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Distinct Adaptations of Mitochondrial Dynamics to Electrical Pulse Stimulation in Lean and Severely Obese Primary Myotubes

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Abstract

Background—Skeletal muscle from lean and obese subjects elicit differential adaptations in response to exercise/muscle contractions. In order to determine whether obesity alters the adaptations in mitochondrial dynamics in response to exercise/muscle contractions and whether any of these distinct adaptations are linked to alterations in insulin sensitivity, we compared the effects of electrical pulse stimulation (EPS) on mitochondrial network structure and regulatory proteins in mitochondrial dynamics in myotubes from lean humans and humans with severe obesity and evaluated the correlations between these regulatory proteins and insulin signaling.

Methods—Myotubes from human skeletal muscle cells obtained from lean humans (BMI 23.8 ± 1.67 kg/m^2) and humans with severer obesity (45.5 ± 2.26 kg/m^2) (n=8/group) were electrically stimulated for 24 hours. Four-hours after EPS, mitochondrial network structure, protein markers of insulin signaling and mitochondrial dynamics were assessed.

Results—EPS enhanced insulin-stimulated Akt^{Ser473} phosphorylation, reduced the number of non-networked individual mitochondria and increased the mitochondrial network size in both groups (P<0.05). Mitochondrial fusion marker mitofusin 2 was significantly increased in myotubes from the lean subjects (P<0.05), but reduced in subjects with severe obesity (P<0.05). In contrast, fission marker dynamin-related protein 1 (Drp1^{Ser616}) was reduced in myotubes from subjects with severe obesity (P<0.05), but remained unchanged in lean subjects. Reductions in Drp^{Ser616} phosphorylation were correlated with improvements in insulin-stimulated Akt^{Ser473} phosphorylation following EPS (r = −0.679, P = 0.004).

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Conflicts of Interest

No potential conflicts of interest relevant to this article were reported. The present study does not constitute an endorsement by ACSM, and results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

Conclusion—Our data demonstrated that EPS induces more fused mitochondrial networks, which are associated with differential adaptations in mitochondrial dynamic processes in myotubes from lean humans and human with severe obesity. It also suggests that improved insulin signaling following muscle contractions may be linked to the reduction in Drp1 activity.

Keywords

Exercise; insulin signaling; mitochondrial adaptation; dynamin-related protein 1

Introduction

Skeletal muscle insulin resistance is a hallmark characteristic of severe obesity (body mass index [BMI] ≥ 40kg/m^2) and is a significant risk factor in the development of metabolic diseases such as type 2 diabetes (1). Lifestyle interventions, such as exercise, are imperative to prevent the deleterious effects of severe obesity on muscle insulin sensitivity. Chronic exercise training has been shown to lead to extensive adaptations in skeletal muscle, which improves whole body and skeletal muscle insulin sensitivity in both healthy and insulin-resistant populations (2–5). Despite these beneficial adaptations, skeletal muscle from lean and obese subjects often elicit differential responses in insulin sensitivity following exercise/muscle contractions (6, 7). Several studies have reported that improvement of insulin sensitivity after exercise was compromised in individuals with obesity (8–10). These data suggest that phenotypic characteristics associated with obesity (i.e., insulin resistance) may influence exercise-induced skeletal muscle adaptations.

Mitochondrial dysfunction has been linked to skeletal muscle insulin resistance (11, 12). The quality and function of the mitochondria are controlled by mitochondrial dynamic processes, fusion, and fission (13, 14). We and others have shown that mitochondrial dynamics are dysregulated in skeletal muscle from obese insulin-resistant individuals (15–17). Interestingly, it has been reported that exercise training-induced improvements in skeletal muscle insulin action are linked to an enhanced mitochondrial network structure through remodeled mitochondrial dynamics (18, 19). Additionally, similar to changes in insulin sensitivity, exercise-induced remodeling of skeletal muscle mitochondrial dynamics also seems to be different between lean humans and humans with obesity. In support of this, it has been reported that mitochondrial fusion markers were upregulated following exercise training with no changes in mitochondrial fission in skeletal muscle from healthy subjects (20, 21). In contrast, in skeletal muscle from obese individuals, there was a reduction in mitochondrial fission markers with no changes in mitochondrial fusion following exercise training (18, 22). However, it remains inconclusive whether the distinct patterns of adaptations in mitochondrial dynamics in response to exercise training is due to obesity per se or different exercise protocols among different studies. Therefore, it remains unknown whether there are differential adaptations in skeletal muscle mitochondrial network structure and regulatory proteins involved in mitochondrial dynamics in response to exercise training between lean humans and humans with severe obesity; and if so, whether these differential adaptations in mitochondrial dynamics are associated with distinct alterations in insulin sensitivity following exercise training.

In primary myotubes, prolonged electrical pulse stimulation (EPS) has been previously validated to mimic endurance-type exercise with comparable physiological adaptations (e.g., improved insulin action, lipid oxidation, and mitochondrial content) (8, 23). We chose the EPS system to compare skeletal muscle-specific adaptations in mitochondrial dynamics as it not only eliminates systemic influences (e.g., hormones and cytokines) but also ensures the same contractile stimulus applied to both lean and severely obese groups, which is often a challenge in human studies due to lower exercise capacity associated with severe obesity (24).

The purpose of the present study was to compare the effects of EPS on mitochondrial network structure and regulatory proteins governing mitochondrial dynamics in myotubes established from lean humans and humans with severe obesity and examine the association between the regulatory proteins of mitochondrial dynamics and insulin signaling following EPS. We hypothesized that 24 hours of EPS would 1) enhance mitochondrial network structure in myotubes from both groups but will induce more interconnected mitochondrial networks in primary myotubes derived from lean humans; 2) elicit differential alterations in regulatory proteins of mitochondrial dynamics in myotubes established from lean humans and humans with severe obesity, which are associated with differential improvement in insulin signaling.

Methods

Human subjects

Women with severe obesity (BMI ≥ 40 kg/m^2) and lean, healthy women (BMI < 25kg/m^2) were recruited at East Carolina University. All participants were sedentary and nonsmokers based on self-reported questionnaire. Subjects were excluded if taking medications known to alter metabolism, involved in regular exercise, or if they had been diagnosed with cardiovascular disease, diabetes, or cancer. The individual metabolic data from a subset of participants in the current study have been presented previously (15). Skeletal muscle biopsies were obtained from the vastus lateralis using a percutaneous needle biopsy with suction (25). Fasting blood samples were taken at the same time as muscle biopsies. All procedures were approved by the East Carolina University and the University of Massachusetts Boston Institutional Review Boards, and informed consent was obtained from all participants.

Human skeletal muscle cell culture

Immediately following the procedure, primary muscle cells were isolated and cultured into myoblast, as previously described (26). After isolation, myoblasts (Passage 3) were thawed and grown in a humidified environment with 5% CO_2 at 37°C on a collagen I TC flask (Greiner Bio-one, Monroe, NC). At a confluence of ~80–90%, myoblasts were subcultured onto either a 6-well type I collagen-coated plate (Corning, Corning, NY) or a 35 mm collagen-coated glass-bottom dish (MatTek, Ashland, MA) for immunoblot and fluorescence microscopy, respectively. At ~80–90% confluency, myoblasts were switched to low-serum (2% horse serum) media to induce differentiation into myotubes.

Electrical pulse stimulation

Electrical pulse stimulation was applied to mature myotubes, as previously described (8). On day 6 of differentiation, myotubes were electrically stimulated continuously for 24 hours. The 24-hr continuous stimulation protocol has been widely used by many groups to mimic chronic exercise training in muscle cell culture (8, 9, 23). It was demonstrated that this type of stimulation induces similar biological adaptations to endurance-oriented exercise training. Therefore, we chose to use this EPS protocol to keep it consistent when comparing the results. The electrical pulse generator (C-Pace EM, IonOptix LLC, Westwood, MA) provided an electrical stimulus at 11.5 V, 1 Hz, and 2 ms. Culture media was changed immediately after EPS to remove floating cells. To minimize the effects of serum starvation on mitochondrial dynamics, fresh regular differentiation media was used. Mitochondrial morphology images and cell lysates were collected 4 hours after the 24 hours of EPS. We waited a 4-hr period after contractile activity to mitigate the immediate effect of EPS on insulin signaling and mitochondrial dynamics as well as permit the myotubes to return to a basal state. We previously reported that EPS did not alter cell viability and myotube differentiation (8).

Fluorescence microscopy and quantification of mitochondrial morphology

Fluorescence microscopy was used for quantification of the mitochondrial morphology (15). Briefly, myotubes cultured on a 35mm Collagen-I coated glass-bottom dish were stained with 100nM concentration of MitoTracker™ RedFM (Thermo Fisher Scientific, Waltham, MA) diluted in differentiation media for 15 minutes. A Zeiss confocal microscopy was then used to image the myotubes mitochondrial network using a 64 × 1.4NA oil objective. Fifteen images were taken for quantification.

Images were analyzed using the mitochondrial network analysis macro (MiNA) tool developed for FIJI distribution of ImageJ as previously described (27). The tool and related documents can be found at http://github.com/ScienceToolkit/MiNA. Images were processed by an unsharp mask with a two-pixel radius, enhancement of local contrast (CLAHE), filter to remove background, binarized, and skeletonized for further analysis. Images of mitochondria morphology were used to measure the number of individual non-networked mitochondria, the number of mitochondrial networks, branch length per network, and the number of branches per network using a previously developed protocol (27). Data for the number of individual non-networked mitochondria and the number of mitochondrial networks from each image was normalized by total MitoTracker intensity per nucleus.

Citrate synthase activity

Citrate synthase (CS) activity was determined from cell homogenates prepared from the myotubes according to the supplier's protocol (Citrate Synthase Activity Colorimetric Assay, #119692. Abcam, Cambridge, MA).

Immunoblot analyses

Myotubes were harvested for protein extraction, as previously described (28). To assess insulin signaling, myotubes were incubated with 100nM insulin for 10 minutes prior to protein extraction. Equal amounts of protein were subjected to SDS-Page electrophoresis

using 4–20% gradient polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane using a Trans-Blot Transfer system (Bio-Rad, Hercules, CA). Membranes were probed with antibodies recognizing: Phosphorylated Drp1 Ser^{616} (cat# 3455, 1:500), Drp1 (cat# 8570, 1:1000), Optic Atrophy 1 (Opa1, cat# 67589, 1:1000), Citrate Synthase (cat# 14309, 1:1000), Voltage-dependent anion channel (VDAC, cat# 4661, 1:1000), Microtubule-associated proteins 1A/1B light chain 3B (LC3B, cat# 3868, 1:1000), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, cat# 2118, 1:5000), Akt (cat# 9272, 1:1000), Phosphorylated Akt Ser^{473} (cat# 9271, 1:1000), (Cell Signaling, Danvers, MA), Mitochondrial fission protein 1 (Fis1, cat# sc-376447, 1:1000), Beclin-1 (cat# sc-48341, 1:1000)(Santa Cruz Biotechnology, Dallas, TX), Mitofusin 2 (Mfn2, cat# ab56889, 1:1000), Mitofusin 1(Mfn1, cat# ab104274, 1:1000), P62 (cat# ab56416, 1:1000), Parkin (cat# ab77924, 1:1000) and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α, cat# ab106814, 1:1000) (Abcam, Cambridge, MA). Membranes were probed with an IRDye secondary antibody (Li-Cor, Lincoln, NE, 1:5000) and quantified using Odyssey CLx software (Li-Cor, Lincoln, NE). All antibodies were diluted in 5% bovine serum albumin (BSA) or 5% non-fat milk for GAPDH. Data were normalized to GAPDH protein expression.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using a repeated measure two-way analysis of variance (ANOVA) comparing the lean vs. the obese group (factor 1) on EPS vs non-EPS (factor 2) groupings. Statistical significance was set at $P<0.05$. When there were statistically group differences indicated by a significant interaction term of EPS and obesity factors, we used pairwise comparisons. Notably, traditional pairwise comparisons were not appropriate because of the limited sample sizes, therefore we employed Mann-Whitney U test for independent samples and the Wilcoxon signed-rank test for dependent samples as substitutes for pairwise comparisons. Spearman correlation analysis was used to assess linear relationships. All calculations were performed with SPSS statistical software (24.0; SPSS, Inc, Chicago, IL).

Results

Subject characteristics

Subject characteristics are presented in Table 1. Subjects with severe obesity were older and had a higher body mass, BMI, fasting plasma insulin level, and HOMA-IR score ($P<0.05$). There was no statistical difference in fasting glucose concentration between the two groups.

Insulin Signaling

EPS significantly reduced basal levels of Akt Ser473 phosphorylation and increased the insulin-stimulated Akt Ser473 phosphorylation from both lean subjects and subjects with severe obesity when compared to the non-EPS groups (Supplemental Fig. 1A. insulin signaling in HSkMCs derived from lean humans and humans with severely obesity with or without EPS, main effect of EPS, $P<0.05$). When data were expressed as relative fold change from basal conditions, both experimental groups displayed significant increases following EPS when compared to the non-stimulated controls (Supplemental Fig. 1B, insulin signaling

in HSkMCs derived from lean humans and humans with severely obesity with or without EPS, main effect of EPS, P<0.05).

Mitochondrial Morphology

We next examined the effect of 24 hours of EPS on mitochondrial morphology in myotubes from lean and severely obese humans. Representative images of the mitochondrial network from myotubes derived from lean subjects and subjects with severe obesity with and without EPS are presented (Fig. 1A). MitoTracker intensity was significantly reduced in myotubes from humans with severe obesity when compared to the myotubes from lean controls, regardless of EPS conditions (Fig. 1B, main effect of obesity, $P<0.05$). Regarding the mitochondrial network morphology, myotubes derived from subjects with severe obesity displayed a trend towards an increase in the volume of individual non-networked mitochondria (a marker of mitochondrial fragmentation) when compared to lean counterparts (Fig. 1C, $P=0.085$). Interestingly, EPS markedly reduced the volume of individual non-networked mitochondria by 35.1% and 14.9% in myotubes from lean and severely obese subjects, respectively (Fig. 1C, main effect of EPS, $P<0.05$). Furthermore, the number of non-networked individual mitochondria was negatively correlated with the fold change of insulin-stimulated Akt Ser^{473} phosphorylation over the basal state (Fig. 1E; $r = -0.517$, $P=0.028$). Mitochondrial network size, measured by the number of branches per network, did not differ between lean subjects and subjects with severe obesity but displayed an overall significant increase after EPS (Fig. 1D, main effect of EPS, $P<0.05$). No obesity or EPS effect was observed on the number of mitochondrial networks (Supplemental Fig. 2A, mitochondrial morphology in primary myotubes derived from lean humans and humans with severe obesity with or without EPS) and average branch length per mitochondrial network (Supplemental Fig. 2B, mitochondrial morphology in primary myotubes derived from lean humans and humans with severe obesity with or without EPS).

Mitochondrial Dynamics

We next evaluated the effect of EPS on regulatory proteins of mitochondrial dynamics in myotubes from lean subjects and subjects with severe obesity. The protein expression of mitochondrial fusion marker Mfn2 did not differ between groups. Rather, a significant interaction of obesity and EPS was revealed. EPS induced a significant increase (11.9%) in Mfn2 protein expression in myotubes from the lean subjects but a significant reduction (24.5%) in subjects with severe obesity (Fig. 2A, $P<0.05$). There were no differences in MFN1 (Fig. 2B) or Opa1 protein (Fig. 2C and Supplemental Fig. 3A–C, OPA1 isoforms expression in primary myotubes derived from lean humans and humans with severe obesity with or without EPS) content between any groups.

While mitochondrial fission marker Drp1 protein content did not differ between any groups (Fig. 2D), the phosphorylation of Drp1 Ser^{616} exhibited an EPS main effect (Fig. 2E, main effect of EPS, $P<0.05$). Also, a significant interaction of obesity and EPS was observed in Drp1 Ser^{616} phosphorylation (Fig. 2E, obesity, and EPS interaction, $P<0.05$). Drp1 Ser^{616} phosphorylation was significantly reduced by 22.1% in myotubes from subjects with severe obesity following EPS when compared to unstimulated myotubes (Fig. 2E, $P<0.05$). In contrast, Drp1 Ser^{616} phosphorylation virtually remained unchanged in myotubes from

lean subjects after EPS (2.1%). Similarly, there was a main effect of EPS on the ratio of phosphorylated Drp1 Ser^{616} to Drp1 protein content (Fig. 2F, main effect of EPS, $P<0.05$). While this reduction following EPS was non-significant in myotubes from lean subjects (4.7%), the myotubes from subjects with severe obesity displayed a significant 17.3% reduction in the ratio of phosphorylated Drp1 Ser^{616} to Drp1 protein content after EPS when compared to unstimulated myotubes (Fig. 2F, $P<0.05$). Mitochondrial fission adaptor protein Fis1 did not differ in myotubes established from lean subjects and subjects with severe obesity. However, there was a significant interaction of obesity and EPS, which revealed a significant increase (26.9%) in myotubes from lean subjects following EPS (Fig. 2G, $P<0.05$).

Mitochondrial Biogenesis, Mitophagy, and Content

Along with mitochondrial dynamics, we examined protein markers involved in mitochondrial turnover as mitochondrial morphology and content are influenced by changes in the biogenesis of new mitochondria, as well as degradation of damaged mitochondria through selective autophagy (mitophagy). Although there was no EPS or obesity main effect on the protein content of mitochondrial biogenesis regulator PGC1α, a significant interaction of obesity and EPS emerged (Fig. 3A, $P<0.05$). EPS induced a significant increase (10.7%) in myotubes from lean subjects that was diminished in myotubes from subjects with severe obesity (Fig. 3A, $P<0.05$). The protein content of mitophagy marker Parkin also did not differ between myotubes from lean subjects and subjects with severe obesity but was significantly reduced by 27.9% in myotubes from humans with severe obesity after EPS while remaining unchanged in lean humans (Fig 3B, $P<0.05$). There was no significant obesity or EPS effect on autophagy markers Beclin-1, P62, and LC3 II:I ratio (Fig. 3C–E) or mitochondrial content markers citrate synthase content (Fig. 3F) and activity (Supplemental Fig. 3D, Citrate synthase activity in primary myotubes derived from lean humans and humans with severe obesity with or without EPS) and VDAC content (Fig. 3G).

Correlations between Skeletal Muscle Insulin Signaling and Various Mitochondrial Quality Control Proteins

There was a negative correlation between the percent changes in the phosphorylation of Drp1 Ser^{616} and fold change of insulin-stimulated phosphorylation of Akt Ser^{473} over the basal state (Fig. 4A; $r = -0.679$, $P=0.004$). No correlation was found between other protein markers of mitochondrial dynamics and mitophagy and the fold change of insulin-stimulated phosphorylation of Akt Ser^{473} over the basal state (Fig. 4B and C).

Discussion

To our knowledge, this is the first study to compare the skeletal muscle-specific adaptations in mitochondrial network morphology and regulatory proteins involved in mitochondrial dynamic processes in response to contractile activity. Our data revealed that 24 hours of EPS resulted in an improved mitochondrial network structure towards fusion in myotubes derived from lean humans and humans with severe obesity, which was associated with improved skeletal muscle insulin signaling. While exercise has been shown to improve mitochondrial dynamics and mitochondrial network structure (22, 29), our study provides

novel insight that the enhanced mitochondrial network structure following exercise/muscle contraction is mediated by distinct alterations in regulatory proteins of mitochondrial dynamics in myotubes from the lean humans and humans with severe obesity. These data suggest that exercise/muscle contraction may improve the mitochondrial network structure in skeletal muscle through different mitochondrial dynamic processes in lean humans and humans with severe obesity and insulin resistance. In addition, among regulatory proteins of mitochondrial quality control, only the phosphorylation of mitochondrial fission protein Drp1 Ser^{616} exhibited a significant correlation with the improvement in insulin signaling following EPS independent of body weight status, which suggests that Drp1-mediated mitochondrial fission may be an essential modulator of exercise-induced improvement in skeletal muscle insulin sensitivity.

Exercise has been demonstrated to improve skeletal muscle insulin signaling *in vivo* from lean and obese insulin-resistant humans (30, 31). These changes have been associated with improvements in mitochondrial morphology, with elongated, interconnected mitochondrial networks coinciding with improved insulin sensitivity (19). In line with this, our data demonstrated that EPS, an *in vitro* model that mimics muscle contraction during exercise, enhanced the mitochondrial structure towards more fused networks, which was correlated with improved skeletal muscle insulin signaling in primary myotubes from both lean humans and humans with severe obesity. It is noteworthy that previous studies have observed a blunted improvement in insulin signaling in response to EPS in subjects with obesity when compared to lean counterparts (8, 9), which is contradictory to our finding. One possible explanation for this discrepancy may involve the media used before insulin stimulation, as we did not utilize starvation media after EPS, as Park and colleagues performed (8). Starvation has been shown to enhance muscle cells' response to insulin stimulation (e.g., insulin-stimulated Akt phosphorylation, glucose transport)(32). Thus, the magnitude of the difference in Akt signaling in myotubes between lean humans and humans with severe obesity may have been masked. However, starvation may also have an independent effect on mitochondrial dynamics, with some reports revealing enhanced mitochondrial fission under starvation conditions (33). Therefore, in order to evaluate the effects of EPS on mitochondrial dynamics without inducing additional stress, we decided not to use serum starvation after EPS in this study. Future work is warranted to examine how starvation affects the beneficial effects of contractile activity on the metabolic characteristics of skeletal muscle. Nonetheless, our findings indicate that contractile activity alone may be sufficient to enhance the mitochondrial network structure, leading to improvements in insulin signaling in skeletal muscle.

The mitochondrial network structure is controlled by mitochondrial dynamic processes, including fusion and fission (13, 14). Fusion is mediated by two outer mitochondrial membrane proteins Mfn1 and Mfn2, which tethers two adjacent mitochondria together (34). Once the outer membrane is fused, dynamin-related protein Opa1 allows the fusion of the inner membrane (35). In the present study, EPS in myotubes from lean insulin-sensitive individuals stimulates Mfn2-mediated mitochondrial fusion (Fig. 2A). This is in accord with previous *in vivo* exercise training interventions in humans that showed aerobic exercise increases Mfn2 protein expression in sedentary healthy individuals (20, 21). In contrast to our hypothesis, 24 hours of EPS reduced the expression of mitochondrial fusion protein

Mfn2 in myotubes from humans with severe obesity (Fig. 2A). This finding suggests that Mfn2 may be a dispensable determinant of mitochondrial fusion and network morphology. In support of this notion, it has been reported that only Mfn1 and Mfn2 double knockout in skeletal muscle, but not Mfn1 or Mfn2 single knockout, resulted in severe mitochondrial fragmentation (36), which may result in part due to the compensatory mechanism of Mfn1 and Mfn2. Collectively, these data suggest that there is a vital role of mitochondrial fusion in the contractile-mediated remodeling of the mitochondrial network in sedentary healthy individuals, but not in humans with severe obesity.

Conversely, mitochondrial fission is regulated by cytoplasmic protein Drp1, which is activated when phosphorylated at the serine residue 616. When activated, Drp1 is translocated to the mitochondria to initiate mitochondrial fission (37, 38). We have previously shown that Drp1-mediated mitochondrial fission is increased in humans with severe obesity and insulin resistance, and the reduction improved the balance in mitochondrial dynamics, restoring the mitochondrial network morphology and insulin action (15). In the current study, myotubes from individuals with severe obesity had a significant reduction in the phosphorylation of Drp1 Ser^{616} following EPS, which was correlated with the improvement in insulin signaling (Fig. 4A). Similarly, *in vivo* exercise training has been reported to reduce skeletal muscle phosphorylation of Drp1 Ser^{616}, which was also associated with improved insulin sensitivity in humans with severe obesity and insulin resistance (18). Altogether, these data suggest that contractile activity may restore the mitochondrial network structure in skeletal muscle from humans with severe obesity and insulin resistance by reestablishing the balance between mitochondrial fusion and fission through the reduction of Drp1-mediated mitochondrial fission. However, whether Drp1 is required for such changes in mitochondrial dynamics and network morphology induced by EPS is unknown and currently being investigated in our laboratory using a genetic approach of Drp1 depletion in myotubes from individuals with obesity.

We further performed correlational analysis between various regulatory proteins in mitochondrial quality control and skeletal muscle insulin signaling following EPS. Our data demonstrated that the reduction of Drp1 Ser^{616} phosphorylation was the only marker that was highly correlated with the improvement in insulin signaling in myotubes derived from both lean humans and humans with severe obesity following EPS. Our data corroborate with the findings from a previous study (18) and extends to the lean, healthy humans. Together, it suggests that Drp1-mediated mitochondrial fission may be an important modulator of exercise training-induced improvement in skeletal muscle insulin sensitivity. Indeed, this notion is supported by a recent mechanistic study in which the authors found that skeletal muscle-specific Drp1-knockout mice exhibited blunted improvements in exercise performance following exercise training (39). Future studies using genetic modification tools to inhibit Drp1 in muscle cell culture and/or animal models should be warranted to assess the mechanistic role of Drp1 in exercise-induced beneficial adaptations in skeletal muscle under obese insulin-resistant conditions.

It has been reported that mitophagy and Drp1-mediated mitochondrial fission are tightly related (40). In the current study, EPS reduced mitochondrial fragmentation through the reduction of Drp1-mediated mitochondrial fission, which also resulted in a reduction in

protein expression of mitophagy marker Parkin in myotubes from humans with severe obesity. This is in agreement with a previous study showing that *in vivo* exercise training reduced mitophagy in skeletal muscle from humans with obesity and insulin resistance (22). Although the precise mechanism responsible for the reduced Parkin expression is unclear, one can speculate that the need for mitophagy to remove damaged mitochondria may be reduced after 24 hours of EPS due to reduced mitochondrial fission and improved mitochondrial network structure and integrity. However, further research is necessary to elucidate the effect of EPS on skeletal muscle mitophagy flux, which is one of the limitations of the current study.

In the current study, we found no increase in PGC1α expression after 24 hours of EPS in myotubes from individuals with severe obesity. This finding is in disagreement with our previous study (8). Further, we found that the magnitude of enhancement in PGC1α expression was reduced by ~50% in myotubes from lean individuals when compared to our recent study (8). Taken together, since the same EPS protocol and cell culture models were used in both studies, we speculate that the blunted response to EPS may be due to the use of regular culture media without serum depletion after EPS in the present study that potentially masked the EPS effect. This hypothesis was corroborated by findings from two previous studies that reported increased PGC1α expression following EPS, both of which utilized serum-depleted media during and after EPS (41, 42). In contrast, Nikolic et al., reported no significant change of PGC1α mRNA expression in primary human myotubes following EPS while using regular culture media (23). Therefore, these findings suggest that serum (e.g., BSA, Horse Serum) in culture media may exert influence on PGC1α expression. Alternatively, we previously found that there is substantial individual variation in PGC1α in response to exercise/muscle contractions in HSkMCs (8, 43), which may also contribute to the inconsistency of PGC1 α protein expression in humans with obesity. Given the discrepant findings in Akt phosphorylation and PGC1α expression following EPS, future studies should consider using serum-depleted culture media to maximize the adaptations to EPS in human myotubes.

Our study has some limitations. First, there was an age difference between lean and obese subjects. While aging has been shown to reduce mitochondrial function and adaptations to exercise/muscle contraction (44, 45), the age difference in our study (28 vs. 34 yrs) is much smaller than those studies the age is much older in those studies (~20. vs. ~70 yrs). In addition, a previous study reported that obesity is more pertinent for changes in mitochondrial dynamics markers rather than chronological age per se (46)., We also performed statistical analysis with age as a covariate. We found that the differential changes between lean and obese humans in mitochondrial dynamics did not attenuate, although significant mitochondrial morphology changes after EPS was diminished. Nonetheless, future research is needed to determine the extent to which aging has on the remodeling of the mitochondrial network structure and dynamics following EPS. Second, we did not collect quantifiable physical activity data from subjects to ensure their physical activity levels are matched. However, given both groups are sedentary, we expect the differences in their physical activity levels are minimal. Lastly, while we and others have demonstrated that prolonged EPS (i.e., 24 hrs) imitates contractile muscle activity during exercise training and induces similar metabolic adaptations following long-term exercise training (8, 47),

there are inevitable limitations of this *in vitro* system (e.g., lack of motor neuron activation, predominantly produce ATP via aerobic glycolysis). Therefore, future research needs to repeat similar studies in human subjects.

In conclusion, our study provides evidence that 24 hours of EPS, a model of muscle contraction, induces a more interconnected mitochondrial network in primary myotubes from both lean humans and humans with severe obesity. However, myotubes from these humans exhibit distinct adaptations in mitochondrial dynamics to EPS. Specifically, EPS promotes a profusion environment by increasing mitochondrial fusion protein Mfn2 in myotubes from lean humans, while reducing mitochondrial fission protein Drp1 Ser^{616} phosphorylation in humans with severe obesity. Interestingly, the reduction in Drp1 Ser^{616} phosphorylation is associated with the improvement in insulin signaling in myotubes independent of BMI, suggesting that Drp1 may be a critical mediator in the regulation of skeletal muscle insulin signaling in response to exercise/muscle contractions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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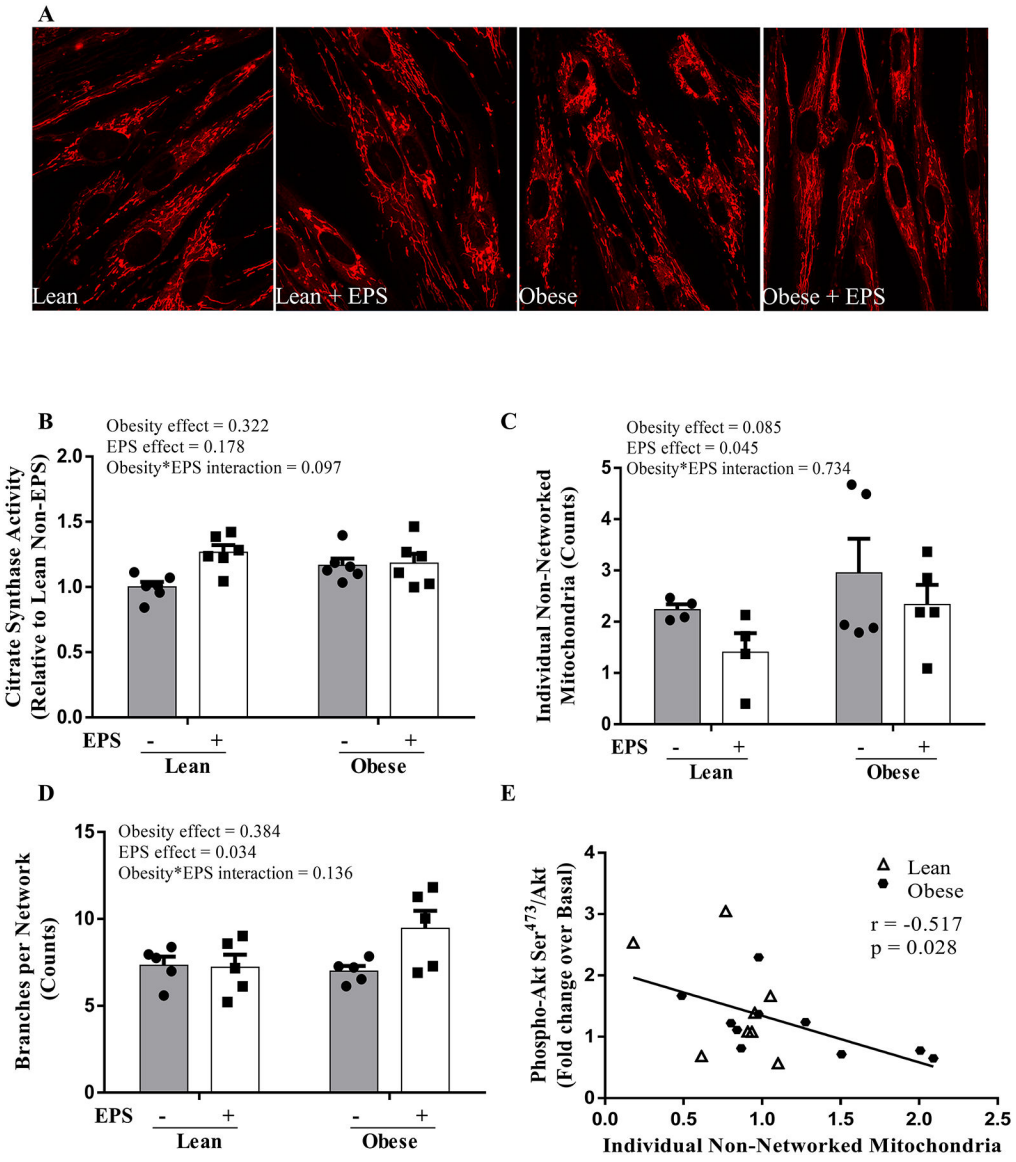

Fig. 1.

Mitochondrial morphology in primary myotubes derived from lean humans and humans with severe obesity with or without EPS. **A)** Representative images of myotubes stained with MitoTraker™ RedFM. **B)** Mitotracker intensity per nucleus. **C)** Number of individual non-networked mitochondria. **D)** Number of branches per network (network size). **E)** Correlation between the number of individual non-networked mitochondria and the fold change of insulin-stimulated phosphorylation of Akt Ser^{473}/Akt over basal state. Data are presented as mean ± SEM. n=4–6/group.

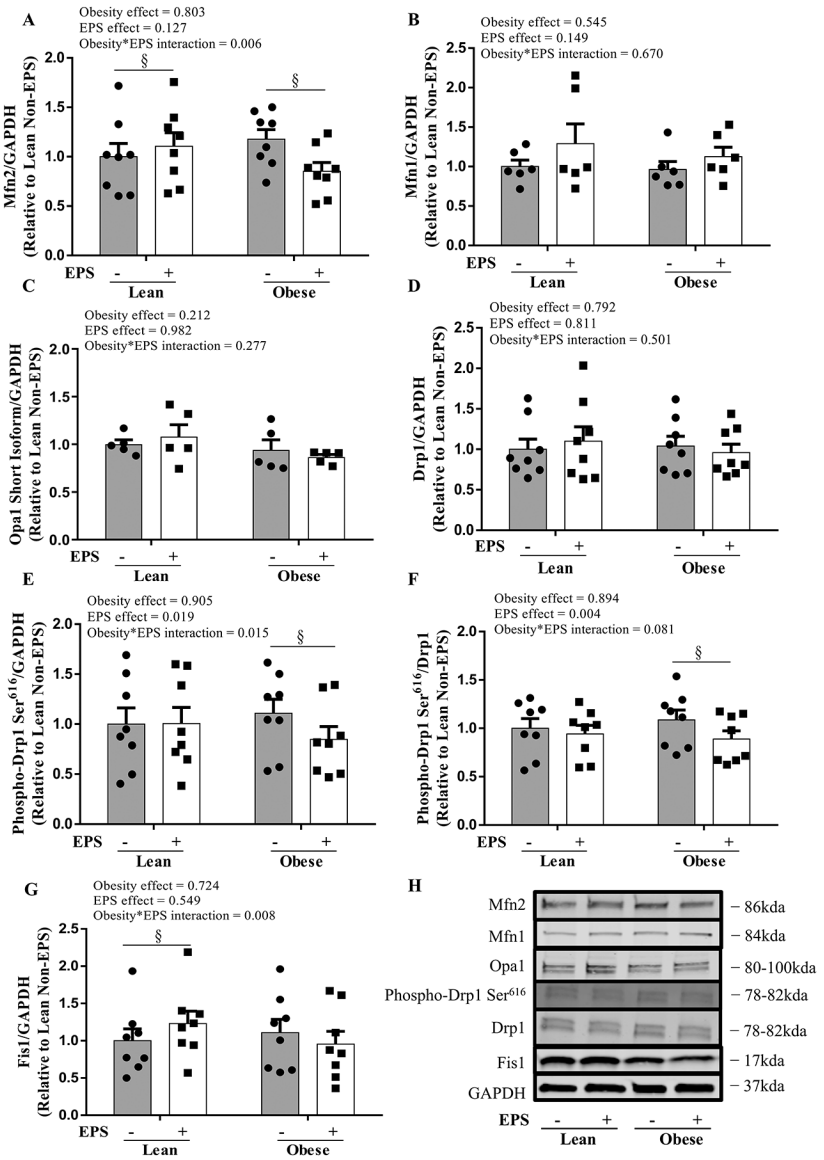

Fig. 2.

Protein expression of mitochondrial dynamics markers in primary myotubes derived from lean humans and humans with severe obesity with or without EPS. **A)** Mfn2. **B)** MFN1. **C)** Opa1. **D)** Drp1. **E)** Phosphorylation of Drp1 Ser^{616}. **F)** Ratio of the phosphorylation of Drp1 Ser^{616} and Drp1. **G)** Fis1. **H)** Representative immunoblots for mitochondrial dynamic markers. Data are presented as mean ± SEM. n=5–8/group. § P<0.05 vs. respective non-EPS.

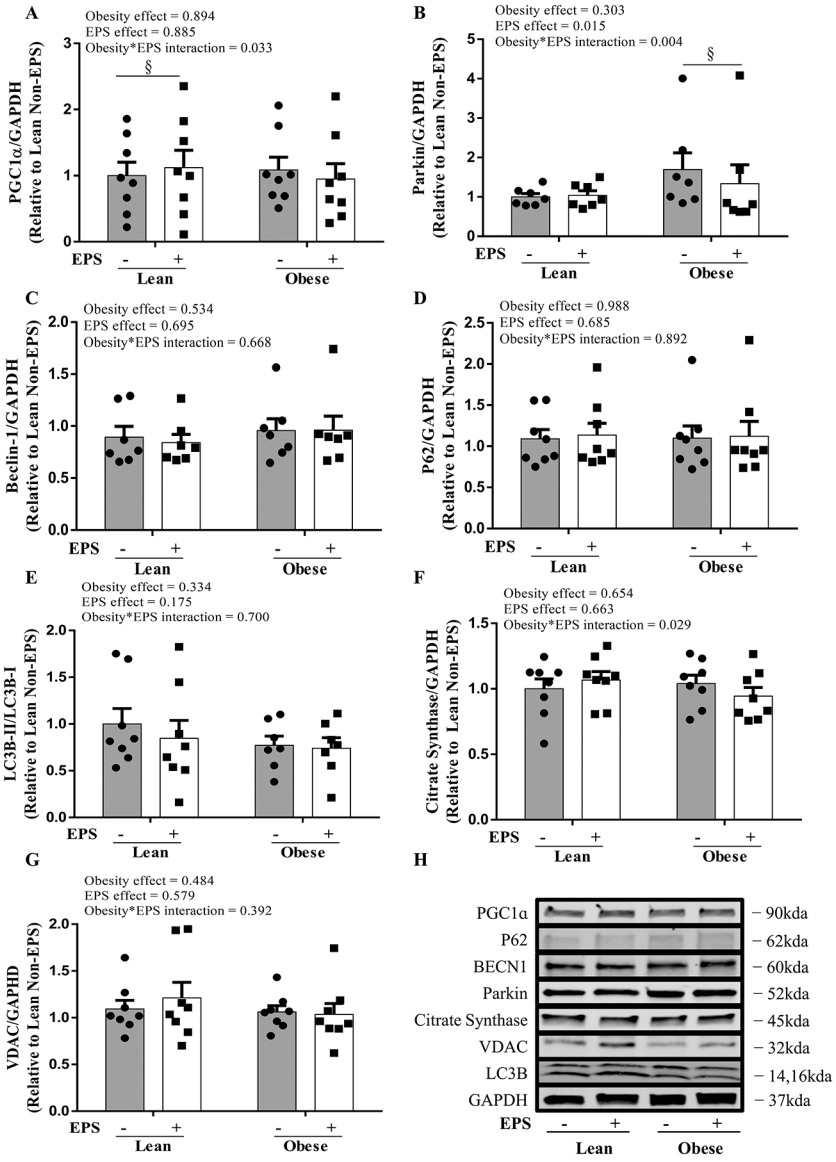

Fig. 3.

Protein expression of mitochondrial biogenesis, mitophagy, and content markers in primary myotubes derived from lean humans and humans with severe obesity with or without EPS. **A)** PGC1α. **B)** Parkin. **C)** Beclin-1. **D)** P62. **E)** Ratio of LC3B II:I. **F)** Citrate Synthase. **G)** VDAC. **H)** Representative immunoblots for mitochondrial biogenesis, mitophagy, and content markers. Data are presented as mean ± SEM. n=7–8/group. § P<0.05 vs. respective non-EPS.

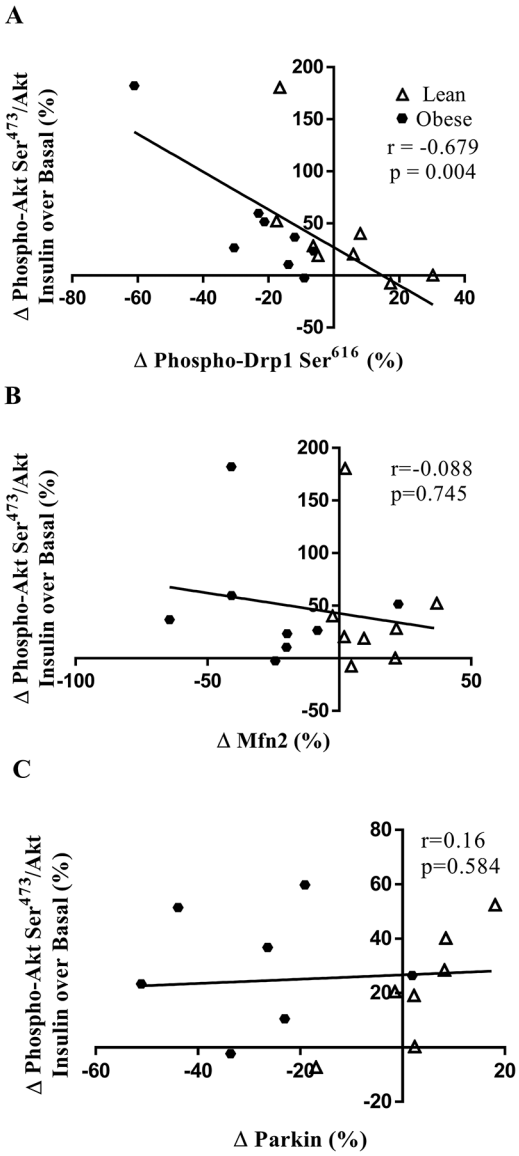

Fig. 4.

A) Correlation between percent change in the phosphorylation of Drp1 Ser^{616} and the fold change of insulin-stimulated phosphorylation of Akt Ser^{473}/Akt over basal state following EPS (r = −0.679, P = 0.004). **B)** Correlation between percent change in Mfn2 and the fold change of insulin-stimulated phosphorylation of Akt Ser^{473}/Akt over basal state following EPS (r = 0.088, P = 0.745). **C)** Correlation between percent change in Parkin and the fold change of insulin-stimulated phosphorylation of Akt Ser^{473}/Akt over basal state following EPS (r = 0.16, P = 0.584).

Table 1.

Subject characteristics

	Lean (*n* = 8)	**Severely Obese (*n* = 8)**	**P-value**
Age	26.3 ± 3.2	34.9 ± 3.4	0.04
Weight (kg)	64.3 ± 3.9	134.9 ± 7.7	0.001
BMI (kg/m^2)	23.8 ± 1.67	45.5 ± 2.26	0.001
Fasting Glucose (mg/dL)	84.4 ± 1.78	91.0 ± 2.73	0.057
Fasting Insulin (uIu/mL)	5.69 ± 0.65	14.8 ± 1.27	0.001
HOMA-IR	1.19 ± 0.14	3.33 ± 0.30	0.001

Data are presented as mean ± SEM

BMI, body mass index

HOMA-IR, Homeostatic Model Assessment of Insulin Resistance (fasting insulin concentration[μIU/mL) x fasting glucose concentration [mg/dl]/ 405)