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Stimulation of insulin release by glyceraldehyde may not be similar to glucose

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Abstract

Glyceraldehyde (GA) has been used to study insulin secretion for decades and it is widely assumed that β-cell metabolism of GA after its phosphorylation by triokinase is similar to metabolism of glucose; that is metabolism through distal glycolysis and oxidation in mitochondria. New data supported by existing information indicate that this is true for only a small amount of GA's metabolism and also suggest why GA is toxic. GA is metabolized at 10–20% the rate of glucose in pancreatic islets, even though GA is a more potent insulin secretagogue. GA also inhibits glucose metabolism to CO_2 out of proportion to its ability to replace glucose as a fuel. This study is the first to measure methylglyoxal (MG) in β-cells and shows that GA causes large increases in MG in INS-1 cells and D-lactate in islets but MG does not mediate GA-induced insulin release. GA severely lowers NAD(P) and increases NAD(P)H in islets. High NADH combined with GA's metabolism to CO_2 may initially hyperstimulate insulin release, but a low cytosolic NAD/NADH ratio will block glycolysis at glyceraldehyde phosphate (GAP) dehydrogenase and divert GAP toward MG and D-lactate formation. Accumulation of D-lactate and 1-phosphoglycerate may explain why GA makes the β-cell acidic. Reduction of both GA and MG by abundant β-cell aldehyde reductases will lower the cytosolic NADPH/NADP ratio, which is normally high.
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Keywords: Glyceraldehyde; Methylglyoxal; NAD(P)/NAD(P)H ratio; Glyceraldehyde metabolism; Aldehyde reductase; Glyoxalase; D-Lactate; Pancreatic islet β-cell

Over 200 publications of D-glyceraldehyde (GA)'s[1] use in studies of insulin secretion have appeared since it was first discovered to mimic the electrophysiologic effect of glucose on the β-cell [1] and to be a potent insulin secretagogue [2,3]. Glucose, the most potent physiologic insulin secretagogue, stimulates insulin secretion by aerobic glycolysis and GA has been assumed by many researchers including ourselves [4] to stimulate insulin release very similarly to glucose; that is solely by entering the glycolytic pathway via the triokinase reaction to form triose phosphates and from this step onward undergoing glycolytic and mitochondrial metabolism identical to that of glucose (Fig. 1). The current work presents evidence to suggest that this pathway accounts for only a small portion of GA's metabolism and uses our new data and data previously reported by us and others to support the idea that GA metabolism by the β-cell differs significantly from glucose metabolism. GA is generally observed to be a more potent insulin secretagogue than glucose at equimolar or lower concentrations [3,5–18] even though, as shown herein, the rate of oxidation of GA to CO_2 by pancreatic islets is much lower than that of glucose. It has been suggested previously that stimulation of insulin release by GA is only partially similar to that of glucose [19,20] or does not require its metabolism [18]. It has also been proposed that GA's insulinotropism is related to its

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[1] *Abbreviations used:* GA, glyceraldehyde; GAPDH, glyceraldehyde phosphate dehydrogenase; MG, methylglyoxal.

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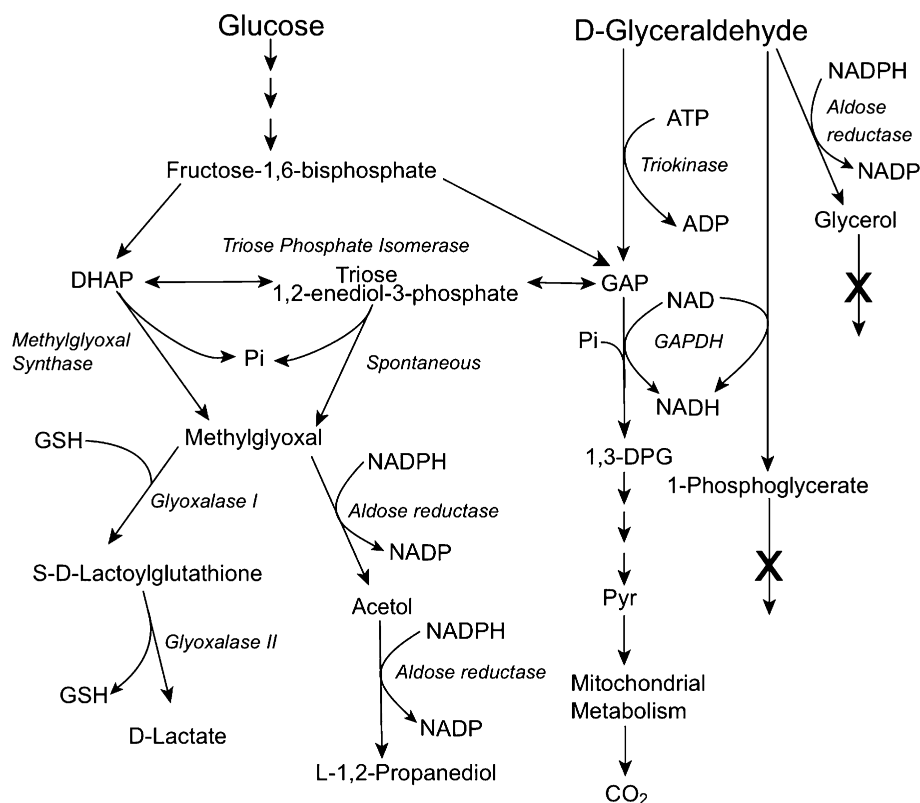

Fig. 1. Pathways of glucose and glyceraldehyde metabolism in the insulin cell.

metabolism to methylglyoxal and/or is caused by methylglyoxal contaminating glyceraldehyde preparations [21–23]. The current work is the first to report measurements of methylglyoxal in the β-cell and shows that although GA forms a massive amount of methylglyoxal, methylglyoxal formation does not account for GA's insulinotropism. The current study also shows that the β-cell contains a high level of glyoxalase I the first enzyme of the glyoxalase pathway [24] and that methylglyoxal formed from GA in the β-cell (Fig. 1) [24] is converted to high levels of D-lactate via this pathway.

A major difference between GA and other secretagogues is that GA is toxic to the β-cell. Pancreatic islets that have been exposed to GA do not release insulin in response to metabolizable secretagogues and monolayers of insulin cell lines maintained in the presence of GA for several hours cells die and may even become detached and floating in the medium. Best et al. [17,18] have shown that GA acidifies the β-cell, while other secretagogues, such as glucose and α-ketoisocaproate, cause a slight alkalinization of the β-cell. We propose this is because GA metabolism forms high concentrations of acidic products 1-phosphoglycerate and D-lactate in the β-cell. GA can be a substrate in the reaction catalyzed by glyceraldehyde phosphate dehydrogenase, an enzyme that is extremely abundant in β-cells. In contrast, the activity of triokinase, the enzyme that allows GA to enter the glycolysis pathway by converting it to glyceraldehyde phosphate (Fig. 1), is very low in the β-cell [19]. By comparing the relative activities of the two enzymes in β-cell cytosol with GA as a substrate it can be calculated that, at insulinotropic concentrations of GA, the rate of product formation from GA in the glyceraldehyde phosphate dehydrogenase reaction is 20-fold higher than in the triokinase reaction. When GA is a substrate in the glyceraldehyde phosphate dehydrogenase reaction, the theoretical product is 1-phosphoglycerate which is not a physiologic intermediate and cannot be further metabolized [19].

The current work shows that GA increases NAD(P)H and lowers NAD(P) levels and increases the L-lactate/pyruvate ratio in the islet without significantly increasing L-lactate. This is consistent with GA lowering the cytosolic NAD/NADH ratio by acting as a substrate in the glyceraldehyde phosphate dehydrogenase reaction. When GA is applied to an islet, the immediate effect of increased NADH plus early metabolism of a small amount of GA completely to CO_2 might explain GA's ability to hyperstimulate insulin release, but the long term effect of a very reduced NAD/NADH ratio will be to block glycolysis at the step catalyzed by glyceraldehyde phosphate dehydrogenase. As shown in the current study, GA's ability to interfere with glucose oxidation out of proportion to its ability to replace glucose as a fuel, plus the increased levels of glyceraldehyde phosphate and dihydroxyacetone phosphate previously observed in GA-exposed pancreatic islets [27], are also consistent with a block of glycolysis at glyceraldehyde phosphate dehydrogenase. Increased glyceraldehyde phosphate will be diverted to methylglyoxal formation (Fig. 1). The extreme decrease in flux through glycolysis will limit

mitochondrial energy production. This and the low pH from the decreased cytosolic NAD/NADH ratio, coupled with the accumulation of acidic products 1-phosphoglycerate and D-lactate, as well as increased levels of the toxic metabolite methylglyoxal, likely account for GA's toxicity to the β-cell.

The activities of aldehyde reductases are very high in the β-cell [28] and these enzymes catalyze the reduction of GA to glycerol and of methylglyoxal to propanediol (Fig. 1). Although as shown herein, these reactions do not contribute to GA's insulinotropism, this will lower the cytosolic NADPH/NADP ratio and since this ratio is usually high, it also is not normal for the cell.

Research design and methods

Materials

Sprague–Dawley rats of either sex weighing 200–300 g were obtained from Harlan Sprague–Dawley, Madison, WI. Methylglyoxal stock solutions were manufactured through acid hydrolysis of dimethylacetal and standardized through HPLC as previously described [31]. D- and D-L-Glyceraldehyde, methylglyoxal D-lactate dehydrogenase and all other chemicals were from Sigma Chemical, St. Louis, MO, in the highest purity available. βTC6-7 F cells were from Shimon Efrat and INS-1 cells were from Claus Wollheim via Barbara Corkey.

Islet studies

Islets were isolated from rats allowed free access to chow and water. Studies of insulin release [32] and secretagogue oxidation [33] were as previously described. Islets were incubated for metabolite measurements also as previously described [34]. Briefly, islets (100) were incubated in 200 ml Krebs Ringer bicarbonate solution, pH 7.3. After 30 min, the Krebs Ringer solution was quickly removed and 50 μl of 6% perchloric acid was added to the islet pellet. The acid-precipitated islet pellet was saved for protein analysis and the perchloric acid extract was neutralized with about 14 μl of 15% KOH and metabolites in the neutralized extract were measured.

Cell culture

INS-1 cells were cultured in RPMI 1640 medium supplemented with 1 mM pyruvate, 50 μM β-mercaptoethanol, 10% dialyzed calf serum, 100 U penicillin/ml, and 100 μg streptomycin/ml. Before each experiment, the cells were grown in 15 cm tissue culture dishes to confluence and switched to fresh RPMI 1640 media containing 0, 10 or 20 mM glyceraldehyde for 0, 8 or 24 h. The culture medium contained 1 mM glucose which provides carbohydrate for any needed synthesis of nucleotides or structural molecules. Cells were harvested and washed three times with cold PBS to minimize extracellular glyceraldehyde. Final pellet volume was generally 100 μl. Samples were resuspended in 500 μl of 130 mM acetic acid, frozen at 70 °C and saved for methylglyoxal analysis. INS-1 cells and βTC6-7F cells were also grown to confluence in RPMI 1640 media (contains 11.1 mM glucose) and then maintained at 5 mM glucose for 24 h, at 3 mM glucose for a second 24 h and then at 3 or 20 mM glucose for a third 24 h. Cells were harvested at the end of the third day and quickly washed three times in cold phosphate buffered saline (PBS) or harvested directly without washing in PBS. Samples of cells were saved for estimates of total protein and cell number. About 2×10^7 cells (80–100 μl cell pellet volume) were suspended in 500 μl of 130 mM acetic acid, frozen at −70 °C and saved for methylglyoxal analysis.

Glyoxalase assay

Glyoxalase I enzyme activity was estimated in a continuous spectrophotometric assay by measuring the formation of *S*-lactoylglutathione as the increase in absorbance at 240 nm [35]. The enzyme reaction mixture contained 2 mM methylglyoxal and 1 mM reduced glutathione in 50 mM sodium phosphate buffer, pH 7.2, maintained at 30 °C. The reaction was started with the addition of cytosol. The background rate of the complete reaction mixture was subtracted from the rate in the presence of cytosol to give the rate attributable to the enzyme. Cytosol was the supernatant fraction from centrifuging a homogenate of pancreatic islets or liver for 20,000*g* for 20 min.

Metabolite assays

L-Lactate and NAD(P)H were estimated as previously described [34]. D-Lactate was estimated by alkali-enhanced fluorescence exactly as previously described for the measurement of L-lactate except that 100 μg/ml D-lactate dehydrogenase (specific enzyme activity 2500 U/mg protein) from *Leuconostoc mesenteroides* was present in the assay mixture instead of L-lactate dehydrogenase [34]. Fluorescence of blanks containing all reagents except D-lactate or L-lactate dehydrogenases was subtracted from total fluorescence to give that attributable to D-lactate or L-lactate, respectively. In addition when indicated, values from assays of incubation mixtures containing all reagents except islets were subtracted from those containing islets.

Methylglyoxal assay

Methylglyoxal was detected as the 2-methylquinoxaline (2-MQ) derivative of methylglyoxal formed with *o*-phenylenediamine (*o*-PD) using a modification of the general approach previously described [31]. Sample volume was increased to 2.5 ml with 100 mM acetic acid and the samples were sonicated (5 s, 30 W). Samples were supplemented with 12.5-nmol 5-methylquinoxaline (5-MQ; internal standard) and 250-nmol *o*-phenylenediamine (derivatizing agent) and reacted at 20 °C for 3.5–4 h. Per-

chloric acid (5 M, 0.25 ml) was added to precipitate macromolecules and the resulting mixture was incubated on ice for 20 min and then centrifuged (12,000*g*, 10 min) to remove precipitated materials. The supernatant fraction was passed through a C-18 solid phase extraction (SPE) cartridge containing 0.5 g of tC18 (Waters Sep-Pak tC18 plus cartridge, Millipore Corp, Marlborough, MA) that had been prepared by flushing with 6–8 ml of acetonitrile followed by 6–8 ml of 10 mM KH_2PO_4, pH 2.5. The cartridge was rinsed with 1–2 ml 10 mM KH_2PO_4 (pH 2.5) and the retentate eluted with 2 ml of acetonitrile. Eluates were evaporated to a volume of 200 μl using a Speed-Vac Concentrator vacuum centrifugation unit (Savant Instruments, Farmingdale, NY) and filtered through 0.2 μm Gelman PVDF filters into sample vials. High performance liquid chromatography (HPLC) was performed as described previously [31] but with a mobile phase consisting of 35% acetonitrile/0.1% trifluoroacetic acid, pH 2.4, and 65% 10 mM phosphate/0.1% trifluoroacetic acid in HPLC grade water, pH 2.4. Under these modified conditions, 2-MQ eluted after 7.5 min and 5-MQ eluted after 11.2 min.

Glyceraldehyde purification

A 0.5 M solution of glyceraldehyde in 100 mM acetic acid was prepared. 2.5 μmol of *o*-PD was added to 10 ml of this solution (final concentration 250 μM) and the resulting mixture incubated at room temperature for 3.5–4 h. The reaction mixture was passed through a C-18 SPE cartridge containing 5 g of tC18 that had been prepared by flushing with 6–8 ml of acetonitrile followed by 6–8 ml of 10 mM KH_2PO_4, pH 2.5. The supernatant fraction was retained and analyzed for methylglyoxal content as described. The cartridges were rinsed with 1–2 ml 10 mM KH_2PO_4, pH 2.5, and the retentate eluted with 2 ml of acetonitrile. The retentate was concentrated and prepared for HPLC analysis.

Statistical analysis

Statistical analyses were done with ANOVA when multiple groups were compared. Pair-wise comparisons were confirmed with student's *t* test.

Results

GA oxidation

At 5–20 mM concentrations GA is metabolized to CO_2 at about 12–20% the rate of similar concentrations of glucose (Fig. 2A). When unlabeled GA was incubated together with [U-^{14}C]glucose, GA inhibited glucose metabolism to $^{14}CO_2$ to an extent that was proportionately much greater than that could be explained by metabolism of GA replacing glucose as a fuel. Methylglyoxal did not affect glucose metabolism (Fig. 2B).

D- and L-Lactate and NAD(P)H levels in islets

Islets were incubated with glucose or GA for 30 min and D-lactate was measured. D-Lactate was at the limits of detection in the absence of an addition or in the presence of glucose, but was increased about 100-fold by GA. *S*-Butylglutathione, an inhibitor of glyoxylase I (Fig. 1), lowered the GA-induced increase in D-lactate (Table 1). This is consistent with D-lactate being formed from GA via methylglyoxal. L-Lactate and pyruvate were increased as is well known to occur when glucose is applied to islets. GA caused a large increase in NAD(P)H and lowered NAD(P) resulting in a severely decreased NAD(P)/NAD(P)H ratio (Table 1). The failure of GA to signifi-

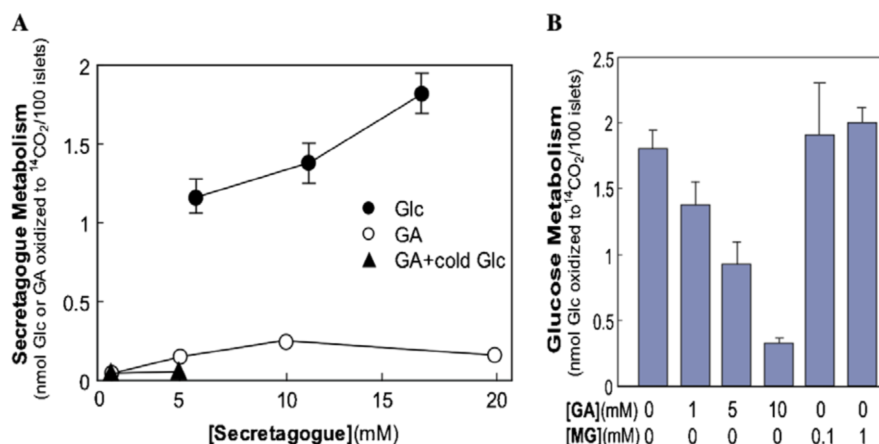

Fig. 2. Comparison of metabolism of glucose and glyceraldehyde by pancreatic islets and inhibition by glyceraldehyde of glucose metabolism. (A) Glyceraldehyde (GA) is oxidized at a much slower rate than glucose. Islets (100 per test tube) were incubated for 90 min at 37 °C with 100 μl of Krebs Ringer bicarbonate buffer, pH 7.4, containing various concentrations of [U-^{14}C]glucose (0.4 μCi/test tube) or [U-^{14}C]D-glyceraldehyde (0.5 μCi/test tube). Results are means ± SE with the number of 6–18 replicate incubations for each data point. Closed triangles show lack of effect of incubation of 5.5 mM unlabeled glucose on metabolism of labeled GA. (B) Inhibition by various concentrations of unlabeled GA or methylglyoxal on oxidation of 16.7 mM [U-^{14}C]glucose to $^{14}CO_2$.

Table 1
D-Lactate, L-lactate, pyruvate, and NAD(P) levels in pancreatic islets in the presence of glucose or glyceraldehyde

Incubation condition	Metabolite content (nmol/mg tissue protein)					Metabolite ratios	
	D-Lactate	L-Lactate	Pyruvate	NAD(P)	NAD(P)H	Lactate/pyruvate	NAD(P)/NAD(P)H
No addition	0.4 ± 0.4 (7)	11 ± 1 (19)	1.7 ± 0.3 (7)	2.4 ± 0.3 (16)	0.3 ± 0.1 (16)	7	8
Glucose	0.9 ± 0.4 (7)	28 ± 4 (11)***	2.7 ± 0.3 (7)*	2.0 ± 0.4 (9)	0.4 ± 0.2 (9)	10	5
Glyceraldehyde	116 ± 23 (4)***	19 ± 2 (22)***	1.9 ± 0.2 (7)	1.2 ± 0.3 (22)**	1.4 ± 0.5 (22)*	10	0.9
Glyceraldehyde and S-butylglutathione	53 ± 4 (5)***,†	NM	NM	NM	NM	NM	NM

Rat pancreatic islets (100 per test tube) were incubated with 16.7 mM glucose and 10 mM D-glyceraldehyde with or without 5 mM S-butylglutathione or no addition in 200 μl Krebs Ringer bicarbonate buffer, pH 7.3, for 30 min. Results are means ± SE with the number of observations in parentheses. NM indicates not measured.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ vs. no addition.

† $p < 0.001$, glyceraldehyde alone vs. with ± S-butylglutathione.

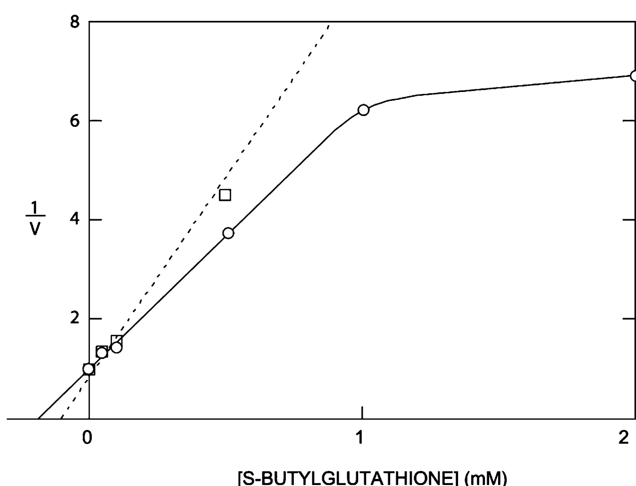

Fig. 3. Inhibition of pancreatic islet cytosol glyoxalase I activity by S-butylglutathione. This figure shows a Dixon plot of glyoxalase I enzyme activity in the presence of various concentrations of S-butylglutathione. Each data point is the average of measurements from four separate preparations of rat pancreatic islet cytosol (circles) or two preparations of liver cytosol (squares).

cantly increase L-lactate as much as glucose increased L-lactate, plus GA causing an increase in the NAD(P)H level, is consistent with GA's inhibition of aerobic glycolysis seen in Fig. 2B.

Glyoxalase activity

Glyoxalase I enzyme activity in cytosol from pancreatic islets was measured with saturating concentrations of the substrates methylglyoxal and reduced glutathione (Fig. 1) and compared with glyoxylase I activity in liver cytosol as a positive control. Activity in islet cytosol and liver cytosol was 337 ± 68 (4) and 1340 ± 53 (3) nmol S-lactoylglutathione formed/min/mg cytosol protein, (means ± SE (*n*)), respectively. The K_is for S-butylglutathione [36] in respect to glyoxalase I activity in islet cytosol and liver cytosol were similar and were 95 and 70 μM, respectively (Fig. 3).

Lack of effect of inhibitors of glyoxalase metabolism on insulin release from islets

Rat pancreatic islets were incubated with S-butylglutathione, an inhibitor of glyoxalase I [36], and insulin release was stimulated with either glucose or GA. Insulin release stimulated by either agent was not inhibited (Table 2). Methylglyoxal is the most effective substrate for aldose and aldehyde reductases which are plentiful in islets [28] and previous studies showed that inhibition of these enzymes does not inhibit glucose-induced insulin release [28]. Table 3 shows that the potent aldose reductase inhibitors Zopelrestat and Pfizer 10668 did not inhibit GA-induced insulin

Table 2
Lack of effect of a glyoxylase inhibitor on glucose- and glyceraldehyde-stimulated insulin release

[S-Butylglutathione]	Insulin release (percent of control) insulin secretagogue		
	Glucose	Glyceraldehyde (4 mM)	Glyceraldehyde (10 mM)
None	100 ± 12 (18)	100 ± 11 (18)	100 ± 9 (18)
50 μM	114 ± 14 (6)	ND	89 ± 9 (12)
100 μM	112 ± 11 (12)	116 ± 9 (12)	104 ± 11 (12)
1 mM	108 ± 13 (6)	117 ± 10 (6)	112 ± 10 (12)
3 mM	ND	ND	105 ± 15 (24)
5 mM	108 ± 14 (6)	104 ± 15 (6)	74 ± 9 (5)

Islets (5 per vial) were incubated for 1 h in 2 ml Krebs Ringer bicarbonate buffer, pH 7.3, in the absence or presence of glucose, D-glyceraldehyde, and the glyoxylase inhibitor S-butylglutathione. Results are expressed as a percentage of the stimulated value in the absence of the inhibitor and are means ± SE with the number of replicate incubations in parentheses. The average insulin release in the presence of 16.7 mM glucose, 4 mM D-glyceraldehyde, 10 mM D-glyceraldehyde, and no addition was 344 ± 39, 110 ± 12, 161 ± 17, and 24 ± μU/5 islets (means ± SE), respectively. ND indicates not done.

Table 3
Lack of inhibition of glyceraldehyde-induced insulin release by aldose reductase inhibition

Addition	Insulin release (% of control) [D-glyceraldehyde]	
	4 mM	10 mM
None	100 ± 12	100 ± 8
Zopelrestat	111 ± 11	103 ± 9
Pfizer 10668	104 ± 14	96 ± 11

Islets were incubated with glyceraldehyde as its D-L-isomers with or without 50 μM Zopelrestat or 50 μM Pfizer 10668. Conditions and 100% control values were the same as in Table 2. Results are means ± SE of 12 replicate incubations and are expressed as a percentage of insulin release in the presence of glyceraldehyde alone.

release. Both of these sets of data are consistent with the idea that methylglyoxal does not mediate insulin release.

Effect of GA exposure on intracellular methylglyoxal levels in INS-1 cells

Contaminant methylglyoxal was measured and found to be approximately 0.01% w/w of D-L-glyceraldehyde levels in D-L-glyceraldehyde stock solutions. Attempts to purify D-L-glyceraldehyde solutions by derivatizing contaminant methylglyoxal with *o*-phenylenediamine and removing the resulting 2-methylquinoxaline adduct through C-18 SPE were unsuccessful. Analysis of the retained fraction from the SPE column showed that approximately 95% of the methylglyoxal originally present in the GA solutions was derivatized and removed. However, within 24 h, the amount of contaminant methylglyoxal present in purified GA solutions returned to prepurification levels (data not shown) indicating methylglyoxal was formed from GA and was present and in equilibrium with GA in solution.

Unpurified GA stock solutions were therefore utilized in experiments with INS-1 cells to determine what influence GA had on intracellular methylglyoxal levels. Cell samples were washed well prior to methylglyoxal analysis to minimize associated extracellular GA and contaminant methylglyoxal. The methylglyoxal assay procedure was modified to provide a partial measure of bound cellular methylglyoxal since washing would have removed much of the free methylglyoxal. From the amounts of protein bound glyoxal it was concluded that untreated INS-1 cells (0 mM D-L-glyceraldehyde, 0 h) contained less than 0.78 μM intracellular methylglyoxal. Treating cells with 10 mM D-glyceraldehyde for 1 min (as an additional control) doubled the methylglyoxal content, but its level was still very low. Cells exposed to 20 mM D-L-glyceraldehyde for 8 and 24 h contained 13 and 7 nmol methylglyoxal/mg tissue protein (123 and 64 μM intracellular concentrations), respectively, while cells exposed to 10 mM D-glyceraldehyde for 8 h contained 3.5 nmol methylglyoxal/mg tissue protein (32 μM intracellular concentration) (Fig. 4).

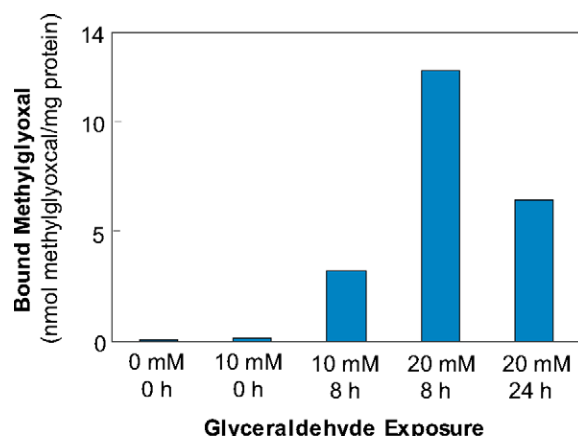

Fig. 4. Effect of glyceraldehyde on intracellular bound methylglyoxal levels. INS-1 cells were grown to confluence in RPMI 1640 medium and then exposed to 10 mM D-glyceraldehyde or 20 mM D-L-glyceraldehyde in fresh RPMI 1640 medium for varying lengths of time before measuring intracellular methylglyoxal. This experiment is representative of four experiments.

Effect of glucose on methylglyoxal levels in insulin cell lines

Four experiments were performed with insulin cell lines, two with the INS-1 cell line and two with the βTC6-7F cell line. In each experiment cells were maintained at 3 and 20 mM glucose for various lengths of time. In one experiment each cell line was washed with cold PBS after harvesting and before homogenization in acetic acid. Because it was queried whether any methylglyoxal present was eluted from the cells by washing, a second set of experiments was performed in which cells were harvested directly into acetic acid. In each case the level of methylglyoxal (1 μM) in cells incubated with high glucose was only slightly higher than that present in cells incubated with low glucose (data not shown).

Methylglyoxal does not increase insulin secretion in pancreatic islets

Rat pancreatic islets were exposed to methylglyoxal at concentrations of 1 μM to 10 mM in the presence or absence of 5.6 mM glucose. This concentration of glucose stimulates a small amount of insulin release and was used to test whether methylglyoxal can potentiate insulin release in the presence of a normal fuel. The insulin released in the presence of methylglyoxal samples was no more than that from the controls incubated without methylglyoxal (Table 4). Methanol was included as a control because methanol is a byproduct of the acid hydrolysis process used to produce methylglyoxal stock solutions [31]. Methanol at 0.25 and 2.5 mM did not alter insulin release in the presence of 5.6 mM glucose.

Discussion

GA metabolism in the insulin cell

In early studies of GA it was assumed (but never studied) that GA stimulates insulin secretion entirely or in part

Table 4
Methylglyoxal does not increase insulin release from isolated rat pancreatic islets

Methylglyoxal (mM)	Glucose (mM)	Methanol (mM)	Insulin release (μU insulin/5 islets/60 min)
—	—	—	23 ± 2 (12)
—	16.7	—	302 ± 39 (11)
0.1	16.7	—	309 ± 23 (6)
1	16.7	—	174 ± 12 (6)*
—	5.6	—	70 ± 20 (5)
—	5.6	0.25	66 ± 14 (5)
—	5.6	2.5	88 ± 11 (6)
0.001	5.6	0.002	58 ± 10 (6)
0.01	5.6	0.02	77 ± 5 (5)
0.1	5.6	0.2	62 ± 9 (4)
1	5.6	2	61 ± 12 (4)
0.001	—	0.002	30 ± 6 (12)
0.01	—	0.02	32 ± 7 (9)
0.1	—	0.2	38 ± 8 (10)
1	—	2	18 ± 2 (9)
10	—	20	21 ± 5 (5)

Islets (5 per vial) were incubated in the absence or presence of various concentrations of methylglyoxal and in the absence or presence of 5.6 mM glucose as a supporting fuel for 60 min. Insulin release in the presence of 16.7 mM glucose is shown as a positive control. Insulin release in the presence of methanol is shown as a control because methanol is a byproduct of the manufacture of methylglyoxal. Results are means ± standard error with the number of replicate incubations in parentheses.

* $p < 0.001$ vs. 16.7 mM glucose alone.

by its metabolism similarly to glucose; that is from glyceraldehyde phosphate through the distal half of the glycolysis pathway to pyruvate which is then metabolized in mitochondria identical to glucose-derived pyruvate. However, since GA is usually observed to be a more potent insulin secretagogue than glucose on either the basis of molarity or triose equivalents [3,5–18] (also cf. Table 4, effect of 5.6 mM glucose vs. Table 2, effect of 4 mM glyceraldehyde) and is metabolized to CO_2 by both islets (Fig. 2A) and insulinoma cells [18] at 10–20% the rate of glucose, it is likely that GA's insulinotropism involves additional pathways than the segment of the aerobic glycolysis pathway shared with glucose (Fig. 1).

GA is toxic to the β-cell and it is possible that GA's hyperinsulinotropism is partially related to the mechanisms that cause its toxicity. We have previously shown that GA can be a substrate in the glyceraldehyde phosphate dehydrogenase (GAPDH) reaction in pancreatic islets and that this can produce NADH at a rate that is 20-fold faster than NADH formed from glyceraldehyde phosphate, which is the normal substrate for the GAPDH reaction and is generated by triokinase [19] (Fig. 1). In further support of this idea, the current work shows that GA drastically increases NAD(P)H and lowers NAD(P) in islets (Table 1). The fact that the catalytic rate with GA as a substrate in the triokinase reaction is 1/3 that with dihydroxyacetone as a substrate, but GA elicits three times or more the insulin release from islets than does dihydroxyacetone [3,6,19] is also consistent with the idea that triokinase is not the only point of entry of GA into β-cell metabolism. There is some evidence that NADH formation is specifically involved in the stimulating normal insulin secretion [26,32,37–43]. The moderately increased lactate/pyruvate ratio in glucose-treated islets (Table 1) is consistent with an increased cytosolic NADH/NAD ratio and agrees with previous observations. The high NAD(P)H fluorescence and slightly increased lactate/pyruvate ratio in GA-treated islets (Table 1) is consistent with a low NAD/NADH ratio in the cytosol. The lowering of total oxidized pyridine nucleotides and the increase in total reduced pyridine nucleotides (Table 1) is most likely due to lowering of NAD and an increase in NADH. This is because the NAD level is much higher than the NADP level in most cells and aldehyde reductases, which are plentiful in the β-cell [28], will reduce GA and methylglyoxal derived from GA to raise the NADP/NADPH ratio. A high NADH level from GA acting as a substrate in the GAPDH reaction combined with the metabolism of GA similarly to glucose may initially potentiate insulin release. However, the persistence of a low NAD/NADH ratio, which is normally high in the cytosol, because cytosolic NADH production is balanced by its oxidation via the mitochondrial hydrogen shuttles [32,39,40], combined with the accumulation of acidic products and other toxic metabolites, would likely contribute to the toxicity of GA in the β-cell.

GA's oxidation in the GAPDH reaction will form 1-phosphoglyceric acid [19] which cannot be further metabolized thus severely limiting mitochondrial ATP production (Fig. 1). In addition, the low cytosolic NAD/NADH ratio caused by oxidation of GA in the GAPDH reaction should block glycolysis at this same step. In agreement with this theory, GA decreases glucose oxidation to CO_2 out of proportion to GA's metabolism to CO_2 (Fig. 2B). This indicates that the decrease in glucose oxidation is primarily due to inhibition of glucose metabolism by GA and not by GA replacing glucose in shared metabolic pathways. Another indication that GA metabolism interferes with glycolysis is the fact that GA does not increase L-lactate in pancreatic islets to the extent that glucose does (Table 1), despite GA producing a very low NAD(P)/NAD(P)H ratio which should increase L-lactate due to the coupling of NADH and lactate levels in the L-lactate dehydrogenase reaction. Although it was not interpreted as evidence for inhibition of glycolysis at the GAPDH step, the idea of GA acting as a substrate in the GAPDH reaction and blocking glycolysis at this step is also supported by data of Taniguchi et al. [27]. They observed marked increases in glyceraldehyde phosphate, the substrate for the GAPDH reaction, and also dihydroxyacetone phosphate, which is in equilibrium with glyceraldehyde phosphate, in GA-treated islets. They also detected a severe decrease in ATP in the presence of GA, consistent with the idea that GA is converted to products that are not precursors for the citric acid cycle.

There are two mitochondrial shuttles that maintain a normal cytosolic NAD/NADH ratio in islets—the glycerol phosphate shuttle and the malate aspartate shuttle [32,39,40]. It has been shown that blockade of the malate aspartate shuttle in islets already deficient in glycerol phos-

phate shuttle activity (due to a low mitochondrial glycerol phosphate dehydrogenase level caused by type 2 diabetes) inhibits GA-induced insulin release more than glucose-induced insulin release [44]. This also supports the idea that the GA-induced insulin release is largely dependent on NADH production and the NAD/NADH ratio is lower in the presence of GA than in the presence of glucose.

Best et al. [17] have shown that GA makes the pancreatic islet acidic while other secretagogues, such as glucose and α-ketoisocaproic acid, make the islet slightly alkaline. In addition to the probable formation of 1-phosphoglycerate, high levels of D-lactate are formed from the metabolism of GA in the glyoxalase pathway (Fig. 1) in islets (Table 1) [20,23] and HIT cells [21]. GA also causes the L-lactate concentration to increase slightly (Table 1) and the accumulation of the three acidic metabolites could explain the low pH of GA-treated cells.

In the insulin cell as in other types of cells, GA and its product methylglyoxal are two of the most efficient substrates for aldehyde and aldose reductases [28] and, therefore, it is likely that in islets these enzymes reduce these two molecules to glycerol and propanediol, respectively, with a concomitant increase in the cytosolic NADP/NADPH ratio (Fig. 1). This is likely detrimental to the cell since this redox couple is usually reduced in the cytosol of the β-cell as in most types of cells [25,45]. Although a low level of glycerol is unlikely to be directly harmful to the cell, its formation cannot provide energy because the β-cell does not possess glycerol kinase [4,29,30], the enzyme that catalyzes phosphorylation of glycerol by ATP to form glycerol phosphate which is metabolizable in the insulin cell.

Methylglyoxal levels in INS-1 cells

One of the purposes of the current work was to examine the role of methylglyoxal in insulin secretion. The formation in GA-treated insulin cells of large amounts of D-lactate, the end product of the glyoxalase pathway [24], (Fig. 1) suggested that methylglyoxal contaminating commercial preparations of GA or methylglyoxal formed from GA metabolism may mediate insulin secretion [21–23]. Methylglyoxal at millimolar concentrations has been reported to cause membrane depolarization and a modest transient stimulation of insulin release in rat pancreatic β-cells [22]. However, methylglyoxal has never been actually measured in insulin cells. The current work demonstrates that methylglyoxal is indeed present in GA solutions at levels approaching 0.01% w/w of that of GA and its attempted removal caused its regeneration as the system re-equilibrated suggesting that GA is in equilibrium with the methylglyoxal contaminant. This indicates that when GA is added to cells, methylglyoxal is also added. However, when GA is incubated with insulin-producing cells over time, methylglyoxal levels are increased dramatically indicating these cells efficiently metabolize GA to methylglyoxal. Methylglyoxal increases steadily to high levels in INS-1 cells incubated for 8–24 h in the presence of 10 mM GA (Fig. 4). In contrast, glucose does not cause significant increases in methylglyoxal levels in insulin producing cells.

Methylglyoxal does not stimulate insulin secretion

Since GA is metabolized to methylglyoxal, it was important to determine what influence methylglyoxal has on insulin secretion. Pancreatic β-cells were exposed to methylglyoxal in the physiological concentration range (~1 μM, Table 4) and in concentrations ranging from 10 μM to 10 mM for 1 h. No increase in insulin secretion was evident at any methylglyoxal concentration not supporting the idea that methylglyoxal itself can act as an insulin secretagogue. Although methylglyoxal is an even more efficient substrate than GA for aldehyde and aldose reductases, inhibitors of these enzymes do not inhibit glucose-induced insulin release [28] indicating that metabolism of aldehydes, including methylglyoxal, through the steps catalyzed by these enzymes (Fig. 1) does not mediate physiologic insulin release. Inhibitors of glyoxalase I (Table 2) or of aldose and aldehyde reductases (Table 3) do not inhibit GA-induced insulin release suggesting that direct reduction of GA by aldehyde reductases or reduction of GA-derived methylglyoxal does not mediate GA-induced insulin release. In short term experiments (<1 h), only a high level of methylglyoxal (1 mM) inhibited insulin release stimulated by 16.7 mM glucose (Table 4). This is consistent with methylglyoxal altering the NADP/NADH ratio, which could inhibit insulin release, or a toxic effect of methylglyoxal. Long-term exposure to even low levels of free methylglyoxal is likely to be quite toxic to β-cells. Methylglyoxal, because of its ability to attack thiols and form advanced glycation end products, is toxic to many types of cells [46–52].

Summary

With the information currently available, it appears that GA stimulates insulin release more efficiently than glucose because of metabolism of a small amount of GA to CO_2 and its initially increasing the cytosolic NADH level more than glucose does. GA drastically increases NADH via its direct oxidation in the GAPDH reaction. This results in increased flux of reducing equivalents through the mitochondrial glycerol phosphate and malate aspartate shuttles. These shuttles are important for coupling fuel metabolism to insulin exocytosis [32,39,40]. However, continued exposure to GA is injurious to the cell because of excessive GA metabolism in the GAPDH reaction. This not only consumes NAD and increases NADH but also forms 1-phosphoglycerate which cannot be converted to any intermediates that can be metabolized via oxidative phosphorylation to produce ATP. Continuation of the enormous unregulated flux of GA through the GAPDH reaction causes the cytosolic NAD/NADH ratio to decrease to a level that severely inhibits glycolysis at the GAPDH step. In addition, the cytosolic NADP/NADPH

ratio, which is normally low, will increase from the reduction of GA and methylglyoxal catalyzed by aldehyde reductases (Fig. 1). The β-cell pH decreases due to the accumulation of 1-phosphoglycerate, D-lactate from glyoxalase and somewhat from accumulation of L-lactate associated with the decreased NAD/NADH ratio. Since methylglyoxal added to islets does not stimulate insulin release, it is unlikely that methylglyoxal produced from GA accounts for the high level of stimulation of insulin release caused by GA.

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