Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:873-886

Accepted: September 09, 2011

Mitochondria-targeted Antioxidants Protect Pancreatic β-cells against Oxidative Stress and Improve Insulin Secretion in Glucotoxicity and Glucolipotoxicity

Sangbin Lim[1,*], Md Abdur Rashid[1,4,*], Miran Jang[1], Yeonghwan Kim[1], Hyeran Won[1], Jeonghoon Lee[1], Jeong-taek Woo[2], Young Seol Kim[2], Michael P. Murphy[3], Liaquat Ali[4], Joohun Ha[1] and Sung Soo Kim[1]

[1]Department of Biochemistry and Molecular Biology, Medical Science and Engineering Research Center for Bioreaction to Reactive Oxygen Species and Biomedical Science Institute (BK-21), School of Medicine, Kyung Hee University, Seoul, Korea, [2]Division of Endocrinology and Metabolism, Department of Internal Medicine, School of Medicine, Kyung Hee University, Seoul, Korea, [3]MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge, CB2 0XY, UK, [4]Department of Biochemistry & Cell Biology, Bangladesh Institute of Health Science, Dhaka, Bangladesh, *these authors contributed equally to this work

Key Words

Mitochondrial electron transport chain • Mitochondrial reactive oxygen species • Lipogenesis • Lipid peroxidation • ER stress • Pancreatic β-cell failure • Type 2 diabetes

Abstract

Mitochondrial oxidative damage is thought to play a key role in pancreatic β-cell failure in the pathogenesis of type 2 diabetes. Despite this, the potential of mitochondria-targeted antioxidants to protect pancreatic β-cells against oxidative stress has not yet been studied. Therefore, we investigated if mitochondria-targeted antioxidants protect pancreatic β-cells such as RINm5F and HIT-T15 cells against oxidative stress under glucotoxic and glucolipotoxic conditions. When β-cells were incubated under these conditions, the expression levels of mitochondrial electron transport chain complex subunits, mitochondrial antioxidant enzymes (such as MnSOD and Prx3), β-cell apoptosis, lipogenic enzymes (such as ACC, FAS and ABCA1), intracellular lipid accumulation, oxidative stress, ER stress, mitochondrial membrane depolarization, nuclear NF-κB and sterol regulatory element binding protein 1c (SREBP1c) were all increased, in parallel with decreases in intracellular ATP content, citrate synthase enzymatic activity and glucose-stimulated insulin secretion. These changes were consistent with elevated mitochondrial oxidative stress, and incubation with the mitochondria-targeted antioxidants, MitoTempol or Mitoquinone (MitoQ), prevented these effects. In conclusion, mitochondria-targeted antioxidants protect pancreatic β-cells against oxidative stress, promote their survival, and increase insulin secretion in cell models of the glucotoxicity and glucolipotoxicity associated with Type 2 diabetes.

Introduction

In eukaryotic cells mitochondria are the major producers of ATP via oxidative phosphorylation, which occurs by coupling electron movement through complexes I-IV of the electron transport to oxygen with ATP synthesis by the FoF1-ATP synthase (complex V) by means of a proton electrochemical gradient [1]. The production of mitochondrial reactive oxygen species (ROS) occurs as a physiological by-product of the action

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/11/0285-0873$38.00/0

Accessible online at:
www.karger.com/cpb

Sung Soo Kim, M.D., Ph.D.
Department of Biochemistry and Molecular Biology, School of Medicine
Kyung Hee University, #1, Hoegi-dong, Dongdaemoon-gu, Seoul 130-701 (Korea)
Tel. +82-2-961-0524, Fax +82-2-959-8168
E-Mail sgskim@khu.ac.kr

of the mitochondrial electron transport chain (mETC) and this ROS production can overwhelm antioxidant defences and lead to oxidative damage under pathological conditions [2]. Among the mETC complexes, complex I and III are considered the major sites that generate superoxide ($O_2^{\cdot-}$). Complex I generates $O_2^{\cdot-}$ only within the mitochondrial matrix, while complex III generates $O_2^{\cdot-}$ in the intermembrane space as well as in the matrix [3, 4]. The proximal ROS generated is $O_2^{\cdot-}$, which leads to the formation of the other ROS hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). As well as being potentially damaging, H_2O_2 can also function directly as a second messenger in a physiologically relevant manner. Manganese superoxide dismutase (MnSOD) is an important anti-oxidant enzyme in the mitochondrial matrix that is encoded by the nuclear SOD2 gene and that catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 [5].

Mitochondrial dysfunction is thought to play a major role in pancreatic β-cell failure in the pathogenesis of type 2 diabetes [6, 7]. Although the exact mechanism underlying β-cell dysfunction is not known, the enhanced mitochondrial ROS production that occurs in pancreatic β-cells under hyperglycemia and hyperlipidemia is considered a major contributing factor for the disruption of β-cell function in type 2 diabetes [8, 9]. In the presence of normal glucose concentrations, pancreatic β-cells metabolize glucose via glycolysis and TCA cycle, and the production of ROS by mitochondria can be detoxified by the endogenous antioxidant pathways. However, when the glucose concentration is elevated, for example following a meal, the mechanism by which β-cells sense the increase in glucose couples it to insulin secretion pushing more electrons into the mETC and thereby increasing the ATP/ADP ratio which leads indirectly to increased insulin secretion. However, the reduced mETC and the increased membrane potential across the mitochondrial membrane can thereby increase until a critical threshold is reached at which electron transfer inside complex III is blocked [10, 11], causing the electrons to generate $O_2^{\cdot-}$. This phenomenon may be further exacerbated in hyperlipidemia, when both β–oxidation of fatty acids and oxidation of FFA-derived acetyl CoA by the TCA cycle generate the electron donors (NADH and FADH2), thereby potentially increasing mitochondrial ROS production [10]. Indeed, obesity, a major risk factor for type 2 diabetes, is associated with increased lipolysis and FFA concentrations, chronic oxidative stress, and inflammation, potentially increasing mitochondrial ROS production [12]. A further consideration is that pancreatic β-cells are particularly susceptible to oxidative damage. Thus, the continual hyperglycemia and hyperlipidemia that occurs chronically in the conditions that lead to type 2 diabetes may lead to mitochondrial oxidative damage in pancreatic β-cells [7, 8] and is a potential mechanism for the marked losses of β-cell function that is found in patients with type 2 diabetes [13].

Consequently, therapies that decrease mitochondrial oxidative damage might be expected to prevent some of this damage [7]. MitoTempol and Mitoquinone (MitoQ) are two promising mitochondria-targeted antioxidants derived from tempol (and antioxidant nitroxide) and ubiquinone, which are targeted to mitochondria by covalent attachment to a lipophilic triphenylphosphonium cation [14-17]. Because of the large mitochondrial membrane potential, the cations are accumulated within mitochondria inside cells. Mitochondria-targeted antioxidants are protective *in vivo* against oxidative damage in many different pathologies, such as diabetes, cardiovascular, cancers, and neurodegenerative disorders [18-21]. In addition, the antioxidant MitoTempol decreases ROS generation, reduces abdominal fat and the weight gain, decreases blood glucose and normalizes blood insulin and creatinine levels in obese/Zucker rats fed a high fat diet [22-24]. However, it was unclear if mitochondria-targeted antioxidants could give greater protection and whether they could also preserve pancreatic β-cells against oxidative stress under glucotoxic and glucolipotoxic conditions. Therefore, we addressed this question in this study and found that mitochondria-targeted antioxidants did protect pancreatic β-cells such as RINm5F and HIT-T15 cells against the glucotoxic and glucolipotoxic conditions found in type 2 diabetes.

Materials and Methods

Materials

RPMI 1640 medium and fetal bovine serum were purchased from Lonza (Walkersville, MD). TO901317 was purchased from Calbiochem (San Diego, CA). MitoQ was provided by Dr. Michael P. Murphy and MitoTempol was purchased from Alexis Biochemicals (San Diego, CA, USA). MitoTempol at 75 μmol/l was used as described previously [25, 26]. 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA), Hoechst 33342, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Oil Red O were acquired from Sigma (St. Louis, MO). Antibodies against each subunit of the mitochondrial complexes were purchased from MitoScience (Eugene, OR, USA) and NDUFS5 was provided from GeneTex (Irvine, CA, USA). Antibodies against acetyl-CoA carboxylase (ACC) was purchased from Upstate Biotechnology and cholesterol transporter (ABCA1) from Novus Biologicals.

Antibodies specific to fatty acid synthase (FAS), 4-hydroxynon-2-enal (HNE), heme oxygenase-1 (HO-1) and malondialdehyde (MDA) were purchased from Abcam (Cambridge, OWN, UK). Antibodies against NDUFAF1, actin, immunoglobulin-binding protein/glucose-regulated protein-78 (Bip/Grp78), insulin, sterol regulatory element binding protein 1c (SREBP1c) and lamin B were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against C/EBP-homologous protein (CHOP), phosphorylation eukaryotic initiation factor 2α (p-eIF2α), poly (ADP ribose) polymerase (PARP) and caspase 3 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against protein disulfide isomerase (PDI) was obtained from Stressgen (Assay Designs, Ann Arbor, MI, USA). The 8-OHdG-EIA kit was from OXIS Health Products (Portland, OR, USA), and DNase I was from Qiagen (Chatsworth, CA, USA).

Cell culture

RINm5F and HIT-T15 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin sulfate (Invitrogen, CA). Glucose at 11.2 mmol/l and 31.2 mmol/l was used as low- and high-glucose condition, respectively, as described previously [27, 28].

MTT assay

Cell viability was assessed using the MTT conversion assay in a 12-well plate. The culture medium was replaced with 1 ml of medium containing 0.5 mg/ml of MTT and cells were incubated for 30 min at 37°C. The blue-colored tetrazolium crystals resulting from mitochondrial enzymatic activity on the MTT substrate were solubilized with 200 µl of dimethyl sulfoxide (DMSO), and the absorbance was read at 595 nm in a microplate reader (Bio-Rad). Cell survival was expressed as the percentage of absorbance relative to that of the untreated cells.

Hoechst 33342 staining

Equal numbers of cells were treated with or without TO901317 (10 µmol/l) in the presence or absence of MitoTempol (75 µmol/l) or MitoQ (100 nmol/l) for 48 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions. Cells were then incubated for 30 min with Hoechst 33342 loading dye (10 µmol/l), fixed for 20 min in 4% formaldehyde, and then washed three times in ice cold phosphate-buffered saline (PBS). The stained cells were monitored using an LSM510 confocal laser microscope (Carl Zeiss, NY), and apoptotic cells were identified by nuclear condensation and fragmentation.

Measurements of cellular triglycerides, free fatty acids and cholesterol

The cellular contents of triglycerides (TG) and cholesterol in RINm5F cells were measured using TG and cholesterol assay kits (ThermoDMA, Louisville, CO, USA). The amount of free fatty acids (FFAs) was determined using a nonesterified fatty acid assay kit (Roche, Indianapolis, IN, USA). Each analysis was performed according to the manufacturer's instruction.

Oil Red O staining

Equal numbers of cells were seeded into a 6-well microplate and grown to 80 % confluency in RPMI medium. The cells were then treated with or without TO901317 (10 µmol/l) in the presence or absence of MitoTempol (75 µmol/l) or MitoQ (100 nmol/l) for 48 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions and fixed for 1 h in 4 % formaldehyde. After fixation, the cells were washed four times with PBS, and Oil Red O solution (3 mg/ml in 60% isopropanol) was added to each well and incubated at room temperature for 15 min. The cells were then washed with PBS and observed under a microscope. The dye was extracted from cell culture dishes with isopropanol (1 ml), and the absorbance was measured spectrophotometrically at 510 nm using previously described methods [29].

Preparation of the mitochondrial fraction and mitochondrial proteins

The preparation of the mitochondrial fraction and mitochondrial proteins was performed as described previously [30]. Cells were suspended in lysis buffer (250 mmol/l sucrose, 0.1 mmol/l EDTA, and 2 mmol/l HEPES, pH 7.4) and homogenized; the mitochondria were then isolated using differential centrifugation, and the mitochondrial H_2O_2 concentration was measured. For Western blotting, the mitochondria were lysed in lysis buffer (Intron Biotechnology, Kyunggi, Korea).

Mitochondrial H_2O_2 measurement

The levels of mitochondrial H_2O_2 were determined using a modified method using absorbance of resorufin, a reaction product of Amplex Red. A total of 100 µl of reaction buffer (120 mmol/l KCl, 3 mmol/l HEPES free acid, 1 mmol/l EGTA, and 0.3% BSA, pH 7.2, at 37°C) with 50 µmol/l Amplex Red (Molecular Probes, Invitrogen, Carlsbad, CA, USA), 6 U/ml HRP and 30 U/ml SOD were added to each microplate well and then pre-warmed at 37°C for 15 min. The reaction was then started by adding 35 µg of the re-suspended mitochondrial fractions in 100 µl reaction buffer. After 30 min, the absorbance of the reaction mixtures was measured at 560 nm using a fluorescence microplate reader (Bio-Rad, Richmond, CA, USA).

Detection of mitochondrial ROS and intracellular ROS level using confocal microscopy

Mitochondrial ROS and intracellular ROS levels were estimated via MitoSOX Red and DCF fluorescence as indicators, respectively, using confocal microscopy as previously described [29, 31]. In brief, equal numbers of RINm5F cells were seeded on glass coverslips in a 12-well microplate and grown to 80% confluency in RPMI medium. The cells were treated with or without TO901317 (10 µmol/l) in the presence or absence of MitoTempol (75 µmol/l) or MitoQ (100 nmol/l) for 48 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions. The cells were then loaded with MitoSOX Red (5 µmol/l) and DCF-DA (10 µmol/l) to detect mitochondrial and intracellular ROS levels, incubated at 37°C for 30 min, and washed five times in ice-cold PBS. After being washed, the cells were fixed in 4% formaldehyde, washed in PBS and

mounted onto slides with histological mounting medium (National Diagnostics, USA). Fluorescence was evaluated using an LSM510 confocal laser microscope (Carl Zeiss, NY). The excitation and emission wavelengths for MitoSOX and DCF-DA were 488 nm and 525 nm, respectively.

Analysis of intracellular ROS and mitochondrial membrane potential using flow cytometry

The intracellular ROS and mitochondrial membrane potential were measured using flow cytometry as previously described [29, 30, 32]. In brief, equal numbers of RINm5F cells were seeded in a 6-well microplate and grown to 80% confluency in RPMI medium. The cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) for 48 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions. The cells were then loaded with DCF-DA (10 μmol/l) and incubated at 37°C for 30 min, after which the cells were washed with PBS, harvested in 1 ml of trypsin, and then suspended in PBS. The supernatant was removed using centrifugation at 1,000 rpm for 5 min, and the cell pellet was resuspended in 0.5 ml of PBS. Fluorescence was assessed via flow cytometry (FACS Calibur; Becton-Dickinson, Franklin Lakes, NJ). The mean DCF fluorescence intensity was assessed with an excitation wavelength of 488 nm and an emission wavelength of 525 nm, and untreated cells were used as a reference for the ROS levels. The mitochondrial membrane potential was measured using the cationic dye $DiOC_6$. Cells were loaded with $DiOC_6$ (40 nmol/l), incubated at 37°C for 40 min, and resuspended in 0.5 ml of PBS before the flow cytometric analysis. $DiOC_6$ was excited at 488 nm, and fluorescence was analyzed at 525 nm.

Alkaline single-cell agarose gel electrophoresis (comet) assay

A comet assay was performed to assess cell oxidative DNA damage as described previously [33]. In brief, equal numbers of RINm5F cells were seeded in a 6-well microplate and grown to 80 % confluency in RPMI medium. The cells were then treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) for 36 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions. The cells were washed with PBS, harvested in 0.5 ml of trypsin, and then resuspended in 100 μl of 0.5% (w/v) low melting agarose (LMA) at 39°C. The cell suspension was applied to a frosted microscope slide that was pre-coated with 200 μl of 1% (w/v) normal melting agarose (NMA). After solidification of the agarose, the slide was covered with another 200 μl of 0.5% (w/v) LMA and then immersed in lysis buffer solution (2.5 mol/l NaCl, 100 mmol/l Na-EDTA, 10 mmol/l Tris, 1% Triton X-100, and 10% DMSO, pH 10) overnight at 4°C. The slides were placed in a horizontal electrophoresis tank, covered with fresh electrophoresis buffer (300 mmol/l NaOH and 10 mmol/l Na-EDTA, pH 13) and incubated for 20 min to allow DNA unwinding and the expression of the alkaline-labile damage. An electric field was applied (300 mA, 25 V) for 25 min at 4°C to draw negatively charged DNA towards an anode. After electrophoresis, the slides were washed with neutralizing buffer (0.4 mol/l Tris, pH 7.5) for 5 min at 4°C and then washed with absolute ethanol for 5 min at 4°C. Finally, the gels were stained with SYBR Gold diluted (1:10,000) in deionized water. The slides were stored in a humidified lightproof box for up to 12 h at 4°C before analysis using fluorescence microscopy (Nikon ECLIPSE 50i, Japan). The percentage of total DNA in the tail was determined using the Comet Software (NIS-Elements F2.20) program and was used as a measure of DNA damage. A total of 50 cells per slide were analyzed, and the results were presented as the mean percentage of DNA in the comet tail ± S.E.M. from 100 cells from two independent experiments.

Determination of 8-hydroxy-2-deoxyguanosine

The 8-hydroxy-2-deoxyguanosine (8-OHdG) content was measured using the Bioxytech 8-OHdG-EIA kit [34]. DNA was isolated from RINm5F cells using DNAzol reagent according to the manufacturer's instructions, and the absorbance was measured spectrophotometrically at 280 nm. Samples containing 200 μg DNA were resuspended in 50 μl of reaction mixture containing 100 mmol/l sodium acetate (pH 5.0) and 5 mmol/l $MgCl_2$ and then digested with 1 μl DNase I for 10 min at room temperature. DNA-digested samples were added to microtiter plates that were precoated with 8-OHdG monoclonal antibody, and the assay was performed according to the manufacturer's instructions. Sample absorbance was measured at 450 nm using a fluorescence microplate reader (Bio-Rad, Richmond, CA, USA).

Measurement of citrate synthase activity

The citrate synthase activity was measured using the citrate synthase assay kit (Sigma). Citrate synthase activity was measured spectrophotometrically at 412 nm using 2 μg isolated mitochondria.

Measurement of intracellular ATP content

ATP concentration was determined using the ATP bioluminescent assay kit supplied by Sigma. The ATP content was calculated as nmoles ATP per microgram protein.

Transmission electron microscopy

Cells were fixed in Karnovsky's fixative solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mmol/l calcium chloride, and 100 mmol/l cacodylate buffer, pH 7.4) for 2 h, washed with cacodylate buffer, and post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. The cells were dehydrated through a graded ethanol series and embedded in propylene oxide and EPON:propylene oxide (1:1). The cells were sectioned using a Reichert Ultracut (Leica, Cambridge, UK). After being stained with uranyl acetate and lead citrate, the cells were observed and photographed using transmission electron microscopy (Zeiss EM 902A, Leo, Oberkohen, Germany).

Preparation of nuclear extracts

Equal numbers of HIT-T15 cells were seeded in 100-mm dishes and grown to 80% confluency in RPMI medium. The cells were then treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ

(100 nmol/l) for 36 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions. The cells were washed with PBS, harvested in 1 ml of trypsin, and then suspended in PBS. The supernatant was removed using centrifugation at 1,000 rpm for 5 min, and the cell pellet was resuspended in cytosolic Buffer A (10 mmol/l Hepes, pH 7.9, 10 mmol/l KCl, 0.1 mmol/l EDTA, 1 mmol/l DTT, and 0.5 mmol/l PMSF) with 1% Nonidet P-40 and then homogenized. The homogenate was centrifuged at 4,500 rpm for 5 min, and the supernatant was retained for cytosolic protein analysis. The nuclear cell pellet was resuspended in nuclear Buffer B (20 mmol/l Hepes, pH 7.9, 0.4 mol/l NaCl, 1 mmol/l DTT, 1 mmol/l PMSF, and 1% Nonidet P-40) and centrifuged at 14,000 rpm for 15 min. The supernatant was then used for nuclear protein analysis.

Measurement of insulin secretion

Equal numbers of HIT-T15 cells were seeded in a 12-well microplate and grown to 80% confluency in RPMI medium. The cells were then treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) for 36 h under low-glucose (11.2 mmol/l) or high-glucose (31.2 mmol/l) conditions and incubated in Krebs-Ringer-bicarbonate-Hepes buffer (KRBH; 140 mmol/l NaCl, 3.6 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l$MgSO_4$, 1.5 mmol/l $CaCl_2$, 2 mmol/l $NaHCO_3$, and 10 mmol/l Hepes) supplemented with 2.8 mmol/l glucose and 0.1% bovine serum albumin (BSA) at 37°C for 1 h under a 95% O_2/5% CO_2 atmosphere. The cells were then incubated with KRBH containing 2.8 mmol/l or 25 mmol/l glucose for 1 h at 37°C, and the medium was collected for the detection of insulin secretion. Insulin secretion was measured using an enzyme immunoassay (ALPCO, Salem, NH, USA) as previously described [29].

Western blot analysis

Western blot analysis was performed using previously described methods [29]. In brief, cell lysates (20 μg protein) were separated via SDS-PAGE and electroblotted onto a PVDF membrane for 1 h at 100 V at 4°C. The membranes were blocked with 3% bovine serum albumin for 1 h in TBST (20 mmol/l Tris-HCl, pH 7.5, 50 mmol/l NaCl, and 0.1% Tween 20). After being blocked, the membranes were incubated overnight at 4°C with primary antibodies diluted 1:1000 in TBST. After being washed in TBST buffer, the membranes were incubated for 1 h in anti-rabbit, anti-goat, or anti-mouse horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies diluted 1:2000 in TBST, and the labeled proteins were detected with chemiluminescence reagents. Unless specified, actin and VDAC were immunoblotted to standardize the quantity of sample proteins for all Western blotting analyses. The SeeBlue pre-stained molecular weight marker standards (Invitrogen, USA) were used in our experiments.

Statistical analysis

The results are expressed as means ± SE and are from at least three independent experiments. Statistical analyses were conducted via Student's *t*-test. Unless otherwise indicated, a P value of < 0.05 was considered significant.

Results

Mitochondria-targeted antioxidants protect against mitochondrial and ER stress under glucotoxic and glucolipotoxic conditions

The liver X receptor (LXR) is a member of the nuclear receptor family of transcription factors and is an important regulator of cholesterol, fatty acid, and glucose homeostasis. The most well characterized LXR target gene is SREBP-1c, which serves as a trigger for down-stream transcriptional events [35]. Several LXR targets through SREBP-1c are genes involved in lipogenesis such as FAS and ACC. TO901317 is a potent and selective agonist for LXR. It was reported that chronic activation of LXR by a synthetic ligand, TO901317, under high-glucose condition contributes to severe glucolipotoxicity-induced β-cells apoptosis [27]. Thereby, to induce glucotoxic and glucolipotoxic condition, the cells were incubated with TO901317 under high-glucose condition. The mitochondrial respiratory chain is a major site of ROS production within the cell. Mitochondrial ROS production in pancreatic β-cells is increased during hyperglycemia and hyperlipidemia, and the elevated oxidative stress causes β-cell dysfunction and apoptosis during the development of type 2 diabetes [8, 36]. Therefore, we examined the effect of mitochondria-targeted antioxidants on the expression levels of mETC complex subunits in pancreatic β-cells. We assessed their expression levels by Western blot analysis. Cells were incubated with or without TO901317 in the presence or absence of mitochondria-targeted antioxidants, MitoTempol or MitoQ, under high-glucose conditions. The cells under low-glucose condition medium were used as the control for all experiments. The expression levels of complex I (NDUFAF1, NDUFB6, NDUFB8, NDUFS3, NDUFS5 and GRIM), complex II (Fp), complex III (Core 1, Core 2 and Rieske) and complex IV (Cox1) subunits and mitochondrial antioxidant enzymes, such as MnSOD and Prx3, were significantly increased under glucotoxic and glucolipotoxic conditions, while these increases were suppressed by treatment with MitoTempol or MitoQ (Fig. 1A and B). Next, we detected ROS in mitochondria via confocal microscopy using the mitochondrial superoxide indicator MitoSOX. The levels of $O_2^{\cdot-}$ were enhanced in mitochondria within cells under glucotoxic and glucolipotoxic conditions, but reduced in cells treated with MitoTempol or MitoQ (Fig. 1C). The levels of H_2O_2 produced by mitochondria isolated from these cells were determined by fluorescence microplate reader using Amplex Red, and showed similar patterns as found for

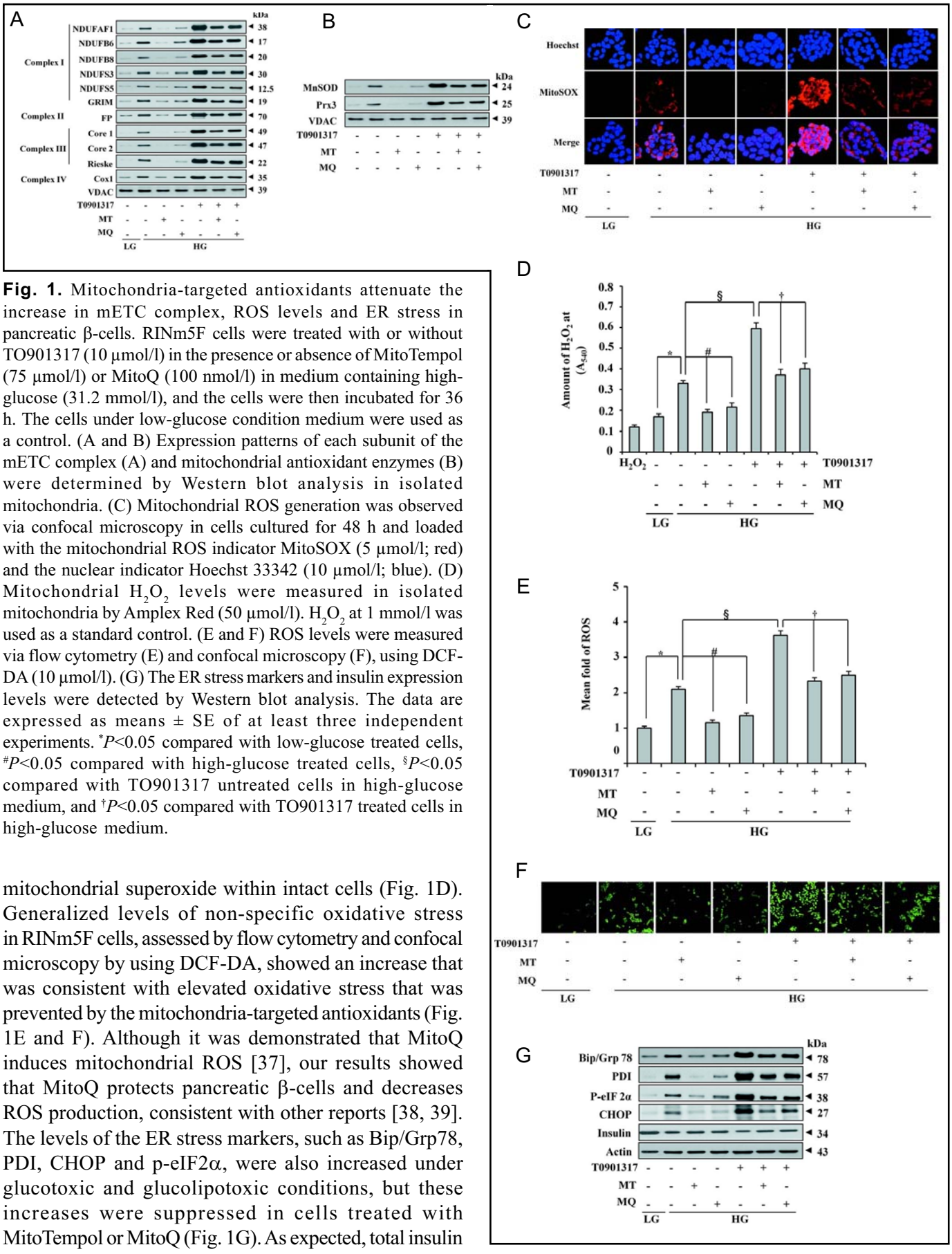


Fig. 1. Mitochondria-targeted antioxidants attenuate the increase in mETC complex, ROS levels and ER stress in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A and B) Expression patterns of each subunit of the mETC complex (A) and mitochondrial antioxidant enzymes (B) were determined by Western blot analysis in isolated mitochondria. (C) Mitochondrial ROS generation was observed via confocal microscopy in cells cultured for 48 h and loaded with the mitochondrial ROS indicator MitoSOX (5 μmol/l; red) and the nuclear indicator Hoechst 33342 (10 μmol/l; blue). (D) Mitochondrial H_2O_2 levels were measured in isolated mitochondria by Amplex Red (50 μmol/l). H_2O_2 at 1 mmol/l was used as a standard control. (E and F) ROS levels were measured via flow cytometry (E) and confocal microscopy (F), using DCF-DA (10 μmol/l). (G) The ER stress markers and insulin expression levels were detected by Western blot analysis. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

mitochondrial superoxide within intact cells (Fig. 1D). Generalized levels of non-specific oxidative stress in RINm5F cells, assessed by flow cytometry and confocal microscopy by using DCF-DA, showed an increase that was consistent with elevated oxidative stress that was prevented by the mitochondria-targeted antioxidants (Fig. 1E and F). Although it was demonstrated that MitoQ induces mitochondrial ROS [37], our results showed that MitoQ protects pancreatic β-cells and decreases ROS production, consistent with other reports [38, 39]. The levels of the ER stress markers, such as Bip/Grp78, PDI, CHOP and p-eIF2α, were also increased under glucotoxic and glucolipotoxic conditions, but these increases were suppressed in cells treated with MitoTempol or MitoQ (Fig. 1G). As expected, total insulin

levels within cells were downregulated under glucotoxic and glucolipotoxic conditions, but upregulated in cells treated with MitoTempol or MitoQ (Fig. 1G). These results suggest that mitochondria-targeted antioxidants protect pancreatic β-cells against the increased mitochondrial ROS, and the loss of insulin in glucotoxicity and glucolipotoxicity.

Mitochondria-targeted antioxidants attenuate β-cell apoptosis under glucotoxic and glucolipotoxic conditions

It has been recently reported that antioxidants protect pancreatic β-cells against apoptosis induced by glucotoxicity and glucolipotoxicity. Therefore, we investigated the effect of mitochondria-targeted antioxidants on apoptosis in pancreatic β-cells. For these experiments, cells were incubated with or without TO901317 in the presence or absence of MitoTempol or MitoQ under high-glucose conditions. We tested the effects of these treatments on cell survival using the MTT assay, Western blot analysis of PARP and caspase 3 cleavages, and Hoechst 33342 staining for detection of DNA fragmentation. The MTT assay showed that cell survival rates were significantly decreased under glucotoxic and glucolipotoxic conditions, but were reversed by treatment with mitochondria-targeted antioxidants (Fig. 2A). The apoptotic markers, cleaved PARP and cleaved caspase, were also elevated under glucotoxic and glucolipotoxic conditions, but this effect was diminished in cells treated with MitoTempol or MitoQ (Fig. 2B). Hoechst 33342 staining also showed a similar pattern of apoptosis under glucotoxic and glucolipotoxic conditions that was prevented by mitochondria-targeted antioxidants (Fig. 2C). These results suggest that mitochondria-targeted antioxidants protect pancreatic β-cells from cell death during glucotoxicity and glucolipotoxicity.

Mitochondria-targeted antioxidants attenuate lipogenesis and lipid accumulation under glucotoxic and glucolipotoxic conditions

TO901317 induces lipogenesis and promotes *de novo* synthesis of FFA and TG under high-glucose conditions in pancreatic β-cells, mimicking the pathology associated with type 2 diabetes [27]. In advance of this study, we tested the effect of TO901317 in low-glucose condition. We got the results that TO901317 can increase expression of genes associated with lipid metabolism in low-glucose condition (data not shown) [40]. Therefore, we examined the effect of mitochondria-targeted antioxidants on lipogenesis in pancreatic β-cells by

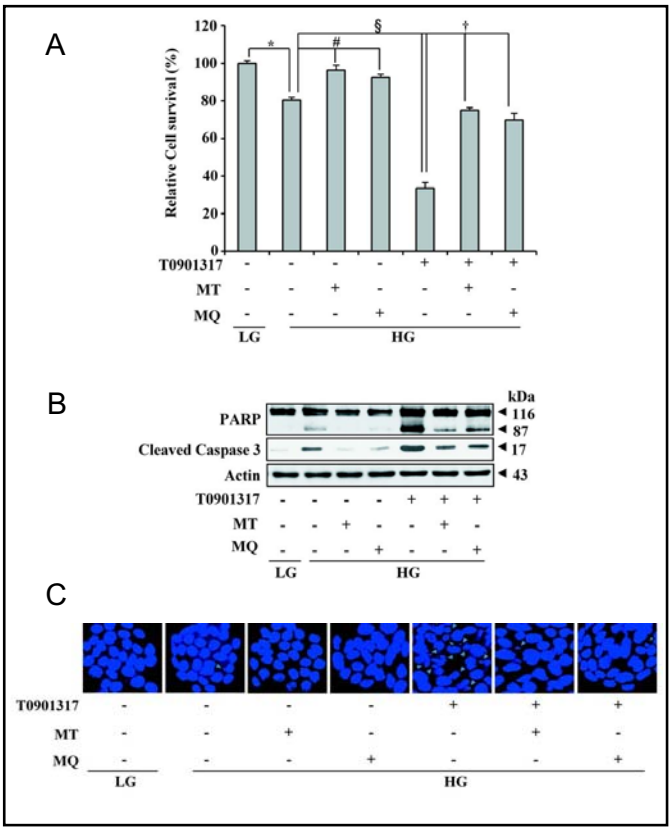
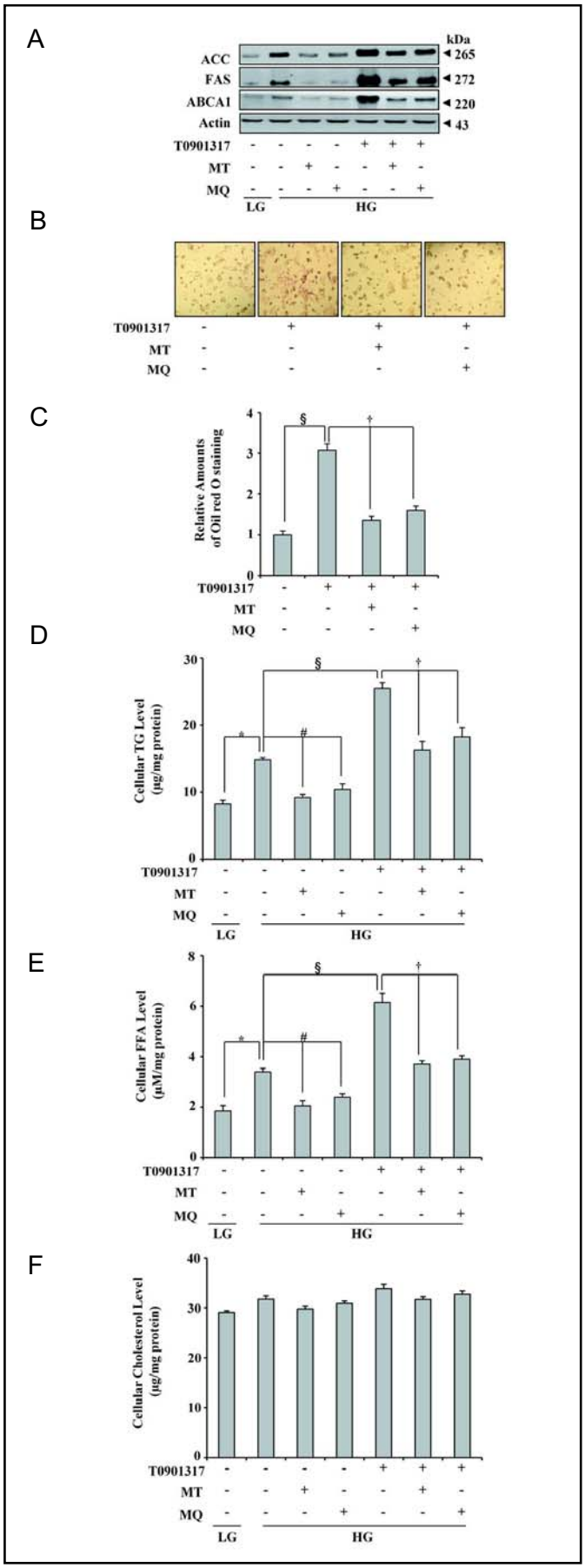


Fig. 2. Mitochondria-targeted antioxidants attenuate apoptosis in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) MTT assays. (B) Apoptotic cell death was monitored by Western blot analysis of processed PARP and caspase 3 cleavages. (C) Cells incubated for 48 h in the above conditions were subjected to Hoechst 33342 staining, and visualized under confocal microscopy. Arrows indicate chromosomal DNA fragmentation. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

Western blot analysis. Cells were incubated with or without TO901317 in the presence or absence of MitoTempol or MitoQ under high-glucose conditions. Then, we observed how these antioxidants affected lipogenesis and fat accumulation in the β-cells. The results showed that the expression levels of lipogenic enzymes, such as ACC, FAS and ABCA1, were significantly increased under glucotoxic and glucolipotoxic conditions, but this was prevented by treatment with MitoTempol or MitoQ (Fig. 3A). Oil Red O staining also showed an increase in lipid accumulation that correlated with the

Fig. 3. Attenuation of lipogenesis by mitochondria-targeted antioxidants in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) The expression levels of lipogenic enzymes were determined by Western blot analysis. Cells were cultured for 48 h and stained with Oil Red O dye. (B) Representative images of Oil Red O staining. (C) Quantification of Oil Red O staining was performed by spectrophotometric analysis. (D) Cellular triglyceride levels. (E) Free fatty acid levels. (F) Cholesterol levels. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.



increased expression of lipogenic enzymes in cells incubated with high-glucose medium containing TO901317, which was prevented in cells treated with MitoTempol or MitoQ (Fig. 3B and C). To further test the effect of mitochondria-targeted antioxidants on lipid accumulation in pancreatic β-cells, we measured the levels of intracellular TG, FFA and cholesterol under the same conditions as described above. Results showed that the levels of cellular TG and FFAs were significantly increased in pancreatic β-cells under glucotoxic and glucolipotoxic conditions, but that treatment with MitoTempol or MitoQ decreased this effect (Fig. 3D and E). Interestingly, the cholesterol level was not changed under both conditions, suggesting that cholesterol metabolism in β-cells is unaffected by redox status and therefore that it may play a minimal role in β-cell dysfunction (Fig. 3F). Taken together, these results suggest that mitochondria-targeted antioxidants attenuate lipogenesis and lipid accumulation in glucotoxicity and glucolipotoxicity.

Mitochondria-targeted antioxidants prevent lipid peroxidation, oxidative stress and oxidative DNA damage under glucotoxic and glucolipotoxic conditions

Lipid peroxidaion products comprise highly reactive lipid aldehydes, such as 4-oxonon-2-enal (ONE), HNE, MDA and acrolein, which are able to cause protein and DNA damage within cells [41]. Therefore, we next investigated the effect of the mitochondria-targeted antioxidants on lipid peroxidation in pancreatic β-cells by Western blot analysis. The levels of the lipid peroxidation products, HNE, MDA and acrolein, were significantly increased under glucotoxic and glucolipotoxic conditions, but were

decreased by treatment with MitoTempol or MitoQ (Fig. 4A). To further verify these observations, we measured the expression levels of a number of oxidative stress-responsive proteins, including HO-1, Hsp60 and Hsp72 by Western blot analysis. These indicators of elevated oxidative stress showed a similar pattern as lipid peroxidation products (Fig. 4A). To test the effect of the mitochondria-targeted antioxidants on oxidative DNA damage in pancreatic β-cells, we detected oxidative DNA damage by using the alkaline comet assay after incubation of cells under the same conditions as described above. These results showed that percentage of DNA in the comet tail was significantly increased under glucotoxic and glucolipoloxic conditions, consistent with elevated DNA damage, and that this damage was decreased in cells treated with MitoTempol or MitoQ (Fig. 4B and C). Furthermore, the magnitude of DNA base modification to 8-OHdG, as determined by ELISA, showed the same patterns as observed with comet assay supporting a role for DNA damage in this system (Fig. 4D). Taken together, these results suggest that mitochondria-targeted antioxidants protect pancreatic β-cell against oxidative damage and promote pancreatic β-cell survival in glucotoxicity and glucolipotoxicity.

Mitochondria-targeted antioxidants prevent the loss of mitochondrial membrane potential under glucotoxic and glucolipotoxic conditions

Sustained elevated oxidative stress within mitochondria can eventually lead to induction of the mitochondrial permeability transition, and thereby lead to the loss of the mitochondrial membrane potential [42] that can in turn lead to the release of cytochrome c, and the activation of the apoptotic pathway [43]. Therefore, we investigated the effect of mitochondria-targeted antioxidants on mitochondrial membrane potential in pancreatic β-cells by flow cytometry, using the cationic dye $DiOC_6$. This analysis showed that the mitochondrial membrane potential was significantly depolarized under glucotoxic and glucolipotoxic conditions, but that mitochondria-targeted antioxidants prevented this loss of potential (Fig. 5A). Furthermore, the loss of membrane potential was correlated with loss of cytochrome c from mitochondria into the cytosol under glucotoxic and glucolipotoxic conditions, and this was also prevented by treatment with MitoTempol or MitoQ (Fig. 5B). We next measured the level of the mitochondrial apoptosis marker Bax by Western blot analysis and found that both MitoTempol and MitoQ attenuated the increase in expression level of Bax in mitochondria under glucotoxic and glucolipotoxic conditions (Fig. 5B).

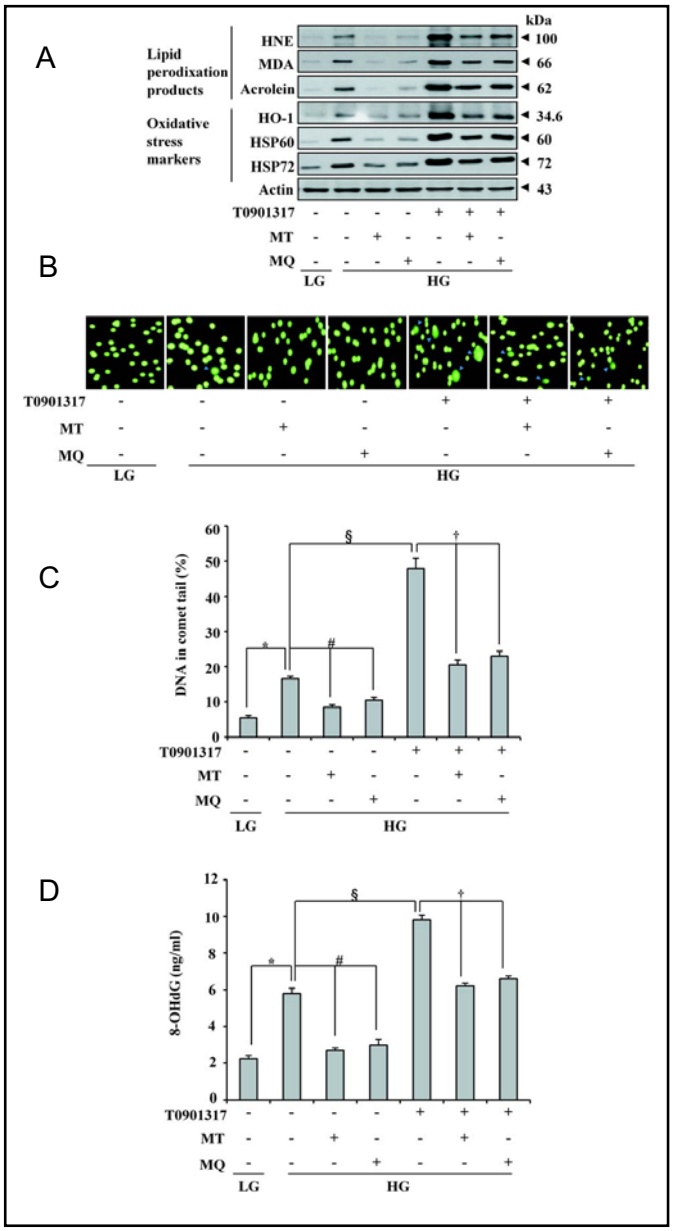


Fig. 4. Mitochondria-targeted antioxidants attenuate lipid peroxidation and oxidative DNA damage in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) The expression levels of lipid peroxidation products and oxidative stress markers were determined by Western blot analysis. (B) Representative images of comet assay. (C) Cellular DNA (%) damage was detected by an alkaline comet assay. (D) The 8-OHdG concentration was measured using an enzyme-immunoassay. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

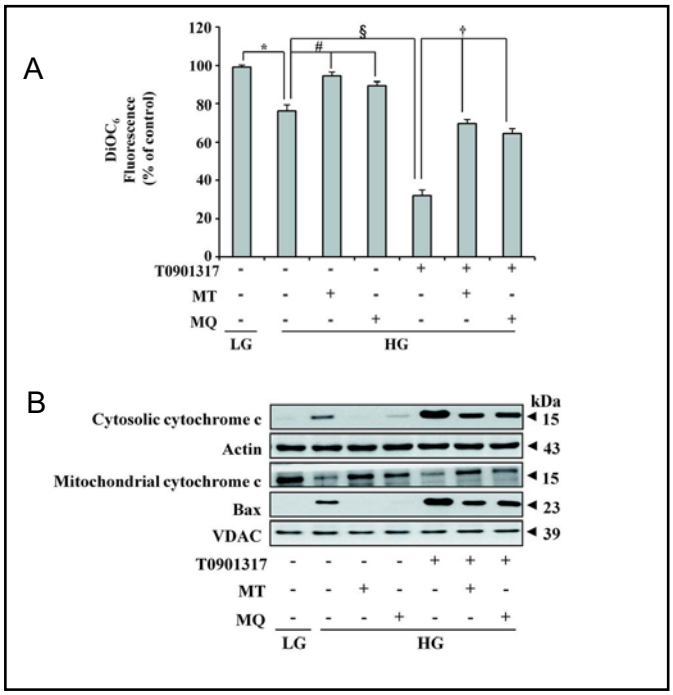


Fig. 5. Mitochondria-targeted antioxidants prevent the loss of mitochondrial membrane potential in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) Mitochondrial membrane potential was assessed via flow cytometry. (B) Cytochrome c and Bax were detected by Western blot analysis. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

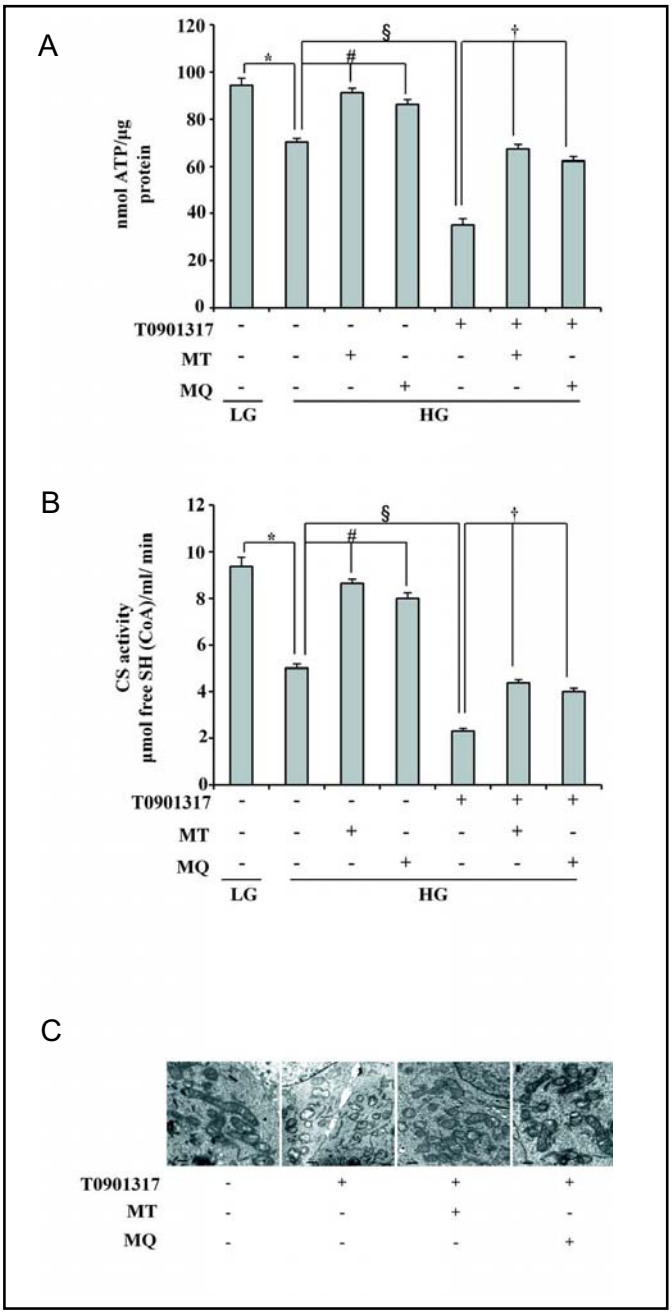


Fig. 6. Mitochondria-targeted antioxidants restore intracellular ATP content and citrate synthase enzymatic activity in pancreatic β-cells. RINm5F cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) ATP content was assayed using a luciferase-luciferin kit. (B) Mitochondrial citrate synthase activity. (C) Mitochondrial ultrastructural features were observed under transmission electron microscopy. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

Mitochondria-targeted antioxidants prevent the decrease in intracellular ATP content and the enzyme activity of citrate synthase that occurs under glucotoxic and glucolipotoxic conditions

It has been shown that elevated concentrations of fatty acids or glucose concentration impair mitochondrial ATP synthesis in pancreatic β-cells [44]. Therefore, we next assessed whether mitochondria-targeted antioxidants could prevent this decrease in mitochondrial ATP production under glucotoxic and glucolipotoxic conditions. We found that the intracellular ATP content was significantly decreased under glucotoxic and glucolipotoxic conditions, but that this loss was prevented by MitoTempol or MitoQ (Fig. 6A). To extend these observations, we

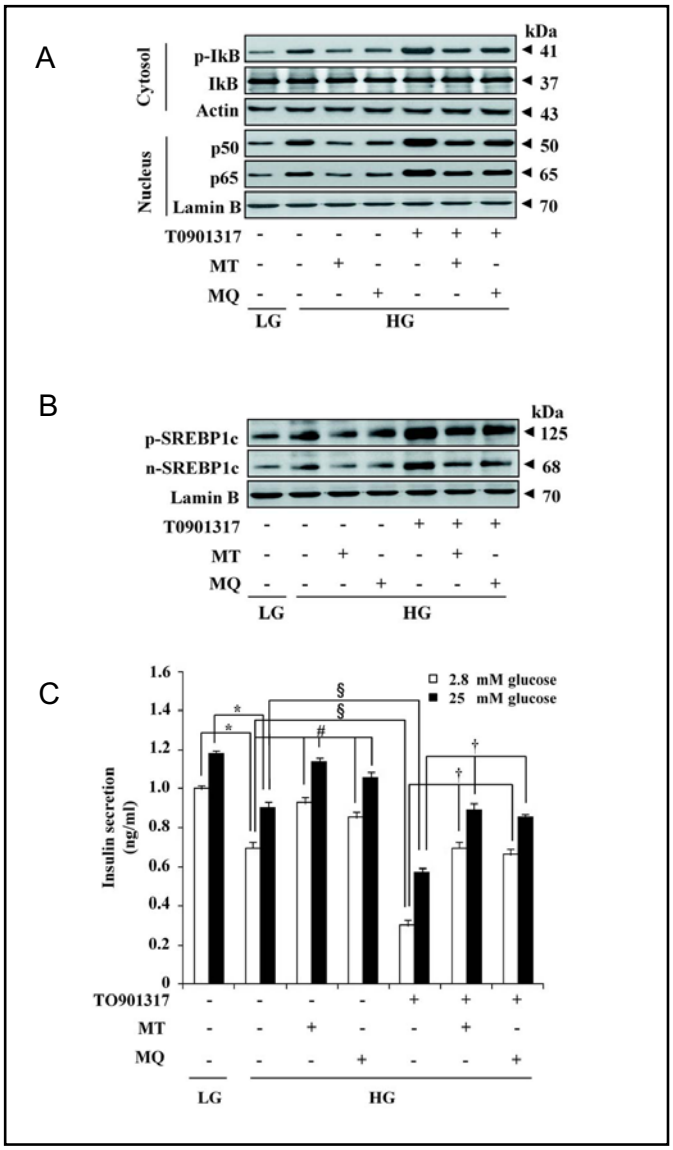


Fig. 7. Mitochondria-targeted antioxidants attenuate the activation of NF-κB and SREBP1c but increase GSIS in pancreatic β-cells. HIT-T15 cells were treated with or without TO901317 (10 μmol/l) in the presence or absence of MitoTempol (75 μmol/l) or MitoQ (100 nmol/l) in medium containing high-glucose (31.2 mmol/l), and the cells were then incubated for 36 h. The cells under low-glucose condition medium were used as a control. (A) Translocation of NF-κB to the nucleus. NF-κB was detected in paired cytoplasmic and nuclear fractions by Western blot analysis. (B) The expression levels of precursor and active nuclear forms of SREBP1c were monitored by Western blot analysis. Lamin B was used as a nuclear protein marker. (C) GSIS was measured using an enzyme-linked immunoassay. Cells were stimulated with KRBH containing 2.8 mmol/l (□) or 25 mmol/l (■) glucose for 1 h. The data are expressed as means ± SE of at least three independent experiments. $^{*}P<0.05$ compared with low-glucose treated cells, $^{\#}P<0.05$ compared with high-glucose treated cells, $^{\S}P<0.05$ compared with TO901317 untreated cells in high-glucose medium, and $^{\dagger}P<0.05$ compared with TO901317 treated cells in high-glucose medium.

assessed the effect of glucotoxicity and glucolipotoxicity on mitochondrial content in the cells by measuring the activity of the mitochondrial matrix marker enzyme citrate synthase. These measurements showed that glucotoxicity and glucolipotoxicity decreased mitochondrial content while the mitochondria-targeted antioxidants prevented this decrease (Fig. 6B). Finally, we assessed the effects of glucotoxicity and glucolipotoxicity on mitochondrial structures by transmission electron microscopy (TEM). The mitochondrial TEM images showed increased numbers and sizes of vacuolated mitochondria with disordered cristae under glucolipotoxic conditions, but these effects were prevented by mitochondria-targeted antioxidants (Fig. 6C). These results suggest that mitochondria-targeted antioxidants preserve mitochondrial structure, content and function during the oxidative stress associated with glucotoxicity and glucolipotoxicity.

Mitochondria-targeted antioxidants inhibit NF-κB and SREBP1c activation, and increase glucose-stimulated insulin secretion under glucotoxic and glucolipotoxic conditions

Glucose-stimulated insulin secretion (GSIS) is defective in RINm5F insulinoma cells [45], but in contrast, Hamster insulinoma HIT-T15 cells secrete insulin in response to glucose and other secretagogues [46]. Therefore, we used the HIT-T15 cell line to determine the effects of glucotoxicity and glucolipotoxicity on GSIS. In parallel, we also assessed the effects of these conditions on the activation of NF-κB and SREBP1c. We found that the levels of p50 and p65 in the cell nuclei increased under glucotoxic and glucolipotoxic conditions, while these increases were prevented by treatment with MitoTempol or MitoQ (Fig. 7A). In contrast, these conditions led to an increase in the phospho-IκB level without any variation of total IκB present in the cytosol (Fig. 7A). The SREBP1c activation was measured by assessing the expression levels of the precursor and active nuclear forms of SREBP1c. In our experiments, the expression levels of both forms of SREBP1c were increased under glucotoxic and glucolipotoxic conditions, but these increases were suppressed by treatment with MitoTempol or MitoQ (Fig. 7B). The magnitude of GSIS, as determined by ELISA, was also decreased by glucotoxicity and glucolipotoxicity, and this was prevented by MitoTempol or MitoQ (Fig. 7C). Taken together, these results suggest that mitochondria-targeted antioxidants decrease the formation of the nuclear active forms of NF-κB and SREBP1c, and increase GSIS in glucotoxicity and glucolipotoxicity.

Discussion

In the present study, we demonstrated that mitochondria-targeted antioxidants, such as MitoTempol and MitoQ, attenuated apoptosis and increased cell survival and insulin secretion in pancreatic β-cell lines under glucotoxic and glucolipotoxic conditions. These effects were mediated by decreases in oxidative damage, ER stress and lipid accumulation as well as the restoration of intracellular ATP content and the preservation of mitochondrial content, function and morphology. In addition, the antioxidants inhibited NF-κB and SREBP1c activation. These data support the possibility that mitochondria-targeted antioxidants can protect pancreatic β-cells from cumulative damage and dysfunction in human type 2 diabetes.

Hyperglycemia and hyperlipidemia are major contributing factors for enhanced mitochondrial ROS production in pancreatic β-cells during the development of type 2 diabetes [8, 9]. Pancreatic β-cells are particularly susceptible to destruction by mitochondrial ROS because the expression level of antioxidant enzymes in pancreatic islets is low [47, 48]. Mitochondrial ROS are also known to cause lipid peroxidation and reactive aldehyde formation in pancreatic β-cells during the development of type 2 diabetes [49, 50]. Lipid peroxidation is a major mechanism of oxygen-free radical toxicity to cellular organelles and membrane-bound enzymes, and aldehydes are cytotoxic products of this lipid peroxidation [51]. In our experiments, we observed that the mitochondria-targeted antioxidants attenuated apoptosis and that this was associated with decreased levels of mitochondrial ROS production, lipid peroxidation, and oxidative DNA damage under glucotoxic and glucolipotoxic conditions. Sustained elevated oxidative stress can lead to the loss of mitochondrial membrane potential [42]. The release of cytochrome c, and the activation of the apoptotic pathway [43]. Indeed, the mitochondrial membrane potential was significantly depolarized under glucotoxic and glucolipotoxic conditions and resulted in the release of mitochondrial cytochrome c to cytosol, whereas mitochondria-targeted antioxidants inhibited the loss of mitochondrial membrane potential. A decrease in mitochondrial content, integrity and consequently a decrease in ATP content were all observed under glucotoxic and glucolipotoxic conditions. Mitochondria-targeted antioxidants restored mitochondrial morphology, content and function, and thus prevented the loss of intracellular ATP in glucotoxicity and glucolipotoxicity. Taken together, these results suggest that mitochondria-targeted antioxidants play a protective role against oxidative damage and promote pancreatic β-cell survival in glucotoxicity and glucolipotoxicity.

There is evidence that ROS are lipogenic factors. First, our previous studies showed that ROS increase lipid accumulation via SREBP1c activation in pancreatic β-cells [29]. Second, Sekiya et al. reported that ROS, especially H_2O_2, increased SREBP1c transcriptional activity and induced lipogenic enzymes to increase lipid accumulation in HepG2 cells [52]. Third, hypoxia, which increased ROS production in human breast cancer cells, was shown to upregulate FAS gene expression *via* activation of Akt and SREBP1c [53]. Finally, ROS, especially H_2O_2, stimulated differentiation of preadipocytes to adipocytes [54, 55]. In our own experiments, we observed that the expression levels of precursor and nuclear forms of SREBP1c, several lipogenic enzymes such as ACC, FAS, ABCA1, and lipid accumulation were increased under glucotoxic and glucolipotoxic conditions, but these increases were reversed by MitoTempol or MitoQ. Taken together, these results suggest that mitochondria-targeted antioxidants attenuate lipid accumulation by reducing mitochondrial ROS in glucotoxicity and glucolipotoxicity.

The relationship between ER stress and ROS still remains to be explained in detail. Thereby, many studies have been focusing on finding out the relation. Recent studies showed that ROS generated at the mitochondria stimulate ER stress [56, 57], indicating that ER stress is closely associated with mitochondrial ROS. Also, it was demonstrated that pancreatic β-cells are susceptible to ROS and ER stress, which in turn interferes with insulin secretion and contributes to β-cell apoptosis in type 2 diabetes [58-61]. Consistent with this concept, we found that the levels of the ER stress markers, such as Bip/Grp78, PDI, CHOP and p-eIF2α, were significantly increased under glucotoxic and glucolipotoxic conditions, but these increases were prevented by treatment with antioxidants. These results were reflected in a reduction in GSIS following glucotoxicity and glucolipotoxicity. Again, it is noteworthy that mitochondria-targeted antioxidants also preserved GSIS, and thus, it may be concluded that mitochondria-targeted antioxidants improve insulin secretion in pancreatic β-cells under glucotoxic and glucolipotoxic conditions by suppressing mitochondrial ROS and reducing ER stress.

In summary, this study demonstrates that mitochondria-targeted antioxidants protect the survival and function of pancreatic β-cell lines under glucotoxic and glucolipotoxic conditions. As mitochondria-targeted

antioxidants, such as an oral formulation of MitoQ, have been shown to be safe and effective in human phase 2 studies [62, 63], these results suggest that mitochondria-targeted antioxidants may be potential therapies in protecting pancreatic β-cells and improving insulin secretion in type 2 diabetes.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST; to S.S.Kim.) (No. 20110030721). Conflicts of interest : The authors confirm that there are no conflicts of interest.

References

1 Koopman WJ, Nijtmans LG, Dieteren CE, Roestenberg P, Valsecchi F, Smeitink JA, Willems PH: Mammalian mitochondrial complex I: Biogenesis, regulation, and reactive oxygen species generation. Antioxid Redox Signal 2010;12:1431-1470.

2 Murphy MP: How mitochondria produce reactive oxygen species. Biochem J 2009;417:1-13.

3 Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 2000;404:787-790.

4 Bell EL, Klimova TA, Eisenbart J, Moraes CT, Murphy MP, Budinger GR, Chandel NS: The qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J Cell Biol 2007;177:1029-1036.

5 Zelko IN, Mariani TJ, Folz RJ: Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-Sod (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 2002;33:337-349.

6 Sivitz WI, Yorek MA: Mitochondrial dysfunction in diabetes: From molecular mechanisms to functional significance and therapeutic opportunities. Antioxid Redox Signal 2010;12:537-577.

7 Green K, Brand MD, Murphy MP: Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. Diabetes 2004;53:S110-118.

8 Evans JL, Goldfine ID, Maddux BA, Grodsky GM: Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. Endocr Rev 2002;23:599-622.

9 Nishikawa T, Araki E: Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Signal 2007;9:343-353.

10 Korshunov SS, Skulachev VP, Starkov AA: High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 1997;416:15-18.

11 Brownlee M: The pathobiology of diabetic complications: A unifying mechanism. Diabetes 2005;54:1615-1625.

12 Dandona P, Aljada A, Chaudhuri A, Mohanty P, Garg R: Metabolic syndrome - a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation 2005;111:1448-1454.

13 Poitout V, Robertson RP: Glucolipotoxicity: Fuel excess and beta-cell dysfunction. Endocrine Reviews 2008;29:351-366.

14 Trnka J, Blaikie FH, Smith RAJ, Murphy MP: A mitochondria-targeted nitroxide is reduced to its hydroxylamine by ubiquinol in mitochondria. Free Radical Bio Med 2008;44:1406-1419.

15 James AM, Sharpley MS, Manas ARB, Frerman FE, Hirst J, Smith RAJ, Murphy MP: Interaction of the mitochondria-targeted antioxidant MitoQ with phospholipid bilayers and ubiquinone oxidoreductases. J Biol Chem 2007;282:14708-14718.

16 Murphy MP, Smith RAJ: Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu Rev Pharmacol 2007;47:629-656.

17 Smith RAJ, Adlam VJ, Blaikie FH, Manas ARB, Porteous CM, James AM, Ross MF, Logan A, Cocheme HM, Trnka J, Prime TA, Abakumova I, Jones BA, Filipovska A, Murphye MP: Mitochondria-targeted antioxidants in the treatment of disease. Ann Ny Acad Sci 2008;1147:105-111.

18 Chacko BK, Reily C, Srivastava A, Johnson MS, Ye YZ, Ulasova E, Agarwal A, Zinn KR, Murphy MP, Kalyanaraman B, Darley-Usmar V: Prevention of diabetic nephropathy in Ins2(+/)(AkitaJ) mice by the mitochondria-targeted therapy MitoQ. Biochem J 2010;432:9-19.

19 Graham D, Huynh NN, Hamilton CA, Beattie E, Smith RAJ, Cocheme HM, Murphy MP, Dominiczak AF: Mitochondria-targeted antioxidant MitoQ(10) improves endothelial function and attenuates cardiac hypertrophy. Hypertension 2009;54:322-328.

20 Lu C, Zhang DW, Whiteman M, Armstrong JS: Is antioxidant potential of the mitochondrial targeted ubiquinone derivative MitoQ conserved in cells lacking mtDNA? Antioxid Redox Sign 2008;10:651-660.

21 Szeto HH: Cell-permeable, mitochondrial-targeted, peptide antioxidants. AAPS J 2006;8:E277-E283.

22 Oprescu AI, Bikopoulos G, Naassan A, Allister EM, Tang C, Park E, Uchino H, Lewis GF, Fantus IG, Rozakis-Adcock M, Wheeler MB, Giacca A: Free fatty acid-induced reduction in glucose-stimulated insulin secretion. Diabetes 2007;56:2927-2937.

23 Ebenezer PJ, Mariappan N, Elks CM, Haque M, Francis J: Diet-induced renal changes in zucker rats are ameliorated by the superoxide dismutase mimetic tempol. Obesity 2009;17:1994-2002.

24 Wilcox CS: Effects of tempol and redox-cycling nitroxides in models of oxidative stress. Pharmacol Ther 2010;126:119-145.

25 Trnka J, Blaikie FH, Smith RA, Murphy MP: A mitochondria-targeted nitroxide is reduced to its hydroxylamine by ubiquinol in mitochondria. Free Radic Biol Med 2008;44:1406-1419.

26 Trnka J, Blaikie FH, Logan A, Smith RA, Murphy MP: Antioxidant properties of mitotempol and its hydroxylamine. Free Radic Res 2009;43:4-12.

27 Choe SS, Choi AH, Lee JW, Kim KH, Chung JJ, Park J, Lee KM, Park KG, Lee IK, Kim JB: Chronic activation of liver X receptor induces beta-cell apoptosis through hyperactivation of lipogenesis: Liver X receptor-mediated lipotoxicity in pancreatic beta-cells. Diabetes 2007;56:1534-1543.

28 Wang H, Maechler P, Antinozzi PA, Herrero L, Hagenfeldt-Johansson KA, Bjorklund A, Wollheim CB: The transcription factor SREBP-1c is instrumental in the development of beta-cell dysfunction. J Biol Chem 2003;278:16622-16629.

29 Rashid MA, Lee S, Tak E, Lee J, Choi TG, Lee JW, Kim JB, Youn JH, Kang I, Ha J, Kim SS: Carbonyl reductase 1 protects pancreatic beta-cells against oxidative stress-induced apoptosis in glucotoxicity and glucolipotoxicity. Free Radical Bio Med 2010;49:1522-1533.

30 Kim J, Choi TG, Ding Y, Kim Y, Ha KS, Lee KH, Kang I, Ha J, Kaufman RJ, Lee J, Choe W, Kim SS: Overexpressed cyclophilin B suppresses apoptosis associated with ROS and Ca^{2+} homeostasis after ER stress. J Cell Sci 2008;121:3636-3648.
31 Jones CI, Han Z, Presley T, Varadharaj S, Zweier JL, Ilangovan G, Alevriadou BR: Endothelial cell respiration is affected by the oxygen tension during shear exposure: Role of mitochondrial peroxynitrite. Am J Physiol Cell Physiol 2008;295:C180-191.
32 Lee J, Choi KJ, Lim MJ, Hong F, Choi TG, Tak E, Lee S, Kim YJ, Chang SG, Cho JM, Ha J, Kim SS: Proto-oncogenic H-ras, K-ras, and N-ras are involved in muscle differentiation via phosphatidylinositol 3-kinase. Cell Res 2010;20:919-934.
33 Singh NP: Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. Mutat Res 2000;455:111-127.
34 Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M, Sbrana S, Torri S, Pollera M, Boggi U, Mosca F, Del Prato S, Marchetti P: Functional and molecular defects of pancreatic islets in human type 2 diabetes. Diabetes 2005;54:727-735.
35 Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, Brown MS: Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. J Biol Chem 2002;277:9520-9528.
36 Robertson RP: Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem 2004;279:42351-42354.
37 Doughan AK, Dikalov SI: Mitochondrial redox cycling of mitoquinone leads to superoxide production and cellular apoptosis. Antioxid Redox Signal 2007;9:1825-1836.
38 Schafer M, Schafer C, Ewald N, Piper HM, Noll T: Role of redox signaling in the autonomous proliferative response of endothelial cells to hypoxia. Circ Res 2003;92:1010-1015.
39 Kalivendi SV, Konorev EA, Cunningham S, Vanamala SK, Kaji EH, Joseph J, Kalyanaraman B: Doxorubicin activates nuclear factor of activated t-lymphocytes and fas ligand transcription: Role of mitochondrial reactive oxygen species and calcium. Biochem J 2005;389:527-539.
40 Ogihara T, Chuang JC, Vestermark GL, Garmey JC, Ketchum RJ, Huang X, Brayman KL, Thorner MO, Repa JJ, Mirmira RG, Evans-Molina C: Liver X receptor agonists augment human islet function through activation of anaplerotic pathways and glycerolipid/free fatty acid cycling. J Biol Chem 2010;285:5392-5404.
41 Oppermann U: Carbonyl reductases: The complex relationships of mammalian carbonyl- and quinone-reducing enzymes and their role in physiology. Annu Rev Pharmacol Toxicol 2007;47:293-322.
42 Cabreiro F, Picot CR, Perichon M, Castel J, Friguet B, Petropoulos I: Over-expression of mitochondrial methionine sulfoxide reductase B2 protects leukemia cells from oxidative stress-induced cell death and protein damage. J Biol Chem 2008;283:16673-16681.
43 Yao K, Ye P, Zhang L, Tan J, Tang X, Zhang Y: Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells. Mol Vis 2008;14:217-223.
44 Kohnke R, Mei J, Park M, York DA, Erlanson-Albertsson C: Fatty acids and glucose in high concentration down-regulates ATP synthase beta-subunit protein expression in INS-1 cells. Nutr Neurosci 2007;10:273-278.
45 Tiedge M, Hohne M, Lenzen S: Insulin secretion, insulin content and glucose phosphorylation in RINm5F insulinoma cells after transfection with human GLUT2 glucose-transporter cDNA. Biochem J 1993;296:113-118.
46 Diem P, Walseth TF, Zhang HJ, Robertson RP: Secretion and degradation of glucagon by hit cells. Endocrinology 1990;127:1609-1612.
47 Lenzen S, Drinkgern J, Tiedge M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic Biol Med 1996;20:463-466.
48 Tiedge M, Lortz S, Drinkgern J, Lenzen S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 1997;46:1733-1742.
49 Chen JJ, Yu BP: Alterations in mitochondrial membrane fluidity by lipid peroxidation products. Free Radic Biol Med 1994;17:411-418.
50 Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S: Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. Endocrinology 2000;141:2767-2772.
51 Esterbauer H, Schaur RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81-128.
52 Sekiya M, Hiraishi A, Touyama M, Sakamoto K: Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochem Biophys Res Commun 2008;375:602-607.
53 Furuta E, Pai SK, Zhan R, Bandyopadhyay S, Watabe M, Mo YY, Hirota S, Hosobe S, Tsukada T, Miura K, Kamada S, Saito K, Iiizumi M, Liu W, Ericsson J, Watabe K: Fatty acid synthase gene is up-regulated by hypoxia via activation of akt and sterol regulatory element binding protein-1. Cancer Res 2008;68:1003-1011.
54 Lee H, Lee YJ, Choi H, Ko EH, Kim JW: Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. J Biol Chem 2009;284:10601-10609.
55 Schroder K, Wandzioch K, Helmcke I, Brandes RP: Nox4 acts as a switch between differentiation and proliferation in preadipocytes. Arterioscler Thromb Vasc Biol 2009;29:239-245.
56 Zhang Y, Soboloff J, Zhu Z, Berger SA: Inhibition of ca^{2+} influx is required for mitochondrial reactive oxygen species-induced endoplasmic reticulum ca^{2+} depletion and cell death in leukemia cells. Mol Pharmacol 2006;70:1424-1434.
57 Guan L, Han B, Li Z, Hua F, Huang F, Wei W, Yang Y, Xu C: Sodium selenite induces apoptosis by ROS-mediated endoplasmic reticulum stress and mitochondrial dysfunction in human acute promyelocytic leukemia NB4 cells. Apoptosis 2009;14:218-225.
58 Evans JL, Goldfine ID, Maddux BA, Grodsky GM: Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes 2003;52:1-8.
59 Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, Kawashima J, Shirotani T, Ichinose K, Brownlee M, Araki E: Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochem Biophys Res Commun 2003;300:216-222.
60 Harding HP, Ron D: Endoplasmic reticulum stress and the development of diabetes: A review. Diabetes 2002;51:S455-S461.
61 Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M: Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. Proc Natl Acad Sci U S A 2001;98:10845-10850.
62 Gane EJ, Weilert F, Orr DW, Keogh GF, Gibson M, Lockhart MM, Frampton CM, Taylor KM, Smith RA, Murphy MP: The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C patients. Liver Int 2010;30:1019-1026.
63 Smith RA, Murphy MP: Animal and human studies with the mitochondria-targeted antioxidant MitoQ. Ann N Y Acad Sci 2010;1201:96-103.