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Glucose-induced glutathione reduction in mitochondria is involved in the first phase of pancreatic β-cell insulin secretion

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ABSTRACT

Glucose can acutely reduce mitochondrial glutathione redox state in rat islets. However, whether glucose-stimulated mitochondrial glutathione redox state relates to glucose-stimulated insulin secretion (GSIS) remains unknown. We used genetically encoded redox-sensitive GFPs to target the mitochondria to monitor glutathione redox changes during GSIS in rat pancreatic β-cells. The results showed that mitochondrial glutathione was more reduced during GSIS, whereas inhibition of this glutathione reduction impaired insulin secretion. In isolated rat pancreatic islets glutathione reduction in mitochondria and the first phase of GSIS were concurrence at the early stage of glucose-stimulation. Our results suggest that the glucose-induced glutathione reduction in mitochondria is primarily required for the first phase of GSIS.

1. Introduction

Type 2 diabetes results from the combination of insulin resistance and defective glucose stimulation of insulin secretion (GSIS) by the endocrine pancreas. GSIS by pancreatic β-cells plays a critical role in blood glucose homeostasis. Insulin secretion after the stimulation of glucose occurs in two phases, in which the first phase release insulin within a few minutes of stimulation then it declines. The second phase begins a few minutes after the first phase and can reach peak at about 30–40 min [1]. This acute glucose effect depends on the acceleration of its oxidation by glycolysis and the mitochondrial Krebs cycle, which result in the increase in the production of various metabolic coupling factors, including NAD(P)H and ATP. Once the ATP: ADP ratio increases in the cytoplasm, the plasmalemmal ATP-sensitive potassium channel (K_{ATP}) closes, resulting in the depolarization of the plasma membrane, which triggers the opening of the voltage-gated Ca^{2+} channel [2]. This results in an influx of Ca^{2+} into the cell that triggers the exocytosis of insulin granules. The K_{ATP}-dependent mechanism may act as the 'triggering signal' responsible for the first phase of insulin secretion. Evidence is emerging that in addition to ATP and the ATP/ADP ratio, reactive oxygen species (ROS) derived from glycolytic and respiratory metabolism inβ-cells, in particular H_2O_2, serve as additional metabolic signals to elicit GSIS [3–5].

Recently, using the redox-sensitive fluorescent probe roGFP2 fused with Grx1, Dr. Jean-Christophe Jonas's group found that glucose and other nutrients acutely reduce mitochondrial but not cytosolic/nuclear E_{GSH} in rat islets. They also confirmed the observation in human islets [6]. But whether there is a relation between mitochondrial glutathione redox state and GSIS still remains unknown. Also, they didn't report where the reduced glutathione comes from during glucose stimulation.

In the present study, we used subcellular redox probe mito-Grx1-roGFP2 to detect redox changes in mitochondria during the

Abbreviations: Grx1, Glutaredoxin-1; GSIS, Glucose-stimulated insulin secretion; roGFP, Reduction-oxidation sensitive green fluorescent protein; ROS, Reactive oxygen species; GSH, reduced glutathione; GSSG, oxidized glutathione; DTT, 1,4-Dithiothreitol; BCNU, Carmustine; BSO, L-Buthionine-sulfoximine; γ-GCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; GR, glutathione reductase.

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GSIS process in INS-1E cells. Furthermore, we analysed the relation between the glutathione reduction state and insulin secretion. We showed that the glucose-induced glutathione reduction in mitochondria is primarily required for the first phase of GSIS. We also found that the reduced glutathione resulted from glutathione synthesis and reduction of oxidised glutathione.

2. Materials and methods

2.1. Materials

1,4-Dithiothreitol (DTT) and puromycin dihydrochloride were purchased from Amresco. Diamide, carmustine (BCNU), L-buthionine-sulfoximine (BSO), Thiazolyl blue tetrazolium bromide (MTT) and D-(+)-glucose were purchased from Sigma–Aldrich.

2.2. Cell culture

Rat insulinoma INS-1E cells, kindly provided by Drs. CB Wollheim and P Maechler (University Medical Center, Switzerland), were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, and 100 U/ml penicillin/streptomycin, as previously described [7,8]. HEK-293 cells (human embryonic kidney cells) were maintained in DMEM supplemented with 10% foetal bovine serum and 100 U/ml penicillin/streptomycin at 37 °C and 5% CO_2.

2.3. Plasmids

pLPCX mito-Grx1-roGFP2 plasmid was kindly provided by Dr. Tobias P. Dick (German Cancer Research Center, Heidelberg, Germany). The cDNA encoding mito-Grx1-roGFP2 was amplified by PCR from pLPCX mito-Grx1-roGFP2 (primer forward: 5′-TTCTAGAGCCACCATGGCCTCCACTCGTGT-3′; primer reverse: 5′-ATTTGCGGCCGCTTACTTGTACAGCTCGTCCAT-3′) and was inserted into the lentiviral expression vector pCDH-CMV.

2.4. Retroviral and lentiviral infection

The HEK-293 cells were co-transfected with a pCL-10A1 retrovirus packaging vector with pLPCX mito-Grx1-roGFP2 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After a 48 h infection, the retrovirus particles were collected from the HEK-293 cells and transfected into INS-1E cells. The INS-1E cell lines that stably expressed mito-Grx1-roGFP2 were identified in the presence of 2 μg/ml puromycin dihydrochloride.

The HEK-293T cells were co-transfected with psPAX2 and pMD2.G lentivirus packaging vectors with pCDH mito-Grx1-roGFP2 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After a 48-h infection, the lentivirus particles were collected from the HEK-293T cells and transfected into rat pancreatic islets. Two days later, the pancreatic islets expressing mito-Grx1-roGFP2 were measured by confocal microscopy.

2.5. MTT assay

The effect of BCNU and BSO on INS-1E mito-Grx1-roGFP2 cell viability was tested using MTT assay [9]. Approximately, 2.4 × 106 cells were plated in a 96-well plate in triplicate and cultured at 37 °C in the presence of 5% CO_2 for 24 h. They were exposed to various concentrations of BCNU and BSO for 24 h. At the end of the treatment, 0.05% MTT reagent (Sigma) was added to each well and kept for 3 h in incubation at 37 °C. Then, 100 μL of iso-propyl alcohol was added to each well of the 96-well plate to dissolve the MTT formazan crystals. Absorbance was measured at a wavelength of 570 nm and absorbance measured at wavelength 690 nm was subtracted as background. Results were calculated as percentages of the BCNU and BSO untreated cells which were set as 100%.

2.6. Fluorescence microscopy

INS-1E cells that stably expressed mito-Grx1-roGFP2 were seeded on a 35-mm culture-dish. After growing for approximately 48 h, the cells were incubated with 100 nM MitoTracker Orange CMTMRos (M-7510; Invitrogen) for 20 min, washed three times with PBS buffer, and incubated with RPMI 1640 medium without phenol red (Invitrogen). Image observations were performed with a Plan-Apochromat 63 × 1.4 oil objective on a Zeiss LSM 710 ConfoCor 3 microscope. A 488-nm laser line was used to excite mito-Grx1-roGFP2 with a 493–578-nm emission window, and a 568-nm laser line was used to excite the Mito Tracker within a 578–712-nm emission window. Scanning was performed using the “line mode” with a 639 × 639 format, 8 bit depth, 2 × line average, and a 0.8-μm pinhole.

Dynamic measurements of mito-Grx1-roGFP2 in rat pancreatic islets were carried out by using confocal microscopy (Nikon A1). The fluorescence ratio of mito-Grx1-roGFP2 (Ex. 405 and 488 nm, Em. 500–550 nm) was measured every 30 s. The results were normalized to the average fluorescence ratio of the first 10 min.

2.7. Live-cell fluorescence measurement with a microplate reader

INS-1E cells that stably expressed mito-Grx1-roGFP2 were seeded on 96-well plates (Corning) and were pre-incubated for 1 h at 37 °C in Krebs–Ringer bicarbonate buffer (KRBH buffer, 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 0.1% BSA, pH 7.4) containing 2.8 mM glucose [7]. Dual-excitation ratios were obtained with a BioTek Synergy NEO microplate spectrophotometer (BioTek) with 405 nm and 488 nm excitation and 520 nm emission for both excitation wavelengths [10]. The cell temperature was maintained at 37 °C during the detection.

2.8. Pancreatic islet isolation

Pancreatic islets were isolated from male Wistar rats (180–220 g) by collagenase (Roche) digestion and subsequent centrifugation in a Histopaque-1077 (Sigma) gradient as described [11]. The islets were hand-picked under a stereomicroscope. They were then either used for islet perifusion or cultured at 37 °C in the presence of 5% CO_2 with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, and 100 U/ml penicillin/streptomycin.

2.9. Islet perifusion and insulin secretion

Islet perifusion experiments were carried out by using a high-capacity, automated perifusion system (Biorep Inc), as described [12,13]. Size-matched 50 islets were loaded onto each column and immobilized in Bio-Gel P-4 Gel (Bio-Rad). The islets were then perifused with KRBH buffer containing 2.8 mM glucose at a perifusion rate of 100 μl/min for 1 h. Next, the islets were perifused with KRBH buffer containing 16.7 mM glucose for 1 h. The perifusate was collected in an automatic fraction collector designed for a 96-well plate format. Perifusates were collected every minute.

Insulin release in the perifusate was measured using a rat insulin ELISA kit (Mercodia, Sweden) following the manufacturer's instructions.

To measure insulin secretion from INS-1E cells, the cells were seeded onto 24-well plates and pre-incubated for 1 h at 37 °C in KRBH buffer containing 2.8 mM glucose. The cells were then incubated in KRBH buffer supplemented with the indicated concentrations of glucose for 1 h. The insulin in the media was measured using a rat insulin ELISA kit (Mercodia, Sweden) [7].

3. Results

3.1. Glucose specifically induces glutathione reduction in the mitochondria

We generated INS-1E cells that stably express mito-Grx1-roGFP2 by retroviral transduction (Materials and methods). We detected the localisation of mito-Grx1-roGFP2 in these stable cell lines. The confocal imaging of the INS-1E cells that expressed mito-Grx1-roGFP2 indicated that this probe exclusively targeted

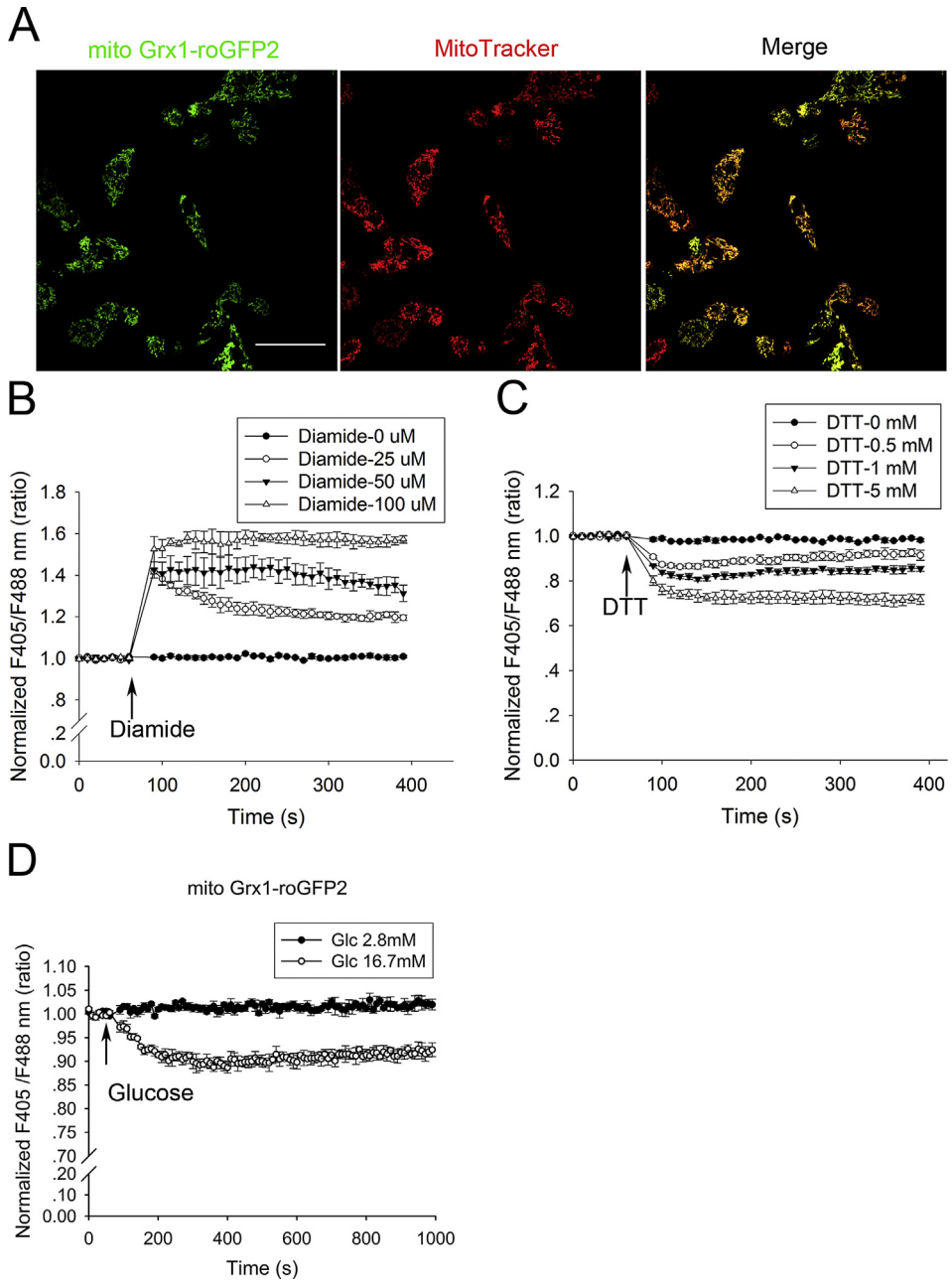

Fig. 1. The effects of glucose on INS-1E cells that stably expressed mito-Grx1-roGFP2. (A) The cultured INS-1E cells that stably expressed mito-Grx1-roGFP2 were incubated with 100 nM MitoTracker for 20 min and then examined by fluorescence microscopy. The overlay of mito-Grx1-roGFP2 and MitoTracker is shown in the right panel. Scale bar, 5 μm. (B and C) INS-1E cells stably expressing mito-Grx1-roGFP2 were excited at 405 and 488 nm in a microplate spectrophotometer, and the ratio of emissions at 525 nm was calculated. The cells were treated with diamide (B) or DTT (C) at the arrow-indicated time points. (D)INS-1E cells stably expressing mito-Grx1-roGFP2 were pre-incubated with KRBH buffer containing 2.8 mM glucose for 1 h and were then stimulated with 16.7 mM glucose at the arrow-indicated time points. The fluorescence intensity of the cells was detected as described in (B and C). The traces were first normalized individually to the average value of the seven earliest time points in each experiment. The results are presented as the mean ± S.E.M. (n = 3).

mitochondria because mito-Grx1-roGFP2 colocalised with the specific mitochondrial marker MitoTracker (Fig. 1A). Next, we examined the ability of mito-Grx1-roGFP2 to monitor changes in the mitochondrial redox of living cells. When the cells were treated with the oxidation reagent diamide, the microplate spectrophotometer detected an increase in the 405/488-nm ratio in the cells that expressed mito-Grx1-roGFP2; this increase occurred in a diamide concentration-dependent manner (Fig. 1B). However, the application of the reducing reagent DTT to the cells caused a decrease in the 405/488-nm ratio in a DTT concentration-dependent manner (Fig. 1C). Taken together, the present results show that the mito-Grx1-roGFP2 probes can specifically detect the redox potentials of the c mitochondria.

By using mito-Grx1-roGFP2-monitoring mitochondrial redox in the present study, we analysed redox changes in the mitochondria of INS-1E cells that were treated with low and high concentrations of glucose. The results detected by microplate spectrophotometer showed that the 405/488-nm ratio of the INS-1E cells expressing mito-Grx1-roGFP2 decreased by approximately 10% under the 16.7 mM glucose treatment (Fig. 1D). The result indicate that the mitochondrial glutathione reduction in INS-1E cells is enhanced by the high glucose treatment.

3.2. Glutathione reduction in mitochondria highly correlates with glucose-stimulated insulin secretion

Because a high glucose concentration is required for insulin secretion in pancreatic beta-cells, we investigated the relation between insulin secretion and mitochondrial glutathione reduction in INS-1E cells under various glucose concentrations. On one hand, the result showed that the insulin secretion levels of INS-1E cells that were treated with 11 mM or higher glucose concentrations were much higher than those treated with low glucose concentrations (2.8 and 5.6 mM) (Fig. 2A). On the other hand, the microplate spectrophotometer measurements indicated that the 405/488-nm ratio of the INS-1E cells expressing mito-Grx1-roGFP2 that were treated with 11 mM or higher glucose concentrations was much lower than that treated with low glucose concentrations (2.8 and 5.6 mM) (Fig. 2B).

Interestingly, the 5.6 mM glucose treatment slightly increased the insulin secretion of INS-1E cells compared with 2.8 mM glucose (Fig. 2A), and it accordingly decreased the 405/488-nm ratio of the cells (Fig. 2B). These results suggest that the degree of glutathione reduction in mitochondria is highly associated with the levels of glucose-stimulated insulin secretion. The lower the glutathione reduction in mitochondria, the higher the insulin secretion will be. Based on these data, we calculated the association coefficient using SigmaPlot. The regression that linked glutathione reduction in mitochondria to insulin secretion ($r = 0.9526$) is highly significant ($P < 0.01$; Fig. 2C).

3.3. The glucose-stimulated glutathione reduction in mitochondria is involved in insulin secretion

Reduced glutathione (GSH) is synthesized from L-glutamate with the help of two enzymes, namely γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) (Fig. 4A). Previous reports showed that GSH synthesis could be impaired by the

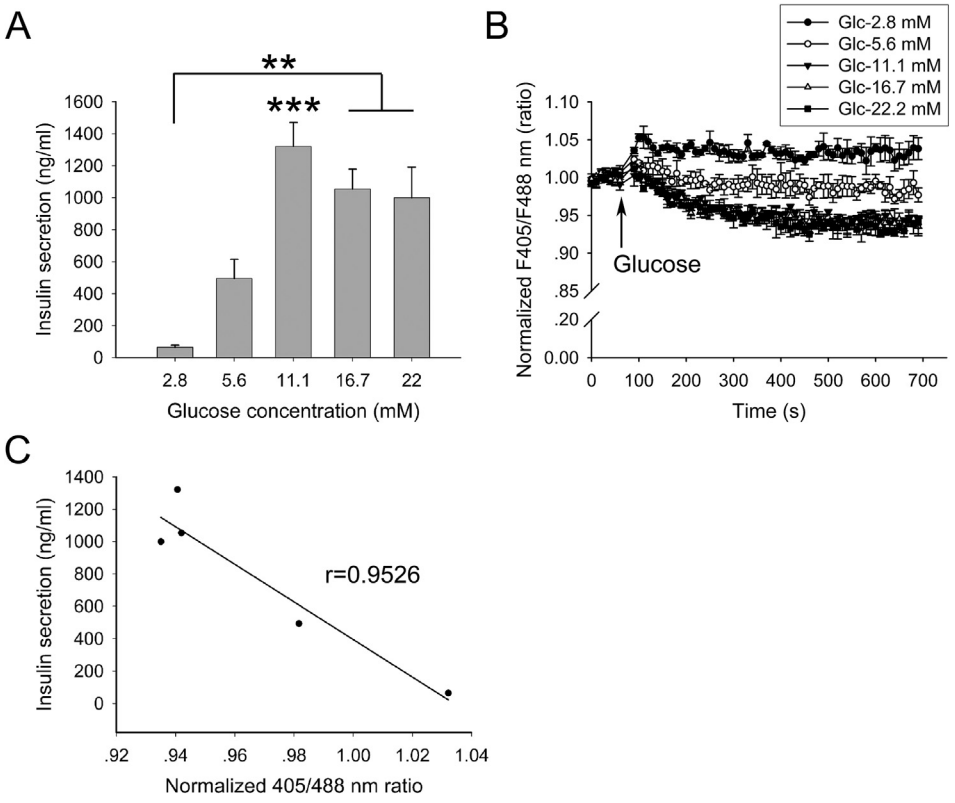

Fig. 2. The relationship between mitochondrial glutathione reduction and GSIS. (A) The insulin secretion of INS-1E cells that stably expressed mito-Grx1-roGFP2 was detected after the cells were incubated in KRBH buffers containing the indicated glucose concentrations for 1 h. The values are the mean ± S.E.M. from three independent experiments. *, $P < 0.05$. (B) The glutathione reduction in mitochondria of INS-1E cells that stably expressed mito-Grx1-roGFP2 were pre-incubated with KRBH buffer containing 2.8 mM glucose for 1 h and were then stimulated by the indicated glucose concentrations at the arrow-indicated time point. The fluorescence intensity of the cells was detected as described in Fig. 1(C) The regression linking insulin secretion to glutathione reduction in mitochondria was calculated ($r = 0.9526$, $P < 0.05$).

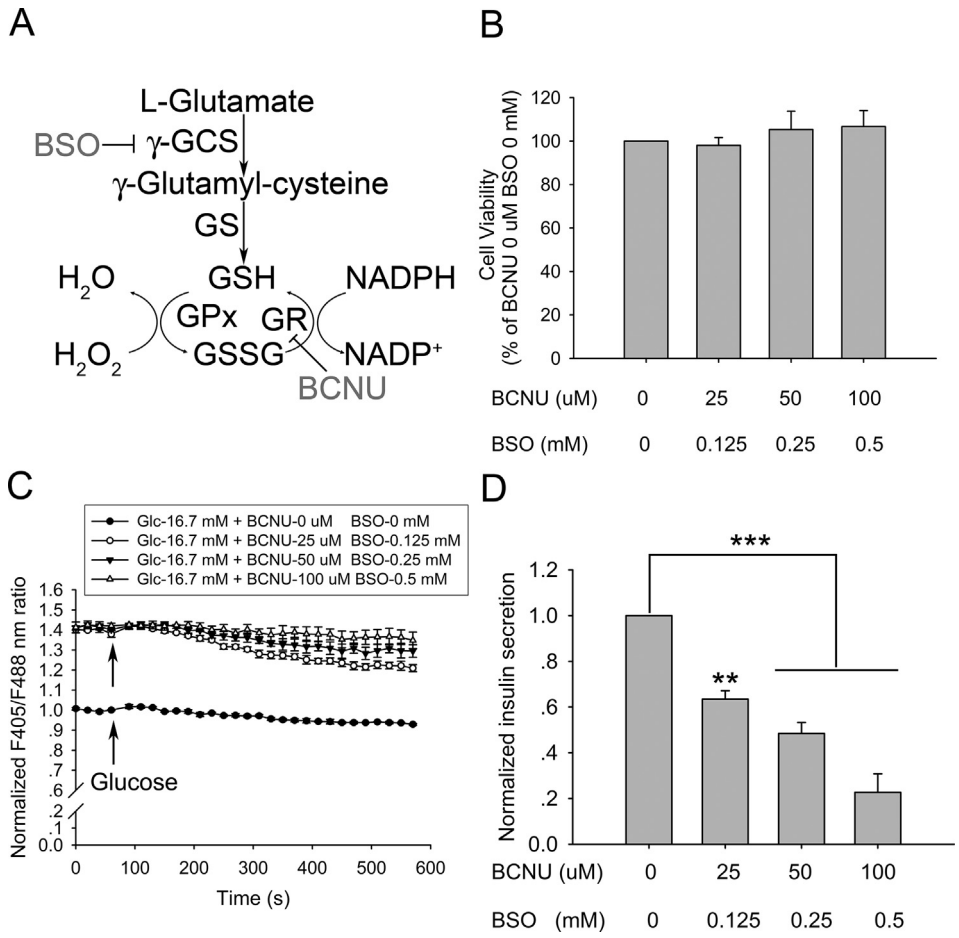

Fig. 3. The inhibition of glutathione reduction-impaired insulin secretion. (A) The glutathione redox pathway and its inhibitors; BSO inhibits GCS and BCNU inhibits GR. (B) Detection of cell viability of INS-1E cells that stably expressed mito-Grx1-roGFP2 after being exposed to BCNU and BSO at indicated concentrations for 24 h (C) INS-1E cells that stably expressed mito-Grx1-roGFP2 were incubated with medium containing the indicated concentrations of both BCNU and BSO for 24 h and were then incubated with KRBH buffer containing 2.8 mM glucose and stimulated with 16.7 mM glucose at the arrow-indicated time point. The fluorescence intensity of the cells was detected as described in Fig. 1. The results are presented as the mean ± S.E.M. (n = 3). (D) The insulin secretion of INS-1E cells stably expressing mito-Grx1-roGFP2 were detected under BSO and BCNU treatment as described in Fig. 4 C. The values are the mean ± S.E.M. from three independent experiments. *, $P < 0.05$ and **, $P < 0.01$.

chemical compound BSO, which inhibits γ-GCS [14,15]. Furthermore, GSH is also derived from the reduction of oxidised glutathione (GSSG) by glutathione reductase (GR), which could be inhibited by the chemical compound BCNU [16] (Fig. 3A). First, we used BSO and BCNU independently to block GSH synthesis or reduction of oxidised glutathione. When BSO was added, the mitochondrial glutathione still became more reduced after 16.7 mM glucose stimutation (Supplementary Fig. A), and didn't affect GSIS (Supplementary Fig. B). After BCNU was added, the mitochondrial glutathione also became more reduced after 16.7 mM glucose stimutation with the insulin secretion the same as control cells (Supplementary Fig. C and D). So, we speculated that the mitochondrial glutathione may have two resources: the GSH synthesis in the cellular and the reduction of oxidised glutathione by glutathione reductase.

Thus, we added both BSO and BCNU to INS-1E cells to inhibit GSH production, which did not decrease cell viability (Fig. 3B). In the presence of both drugs, the 405/488-nm ratio of INS-1E cells expressing mito-Grx1-roGFP2 under 16.7 mM glucose treatment was significantly higher than the cells without the drug treatment (Fig. 3C), indicating that glucose-stimulated glutathione reduction is impaired by these drugs.

We measured the levels of glucose-stimulated insulin secretion in the presence of the indicated concentrations of both drugs. The results indicated that insulin secretion was inhibited by the drug treatments in a dose-dependent manner (Fig. 3D). The inhibitory effects of insulin secretion were inversely correlated with the glutathione reduction levels (compare Fig. 3C with D). Taken together, these observations suggest that glutathione reduction in mitochondria is required by glucose-stimulated insulin secretion and the reduced glutathione resulted from both synthesis in the cellular and the reduction of oxidised glutathione by glutathione reductase.

3.4. Glucose-induced glutathione reduction in mitochondria is primarily required for the first phase of GSIS

GSIS is known to occur in two phases. The first phase occurs within a few minutes of glucose stimulation and initiates insulin secretion that is controlled by the triggering signalling pathway. Insulin secretion in the second phase can peak at approximately 30–40 min and is regulated by the amplifying signalling pathway [1,17]. We realized that the time points for measuring intracellular redox state were important. For example, ROS changes were varied across the previous experiments as measured at different time points. Dr. Rebelato's group observed that the intracellular ROS levels were significantly decreased within 15 min after glucose stimulation [18], whereas the increased ROS levels in the

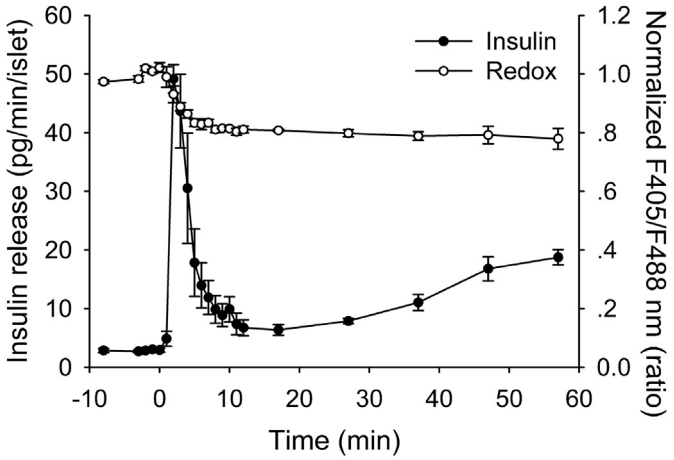

Fig. 4. Glutathione reduction in mitochondria and the first phase of GSIS at the early stage of glucose-stimulation. Rat pancreatic islets were isolated and transfected with lentivirus particles containing mito-Grx1-roGFP2. The fluorescence intensity was monitored by confocal fluorescence microscopy. The pancreatic islets were perifused with KRBH buffer containing 2.8 mM glucose for 1 h and were then stimulated with 16.7 mM glucose at the arrow-indicated time point. The values are the mean ± S.E.M. from three independent islet isolation.

controversial study were observed only after a 30-min glucose treatment [3].

In the present study, we found that the significant increase in reduced glutathione in mitochondria was detected within 2 min after the high glucose treatment, but no such significant redox change was detected in glutathione after that time point (Figs. 2 and 3B). These results suggest that the mitochondrial redox state in the earliest stage of glucose stimulation must be adjusted to a more reduced state. We therefore investigated the relationship between the first phase of insulin secretion and glutathione reduction in mitochondria under glucose stimulation in rat pancreatic islets (Materials and Methods). After the stimulation of 16.7 mM glucose, the first phase of insulin secretion in the isolated islets reached its peak at about 2 min and then fell down within 10 min (Fig. 4). Importantly, mito-Grx1-roGFP2 fluorescence ratio quickly decreased within 5 min and then kept at the similar level within 1 h (Fig. 4). These results indicate that glutathione reduction in mitochondria and the first phase of GSIS are concurrence at the early stage of glucose-stimulation. In conclusion, the present results suggest that the glucose-induced glutathione reduction in mitochondria is primarily required for the first phase of GSIS.

4. Discussion

In the present study, we demonstrate that glucose could induce glutathione reduction in mitochondria (Fig. 1D) in INS-1E cells, and this phenomenon is tightly associated with insulin secretion (Fig. 2). Once the mitochondrial glutathione reduction was blocked, the insulin secretion in INS-1E cells was reduced (Fig. 3). Glutathione reduction in mitochondria is required by glucose-stimulated insulin secretion and the reduced glutathione resulted from both synthesis in the cellular and the reduction of oxidised glutathione by glutathione reductase (Fig. 3). The glucose-induced glutathione reduction in mitochondria is primarily involved in triggering the signalling pathway to regulate the first phase of GSIS (Fig. 4).

Glutathionylation, a form of S-thiolation where protein cysteines form a disulfide bond with the cysteine of the tripeptide glutathione (GSH) [19], has been known for a long time. Glutathionylation is reversible and reduction of PSSG can take place spontaneously, when the GSH/GSSG ratio is high, or can be catalysed by protein thiol-disulfide oxidoreductases [20]. Regardless the molecular mechanisms, S-glutathionylation is one of the most important protein post-translational modifications and is viewed as a regulatory device for proteins involved in energy metabolism, redox signaling, and apoptosis [21–23]. In mitochondria, aconitase [24], α-ketoglutarate dehydrogenase [25], isocitrate dehydrogenase [26], succinyl-CoA transferase [27] can be inhibited upon glutathionylation. Glutathionylation of UCP2 can deactivate it and inhibit proton leak through UCP2 [28]. It was found that UCP2 became deglutathionylated following a 60-min glucose treatment [29]. Until now, UCP2 is the first and the only protein that undergos glutathionylation control during GSIS. As gultathionylation can impact the activity of many proteins, including UCP2 which have a negative influence in GSIS, we propose that the glutathiony reduction in mitochondria during GSIS may lead to the deglutathionylation of some proteins which play important roles in GSIS. We used anti-GSH antibody to immunoprecipitate glutathionylated proteins from mitochondria of INS-1E cells and identified by LC–MS/MS analysis. We found most of the glutathionylated proteins located at mitochondria complexes (data not shown). Further study should be carried out focusing on individual proteins which deglutathionylated after glucose stimulation and thus influence insulin secretion.

In conclusion, we propose that the intracellular redox state in the earliest stage of glucose stimulation must be adjusted to a more reduced state. This reduced state mainly retained by reduced glutathione which comes from GSH synthesis or the reduction of oxidised glutathione. The present data also suggest that the glucose-induced glutathione reduction in mitochondria is primarily involved in triggering the signalling pathway to regulate the first phase of GSIS.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Transparency document

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Appendix A. Supplementary data

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