mean MG production in cultured RBCs from the small number of subjects with type 2 diabetes and proliferative retinopathy ($N=4$) compared to no retinopathy ($N=9$) ($P=0.02$ by ANOVA).

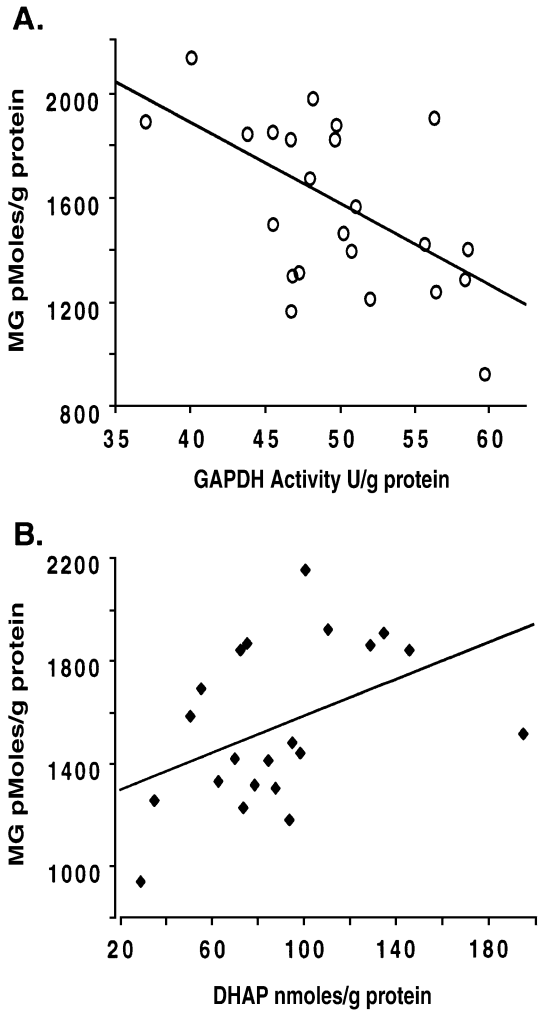

Fig. 4. (A) GAPDH activity determines methylglyoxal levels. RBCs from patients with type 2 diabetes were cultured for 32 h under conditions described in Materials and methods. Intracellular MG and DHAP levels and GAPDH activity were measured in the RBCs. A significant inverse correlation is seen between GAPDH activity and MG levels ($P=0.005$, $R=0.57$). (B) Intracellular DHAP concentrations determine methylglyoxal levels. A significant correlation is seen between DHAP concentrations and MG levels ($P=0.04$, $R=0.45$).

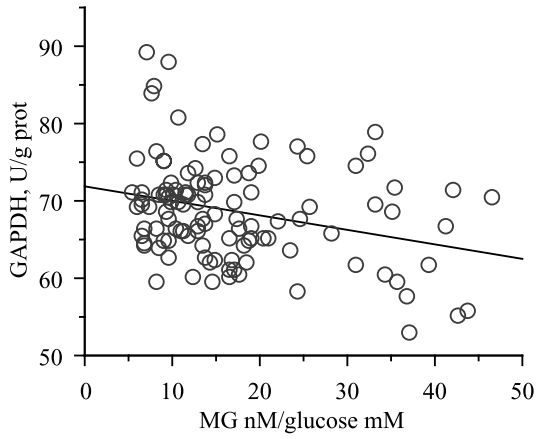

Fig. 5. GAPDH activity correlates negatively with MG production. RBC GAPDH activity, plasma MG levels, and plasma glucose levels were determined in a group of 84 patients with type 1 diabetes and 29 non-diabetic controls. MG is expressed as MG concentration in nanomolar divided by plasma glucose levels in millimolar to correct MG levels for the ambient glucose concentration. Regression analysis reveals a significant negative correlation ($P=0.0018$, $R=0.291$) between GAPDH activity and corrected MG levels.

We also found that GAPDH activity in RBCs from patients with evidence of coronary artery disease tended to be lower than in patients with no history of disease although significance at the $P<0.05$ level was not quite achieved ($P=0.069$).

3.5. GAPDH activity and MG levels in type 1 diabetes

To further characterize GAPDH activity in the clinical setting we measured plasma MG and glucose levels, and RBC GAPDH activity in 110 subjects with type 1 diabetes and in 29 non-diabetic controls. To adjust for the effect of plasma glucose on MG we expressed MG production per millimolar of glucose. This combined population regression analysis showed a significant negative correlation between GAPDH activity and the ratio of MG/glucose ($P=0.0018$, $R=-0.29$) (Fig. 5). These results suggest a measurable in vivo relationship between GAPDH enzyme activity in RBCs and the rate of production of MG from glucose.

4. Discussion

Since methyglyoxal is a potent glycating agent associated with diabetic complications [2,50–52], detailed knowledge of the biology of production and detoxification of this compound is essential to understanding its role in diabetes, aging and other diseases.

GAPDH controls a crucial metabolic step that determines the levels of MG precursors and can therefore regulate its production [11]. To study the effect of GAPDH inhibition we utilized a RBC culture system and demonstrated that inhibition by KA predictably increases media MG and DL concentrations (Fig. 1A and B). Detectable increases in MG accumulation were seen at 30% inhibition of enzyme activity while 79% inhibition led to a sixfold increase in MG production. Intracellular GSH, an important indicator of cellular health and oxidative stress, did not show significant change supporting a direct effect for KA on GAPDH activity (Fig. 1B). The observed increase in MG relative to DL in cells exposed to 5 μM KA could be attributed to a decrease in the efficiency of MG detoxification by the GSH-dependent glyoxylase system. We did observe a small decrease in GSH concentration after 5 μM KA treatment but it is not clear if this or other factors influence the conversion of MG to DL.

Since KA appears to have specificity for certain S-loop configurations, and the sequence of the S-loop in the enzyme can be crucial to inhibitor action [49], the literature was examined for its potential interaction with other important enzymes in the glycolytic pathway. In the initial work done on KA it was not found to inhibit *triosephosphate isomerase* or *phosphoglycerate kinase* [53]. G6PD does not have an active site susceptible to attack [54] and LDH has no potential sites suitable for modification by KA. The enzyme one step downstream from GAPDH, phosphoglyceromutase, does have a Cys residue that could effect catalytic activity. This residue is outside the active site of the enzyme, however, and no data is available for the effect of KA on its activity [55].

Metabolic perturbations of the cell redox state can also influence GAPDH activity and the accumulation of MG [16]. We have validated this in our RBC incubation system by showing that a modest increase to pH 7.5 leads to a substantial 2.4-fold accumulation of MG while more dramatic 13.4-fold increases in MG production are seen as pH approaches 7.8 (Fig. 2). Increasing pH was associated with significant decreases in intracellular pyruvate levels, suggesting that changes in L-lactate-to-pyruvate ratio may be at least partially responsible for the observed increases in MG. These observations also suggest that episodes of mild alkalosis could lead to burst of increased MG production in the clinical setting.

Our data characterize the in vitro production of MG and DL after GAPDH inhibition by chemical modification or by modification of the NAD/NADH ratio. Both results confirm the predicted accumulation of MG and document the ability of our cell culture system to quantify the relationship between GAPDH activity and the subsequent accumulation of metabolites.

When RBCs from diabetic subjects were cultured under hyperglycemic conditions we saw wide individual variation in production of MG and DL (Fig. 3). There was no correlation between the preceding level of glycemic control as measured by HbA_{1c} and the production of MG and DL in culture. MG and DL production increased with declining GAPDH activity and were directly related to the intracellular levels of DHAP, indicating the physiologic importance of GAPDH activity on ambient MG levels (Fig. 4A and B). In a large sample of type 1 diabetics and non-

diabetic controls, the amount of MG corrected for glucose concentration was inversely correlated with GAPDH activity (Fig. 5). This data again demonstrates the biological relevance of GAPDH regulation in vivo, and suggests that individual variations in GAPDH activity could lead to variable MG production on exposure to hyperglycemia.

In diabetes, elevated glucose concentration is an important factor in determining the appearance of diabetic complications [56]. The direct effects of glucose have been extensively studied, and have been shown to produce secondary metabolic changes that may have important implications for the ultimate appearance of complications. We believe that increased production of MG is one of the most important of these secondary effects.

The majority of published studies have demonstrated that diabetes-related hyperglycemia is associated with elevated levels (3–10 fold) of intracellular TPs (GAP and DHAP) in cardiac muscle [57], RBCs [58] and lens [59,60], although two other studies did not confirm these findings [61,62]. This accumulation of TPs and the subsequent production of MG could be a consequence of increased glycolytic flux and/or a reduced ability of the glycolytic enzymes downstream from TP to handle the increased flux. In all of the studies reported above, diabetes-related increased glycolytic flux was supported by the consistent elevation of glucose-6-phosphate and fructose-6-phosphate levels. Our observations of diabetes-related reductions in GAPDH activity supports a role for this enzyme in greater MG production.

Since GAPDH plays a significant regulatory role in the production of MG, it is important to understand the mechanisms by which hyperglycemia can regulate GAPDH activity. It has been shown that its activity is readily influenced by oxidative stress [63–65], direct glycation by glucose [35] or modification by MG [32], as well as changes in the NAD/NADH ratio [16]. Fig. 6 illustrates the major metabolic pathways that influence MG production and points out the possible mechanisms by which diabetes-related hyperglycemia might influence the activity of GAPDH. One possible mechanism is the increased flux through the sorbitol pathway produced by elevated glucose levels [66]. Since the conversion of sorbitol to fructose requires NAD, this increased flux would reduce the amount of this nucleotide available for GAPDH activity, leading to GAPDH inhibition and enhanced production of MG [16]. Increased polyol flux also produces a greater demand for NADPH during the conversion of glucose to sorbitol, thus competing for NADPH in the hexose monophosphate shunt and ultimately reducing regeneration of GSH from GSSG [66]. Depletion of GSH through this mechanism could adversely effect the cell by inhibiting the GSH-dependent glyoxylase system, or by lowering the cellular GSH related antioxidant defenses leading to oxidative modification and inactivation of GAPDH [49,67]. Increased glucose concentration can also enhance oxidative stress though the production of glycation-derived free radicals

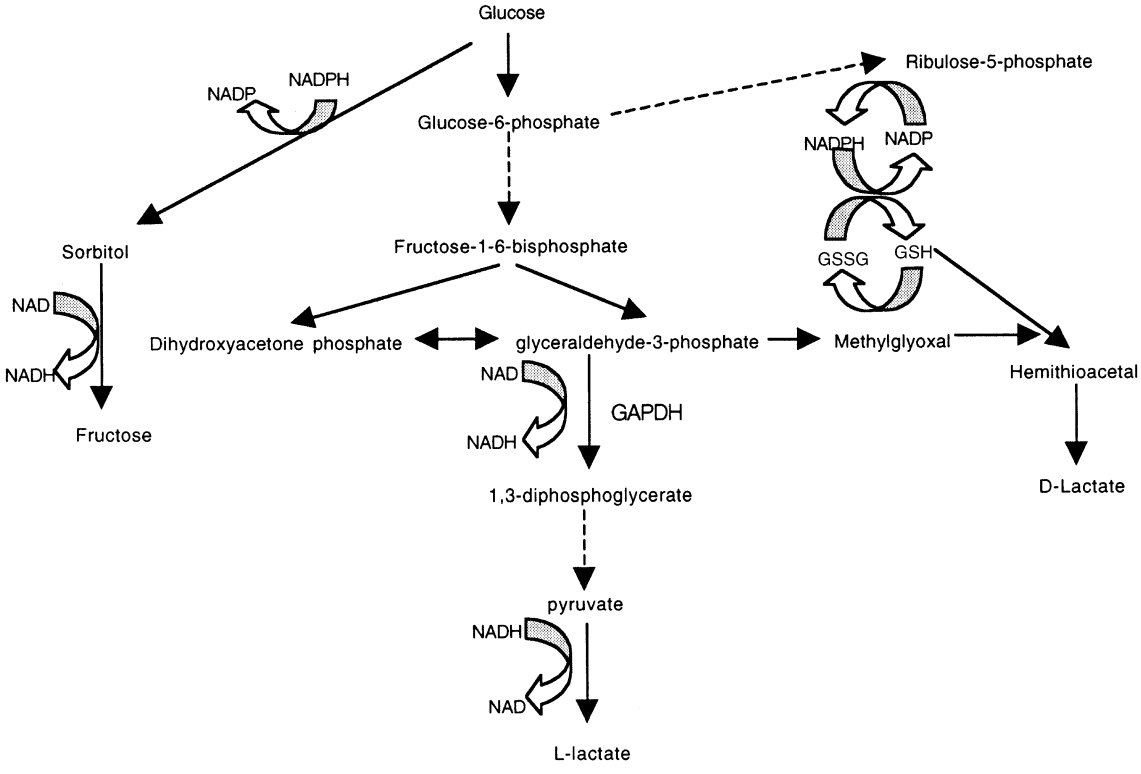

Fig. 6. Major metabolic pathways influencing methylglyoxal production.

that in turn can produce reactive aldehydes and ketones from lipid substrates [31,63,66]. One possible mechanism for this has been proposed by Nishikawa et al. [64] who hypothesize that elevated glucose can alter mitochondrial metabolism thus enhancing the production of reactive superoxide. This in turn impacts the activity of protein kinase C, aldose reductase, and increases MG production via GAPDH inhibition [6,68]. All of these studies show that regulation of GAPDH by environmental factors has the potential to be an important independent modifier of MG levels and potentially of diabetic complications.

GAPDH has traditionally been considered to be a constitutive "housekeeping" enzyme, but recent studies have documented a variety of novel functions for this protein including a role in endocytosis, the translational control of gene expression, nuclear tRNA export, DNA replication and repair as well as cell apoptosis [17]. GAPDH inhibition has been implicated as a potential factor in the pathogenesis of several inherited degenerative neuorological diseases, [20–22]. The mechanism of tissue damage is not clear but one possibility is that cell toxicity via GAPDH inhibition and overproduction of MG could play a role. Amelioration of neurotoxicity by MG inactivating agents such as metformin, aminoquanidine and carnosine also supports this hypothesis [69–71]. Decreased GAPDH activity may also play a role in the high rates of atherosclerosis associated with diabetes and the insulin resistance syndrome [72]. Based on the data presented in this manuscript and the diverse studies implicating the role of GAPDH in the pathogenesis of vascular and neural damage, we propose that excess MG production may be at least partially responsible for some of the observed toxic effects seen with GAPDH inhibition. We are currently investigating the possible role of GAPDH in diabetic complications and are exploring more detailed mechanisms of GAPDH inhibition using molecular techniques.

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