Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle

Huei-Fen Jheng[1], Pei-Jane Tsai[2], Syue-Maio Guo[3], Li-Hua Kuo[1], Cherng-Shyang Chang[1], Ih-Jen Su[4,5], Chuang-Rung Chang[6], and Yau-Sheng Tsai[1,3,7]

[1]Institute of Basic Medical Sciences, [2]Department of Medical Laboratory Science and Biotechnology, [3]Institute of Clinical Medicine, [4]Department of Pathology, and [7]Cardiovascular Research Center, College of Medicine, National Cheng Kung University, Tainan; [5]Division of Infectious Diseases, National Health Research Institues, Tainan; [6]Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan, ROC

Address correspondence to:

Yau-Sheng Tsai, 1 University Rd, National Cheng Kung University, Tainan 701, Taiwan, ROC. Phone: 886-6-2353535 ext 4242; Fax: 886-6-2758781; Email: yaustsai@mail.ncku.edu.tw

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Abstract

Mitochondrial dysfunction in skeletal muscle has been implicated in the development of insulin resistance and type 2 diabetes. Considering the importance of mitochondrial dynamics in mitochondrial and cellular functions, we hypothesized that obesity and excess energy intake shift the balance of mitochondrial dynamics, further contributing to mitochondrial dysfunction and metabolic deterioration in skeletal muscle. First, we revealed that excess palmitate (PA), but not hyperglycemia, hyperinsulinemia, or elevated TNFα, induced mitochondrial fragmentation and increased mitochondria-associated Drp1 and Fis1 in differentiated C2C12 muscle cells. This fragmentation was associated with increased oxidative stress, mitochondrial depolarization, loss of ATP production, and reduced insulin-stimulated glucose uptake. Both genetic and pharmacological inhibition of Drp1 attenuated PA-induced mitochondrial fragmentation, mitochondrial depolarization, and insulin resistance in C2C12 cells. Furthermore, we found smaller and shorter mitochondria and increased mitochondrial fission machinery in the skeletal muscle of both genetic and diet-induced obese mice. Inhibition of mitochondrial fission improved muscle insulin signaling and systemic insulin sensitivity of obese mice. Our findings indicated that aberrant mitochondrial fission is causally associated with mitochondrial dysfunction and insulin resistance in skeletal muscle. Thus, disruption of mitochondrial dynamics may underlie the pathogenesis of muscle insulin resistance in obesity and type 2 diabetes.

Keywords

mitochondrial dynamics; reactive oxygen species; skeletal muscle; obesity; insulin resistance

Introduction

The prevalence of obesity and type 2 diabetes is increasing at an alarming rate in industrialized countries, partly due to excess food intake and physical inactivity. Excess dietary fat and sugar leads to increased flux of energy fuel substrates and increased lipid burden in peripheral tissues. Skeletal muscle is the major site of glucose uptake and metabolism. Increased fatty acid uptake contributes to increased lipid accumulation in skeletal muscle, leading to lipotoxicity, which is known to impair muscle insulin sensitivity (2, 20). In addition, the intracellular lipid metabolites have been shown to activate serine/threonine protein kinases and suppress insulin actions (37).

Mitochondria are important organelles for cellular function through regulation of energy metabolism, ATP generation, and calcium handling. Substantial evidence shows that mitochondrial dysfunction and impairment of the oxidative capacity in skeletal muscle are key mechanisms mediating insulin resistance (24, 34). A reduction in mitochondrial number and function has been documented in the skeletal muscle of type 2 diabetic patients and animals. For example, the activity of the electron transport chain in subsarcolemmal mitochondria is dramatically reduced in type 2 diabetic and obese subjects, compared with that in lean subjects (36). Furthermore, patients with severe insulin resistance exhibit decreased mitochondrial oxidative activity and ATP synthesis in skeletal muscle (22, 34). High-fat diets downregulate the genes related to mitochondrial biogenesis and the electron transport chain in muscle tissues from mice and humans (3, 40), suggesting that excess dietary fat impairs mitochondrial biogenesis

and function.

Mitochondria constantly fuse and divide, processes known as fusion and fission, leading to dynamic networks of mitochondria. The frequencies of fusion and fission events are balanced to maintain the overall morphology of the mitochondrial population (8, 41). A high fusion-to-fission ratio leads to elongated, tubular, interconnected mitochondrial networks, whereas a low ratio results in fragmented, discontinuous mitochondria. These two opposing processes are finely regulated by the mitochondrial fusion proteins, mitofusins 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (Opa1), and the mitochondrial fission proteins, dynamin-related protein 1 (Drp1) and fission protein 1 (Fis1).

Recent work has highlighted the importance of mitochondrial fusion and fission in cellular function and animal physiology (13, 41). For example, fibroblasts lacking Mfn1 and Mfn2 completely lack mitochondrial fusion and show severe cellular defects, including poor growth, heterogeneity of mitochondrial membrane potential, and decreased respiration (11). Lack of fission by down-regulating Drp1 expression leads to loss of mtDNA and a decrease of mitochondrial respiration in HeLa cells (33). However, another study demonstrated that inhibition of Drp1 prevents the decrease of mitochondrial membrane potential and release of cytochrome C in COS-7 cells (16). Nevertheless, balanced mitochondrial dynamics is critical to maintenance of functional mitochondria, energy generation, and prevention of apoptosis.

Although decreased mitochondrial function and activity in skeletal muscle has been documented in obesity and type 2 diabetes, the involvement of mitochondrial dynamics in the pathogenesis of metabolic disorders remains unclear. In this study, we hypothesized that obesity and excess energy intake shift the balance of mitochondrial dynamics, further contributing to mitochondrial dysfunction and metabolic deterioration in skeletal muscle. Therefore, we designed experiments to examine the cellular and physiological significance of the continual fusion and fission of mitochondria in response to metabolic overload.

Materials and Methods

Mice Leptin-deficient (*ob/ob*) mice and control littermates, obtained from The Jackson Laboratory, were fed regular chow (Purina Laboratory Rodent Diet 5001, PMI Nutrition International, Richmond, IN). For the diet-induced obese group, eight-week-old male C57BL/6 mice, obtained from National Laboratory Animal Center (Tainan, Taiwan), were fed with high-fat diet (HF) (58R2; TestDiet, Richmond, IN) and its control low-fat diet (LF) (58R0; TestDiet). Animals were housed in a specific-pathogen-free barrier facility and handled following procedures approved by the Institutional Animal Care and Use Committees of National Cheng Kung University.

Cell culture Mouse C2C12 myoblasts were maintained in DMEM supplemented with 10% FBS. Cells were differentiated by replacing the medium with DMEM containing 2% horse serum. After 3 days of differentiation, C2C12 cells expressing muscle marker desmin were starved for 4 hr, and then changed into serum-free DMEM containing 2% BSA with or without fatty acids (FA) (Sigma-Aldrich, St. Louis, MO). No effect of FAs on cell viability in this experimental condition was identified. C2C12 cells on the Day 2 after differentiation were transfected with plasmids expressing Drp1-K38A (9) or control plasmids (pCDNA3) using Neon transfection system (Invitrogen, Carlsbad, CA). Knockdown of Drp1 was performed on 1-day post-differentiated C2C12 cells transfected with *Drp1* shRNA (TRCN0000321167) or control shRNA (pLKO.1) plasmids using TurboFect transfection system (Fermentas, Glen Burnie, MD).

Mitochondrial morphology Transmission electron microscopy was performed on 90 nm sections from mouse gastrocnemius muscle with a Hitachi 7000 TEM. Mitochondrial area and length in the gastrocnemius skeletal muscle were measured in 400 mitochondria per mouse using ImageJ software. Mitochondrial morphology was examined in C2C12 cells stained with 200 nM MitoTracker Green FM (Molecular Probes, Eugene, OR) by a fluorescence microscope (Olympus, Tokyo, Japan) or a confocal microscope (C1-Si, Nikon, Tokyo, Japan). For the quantification of tubular mitochondrial morphology, at least 100 randomly chosen cells per treatment group were designated as containing either elongated all over (100%), predominantly elongated (80%), modestly elongated (60%), predominantly fragmented (40%), or fragmented all over (20%) over three independent experiments by two investigators blinded to the treatment. For the real-time recording of mitochondrial morphology, cells were visualized by a confocal microscope with 60X objective lens, and the images were taken each 30 sec after treatment with different medium for 1 hr.

Mitochondria extraction Mitochondrial fraction was isolated as previously described (6). Briefly, tissues and cells were collected and homogenized in the buffer (250 mM Sucrose, 0.5 mM EGTA, 0.5 mM EDTA, 3 mM HEPES-NaOH; pH 7.2). The homogenate was centrifuged at 800 g for 10 min at 4^{o}C. The supernatant was transferred and centrifuged again at 10000 g for 10 min at 4^{o}C. The pellet containing mitochondria was resuspended.

Immunoblot analysis Proteins were subjected to electrophoresis, transferred to PVDF membranes, and probed with antibodies against Mfn1 and Opa1 (Abnova, Taipei, Taiwan); Mfn2 (Sigma-Aldrich); Drp1 (BD Biosciences, Franklin Lakes, NJ); Fis1 (Biovision, Mountain View, CA); IRS-1-phospho-Tyr608 (Abcam, Cambridge, MA); IRS-1 (Millipore, Billerica, MA); Akt-phospho-Ser473, Akt, GSK-3-phospho-Ser21, GSK-3, ERK1/2-phospho-Thr202/204, ERK1/2, p38-phospho-Thr180/Tyr182, p38, JNK-phospho-Thr183/Tyr185, and JNK (Cell Signaling, Danvers, MA). Immunoreactive proteins were detected using an enhanced chemiluminescence western blotting detection system (Millipore).

Quantitative RT-PCR and mtDNA content analyses Total RNA was extracted using REzol (PROtech, Mukilteo, WA). Samples of mRNA were analyzed with SYBR Green-based real-time quantitative RT-PCR (Applied Biosystems, Foster City, CA), with *cyclophilin A* as the reference gene in each reaction. Total DNA was extracted from cells using a genomic DNA isolation kit (Geneaid, Taipei, Taiwan). The content of mtDNA was calculated using real-time quantitative PCR by measuring a mitochondria-encoded gene (*Cox1*) versus a nuclear-encoded gene (*Gapdh*).

ROS production, mitochondrial polarization and ATP content The intracellular level of ROS was detected using the fluorescent probe H_2DCFDA (25 μM; Sigma-Aldrich). Mitochondrial polarization in cells was analyzed using a cationic fluorescent dye JC-1 (1.5 μM; Molecular probe). Total ATP content was determined using ATP bioluminescent assay kit (Molecular probes).

Glucose uptake assay After treated with or without PA, C2C12 cells were incubated in PBS in the presence or absence of 10 nM insulin for 30 min. Glucose uptake was determined by adding fluorescent $_D$-glucose analog 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; 200 μM; Molecular probes) for 15 min. After washing with PBS, the glucose uptake was measured by a microplate fluorometer (Fluoroskan Ascent, Thermo), and images were captured by a fluorescence microscope (Olympus) with a 10X objective lens.

Glucose tolerance test Mice were fasted for 4 hr and given an oral glucose bolus (2 g/kg body weight). Blood samples were collected before and at indicated times after injections. Plasma glucose concentration was determined by a glucose colorimetric test (Autokit Glucose, Wako, Osaka, Japan). Insulin was measured using mouse insulin ELISA (Mercodia, Uppsala, Sweden). The insulin resistance index was calculated as the product of the areas under glucose and insulin curves in the glucose tolerance test.

Mdivi-1 preparation Mdivi-1 (Enzo Life Sciences, Plymouth Meeting, PA) was dissolved in DMSO. For the *in vitro* experiment, C2C12 cells were incubated with mdivi-1 for 1 hr before analyses of mitochondrial morphology, ROS production, or mitochondrial polarization. For the *in vivo* experiment, mice were fasted for 16 hr and intraperitoneally received mdivi-1 (44 mg/kg in PBS) twice (16 hr and 1 hr) prior to insulin stimulation or glucose overload.

Data analysis Values are reported as mean ± SEM. Statistical analyses were conducted by two-way ANOVA with treatment and set of experiment as factors. Student's *t* test was used for comparisons between groups within each experiment, and differences were considered to be statistically significant at $P < 0.05$.

Results

External factors involved in the change of mitochondrial morphology

Alterations in the extracellular milieu, including hyperglycemia, hyperinsulinemia, elevated free fatty acids (FFAs), and elevated proinflammatory cytokines, cause muscle insulin resistance in obesity and type 2 diabetes (18, 31). To investigate whether these external factors directly alter mitochondrial dynamics, we used differentiated C2C12 skeletal muscle cells and screened for putative factors involved in the alteration of mitochondrial dynamics. After staining with MitoTracker Green, C2C12 muscle cells exhibited an interconnected network of tubular, elongated structures. No difference in the tubular feature of mitochondrial morphology was observed between cells treated with low glucose (5.6 mM) and those treated with high glucose (25 mM) for 12 hr (Fig. 1A). The tubular feature was maintained when cells were treated with a higher glucose concentration of 33 mM for 84 hr (data not shown). Similarly, high concentrations of insulin (upto 1.2 μmol/l) and TNFα (upto 1 pmol/l) incubated for 12 hr were without effect on the change of mitochondrial tubular feature (data not shown). In contrast, mitochondrial morphology was shifted toward a fragmented, discontinuous network, with a higher proportion of smaller and rounder mitochondria, when cells were treated with one of the most abundant FAs, palmitate (PA) (Fig. 1A). Quantification of the mitochondrial tubular feature, according to the method described by Brooks *et al*. (5), revealed that treatment with PA shifted mitochondrial morphology toward a fission type in a time- and dose-dependent manner (Fig. 1B). Time lapse recording demonstrated that mitochondria of the vehicle-treated group exhibited frequent fusion and fission, and the tubular feature of mitochondria was maintained within the

time of recording (see supplemental video). In contrast, normal tubular mitochondria underwent fission and became short and small in response to PA treatment. These data suggest FA, particularly PA, as an external factor altering mitochondrial dynamics and shifting the balance towards fission in muscle cells

Differential effect of saturated and unsaturated FAs on mitochondrial morphology

To determine whether different FAs exhibited differential effect on the change of mitochondrial morphology and dynamics, we treated cells with saturated FAs, including myristate (MA, C14:0) and stearate (SA, C18:0); unsaturated FAs, including palmitoleate (PLA, C16:1), oleate (OA, C18:1), and linoleate (LA, C18:2); and ω-3 polyunsaturated FA docosahexaenoate (DHA, C22:6) at 200 μM for 6 hr or 12 hr. Treatment of C2C12 cells with SA and all unsaturated FAs (PLA, OA, and LA) did not alter mitochondrial tubular morphology (Fig. 2A). However, mitochondrial fragmentation was observed in the groups treated with MA for 6 hr and 12 hr, which was similar with the groups treated with PA. Co-treatment with unsaturated FAs, OA and LA, and polyunsaturated FA, DHA, but not saturated FA, SA, attenuated PA-induced mitochondrial fragmentation in C2C12 cells (Fig. 2B). These data indicate that saturated FAs, including MA and PA, lead to mitochondrial fragmentation, whereas unsaturated and polyunsaturated FAs protect against PA-induced mitochondrial fragmentation.

Mitochondrial dysfunction but not change in mitochondrial content in the treatment of PA

To investigate whether the occurrence of mitochondrial fragmentation is associated with mitochondrial

dysfunction, we examined mitochondrial membrane potential and total ATP content. Treatment with PA for 6 hr and 12 hr significantly decreased mitochondrial membrane potential of C2C12 cells. Co-treatment with DHA completely reversed the decrease in mitochondrial membrane potential (Fig. 3A). Consistently, the total ATP content, reflecting cellular energy production, was decreased in the presence of PA, and this phenomenon was recovered by co-treatment with DHA (Fig. 3B). Thus, these results suggest that mitochondrial fragmentation induced by PA is accompanied by mitochondrial depolarization and loss of ATP production, the characteristics of mitochondrial dysfunction.

Next, we tested whether PA-induced mitochondrial fragmentation and dysfunction were the consequence of reduced mitochondrial content. The ratio of mitochondrial DNA (mtDNA) to nuclear DNA was not different between the groups treated with and without PA. Co-treatment with DHA also did not alter the ratio of mtDNA to nuclear DNA, compared to the other two groups (Fig. 3C). Expression of genes related to mitochondrial biogenesis, including peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Ppargc1a*), mitochondrial transcription factor A (*Tfam*) and estrogen-related receptor alpha (*Esrra*), and mtDNA replication and repair, including single-stranded DNA binding protein 1 (*Ssbp1*) and polymerase gamma 2 (*Polg2*), were increased upon PA treatment and reversed by co-treatment with DHA (Fig. 3D). These results suggest that PA-induced mitochondrial fragmentation and dysfunction are not the consequence of reduced mitochondrial content and biogenesis. On the contrary, the decrease in mitochondrial function upon PA exposure was compensated by upregulation of genes related to mitochondrial biogenesis and mtDNA replication and repair.

Involvement of ROS in PA-induced mitochondrial fragmentation

Because ROS has been suggested as a mediator in mitochondrial fragmentation in other cell types (26, 48), we next investigated whether ROS is involved in PA-induced mitochondrial fragmentation in C2C12 cells. First, we observed that treatment with PA significantly increased intracellular ROS levels, reflected by the stain of the fluorescent probe H_2DCFDA. Co-treatment of DHA or α-tocopherol (TCP), a ROS scavenger, significantly attenuated the increased ROS levels induced by PA (Fig. 4A). Interestingly, co-treatment with TCP ameliorated PA-induced mitochondrial fragmentation (Fig. 4B). The amelioration of PA-induced mitochondrial fragmentation by co-treatment of TCP was associated with the recovery of ATP production efficiency (Fig. 4C). Thus, our data indicated that attenuation of ROS generation protected against PA-induced mitochondrial fragmentation and dysfunction, implicating a link between ROS generation and mitochondrial fragmentation. To examine whether PA-induced ROS generation is the consequence of changes in fatty acid oxidation, we measured expression of genes related to FA oxidation. Except for medium-chain acyl-CoA dehydrogenase (*Mcad*), expression of carnitine palmitoyltransferase 1b (*Cpt1b*), long-chain-acyl-CoA dehydrogenase (*Lcad*), long-chain-fatty-acid-CoA ligase 1 (*Acsl1*), and acyl-coenzyme A oxidase 1 (*Acox1*) was significantly increased by PA treatment. While co-treatment of DHA significantly ameliorated PA-induced expression of *Cpt1b*, *Acsl1*, and *Acox1*, co-treatment of TCP only attenuated PA-induced expression of *Cpt1b* (Fig. 4D).

Increased mitochondria-associated Drp1 and Fis1 under PA treatment

To determine which components of the mitochondrial fusion and fission machinery mediated mitochondrial fragmentation under PA treatment, we isolated mitochondria from PA-treated C2C12 cells and performed immunoblot analysis. While no difference in mitochondria-associated protein levels of Mfn1, Mfn2 and Opa1 was detected between PA and vehicle-treated C2C12 cells, mitochondria-associated protein levels of Drp1 and Fis1 were greatly increased in the PA-treated group (Fig. 5A). These results indicated that the component regulating mitochondrial fission was increased in the treatment of PA, contributing to the imbalance of mitochondrial dynamics favoring fission.

Attenuation of PA-induced mitochondrial dysfunction and reduction in cellular glucose metabolism by inhibition of mitochondrial fission

We next asked whether inhibition of Drp1 by genetic manipulation would attenuate PA-induced mitochondrial fragmentation and dysfunction. Overexpression of dominant negative Drp1 (DN-Drp1; Drp1-K38A) or downregulation of Drp1 protein level by *Drp1*-shRNA (Fig. 5B) significantly restored PA-induced mitochondrial fragmentation (Fig. 5C and 5F) and mitochondrial depolarization (Fig. 5D and 5G). To directly address whether PA-induced mitochondrial fragmentation is correlated with cellular metabolic deterioration, we examined glucose uptake under insulin stimulation in C2C12 cells. While the presence of PA significantly decreased insulin-stimulated glucose uptake in C2C12 cells, inhibition or knockdown of Drp1 restored the PA-induced reduction of insulin-stimulated glucose uptake (Fig. 5E and

5H).

Mitochondrial division inhibitor-1 (mdivi-1) is a chemical compound which attenuates mitochondrial fission by selectively blocking GTPase activity of Drp1 (7) and provides the potential for therapeutic use. Although treatment with mdivi-1 partially reversed PA-induced mitochondrial fragmentation (Fig. 6A), it effectively ameliorated PA-induced ROS generation and mitochondrial depolarization, as well as PA-induced reduction of insulin-stimulated glucose uptake, in a dose-dependent manner (Fig. 6B~6D). Thus inhibition of mitochondrial fission attenuates PA-induced mitochondrial fragmentation, ROS generation, mitochondrial depolarization and suppression of insulin-stimulated glucose uptake.

Altered mitochondrial morphology and proteins involved in mitochondrial dynamics in the skeletal muscle of obese mice

We next studied whether mitochondrial fission was exhibited in skeletal muscle *in vivo* in response to metabolic overload. 3-mo-old *ob/ob* mice exhibited morbid obesity and severe insulin resistance (data not shown). Similarly, HF treatment for 10 wks on wild-type C57BL/6 mice resulted in increased body and fat weight, hyperglycemia, hyperinsulinemia, and increased plasma FFA and triglyceride levels, compared to LF fed mice (data not shown). Both genetic-induced *ob/ob* (Fig. 7A and 7B) and HF diet-induced obese mice (data not shown) exhibited smaller and shorter mitochondria in the gastrocnemius skeletal muscle than those from their respective control lean mice. To further evaluate whether this phenomenon was associated with proteins involved in mitochondrial dynamics, we measured protein levels in the mitochondrial fraction

from the gastrocnemius muscle of obese and lean mice. No difference in mitochondria-associated protein levels of Mfn1, Mfn2 and Opa1 of *ob/ob* mice was observed compared to their control lean mice (Fig. 7C). Drp1 and Fis1 were significantly increased in the mitochondrial fraction of *ob/ob* muscle. Consistently, levels of proteins related to mitochondrial fusion, including Mfn1, Mfn2 and Opa1, were not altered in the muscle from HF fed mice (Fig. 7D). While Drp1 level was not altered in the mice fed HF for 10 wks, it was significantly increased in the mice fed HF for 16 wks (Fig. 7E). Fis1 level was significantly increased in the mitochondrial fraction of the muscle from both 10- and 16-wk HF fed mice.

Improved muscle insulin resistance by inhibition of mitochondrial fission in obese animals

We further tested the effects of inhibiting mitochondrial fission on the skeletal muscle of *ob/ob* mice. Treatment of *ob/ob* mice with 44 mg/kg mdivi-1 prior to insulin stimulation increased insulin-stimulated phosphorylation at Tyr608 of insulin receptor substrate-1 (IRS-1), Ser473 of protein kinase B (Akt), and Ser21 of glycogen synthase kinase-3 α-subunit (GSK-3α), compared to those in the vehicle-treated group (Fig. 8A). Furthermore, we performed the oral glucose tolerance test (OGTT) to assess the effect of mdivi-1 on whole-body glucose metabolism. Although the treatment of mdivi-1 did not change clearance of glucose after a glucose load, it modestly decreased plasma insulin levels during OGTT (Fig. 8B). The insulin resistance index calculated from the OGTT was significantly lower in the mdivi-1-treated *ob/ob* mice than that of the vehicle-treated *ob/ob* mice. Thus, the inhibition of mitochondrial fission machinery by pharmacological inhibitor improved insulin signaling in the skeletal muscle and systemic insulin sensitivity

of *ob/ob* mice.

Attenuation of protein kinases by inhibition of mitochondrial fission in obese animals

To address how changes in mitochondrial morphology can modulate insulin signaling pathway, we detected several serine/threonine protein kinases that are known to be activated by intracellular lipid metabolites and ROS and inhibit insulin signaling. The phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) was increased in the skeletal muscle of *ob/ob* mice, compared to those of control mice (Fig. 9A). Mdivi-1 treatment attenuated the increased phosphorylation of ERK1/2 and p38 in the skeletal muscle of *ob/ob* mice. No difference in phosphorylation of c-Jun N-terminal kinases (JNK) was detectable among the three groups. We further examine the role of fatty acid oxidation in the skeletal muscle of *ob/ob* mice. Expression of *Cpt1b*, *Lcad*, *Mcad*, and *Acsl1* was up-regulated in the skeletal muscle of *ob/ob* mice, whereas mdivi-1 treatment showed the tendency to restore the increased gene expression in *ob/ob* mice (Fig. 9B).

Discussion

The idea that disruption of mitochondrial dynamics underlies the pathogenesis of metabolism-related diseases is gaining support. For example, two studies demonstrated that an imbalance of mitochondrial networks in neuron favoring mitochondrial fission plays a critical role in the pathogenesis of diabetic neuropathy both *in vivo* and *in vitro* (14, 45). Another study revealed mitochondrial fragmentation in coronary endothelial cells from diabetic mice (26). Several types of cultured cells from the cardiovascular system exhibited mitochondrial fission in the hyperglycemic condition (48). These studies all support the notion that sustained hyperglycemia is the cause of mitochondrial fission. Alteration in mitochondrial morphology was also reported to mediate tissue injury upon ischemic stress. For example, mitochondrial fission occurs in the kidney and heart after acute ischemia/reperfusion injury in mice, and prevention of this process is beneficial (5, 32). Finally, exposure to high levels of glucose and PA induced pancreatic β-cell mitochondrial fragmentation, and preserving mitochondrial dynamics protected β-cells from apoptosis (28, 29). Thus, the fine balance between mitochondrial fusion and fission can be upset by a variety of stress responses, including nutrient stress and simulated ischemia. Furthermore, amelioration of imbalanced mitochondrial dynamics reduces cellular damage and disease severity, highlighting the importance of mitochondrial dynamics in the pathogenesis of diseases of the neuron, heart, kidney, and β-cells, which rely heavily on functional, healthy mitochondria.

Our results suggest that the fusion machinery is not much affected in the skeletal muscle from both

obese rodents and in PA-treated C2C12 cells. Instead, the fission machinery is likely the main component involved in the regulation of mitochondrial dynamics in our *in vivo* and *in vitro* models. In agreement with our hypothesis, fusion-related proteins, including Mfn1, Mfn2 and Opa1, were not changed in the muscle of both *ob/ob* and HF-induced obese mice, as well as the PA-treated C2C12 cells. In contrast, the fission-related protein Fis1 was significantly increased in the muscle from both obese rodents and in PA-treated C2C12 cells. Another fission-related protein, Drp1, was increased in PA-treated C2C12 cells and the muscle from *ob/ob* mice and mice fed HF for 16 wks, but not altered in the muscle from mice fed HF for 10 wks. The lack of a detectable increase in mitochondria-associated Drp1 in the muscle from mice fed HF for 10 wks may be due to the relatively lower body weight increase in mice fed HF for 10 wks (mean body weight 39 g in HF-10wk vs 28 g in LF) than those of *ob/ob* mice (56 g in *ob/ob* vs 26 g in control) and mice fed HF for 16 wks (48 g in HF-16wk vs 29 g in LF). Thus, it is possible that the increase of mitochondria-associated Drp1 level is correlated with the degree of body weight increase. Nevertheless, the consistent increases of mitochondria-associated Fis1 both *in vivo* and *in vitro* support the idea that mitochondria are equipped with fission machinery under this circumstance. These findings further suggest that Fis1, at least in part, is the cause of increased mitochondrial fission in the muscle of obese mice and PA-treated cultured muscle cells.

Mdivi-1 attenuates mitochondrial fragmentation by selectively inhibiting the assembly and GTPase activity of Drp1 (7). It affects neither GTPase activity of Dynamin-1 nor that of yeast homologs of Mfn1/2

and Opa1. In our study, inhibition of Drp1 by mdivi-1 *in vitro* rescued PA-mediated mitochondrial injuries, as indicated by diminished mitochondrial depolarization and ROS generation. Consistently, we found that inhibition of Drp1 rendered C2C12 cells resistant to PA-mediated suppression of insulin-stimulated glucose transport. Furthermore, Drp1 inhibition with mdivi-1 ameliorated the impairment in insulin signal transduction in obese rodent muscle. Because mitochondria-associated Drp1 was increased in *ob/ob* muscle, this might explain the efficacy of direct inhibition of Drp1 GTPase activity with mdivi-1 on attenuation of metabolic deterioration *in vivo*.

In the search for extracellular stimuli that induce mitochondrial fission, we did not detect an effect of hyperglycemia, hyperinsulinemia, or elevated TNFα at supraphysiological and pathological concentrations on changes in mitochondrial morphology. In humans, the average concentration of FFA in postabsorptive state is 500~1000 μM in the plasma, and PA can reach 200 μM (17). Although many studies demonstrated the effect of hyperglycemia on increased generation of ROS and mitochondrial fission in a variety of cell types (26, 48), elevated circulating lipid and inflammatory cytokines usually occurs prior to the development of hyperinsulinemia and hyperglycemia during the progression of type 2 diabetes (27, 38). Lipid overload impairs oxidative capacity and increase intracellular accumulation of FA-derived metabolites, such as long-chain acyl-CoA, diacylglycerol, ceramide and triacylglycerol, in skeletal muscle (1, 30, 35, 37). These metabolites are associated with insulin resistance by impairing the insulin-signaling pathways. Thus, our results provide a rationale for the development of muscle insulin resistance in response to lipid flux.

In the time course study, we found similar effect of PA on alteration of mitochondrial morphology when cells were treated with 200 μM PA for 24 or 48 hr (data not shown). Because the cell toxicity, examined by lactate dehydrogenase assay and crystal violet stain, was exhibited at 48 hr of treatment with 200 μM PA (data not shown), we performed treatment for 6 or 12 hr in this study. Similarly, no evidence of apoptosis, revealed by immunoblotting of cleaved caspase 3, was observed in the cells treated with 200 μM PA for 6 hr (data not shown). Thus, our data suggest that PA does not largely affect signaling and cellular processes that could be linked to cellular death in the experimental condition and time course we performed.

In our study, we noticed that the results from muscle cells are in acute treatments, whereas data from animals are due to chronic effects. A speculation on the association between acute HF feeding and mitochondrial morphology *in vivo* is raised. Short-term lipid infusion for 6~8 hr in healthy individuals does not change mitochondrial content, morphology and respiration rates in skeletal muscle despite lower mitochondrial membrane potential (4, 10). Similarly, HF feeding for 4 wks in mice does not alter mitochondrial content and respiration rates (3). These suggest that acute HF feeding might not cause the same effect on mitochondrial morphology and dysfunction prior to demonstrable obesity. However, the question is still left whether the findings obtained in culture are relevant to adult muscle tissue in this study. For example, the muscle tissue, with the myocytes and other accessory cells, in obese mice encountered long-term excess FFAs as well as other nutrient and inflammatory stimuli. The effect *in vivo* is also

influenced by inputs from other organs. In contrast, the cultured cells encountered relatively short-term excess FFAs alone. Nevertheless, our results at least suggest that the presence of PA to muscle cells is deleterious to mitochondrial architecture.

It is generally recognized that saturated, unsaturated and polyunsaturated FAs mediate quite diverse effects. For example, saturated FAs reduce mitochondrial membrane potential as well as ATP generation in C2C12 cells, while unsaturated and ω-3 polyunsaturated FFAs do not alter these functions (21). Comparisons of several FAs yielded interesting findings in our study. For example, while the saturated FAs, MA and PA, induced mitochondrial fission, SA had no effect despite being only two carbon atoms longer than PA. None of the unsaturated and polyunsaturated FAs we tested affected mitochondrial fission. Interestingly, co-treatment with unsaturated and polyunsaturated FAs alleviated PA-induced mitochondrial fission. Thus, the diverse effects of different FAs on mitochondrial morphology correlated with their impacts on mitochondrial and cellular functions. Furthermore, unsaturated FAs added to the diet have protective effects on metabolic disorders. For example, supplementation of eicosapentaenoate (EPA) and DHA protects mice from HF-induced body weight gain, dyslipidemia, and glucose intolerance (23). Dietary supplementation of monounsaturated FAs improves insulin sensitivity and adipokine and lipid profiles in the HF-fed mice and healthy young subjects (44, 47). Although no evidence directly addresses the relationship between dietary unsaturated FAs and *in vivo* mitochondrial morphology, our results, together with other studies, suggest that supplementation of unsaturated FAs can reverse the insulin resistance and *in*

vivo mitochondrial morphology defects.

The relationship between fatty acid oxidation and mitochondrial function remains unclear. While reduced fatty acid oxidation was observed in obese human muscle (39, 42), increased fatty acid oxidation in muscle tissue was found in several HF-fed rodent models (19, 43). Our data showed that genes related to fatty acid oxidation were up-regulated in PA-treated cells and *ob/ob* muscles, suggesting that fatty acid oxidation is increased in response to PA or lipid overload. Thus, it is likely that excessive fatty acid oxidation due to lipid overload leads to the formation of free radicals and ROS that can compromise mitochondrial function. Interestingly, co-treatment of DHA in PA-treated cells or inhibition of fission by mdivi-1 in *ob/ob* mice reversed up-regulated genes, implicating the attenuation of increased fatty acid oxidation in the presence of DHA or mdivi-1.

Substantial evidence shows that ROS is a key mechanism linking metabolic disturbance to nutrient excess. Thus, obesity induced by a HF diet leads to enhanced oxidative stress in rodents (3, 46). Our study demonstrated that increased ROS levels in response to excess PA is the direct cause of mitochondrial fragmentation, because decreasing ROS levels with a scavenger prevented PA-induced mitochondrial fission (Fig. 4B). Furthermore, blocking mitochondrial fission significantly alleviated PA-induced ROS generation (Fig. 6B). These data imply a tight association and interplay between ROS generation and mitochondrial fission. ROS can function as signaling molecules to activate the MAPK family, including

ERK, p38 and JNK (15, 25). Other studies demonstrated that activation of these protein kinases phosphorylates IRS-1 at its serine residue(s), which further interrupts tyrosine phosphorylation on IRS-1 and suppresses downstream insulin signaling (12). Thus, our study indicated that ROS resulted from nutrient excess or PA exposure is deleterious to mitochondrial architecture and dynamics in muscle tissue/cells. Imbalance in the mitochondrial dynamics would accelerate ROS accumulation, which may further activate signaling molecules including the MAPK family and suppress insulin signaling. Our data showed that obesity increased phosphorylation of ERK1/2 and p38 in the skeletal muscle, and inhibition of mitochondrial fission reversed that, which is accompanied by amelioration of insulin resistance. These results support the link between mitochondrial morphology, ROS generation, and activation of the MAPK family in the regulation of insulin signaling pathway.

In conclusion, we provide evidence that mitochondrial fission occurs in the skeletal muscle of obese animals and in cultured muscle cells in response to high levels of some saturated FAs. Inhibition of mitochondrial fission protected muscle cells against mitochondrial dysfunction and insulin resistance *in vitro*, and, more importantly, improved muscle insulin signaling and systemic insulin sensitivity *in vivo*. Thus, our results establish a causative link between mitochondrial dynamics and metabolic deterioration, and implicate that disruption of mitochondrial dynamics in skeletal muscle may underlie the pathogenesis of insulin resistance. Finally, manipulating mitochondrial morphology may provide a novel therapeutic strategy for insulin resistance and type 2 diabetes.

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Reference

1. **Abdul-Ghani, M. A., F. L. Muller, Y. Liu, A. O. Chavez, B. Balas, P. Zuo, Z. Chang, D. Tripathy, R. Jani, M. Molina-Carrion, A. Monroy, F. Folli, H. Van Remmen, and R. A. DeFronzo.** 2008. Deleterious action of FA metabolites on ATP synthesis: possible link between lipotoxicity, mitochondrial dysfunction, and insulin resistance. Am J Physiol Endocrinol Metab **295:**E678-685.

2. **Bonen, A., M. L. Parolin, G. R. Steinberg, J. Calles-Escandon, N. N. Tandon, J. F. Glatz, J. J. Luiken, G. J. Heigenhauser, and D. J. Dyck.** 2004. Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. Faseb J **18:**1144-1146.

3. **Bonnard, C., A. Durand, S. Peyrol, E. Chanseaume, M. A. Chauvin, B. Morio, H. Vidal, and J. Rieusset.** 2008. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. J Clin Invest **118:**789-800.

4. **Brands, M., J. Hoeks, H. P. Sauerwein, M. T. Ackermans, M. Ouwens, N. M. Lammers, M. N. van der Plas, P. Schrauwen, A. K. Groen, and M. J. Serlie.** 2011. Short-term increase of plasma free fatty acids does not interfere with intrinsic mitochondrial function in healthy young men. Metabolism.

5. **Brooks, C., Q. Wei, S. G. Cho, and Z. Dong.** 2009. Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest **119:**1275-1285.

6. **Cartoni, R., B. Leger, M. B. Hock, M. Praz, A. Crettenand, S. Pich, J. L. Ziltener, F. Luthi, O. Deriaz, A. Zorzano, C. Gobelet, A. Kralli, and A. P. Russell.** 2005. Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. J Physiol **567:**349-358.

7. **Cassidy-Stone, A., J. E. Chipuk, E. Ingerman, C. Song, C. Yoo, T. Kuwana, M. J. Kurth, J. T. Shaw, J. E. Hinshaw, D. R. Green, and J. Nunnari.** 2008. Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev Cell **14:**193-204.

8. **Chan, D. C.** 2006. Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol **22:**79-99.

9. **Chang, C. R., and C. Blackstone.** 2007. Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem **282:**21583-21587.

10. **Chavez, A. O., S. Kamath, R. Jani, L. K. Sharma, A. Monroy, M. A. Abdul-Ghani, V. E.**

Centonze, P. Sathyanarayana, D. K. Coletta, C. P. Jenkinson, Y. Bai, F. Folli, R. A. Defronzo, and D. Tripathy. 2010. Effect of short-term free Fatty acids elevation on mitochondrial function in skeletal muscle of healthy individuals. J Clin Endocrinol Metab **95:**422-429.

11. **Chen, H., A. Chomyn, and D. C. Chan.** 2005. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem **280:**26185-26192.

12. **De Fea, K., and R. A. Roth.** 1997. Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. J Biol Chem **272:**31400-31406.

13. **Detmer, S. A., and D. C. Chan.** 2007. Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol **8:**870-879.

14. **Edwards, J. L., A. Quattrini, S. I. Lentz, C. Figueroa-Romero, F. Cerri, C. Backus, Y. Hong, and E. L. Feldman.** 2010. Diabetes regulates mitochondrial biogenesis and fission in mouse neurons. Diabetologia **53:**160-169.

15. **Evans, J. L., I. D. Goldfine, B. A. Maddux, and G. M. Grodsky.** 2003. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes **52:**1-8.

16. **Frank, S., B. Gaume, E. S. Bergmann-Leitner, W. W. Leitner, E. G. Robert, F. Catez, C. L. Smith, and R. J. Youle.** 2001. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell **1:**515-525.

17. **Fraser, D. A., J. Thoen, A. C. Rustan, O. Forre, and J. Kjeldsen-Kragh.** 1999. Changes in plasma free fatty acid concentrations in rheumatoid arthritis patients during fasting and their effects upon T-lymphocyte proliferation. Rheumatology (Oxford) **38:**948-952.

18. **Guilherme, A., J. V. Virbasius, V. Puri, and M. P. Czech.** 2008. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol **9:**367-377.

19. **Hancock, C. R., D. H. Han, M. Chen, S. Terada, T. Yasuda, D. C. Wright, and J. O. Holloszy.** 2008. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. Proc Natl Acad Sci U S A **105:**7815-7820.

20. **Hegarty, B. D., G. J. Cooney, E. W. Kraegen, and S. M. Furler.** 2002. Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats. Diabetes **51:**1477-1484.

21. **Hirabara, S. M., R. Curi, and P. Maechler.** 2010. Saturated fatty acid-induced insulin resistance is

associated with mitochondrial dysfunction in skeletal muscle cells. J Cell Physiol **222:**187-194.

22. **Kelley, D. E., J. He, E. V. Menshikova, and V. B. Ritov.** 2002. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes **51:**2944-2950.

23. **Kuda, O., T. Jelenik, Z. Jilkova, P. Flachs, M. Rossmeisl, M. Hensler, L. Kazdova, N. Ogston, M. Baranowski, J. Gorski, P. Janovska, V. Kus, J. Polak, V. Mohamed-Ali, R. Burcelin, S. Cinti, M. Bryhn, and J. Kopecky.** 2009. n-3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet. Diabetologia **52:**941-951.

24. **Lowell, B. B., and G. I. Shulman.** 2005. Mitochondrial dysfunction and type 2 diabetes. Science **307:**384-387.

25. **Maddux, B. A., W. See, J. C. Lawrence, Jr., A. L. Goldfine, I. D. Goldfine, and J. L. Evans.** 2001. Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by mircomolar concentrations of alpha-lipoic acid. Diabetes **50:**404-410.

26. **Makino, A., B. T. Scott, and W. H. Dillmann.** 2010. Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes. Diabetologia **53:**1783-1794.

27. **McGarry, J. D.** 2002. Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. Diabetes **51:**7-18.

28. **Men, X., H. Wang, M. Li, H. Cai, S. Xu, W. Zhang, Y. Xu, L. Ye, W. Yang, C. B. Wollheim, and J. Lou.** 2009. Dynamin-related protein 1 mediates high glucose induced pancreatic beta cell apoptosis. Int J Biochem Cell Biol **41:**879-890.

29. **Molina, A. J., J. D. Wikstrom, L. Stiles, G. Las, H. Mohamed, A. Elorza, G. Walzer, G. Twig, S. Katz, B. E. Corkey, and O. S. Shirihai.** 2009. Mitochondrial networking protects beta-cells from nutrient-induced apoptosis. Diabetes **58:**2303-2315.

30. **Montell, E., M. Turini, M. Marotta, M. Roberts, V. Noe, C. J. Ciudad, K. Mace, and A. M. Gomez-Foix.** 2001. DAG accumulation from saturated fatty acids desensitizes insulin stimulation of glucose uptake in muscle cells. Am J Physiol Endocrinol Metab **280:**E229-237.

31. **Muoio, D. M., and C. B. Newgard.** 2008. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Biol **9:**193-205.

32. **Ong, S. B., S. Subrayan, S. Y. Lim, D. M. Yellon, S. M. Davidson, and D. J. Hausenloy.** 2010. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation **121:**2012-2022.

33. **Parone, P. A., S. Da Cruz, D. Tondera, Y. Mattenberger, D. I. James, P. Maechler, F. Barja, and J. C. Martinou.** 2008. Preventing mitochondrial fission impairs mitochondrial function and leads to loss of mitochondrial DNA. PLoS One **3:**e3257.

34. **Petersen, K. F., S. Dufour, D. Befroy, R. Garcia, and G. I. Shulman.** 2004. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med **350:**664-671.

35. **Powell, D. J., S. Turban, A. Gray, E. Hajduch, and H. S. Hundal.** 2004. Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. Biochem J **382:**619-629.

36. **Ritov, V. B., E. V. Menshikova, J. He, R. E. Ferrell, B. H. Goodpaster, and D. E. Kelley.** 2005. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes **54:**8-14.

37. **Sabin, M. A., C. E. Stewart, E. C. Crowne, S. J. Turner, L. P. Hunt, G. I. Welsh, M. J. Grohmann, J. M. Holly, and J. P. Shield.** 2007. Fatty acid-induced defects in insulin signalling, in myotubes derived from children, are related to ceramide production from palmitate rather than the accumulation of intramyocellular lipid. J Cell Physiol **211:**244-252.

38. **Schenk, S., M. Saberi, and J. M. Olefsky.** 2008. Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest **118:**2992-3002.

39. **Simoneau, J. A., J. H. Veerkamp, L. P. Turcotte, and D. E. Kelley.** 1999. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. Faseb J **13:**2051-2060.

40. **Sparks, L. M., H. Xie, R. A. Koza, R. Mynatt, M. W. Hulver, G. A. Bray, and S. R. Smith.** 2005. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes **54:**1926-1933.

41. **Suen, D. F., K. L. Norris, and R. J. Youle.** 2008. Mitochondrial dynamics and apoptosis. Genes Dev **22:**1577-1590.

42. **Thyfault, J. P., R. M. Kraus, R. C. Hickner, A. W. Howell, R. R. Wolfe, and G. L. Dohm.** 2004. Impaired plasma fatty acid oxidation in extremely obese women. Am J Physiol Endocrinol Metab **287:**E1076-1081.

43. **Turner, N., C. R. Bruce, S. M. Beale, K. L. Hoehn, T. So, M. S. Rolph, and G. J. Cooney.** 2007. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. Diabetes **56:**2085-2092.

44. **Uusitupa, M., U. Schwab, S. Makimattila, P. Karhapaa, E. Sarkkinen, H. Maliranta, J. Agren, and I. Penttila.** 1994. Effects of two high-fat diets with different fatty acid compositions on glucose and lipid metabolism in healthy young women. Am J Clin Nutr **59:**1310-1316.

45. **Vincent, A. M., J. L. Edwards, L. L. McLean, Y. Hong, F. Cerri, I. Lopez, A. Quattrini, and E. L. Feldman.** 2010. Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta Neuropathol **120:**477-489.

46. **Wellen, K. E., and C. B. Thompson.** 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. Mol Cell **40:**323-332.

47. **Yang, Z. H., H. Miyahara, T. Mori, N. Doisaki, and A. Hatanaka.** 2011. Beneficial effects of dietary fish-oil-derived monounsaturated Fatty acids on metabolic syndrome risk factors and insulin resistance in mice. J Agric Food Chem **59:**7482-7489.

48. **Yu, T., S. S. Sheu, J. L. Robotham, and Y. Yoon.** 2008. Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. Cardiovasc Res **79:**341-351.

Legends for figures

FIG. 1.

External factors altering mitochondrial morphology in C2C12 cells. (**A**) Images of C2C12 cells stained with Mitotracker Green after 12 hr incubation in medium containing 5.6 mM or 25 mM glucose with various concentrations of PA from 0 to 200 μM. Each scale bar is 20 μm. (**B**) Percentage of tubular mitochondria in C2C12 cells incubated in medium with various concentrations of glucose and PA for 2, 6, or 12 hr. Results are average of three individual experiments with at least 100 cells per treatment group in each experiment. $^{**}P$ <0.01 and $^{***}P$ <0.001 *versus* the cells treated with 5.6 mM glucose alone, and $^{\#\#}P$ <0.01 *versus* the cells treated with 25 mM glucose alone.

FIG. 2.

Differential effect of saturated and unsaturated FAs on mitochondrial morphology. (**A**) Percentage of tubular mitochondria in C2C12 cells treated with various FAs at 200 μM for 6 or 12 hr. Saturated FAs include MA, PA and SA; unsaturated FAs include PLA, OA and LA; and polyunsaturated FA includes DHA. (**B**) Percentage of tubular mitochondria in C2C12 cells treated with PA in the presence or absence of other indicated FAs (200 μM) for 6 or 12 hr. $^{*}P$ <0.05, $^{**}P$ <0.01 and $^{***}P$ <0.001 *versus* vehicle. $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 and $^{\#\#\#}P$ <0.001 *versus* PA alone.

FIG. 3.

Mitochondrial function and content in the treatment of PA. (**A**) Membrane potential (n=8 in each group) and (**B**) total ATP content (n=3~4 in each group) of C2C12 cells treated with 200 μM PA in the presence or absence of DHA for 6 or 12 hr. Data are normalized to the average of the vehicle-treated group. (**C**) mtDNA content calculated as the ratio of *COX1* to *Gapdh* DNA levels measured by quantitative PCR in C2C12 cells treated with PA in the presence or absence of DHA (n=3 in each group). (**D**) Expression of genes for mitochondrial biogenesis and mtDNA replication and repair by quantitative RT-PCR (n=4) after 6 hr of treatment. mRNA amount is expressed relative to the average level of the vehicle-treated group. **P*<0.05, ***P*<0.01, and ****P*<0.001 *versus* vehicle. $^{\#}P$<0.05, $^{\#\#}P$<0.01, and $^{\#\#\#}P$<0.001 *versus* PA alone.

FIG. 4.

Involvement of ROS in PA-induced mitochondrial fragmentation. (**A**) Intracellular ROS levels were measured by oxidation of H_2DCFDA in C2C12 cells treated with 200 μM PA, as well as in the group co-treated with DHA (200μM) or TCP (500μM), for 6 or 12 hr. n=8 in each group. (**B**) Percentage of tubular mitochondria and (**C**) total ATP content in C2C12 cells treated with PA in the presence or absence of TCP for 6 or 12 hr. (**D**) Expression of genes related to fatty acid oxidation by quantitative RT-PCR (n=4) after 6 hr of treatment. mRNA amount is expressed relative to the average level of the vehicle-treated group **P*<0.05, ***P*<0.01, and ****P*<0.001 *versus* vehicle. $^{\#}P$<0.05, $^{\#\#}P$<0.01, and $^{\#\#\#}P$<0.001 *versus* PA alone.

FIG. 5.

Change in protein levels and effects of genetically inhibiting Drp1 in PA-treated C2C12 cells. (**A**) Immunoblot analyses on protein content from the mitochondrial fraction of C2C12 cells treated with 200 μM PA. Porin was used as a loading control for mitochondrial extracts. (**B**) Protein levels of overexpressed DN-Drp1 (left panel) and effect of Drp1 knockdown (right panel) in C2C12 cells. (**C**) Percentage of DsRed2-Mito-labeled C2C12 cells displaying tubular mitochondria after transfection with DN-Drp1 plasmids. Results are average of four individual experiments with at least 100 cells per treatment group in each experiment. (**D**) Membrane potential of C2C12 cells transfected with DN-Drp1 or control plasmids. n=7 in each group. Data are normalized to the average of the vehicle-treated cells transfected with control plasmids. (**E**) Quantification of uptake of florescent glucose analog in C2C12 cells transfected with DN-Drp1 or control plasmids. n=6 in each treatment group. Results are normalized to the average of basal uptake. (**F**) Percentage of tubular mitochondria, (**G**) membrane potential, and (**H**) uptake of florescent glucose analog of C2C12 cells transfected with *Drp1*-specific shRNA or control shRNA plasmids. Results for (F) are average of three individual experiments with at least 100 cells per treatment group in each experiment. n=8 in each treatment group of (G) and (H). **P*<0.05, ***P*<0.01, and ****P*<0.001.

FIG. 6.

Effects of pharmacological inhibition of mitochondrial fission *in vitro*. (**A**) Percentage of tubular mitochondria, (**B**) intracellular ROS levels and (**C**) membrane potential of C2C12 cells treated with 200 μM PA in the presence or absence of various concentrations of mitochondrial fission inhibitor mdivi-1. n=8 in

each treatment group of (**B**) and (**C**). *P<0.05 and ***P<0.001 *versus* vehicle. $^{\#}P$<0.05, $^{\#\#}P$<0.01, and $^{\#\#\#}P$<0.001 *versus* PA alone. (**D**) The representative images (left panels) and quantification (right panel) of uptake of florescent glucose analog in C2C12 cells treated with 200 μM PA in the presence or absence of various concentrations of mdivi-1 for 6 hr. The scale bar is 200 μm. n=14 in each treatment group. Results are normalized to the average of basal uptake. *P<0.05, **P<0.01, and ***P<0.001.

FIG. 7.

Mitochondrial morphology and proteins related to mitochondrial dynamics in the skeletal muscle of obese mice. (**A**) Mitochondrial morphology imaged by transmission electron microscope and (**B**) distribution of mitochondrial area and length in the gastrocnemius muscle of *ob/ob* and their control mice. Each scale bar is 500 nm. Immunoblot analyses on protein content from the mitochondrial fraction of the muscle tissues of (**C**) *ob/ob* and their control mice at age of 13 wks, (**D**) HF and LF fed mice for 10 wks, and (**E**) HF and LF fed mice for 16 wks. Samples from representative animals are shown in the western blot, with each lane representing one animal. The intensities of the bands, quantified densitometrically relative to their respective controls, are shown with the sample number in parentheses. *P<0.05 and **P<0.01. Porin was used as a loading control for mitochondrial extracts.

FIG. 8.

Effects of mitochondrial fission inhibition *in vivo*. (**A**) Immunoblot analyses on phosphorylation at Tyr608

of IRS-1, Ser473 of Akt, and Ser21 of GSK-3α from the whole lysate of gastrocnemius muscle of *ob/ob* mice under mdivi-1 treatment. Muscle tissues were collected 2 min after injection with 5 U/kg insulin through the vena cava for analyses of phosphorylation. Each band represents a tissue extract from a single mouse. (**B**) Plasma glucose (left panel) and insulin levels (middle panel) and insulin resistance (IR) index (right panel) during OGTT in 13-wk-old *ob/ob* mice received mdivi-1 (n=7) or vehicle (n=6). **P*<0.05 *versus* vehicle.

FIG. 9.

Protein kinases and fatty acid oxidation in the skeletal muscle of obese mice. (**A**) Immunoblot analysis on phosphorylation of ERK1/2, p38, and JNK in the skeletal muscle of control and *ob/ob* mice treated with vehicle or mdivi-1. (**B**) Expression of genes related to fatty acid oxidation by quantitative RT-PCR. Control mice received vehicle (Control-vehicle), n=9; *ob/ob* mice received vehicle (*ob/ob*-vehicle), n=5; *ob/ob* mice received mdivi-1 (*ob/ob*-mdivi-1), n=7. mRNA amount is expressed relative to the average level of the vehicle-received control mice. **P*<0.05 and ****P*<0.001 *versus* control-vehicle. $^{\#}P$<0.05 *versus* *ob/ob*-vehicle.