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Linoleic Acid Promotes Mitochondrial Biogenesis and Maintains Mitochondrial Structure for Prevention of Streptozotocin Damage in RIN-m5F Cells

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Linoleic acid (LA) improves insulin resistance and prevents diabetes. To investigate whether linoleic acid could protect against streptozotocin (STZ)-induced cell death, rat RIN-m5F cells were exposed to STZ. SL and SO groups consisted of cells treated with STZ and then LA or oleic acid (OA) respectively. STZ treatment decreased the mitochondrial membrane potential in the STZ, SO, and SL groups. Cells of the SL group had more intact mitochondria. Increased mRNA expression of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), as well as of the mitochondrial biogenesis regulators peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α), and mitochondrial transcription factor A (Tfam), were found in the LA group. The insulin content was significantly decreased in all three groups. These results suggest that the effects of LA on cell viability after STZ damage occur through maintenance of mitochondrial structure and increased mitochondrial biogenesis.

Key words: linoleic acid; oleic acid; mitochondrial biogenesis; mitochondrial transcription factor; streptozotocin

The diabetogenic agent STZ is a D-glucopyranose derivative of *N*-methyl-*N*-nitrosourea MNU. STZ has broad-spectrum antibiotic activity and is often used to induce diabetes mellitus in experimental animals through its toxic effects on pancreatic β cells. The pathogenic effect is mediated to a significant extent *via* increased production of reactive oxygen species and/or reactive nitrogen species and subsequent oxidative stress.[1,2)]

Olive oil is an integral ingredient of the Mediterranean diet. Recent, accumulating evidence suggests that it contributes to lower risk of cardiovascular diseases and several types of cancers. OA (18:1 n-9) is the most important monounsaturated fatty acid (MUFA). It represents more than 90% of ingested MUFAs in the human diet, and olive oil has a high oleic acid content. It has long been known that n-6 fatty acids reduce serum total and low-density lipoprotein cholesterol.[3)] LA (18:2 n-6) is the most abundant essential dietary fatty acid. Elevated polyunsaturated fatty acid (PUFA) consumption improved insulin resistance in a controlled feeding study.[4)] Intake of PUFA is inversely related to the incidence of type 2 diabetes, as reported in a large prospective study.[5)]

Human mtDNA is a circular, intronless 16.5-kb molecule present in 1 to 10 copies in each mitochondrion. It encodes 13 of about 80 protein subunits constituting the oxidative phosphorylation system, as well as two rRNAs and 22 tRNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes. While mtDNA encodes 13 polypeptides involved in cellular respiration, nuclear DNA encodes the majority of respiratory chain proteins and all the proteins and enzymes that regulate replication and transcription of mtDNA.[6)]

In mammals, Tfam regulates both mitochondrial transcription and replication by permitting commencement of RNA transcription from the unwinding L-strand of DNA.[7)] Tfam is a nuclear-encoded 25-kDa protein that bends and unwinds mtDNA upon binding. It belongs to the high-mobility-group-box family of proteins.[8)] Knockdown of Tfam gene expression may reduce mtDNA copy number and expression, thereby compromising mitochondrial function and subsequent cell growth and morphology.[9)] PGC-1α has been reported to regulate various PGC-1α targets, including gluconeogenic-related genes and mitochondrial oxidative phosphorylation genes.[10)]

In our previous study, we found increased oxidative mtDNA damage in multiple tissues of rats with STZ-induced diabetes. Intervention with rice bran oil, which contains a high proportion of LA, prevented induction of oxidative damage by STZ.[11)] This rice bran oil-containing diet also significantly suppressed hyperlipidemic and hyperinsulinemic responses in diabetic rats.[12)] In the present study, the cell protective potentials of LA and OA were determined by studying rat RIN-m5F cells exposed to STZ. The effects on mitochondrial biogenesis, membrane potential, and mitochondrial structure were determined.

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Materials and Methods

Cell culture and drug treatment. Rat RIN-m5F islet cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2 for 3 d before the experimental treatment began. Based on the literature and the manufacturer's instructions, STZ, LA-albumin complex, and OA-albumin complex were dissolved in the medium. STZ, LA-albumin complex, and OA-albumin complex were purchased from Sigma-Aldrich (St. Louis, MO). Cells were seeded in 96-well culture plates for 24 h and then treated with 4 mM STZ for 2 h, followed by 0.4 mM OA or LA treatment for 24 or 48 h.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay of cell viability. Metabolic reduction of a tetrazolium compound (MTS) was used to evaluate the viability of RIN-m5F islet cells. The MTS colorimetric assay is based on the reduction of MTS to a colored formazan product. Absorbance was measured at 492 nm. It was directly proportional to the number of living cells in the tested samples. Cells were seeded in 96-well plates for 24 h. To determine the effects of STZ, LA, and OA on cell viability, several concentrations of the drugs were added to the culture medium to a total volume of 100 µl in each well. After 24 or 48 h of incubation, the medium was aspirated, and the cells were washed twice with 100 µl PBS. MTS reagent (20 µl) mixed with 100 µl of fresh medium without drugs was added to each well, and the cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 2 h. The plates were read on a Microplate Reader (VersaMax, Molecular Devices, Sunnyvale, CA). Cell viability was expressed as the percentage of viable cells in treated samples relative to the non-treated control.

RNA extraction and real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the cells with the Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Total RNA (1–5 µg) was reverse-transcribed into complementary DNA (cDNA) using oligo (dT) 18 (Protech Technology, Taipei, Taiwan) as a primer and MMLV reverse transcriptase (Epicentre Biotechnology, Madison, WI), and 2 µl of the cDNA template was separately used to amplify the different mRNAs. A LightCycler PCR machine (Roche Diagnostics, Mannheim, Germany) was used to perform real-time PCR. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s. The primers were designed using Primer Express (Applied Biosystems). The sequences of the oligonucleotide primers used in this study were as follows: NADH dehydrogenase subunit 1 (ND1) (forward, CCCAACCCTCTCCCTTACA; reverse, ATTTGAGGCTCATCCCG), ND6 (forward, CCAGCCACCACTATCATTC; reverse, GAGTTGGTAGTGTTCTACTTGT), Cytochrome *c* oxidase subunit VIc (COXVIc) (forward, CGTCTGCGGGTTCATATT; reverse, GCCTGCCTCATCTCTTCAA), PGC-1α (forward, CGTTCAAGGTCACCCTACA; reverse, TGCTTTCTGCTTCTGCC), uncoupling protein 2 (UCP2) (forward, GGTCGGAGATACCAGAGCA; reverse, ATGAGGTTGGCTTTCAGG), insulin (forward, CCGTCGTGAAGTGGAGGA; reverse, TTGGTAGAGGGAGCAGATG), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (forward, ATGGAGTCTACTGGCGT; reverse, CTCTTGAGGGAGTTGTCATATTTC).

Determination of protein expression. Total protein was extracted from the harvested cells using lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 10% glycerol, and 1% Triton X-100) with protease inhibitors. The cell lysate was cleared of cell debris by centrifugation at 10,000 × *g* for 5 min. The protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved on polyacrylamide gels, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia, Piscataway, NJ). The membranes were blocked for 1 h at 4 °C with 10% skimmed milk in TBST buffer (1 M Tris–HCl, 100 mM NaCl, and 1% Tween-20). Blots were probed with the following primary antibodies: monoclonal antibodies against PGC-1α (Santa Cruz, CA), COXVIc (Invitrogen), and Tfam (GlycoNex, Taipei, Taiwan), or a polyclonal antibody against GAPDH (Santa Cruz Biotechnology). Blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) antibody. Antibody-bound protein was detected using the western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology), followed by exposure to radiographic film. The insulin content of RIN-m5F cells was quantified with an ELISA kit (Mercodia, Uppsala, Sweden).

Assessment of the mitochondrial membrane potential. The mitochondrial membrane potential was verified with the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) cationic fluorophore. The JC-1 dye is redistributed between the inside and outside of the mitochondrial matrix depending on the membrane potential between the inner and outer membranes. With polarized membranes, JC-1 forms red fluorescent aggregates, but when the membranes depolarize, it stays dispersed as a monomer and fluoresces green. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) causes mitochondrial uncoupling and produces a drastic decrease in JC-1 fluorescence. After 24 h of incubation, cells were trypsinized, centrifuged, and resuspended in 1 ml of PBS. JC-1 (50 µM) was added for 20 min at 37 °C. CCCP (5 µg/ml) was used as a positive control for mitochondrial depolarization. The cells were illuminated with blue (488 nm) excitation, and green and red fluorescence emissions were detected with 530/30- and 585/42-nm filters respectively in a FACS Calibur system using CellQuest software (BD Biosciences, San Jose, CA). The proportion of red-to-green fluorescence of individual cells was calculated.

Determination of mitochondrial ultrastructure. Treated cells were fixed for 2 h in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M cacodylate buffer, washed in 0.1 M cacodylate buffer containing 0.2 M sucrose 3 times, and post-fixed for 2 h in 1% osmium tetroxide. Dehydration was achieved by a graded series of 35%, 50%, 75%, 95%, and 100% ethanol. The samples were then incubated in a mixture of ethanol and Spurr resin (Electron Microscopy Sciences, Hatfield, PA) and embedded in Spurr resin. Ultrathin sections were cut on Leica AG ultramicrotome, placed on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and photographed with a Hitachi T-600 electron microscope.

Results

Effects of STZ, LA, and OA on cell viability

RIN-m5F cells were treated with 4 mM STZ for 2 h, followed by 0.4 mM LA or OA for 24 or 48 h. Cells treated with STZ and then LA or OA comprised the SL and SO groups respectively. Significantly decreased cell survival was observed in the STZ and SO groups as compared to the control cells. LA treatment prevented STZ-induced cell death (Fig. 1A). With 48 h of LA treatment, cell viability significantly increased, to the level of the control group (Fig. 1B). No significant differences were observed for OA treatment as compared with STZ group (Fig. 1A, B).

Determination of mitochondrial membrane potential and mitochondrial ultrastructure

Mitochondrial membrane potential and mitochondrial ultrastructure were determined in order to detect latent mitochondrial damage. STZ treatment decreased the mitochondrial membrane potential in the STZ, SO, and SL groups (Fig. 1C). The mitochondrial ultrastructure was examined by electron microscopy. The control cells showed multiple cristae and maintenance of fine ultrastructure (Fig. 2A). Disrupted mitochondrial ultrastructure was observed in the STZ and SO groups (Fig. 2B, C). There were more intact mitochondria in the cells of the SL group (Fig. 2D).

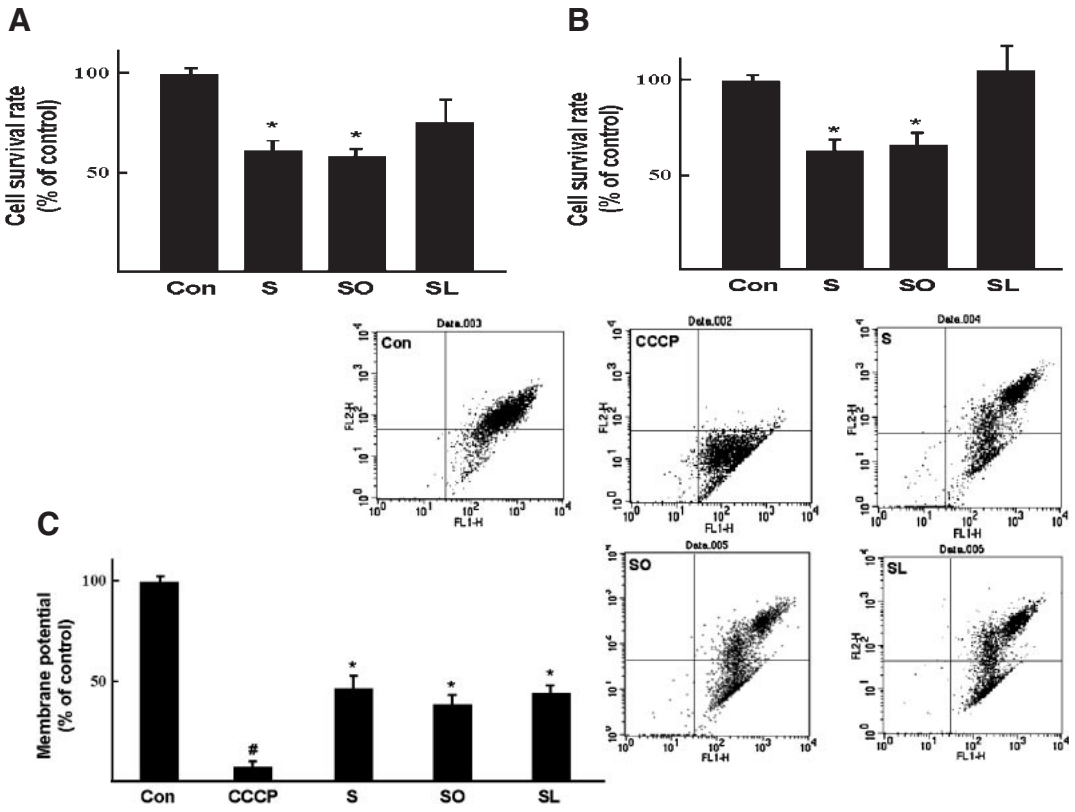

Fig. 1. Cell Viabilities Were Determined by MTS Assay after STZ, OA, and LA Treatment.

RIN-m5F cells were treated with 4 mM STZ for 2 h followed by 0.4 mM OA or LA treatment for (A) 24 or (B) 48 h. (C) Mitochondrial membrane potential was determined. Cells were incubated in 4 mM STZ for 2 h followed by 0.4 mM OA or LA treatment for 24 h, then labeled with JC-1 fluorescent dye and analyzed by flow cytometry. Data are expressed as the mean ± SD of three separate experiments. $^{*}p < 0.05$, significantly different from control cells.

mtRNA expression in LA- and OA-treated cells

To determine whether mitochondrial biogenesis was induced by LA, the expression of multiple genes associated with mitochondria was measured in the STZ, SO, and SL groups. Mitochondrial gene expression of ND1 and ND6 was induced in the LA group (Fig. 3A, B). Expression of nDNA-encoded COXVIc was also elevated in the LA group (Fig. 3C). Increased gene expression of the mitochondrial biogenesis regulators Tfam and PGC-1α was observed in the LA group (Fig. 3D, E). There were no differences in UCP2 expression in the three groups as compared to the control group (Fig. 3F). Downregulation of insulin was detected in the STZ and SO groups, and increased expression was found in the SL group as compared to the STZ and SO groups (Fig. 3G).

Mitochondrial protein expression in LA- and OA-treated cells

The protein levels of PGC-1α, Tfam, and the electron transfer chain subunit of nuclear-encoded COXVIc were determined in order to characterize mitochondrial biogenesis. Increased protein levels of PGC-1α were found in the LA-treated cells as compared to the STZ and OA groups (Fig. 4A). Tfam and COXVIc expression was significantly decreased in the cells treated with STZ and OA, but no significant difference was found between cells treated with LA and the control cells (Fig. 4B, C). Insulin content was significantly decreased in the STZ, SO, and SL groups (Fig. 4D).

Discussion

The results of this study indicate that STZ treatment decreased cell viability, and that LA treatment for 24 and 48 h increased cell viability as compared to the STZ and OA treatments (Fig. 1). Decreased viability in various types of cells treated with STZ has been reported.[13,14] OA promotes apoptosis and necrosis of human lymphocytes.[15] Shen *et al.*[16] also reported increased intracellular oxidant formation in MIN6 cells chronically exposed to OA. In a study of mitochondrial membrane potential, the results showed that STZ treatment decreased the membrane potential and this effect was not improved by OA or LA treatment (Fig. 1C). Loss of mitochondrial membrane potential in cells treated with STZ has been reported.[17,18] Saturated fatty acids and PUFAs decrease membrane potential through their lipotoxic effects.[16,19] These previous observations of mitochondrial membrane potential loss induced by STZ and fatty acids are consistent with the results of our study.

STZ treatment also disrupted the mitochondrial ultrastructure (Fig. 2B). LA treatment prevented STZ-nduced mitochondrial structural damage (Fig. 2D), but no significant protective effect was detected in the OA-treated group. LA was better able to maintain mitochondrial morphology than OA. Different fatty acids have various effects on lipotoxic stress. Long-term exposure of pancreatic beta cells to saturated fatty acid palmitate induced lipotoxic stress that was mediated by the mitochondrial permeability transition, but co-application

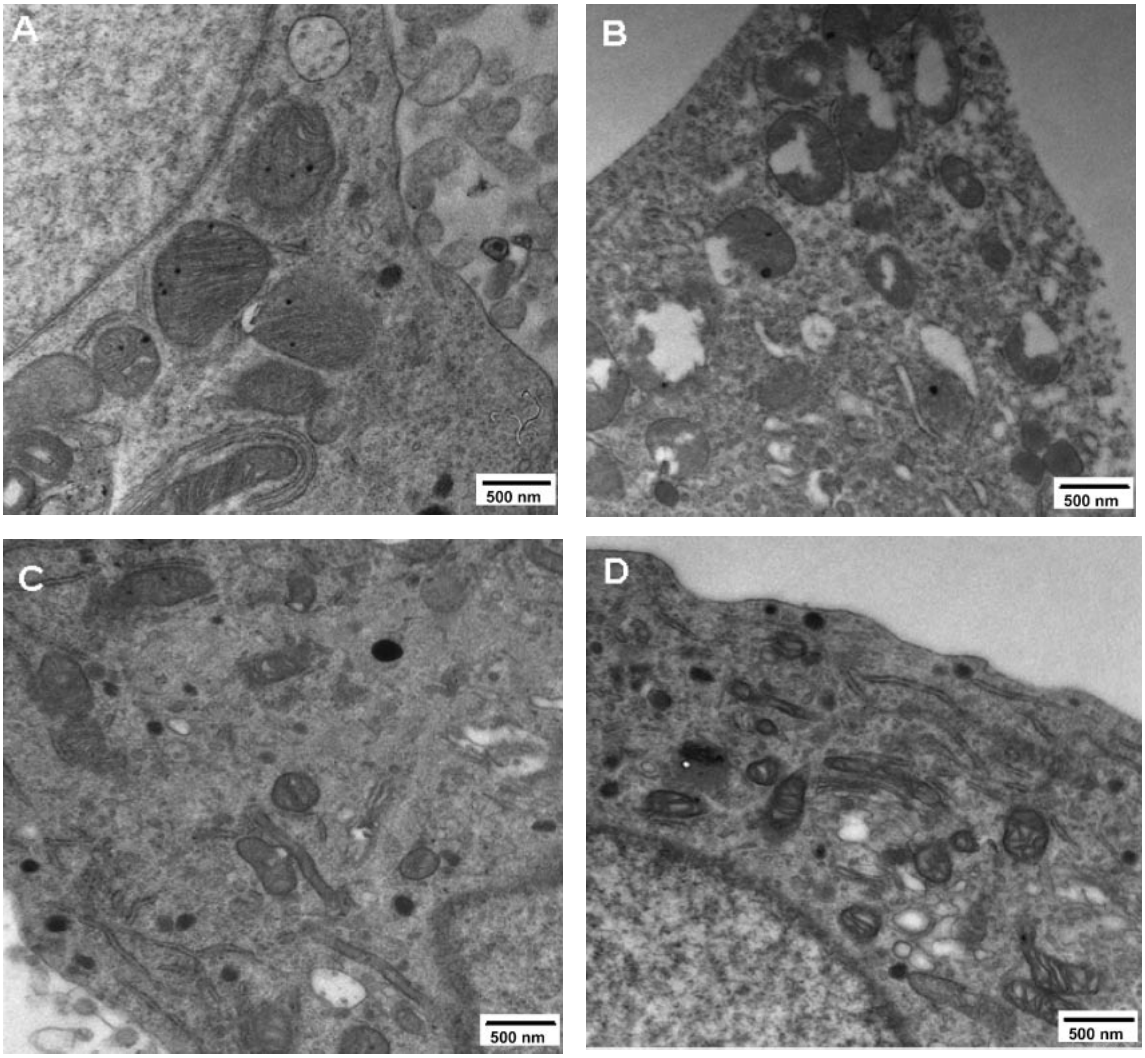

Fig. 2. Ultrastructure of Mitochondria in RIN-m5F Cells.

(A) Fine mitochondrial ultrastructure with multiple cristae in control cells was determined by electron microscopy. (B) Disrupted mitochondrial ultrastructure was observed in cells treated with (B) 4 mM STZ for 2 h or (C) 4 mM STZ for 2 h followed by 0.4 mM OA for 24 h. (D) Intact mitochondrial ultrastructure with multiple cristae was observed in cells treated with 4 mM STZ for 2 h followed by 0.4 mM LA for 24 h. Original magnification ×25,000. Data are from three separate experiments.

of the unsaturated fatty acid oleate provided significant protection from lipotoxic stress.[16] In the present study, OA did not prevent mitochondrial damage caused by STZ.

LA treatment enhanced RNA expression of mtDNA-encoded ND1 and ND6, as well as that of nuclear-encoded COXVIc, Tfam, and PGC-1α. These findings suggest that the maintenance of cell survival was due to enhanced mitochondrial biogenesis (Fig. 3). In addition, LA treatment also increased PGC-1α, Tfam, and COXVIc protein expression in STZ-treated cells (Fig. 4), no significant elevation of insulin levels due to LA was detected. Decreased insulin and mitochondrial protein expression after STZ treatment have been reported in similar studies. Mulder *et al.*[20] showed that islet amyloid polypeptide and insulin mRNA levels were reduced to 45% of that of controls with low-dose STZ treatment. Mouse islets exhibited decreased mRNA levels of mtDNA-encoded cytochrome b following STZ exposure.[21] In contrast, many studies have found that fatty acids can enhance mitochondrial biogenesis. Increased plasma concentrations of fatty acids induce mitochondrial biogenesis, including upregulation of fatty acid oxidative genes, the TCA cycle, and electron transfer chain-related genes in muscles. An increased mtDNA copy number was also reported.[22] Eicosapentaenoic acid induces mitochondrial proliferation and reduces intracellular lipid levels.[23,24] The present study found upregulation of PGC-1α and Tfam mRNA and protein expression in the SL group (Figs. 3, 4). A 3-fold stimulation of the expression of the mitochondrial regulatory factors PGC-1α and nuclear respiratory factor-1 has also been reported.[24] Fatty acids regulate gene expression through activation of peroxisome proliferator-activated receptors (PPARs),[25] and increased PPARδ activity induces mitochondrial biogenesis.[26] Free fatty acids increased PGC-1α expression levels in isolated islets and the mouse beta-cell-derived beta TC3 cell line after chronic incubation with OA and palmitic acid.[27] This study indicated that upregulation of mitochondrial biogenesis occurred through the PGC-1α and Tfam pathways. UCP-2 expression is modulated by a PPAR-dependent pathway. Overexpression of UCP-2 and PPAR-γ proteins and a consequent decrease in ATP production were demonstrated in islets cultured at high free fatty acid levels.[28] There were no significant

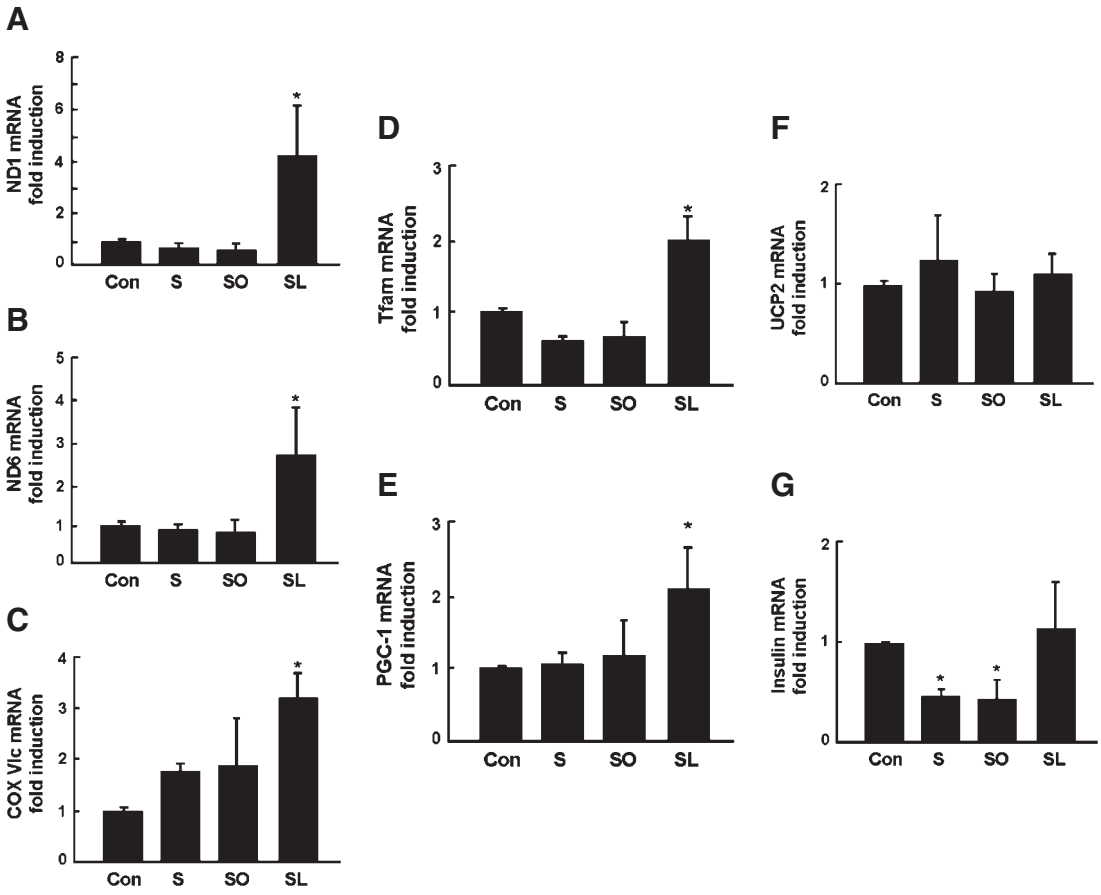

Fig. 3. Expression of mtRNA.

Cells were incubated in 4 mM STZ for 2 h followed by 0.4 mM OA or LA treatment for 24 h. ND1 (A), ND6 (B), COX VIc (C), Tfam (D), PGC-1α (E), UCP2 (F), and insulin (G) gene expression was determined by real-time RT-PCR and quantified by relation to GAPDH. Data are expressed as the mean $\pm$ SD of three separate experiments. $^{*}p < 0.05$, significantly different from control cells.

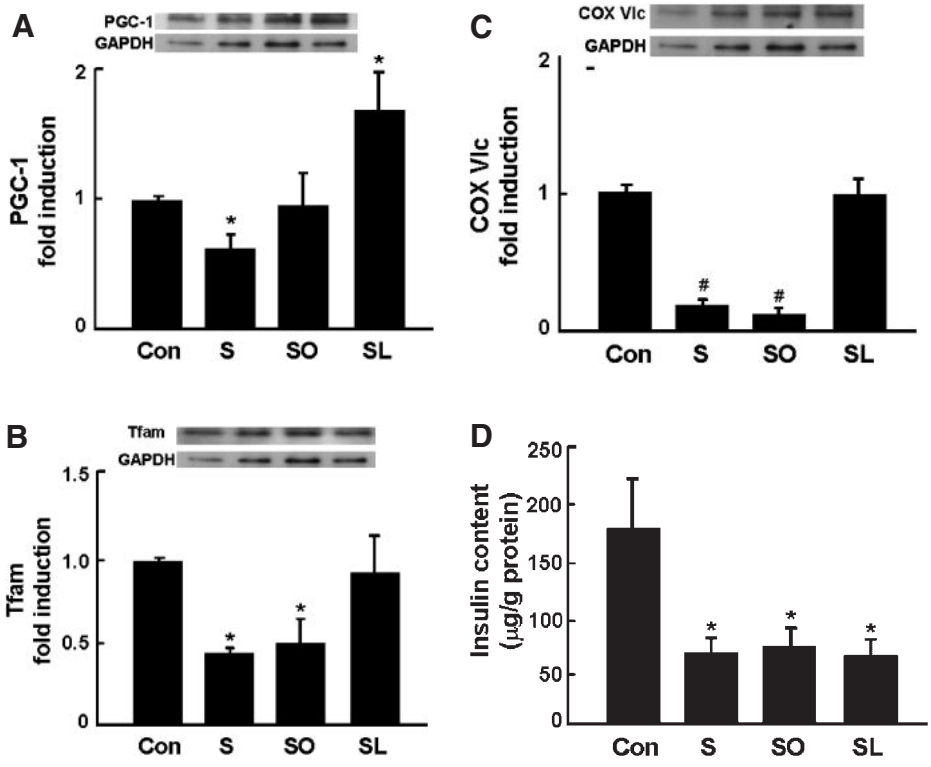

Fig. 4. Expression of Protein.

Cells were incubated in 4 mM STZ for 2 h followed by 0.4 mM OA or LA for 24 h. Protein levels of PGC-1α (A), Tfam (B), and COX VIc (C) were determined by western blotting. Insulin content was determined by ELISA (D). Data are expressed as the mean $\pm$ SD of three separate experiments. $^{*}p < 0.05$, significantly different from control cells.

differences in UCP2 expression among the three groups in our study.

LA may thus prevent cell death induced by STZ, but insulin production was unaffected. This may be correlated with the inability to maintain the mitochondrial membrane potential. A decreased mitochondrial membrane potential might result in reduced production of energy by the mitochondrial oxidative phosphorylation system, as well as ATP depletion, this might further affect insulin production. The effects of LA on cell viability after STZ damage thus occurred through maintenance of the mitochondrial structure and increased mitochondrial biogenesis.

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