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Molecules and Cells

ER Stress Is Implicated in Mitochondrial Dysfunction-Induced Apoptosis of Pancreatic Beta Cells

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Mitochondrial dysfunction induces apoptosis of pancreatic β-cells and leads to type 2 diabetes, but the mechanism involved in this process remains unclear. Chronic endoplasmic reticulum (ER) stress plays a role in the apoptosis of pancreatic β-cells; therefore, in current study, we investigated the implication of ER stress in mitochondrial dysfunction-induced β-cells apoptosis. Metabolic stress induced by antimycin or oligomycin was used to impair mitochondrial function in MIN6N8 cells, which are mouse pancreatic β-cells. Impaired mitochondria dysfunction increased ER stress proteins such as p-eIF2α, GRP78 and GRP 94, as well as ER stress-associated apoptotic factor, CHOP, and activated JNK. AMP-activated protein kinase (AMPK) was also activated under mitochondria dysfunction by metabolic stress. However, the inhibition of AMPK by treatment with compound C, inhibitor of AMPK, and overexpression of mutant dominant negative AMPK (AMPK-K45R) blocked the induction of ER stress, which was consist-ent with the decreased β-cell apoptosis and increase of insulin content. Furthermore, mitochondrial dysfunction increased the expression of the inducible nitric oxide synthase (iNOS) gene and the production of nitric oxide (NO), but NO production was prevented by compound C and mutant dominant negative AMPK (AMPK-K45R). Moreover, treatment with 1400W, which is an inhibitor of iNOS, prevented ER stress and apoptosis induced by mitochondrial dysfunction. Treatment of MIN6N8 cells with lipid mixture, physiological conditions of impaired mitochondria function, activated AMPK, increased NO production and induced ER stress. Collectively, these data demonstrate that mitochondrial dysfunction activates AMPK, which induces ER stress via NO production, resulting in pancreatic β-cells apoptosis.

INTRODUCTION

Mitochondrial dysfunction is associated with type 2 diabetes and insulin resistance (Petersen et al., 2003; Stump et al., 2003). However, the molecular mechanisms underlying these individual disorders are currently not well understood. Type 2 diabetes is initially characterized by peripheral insulin resistance, defined as decreased insulin sensitivity of peripheral tissues (liver, skeletal muscle, and adipose tissue) 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 (Petersen et al., 2006). Mitochondria dysfunction in skeletal muscle has been shown to cause insulin resistance and type 2 diabetes (Lim et al., 2006; Park et al., 2005). Furthermore, several lines of evidence suggest that mitochondria dysfunction in pancreatic β-cell induces failure of β-cell function and contribute to the pathogenesis of type 2 diabetes (Hayakawa et al., 1998; Kennedy et al., 1998; Robey et al., 2006; Simmons et al., 2005; Tsuruzoe et al., 1998). Mitochondrial dysfunction impaired insulin secretion and induced β-cell apoptosis. However, the molecular mechanisms responsible for mitochondria dysfunction-induced β-cell apoptosis are currently not well characterized.

The endoplasmic reticulum (ER) is a highly dynamic organelle responsible for multiple cellular functions. The ER produces the transmembrane proteins and lipids for most cell organelles and is responsible for the synthesis of almost all secreted proteins. The ER also plays an important role in Ca^{2+} storage and signaling. However, the factors that perturb ER function lead to the accumulation of misfolded protein in ER, leading to ER stress and subsequent activation of a signal response known as UPR. The UPR functions to reduce the amount of nascent protein that enters the ER lumen, to increase the ER capacity to fold protein through transcriptional upregulation of ER chaperones and to induce degradation of misfolded proteins. However, chronic or overwhelming ER stress induces pathogenesis of multiple human disease (Kim et al., 2008). Persistent ER stress also induces insulin resistance in liver and adipose tissue, reduces insulin secretion and causes apoptosis in β-cells; therefore, it plays a critical role in the pathogenesis of diabetes (Fonseca et al., 2009).

Previously, we demonstrated that mitochondrial dysfunction induced by treatment with antimycin or oligomycin, which are

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inhibitors of mitochondrial respiratory chains, leads to the apoptosis of pancreatic β-cells through decreased interaction of glucokinase with the mitochondria (Lee et al., 2009). However, the molecular mechanisms involved remain unclear. Therefore, in the current study, we attempted to elucidate another molecular mechanism underlying these events. Here, we investigated the involvement of ER stress in mitochondrial dysfunction induced-pancreatic β-cell apoptosis. Our findings show that mitochondrial dysfunction activates AMPK, which induces ER stress through increased NO production, resulting in apoptosis of pancreatic β-cells.

MATERIALS AND METHODS

Cell line and reagents

MIN6N8 cells, which are S0V40 T-transformed insulinoma cells derived from NOD mice, were grown in DMEM containing 15% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, USA). Antibodies used in this study were obtained from either Cell Signaling Technology (USA) or Santa Cruz Biotechnology Inc. (USA). Antimycin, oligomycin, and other chemicals were obtained from Sigma (USA). A Griess reagent kit for nitrite quantitation was purchased from Invitrogen.

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 12 h. In the case of the lipid mix treatment, cells were grown in the presence of 1% lipid mix (Sigma) for 24 h after 1 h of serum starvation.

Western blot

Cell were lysed in RIPA buffer (50 mM Tris, pH 7.5, 1% NP-40, 150 mM sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) at 4°C, after which immunoblot was performed as described previously (Lee et al., 2009).

Flow cytometric analysis of apoptosis

After cells were trypsinized, approximately 10^6 cells were collected by centrifugation at 1000 × *g* for 5 min. Cells were then washed in phosphate-buffered saline (PBS) followed by resuspension and fixation in 70% ethanol for approximately 2 h. Next, cells were washed with PBS and then resuspended in 500 μl of PBS containing 100 μg RNase, followed by incubation at 37°C for 30 min. Cellular DNA was then stained by the addition of 10 μg propidium iodide, and cells were analyzed by FACScan analysis using Cellquest software (Becton Dickinson, USA).

Stable transfection

The AMPK-WT and AMPK-K45R pcDNA3 expression vectors for wild-type and mutant rat AMPKα2 were a generous gift from Dr. M. Birnbaum (University of Pennsylvania School of Medicine, USA). MIN6N8 cells were grown in 6-well culture plates to about 60% to 70% confluence. The cells were then transfected with pcDNA-AMPK-WT or pcDNA-AMPK-K45R and vector control DNA using a lipofectin reagent (Invitrogen). The stably transfected cells were isolate in the presence of 400 μg/ml of geneticin.

Measurement of insulin secretion

Insulin release in response to glucose stimulation was determined as previously described (Lee et al., 2009), after treating MIN6N8 cells with antimycin or without antimycin treatment.

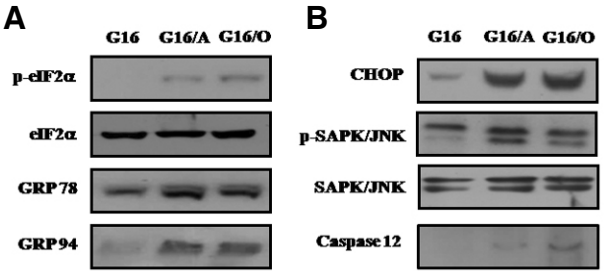

Fig. 1. Mitochondrial dysfunction induced ER stress in pancreatic β-cells. MIN6N8 cells were incubated for 1 h in glucose free DMEM medium containing 2% FBS and then treated with antimycin (3 μM) or oligomycin (10 μM) for 12 h in medium containing 16 mM glucose. (A) ER stress proteins including phospho-eIF2α, GRP78 and GRP94 were measured by Western blot (B) ER stress-associated apoptotic proteins including CHOP and phospho-SAPK/JNK were examined by Western blot.

Measurement of nitrite

The level of NO derived from the MIN6N8 cell culture supernatant was determined using commercially available Griess reagent as described by the manufacturer (Invitrogen). The nitrite levels were assayed in triplicate after treating MIN6N8 cells with or without antimycin.

Statistical analysis

All data are expressed as means ± SEM. ANOVA was used to determine significant differences between the groups and was performed using the Duncan test. $P < 0.05$ was considered statistically significant. All experiments were performed at least three times.

RESULTS

Mitochondrial dysfunction induces ER stress and increases ER stress-associated apopotic factors in pancreatic β-cells

It has been reported that ER stress plays a critical role in the development of type 2 diabetes through induction of insulin resistance and apoptosis of pancreatic β-cells (Fonseca et al., 2009). ER stress-mediated apoptosis is caused by JNK activation, induction of CHOP, and activation of caspase 12 in the lumen of ER (Park et al., 2006). Therefore, to evaluate the involvement of ER stress in the apoptosis of β-cells induced by mitochondria dysfunction, we examined ER stress proteins and ER stress-associated apoptotic factors in β-cells with dysfunctional mitochondria. As shown in Fig. 1A, ER stress proteins such as p-eIF2α, GRP78 and GRP94 were upregulated by treatment with antimycin or oligomycin. Consistent with the increase in ER stress proteins, the levels of CHOP, phosphorylated JNK and activated caspase 12 were increased (Fig. 1B). These results indicate that mitochondrial dysfunction induces ER stress and increases ER stress-associated apoptotic factors, which may contribute to the apoptosis of β-cells.

Mitochondrial dysfunction activates AMP-protein kinase (AMPK), which plays a role in the induction of ER stress and apoptosis by mitochondrial dysfunction

Next, to characterize the upstream molecular signaling associated with the induction of ER stress by mitochondrial dysfunc-

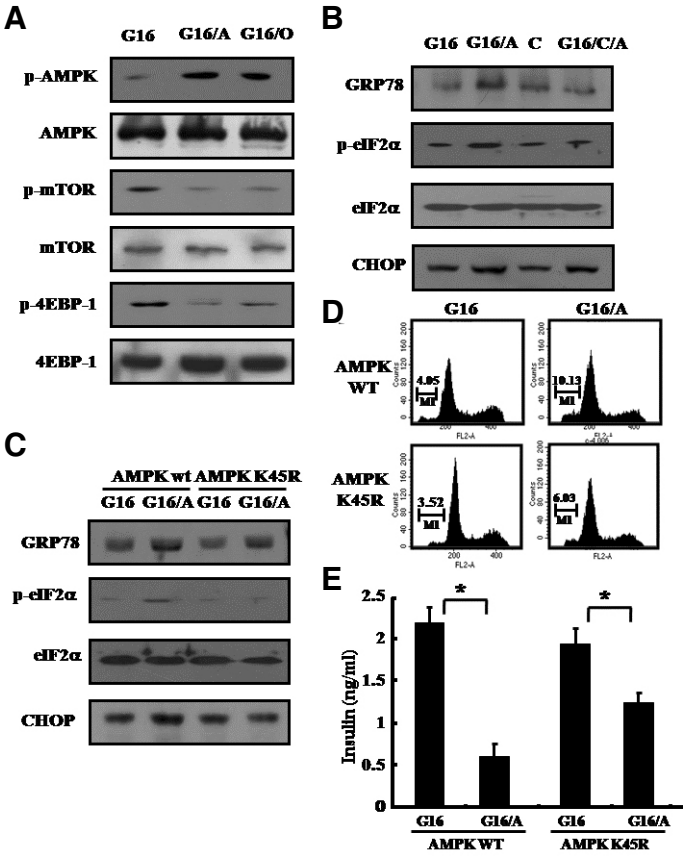

Fig. 2. AMPK was activated by mitochondria dysfunction, which plays a role in the induction of ER stress and apoptosis by mitochondrial dysfunction. (A) MIN6N8 cells were incubated for 1 h in glucose free DMEM medium containing 2% FBS and then treated with antimycin or oligomycin for 30 min in medium containing 16 mM glucose. The phospho-AMPK, phospho-mTOR and phospho-4EBP-1 levels were measured by Western blot. (B) MIN6N8 cells were pretreated with compound C (20 μM) for 2 h before treatment with antimycin in medium containing 16 mM glucose. ER stress proteins were measured by Western blot. (C) The wild type AMPK or dominant mutant AMPK K45R stably transfected cells were incubated for 1 h in glucose free DMEM medium containing 2% FBS and then treated with antimycin for 12 h in medium containing 16 mM glucose. ER stress proteins were measured by Western blot. (D) Apoptotic cells were quantified by FACScan flow cytometry. Apoptosis (M1, sub-G1 peak) was measured by PI staining. The numbers gated in each phase area are the means ± SEM of three experiments. (E) The insulin contents were measured by an enzymatic immunoassay of cell. The asterisks indicate a P value < 0.05 for the bracketed comparisons.

tion, we investigated the possible involvement of AMPK. As shown in Fig. 2A, AMPK phosphorylation was increased in antimycin or oligomycin-treated cells. Consistent with AMPK activation, mTOR phosphorylation and 4E-BP1 phosphorylation, which are downstream target pathways of AMPK, were decreased. Then, to confirm the direct involvement of AMPK in mitochondrial dysfunction-induced ER stress and β-cell apoptosis, we examined the induction of ER stress and β-cell apoptosis in cells that were pretreated with compound C, a cell permeable specific inhibitor of AMPK activity, and AMPK dominant mutant overexpressed cells. As shown in Fig. 2B, compound C effectively prevented antimycin-increased ER stress proteins containing GRP78, p-eIF2α and CHOP (Fig. 2B). To further clarify the essential role of AMPK, MIN6N8 cells were stably transfected with expression vectors for wild type AMPK or mutant AMPK-K45R, after which they were treated with antimycin. GRP78, p-eIF2α and CHOP were increased in wild AMPK-transfected cells by antimycin treatment, whereas the mutant AMPK-K45R reduced the increase of ER stress (Fig. 2C). Concomitant with the reduction of ER stress, mutant AMPK-K45R blocked the apoptosis induced by antimycin in wild AMPK-transfected cells (Fig. 2D) and recovered the level of insulin reduced by antimycin treatment (Fig. 2E).

Nitric oxide (NO) is produced via AMPK activation and leads to induction of ER stress

It has been reported that NO disturbs ER functions and activates ER stress-mediated apoptosis (Oyadomari et al., 2001); therefore, we measured the expression of inducible nitric oxide synthase (iNOS) in antimycin treated-cells, which is responsible for endogenous NO production and the formation of nitrite, as a reflection on NO production. As shown in Fig. 3A, treatment with antimycin or oligomycin increased the expression of iNOS and production of nitrite, suggesting that mitochondrial dysfunction increases NO production. Next, to determine if AMPK plays a role in NO production, we examined the expression of iNOS and nitrite formation in cells that were pretreated with compound C or transfected with mutant AMPK-K45R. Both compound C treatment (Fig. 3B) and mutant AMPK-K45R (Fig. 3C) prevented nitrite formation. These results demonstrate that mitochondrial dysfunction induces NO production through activation of AMPK.

Next, to determine the involvement of NO in ER stress and ER stress-mediated apoptosis induced by mitochondrial dysfunction, we measured ER stress proteins and apoptosis in cells that were pretreated with 1400W, an iNOS inhibitor. Treatment with 1400W reduced the nitrite formation induced by antimycin treatment (Fig. 3D) and efficiently prevented the increase of ER stress proteins such as GRP 78, p-eIF2α and CHOP (Fig. 3E). Consistent with the reduction of ER stress, 1400W blocked the apoptosis induced by antimycin (Fig. 3F).

Mitochondrial dysfunction induced by lipids also leads to the induction of ER stress and causes the apoptosis of pancreatic β-cells

Elevation of intracellular lipid levels is a characteristic of impaired mitochondria function. Previously, we demonstrated that lipid mixtures impair mitochondria membrane potentials and induce the apoptosis of β-cells (Lee et al., 2009). Here, we evaluated that mitochondria dysfunction induced by the elevation of intracellular lipids could also activate AMPK and induce ER stress. To accomplish this, we treated MIN6N8 cells with a

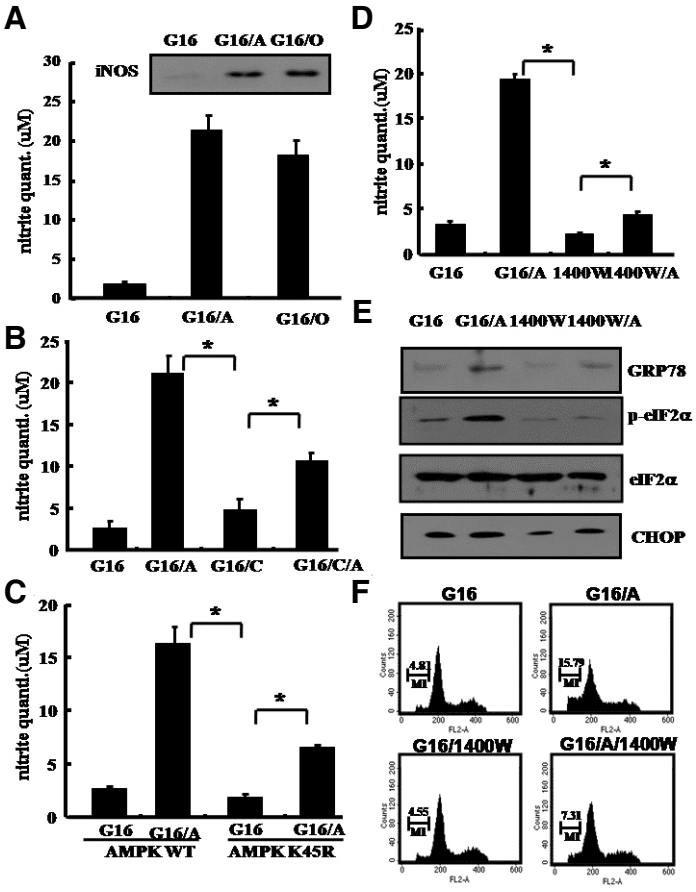

Fig. 3. NO production was increased via AMPK activation in mitochondria dysfunctional β-cells and its inhibition prevented ER stress and apoptosis induced by mitochondrial dysfunction. (A) MIN6N8 cells were incubated for 1 h in glucose free DMEM medium containing 2% FBS and then treated with antimycin or oligomycin for 30 min in medium containing 16 mM glucose. The level of iNOS protein was analyzed by Western blot, and nitrite was quantified using Griess reagent. (B) MIN6N8 cells were pretreated with compound C for 2 h before antimycin treatment in the medium containing 16 mM glucose. Nitrite was quantified using Griess reagent. (C) The wild type AMPK and dominant mutant AMPK K45R were incubated for 1 h in glucose free DMEM medium contained 2% FBS and then were treated with antimycin for 12 h in the medium containing 16 mM glucose. Nitrite was quantified using Griess reagent. (D) MIN6N8 cells were pretreated with 1400W (10 μM) for 2 h before being treated with antimycin in medium containing 16 mM glucose. Nitrite was quantified using Griess reagent. (E) ER stress proteins were analyzed by Western blot. (F) Apoptotic cells were quantified by FACScan flow cytometry. Apoptosis (M1, sub-G1 peak) was measured by PI staining. The numbers gated in each phase areas are the means ± SEM of three experiments. The asterisks indicate a *P* value < 0.05 for the bracketed comparisons.

lipid mixture containing palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and cholesterol. As shown in Fig. 4A, treatment with a lipid mixture increased the levels of phosphorylated AMPK and then inhibited the mTOR signaling pathway. Furthermore, the lipid mixture significantly increased the expression of iNOS and NO production (Fig. 4B). However, compound C treatment blocked the increase in NO production by lipid mixture. The lipid mixture increased ER stress proteins such as p-eIF2α, GRP 78, GRP 94 and CHOP. Based on these results, we postulated the general mechanism by which disturbance of mitochondrial function could activate AMPK, which induced ER stress via NO, resulting in the apoptosis of pancreatic β-cells.

DISCUSSION

Several reports have demonstrated that mitochondrial dysfunction contributes to insulin resistance in skeletal muscle and the liver as well as β-cell apoptosis in the pancreas (Lim et al., 2006; Park et al., 2005; Petersen et al., 2003; 2006; Stump et al., 2003). Previously, we reported that mitochondrial dysfunction by metabolic stress in pancreatic β-cells reduces GCK expression through PDX-1 downregulation via the production of ROS, which then decreases the association of GCK with the mitochondria, resulting in pancreatic β-cell apoptosis (Lee et al., 2009). In the current study, we demonstrated that ER stress is involved in β-cell apoptosis induced by mitochondrial dysfunction via AMPK activation.

Prolong ER stress has been implicated in the development of type 2 diabetes, affecting both insulin production by pancreatic β-cells and insulin sensitivity in insulin tissues (Eizirik et al., 2008). It has been suggested that three signaling components of ER stress, IRE1-JNK, CHOP, and caspase 12, play an important role in β-cell apoptosis mediated by ER stress (Fonseca et al., 2009). In this study, the treatment of β-cells with a metabolic stress inducer or lipid mixture to impair mitochondrial function increased the ER stress proteins, GRP78 and p-eIF2α, induced the ER stress-associated apoptotic factor CHOP and phosphorylated JNK with increasing β-cell apoptosis, suggesting that mitochondrial dysfunction-mediated apoptosis may be associated with the induction of ER stress.

The pathological and physiological factors that lead to the induction of ER stress include hypoxia, environmental toxins, hypoglycemia, viral infection, inflammatory cytokines, aging and a large biosynthetic load. However, the signaling involved in induction of ER stress is not well understood. AMPK is activated by an increase in the intracellular AMP/ATP ratio under different stress conditions such as glucose deprivation, hypoxia whose conditions are same as pathological ER stress conditions (Zhang et al., 2009). Even though the role of AMPK in the function of pancreatic β-cells is still controversial, sustained AMPK activation is involved in β-cell apoptosis induced by cytokines or hyperglycemia (Kefas et al., 2004; Meisse et al., 2002). Moreover, because mitochondrial dysfunction impairs ATP production, we examined AMPK activation in β-cells with dysfunctional mitochondria. We found that mitochondrial dysfunction of β-cells occurred by treatment with metabolic stress or lipid mixture activated AMPK. Inhibition of AMPK activation via pretreatment with compound C or dominant negative AMPK prevented the induction of ER stress concomitantly with a decrease in β-cells apoptosis. These results suggest that AMPK activation induced by mitochondria dysfunction contributes to

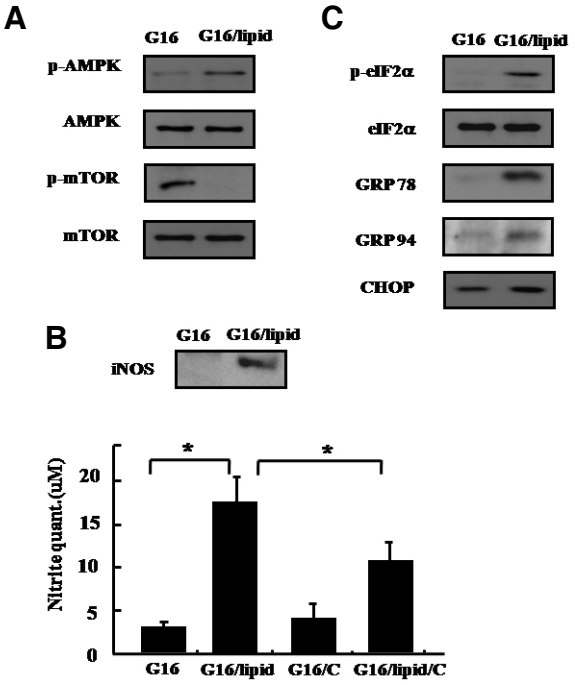

Fig. 4. Mitochondria dysfunction by lipid mixture activated AMPK and induced ER stress. (A) MIN6N8 were grown in the presence of 1% lipid mix for 24 h after 1 h of serum starvation. The levels of phospho-AMPK, and phospho-mTOR were measured by Western blot. (B) The level of iNOS protein was analyzed by Western blot, and nitrite was quantified using Griess reagent. The asterisks indicate a P value < 0.05 for the bracketed comparisons. (C) ER stress proteins including phospho-eIF2α, GRP78 and GRP94 were measured by Western blot.

ER stress induction.

NO plays an important role in ER stress-mediated β-cell apoptosis, especially when it is induced by inflammatory cytokines such as interferon and interleukin-1b (Oyadomari et al., 2001). NO production decreases the expression of the sarcoendoplasmic reticulum pump Ca+2ATPase 2B (SERCA2b), leading to a decrease in Ca+2 in the ER. This Ca+2 depletion causes a high level of ER stress in β-cells and induces CHOP, leading to β-cell failure and apoptosis. We also determined that NO was significantly upregulated in response to metabolic stress or lipid mixture-incubated β-cells. However, these events were blocked by compound C and dominant mutant AMPK, suggesting that AMPK activated by mitochondrial dysfunction stimulates NO production in β-cells. Pretreatment with iNOS inhibitor efficiently prevented the induction of ER stress and CHOP. In addition to NO, as ROS induces ER stress, mitochondrial dysfunction-induced ROS may also play an important role in induction of ER stress with NO in MIN6N8 cells with dysfunctional mitochondria.

Taken together, these results suggest that mitochondrial dysfunction persistently activates AMPK, which stimulates NO production, leading to ER stress and subsequent β-cells apoptosis.

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