Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes

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\section*{A B S T R A C T}
Hyperglycemia and high free fatty acids (FFAs) are two well-known characteristics of type 2 diabetes, and are also implicated in the etiology of insulin resistance. However, their roles in mitochondrial dysfunction of white adipocytes are not well-studied. In this study, we investigated the effects of high glucose (25 mM), high free fatty acids (FFAs, 1 mM), or a combination of both on mitochondrial dysfunction in differentiated 3T3-L1 adipocytes after 48 h of treatment. We found that high glucose, high FFAs, or high glucose + high FFAs reduced insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes. Additionally, the mitochondria became smaller and more compact. Levels of the mitofusion protein mfn1 decreased and levels of the mitofission protein Drp1 increased as compared to controls. NRF1 was downregulated, and PGC-1\textbeta levels were diminished in the high glucose and high glucose + high FFAs conditions. Levels of PGC-1\textalpha and mtTFA mRNA were greatly downregulated. No difference was found in the mitochondrial DNA (mtDNA) and intracellular ATP levels of treated cells compared to controls. The results of this study demonstrate that high-glucose and high FFAs can regulate insulin sensitivity, and mitochondrial dysfunction may occur in this process.

\section*{1. Introduction}
As a consequence of the increased prevalence of obesity, related problems such as type 2 diabetes have become a global health threat. Although the pathogenesis of type 2 diabetes is unclear, it is obvious that insulin resistance in target tissues and organs plays a role in the development of the condition, which is commonly accompanied by hyperglycemia and hyperlipidemia. Adipose tissue is classically viewed as an inert storage depot for excess calories, while metabolism of glucose and free fatty acids (FFAs) occurs in the mitochondria. There is growing evidence that mitochondrial dysfunction plays a crucial role in the pathogenesis of insulin resistance. Many studies (Mogensen et al., 2007; Mootha et al., 2003; Patti et al., 2003; Hammarstedt et al., 2003; Semple et al., 2004; Szendroedi et al., 2009) report pronounced mitochondrial dysfunction in myocytes and adipocytes in patients with insulin resistance or type 2 diabetes, in subjects with a positive family history of diabetes, and in animals with obesity-associated type 2 diabetes (Choo et al., 2006). However, these investigations utilized severely insulin-resistant models, and given the complexity of the pathophysiological process, it is difficult to ascertain the relationship among hyperglycemia, hyperlipidemia, mitochondria dysfunction and insulin resistance. The results of Sutherland et al. (2008) demonstrated that high-fat diet-induced reductions in adipose tissue mitochondrial content occurred after the development of impaired glucose homeostasis (Sutherland et al., 2008). Additionally, TNF-\textalpha impairs mitochondrial biogenesis and function in different tissues of obese rodents by downregulating ENOS expression (Valerio et al., 2006). Thus, reductions in adipose tissue mitochondrial proteins are most likely not a causal event in the development of insulin resistance.

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The aim of this study was to determine whether treatment of adipocytes with high glucose and/or high FFAs affects the following: (1) insulin sensitivity of adipocytes, mitochondrial morphology and mitochondrial DNA (mtDNA) copy number; (2) expression of genes and proteins involved in mitochondrial biogenesis; and (3) intracellular reactive oxygen species (ROS) levels, ATP levels, intramitochondrial Ca\(^{2+}\) concentration, and the mitochondrial membrane potential.

We showed that high glucose and high FFAs induced obvious insulin resistance and mitochondrial dysfunction in differentiated 3T3-L1 adipocytes. During this treatment, mitochondrial morphology and biogenesis, but not mtDNA copy number, were altered. Increased levels of intracellular ROS and the loss of the mitochondrial membrane potential (\(\Delta \Psi\)) may have contributed to mitochondrial dysfunction.

2. Materials and methods

2.1. Antibodies

Primary rabbit polyclonal nuclear respiratory factor-1 (NRF1) antibody and mfn2 antibody were purchased from Abcam (MA, USA). Rabbit polyclonal antibodies to PGC-1\(\beta\) and Drp1, mfn1, SIRT1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The \(\beta\)-Actin antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated AffiniPure goat anti-rabbit secondary antibodies were from Zhongshan Gold Bridge Biotechnology (Beijing, China). Oleic acid (O1383), linoleic acid (L11012), arachidonic acid (A3555), myristic acid (M3128), and lauric acid (W264108) were purchased from Sigma (St. Louis, MO, USA). The high FFAs solution was prepared according to a previously published method (Subauste and Burant, 2007). All of the above fatty acids, dissolved in 2% (v/v) fatty acid-free bovine serum albumin (BSA) with a stock concentration of 100 mM or an equivalent volume of vehicle, were diluted 1:100 in DMEM to a final concentration of 1 mM.

2.2. Cell culture and differentiation

3T3-L1 preadipocytes were cultured as follows. Two days after complete confluence (day 0), the cells were cultured for 48 h in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 0.5 mM/L 1-methyl-3-isobutylxanthine (Sigma, St. Louis, MO, USA), 1 \(\mu\)g/mL dexamethasone (Sigma, St. Louis, MO, USA), and 10 \(\mu\)g/mL insulin (Sigma, St. Louis, MO, USA). From day 2 to day 4, the medium was supplemented with 100 mM/L 1-methyl-3-isobutylxanthine, 1 \(\mu\)g/mL dexamethasone, and 10 \(\mu\)g/mL insulin only. The cells were then transferred to DMEM containing 10% FBS for the remaining culture period. The cultures were replenished every 2 days. After induction for 10 days, more than 90% of the cells exhibited typical adipocyte morphology. We treated adipocytes with low glucose (5 mM) in DMEM (control group), high FFAs (1 mM; FFAs group), high glucose (25 mM; high glucose group), or high glucose along with high FFAs (high glucose + high FFAs group). After 48 h of incubation, the adipocytes were collected for the next experiment.

2.3. Glucose uptake

2-Deoxy-d-[\(\beta\)-3H] glucose (CIC, Beijing, China) uptake was assayed as described previously but with minor modifications (Cedda et al., 2005). The cells were cultured in six-well plates and induced into differentiated adipocytes. After treatment with high glucose and high FFAs for 48 h, the cells were serum starved for 3 h in DMEM containing 0.5% FBS. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated in KRP-HEPES buffer (30 mM/L HEPES (pH 7.4), 10 mM/L NaHCO\(_3\), 120 mM/L NaCl, 4 mM/L KH\(_2\)PO\(_4\), 1 mM/L MgSO\(_4\), and 1 mM/L CaCl\(_2\) in the presence or absence of 100 mM/L insulin for 30 min at 37\(^\circ\)C. Labeled 2-deoxy-d-[\(\beta\)-3H] glucose was added to a final concentration of 2 \(\mu\)Ci/mL. After 10 min at 37\(^\circ\)C, the reaction was terminated by washing 3 times with ice-cold PBS supplemented with 10 mM/L L-glucose. The cells were solubilized by adding 200 \(\mu\)L of 1 mM/L NaOH to each well, and aliquots of the lysate were transferred to scintillation vials for radioactivity counting. The remainder of the lysate was used for the protein assay by the bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA), and the radioactivity was normalized by protein concentration.

2.4. Electromicrograph morphometry

The adipocytes were collected after trypsin digestion and fixed in a mixture of 2.5% glutaraldehyde, 1.25% paraformaldehyde, and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were then washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 h, washed in water, and stained in 1% aqueous uranyl acetate for 30 min, followed by dehydration in different concentrations of alcohol (5 min in 70% alcohol, 5 min in 90% alcohol, and 5 min in 100% alcohol). The samples were then infiltrated and embedded in TAAB Epon (Marivac Canada Inc., St. Laurent, Canada). Ultrathin sections (about 60 nm) were cut on a Reichert Ultracut-S microtome, placed onto copper grids stained with uranyl acetate and lead citrate, and examined in a JEOL 1200EX.

2.5. Western blot analysis

Treated cells were washed with ice-cold PBS and lysed in protein lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 200 mM sodium fluoride, 4 mM sodium orthovanadate-containing protease inhibitors, pH 7.5) for 1 h on ice. Proteins were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer’s instructions. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS (50 mM Tris, pH 7.5, 0.15% NaCl, 0.05% Tween-20). The membranes were incubated at 4\(^\circ\)C overnight in 5% BSA in TBS containing primary antibodies to one of the following: Drp1 1:200, mfn1 1:1000, mfn2 1:200, SIRT1 (1:250), NRF1 (1:500), PGC-1\(\beta\) (1:200), or \(\beta\)-actin (1:1000). The membrane was washed 5 times with TBS for 5 min each wash. After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit secondary antibodies for 1 h at room temperature, washed with TBS, and developed with the enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA). Western blot results were analyzed by using the Gelpro32 Analyzer (Media Cybernetics, Inc., Silver Spring, MD, USA).

2.6. Real-time PCR of mitochondrial DNA

Relative amounts of nuclear DNA and mtDNA were determined by quantitative real-time PCR as previously described (He et al., 2002). Briefly, DNA was isolated from adipocytes by using a DNA extraction kit (Baitake, Beijing, China), quantified by spectrophotometry at 260 nm. Two primer sets were used for PCR analysis. A 110 bp mtDNA fragment within the CYTB gene was used for quantification of mtDNA. The PCR product was previously cloned into the plasmid pMD-T18 and verified by DNA sequencing. Plasmid standards of known copy number were used to generate a log-linear standard curve, from which the CYTB copy numbers of studied samples could be determined by qRT-PCR. Real-time quantitative PCR was performed on an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA). A 291 bp region of the nuclear gene for 28S was used to normalize results. The plasmid standard curve containing the 28S fragment was used to determine copy numbers of test samples. The ratio of mtDNA to nuclear DNA reflects the concentration of mitochondria per cell. The sequences of primers and Taqman probes (Shengong, Shanghai, China) are shown in Table 1.

2.7. Quantitative real-time PCR

Total RNA from adipocytes was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). First-strand cDNA was generated with random primers using reverse transcription kits (Invitrogen Corp.). Real-time analysis was performed on an ABI 7300 RT-PCR system (Foster City, CA, USA) using the following conditions: an initial denaturation for 10 min at 95\(^\circ\)C, followed by 40 cycles of 15 s denaturation at 95\(^\circ\)C, 30 s annealing at the optimal primer temperature (Table I) and 36 s extension at 72\(^\circ\)C. Each sample was assayed in duplicate in a 25 \(\mu\)L reaction volume containing 1 \(\mu\)L cDNA, 12.5 \(\mu\)L SYBR Green Master mix (Qiagen, Valencia, CA), and 0.2 \(\mu\)L of each primer. Negative controls (no template or selected untranscribed RNA) were run as well to ensure the absence of contamination. Analysis was performed according to the \(\Delta \Delta C\) method using \(\beta\)-actin as the housekeeping gene. Specific primers for each gene were designed to amplify a single product (see Table I), as confirmed by regular PCR and dissociation curve analysis after the real-time PCR run. The primer sequences are available upon request.

2.8. ATP content

The ATP content of the adipocytes was measured with ATP lite-glo, a luciferase-based luminescence assay kit (PerkinElmer, Norwalk, CT, USA). Briefly, treated cells were mixed with the detection reagent for 5 min, and luminescence was measured with the VERITAS Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). The luminescence then was normalized by protein concentration.

2.9. Determination of intracellular reactive oxygen species (ROS) generation

Intracellular ROS generation was assessed using 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (H\(_2\)-DCFDA) as described previously (Park et al., 2005). Cells were washed twice in KRP buffer and then incubated in pre-warmed KRP containing 25 mM glucose and 5 \(\mu\)M CM-DCF at 37\(^\circ\)C. After 30 min, the cells were washed twice with KRP, and fluorescence was immediately measured in a plate reader using fluorescence assisted cell sorting (FACS, excitation at 488 nm, emission at 525 nm), and the images were captured on a fluorescence microscope. The fluorescence intensity represented intracellular ROS levels.
Table 1  
Oligonucleotide sequences for primer/probe sets used in TaqMan analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYTB</td>
<td>TTTTATCTGCATCTGAGTTTAATCCTGT</td>
<td>CCACCTCACCTACATTTATATCCG</td>
<td>AGCAAATTCGTTCACCTCCTTCCTCCAC</td>
</tr>
<tr>
<td>28S</td>
<td>GCGGGGCAACGGTGTCAG</td>
<td>AGCCGTTGAGCTATAACCCACAG</td>
<td>TGGTACGTTCGCCCCATGGCTCT</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>CGGAATTACATATCCACCAG</td>
<td>TGGAGAACGTACAGTGGTGGT</td>
<td>TGGTACGTTCGCCCCATGGCTCT</td>
</tr>
<tr>
<td>mtTFA</td>
<td>GGATATGGCGGCTGCTAAA</td>
<td>TGGTACGTTCGCCCCATGGCTCT</td>
<td>TGGTACGTTCGCCCCATGGCTCT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CCTGAGGCTTITTTCTGAGG</td>
<td>TAGAGGTCTTTACGGTACTCA</td>
<td>TGGTACGTTCGCCCCATGGCTCT</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of high glucose and high FFAs on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Basal and insulin-stimulated glucose uptake was quantified by measuring the radioactivity of 2-deoxy-d-[3H] glucose absorbed by adipocytes. Values represent the means ± S.D. from three independent experiments (n = 3). (1) Control; (2) high FFAs group; (3) high glucose group; (4) high glucose + high FFAs group.

* P < 0.05 vs. insulin-stimulated control. # P < 0.01 vs. insulin-stimulated control.

2.10. Measurement of intramitochondrial Ca2+

Intramitochondrial calcium ([Ca2+]m) in adipocytes was measured using a rhod-2 fluorescence imaging system (Antigny et al., 2009). Adipocytes were first washed several times with PBS. Before [Ca2+]m measurement, adipocytes were incubated in serum-free medium for 2 h and then rinsed with PBS. Adipocytes were loaded with 10 mmol/L rhod-2 acetoxymethyl ester (Molecular Probes, Invitrogen) in the same buffer in the dark for 1 h at 37 °C. The adipocytes were then rinsed three times with PBS to remove extracellular dye and washed three times with pre-warmed PBS. The spectral characteristics of the harvested cells were analyzed by FACS (excitation at 543 nm, emission at 615 nm). The fluorescence intensity reflects the [Ca2+]m.

2.11. Mitochondrial membrane potential (∆Ψ)

Adipocytes were incubated with 150 nM of Mitotracker, a red mitochondria-specific cationic fluorescent dye (Molecular Probes, Invitrogen) which accumulates in mitochondria depending on the ∆Ψ, for 30 min at 37 °C, and then washed 3 times with pre-warmed PBS. Once inside the mitochondria, the dye cannot flow back out irrespective of the mitochondrial membrane potential. The spectral characteristics of the harvested cells were analyzed by FACS (excitation at 579 nm, emission at 644 nm), and the images were captured on a fluorescence microscope. The fluorescence intensity reflects the mitochondrial membrane potential.

2.12. Statistical analysis

All data are expressed as means ± S.D. Statistical analysis was performed using one-way ANOVA with the SPSS 10.0 statistical software package (SPSS Inc., Chicago, IL, USA). The threshold of significance was defined as P < 0.05.

3. Results

3.1. Mitochondria morphology and insulin sensitivity are altered in adipocytes incubated with high glucose and high FFAs

Since high glucose and FFA levels are independent risk factors for type 2 diabetes and are commonly found in patients with this
disease, we tested whether treatment with high glucose and FFAs affected the insulin sensitivity of adipocytes. We incubated differentiated 3T3-L1 adipocytes with 25 mM glucose, a 1 mM solution of a mixture of long chain FFAs (including oleic acid, linoleic acid, arachidonic acid, myristic acid, and lauric acid). The FFA concentration applied here was selected by Abbasi et al. (1998), or both glucose and FFAs together. As expected, these treatments caused a significant decrease in insulin sensitivity as measured by insulin-stimulated glucose uptake. The ratios of insulin-stimulated glucose uptake to basal levels for each treatment group were 4.7 (control), 2.1 (high FFAs), 2.0 (high glucose), and 1.2 (high glucose + high FFAs) (Fig. 1). These data show that high glucose and FFA levels can induce appreciable insulin resistance levels in 3T3-L1 adipocytes. Previous studies have indicated that mitochondrial dysfunction contributes to insulin resistance (Kim et al., 2008). To determine whether the mitochondria play a role in the insulin resistance of adipocytes, we assessed the ultrastructure of mitochondria in adipocytes after treatment with 25 mM glucose, 1 mM FFAs, or glucose + FFAs. Notably, cells incubated with high glucose and/or high FFAs displayed condensed mitochondria with twisted and condensed cristae, which were smaller compared to those in control adipocytes (Fig. 2). The abnormal morphology of these mitochondria suggests that significant cytological changes had occurred during of the treatments with high levels of glucose and/or high FFAs treatments.

3.2. Mitochondrial fusion and fission is impaired and mitochondrial biogenesis is reduced after treatment with high glucose and high FFAs

To further clarify the mechanisms underlying the effects of high glucose and FFA treatment on mitochondrial morphology, we examined the levels of the mitochondrial fusion and fission proteins mfn1, mfn2, and Drp1 (Fig. 3a and a-1). Compared to controls (cells incubated with 5 mM glucose), mfn1 protein levels were considerably reduced in adipocytes treated with high FFAs and high glucose + high FFAs. No difference was observed in adipocytes treated with high glucose. Mfn2 protein levels remained unchanged after all treatments. Levels of the fission protein Drp1 were strikingly elevated upon treatment with high FFAs, but were only slightly increased in adipocytes treated with high glucose and high glucose + high FFAs. Although the proteins impacted the mito-

![Fig. 3.](image-url)

Fig. 3. Effects of high glucose and high FFAs on mitochondrial biogenesis. The protein levels of Drp1, mfn1, mfn2, NRF1, SIRT1 and PGC-1β of differentiated 3T3-L1 were analyzed by Western blot. (a) (1) Control; (2) high FFAs group; (3) high glucose group; (4) high glucose + high FFAs group. (a-1 and a-2) The grey scales were determined by GELPRO32. (b) The expression levels of PGC-1α and mtTFA mRNA was examined by quantitative real-time PCR (n = 6). Values represent the means ± S.D. from three independent experiments. *P < 0.05 vs. control adipocytes. **P < 0.01 vs. control adipocytes.
are representative of those obtained from three independent experiments (FFAs group; (3) high glucose group; (4) high glucose + high FFAs group. The results in gene expression of PGC-1α, were reflected by the ratio of mtDNA to nuclear DNA. (c) The housekeeping gene 28S, detected as internal controls, remained the same (Fig. 4c). These data indicate that mitochondrial biogenesis is greatly altered by treatment with high glucose and FFAs.

3.3. MtDNA and ATP content are not affected by treatment with high glucose and high FFAs

As mitochondrial biogenesis was affected by treatment with high glucose and high FFAs, we next investigated whether these treatments affected mtDNA copy number and ATP production. We found that neither of these properties was altered after treatment with high glucose and high FFAs (Fig. 4a and b), while the housekeeping gene 28S, detected as the internal control, remained the same (Fig. 4c).

3.4. Intracellular ROS levels increased and mitochondrial membrane potential and [Ca^{2+}]_{im} concentration decreased in adipocytes after treatment with high glucose and high FFAs

We next sought to determine whether high FFAs and high glucose affects ROS content, [Ca^{2+}]_{im} concentration, and mitochondrial membrane potential. ROS levels in adipocytes treated with high FFAs and high glucose were much higher than that of control adipocytes, with high glucose + high FFAs treatment producing the largest increase in ROS levels, as indicated by more intense fluorescence signals in the presence of the compound DCFDA (Fig. 5). Conversely, high glucose or high glucose + high FFAs led to a large reduction in [Ca^{2+}]_{im}, while high FFAs alone had no effect (Fig. 6). This reduction in [Ca^{2+}]_{im} might therefore be mediated by high glucose. Mitochondrial membrane potential in adipocytes was greatly decreased upon treatment with high FFAs, high glucose, or high glucose + high FFAs, with the combined treatment condition producing the largest effect (Fig. 7). These results clearly demonstrate that high FFAs and high glucose levels induce insulin resistance accompanied by mitochondrial dysfunction in adipocytes.

4. Discussion

Elevated circulating concentrations of fatty acids and glucose are two well-known characteristics of type 2 diabetes and are also implicated in the etiology of insulin resistance. Insulin resistance is thought to arise from impaired insulin signaling in target tissues and involves the augmentation of phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1 and IRS-2), a reduction of activated PI3-kinase, and an inhibition of insulin-stimulated GLUT4 translocation (Shepherd, 2005; Lim et al., 2006). However, many details of the mechanisms remain unknown. A growing body of evidence has linked mitochondrial dysfunction to insulin resistance and type 2 diabetes (Choo et al., 2006; Duchen, 2004). Mitochondria are energy-producing organelles and are the main sites of lipid and glucose metabolism. Currently, the role of mitochondrial dysfunction in insulin resistance is thought to arise from the overloading of mitochondria by lipid and carbohydrate metabolism in conditions with a surplus of nutrients (Lowell and Shulman, 2005; Parish and Petersen, 2005). Since insulin resistance in white adipose tissue has a direct effect on the body’s energy storage, and because mitochondria in white adipose tissue are the organelles responsible for energy metabolism, we investigated the...
characteristics and causes of mitochondrial dysfunction in insulin-resistant white adipocytes.

We found that adipocytes became insulin resistant after 48 h of incubation with high glucose or high FFAs, conditions that are commonly observed in patients with obesity or diabetes. We then examined mitochondria in conditions with an excess of nutrients, and found that mitochondrial morphology was greatly altered. The presence of high glucose, high FFAs, or both had similar effects on mitochondria morphology, although it is unknown whether these effects arise from common or different pathways. As the matrix and cristae of mitochondria are the main sites for metabolism, it is conceivable that the condensed mitochondria did not contain enough space to maintain normal or excessive metabolic needs. More mitochondria of smaller size were also present. Normally, mitochondria form a network in the cell that allows them to effectively transmit energy and content between remote parts of the cell; small, isolated mitochondria cannot participate in this network (Mijaljica et al., 2007). In addition, mitochondria frequently undergo fusion and fission so that they can effectively exchange power and content. mfn1 and mfn2 are mitochondrial GTPases required for mitochondrial outer membrane fusion in mammals, disruption of mitochondrial morphology required for mitochondrial outer membrane fusion in mammals, disruption of mitochondrial function by knockdown of mfn1 and mfn2 is caused by downregulation of DRP1 (Smirnova et al., 1998). At present, adipocytes treated with high FFAs and high glucose + high FFAs contained less mfn1 protein than control, but high glucose alone had little effect on mfn1.

**Fig. 5.** Effects of high glucose and FFAs on intracellular ROS content. ROS levels were determined by measuring the fluorescence of DCF with FACS (excitation at 488 nm, emission at 530 nm). (a) Fluorescence images of ROS in adipocytes were shown by fluorescence microscope ((a) control; (b) high FFAs group; (c) high glucose group; (d) high glucose + high FFAs group), and by (b) FACS ((1) control; (2) high FFAs group; (3) high glucose group; (4) high glucose + high FFAs group). The results are representative of those obtained from three independent experiments (n=6). Values represent the means ± S.D. *P<0.05 vs. control adipocytes. **Fig. 6.** Effects of high glucose and high FFAs on intramitochondrial Ca²⁺ levels. Measurement of intramitochondrial Ca²⁺ levels were performed using rhod-2 fluorescence probes with FACS ((1) control; (2) high FFAs group; (3) high glucose group; (4) high glucose + high FFAs group). The results are representative of those obtained from three independent experiments (n=6). Values represent the means ± S.D. *P<0.01 vs. control adipocytes.
Fig. 7. Effects of high glucose and high FFAs on mitochondrial membrane potential. Mitochondrial membrane potential was measured using the Mitotracker-red fluorescence probe with FACS. (a) Fluorescence images of the mitochondrial membrane potential in adipocytes were shown by using a fluorescence microscope ((a) control; (b) high FFAs group; (c) high glucose group; (d) high glucose + high FFAs group, 200×), and by (b) FACS ((1) control; (2) high FFAs group; (3) high glucose group; (4) high glucose + high FFAs group). The results are representative of those obtained from three independent experiments (n = 6). Values represent the means ± S.D. *P < 0.05 vs. control adipocytes. #P < 0.01 vs. control adipocytes.

Protein levels. High glucose + high FFAs had the greatest impact on mfn1 expression. Levels of Drp1 increased slightly after treatment with high glucose and high glucose + high FFAs, but was greatly increased after incubation with high FFAs. As the program and regulation of mitochondrial fusion and fission was so complexity, it was difficult to explain the inconsistencies of the proteins regulating mitochondrial fission and fusion, perhaps they played minor roles in regulating mitochondrial morphous here. We believed the disturbances of mitochondrial fusion and fission were important informations but needed further investigation.

White adipocytes express low levels of PGC-1α, but PGC-1β expression increases during adipocyte differentiation (Kamei et al., 2003). Both factors belong to the PGC-1 family and have important roles in mitochondrial biogenesis (Savagner et al., 2003). We found that PGC-1α mRNA (because it is expressed at a low level in white adipocytes, we could not test its protein expression) and PGC-1β protein (tested using a specific commercial antibody) decreased drastically upon treatment with high glucose and high FFAs. We also observed reduced levels of NRF1 protein and mtTFA mRNA, both targets of PGC-1. NRF1 is a transcription factor that stimulates the transcription of many nuclear-encoded mitochondrial genes, such as OXPHOS genes and mtTFA. MtTFA can bind to the D-loop of the mitochondrial genome and promotes transcription of mitochondrial genes and replication of mitochondrial DNA (Vianna et al., 2006). However, the mtDNA content was not altered after high glucose or high FFAs treatment. It is possible that moderate reduction of mtTFA, between 33 and 57%, could keep mtDNA at normal levels in the resting state. Other unknown or undetected factors could also function in white adipose cells to compensate for the disruptive effects of high glucose or high FFAs treatment.

Mitochondria are the main sites for fat and carbohydrate metabolism and are also the major source of ROS, a byproduct of the mitochondrial electron transport chain. ROS can react with multiple cellular components, such as proteins, lipids, and nucleic acids, leading to reversible or irreversible oxidative modifications and organism dysfunction. Adipocytes exposed to high glucose or high FFAs for 48 h displayed elevated ROS content, in contrast to the 18 h observation period in other cell lines. A role for mito-
drial shape changes in determining ROS content has been proposed (Yu et al., 2006). Additionally, short-term treatment with hydrogen peroxide has also been shown to cause a transient increase in ROS and temporary changes in mitochondrial morphology in human endothelial cells (HUVEC) (Jendrach et al., 2008). Here, we showed that treatment with high FFAs affects mitochondrial morphology and biogenesis. However, there are varying reports in the literature as to how insulin resistance or obesity affects mitochondrial bio-
genesis; most papers reported that mtDNA were reduced, but some were elevated (Morino et al., 2006) or remained unaltered (Boushel et al., 2007). In our report, increasing ROS levels was accompanied by decreased PGC-1α levels; however, St-Pierre et al. previously indicated that PGC-1α can be induced by ROS (St-Pierre et al., 2006). This difference may be due to the use of different cells, experimental designs, or test time points (2 h vs. 48 h). Mitochondrial dysfunction was also apparent in the loss of ΔΨ and the decrease in [Ca^{2+}]_m ion concentrations. Our results illustrated that both high glucose and high FFAs had a negative effect on mitochondrial membrane potential, but only high glucose affected [Ca^{2+}]_m ion concentrations. It has been reported that mitochondrial morphology is controlled by the ΔΨ across the inner membrane (Legros et al., 2002; Ishihara et al., 2003). The mitochondria is also a major Ca^{2+} storage organelle and can take up Ca^{2+} from the endoplasmic reticulum (ER). In mito-
chondria, the uptake of Ca^{2+} is driven by ΔΨ and mediated by an electrogenic uniporter, the activity of which is triggered by a rise in intracellular calcium concentration ([Ca^{2+}]_i) and depends on the high inner ΔΨ (Antigny et al., 2009).

Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) is a chemical uncoupler that depolarizes ΔΨ. It was reported that FCCP could inactivate [Ca^{2+}]_m uptake by collapsing of ΔΨ and causing depolarization and Ca^{2+} release from the ER and mitochondria in brown adipocytes, since the mitochondrial Ca^{2+} uniporter (UP) is sensitive to variations of ΔΨ (Masako et al., 2007). Thus the decreased [Ca^{2+}]_m partly resulted from the loss of ΔΨ. Meanwhile, the [Ca^{2+}]_m level in the high FFAs group was higher than that of high glucose groups (high glucose group or high glucose + high FFAs group). The reason for this observation was that the high glucose groups displayed a greater degree of decrease in ΔΨ; only when the ΔΨ reached a threshold value could the electrogenic uniporter be activated. Studies on cultured mammalian cells have shown that formation of a reticular mitochondrial network is important for proper mitochondrial calcium buffering and for propagating [Ca^{2+}]_m waves (Szabadkai et al., 2004; Frieden et al., 2004). Therefore, we believe that treatment with high glucose and high FFAs could deform adipocyte mitochondria and impair Ca^{2+} uptake and buffering. Meanwhile, elevated ROS levels, the disrupted membrane potential, and the alteration in calcium concentration could cause further deformation of the mitochondria, resulting in a vicious cycle. Nevertheless, only treatment with high glucose suppressed [Ca^{2+}]_m levels. Early in 2004, it was reported that hyperglycemic conditions affected the shape of mitochondria in endothelial cells, and that the mitochondrial shape affects [Ca^{2+}]_m homeostasis (Paltauf-Dobrzynska et al., 2004).

In the present study, we showed decreases in ΔΨ but the ATP content was not changed. It was difficult to reconcile these inconsistencies. Abdul-Ghani et al. (2008) reported in isolated mitochondria of muscle that 0.5–2 μM FFA metabolites stimulated ATP synthesis; however, there was a dose–response inhibition of ATP synthesis above a concentration of 5 μM. On the other hand, FFA metabol-
ites decreased ΔΨ, and the inhibitory effect of FFA on ΔΨ was dose-dependent (Abdul-Ghani et al., 2008). In our results, the discrepancy of ATP content and ΔΨ may reflect a different underlying mechanism. In another report, mild mitochondrial uncoupling resulted in a slight decrease in ΔΨ but did not modify the ATP con-
tent (Tejerina et al., 2009). Perhaps some yet unknown factors may regulate ATP synthesis and ΔΨ independently.

In conclusion, the results of this study demonstrated that treatment of adipocytes with both high glucose and high FFAs can induce insulin resistance. During this process, the mitochondria become deformed, the ΔΨ is decreased, mitochondrial calcuium is released, and intracellular ROS are produced. Abnormal mitochondrial biogenesis is also involved in the pathogenesis. We believe that there exists some common pathways of high glucose and/or FFAs to insulin resistance, for example, ROS and mitochondrial function defect, whereas, the inconsistence results among high glu-
cose and/or FFAs implies there maybe some different pathways existed. Additionally, to determine whether these defects in insulin sensitivity and mitochondrial dysfunction occur independently, yet in parallel, will require further study.

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