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Mitochondrial dysfunction: Glucokinase downregulation lowers interaction of glucokinase with mitochondria, resulting in apoptosis of pancreatic β-cells

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ABSTRACT

Mitochondrial dysfunction has been considered a critical component in the development of diabetes. In pancreatic β-cells especially, mitochondrial dysfunction impairs insulin secretion and the eventual apoptosis of β-cells. The aim of this study was to elucidate the molecular mechanism underlying these events. Metabolic stress induced by antimycin or oligomycin was used to impair mitochondrial function in MIN6N8 cells, a mouse pancreatic β-cells, and the effects of glucokinase (GCK) and mitochondria were investigated. Concurrent with reduction in mitochondrial membrane potential ($\Delta\Psi m$) and cellular ATP content, impaired mitochondrial function reduced GCK expression and resulted in decreased insulin secretion and β-cell apoptosis. Specifically, lowered GCK expression led to decreased interactions between GCK and mitochondria, which increased Bax binding to mitochondria and cytochrome *C* release into cytoplasm. However, these events were blocked by treatment with the antioxidant, N-acetyl-cysteine (NAC), as well as GCK overexpression. Moreover, examination of the GCK promoter in antimycin-treated cells demonstrated that the promoter region within −287 bases from transcription site is involved in the transcriptional repression of GCK by mitochondrial stress, whose region contains a putative binding site for pancreatic duodenal homeobox-1 (PDX-1). Mitochondrial stress reduced PDX-1 expression, and increased ATF3 expression dependent on reactive oxygen species (ROS). Collectively, these data demonstrate that mitochondrial dysfunction by metabolic stress reduces GCK expression through PDX-1 downregulation via production of ROS, which then decreases the association of GCK with mitochondria, resulting in pancreatic β-cell apoptosis and reduction of insulin secretion.

1. Introduction

Mitochondrial dysfunction has been implicated in a large number of diseases, including neurodegenerative and retinal disorders, diabetes, aging, and other age-related diseases [1]. The molecular mechanisms underlying these individual disorders have not been well understood. Type 2 diabetes is characterized by peripheral insulin resistance and pancreatic β-cell dysfunction, which are defined as impaired insulin secretion and decreased β-cell mass associated with increased rates of β-cell apoptosis [2]. A number of studies have suggested that the prominent features of type 2 diabetes are caused by mitochondrial dysfunction [3–7]. For example, studies have shown that depletion of mitochondrial DNA or treatment with mitochondrial inhibitors caused insulin resistance by impairing insulin signaling in skeletal muscle cells [8,9]. Moreover, impaired insulin secretion and β-cell apoptosis due to mitochondrial dysfunction was especially apparent in pancreatic β-cells [10–13]. However, the molecular mechanisms responsible for these events are currently not well characterized.

Several published studies have linked glucose metabolism with apoptosis, as hexokinase (HK), which is found in the mitochondria, is known to play a role in integrating glucose metabolism and apoptosis [14–17]. Under normal conditions, the HK enzyme catalyzes the initial step of intracellular glucose metabolism. Although often associated with regulating glycolysis, both HK I and HK II can bind mitochondria with high affinity at outer mitochondrial

Abbreviation: ATF3, activating transcription factor 3; GCK, glucokinase; GLUT2, glucose transporter 2; HK, hexokinase; NAC, N-acetyl-cysteine; OMM, outer mitochondrial membrane; PDX-1, pancreatic duodenal homeobox-1; ROS, reactive oxygen species; TUNEL, Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling; VDAC, voltage dependent anion channel.

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membrane (OMM) contact sites where they interact with voltage dependent anion channels (VDAC). The association between mitochondrial HK (mtHK) and the OMM is dynamic and greatly influences the structure and conductance of VDAC, which may have regulatory implications for cytochrome *C* release. In addition to regulating VDAC activity, mtHK may directly antagonize the recruitment of Bax to the mitochondria, a potential mechanism for the antiapoptotic activity of mtHK [14–17]. Glucokinase, also known as HKIV, catalyzes the phosphorylation of glucose to glucose-6-phosphate, the first step in glucose metabolism leading to glycolysis and storage of excess glucose as glycogen in the liver and pancreas [18]. Insulin secretion stimulated by glucose is regulated by the rate of glucose metabolism within β-cells, and a key event in this process is the phosphorylation of glucose by GCK [18]. Therefore, optimal β-cell function may be dependent on the expression of genes involved in glucose sensing such as GCK. Similar to HK I and II, GCK also binds to mitochondria depending on glucose concentration, and gains preferential access to mitochondria-produced ATP and consequently increases the rate of glucose metabolism for insulin secretion [19,20]. Furthermore, it has been demonstrated that the interaction of GCK with mitochondria regulates β-cell apoptosis [20]. The binding of GCK to mitochondrial VDAC interferes with the ability of Bax to bind to mitochondria and release cytochrome *C*, and therefore prevents Bax-induced apoptosis in pancreatic β-cells. Thus, the association of GCK with mitochondria serves to link glycolysis with oxidative phosphorylation and prevention of apoptosis in pancreatic β-cells. Furthermore, it has been suggested that the binding of HKs to mitochondria is controlled in part by phosphorylation of Bad via Akt/protein kinase B [21]. Phosphorylation of Bad enhances glycolysis, as well as preventing apoptosis where glucose promotes Bad phosphorylation.

Pancreatic duodenal homeobox-1 (PDX-1), also known as IPF-1, IDX-1, and STF-1, is a homeodomain-containing transcription factor that plays a critical role in pancreatic development, β-cell differentiation, and maintenance of mature β-cell function through regulating the expression of insulin, islet amyloid polypeptide, GCK, and glucose transporter 2 (GLUT2) [22]. Interestingly, PDX-1 is a common target in glucotoxicity and lipotoxicity [23]. Glucotoxicity is associated with decreased PDX-1 protein expression, whereas PDX-1 is expressed, but retained in the cytoplasm in lipotoxicity. It is therefore likely that these toxic conditions, which occur concomitantly in most patients with type 2 diabetes, have deleterious effects on insulin gene expression [23].

Here, we investigated the effects of mitochondrial dysfunction on insulin secretion and apoptosis in pancreatic β-cells and the molecular mechanisms involved with these effects, with a particular focus on GCK. Our findings show that mitochondrial dysfunction represses GCK expression through PDX-1 downregulation in an ROS-dependent manner and decreased GCK interactions with mitochondria, leading to reduced insulin secretion and enhanced apoptosis of pancreatic β-cells.

2. Materials and methods

2.1. Cell line and reagents

MIN6N8 cells, S0V40 T-transformed insulinoma cells derived from NOD mice, were kindly provided by Dr. M. S. Lee (Sungkyunkwan University School of medicine, Seoul, Korea). These cells were grown in DMEM containing 15% fetal bovine serum, 2 mM glutamine, and 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBco Life Technologies, GrandIsland, NY, USA). Antibodies used in this study were obtained either from Cell Signaling Technology (Beverly, MA, USA) or Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Glucose was purchased from Biosource International (Camarillo, CA, USA). Antimycin, oligomycin, NAC, the ATP assay kit, and other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Induction of mitochondrial dysfunction

To induce mitochondrial dysfunction, MIN6N8 cells were treated with a mitochondrial respiratory chain inhibitor, antimycin (3 μM) or oligomycin (10 μM) for 24 h. In the case of lipid mix treatment, cells were grown in the presence of 1% lipid mix (Sigma, St Louis, MO, USA) for 24 h after 1 h serum starvation.

2.3. Plasmids

The GCK expression vector (pcDNA3-GCK) was generated as described previously [20]. Rat GCK promoter reporter constructs including pRGP-1003, pRGP-404, pRGP-287, and pRGP-84 were kindly provided by Dr. Y. H. Ahn (Yonsei University College of medicine, Seoul, Korea).

2.4. Measurement of mitochondrial membrane potential ($\Delta\Psi m$) and intracellular ATP contents

Mitochondrial membrane potential was determined using Mito-Tracker, a mitochondrially selective probe. Antimycin or oligomycin treated MIN6N8 cells in serum free medium were incubated with 100 nM Mitotracker Green FM (Molecular Probes-Invitrogen, Eugene, OR, USA) for 30 min. Fluorescent signals were detected using a confocal microscopy (Bio-Rad, Radiance 2000, Hercules, CA, USA) at Ex 490 nm/Em 516 nm and fluorescence intensity was measured using a FACSCalibar flow cytometer (Becton Dickinson Biosciences, San Jose, CA). Intracellular concentrations of ATP were determined using the ATP-dependent luciferin–luciferase bioluminescence assay (Sigma, St. Louis, MO, USA) as described previously [9].

2.5. Measurement of insulin secretion

Insulin release in response to glucose stimulation was determined as previously described [20], after treating MIN6N8 cells with antimycin or without antimycin treatment.

2.6. Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) assay

Apoptotic cells were detected using DeadEnd™ colorimetric TUNEL system (Promega, Madison, WI, USA). Briefly, cells were fixed on slides and then incubated with terminal transferase and Biotinylated Nucleotide Mix. After washing, cells were incubated with 20× DAB substrate, 20× DAB Chromogen and 20× hydrogen peroxide until there is a light brown background (typically approximately 10 min). The slides were then directly analyzed under a light microscope to observe staining.

2.7. Immunoblots and co-immunoprecipitation

The cell lysates were subjected to electrophoresis through 10% SDS-PAGE and blotted with respective antibodies. For endogeneous complexes, mitochondrial lysates (1 mg) fractionated from the cells were immunoprecipitated with 2 μg of anti-GCK or anti-VDAC antibody, and then immunoblotted with anti-VDAC or anti-Bax antibody. The immunoblots were visualized by chemiluminescence using the ECL Western Blotting System (Amersham Biosciences, Freiburg, Germany).

2.8. Measurement of reactive oxygen species (ROS)

Production of ROS was determined by CM-H_2DCFDA fluorescence assay. In brief, after treatment with antimycin or oligomycin, the cells were incubated with 5 μM of CM-H_2DCFDA probe (Molecular Probes-Invitrogen, Eugene, OR, USA) for 30 min at 37 °C, and then detached with trypsin. After washing the cells twice with PBS, fluorescence intensity

was measured using a FACSCalibar flow cytometer (Becton Dickinson Biosciences, San Jose, CA).

2.9. Stable transfection

MIN6N8 cells were grown in 6-well culture plates to about 60% to 70% confluence. MIN6N8 cells were transfected with pcDNA-GCK and vector control DNA using a lipofectin reagent (Invitrogen, Carlsbad, CA, USA). After a 16 h incubation period, the media was replaced with normal growth media and cells were grown for an additional 48 h. The cells were then exposed to a selective concentration of 400 μg/ml of geneticin (G418-sulfate) (GIBCo) to isolate stably transfected cells. Positive colonies were passed three times before selecting unique clones. The stably transfected cell lines were maintained under constant selective pressure in the continued presence of 400 μg/ml of geneticin.

2.10. Transient transfection and luciferase activity assay

MIN6N8 cells with 80% confluency were grown in six-well plates and subjected to transient transfection using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, the cells were lysed in lysis buffer (Promega), and luciferase activity was measured using the Luciferase Assay System (Promega).

2.11. Statistical analysis

All the data are expressed as means±SEM. Differences between the groups were determined by one way analysis of variance (ANOVA) using the SAS statistical analysis program (SAS Institute, Cray, NC, USA). Duncan's multiple range test was performed to evaluate any differences between the groups.

3. Results

3.1. Acute glucose treatment stimulates insulin secretion related to increased interaction between GCK with mitochondria

Since increasing glucose stimulates insulin secretion, we first examined the effect of glucose on insulin secretion and expression of GCK in MIN6N8 pancreatic β-cells. As shown in Fig. 1, when glucose concentrations increased from 2.2 mM to 16 mM, 16 mM glucose stimulated insulin secretion (Fig. 1A) and increased GCK expression significantly (Fig. 1B). As the interactions between GCK and mitochondria reportedly regulate both glucose metabolism and apoptosis in pancreatic β-cells, GCK and mitochondrial interactions were investigated by the co-immunoprecipitation assay using antibodies for GCK and mitochondrial VDAC. Consistent with upregulated GCK, 16 mM glucose increased interactions between GCK and mitochondrial VDAC (Fig. 1C). Furthermore, as it has been reported that Bad phosphorylation is necessary for GCK to interact with mitochondria, we investigated Bad phosphorylation and Akt phosphorylation, upstream of Bad phosphorylation, in glucose-stimulated MIN6N8 cells. As shown in Fig. 1D, Bad phosphorylation as well as Akt phosphorylation increased in 16 mM glucose-stimulated cells. These results suggest that acute glucose treatment triggers insulin secretion, which is associated with upregulated GCK expression and increased GCK and mitochondrial interaction.

3.2. Mitochondrial dysfunction reduces insulin secretion and induces apoptosis in pancreatic β-cells

Next, we elucidated the effects of mitochondrial dysfunction on the function of pancreatic β-cells and apoptosis. To this end, we examined insulin secretion and apoptosis in MIN6N8 cells with dysfunctional mitochondria induced by treatment with antimycin or oligomycin,

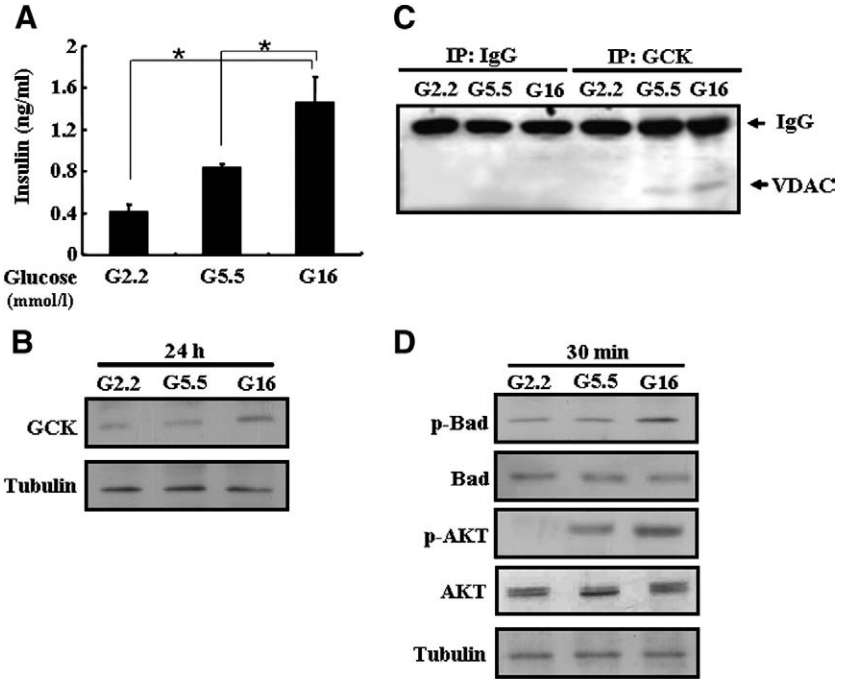

Fig. 1. Acute glucose treatment stimulated insulin secretion through increased interaction of GCK within MIN6N8 cells. MIN6N8 cells were incubated for 1 h in glucose free DMEM medium contained 2% FBS and subsequently were treated with different glucose concentrations of 2.2 mM (G2.2), 5.5 mM (G5.5) and 16 mM (G16) for 24 h. (A) The insulin content was measured by enzymatic immunoassay as described previously. Each bar represents ±SEM of three independent experiments performed in triplicate(*$p<0.0060$). (B) GCK expression was measured by western blot analysis. (C) Co-immunoprecipitation assay was carried out with the mitochondrial lysates isolated from the cells cultured for 24 h in indicated glucose concentrations. Mitochondrial lysates (1 mg) were immunoprecipitated with GCK antibody, and immunoblotted with VDAC antibody. (D) Phosphorylation of Bad was measured by immunoblotting with ser136 Bad after 30 min incubation in indicated glucose concentration. Figures in B, C, and D are representative of three independent experiments. Each experiment displayed similar results.

inhibitors of mitochondrial respiratory chains. Mitochondrial membrane potential was dramatically reduced in antimycin or oligomycin-treated cells (Fig. 2A) as revealed by FACS analysis (upper) and confocal microscopy (lower) after staining with Mitotracker. Concomitantly, total cellular ATP from antimycin or oligomycin-treated cells was reduced compared to control cells (Fig. 2B). In contrast to the ability of glucose to stimulate insulin secretion acutely, treatment with antimycin or oligomycin decreased insulin secretion induced by 16 mM glucose (Fig. 2C). Additionally, antimycin or oligomycin treatment induced marked genomic DNA fragmentation (Fig. 2D) and caused a significant increase in the number of TUNEL positive MIN6N8 cells relative to cells treated with only 16 mM glucose (Fig. 2E), suggesting that mitochondrial dysfunction induces apoptosis in pancreatic β-cells. Treatment with animycin or oligomycin also decreased levels of Bcl-2, whereas Bax levels increased, thereby increasing the Bax:Bcl-2 ratio (Fig. 2F). Furthermore, caspase 3 activation was observed in mitochondrial stress-treated β-cells (Fig. 2F). All together, these results reveal that mitochondrial dysfunction results in β-cell dysfunction and ultimately β-cell apoptosis.

3.3. Mitochondrial dysfunction downregulates GCK expression and consequently decreases GCK and mitochondrial interaction

To elucidate the molecular mechanisms involved in β-cell dysfunction induced by mitochondrial dysfunction, we examined GCK expression and its interaction with mitochondria. As shown in Fig. 3A, treatment with antimycin or oligomycin decreased GCK expression concomitant with reduced Glut2 levels, whereas tubulin expression was unaffected. Furthermore, after mitochondrial stress,

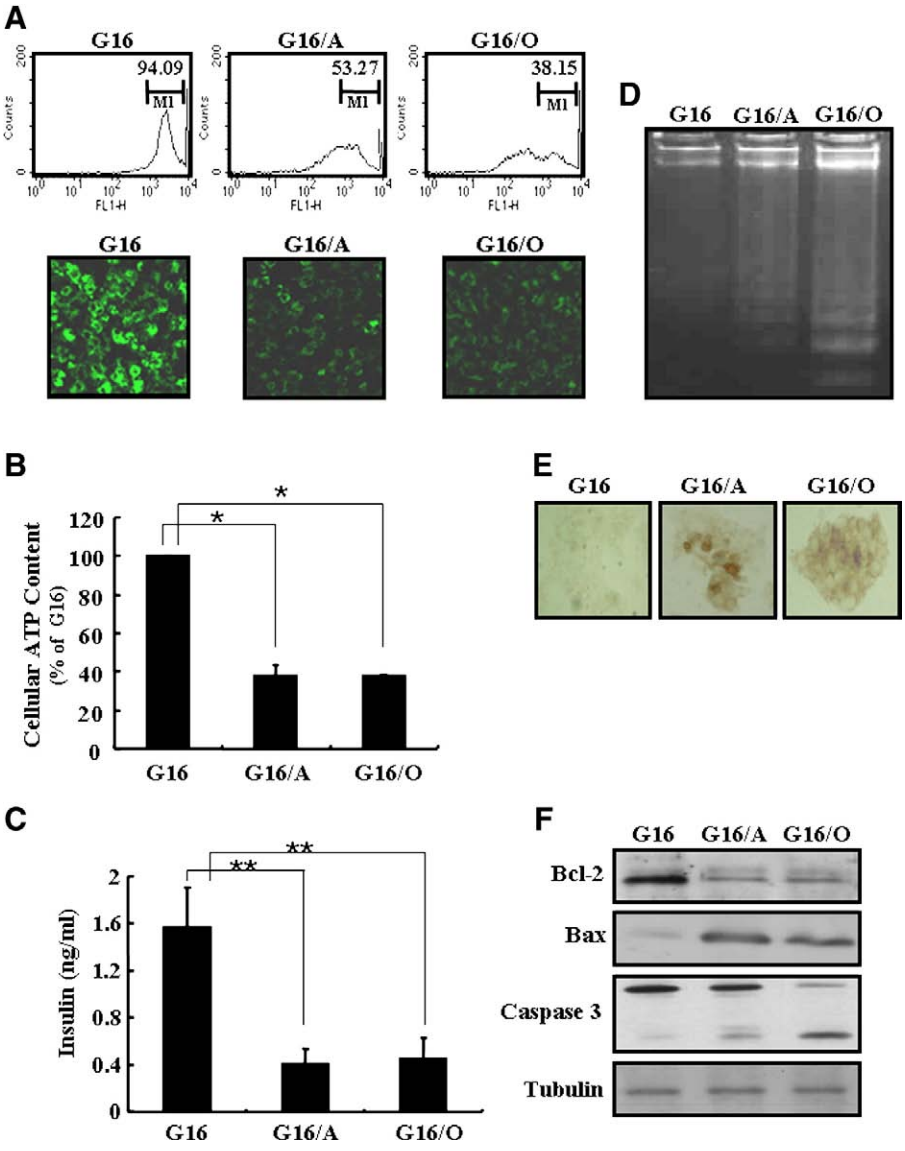

Fig. 2. Mitochondrial dysfunction induced apoptosis as well as reduced insulin secretion in pancreatic β-cells. MIN6N8 cells were incubated for 1 h in glucose free DMEM medium contained 2% FBS and then were treated with antimycin or oligomycin for 24 h in the medium containing 16 mM glucose. (A) The mitochondrial membrane potential was measured using MitoTracker. Fluorescence intensity of cells stained with MitoTracker Green FM was measured using a FACSCalibar flow cytometer for changes in mitochondrial membrane potential (M1, the percentage of mitochondrial membrane potential. The numbers gated in each phase areas are mean±SEM of three experiments (upper). Fluorescence of MitoTracker Green FM was detected using a confocal microscopy (lower). (B) The cellular ATP content was measured based on ATP-driven luciferin luciferase activity. Each value is expressed as the relative percentage compared to ATP content from G16 cells. Each bar represents ±SEM of three independent experiments performed in triplicate(*$p<0.0001$). (C) The insulin content was measured by enzymatic immunoassay. Each bar represents ±SEM of three independent experiments performed in triplicate (**$p<0.0170$). (D) Apoptosis was analyzed by DNA fragmentation on agarose gel electrophoresis. (E) Apoptosis was also assessed by TUNEL assay. TUNNEL positive cells showed a typical apoptotic configuration. (F) The levels of apoptotic-related proteins were measured by western blot analysis. Figures in D, E, and F are representative of three independent experiments. Each experiment displayed similar results.

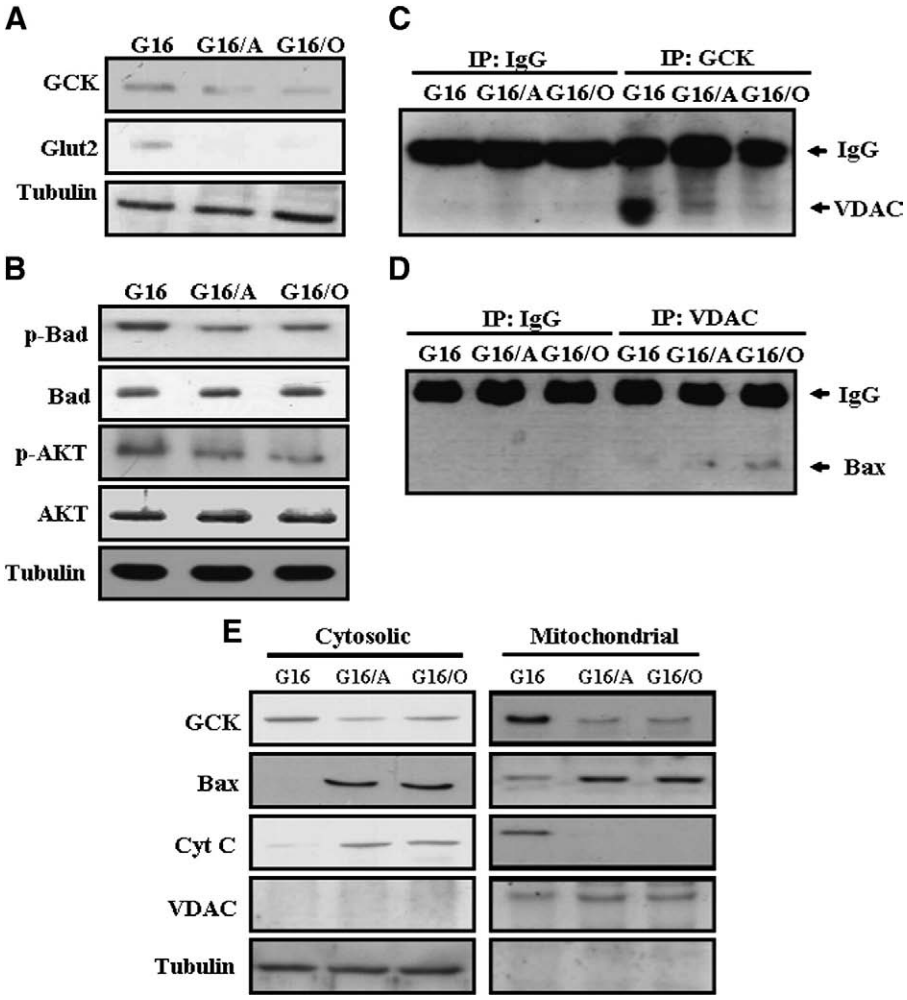

Fig. 3. Mitochondrial dysfunction reduced GCK expression and the interaction of GCK with mitochondria in MIN6N8 cells. (A) GCK level was measured by western blot analysis after treatment with antimycin or oligomycin for 24 h. (B) Phosphorylated Bad at ser 136 was measured after incubation with antimycin A or oligomycin for 30 min. (C) Interaction of GCK with mitochondria was analyzed by co-immunoprecipitation of GCK and mitochondrial VDAC with the mitochondrial lysates. Mitochondrial lysates (1 mg) were immunoprecipitated with GCK antibody, and immunoblotted with VDAC antibody. (D) Interaction of Bax with mitochondria was analyzed by co-immunoprecipitation of Bax and mitochondrial VDAC with the mitochondrial lysates. (E) The levels of cytochrome *C* and Bax were measured from mitochondrial fractions or cytosolic fractions from the cells incubated with antimycin or oligomycin for 24 h by western blot analysis. All figures are representative of three independent experiments. Each experiment displayed similar results.

Bad phosphorylation was also reduced in the absence of changes to protein levels (Fig. 3B). Consistent with reduced GCK expression and Bad phosphorylation, treatment with antimycin or oligomycin lowered GCK interactions with mitochondria in stress-treated cells (Fig. 3C). It has been demonstrated that the association between GCK with mitochondria prevents Bax from interacting with mitochondrial VDAC and therefore inhibits Bax-induced apoptosis. We therefore investigated interactions between Bax and mitochondrial VDAC in stress-treated MIN6N8 cells where the interactions between GCK and mitochondria have been significantly reduced. As shown in Fig. 3D, Bax interactions with mitochondrial VDAC increased after treatment with mitochondrial inhibitors (Fig. 3D). Next, to clarify changes in mitochondrial interactions with GCK or Bax induced by mitochondrial dysfunction, we measured protein levels of GCK or Bax in mitochondrial fractions from antimycin or oligomycin-treated cells. In agreement with results from the co-immunoprecipitation assay, GCK decreased while Bax levels increased in mitochondrial fractions from cells treated with mitochondrial inhibitors (Fig. 3E). Consequently, cytochrome *C* increased in the cytosolic fractions. These results indicate that mitochondrial dysfunction reduces interactions between GCK and mitochondria, resulting in enhanced Bax binding to mitochondria and release of cytochrome *C* into the cytosol, contributing to β-cell apoptosis.

3.4. ROS is involved in apoptosis of β-cells induced by mitochondrial dysfunction

Mitochondrial dysfunction can lead to increased production of ROS, and therefore we investigated the involvement of ROS in β-cell dysfunction and apoptosis in antimycin or oligomycin-treated MIN6N8 cells. As shown in Fig. 4A, treatment with antimycin or oligomycin increased ROS production as revealed by FACS analysis after staining with CM-H_2DCFDA. Next, to examine the involvement of ROS in insulin secretion and apoptosis, we investigated the effects of NAC, the antioxidant, insulin secretion and apoptosis in antimycin-treated cells. As shown in Fig. 4B, C, pretreatment with NAC significantly recovered insulin secretion (Fig. 4B) and prevented the apoptosis induced by antimycin (Fig. 4C). This suggests that ROS is responsible for the apoptosis induced by mitochondrial dysfunction. Next, we investigated whether ROS plays a role in decreased interactions between GCK and mitochondria though GCK downregulation. As shown in Fig. 4D, NAC treatment restored the reduction in both GCK expression and Bad phosphorylation. It appears then that NAC treatment restored GCK interactions with mitochondrial VDAC that were reduced by antimycin treatment (Fig. 4E). To further confirm these results, we examined protein levels of GCK, Bax, and cytochrome *C* in mitochondrial or cytosolic fractions from cells pretreated with NAC. Similar to previous

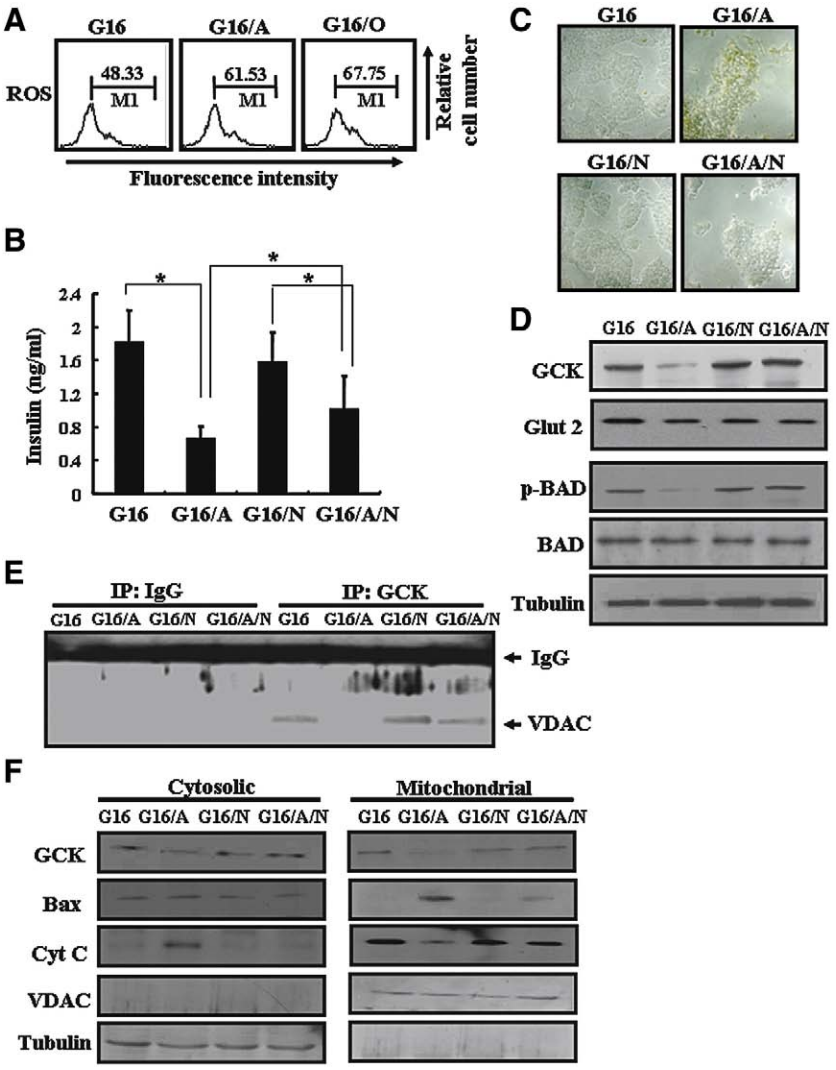

Fig. 4. ROS was involved in the apoptosis and decreased interaction of GCK with mitochondria induced by mitochondrial dysfunction. (A) ROS produced by mitochondrial dysfunction was measured with incubation of CM-H_2DCFDA for 30 min after treatment with antimycin or oligomycin for 6 h. Intracellular fluorescence intensity was detected by FACScan flow cytometry (M1, the percentage of ROS production). The numbers gated in each phase areas are mean ± SEM of three experiments. (B) Insulin contents were measured with enzymatic immunoassay from the cells pretreated with NAC for 1 h and then incubated with antimycin for 24 h. Each bar represents the mean ± SEM of three independent experiments (*$p<0.0105$). (C) The apoptosis was analyzed by TUNEL assay. The cells were pretreated with NAC for 1 h and then were incubated with antimycin for 24 h. One representative of three experiments is shown. (D) The levels of GCK and phosphorylated Bad were evaluated by western blot analysis with GCK or ser 136 Bad antibody. (E) The interaction of GCK with mitochondria was analyzed by co-immunoprecipitation of GCK and mitochondrial VDAC with the mitochondrial lysates fractioned from the cells pretreated with NAC for 1 h and then incubated with antimycin for 24 h. (F) The levels of GCK, Bax, and cytochrome *C* were determined from cytosolic fraction or mitochondrial fractions by western blot analysis. The cytosolic fractions or mitochondrial fractions were isolated from the cells pretreated with NAC for 1 h and then incubated with antimycin for 24 h. Figures in D, E, and F are representative of three independent experiments.

results, expression of GCK was restored in mitochondrial fractions by NAC pretreatment, while Bax decreased in mitochondrial fractions and accordingly, release of cytochrome *C* to cytosol was reduced (Fig. 4F). These results provide evidence that ROS generated by mitochondrial dysfunction decreases interactions between GCK and mitochondria through reduced GCK expression and Bad phosphorylation, causing apoptosis of MIN6N8 cells.

3.5. GCK overexpression prevents mitochondrial dysfunction-induced apoptosis

To examine the essential role of GCK in mitochondrial dysfunction-induced apoptosis, MIN6N8 cells were transfected with the pcDNA-GCK that overexpresses GCK, followed by treatment with antimycin. As shown in Fig. 5A, while GCK expression was reduced in Neo-control cells after antimycin treatment, GCK expression was sustained in GCK-overexpressing cells (Fig. 5A). When insulin secretion and apoptosis were investigated in GCK-overexpressing cells, expression of GCK restored insulin secretion reduced by antimycin treatment (Fig. 5B). Additionally, expression of GCK restored ATP content by antimycin treatment (Fig. 5C) and prevented antimycin-induced apoptosis (Fig. 5D). We then investigated whether this reversal was associated with interactions between GCK and mitochondria. Results from co-immunoprecipitation assays showed that antimycin decreased GCK interactions with mitochondrial VDAC in Neo-transfected cells, whereas GCK interactions were not affected in GCK overexpressing cells after antimycin treatment compared with untreated GCK-overexpressing cells (Fig. 5E). Consistent with these results, GCK levels were unaffected in mitochondrial fractions from GCK-overexpressing cells treated with antimycin. Correspondingly, Bax levels were not increased in mitochondrial fractions from GCK-overexpressing cells as much as in neo-transfected cells, and cytochrome *C* release into cytoplasm was significantly reduced in GCK-overexpressing cells (Fig. 5F). All of these results indicate that GCK overexpression inhibits apoptosis induced by

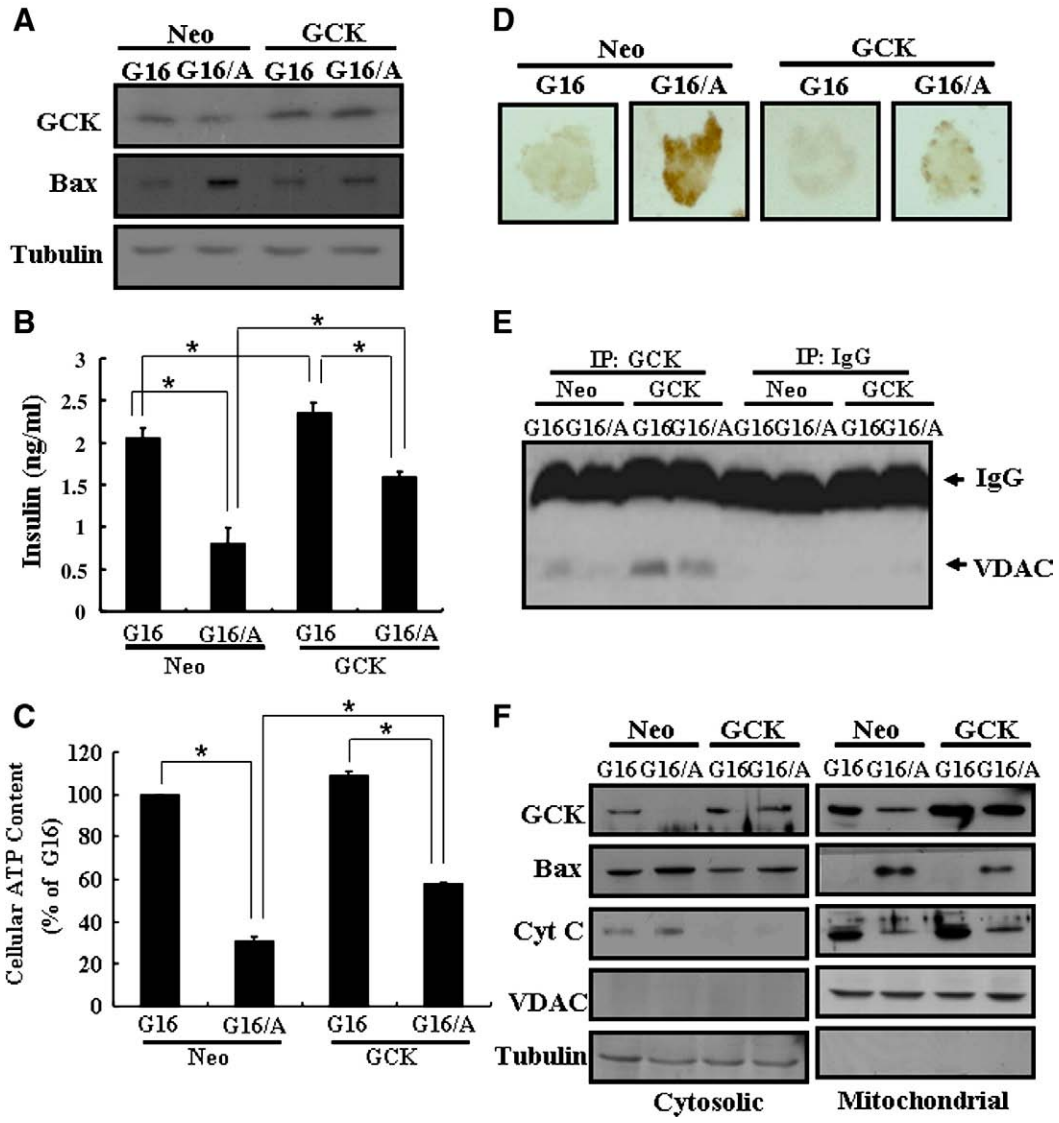

Fig. 5. GCK overexpression prevented the apoptosis induced by mitochondrial dysfunction. MIN6N8 cells were stably transfected with either Neo or wild type GCK expression vector. The transfected MIN6N8 cells were incubated for 1 h in glucose free DMEM medium contained 2% FBS and were subsequently treated with antimycin for 24 h in the medium containing 16 mM glucose (A) GCK levels were measured by western blot analysis. (B) The insulin contents were measured by enzymatic immunoassay. Each bar represents the mean ± SEM of three independent experiments(*$p<0.0001$). (C) The cellular ATP content was measured based on ATP-driven luciferin luciferase activity(*$p<0.0001$). (D) Apoptosis was analyzed by TUNEL assay. (E) The interaction of GCK with mitochondria was analyzed by co-immunoprecipitation of GCK and mitochondrial VDAC with the mitochondrial lysates. (F) The levels of GCK, Bax, and cytochrome *C* from cytosolic fractions or mitochondrial fractions were measured by western blot with respective antibody. The figures are representative of three independent experiments.

mitochondrial dysfunction through increased GCK interaction with mitochondria, thereby inhibiting Bax translocation into the mitochondria and cytochrome *C* release.

3.6. Repression of PDX-1 induced by mitochondrial dysfunction plays an important role in transcriptional downregulation of GCK expression

In an effort to elucidate the mechanism responsible for the downregulation of GCK by mitochondrial dysfunction in MIN6N8 cells, we analyzed rat GCK promoters by reporter assay after treating with antimycin. The various 5′ flanking sequences of rat GCK gene were fused to luciferase reporter gene and the promoter activities were investigated in antimycin-treated cells. As shown in Fig. 6A, the promoter activity of −1003 bp region exhibited approximately 5 fold reduction after treatment with antimycin. However, this reduction was significantly reversed by NAC treatment. When the promoter activities of constructs containing sequential deletions, −404, −287, −84 were investigated, the promoter activities of mutants containing deletions within the −404 and −287 promoter regions were also reduced from mitochondrial dysfunction, and whose repression was significantly restored by NAC treatment. In contrast, deletions beyond the −84 reduced promoter activity to basal levels and abolished the reduction of GCK transcription by mitochondrial dysfunction, indicating that the transcription factor responsible for repression of GCK expression is located within the −287/−84 region. We then searched putative transcription factor binding sites present between −287 and −84 using TESS, a web-based tool, and found a putative binding site for PDX-1 within the −208/−202 region, a transcription factor important for GCK expression (Fig. 6B). To see whether PDX-1 is regulated by mitochondrial inhibitors and ROS, we examined the expression of PDX-1 and related transcription factors in antimycin-treated cells. As shown in Fig. 6C, PDX-1 levels decreased in antimycin-treated MIN6N8 cells, whereas ATF3 levels increased. However, these changes were diminished after pretreatment with NAC (Fig. 6C). Since PDX-1

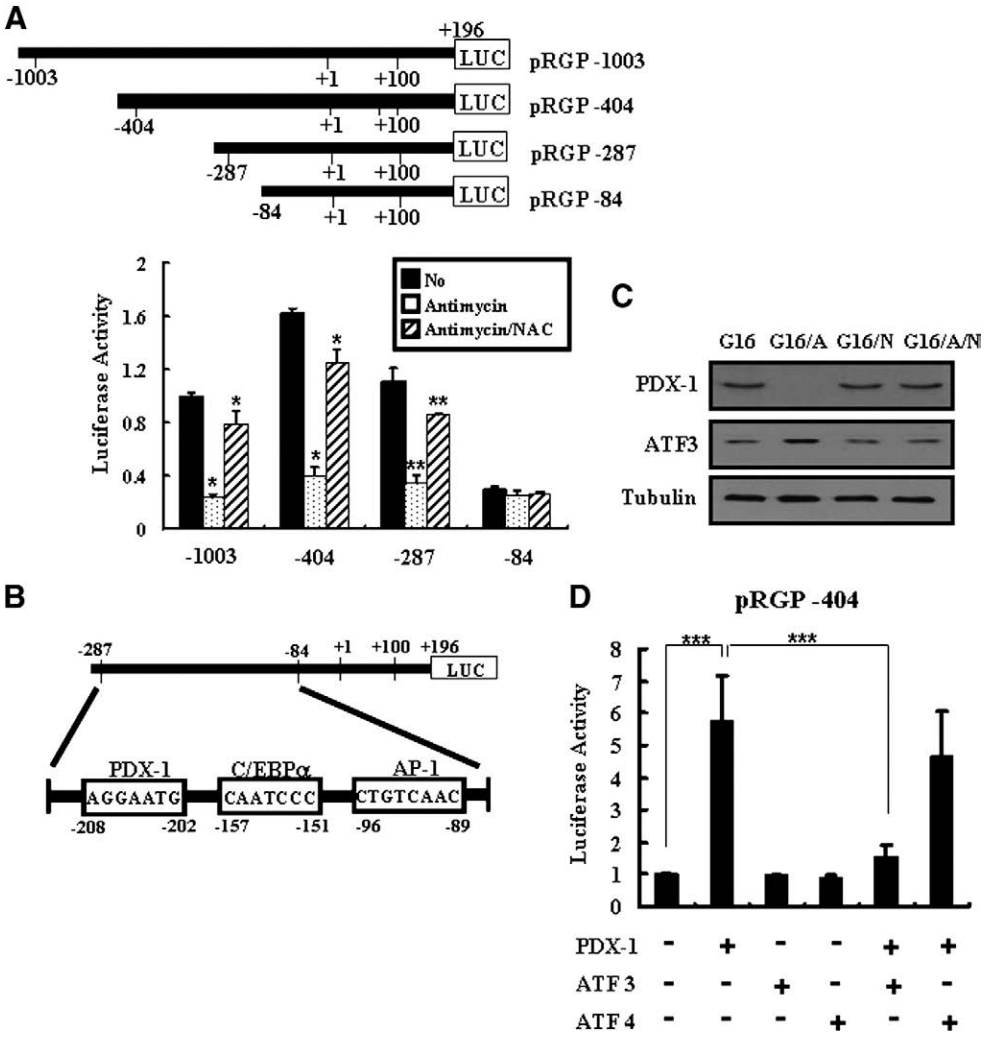

Fig. 6. PDX-1 and ATF3 were involved in downregulation of GCK expression by mitochondrial dysfunction. (A) The promoter activities were analyzed by luciferase assay. Various serial deleted 5′ flanking regions of the rat glucokinase gene were cloned into pGL3 basic reporter plasmid and were transfected into MIN6N8 cells. After transfection for 48 h, the cells were incubated with antimycin for 24 h, and then was collected for promoter assay. NAC was pretreated for 1 h before incubation with antimycin. Each value is expressed as the relative compared to luciferase activity of −1003 GCK promoter with no treatment. Each bar represents ±SEM of three independent experiments performed in triplicate (*$p<0.0001$, **$p<0.0005$). (B) Schematic representation of rat glucokinase −287 and −84. (C) The levels of PDX-1 or ATF-3 were determined by western blot. After incubation with antimycin for 24 h, the cell lysates were immunoblotted with PDX-1 or ATF3 antibody. (D) The promoter activities were analyzed by luciferase assay. After −404 GCK promoter were transfected into HepG2 cells with PDX-1, ATF3, or ATF4 expression vectors, the promoter activities were measured by luciferase assay. Each value is expressed as the relative compared to luciferase activity of −404 GCK promoter without cotransfection. Each bar represents the mean±SEM of three independent experiments (***$p<0.0027$).

has been demonstrated to stimulate expression of β-cell specific genes including insulin, GCK and Glut2, we further examined the effects of PDX-1 expression on GCK promoter activity in MIN6N8 cells cotransfected with the PDX-1 expression and −404 GCK reporter construct. As shown in Fig. 6D, PDX-1 expression significantly increased promoter activity of the −404 bp region, whereas expression of ATF3 or ATF4 did not affect promoter activities directly. These results suggest that mitochondrial dysfunction represses expression of PDX-1, resulting in GCK downregulation. Furthermore, even though ATF3, whose expression was induced by antimycin treatment, did not repress GCK promoter directly as shown in Fig. 6D, ATF3 significantly inhibited GCK promoter activities stimulated by PDX-1 (Fig. 6D). Therefore, these results indicate that ATF3 also plays a role in downregulating GCK expression by mitochondrial dysfunction through inhibition of PDX-1 transcriptional activity. Taken together, we postulate a mechanism for repression of GCK expression by mitochondrial stress in MIN6N8 cells, whereby elevation of ROS decreases PDX-1 expression and increases ATF3 expression, resulting in the subsequent reduction of PDX-1-mediated transcriptional activation of GCK, which in turn downregulates GCK expression.

3.7. Mitochondrial dysfunction induced by lipid also leads to lower interaction of glucokinase with mitochondria, and causes the apoptosis of pancreatic β-cells

Elevation of intracellular lipid is more physiological conditions of impaired mitochondria function. We asked whether elevation of intracellular lipid could induce mitochondrial dysfunction, and subsequent apoptosis. We treated MIN6N8 cells with lipid mixture containing palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and cholesterol, respectively. As shown in Fig. 7A, mitochondrial membrane potential was decreased by high lipid contents, and cellular ATP level was reduced similar to oligomycin treatment (Fig. 7B). Concomitantly, treatment with lipid mixture decreased insulin secretion (Fig. 7C). Additionally, lipid mixture treatment caused a significant increase in the apoptosis of MIN6N8 cells relative to cells treated with only 16 mM glucose (Fig. 7D), suggesting that lipid mixture also induces apoptosis in pancreatic β-cells. Treatment with lipid mixture decreased GCK expression (Fig. 7E) and Bad phosphorylation (Fig. 7F). Consistent with reduced GCK expression and Bad phosphorylation, treatment with lipid mixture lowered GCK interactions with mitochondria in stress-treated

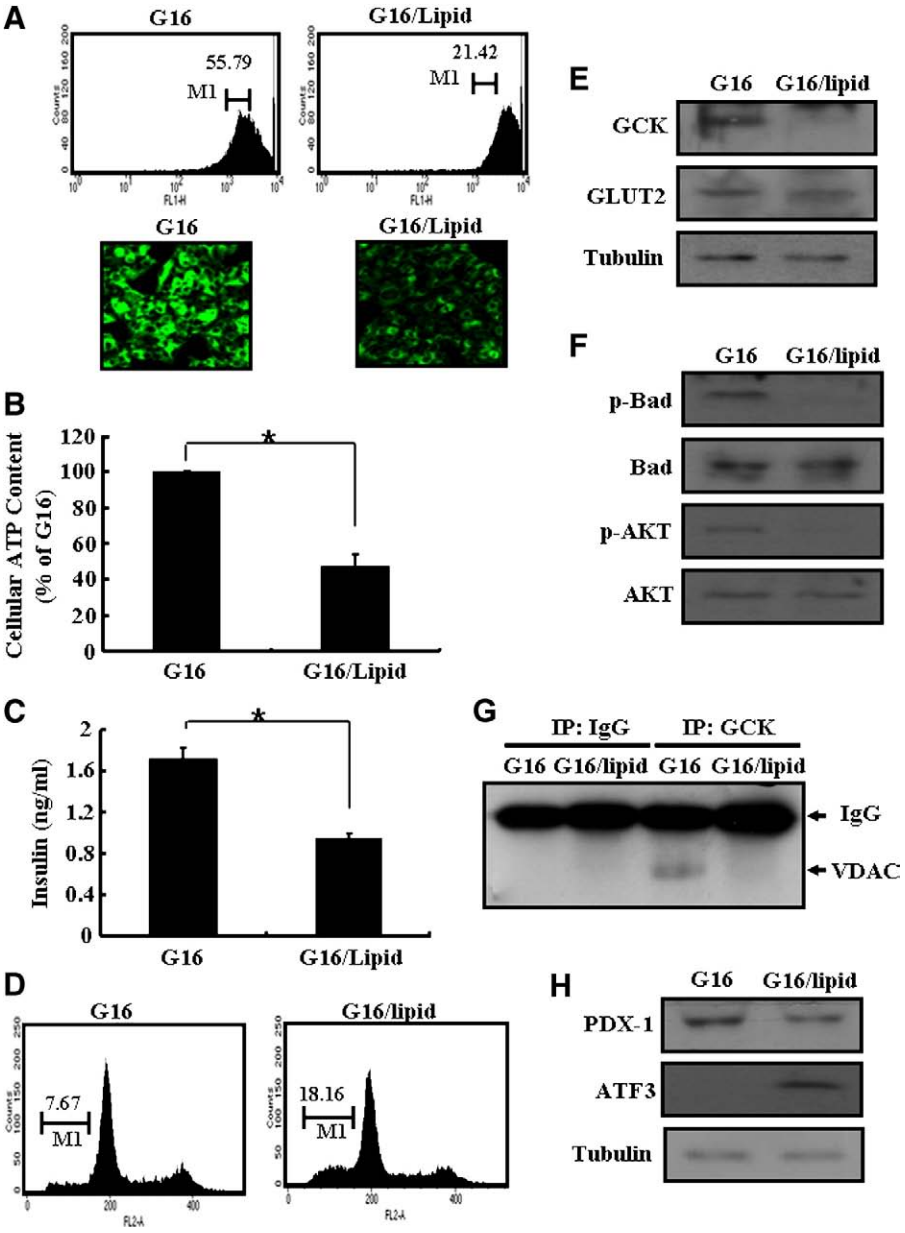

Fig. 7. Mitochondrial dysfunction induced by lipid also leads to lower GCK interaction with mitochondria and induces apoptosis of β-cells. (A) The mitochondrial membrane potential was measured by fluorescence intensity of cells stained with MitoTracker Green FM using a FACSCalibar flow cytometer and confocal microscopy. (B) The cellular ATP content was measured based on ATP-driven luciferin luciferase activity. Each bar represents ±SEM of three independent experiments performed in triplicate(*$p<0.0001$). (C) The insulin content was measured by enzymatic immunoassay. Each bar represents ±SEM of three independent experiments performed in triplicate(*$p<0.0001$). (D) Apoptosis was analyzed by FACS. (M1, the percentage of apoptosis) (E) GCK level was measured by western blot analysis after treatment with lipid mixture for 24 h. (F) Phosphorylated Bad at ser 136 was measured after incubation with lipid mixture for 6 h. (G) Interaction of GCK with mitochondria was analyzed by co-immunoprecipitation of GCK and mitochondrial VDAC with the mitochondrial lysates. (H) PDX-1 or ATF3 levels were measured by western blot analysis after treatment with lipid mixture.

cells (Fig. 7G). Furthermore, PDX-1 levels decreased in lipid mixture-treated MIN6N8 cells, whereas ATF3 levels increased (Fig. 7H). From these results, we postulated the general mechanisms by which disturbance of mitochondrial function could lower glucokinase interaction with mitochondria and induce apoptosis of pancreatic β-cells.

4. Discussion

Mitochondrial dysfunction caused by mutation and reduction of mitochondrial DNA (mtDNA) have been implicated in the pathogenesis of diabetes as well as in all the major neurodegenerative and aging-related diseases [24]. Several reports have demonstrated that impaired mitochondrial function produces insulin resistance in skeletal muscle and the liver [25,26]. Previously, we reported that mitochondrial dysfunction induced by treatment with respiratory inhibitors in C2C12 myocytes represses IRS-1 through ATF3 in a Ca^{2+} and c-*jun* N-terminal kinase (JNK)-dependent manner, and decreases tyrosine phosphorylation through increased serine phosphorylation, resulting in aberrant insulin signaling and abnormal glucose utilization observed in many insulin resistance states [9]. It was also shown that reduced IRS-1 expression and insulin-stimulated phosphorylation of IRS-1 and akt2 were associated with insulin resistance in L6Glut4 mys myocytes, where 95% of the mitochondria had been depleted by treatment with ethidium bromide (EtBr) [8]. Results from another in vivo study demonstrated that insulin resistance in the elderly is related to increased intramyocellular fatty acid metabolites that may be a result of an age-associated reduction in mitochondria oxidation and phosphorylation [27,28].

It has been established that mitochondrial dysfunction caused by mitochondrial poisons and EtBr in β-cells inhibits glucose-stimulated insulin secretion, and induces apoptosis with reduced proliferation [12]. Moreover, an A3243G mutation has been associated with reduction of islet mass involving both β-cells and neighboring cells [11]. Since in type 2 diabetes, pancreatic β-cells fail to produce enough insulin for the body's demand due to loss of β-cell mass caused by an increased rate of β-cell apoptosis, it is suggested that mitochondrial damage in β-cells may contribute to development of type 2 diabetes [28]. However, the molecular mechanisms underlying these events induced by mitochondrial dysfunction are not well characterized in β-cells. In the current study, we demonstrated that mitochondrial dysfunction caused by respiratory inhibitors resulted in reduced insulin secretion and apoptosis of pancreatic β-cells. Under these circumstances, the expression of GCK and its interactions with mitochondria, which play an important role in regulating β-cell apoptosis, decreased consistent with decreased Bad phosphorylation. However, overexpression of GCK to compensate for reduced GCK expression induced by mitochondrial dysfunction resulted in an increase in the interactions of GCK with mitochondria and then prevented the induction of apoptosis as well as reduction of insulin secretion by treatment with antimycin. Consequently, decreased interactions between GCK and mitochondria through downregulated GCK and decreased Bad phosphorylation plays a role in inducing apoptosis in MIN6N8 cells with defunct mitochondria in addition to regulating glucose metabolism for insulin secretion. These results indicate that GCK involvement is integral for the events between impaired insulin secretion and apoptosis induced by mitochondrial dysfunction. Reportedly, β-cells are especially vulnerable to attacks by ROS, because expression of antioxidant enzymes in pancreatic islets is very low [23]. Increased ROS levels impair glucose-stimulated insulin secretion, decrease gene expression of key β-cell genes, and induce cell death [23]. In this study, we observed production of ROS in MIN6N8 cells treated with mitochondrial inhibitor. However, NAC treatment prevented reduction of both GCK expression and Bad phosphorylation, and then restored GCK binding to mitochondria reduced by mitochondrial dysfunction. Therefore, these results suggest that ROS is responsible for decreased interactions between GCK and mitochondria through reduced GCK expression, and for apoptosis in MIN6N8 cells with dysfunctional mitochondria.

It has been shown that mitochondrial dysfunction can greatly modify nuclear gene expression via transcription factor regulation. Retrograde regulation, a general term for mitochondrial signaling, is broadly defined as cellular responses to changes in the functional status of mitochondria [29]. Much of our understanding of the regulation of the retrograde response has been derived from studies with *Saccharomyces cerevisiae* [30]. In mammalian systems, mitochondrial retrograde signaling has been described in C2C12 skeletal myoblasts and in human lung carcinoma A549 cells [31,32]. Mitochondrial dysfunction in both cell types resulted in elevated cytosolic free Ca^{2+} which activates factors such as NFkB, NFAT, and ATF3 dependent on Ca^{2+}-dependent protein kinases, including PKC and CaMK, and enhanced expression of genes involved in Ca^{2+} transport and storage, including ryanodine receptor I and II. Here, we determined which transcription factor is involved in downregulating GCK to characterize retrograding signaling relating to downregulated GCK in pancreatic β-cells. From the data obtained from an examination of the GCK promoter in antimycin-treated cells, the −83 GCK promoter, which does not have the PDX-1 binding site, nearly abolished the response triggered by mitochondrial dysfunction, suggesting that PDX-1 may play a critical role in downregulating GCK by mitochondrial dysfunction. Since PDX-1 is essential for activating several genes important for β-cell function including GCK, insulin, and Glut2, we investigated PDX-1 level in antimycin-treated cells. Treatment with the mitochondrial inhibitor reduced expression of PDX-1 dependent on ROS, suggesting that GCK downregulation by mitochondrial dysfunction is due to reduced PDX-1 expression. Furthermore, we investigated the involvement of ATF3 in GCK downregulation because ATF3 expression was increased in antimycin-treated cells. While expression of ATF3 did not repress promoter activity of GCK, it inhibited PDX-1-stimulated promoter activity of GCK. Since PDX-1 functionally interacts with other transcription factors and regulates its target gene expression [33], ATF3 induced by mitochondrial dysfunction may interact with PDX-1 and then inhibit GCK expression through suppression of PDX-1 transcriptional activity. We have now demonstrated the interaction of ATF3 with PDX-1.

5. Conclusion

In conclusion, we demonstrated the role of GCK, a critical enzyme for glucose metabolism in pancreatic β-cells, in inducing apoptosis after mitochondrial dysfunction. We also characterized the retrograde signaling associated with GCK downregulation in β-cells. Mitochondrial dysfunction reduced GCK expression concomitant with decreased Bad phosphorylation in an ROS dependent manner, and led to decreased interactions between GCK and mitochondria, resulting in impaired insulin secretion and apoptosis in pancreatic β-cells. Additionally, mitochondrial dysfunction suppressed PDX-1 transcriptional activity through reduced PDX-1 expression and increased ATF3 expression, thereby downregulating GCK expression.

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