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Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic β-cells

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Abstract

Pancreatic β-cells exposed to hyperglycemia produce reactive oxygen species (ROS). Because β-cells are sensitive to oxidative stress, excessive ROS may cause dysfunction of β-cells. Here we demonstrate that mitochondrial ROS suppress glucose-induced insulin secretion (GIIS) from β-cells. Intracellular ROS increased 15 min after exposure to high glucose and this effect was blunted by inhibitors of the mitochondrial function. GIIS was also suppressed by H_2O_2, a chemical substitute for ROS. Interestingly, the first-phase of GIIS could be suppressed by 50 μM H_2O_2. H_2O_2 or high glucose suppressed the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme, and inhibitors of the mitochondrial function abolished the latter effects. Our data suggested that high glucose induced mitochondrial ROS, which suppressed first-phase of GIIS, at least in part, through the suppression of GAPDH activity. We propose that mitochondrial overwork is a potential mechanism causing impaired first-phase of GIIS in the early stages of diabetes mellitus.

Keywords: Islets of Langerhans; Hydrogen peroxide; Oxidative stress; Diabetes mellitus; Iodoacetates; Glyceraldehyde 3-phosphate dehydrogenase; Glycolysis; Insulin secretion; Electron transport system; Reactive oxygen species

Type 2 diabetes is a polygenic disease aggravated by environmental factors. Although patients with this form of diabetes show failure of glucose-induced insulin secretion (GIIS), the molecular nature of β-cell dysfunction in type 2 diabetes is still unknown.

Failure of insulin secretion in type 2 diabetic patients is characterized by decreased first-phase of GIIS, delayed hyperinsulinemia, and late development of failure of insulin synthesis [1–5]. Several studies have shown that failure of GIIS in pancreatic β-cells is caused by both genetic factors and acquired factors. To explain how acquired factors lead to dysfunction of β-cells, the following changes have been identified [5–8]: (1) the first-phase of GIIS is relatively intact in nondiabetic people destined to develop type 2 diabetes, (2) first-phase of GIIS is no longer present when fasting glucose level reaches 6.4 mM, (3) tight metabolic control partially restores the first-phase of GIIS, and (4) insulin responses to secretagogues other than glucose are relatively unimpaired in subjects with type 2 diabetes.

Although type 2 diabetes has a polygenic background, why do type 2 diabetes patients show a common failure of β-cells as described above? One possibility is that several stressors to pancreatic β-cells during the development of diabetes or impaired glucose tolerance might lead to such failure through a common mechanism. In the presence of insulin resistance in the muscle and liver, pancreatic β-cells have to compensate for such resistance by secreting more insulin to maintain normal blood glucose levels. When more insulin is demanded, more glucose would flow into pancreatic β-cells.

Recent studies have shown that the mitochondria produce ROS under physiological and pathological levels of glycemia via electron transport system [9,10].

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Other reports indicate that ROS are involved in pancreatic β-cell dysfunction and apoptosis in a rodent model of type 1 diabetes, and in the failure of insulin synthesis after chronic hyperglycemia in type 1 and type 2 diabetes [11–14]. However, these reports were not designed to study the process underlying the early stage of β-cell failure and the mechanism by which ROS are generated was not defined.

To address these questions, we exposed β-cells to transient hyperglycemia and studied the effect on ROS production from mitochondrial electron transport system. Under such condition, we further assessed the impact of ROS on GIIS and found that low concentration of ROS from mitochondria could deteriorate GIIS via suppression of GAPDH.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) 1640 were purchased from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) was from Intergen (NY). Bovine serum albumin (BSA), glybenclamide, rotenone, thenoyltrifluoroacetone (TTFA), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), collagenase XI, dithiothreitol (DTT), leupeptin, aprotinin, antipain, phenylmethylsulfonyl fluoride (PMSF), sodium arsenate, iodoacetate, glyceraldehyde 3-phosphate, and enzymes for metabolite concentration assays were obtained from Sigma (St. Louis, MO). EDTA was from Kanto Chemical (Tokyo, Japan). NAD, NADH, and NAD^+ were from Wako (Osaka, Japan). Manganese (III) tetrakis (4-benzoic acid) porphyrin (TBAP) and M-α-tosyl-L-lysine-chloromethyl-thyketone were from Calbiochem (San Diego, CA). 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was from Molecular Probes (Eugene, OR). H_2O_2 was from Santoku Chemical (Tokyo, Japan).

Cell culture condition. MIN6 cells were a gift from Professor Susumu Seino (Department of Cellular and Molecular Medicine, Chiba, Japan) and were grown in DMEM in the presence of 25 mM glucose, 15% FBS, and 50 μg/ml ampicillin under 5% CO_2–95% air at 37 °C.

Preparation of mouse and human pancreatic islets. Pancreatic islets were isolated from CD-1/ICR mouse (7–9 weeks old) as described previously [15]. Mouse islets were cultured in RPMI 1640 with 5.6 mM glucose and 20% FBS at 37 °C for 48 h before use. Human adult pancreatic islets were prepared at the Islet Transplantation Facility at the University of Giessen, Germany. Pancreata were obtained within 8 h of death and islets were prepared as described previously [16].

Measurement of intracellular ROS generation. MIN6 cells were incubated in Krebs–Ringer bicarbonate buffer (KRBB) (in mM, NaCl 135, KCl 3.6, Hepes 10, pH 7.4, $NaHCO_3$ 5, NaH_2PO_4 0.5, $MgCl_2$ 0.5, and $CaCl_2$ 1.5) containing 30 mM glucose for 0–96 h after basal incubation at 5 mM glucose. In some experiments, MIN6 cells or human islets were incubated for 3 h in 5 or 30 mM glucose in the presence or absence of 5 μM rotenone, 10 mM TTFA, 0.1 mM CCCP or 100 μM TBAP. ROS was measured as described previously [10].

Measurement of insulin secretion. GIIS was determined by static incubation. MIN6 cells or 15 mouse islets were incubated in KRBB containing 2.8 mM glucose and 0.5% BSA at 37 °C for 60 min in 24-well plate or 1.5 ml Eppendorf tube. Subsequently, they were incubated in KBB containing 2.8 mM glucose in the absence or presence of H_2O_2 (0–200 μM) for 30 min. They were further incubated in KRBB containing 2.8 mM glucose or 25 mM glucose in the absence or presence of H_2O_2 for 30 min. After incubation, the medium was centrifuged and the supernatant was assayed by radioimmunoassay (RIA, Eiken Chemical, Tokyo). GIIS was expressed as fold-increase of that in response to low glucose in MIN6 cells. In case of mouse islets, GIIS was expressed as a percentage of that secreted in response to low glucose without H_2O_2.

Perifusion studies. One hundred and fifty mouse islets were loaded into a Pharmacia C10/10 column. Insulin secretion was stimulated with 25 mM glucose and 0.5% BSA for 20 min after basal perifusion with 2.8 mM glucose for 40 min at a flow rate of 200 μl/min. In some experiments, 50 μM H_2O_2 or 100 μM iodoacetate was added. Ten μM glybenclamide was added in the last 10 min of perifusion. The effluents were collected every 2 min and immunoreactive insulin (IRI) in the samples was assayed by RIA.

GAPDH activity. Mouse islets were preincubated with RPMI 1640 containing 5 or 25 mM glucose in the presence or absence of 10 mM TTFA or 0.1 mM CCCP for 48 h. Fifty μM H_2O_2 or 100 μM iodoacetate was added in the last 30 min. GAPDH activity was determined as described previously [17,18].

Metabolite concentration assays. Concentrations of various glucose metabolites were assayed in MIN6 cells. Total cell lysates were obtained to the cells after 30 min stimulation with 25 mM glucose (normal control) with or without 50 μM H_2O_2 or 100 μM iodoacetate. Each metabolite was measured as previously described [19].

Statistical analysis. Data were expressed as means ± SD. Differences between two groups were evaluated by unpaired Student's *t* test. A P value <0.05 denoted the presence of a statistically significant difference.

Results

Glucose increases intracellular ROS derived from the mitochondrial electron transport chain

We examined whether incubation of MIN6 cells and human islets under high glucose results in increased intracellular ROS production. As shown in Figs. 1A and B, high glucose significantly increased intracellular ROS concentrations after 15 min of exposure and such an increase was observed at all other time intervals except at 6 and 24 h.

To confirm that ROS was derived from the mitochondrial electron transport chain, we examined the effects of several inhibitors of the mitochondrial electron transport system or intracellular antioxidant mimics, including rotenone, an inhibitor of complex I, TTFA, an inhibitor of complex II, CCCP, an uncoupler of oxidative phosphorylation, and TBAP, a manganese superoxide dismutase mimetic.

In MIN6 cells, 3 h of exposure to 30 mM glucose increased intracellular ROS and this effect was completely blunted by the addition of any of the inhibitors of the mitochondrial electron transport system (Fig. 1C). Furthermore, in human isolated islets, 30 mM glucose also induced an increase in intracellular ROS, which was completely suppressed by the addition of TTFA, CCCP or TBAP (Fig. 1D). These results suggest that high glucose increase intracellular ROS as a result of increased generation of NADH and $FADH_2$ which

augment the production of superoxide by the electron transport chain in pancreatic β-cells.

Effect of ROS on GIIS

In the next series of experiments, we examined the effects of ROS on GIIS. Inhibitors of the mitochondrial electron transport system suppress not only glucose-induced increase of intracellular ROS but also ATP synthesis. Because ATP is an important signal for GIIS per se, these inhibitors are inappropriate for examination of the effects of ROS. Accordingly, we used H_2O_2, a chemical substitute for ROS, to assess the effects of ROS on GIIS from β-cells.

First, MIN6 cells were cultured with either 2.8 or 25 mM glucose with various concentrations of H_2O_2. As shown in Fig. 2A, 25 mM glucose resulted in approximately 3-fold increase in GIIS compared with 2.8 mM glucose in H_2O_2-free medium. H_2O_2 suppressed GIIS in a dose-dependent manner. On the other hand, GIIS with 2.8 mM glucose was not affected by H_2O_2 (date not shown).

In mouse islets, 25 mM glucose resulted in approximately 7-fold increase in GIIS when islets were incubated in H_2O_2-free medium. H_2O_2 suppressed GIIS in a dose-dependent manner, although this was significant only at 50 μM and higher concentrations of H_2O_2, as observed in MIN6 cells. GIIS in response to 2.8 mM glucose was not affected by H_2O_2 in mouse islets (Fig. 2B). To confirm the fact that H_2O_2 does not affect cell viability, trypan blue staining was performed. No significant change in cell viability was observed following exposure of the cells to various concentrations of H_2O_2 (0–200 μM) (data not shown). These results suggest that the relatively low concentrations of H_2O_2 used in our experiments had deleterious effect on glucose-derived intracellular signal per se but had no effect on cell viability and thus maintain a normal secretory machinery.

Next, we examined the effects of ROS on the dynamics of GIIS in perifusion studies using isolated mouse islets. When glucose concentration in the perifusate was increased from 2.8 to 25 mM, a biphasic pattern of GIIS was observed (Fig. 2C, open circles). Interestingly, the first-phase of GIIS was significantly blunted when islets were exposed to 50 μM H_2O_2 (Fig. 2C, closed squares), although the H_2O_2-induced suppression of the second-phase of GIIS was not significant. When both groups of islets were stimulated sequentially with 10 μM glybenclamide at the end of perifusion, a similar degree of potentiation of insulin secretion was observed. These results suggest that 50 μM H_2O_2 does not have any inhibitory effects on sulfonyl-

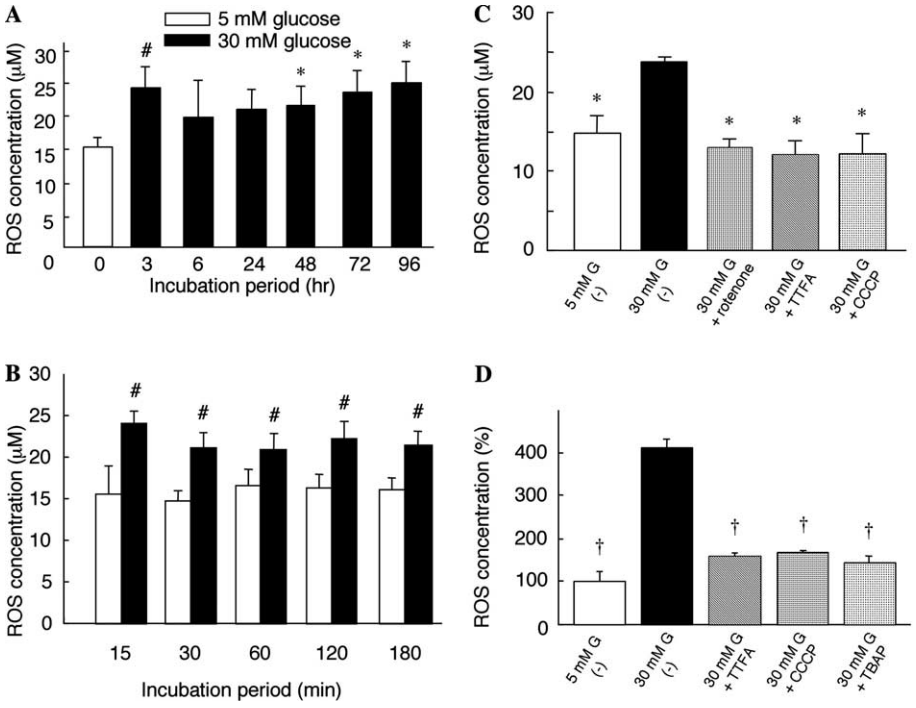

Fig. 1. Effects of glucose on intracellular ROS in MIN6 cells and human islets. MIN6 cells were incubated in KRBB containing 5 or 30 mM glucose for indicated times (A,B). MIN6 cells (C) or human islets (D) were preincubated in KRBB containing 5 or 30 mM glucose in the absence or presence of rotenone, TTFA, CCCP or TBAP for 3 h. Intracellular ROS was determined as described in "Materials and methods." Data are means ± SD of 8 (A), 4 (B), 8 (C), and 4 (D) experiments. $*P < 0.01$ and $\#P < 0.05$ compared with values incubated with 30 mM glucose for 0 h (A). $\#P < 0.05$ compared with values incubated with 5 mM glucose each time (B). $*P < 0.01$ and $\dagger P < 0.001$ compared with values incubated with 30 mM glucose alone.

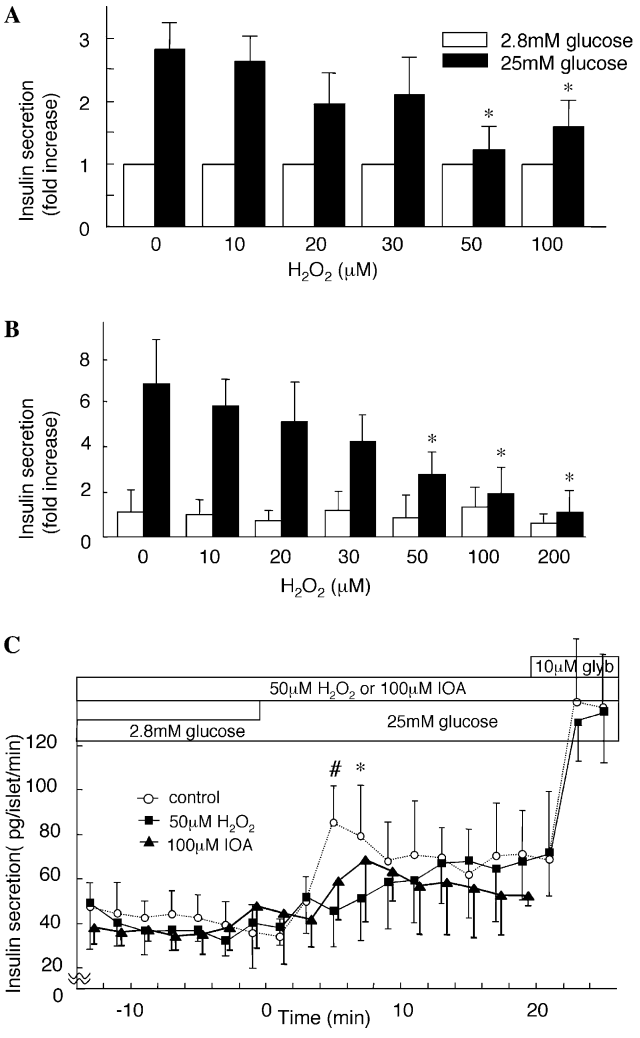

Fig. 2. Effect of H_2O_2 on GIIS in MIN6 cells (A) and mouse islets (B,C). MIN6 cells or mouse islets were incubated in KRBB containing 2.8 mM glucose with or without various concentrations of H_2O_2 at 37 °C for 30 min. Subsequently, cells were incubated in KRBB containing 2.8 or 25 mM glucose with or without various concentrations of H_2O_2 for further 30 min (A,B). One hundred and fifty islets were perifused with KRBB containing 2.8 mM glucose for 40 min without (open circles) or with (closed squares) 50 μM H_2O_2 or 100 μM iodoacetate (IOA) (closed triangles) and then with KRBB containing 25 mM glucose. At the end of perifusion, islets were perifused with KRBB containing 10 μM glybenclamide (C). Data are means ± SD of 10 (A,B), 6 (C) experiments. *$P < 0.05$ compared with incubation without H_2O_2 (A,B). *$P < 0.05$ and #$P < 0.001$ compared with medium containing 50 μM H_2O_2 (C).

urea-induced insulin secretion. They also suggest that the primary target of H_2O_2 in our experiments is present upstream of ATP-sensitive K^+ channel.

Effect of high glucose-induced mitochondrial superoxide overproduction on GAPDH activity

It has been reported that the activities of mitochondrial aconitase, a TCA cycle enzyme [20], mitochondrial adenine-nucleotide translocase [21], and GAPDH are susceptible to oxidative modification [22,23]. In the next series of experiments, we examined the effects of ROS on GAPDH activity in isolated mouse islets (Fig. 3A). GAPDH activity was significantly reduced to 68% by incubation of the cells with 5 mM glucose and 50 μM H_2O_2, relative to the activity under 5 mM glucose. In addition, 100 μM iodoacetate (IOA) also reduced GAPDH activity to 50%. GAPDH activity was also reduced to 64% by 25 mM glucose, and inhibitors of the mitochondrial electron transport system completely abrogated the reduction of GAPDH activity induced by 25 mM glucose. These results confirm the fact that high glucose-induced increase in intracellular mitochondrial ROS inhibits GAPDH activity.

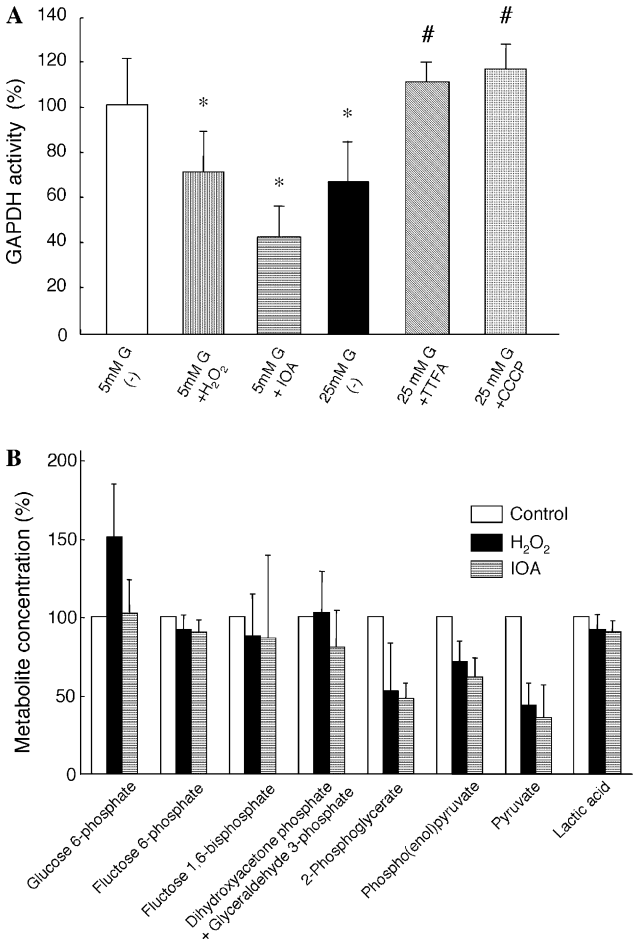

Fig. 3. Effect of ROS overproduction on GAPDH activity and metabolite concentrations. Islets were preincubated with 5 or 25 mM glucose in the presence or absence of TTFA or CCCP for 48 h. Fifty μM H_2O_2 or 100 μM iodoacetate (IOA) was added during the last 30 min. GAPDH activity was measured as described in "Materials and methods," and expressed relative to the activity at 5 mM glucose (A). MIN6 cells were incubated with 25 mM glucose in the presence or absence of 50 μM H_2O_2 or 100 μM IOA for 30 min, and metabolite concentrations were measured (B). Data are shown as percent of control. Data are means ± SD of six experiments (A) and four experiments (B). *$P < 0.05$ compared with values incubated with 5 mM glucose alone. #$P < 0.05$ compared with values incubated with 25 mM glucose alone.

Metabolite concentrations

As shown in Fig. 3B, the downstream metabolites of GAPDH, 2-phosphoglycerate, phosphoenolpyruvate, and pyruvate were decreased by H_2O_2 as well as by IOA. Unexpectedly, the upstream metabolites of GAPDH, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate were not changed by H_2O_2 or IOA, except that glucose 6-phosphate was increased by H_2O_2.

The effect of iodoacetate on GIIS

Finally we examined the effect of IOA on the dynamics of GIIS in perifusion studies. As shown in Fig. 2C closed triangles, the first- and second-phase of GIIS tended to be decreased when islets were exposed to 100 μM IOA, although the decrement was not statistically significant. These results further suggest that inhibition of GAPDH might be one of the mechanisms on the deterioration of GIIS by H_2O_2.

Discussion

The aim of the present study was to provide evidence that high glucose increase intracellular ROS via the mitochondria in pancreatic β-cells and that ROS play a crucial role on dysfunction of GIIS.

First we examined whether high glucose could increase mitochondrial ROS in pancreatic β-cells. Our results demonstrated a biphasic increase in intracellular ROS concentrations after exposure of these cells to 30 mM glucose. Although long exposure to high glucose concentrations (more than five weeks in rodents, and more than 30 passages in cell lines) [12,14,24,25] or to D-ribose (more than 72 h) [13] has been reported to increase intracellular ROS, our study is the first to report that intracellular levels of ROS could increase within a shorter time frame (15 min to 3 h) in β-cells. Why does the increase in ROS concentrations show a biphasic pattern? Previous studies indicated that transient exposure of pancreatic islets to high glucose increases the activities of antioxidant enzyme, such as Cu/Zn-SOD [26]. Therefore, up-regulation of this antioxidant enzyme may be one of the reasons why ROS transiently decreased within 6 and 24 h of exposure to high glucose. On the other hand, Laybutt et al. [27] reported the limitation of Mn-SOD and Cu/Zn-SOD induction by high glucose, suggesting that ROS production might surpass the elimination of ROS after long-term exposure to high glucose, with subsequent increase in intracellular ROS concentrations.

Previous studies demonstrated that intracellular ROS is generated exclusively from the mitochondrial electron transport chain in other cells such as cultured bovine aortic endothelial cells [10] and peritoneal mesothelial cells [28]. To our knowledge, however, mitochondria derived ROS production in pancreatic islets has not been reported previously. Therefore we examined the origin of intracellular ROS and demonstrated for the first time that ROS was generated from the mitochondrial electron transport chain in MIN6 cells and human islets by using inhibitors of the mitochondrial electron transport system and intracellular antioxidants.

Next, we examined whether intracellular ROS induced by high glucose could influence GIIS. Previous studies reported that high H_2O_2 concentrations (200 μM to 1 mM) could induce dysfunction of pancreatic β-cells mainly by hyperpolarization of mitochondrial membrane potential and consequent decrease in ATP/ADP ratio [29,30]. In contrast to these reports, we investigated the effects of much lower concentrations of H_2O_2 (50 μM) for the following: (1) intracellular concentration of ROS in MIN6 cells exposed to <30 mM glucose was 24.4 ± 2.1 μM in our study, (2) Anderson [31] reported that the H_2O_2 level during activation of phagocytes in vitro was 200 μM, and (3) 200 μM H_2O_2 potentiated insulin secretion under basal glucose concentration in a β-cell line INS-1 cell [29]. Therefore it was suggested that 200 μM or higher H_2O_2 could be considered as supraphysiological concentration for pancreatic β-cells and that such high concentration of H_2O_2 might be harmful not only to secretory machineries but also viability of β-cells. Our results showed that even at 50–100 μM, H_2O_2 significantly suppressed GIIS and that such suppression was observed under lower concentration of H_2O_2 (20–30 μM) in MIN6 cells and mouse islets. These results suggest that physiological levels of ROS observed under transient hyperglycemia could reduce GIIS.

We also examined the effects of ROS on the dynamics of GIIS in mouse islets. In this perifusion study, we used mouse islets, because mouse islets respond to glucose stimulation with a marked first-phase but relatively low second-phase, of GIIS increase [32]. The data shown in Fig. 2C demonstrate that the first-phase, but not the second-phase of GIIS was significantly suppressed by 50 μM H_2O_2. In contrast to glucose, insulin secretion in the presence of glybenclamide was not affected by 50 μM H_2O_2. Our results are similar to the clinical observation in early steps of type 2 diabetes, where first-phase of GIIS is defective, but insulin secretion to sulfonylurea is not [7].

Next, we investigated the target molecules for H_2O_2 effects on the first-phase of GIIS. Several studies have shown that oxidative stress or H_2O_2 negatively influenced pancreatic β-cells [14,20–22,29,30,33–36]. Chatham et al. [34] reported that in isolated rat heart, H_2O_2 caused inactivation of GAPDH. On the other hand, it is thought that abnormal GIIS in type 2 diabetes could be due to abnormalities between GLUT2 and ATP-sensitive K^+ channel. Mertz et al. [37] also reported that

glycolytic but not TCA cycle metabolism of glucose might be critically involved in insulin secretion signaling process. More recently, Eto et al. [38] reported that not only TCA cycle but also NADH shuttle system was pivotal to GIIS. In their model, GAPDH plays critical roles in both the catalysis of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway and the generation of NADH, which is transported into the mitochondria. Based on these reports, we hypothesized that GAPDH is a potential target molecule for H_2O_2 in suppressing the first-phase of GIIS from pancreatic β-cells. Our results indicated that mitochondrial ROS induced by high glucose suppressed the activity of GAPDH. It was also supported by the observation that the downstream metabolites of glucose were decreased by H_2O_2. The reason why the upstream metabolites of glucose were not increased is not clear at present. However one possibility is that upstream metabolites could flow into hexosamine pathway because it was reported that hyperglycemia induced 2.4-fold increase in hexosamine pathway activity via increased production of mitochondrial superoxide [23].

This inhibitory effect of high glucose and H_2O_2 on GAPDH activity could result in the suppression of both glycolysis and NADH shuttle, and consequently suppress the mitochondrial ATP synthesis. This could be one of the underlying mechanisms of the blunted first-phase of GIIS observed in the perifusion study using H_2O_2. Our results showed that in contrast to the first-phase, second-phase of GIIS increase in insulin secretion was not affected by 50 μM H_2O_2. Even when GAPDH activity was partially suppressed, a sufficient amount of ATP necessary to maintain the second-phase of GIIS could be synthesized by the remaining glycolytic activity in cells cultured under high glucose.

Our results suggest that suppression of oxidative stress in β-cells could prevent the progression of impaired glucose tolerance in vivo. In this regard, Kaneto et al. [39] reported that antioxidant treatment could protect against the onset of diabetes in rodent diabetic model, GK rats. Furthermore, STOP-NIDDM trial [40] reported that acarbose effectively reduced the risk of developing diabetes in subjects with impaired glucose tolerance. Because α-glucosidase inhibitor relieves postprandial hyperglycemia, protection of β-cells against ROS during transient hyperglycemia could be a possible mechanism by which acarbose prevents the progression of diabetes mellitus.

In conclusion, we have demonstrated in the present study that high glucose increases intracellular ROS production from the mitochondria in pancreatic β-cells. Excess ROS reduced GIIS, especially the first-phase of GIIS from pancreatic β-cells. Our results suggested that ROS-induced deterioration of GIIS is mediated, at least in part, by down-regulation of GAPDH activity. We propose that mitochondrial overwork could potentially occur in the early stages of glucose intolerance, which may cause common dysfunction of β-cells and the development of type 2 diabetes.

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