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Protective Effects of *R*-Alpha-Lipoic Acid and Acetyl-L-Carnitine in MIN6 and Isolated Rat Islet Cells Chronically Exposed to Oleic Acid

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Abstract Mitochondrial dysfunction due to oxidative stress and concomitant impaired β-cell function may play a key role in type 2 diabetes. Preventing and/or ameliorating oxidative mitochondrial dysfunction with mitochondria-specific nutrients may have preventive or therapeutic potential. In the present study, the oxidative mechanism of mitochondrial dysfunction in pancreatic β-cells exposed to sublethal levels of oleic acid (OA) and the protective effects of mitochondrial nutrients [*R*-alpha-lipoic acid (LA) and acetyl-L-carnitine (ALC)] were investigated. Chronic exposure (72 h) of insulinoma MIN6 cells to OA (0.2–0.8 mM) increased intracellular oxidant formation, decreased mitochondrial membrane potential (MMP), enhanced uncoupling protein-2 (UCP-2) mRNA and protein expression, and consequently, decreased glucose-induced ATP production and suppressed glucose-stimulated insulin secretion. Pretreatment with LA and/or ALC reduced oxidant formation, increased MMP, regulated UCP-2 mRNA and protein expression, increased glucose-induced ATP production, and restored glucose-stimulated insulin secretion. The key findings on ATP production and insulin secretion were verified with isolated rat islets. These results suggest that mitochondrial dysfunction is involved in OA-induced pancreatic β-cell dysfunction and that pretreatment with mitochondrial protective nutrients could be an effective strategy to prevent β-cell dysfunction. J. Cell. Biochem. 104: 1232–1243, 2008. © 2008 Wiley-Liss, Inc.

Key words: acetyl-L-carnitine; insulin secretion; *R*-alpha-lipoic acid; mitochondria; oxidative stress; uncoupling protein (UCP)-2; β-cell

The prevalence of metabolic syndrome, including obesity, insulin resistance, dyslipidemia, and hypertension, is increasing worldwide, especially among the elderly [Bechtold et al., 2006]. Mitochondrial dysfunction due to oxidative stress is a major contributor to aging and age-associated diseases [Moreira et al., 2007; Rodriguez et al., 2007]. Increasing evidence shows that mitochondrial functional impairment plays an important role in metabolic syndrome such as type 2 diabetes [Toledo et al., 2007]. Mitochondrial oxidant production associated with hyperlipemia disrupts glucose-stimulated insulin secretion by pancreatic β-cells, which are particularly susceptible to oxidative damage. Mitochondrial oxidant production in response to hyperglycemia may contribute to both the progression and pathological complications of diabetes, suggesting that prevention of mitochondrial

Abbreviations used: OA, oleic acid; KRBH, Krebs–Ringer HEPES buffer; UCP-2, uncoupling protein 2; LA, (*R*)-alpha-lipoic acid; ROS, reactive oxygen species; MMP, mitochondrial membrane potential; ALC, acetyl-L-carnitine; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; JC-1, tetraethylbenzimidazolecarbocyanine iodide.

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oxidative damage might be a therapeutic strategy for diabetes [Tang et al., 2007].

Recent studies demonstrate that long-term exposure of excessive free fatty acids (FFAs), mimicking hyperlipemia, leads to an oversupply of electrons in the mitochondrial electron transfer chain resulting in membrane hyperpolarization and formation of reactive oxygen species (ROS). ROS, in turn, induces β-cell dysfunction and ultimately cell death [Moriscot et al., 2007]. Understanding how hyperlipemia-induced oxidative stress leads to β-cell dysfunction has advanced considerably in recent years [Lameloise et al., 2001] and uncoupling protein 2 (UCP-2) may act as an important link to impaired insulin secretion [Chan et al., 2004].

Mitochondria provide energy to cells but are also both a source and a target of oxidants. Protecting mitochondria from oxidative damage such as that mediated by exposure to FFA might maintain β-cell function. We define mitochondrial nutrients as those which protect mitochondria from oxidative damage and improve mitochondrial function [Liu and Ames, 2005]. Well-known mitochondrial nutrients or prosthetic groups are *R*-alpha-lipoic acid (LA), acetyl-L-carnitine (ALC), and coenzyme Q10. These are mitochondrial components that can all enter cells and mitochondria following exogenous treatment [Liu and Ames, 2005]. LA is a coenzyme that is involved in carbohydrate and amino acid utilization and in the production of ATP by mitochondria. LA is reduced in mitochondria to dihydrolipoic acid (DHLA), a potent antioxidant which scavenges free radicals and recycles other antioxidants to reduce the oxidative stress to mitochondria [Packer and Tritschler, 1996; Packer et al., 1997; Liu et al., 2002a]. ALC is a betaine required for the transport of long-chain fatty acids into the mitochondria for β-oxidation, ATP production, and for the removal of excess short- and medium-chain fatty acids [Head et al., 2002; Ames and Liu, 2004]. LA has been used in the treatment of diabetic neuropathy and has pancreatic and peripheral effects that improve glucose transport and metabolism [Konrad, 2005]. ALC also has been used as treatment for diabetes and chronic diabetic neuropathy in animal experiments [Alves et al., 2006] and clinical studies [Sima et al., 2005]. LA and ALC improve mitochondrial function in aging and degenerative diseases and in combination seem more potent owing to complementary effects [Liu et al., 2002a; Liu et al., 2002b]. Beneficial effects on delaying or protecting mitochondrial function might be obtained by combinations of a number of these mitochondrial nutrients. Thus, optimal doses of a combination of mitochondrial nutrients could be a strategy for delaying and treating cellular dysfunction.

The purpose of this study was to investigate the protective effects of LA or/and ALC on β-cell dysfunction in insulinoma MIN6 cells and isolated rat islets, induced by chronic sublethal levels of FFAs.

MATERIALS AND METHODS

Materials

Oleic acid (OA), anti-β-actin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA-fatty acid free), 1,4-dithio-DL-threitol (DTT), and ATP Bioluminescent Assay Kit were obtained from Sigma (St. Louis, MO); ALC (hydrochloride salt) from Sigma Tau (Pomezia, Italy); tetraethylbenzimidazolecarbocyanine iodide (JC-1) from Molecular Probes (Leiden, Netherlands); 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) from Calbiochem (Darmstadt, Germany); anti-UCP-2 antibody from Chemicon (Temecula, CA); mouse insulin ELISA kit from Linco Research (St. Charles, MO); TRIzol from Invitrogen (Carlsbad); Reverse Transcription System kit and SYBR Green from Promega (Manheim, Germany); HotStarTaq from TaKaRa (Otsu, Shiga, Japan), and UCP-2 and β-actin primers synthesized by Bioasia Biotech (Shanghai, China). MIN6 cells were a generous gift from Dr. Kazutomo Inoue (Kyoto University, Japan) and LA (Tris salt) from Dr. K. Wessel, Viatris, Germany.

MIN6 Cell Culture and Treatments

MIN6 cells retain insulin response to glucose and other secretagogues, and have been used extensively in studies of the mechanisms controlling insulin secretion. MIN6 cells (passages between 30 and 40) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere (5% CO_2, 95% air), and grown in monolayers to 80% confluence in 100-mm^2 dishes or 24-well plates. For experiments, cells

were incubated overnight in DMEM containing 11.1 mM glucose, and then treated with various concentrations of LA and/or ALC for 6 h followed by treatment with or without 0.4 mM OA for 72 h in the presence of LA and/or ALC. The selection of 6 h pretreatment with LA and/or ALC 72 h before OA treatment was based on the following considerations: (1) the purpose of this study is to test the preventive, not therapeutic effects of mitochondrial nutrients to show that mitochondrial dysfunction is involved in OA-induced β-cell dysfunction; (2) the possible direct reaction of LA and ALC with OA, if any, should be avoided; and (3) a pilot time-dependent study of pretreatment of MIN6 cells with LA from 2 to 24 h showed that the optimal inhibiting effect on ROS levels (determined by DCF fluorescence) was at 6 h. A solution of OA bound to BSA was prepared by stirring 4 mM OA with 5% (w/v) BSA in Krebs–Ringer HEPES (KRBH) buffer (115 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM HEPES, pH 7.4) for 30 min at 50°C. At the end of the incubation, the pH of the fatty acid solution was adjusted to 7.4. The stock solution was filtered through a 0.2-μm filter and stored at −20°C under nitrogen to prevent oxidation. When BSA-bound fatty acids were added to serum-free culture medium, the final concentration of BSA was adjusted to 0.5% [Wang et al., 2004].

Islet Isolation and Culture

Pancreatic islets were isolated from Male Sprague–Dawley rats (220–250 g). The experimental procedures were performed according to the guidelines for the care and use of laboratory animals as established by our institute. Perfusion of the pancreatic duct and in situ collagenase XI digestion were performed as described [Gotoh et al., 1987]. Islets were subsequently purified on dextran-70 gradient solutions. Purified islets were handpicked and first cultured overnight at 5.5 mM glucose in DMEM; after pretreatment with 11.1 mM glucose in DMEM, they were exposed to various concentrations of LA and/or ALC for 6 h followed by treatment with or without 0.4 mM OA for 72 h.

Cell Viability Assay

Cultured cells in 96-well dishes were washed twice with 0.2 ml KRBH buffer supplemented with 0.1% BSA (w/v) and then incubated with 0.2 ml of the same buffer containing 100 μg/ml MTT for 4 h at 37°C. After washing, the formazan (MTT metabolic product) was resuspended in 200 μl DMSO. The absorbance of the dissolved formazan was measured at 560 nm in a microplate reader. The background absorbance of the reaction solution alone (without cells) was subtracted from the sample absorption values before data analysis [Busch et al., 2005].

Insulin Secretion Assay

After culture for 72 h under the various experimental conditions, MIN6 cells or islets were washed and preincubated for 1 h in a modified KRBH buffer with 0.1% BSA. Cells were subsequently incubated for 1 h in the same buffer containing 2.8 or 30 mM glucose. Incubation media were collected, and insulin secretion was quantified using a mouse insulin ELISA kit according to the manufacturer's instructions and normalized by cellular protein content.

Glucose-Induced ATP Generation in MIN6 Cells

ATP generation in MIN6 was determined by chemiluminescence using luciferin–luciferase [Patane et al., 2002]. To measure cellular ATP, MIN6 cells were cultured in white-walled 96-well microtiter plates at a density of 2×10^4 cells/well and pre-equilibrated by incubation (2 h, 37°C) in medium in the absence of glucose, followed by incubation (1 h, 37°C) in the presence or absence of a stimulatory concentration of glucose (2.8 or 30 mM). Cells were lysed with 100 mM glycine buffer (pH 7.4) containing 0.05% Triton X-100 (10^6 cells/100 μl). After brief centrifugation, 80 μl of the supernatant was added to 100 μl of luciferin–luciferase solution (10 mg/ml), and the chemiluminescence was read by Luminometer.

Evaluation of the ATP/ADP Ratio in Islets

The ATP/ADP ratio in islets was determined by high-pressure liquid chromatography (HPLC). Groups of 50 islets were incubated in experimental medium for 72 h. Then 250 μl of 0.6 M $HClO_4$ with 25 mM of bis(2-aminoethyl)ethyleneglycoltetraacetic acid (EGTA) was added to islets cultured in 10 cm plates. To obtain a protein-free sample, the mixture was vortexed for 5 min, and centrifuged for 2 min at 10,000*g* in an Eppendorf centrifuge at 4°C. The supernatant was neutralized with 3 M KOH

and then centrifuged at 10,000*g* for another 2 min. All procedures were conducted at 4°C. The adenine nucleotides were separated by reverse phase HPLC in an Agilent 1100 chromatograph with a model quadruple pump and a DAD detector. The detection wavelength was 254 nm, using a Zorbax RP C-18 column (5 μM) made by Agilent. The protocol used consisted of isocratic elution with potassium phosphate buffer (100 mM; pH 5.5). The flow rate was 0.8 ml/min for 15 min. The levels of ATP and ADP were quantified and the ratio was calculated.

Real-Time PCR

MIN6 cells were washed twice with ice-cold PBS after pretreatment with LA and/or ALC and treatment with OA. Total RNA was isolated using the single-step TRI reagent and 1 μg RNA was reverse transcribed into cDNA. In brief, the isolated RNA was dissolved in sterile water and 2.5 mM Mg^{2+}, 1 mM dNTPs, 0.5 μg oligodT_{15}, 25 U AMV reverse transcriptase, 10 × RT buffer, giving a final volume of 20 μl. The sample was incubated at 25°C (10 min), 42°C (60 min), and 99°C (5 min). All Real-Time PCR quantifications were performed using the BioRad iCycler iQ system (BioRad, Hercules, CA) and expressed relative to β-actin mRNA levels in the same samples. Primers for mouse UCP-2 and β-actin mRNAs were as follows: UCP-2 sense 5′-GTC GGA GAT ACC AGA GCA CT-3′; UCP-2 antisense 5′-GTG ACC TGC GCT GTG GTA CT-3′; β-actin sense 5′-ACG GCC AAG TCA TCA CTA TTG-3′; and β-actin antisense 5′-AGC CAC CGA TCC ACA CAG A-3′. The predicted sizes of the UCP-2 and β-actin PCR products were 308 and 300 bp, respectively. The real time PCR conditions were 2 min at 50°C, 10 min at 95°C and then 40 cycles for 15 s at 95°C and 1 min at 53°C. Serial dilutions of the template were prepared to verify that detection occurred in the linear range of amplification. Standard curves were generated for each primer set using untreated MIN6 RNA as control; cycle threshold values were plotted as a function of starting reverse transcribed RNA levels, the slope of the resulting line was used to calculate expression of the target gene. The linear regression formula is $Y = -3.421 \times \text{Log}(X) + 36.66$, $R^2 = 0.9998$. UCP-2 mRNA levels were corrected for β-actin mRNA levels. There were no differences in β-actin levels between treatments.

Western Blot Analysis

After pretreatment with LA and/or ALC and treatment with OA, MIN6 cells were washed twice with ice-cold PBS, lysed in sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 5 mM DTT) at room temperature and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000 rpm, 10 min at 4°C). Protein concentration was determined using the BioRad DC protein assay. The soluble lysates (20 μg per lane) were subjected to 12% SDS-PAGE; after electrophoresis, the protein was transferred onto nitrocellulose membranes. The membranes were treated with UCP-2 polyclonal antibody (diluted 1:2000) directed toward the C-terminal domain of UCP-2, followed by incubation with a secondary antibody (goat antirabbit Ig conjugated to horseradish peroxidase, diluted 1:5,000). Western blots were developed using ECL (Amersham Biosciences), quantified by scanning densitometry (BioRad), and normalized by β-actin content in parallel samples.

Assessment of Mitochondrial Membrane Potential (MMP)

JC-1 was used to qualitatively determine alterations in MMP. Mitochondrial depolarization was indicated by a decrease in the red to green fluorescence intensity ratio. MIN6 cells were cultured for 72 h with OA, preincubated for 6 h with or without different concentrations of ALC and LA. After aspiration of medium and washing, cells were detached by treating with 0.05% EDTA (in PBS) and resuspended in Hanks' solution, followed by incubation with JC-1 (10 μg/ml) at 37°C for 10 min. The dispersed cells were resuspended in KRBH containing 5 mM glucose, and aliquots (100 μl) of the cell suspensions were applied to a 96-well plate. Measurements were made using a fluorescence spectrometer with excitation wavelength at 490 nm and emission wavelengths at 530 and 590 nm [Carlsson et al., 1999].

Statistical Analysis

All quantitative data are representative of at least three independent experiments. Data are presented as means ± SEM. Statistical significance was determined by using one-way ANOVA with Bonferroni's post hoc tests between groups. The criterion for significance was set at $P < 0.05$.

RESULTS

Cell Viability

Free fatty acids or protective agents may cause changes in cell viability, which is relevant to the pro-apoptotic effects of FFAs on β-cells. Therefore, it is necessary to control cell viability in the absence of treatments with OA, LA, and/or ALC. We have chosen OA, not palmitic, because OA is well incorporated into triglycerides and well tolerated whereas palmitic acid is poorly incorporated into triglyceride and causes apoptosis in non-adipose cells [Listenberger et al., 2003]. Using the MTT assay, it was found that no obvious changes in cell viability were caused by the various treatments with OA, LA, and/or ALC in the presence of 11.1 mM glucose in DMEM, that is, pretreatments with LA and/or ALC for 6 h followed by treatment with or without 0.4 mM OA for 72 h (data not shown).

Insulin Secretion

MIN6 cells in the presence of 2.8 mM glucose did not exhibit a significant change in insulin secretion. At high glucose levels (30 mM), insulin secretion was increased 3.4-fold compared to that at 2.8 mM glucose; incubation with 0.4 mM OA for 72 h significantly decreased glucose-induced insulin secretion (Fig. 1a). Pretreatment with LA (10 μM) prevented the OA-induced decrease in insulin secretion ($P < 0.05$ vs. OA-stimulation), whereas ALC did not show any effect. Pretreatment with the combination of LA and ALC provided further protection against OA-induced decreases in insulin secretion ($P < 0.01$ vs. OA-stimulation; Fig. 1a).

Insulin secretion responds similarly in isolated rat islets. As shown in Figure 1b, high glucose (30 mM) caused an increase of 4.5-fold compared to that of 2.8 mM glucose, and incubation with 0.4 mM OA for 72 h significantly decreased glucose-induced insulin secretion. Although the pretreatments with LA or ALC individually only showed a small trend toward preventing the OA-induced decrease in insulin secretion, pretreatment with the combination of LA (10 μM) and ALC (10 μM) significantly prevented the OA-induced decrease in insulin secretion (Fig. 1b) as is the case in MIN6 cells (Fig. 1a).

Control experiments were performed to measure total cellular insulin content in control β-cells and cells exposed to the various conditions of LA and ALC alone; no significant differences in insulin content were found (data not shown).

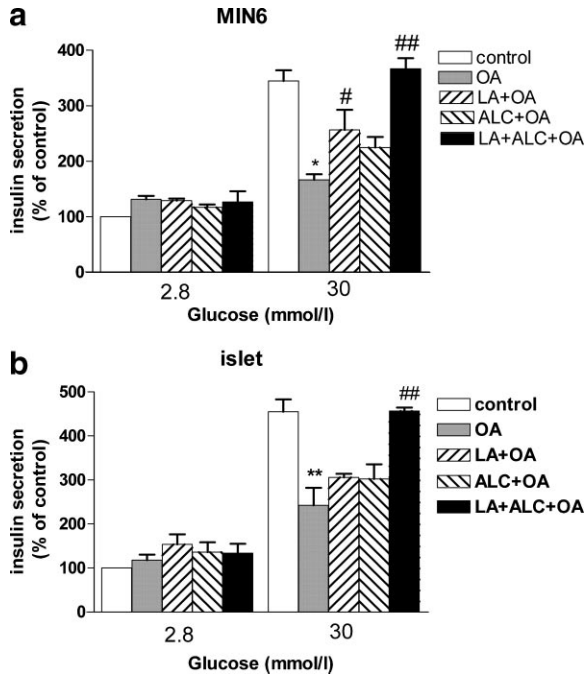

Fig. 1. Effects of OA and/or LA, ALC treatments on insulin secretion in MIN6 cells (**a**) and isolated rat islets (**b**). Cells were preincubated with LA (10 μM) and/or ALC (10 μM) for 6 h, treated with OA (0.4 mM) for 72 h in the presence of LA and/or ALC, and then insulin secretion was measured. Results are mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. OA.

Glucose-Induced ATP Generation

At low glucose levels (2.8 mM, 1 h), neither OA nor pretreatment with LA or/and ALC resulted in changes in ATP content in MIN6 cells. High levels of glucose (30 mM, 1 h) resulted in an increase in ATP content of control MIN6 cells (2.30 ± 0.36 and 4.10 ± 0.19 nmol/mg protein). OA treatment inhibited the glucose-stimulated ATP increase in MIN6 cells (Fig. 2a). Pretreatment with LA (10 μM) or ALC (10 μM) significantly elevated ATP in OA-treated MIN6 cells, and pretreatment with the combination of LA and ALC similarly restored the ATP level but did not show an additive effect (Fig. 2a).

The effects of OA and LA and/or ALC on ATP have also been examined in isolated rat islets by calculating the ratio between ATP and ADP. This ratio is considered more important for glucose-stimulated insulin secretion than the level of ATP. Similar to the effect on ATP content in MIN6 cells, high levels of glucose

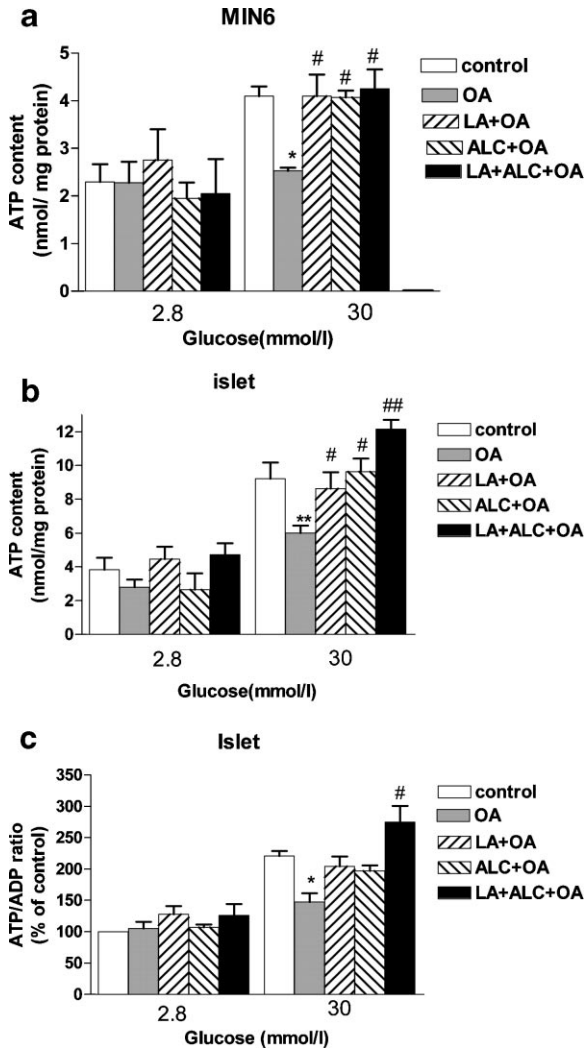

Fig. 2. Glucose-induced ATP production in MIN6 cells (**a**) and ATP content (**b**) and ATP/ADP ratios in isolated rat islets (**c**). Cells were preincubated with LA (10 μM) or/and ALC (10 μM) for 6 h, treated with OA (0.4 mM) for 72 h in the presence of LA and/or ALC. Values are the mean ± SEM of three independent experiments performed in duplicate. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. OA; $^{\#\#}P < 0.01$ vs. OA.

(30 mM, 1 h) resulted in an increase in ATP content of control islet cells (3.83 ± 0.70 and 9.21 ± 0.96 nmol/mg protein) and a twofold increase in the ATP/ADP ratio in islets, and OA treatment significantly inhibited the increase in ATP content and ATP/ADP ratio. Pretreatment with LA (10 μM) or ALC (10 μM) prevented the OA-induced decrease in ATP content ($P < 0.05$ vs. OA-stimulation), pretreatment with the combination of LA and ALC provided further protection against OA-induced decreases in ATP content ($P < 0.01$ vs. OA-stimulation; Fig. 2b).

LA and/or ALC pretreatments showed trend on preventing OA-induced reduction of ATP/ADP ratio, but only pretreatment with the combination of LA and ALC showed a significant protection against the OA-induced ATP/ADP reduction (Fig. 2c).

UCP-2 Expression

Exposure of the MIN6 cells to OA (0.2–0.8 mM) for 72 h stimulated UCP-2 protein expression, detected by immunoblot as a 32 kDa band. Maximum stimulation was reached at 0.4 mM OA (Fig. 3a). Pretreatment with LA showed a trend toward decreasing OA-induced UCP-2 protein levels, but ALC at 100 μM inhibited OA-induced UCP-2 protein levels. Nutrient combination pretreatment also significantly decreased the UCP-2 protein level dose-dependently (Fig. 3b).

mRNA of UCP-2 expression was examined using real time PCR techniques. Exposure of the MIN6 cells to OA (0.2–0.8 mM) for 72 h stimulated UCP-2 mRNA expression, and the maximum stimulation was reached at 0.4 mM OA (305 ± 22%, $P < 0.05$ OA vs. BSA; Fig. 3c), similar to the results of UCP-2 protein expression. Pretreatment with either LA or ALC significantly decreased OA-induced stimulation of UCP-2 mRNA expression: by 117 ± 9.8% (LA 10 μM) and by 77.5 ± 20% (ALC 10 μM), respectively ($P < 0.05$ vs. OA; Fig. 3d). However, the combination of LA (10 μM) and ALC (10 μM) did not lead to an inhibition in UCP-2 mRNA expression after OA treatment.

Mitochondrial Membrane Potential

Long term (72 h) exposure to OA moderately decreased MMP (Fig. 4a). As shown in Figure 4b, OA produced an inhibitory effect on membrane potential over the concentration range from 0.2 to 0.8 mM. The protective effects of LA or/and ALC, measured at 0.4 mM OA for 72 h, induced a 40% decrease in JC-1 fluorescence in the presence of 11.1 mM glucose ($P < 0.01$ for OA vs. BSA). LA prevented OA-induced decreases in MMP at 100 μM, whereas ALC was effective at both 10 and 100 μM. The combination of LA and ALC also showed significant protection against the OA-induced decrease in MMP at both 10 and 100 μM (Fig. 4c).

ROS Production

Exposure of MIN6 cells to increasing concentrations of OA (0.2–0.8 mM) for 72 h increased

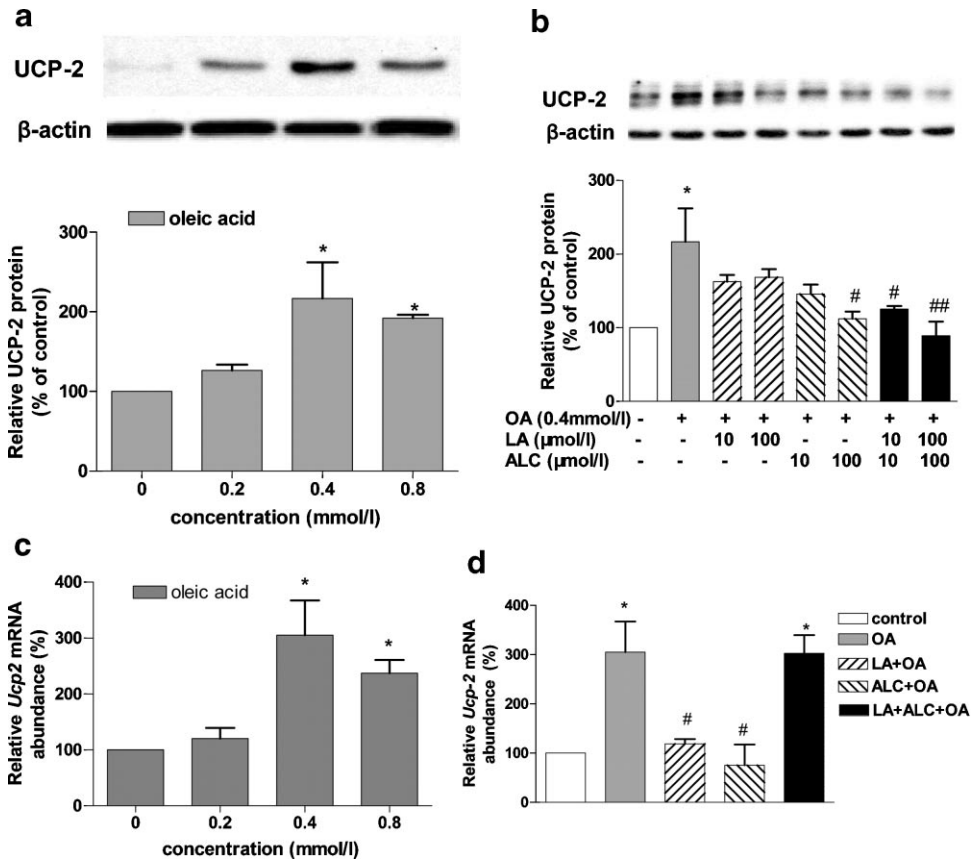

Fig. 3. UCP-2 protein and mRNA expression in MIN6 cells. **a**: UCP-2 protein expression of MIN6 cells exposed to various concentrations of OA for 72 h. **b**: Representative Western blot image (**upper**) and quantification (**lower**) of UCP-2 protein expression of MIN6 cells preincubated with LA or/and ALC for 6 h, then exposed to OA (0.4 mM) for 72 h in the presence of LA and/or ALC. Values are mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. OA. **c**: UCP-2 mRNA expression of MIN6 cells treated with various concentrations of OA for 72 h. Values are mean ± SEM (n = 4). $^{*}P < 0.05$ vs. control. **d**: UCP-2 mRNA expression of MIN6 cells preincubated with LA (10 μM) and/or ALC (10 μM) for 6 h then exposed to 0.4 mM OA for 72 h. Values are mean ± SEM of four independent experiments. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. OA.

ROS production in a concentration-dependent manner (Fig. 5a, b). The effects of LA or/and ALC were examined at 0.4 mM OA for 72 h; this concentration of OA caused more than a 100% increase in ROS production. Concentrations of LA from 1 to 100 μM inhibited the OA-induced ROS increase, whereas ALC did not show inhibition (Fig. 5c). Combinations of LA and ALC were as inhibitory as LA alone over the range of concentrations used (1–100 μM; Fig. 5c).

DISCUSSION

The Glucolipotoxicity Hypothesis

The glucolipotoxicity hypothesis suggests that elevated FFAs together with hyperglycemia are synergistic in causing β-cell damage possibly due to the fact that high glucose inhibits fat oxidation and consequently lipid detoxification [El-Assaad et al., 2003]. However, the underlying mechanisms are unknown [Ravnskjaer et al., 2005]. Several mechanisms are suggested, including the mediation by peroxisome proliferator-activated receptor (PPAR) family via induction of UCP-2 [Lameloise et al., 2001; Ravnskjaer et al., 2005], mediation via formation of ceramide and activation of the mitochondrial apoptotic pathway [Maedler et al., 2003], and the involvement of mitochondrial dysfunction characterized by increased superoxide production and reduced MMP [Maestre et al., 2003]. The phenomenon of fatty acid-induced mitochondrial oxidant stress and impairment of antioxidant response is also evident in proximal tubular cells [Ishola et al., 2006]. However, some studies showed that cytotoxicity of FFAs to normal rat islet cells

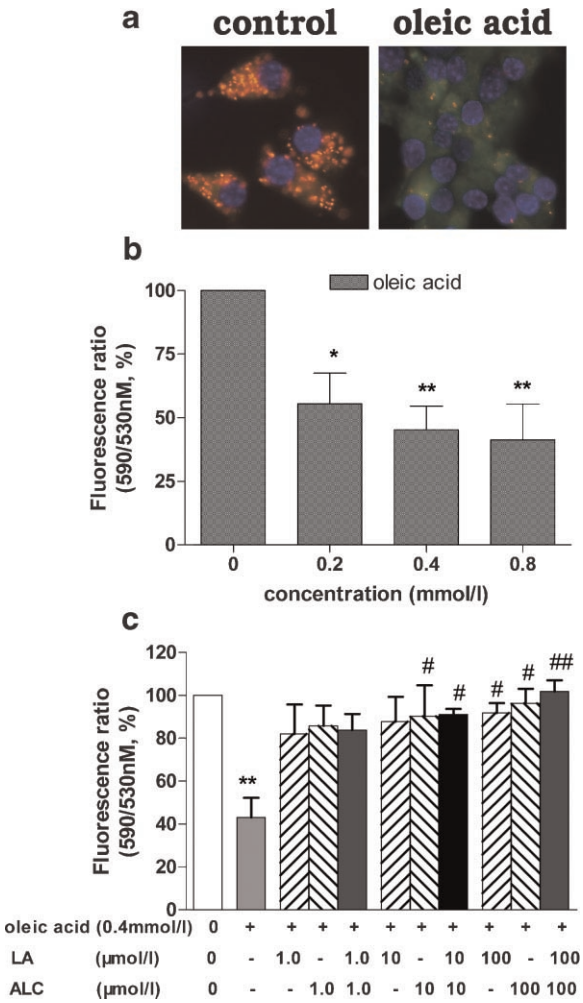

Fig. 4. Changes in mitochondrial membrane potential in MIN6 cells. **a**: Microscopic photographs of MIN6 cells treated with or without OA for 72 h and stained with JC-1 for membrane potential and DAPI for nuclei. **b**: Quantification of membrane potential (the ratio of FL1:590 nm/FL2: 530 nm) of MIN6 cells treated with various concentrations of OA for 72 h. **c**: Quantification of membrane potential of MIN6 cells pretreated with LA or/and ALC for 6 h and then exposed to OA (0.4 mM) for 72 h in the presence of LA and/or ALC. Results are mean ± SEM of three independent experiments performed in duplicate.*$P < 0.05$ vs. control, **$P < 0.01$ vs. control; and $^{\#}P < 0.05$ vs. OA, $^{\#\#}P < 0.01$ vs. OA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

was not suppressed by etomoxir (an inhibitor of mitochondrial carnitine palmitoyltransferase I) or by antioxidants and was not associated with inducible nitric oxide synthase expression or nitric oxide formation, but showed an inverse relationship to cellular triglyceride accumulation [Maedler et al., 2003]. Recently, mitochondrial dysfunction resulting from chronic treatment with OA has been examined [Frigerio et al., 2006]. In the present study, we have

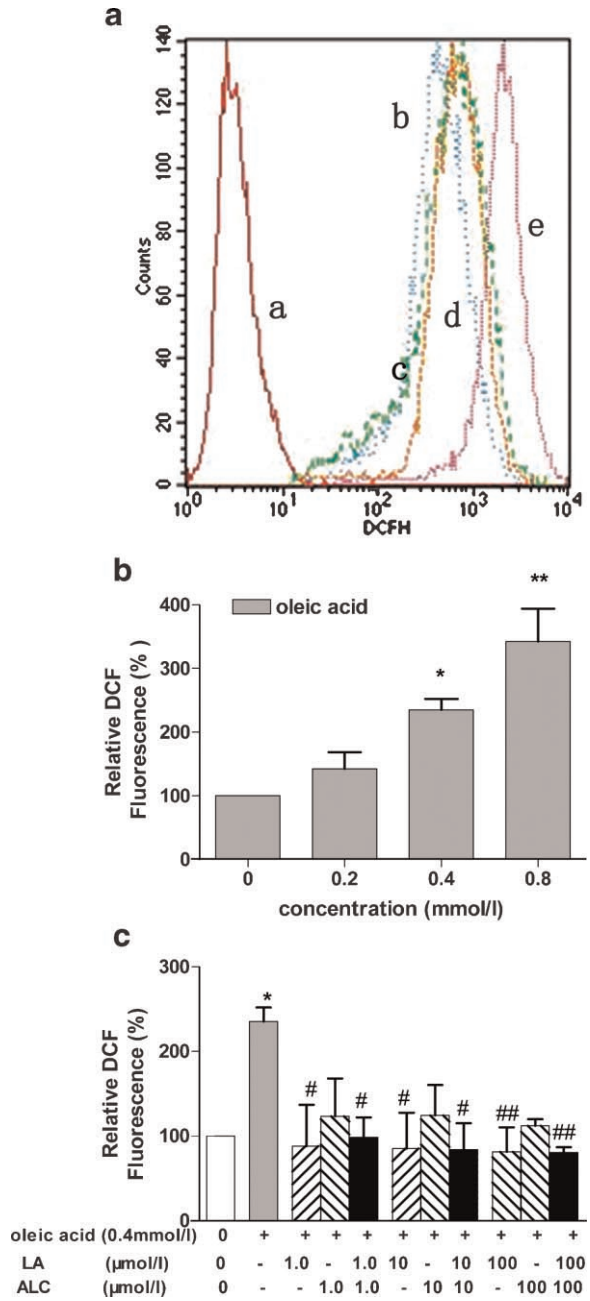

Fig. 5. Generation of ROS in MIN 6 cells. **a**: Flow cytometric assay for ROS levels in MIN6 cells exposed to various concentrations of OA for 72 h and stained with H_2DCF-DA. The rightward shift in the OA curve represents an increase in ROS generation. a, negative control; b, control (without treatment); c, 0.2 mM OA; d, 0.4 mM OA; e, 0.8 mM OA. **b**: Quantification of ROS levels of MIN6 cells treated with OA. Values are mean ± SEM of five independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. control without OA treatment. **c**: Quantification of ROS levels of MIN6 cells pretreated with LA and/or ALC for 6 h then exposed OA (0.4 mM) for 72 h in the presence of LA and/or ALC. Values are mean ± SEM of four independent experiments. *$P < 0.05$ vs. control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. OA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shown that chronic exposure of pancreatic β-cells to sublethal OA levels causes a suppression of glucose-stimulated insulin secretion accompanied by an increase in intracellular oxidant formation, a decrease in MMP, enhancement of UCP-2 protein expression and decreased glucose-induced ATP production. Results suggest that mitochondrial dysfunction may play a key role in FFA-induced β-cell dysfunction. The protective effect of pretreatment with mitochondrial nutrients LA and/or ALC further supports this suggestion and also suggests that dietary supplementation of these two nutrients may prevent or delay β-cell dysfunction induced by exposure to the combination of high oleate and high glucose.

Inhibition of Insulin Secretion in β-Cells as a Consequence of Mitochondrial Dysfunction

Hyperglycemia, high levels of lipids, and obesity all lead to impairments in glucose-stimulated insulin secretion, resulting in β-cell dysfunction characterizing type 2 diabetes [Lowell and Shulman, 2005]. The underlying mechanisms of hyperglycemia, the role of lipids, and obesity-induced β-cell dysfunction remain unclear. All of these factors may cause mitochondrial dysfunction, including up-regulation of UCP-2 activity and ATP decrease [Lowell and Shulman, 2005]. Our results with both MIN6 cells and isolated rat islets exposed to OA at high glucose levels led to suppression of insulin secretion, which was prevented by pretreatment with LA and/or ALC, suggesting that the inhibition of insulin secretion is a consequence of OA-induced mitochondrial dysfunction. It is intriguing that, as opposed to other studies, oleate treatment did not increase basal insulin release. This might be MIN6 cell-specific as others used INS-1 cells [Segall et al., 1999; Alstrup et al., 2004].

LA and ALC are two nutrients known to protect mitochondrial dysfunction by different mechanisms [Liu and Ames, 2005]. The measurements of the OA-induced decrease in MMP and increase in ROS, and the protective effects of LA and/or ALC on these mitochondrial parameters, further suggest that high levels of FFAs play an important role in causing mitochondrial dysfunction in β-cells.

ROS and ATP Production in β-Cell Dysfunction

ATP production from glucose is a key modulator of glucose-stimulated insulin secretion. Mitochondrial oxidative metabolism has been estimated to produce 98% of β-cell ATP. Direct measurements of cellular ATP content or ATP/ADP ratio confirmed that a defect exists in glucose-induced ATP generation in the OA-treated MIN6 cells and isolated rat islets, and that pretreatment with mitochondrial nutrients LA or/and ALC inhibited the ATP decrease in both MIN6 cells and islets. These results suggest that insulin secretion suppression may result from mitochondrial dysfunction.

Oxidative stress is an important component of disorders related to high levels of FFA, and ROS are thought to significantly contribute to defective β-cell function in type 2 diabetes. The mitochondrial respiratory chain is the most significant generator of ROS, hence strategies to decrease mitochondrial free radical production and oxidative damage may have therapeutic potential [Poitout and Robertson, 2002; Lowell and Shulman, 2005]. Previous experiments suggested that the mitochondrial respiratory chain is the principal source of ROS in MIN6 cells [Koshkin et al., 2003]. A radical explanation for glucose-induced β-cell dysfunction is that hyperglycemia-induced mitochondrial oxidant production activates UCP-2, which decreases the ATP/ADP ratio and thus reduces the insulin-secretory response [Brownlee, 2003]. In our experiments, we showed clearly that chronic exposure of β-cells to OA resulted in increased ROS production, which is responsible for the mitochondrial dysfunction characterized by a decrease in membrane potential, decreased ATP production, and consequent decrease in insulin secretion in β-cells. We should emphasize that the role of ROS in β-cells is simplified here because: (1) as in other situations such as stress [Liu and Mori, 1999], the role of ROS is very complex, especially so because a delicate balance of oxidants and antioxidants must be kept in order to maintain optimal β-cell function, and (2) it is hard to know whether ROS production causes mitochondrial dysfunction or mitochondrial dysfunction causes ROS generation, or they both stimulate each other to form a vicious cycle.

UCP-2 Regulation in β-Cell Dysfunction

By catalyzing proton leak, UCPs are involved in regulating cellular ATP production and are localized in the inner mitochondrial membrane [Chan et al., 2001]. The uncoupling activity of UCP-2 is physiologically important for β-cell

defense against oxidants [Chan et al., 2004]. Fatty acid oxidation possibly enhances UCP-2 expression in response to increased oxidative stress. However, the theory about UCP-2 and its role in β-cells is highly controversial in the case of pancreatic β-cells. Enhanced UCP-2 expression could also decrease glucose-induced ATP production by uncoupling oxidative phosphorylation [Lamson and Plaza, 2002; Krauss et al., 2003]. Further, overexpression of UCP-2 in β-cells decreases glucose-stimulated insulin secretion [Chan et al., 1999]. In parallel, mice lacking UCP-2 secrete more insulin and recover glucose-stimulated insulin secretion [Joseph et al., 2002]; thus, UCP-2 exerts a substantial negative control over glucose-stimulated insulin secretion. However, results obtained in transgenic mice and in a β-cell line do not support this hypothesis [Nicholls, 2006; Produit-Zengaffinen et al., 2007]. Our results with MIN6 cells clearly demonstrate that OA stimulates expression of both mRNA and protein levels of UCP-2 and pretreatment with a combination of LA and ALC at both 10 and 100 μM concentrations effectively inhibited the OA-stimulated overexpression of UCP-2 protein. It is interesting to note that both LA and ALC also inhibited OA-induced overexpression of UCP-2 mRNA, but the combination of LA and ALC, although it also inhibited UCP-2 protein overexpression, did not affect the overexpression of UCP-2 mRNA. The underlying mechanism is unknown. It suggests that the combination may cause an interaction leading to a different inhibition mechanism from that of LA and ALC individually: LA or ALC may work at the transcription level while the combination may work at the translation level. The underlying mechanism requires further study.

Mechanism of the Protective Effects of LA and ALC

LA and ALC have been considered as mitochondrial nutrients that protect mitochondrial dysfunction by different mechanisms, such as (1) preventing oxidant production or scavenging free radicals, processes that prevent oxidative stress in mitochondria; (2) enhancing mitochondrial antioxidant defenses; (3) enhancing mitochondrial metabolism to facilitate both the repair of less damaged and the degradation of more damaged mitochondria, and (4) protecting mitochondrial enzymes and/or stimulating enzyme activity as enzyme substrates and cofactors [Liu and Ames, 2005]. The beneficial effects of supplementation with LA and ALC that tend to ameliorate FFA-related insulin secretion in β-cells may be attributed to their ability to act either as direct mitochondrial antioxidants, phase 2 antioxidant enzyme inducers, energy enhancers, or as enzyme cofactors [Liu and Ames, 2005]. In apparent contradiction, Targonsky et al. [2006] showed that, after either acute or chronic treatment, LA both inhibited glucose-stimulated insulin secretion and increased ROS production in MIN6 cells. However, the concentrations of LA used in Targonsky's experiments (mM) are much higher than those used in our experiments (μM). Similar pro-oxidant effects of LA at high concentrations have also been observed in our experiments (data not shown).

We should point out that the purpose of the present study was to show that high levels of FFAs may cause mitochondrial dysfunction as a general mechanism in β-cell dysfunction. Although we only selected one FFA, OA, due to its lower toxicity [El-Assaad et al., 2003] and reduced ability to cause apoptosis [Listenberger et al., 2003] when compared to palmitic acid, other fatty acids, such as palmitic acid, may show similar effects on mitochondrial function in β-cells, but possibly at different concentrations or treatment lengths. For example, palmitate is more potent than OA in causing a number of indices of β-cell dysfunction, such as the induction of apoptosis in pancreatic cells and other non-adipose cells [El-Assaad et al., 2003; Listenberger et al., 2003], the alteration in gene expression in MIN6 cells [Busch et al., 2002], the decreased survival of isolated normal rat β-cells [Cnop et al., 2001] and of rat insulinoma INS-1 cells [Higa et al., 2006], and the induction of endoplasmic reticulum stress in INS-1 pancreatic β-cells [Karaskov et al., 2006]. On the other hand, we should also be aware that saturated palmitic acid and monounsaturated OA may function differently in β-cells. One study shows that both monounsaturated palmitoleic acid and OA prevent the deleterious effects of palmitic acid and high glucose on human pancreatic β-cell turnover and function [Maedler et al., 2003]. Therefore, the effects of sublethal doses of palmitic acid on mitochondrial dysfunction in β-cell dysfunction and protection by mitochondrial nutrients warrant further study.

CONCLUSION

The present study clearly demonstrates that high levels of FFAs, such as OA, cause increases in ROS generation, mitochondrial dysfunction (decreases in MMP, increases in UCP-2 protein, and decreases in ATP), and consequently, decreases in insulin secretion; and that mitochondrial protective nutrients, such as LA and ALC, effectively protect β-cells from FFA-induced mitochondrial dysfunction and from decreases in glucose-stimulated insulin secretion. These results suggest that supplementation with mitochondrial nutrients might be an effective strategy in preventing β-cell function.

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