D-Glyceraldehyde Causes Production of Intracellular Peroxide in Pancreatic Islets, Oxidative Stress, and Defective Beta Cell Function via Non-mitochondrial Pathways

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D-Glyceraldehyde (D-GLYC) is usually considered to be a stimulator of insulin secretion but theoretically can also form reactive oxygen species (ROS), which can inhibit beta cell function. We examined the time- and concentration-dependent effects of D-GLYC on insulin secretion, insulin content, and formation of ROS. We observed that a 2-h exposure to 0.05–2 mM D-GLYC potentiated glucose-stimulated insulin secretion (GSIS) in isolated Wistar rat islets but that higher concentrations inhibited GSIS. A 24-h exposure to 2 mM D-GLYC inhibited GSIS, decreased insulin content, and increased intracellular peroxide levels (2.14 ± 0.31-fold increase, n = 4, p < 0.05). N-Acetylcycteine (10 mM) prevented the increase in intracellular peroxides and the adverse effects of D-GLYC on GSIS. In the presence of 11.1 but not 3.0 mM glucose, koningic acid (10 μM), a specific glyceraldehyde-3-phosphate dehydrogenase inhibitor, increased intracellular peroxide levels (1.88 ± 0.30-fold increase, n = 9, p < 0.01) and inhibited GSIS (control GSIS = p < 0.001; koningic acid GSIS, not significant). To determine whether oxidative phosphorylation was the source of ROS formation, we cultured rat islets with mitochondrial inhibitors. Neither rotenone or myxothiazol prevented D-GLYC-induced increases in islet ROS. Adenoviral overexpression of manganese superoxide dismutase also failed to prevent the effect of D-GLYC to increase ROS levels. These observations indicate that exposure to excess D-GLYC increases reactive oxygen species in the islet via non-mitochondrial pathways and suggest the hypothesis that the oxidative stress associated with elevated D-GLYC levels could be a mechanism for glucose toxicity in beta cells exposed chronically to high glucose concentrations.

Chronic exposure of pancreatic islets to supraphysiologic concentrations of glucose adversely affects beta cell function, a process known as glucose toxicity, and thereby plays a secondary pathogenic role in abnormal beta cell function in type 2 diabetes (1–7). We have reported that exposure to high glucose concentrations increases intracellular peroxide levels in human islets and that ROS accumulation causes defective beta cell function (8). This effect was prevented by mannoheptulose, indicating that glucose metabolism is essential for generation of ROS. ROS can be produced in the presence of high glucose concentrations by multiple mechanisms, including oxidative phosphorylation, glucose auto-oxidation via enolization, the Schiff reaction during glycation, protein kinase activation, methylglyoxal formation, and hexosamine metabolism (9–15). D-Glyceraldehyde (D-GLYC) is formed during glucose metabolism and characteristically stimulates insulin secretion (16–19). It enters the glycolytic pathway after phosphorylation to form the glycolytic intermediate, glyceraldehyde-3-phosphate (17). However, hydroxaldehydes such as glyceraldehyde can also undergo enolization in the presence of heavy metals to produce an endioli radical anion, which in the presence of oxygen generates superoxide, hydrogen peroxide, and hydroxyl radicals (9). Although brief exposures of islets to D-GLYC have been shown to stimulate insulin secretion (16–19), the effects of a full range of D-GLYC concentrations on insulin secretion and generation of ROS in islets after 2–24 h have not been examined in detail.

In this work we designed experiments to ascertain 1) the time- and concentration-dependent effects of D-GLYC on intraislet peroxide levels, glucose-stimulated insulin secretion (GSIS), and intraislet insulin content; 2) whether N-acetyllyssteine (NAC), a potent antioxidant, prevents D-GLYC from forming ROS and inhibiting GSIS; and 3) whether the mitochondrial electron transport chain mediates ROS production by D-GLYC and thereby causes adverse effects on beta cell function.

MATERIALS AND METHODS

Materials—D-GLYC, NAC, rotenone, thienyltrifluoroacetone, myxothiazol, and carbonyl cyanide m-chlorophenylhydrazone were obtained from Sigma. Koningic acid, a specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibitor, was a gift from Dr Keiji Hasumi (Tokyo Noko University, Tokyo, Japan).

Pancreatic Islet Isolation—Pancreata from male Wistar rats were infused with 10 ml of a 1.5 mg/ml collagenase type XI (Sigma)/1% fetal bovine serum/2 units/ml RQ1 DNase (Promega) solution in Medium 199 (Sigma). After surgical excision, the pancreas was incubated in the collagenase solution at 37 °C. Undigested tissue was removed by using a 500-μm screen, and the recovered tissue was washed twice with ice-cold Hanks’ balanced salt solution containing 0.1% bovine serum albumin.

The abbreviations used are: ROS, reactive oxygen species; n-GLYC, n-glyceraldehyde; GSIS, glucose-stimulated insulin secretion; NAC, N-acetyllyssteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; carboxy-H2DCFDA, carboxylated dichlorodihydrofluorescein diacetate; PBS, phosphate-buffered saline; KA, koningic acid.
experiments with triplicate determinations.

Wistar rat islets were preincubated for 2 h in RPMI media containing increasing concentrations of d-GLYC for 2 or 24 h prior to study. Islets were then harvested, washed twice with PBS, and resuspended in trypsin-EDTA (0.25% trypsin, 1 mM Na4-EDTA, Invitrogen) for 5 min at 37 °C. To disperse the cell clumping of the dye, the islets were washed twice with PBS and then put back into culture conditions for 2 h. Islets were then harvested, washed twice with PBS, and resuspended in trypsin-EDTA. The pellet was then resuspended in ice-cold PBS, and 2 µg/ml propidium iodide was added.

Cells were analyzed using a 488 nm argon laser EPICS XL-MCL flow cytometer controlled by EXPO 32-ADC software (Beckman Coulter). A single cell population gate was created using a biparametric plot of side scatter and propidium iodide (log fluorescence) of the single cell population. ROS values were analyzed using a histogram plot of carboxy-H2DCFDA (log fluorescence). Results were calculated as the -fold difference from control untreated cells. Cell viability was calculated as the ethanol-hydrochloric acid solution were measured by using a sensitive rat insulin radioimmunoassay kit (Linco Research Immunoassay, St. Charles, MO).

Measurement of Insulin and β-Actin mRNAs—One-step reverse transcription-PCR was carried out by using Gold reverse transcription-PCR kit from PerkinElmer Life Sciences and an ABI Prism 7700 sequence detector equipped with a thermocycler (TaqMan Technology) and a cooled charged-coupled device camera to detect fluorescence emission over a range of wavelengths (500–650 nm) as described previously (8).

Evaluation of ROS and Cell Viability with Flow Cytometry—Intracellular peroxide levels were detected by flow cytometric analysis using an oxidation-sensitive fluorescein-labeled dye, carboxylated dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA, Molecular Probes, Ref. 8). The carboxy-acetoxyethyl ester derivative facilitates diffusion across the cell membranes, and upon cleavage of the acetate groups by endogenous esterase activity, the leakage of the dye is impeded by the two additional negative charges. Upon oxidation by intracellular ROS, the non-fluorescent dye is converted to its fluorescent form. Islets were labeled with 100 µM carboxy-H$_2$DCFDA for 1 h at 37 °C. Following the cell loading of the dye, the islets were washed twice with PBS and then put back into culture conditions for 2 h. Islets were then harvested, washed twice with PBS, and resuspended in trypsin-EDTA (0.25% trypsin, 1 mM Na4-EDTA, Invitrogen) for 5 min at 37 °C. To disperse the islets into a single cell suspension, islets were gently passed 20 times in ethanol:hydrochloric acid solution were measured by using a sensitive rat insulin radioimmunoassay kit (Linco Research Immunoassay, St. Charles, MO).

**TABLE I**

| Glucose | Koningic acid | GAPDH activity |
|---------|---------------|----------------|
| mM      | µM            | -fold control  |
| 3.0     | 0             | 1.00          |
| 3.0     | 10            | 0.09 ± 0.04   |
| 16.7    | 0             | 1.56 ± 0.18   |
| 16.7    | 10            | 0.09 ± 0.05   |

*Significantly different, $p < 0.001$.

**FIG. 1.** Effects of d-GLYC on glucose-stimulated insulin secretion and insulin content. Wistar rat islets were preincubated for 2 h in RPMI media containing increasing concentrations of d-GLYC for 2 or 24 h and incubated in Krebs-Ringer buffer containing 2.8 or 16.7 mM glucose for 1 h to measure insulin secretion. A, GSIS after a 2-h preincubation with d-GLYC. B, GSIS after a 24-h culture with d-GLYC. C, intracellular insulin content after a 2- or 24-h incubation with d-GLYC. All data are presented as means ± S.E. for 3–7 independent experiments with triplicate determinations.

**FIG. 2.** Effect of d-GLYC on intracellular peroxide levels and its prevention by NAC. Rat islets were incubated for 2, 8, 16, or 24 h with 2 mM d-GLYC with or without 10 mM NAC. Comparisons regarding the effects of d-GLYC on islets were calculated as the -fold difference from control islets not treated. (*, $p < 0.05$ versus control; one-way analysis of variance). All data are presented as means ± S.E. for 3–4 independent experiments with duplicate determinations.
number of propidium iodide-negative cells within the single cell population of 10,000 total ungated events. Experimental conditions are expressed as the percentage of viable cells compared with control conditions.

**Measurement of ATP Content**—Rat islets (100 islets/each condition) were washed twice with ice-cold PBS and lysed with 50 μl of cell lysis buffer (Promega). ATP levels were determined by using a luciferase-based assay (ATP determination kit, Molecular Probes), according to Molecular Probes’ directions. Luminescence was read by FUSION (Packard).

**Measurement of GAPDH Activity**—GAPDH activity was measured by monitoring the conversion of NADH to NAD⁺ at 340 nm (21). Islets were sonicated in homogenization buffer containing 100 mM KH₂PO₄, pH 7.5, and 2 mM dithiothreitol. After a brief centrifugation to collect cell debris, the supernatant was collected and assayed for GAPDH activity. Supernatant was added to reaction buffer containing 79.1 mM triethanolamine, 6 mM glycerate 3-phosphate, 1.6 mM MgSO₄, 0.9 mM EDTA, 1.1 mM ATP, and 3 units of phosphoglycerate kinase and incubated at 37 °C for 5 min before the addition of 0.2 mM NADH. Spectrophotometric measurements were made at 340 nm every 30 s for 3 min.

**Statistics**—Comparisons between the results of the experiments were performed using either unpaired or paired analyses and analysis of variance or Student’s t test where appropriate. p < 0.05 was considered significant.

**RESULTS**

**d-Glyceraldehyde Effects on Insulin Secretion and Insulin Content in Rat Islets**—Over a 2-h period, 0.05–2 mM d-GLYC-augmented glucose-stimulated insulin secretion and insulin content (Fig. 1, A and C). However, at concentrations higher than 2 mM, d-GLYC decreased GSIS in a concentration-dependent manner. In 24-h incubations, concentrations of 1–2 mM d-GLYC decreased GSIS (Fig. 1B) and insulin content (Fig. 1C). These adverse effects were even more evident when higher concentrations of d-GLYC were used for 24 h.

**d-Glyceraldehyde Effects on Intracellular Peroxide Levels**—Exposure of rat islets to exogenous d-GLYC, 2 mM, for 2–8 h
had no effect on intracellular ROS levels, but 16- and 24-h exposure increased intracellular ROS 2.05 ± 0.36-fold (16 h) and 2.14 ± 0.31-fold (24 h) (Fig. 2, both n = 4, p < 0.05). Islets were exposed to 10 μM konic acid (KA) to increase endogenous D-GLYC levels. KA in the presence of either 3.0 or 16.7 mM glucose for 72 h decreased GAPDH activity to virtually zero (Table I). In the presence of 11.1 but not 3.0 mM glucose, KA increased intracellular ROS to a level (1.88 ± 0.30-fold increase, Fig. 3A) that was not statistically different from that observed with 2 mM D-GLYC. KA completely inhibited GSIS (Fig. 3B).

Effect of NAC on D-Glyceraldehyde-induced Beta Cell Dysfunction—NAC, a potent antioxidant, was added to the culture media 2 h prior to 2 mM D-GLYC. NAC prevented a D-GLYC-induced increase of intracellular ROS levels after 16- and 24-h culture (Fig. 2) and prevented the adverse effects on GSIS (Fig. 4A). NAC did not prevent D-GLYC from decreasing insulin content (Fig. 4B). D-GLYC caused an increase in insulin mRNA levels (Fig. 4C), an effect attenuated but not blocked by NAC.

Effects of Mitochondrial Electronic Transport Chain Inhibitors and Manganese Superoxide Dismutase Overexpression on D-Glyceraldehyde-induced ROS Generation—Neither rotenone (150 nM) nor myxothiazol (3 ng/ml) prevented the stimulatory effects of D-GLYC on intracellular ROS levels (Fig. 6). Adenoviral overexpression of manganese superoxide dismutase, the mitochondrial form of the antioxidant enzyme, increased islet manganese superoxide dismutase protein but failed to prevent the stimulatory effect of D-GLYC on ROS formation (Fig. 7).

**DISCUSSION**

We previously reported that exposure to high glucose concentrations increased intracellular peroxide levels in human islets and that ROS accumulation caused defective beta cell function (8). This effect was prevented by coincubation with a glucokinase inhibitor, mannoheptulose, indicating that glucose metabolism is essential for generation of ROS in islets. The current studies were designed to examine the hypothesis that the accumulation of excess intracellular concentrations of D-GLYC during the exposure of islets to supraphysiological concentrations of glucose might contribute to beta cell cytotoxicity via ROS formation. We observed that D-GLYC has time- and concentration-dependent biphasic effects on GSIS and insulin content and that it increases the intracellular oxidant load of the islet. Koningic acid, a GAPDH inhibitor (21–23) used to increase endogenous D-GLYC levels, increased the intracellular oxidant load under high but not low glucose conditions to the same extent as 2 mM D-GLYC and inhibited GSIS. The adverse effects of D-GLYC on glucose-stimulated insulin secretion were blocked by the potent antioxidant NAC. However, NAC did not prevent the D-GLYC-induced decline in insulin content. This dichotomy may be related to the stimulatory effect D-GLYC had on insulin mRNA levels, which was only attenuated by NAC. The ROS-generating effect of D-GLYC was not affected by mitochondrial electron transport chain inhibitors or overexpression of manganese superoxide dismutase, which suggests generation of ROS by D-GLYC occurred via non-mitochondrial pathways.

The concept of glucose auto-oxidation via enolization and...
FIG. 5. Effects of mitochondrial electron transport chain inhibitors on cell viability and intracellular ATP contents. Rat islets were incubated in RPMI media containing increasing concentration of mitochondrial inhibitors for 24 h. A, 150 nM rotenone at a <10% decrease in cell viability decreased ATP content >10%. B, 400 μM thenoyltrifluoroacetone at a <10% decrease in cell viability did not decrease ATP content >10%. C, 3 ng/ml myxothiazol at a <10% decrease in cell viability decreased ATP content >10%. D, 1.5 mM carbonyl cyanide m-chlorophenylhydrazone at a <10% decrease in cell viability did not decrease ATP content. All data are presented as means ± S.E. for 3–8 independent experiments with duplicate determinations.
consequent excess generation of ROS in relation to diabetes was proposed as early as 1987 by Wolff and Dean (9) who studied this issue using bovine serum albumin. In 1988, Hunt et al. (10) demonstrated that α-hydroxylaldehydes, such as glyceraldehyde, can undergo enolization to produce an enediol radical anion in the presence of heavy metals. In the presence of oxygen, these anions generate superoxide anions that combine to form hydrogen peroxide in a reaction catalyzed by superoxide dismutase. Hydrogen peroxide in turn forms hydroxyl radicals (10). Although it has been reported that D-GLYC can generate ROS in bovine serum albumin, to our knowledge it has not previously been demonstrated that D-GLYC generates ROS in islets or other tissues nor is it generally appreciated that D-GLYC can inhibit glucose-stimulated insulin secretion. That the antioxidant NAC can prevent these adverse effects suggests that the generation of ROS is one mechanism of action for D-GLYC-induced beta cell dysfunction. Other potential mechanisms include the formation of a Schiff base adduct between D-glyceraldehyde and a protein that regulates insulin exocytosis.

Whether 2 mM D-GLYC is a physiologically relevant concentration to examine is an important issue. Taniguchi et al. (17) demonstrated that islets exposed to 2.8 and 20 mM glucose accumulated 0.01 and 0.025 pmol/islet D-GLYC, respectively.

![Fig. 6. Effects of mitochondrial electron transport chain inhibitors on D-GLYC-induced ROS formation in rat islets.](image)

Rat islets were incubated for 24 h with 2 mM D-GLYC with or without mitochondrial electron transport chain inhibitors. Comparisons of the effects of D-GLYC on islets were calculated as the fold difference from control islets not treated. A, 150 nM rotenone did not prevent but augmented the effects of D-GLYC on intracellular peroxide levels (p < 0.01). B, 3 ng/ml myxothiazol did not prevent the effect of D-GLYC on intraislet peroxide levels (p = ns, not significant). All data are presented as means ± S.E. for 4–6 independent experiments with duplicate determinations.
whereas exposure to 10 mM D-GLYC caused an accumulation of 0.12 pmol/islet D-GLYC. Because we used one-fifth of this concentration of D-GLYC in our experiments, we estimate it caused intraislet levels of ~0.025 pmol/islet, a concentration similar to that observed using 20 mM glucose (17). Taniguchi et al. (17) also demonstrated that D-GLYC did not increase glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, and dihydroxyacetone in isolated rat islets. In our work, exposure of rat islets to KA to increase D-GLYC levels caused an increase in intracellular ROS under 11.1 but not 3.0 mM glucose conditions. This increase in ROS was not significantly different from that observed with 2 mM D-GLYC. It has been also reported that long term to exposure to high glucose concentrations decreases GAPDH activity in islets (15), which could lead to excess D-GLYC accumulation. We acknowledge, however, that results from the KA studies are not conclusive, because KA may not be completely specific for GAPDH, and the inhibition of GAPDH may have caused accumulation of dihydroxyacetone, which in turn could cause depletion of cytosolic NAD via the glycerol 3-phosphate shuttle. Pathways other than enolization, such as protein kinase C activation and/or the formation of methylglyoxal, could also mediate D-GLYC-induced oxidative stress during the exposure of islets to chronically high glucose concentrations. For example, Du et al. (24) reported recently that GAPDH antisense oligonucleotides activated protein kinase C, NF-xB, and advanced glycation end product formation in bovine aortic endothelial cells but only in high (50 mM) glucose concentrations. However, whether our experiments using KA and 11.1 mM glucose and other reported experiments using 50 mM glucose are necessarily relevant to clinical diabetes mellitus remains to be determined.

Nishikawa et al. (12) demonstrated that long time exposure to a high glucose concentration increased ROS production in cultured bovine aortic endothelial cells and through the use of mitochondrial inhibitors concluded that ROS was formed via oxidative phosphorylation. This prompted us to ascertain whether D-GLYC generates ROS via oxidative phosphorylation in islets. Because exposure to electron transport chain inhibitors can be toxic for cells, we first performed cell sorting flow cytometry to separate and quantify dead cells. We considered this step essential, because unappreciated cell death confounds efforts to quantify a decrease in ROS levels, i.e. drugs that kill cells cause an artifactual decrease in apparent intracellular ROS levels. We sought drug concentrations that would decrease ATP content >10% with <10% cytotoxicity. Rotenone and myxothiazol decreased ATP content significantly and still allowed >90% cell viability. Neither drug prevented the ability of D-GLYC to increase intraislet ROS levels. Thienoyltrifluoroacetone and carbonyl cyanide m-chlorophenylhydrazone both failed to decrease ATP content >10% without causing >10% cytotoxicity and therefore could not be used in these islet experiments. As pharmacological inhibitors are not absolutely specific for cellular targets, we also examined adenoviral overexpression of manganese superoxide dismutase. This caused an increase in islet manganese superoxide dismutase content but failed to prevent D-GLYC-induced production of ROS. These results strongly suggest that D-GLYC generates ROS via non-mitochondrial pathways.

Recently, Krauss et al. (25) reported that a high glucose concentration (25 mM) increased mitochondrial superoxide production in islets from wild type and UCP-2 knock-out mice. This led to proton leak, lowered ATP levels, and impaired glucose-induced insulin secretion in islets from the wild type but not from the knock-out animals. Our work suggested that the accumulation of excess endogenous D-glyceraldehyde during exposure to high glucose levels increases intraislet ROS via non-mitochondrial pathways and that this also might be responsible for adverse effects of high glucose levels on beta cell function. Taken together, our work and the reports cited above suggest that both mitochondrial and non-mitochondrial pathways may contribute ROS to the glucotoxic process, which may play an important role in the continual deterioration of beta cell function characteristic of type 2 diabetes.

**Acknowledgments**—We thank Scott Holland, Zhou Huarong, M.D., Ph.D., and Elizabeth Oseid for excellent technical assistance. We thank the University of Iowa Adenoviral Facility for the adenovirus encoding manganese superoxide dismutase, Jill McCuaig in the Pacific Northwest Research Institute Adenoviral Core for technical support, and Dr. Keiji Hasumi (Department of Applied Biological Science, Tokyo Noko University, Tokyo, Japan) for koningic acid.
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