Compensatory increases in nuclear PGC1α protein are primarily associated with subsarcolemmal mitochondrial adaptations in ZDF rats

Running title: Nuclear PGC1α and mitochondrial sub-populations

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**Objective:** We examined in insulin resistant muscle if, in contrast to long-standing dogma, mitochondrial fatty acid oxidation is increased, and whether this is attributed to an increased nuclear content of peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α) and the adaptations of specific mitochondrial sub-populations.

**Research design and methods:** Skeletal muscles from male control and ZDF rats were used to determine; 1) intramuscular lipid distribution, 2) subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial morphology, 3) rates of palmitate oxidation in SS and IMF mitochondria, and 4) the sub-cellular localization of PGC1α. Electrotansfection of PGC1α-cDNA into lean animals tested the notion that increased nuclear PGC1α preferentially targeted SS mitochondria.

**Results:** TEM analysis revealed that in ZDF animals the number (+50%), width (+69%) and density (+57%) of SS mitochondria were increased (P<0.05). In contrast, IMF mitochondria remained largely unchanged. Rates of palmitate oxidation were ~40% higher (P<0.05) in ZDF SS and IMF mitochondria, potentially as a result of the increased PPAR-targeted proteins, carnitine palmitoyltransferase-I and FAT/CD36. PGC1α mRNA and total protein were not altered in ZDF animals, however a greater (~70%; P<0.05) amount of PGC1α was located in nuclei. Over-expression of PGC1α only increased SS mitochondrial oxidation rates.

**Conclusions:** In ZDF animals, intramuscular lipids accumulate in the IMF region (increased size and number), and this is primarily associated with increased oxidative capacity in SS mitochondria (number, size, density and oxidation rates). These changes may result from an increased nuclear content of PGC1α, as under basal conditions, over-expression of PGC1α appears to ‘target’ SS mitochondria.
Skeletal muscle, due to its mass and high rate of glucose disposal, is an important tissue in the development of insulin resistance. While the etiology of skeletal muscle insulin resistance remains uncertain, it has been proposed that an accumulation of intramuscular lipids, particularly diacylglycerol (DAG) (1) and ceramides (2; 3), may attenuate the insulin-signaling cascade. Kelley (4; 5) and others (6-8) have speculated that a dysfunction in mitochondrial fatty acid oxidation, due to either a reduction in the number of mitochondria and/or a reduction in their intrinsic activity, may account for intramuscular lipid accumulation. However, support for mitochondrial dysfunction as a mechanism to induce lipid accumulation and insulin resistance has begun to wane, as recent reports have shown that despite the presence of skeletal muscle insulin resistance in animals (9-11) and in humans (12-14), the capacity for fatty acid oxidation by mitochondria is not down-regulated. In addition, reductions in mitochondrial content have not been consistently observed in insulin resistant muscle (10; 15). Clearly, whether compromised fatty acid oxidation can account for intramuscular lipid accumulation has been questioned. However, whether there are alterations in fatty acid oxidation in sub-populations of mitochondria with insulin resistant muscles has received little attention.

Mitochondria in skeletal muscle are present in two distinct locations, below the sarcolemma and between the myofibrils, and are known as subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, respectively. Their sub-cellular distribution and lipid accumulation near IMF mitochondria (16) suggest that the metabolic roles of SS and IMF mitochondria may differ. Functional studies have shown mitochondrial sub-populations do not respond uniformly to selected physiological stimuli (17; 18), and therefore it is possible that divergent metabolic responses could occur in mitochondrial sub-populations from insulin resistant muscle, particularly with respect to fatty acid oxidation. Although Kelley and colleagues suggested, based on changes in mitochondrial size and enzymatic ratios (5) in insulin resistant muscle, that there was a preferential dysfunction in SS mitochondrial fatty acid oxidation, it is unknown whether these parameters scale with rates of mitochondrial fatty acid oxidation. Studies in insulin resistant muscles of obese Zucker rats indicated that fatty acid oxidation was increased in red skeletal muscle SS not IMF mitochondria (10). Thus, the muscle fiber type as well as the sub-population of mitochondria may influence fatty acid oxidation in insulin resistant muscle. Whether such differences are related to peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), which is well-known to differ in red and white muscle, is uncertain.

PGC1α, a transcriptional co-factor regulating mitochondrial biogenesis, has been linked to mitochondrial proliferation (19; 20), and lipid oxidation (20). This co-activator may therefore represent an important mechanism in the context of mitochondrial oxidative capacity and the development of insulin resistance. Indeed, PGC1α mRNA is reduced in selected models of insulin resistance (21; 22), although others have shown that PGC1α mRNA (6; 23) and protein contents (23; 24) are not reduced with insulin resistance, and that PGC1α over-expression in muscle has not improved insulin sensitivity (25). In contrast, more recent work in healthy muscle has shown that over-expression of PGC1α within physiologic limits improved insulin-stimulated glucose transport and selected steps in the insulin-signaling cascade (20). Interestingly, PGC1α also upregulated fatty acid oxidation, but only in SS
mitochondria (20). This suggested that PGC1α preferentially targeted this mitochondrial sub-population. Since PGC1α can be induced to translocate from the cytosol into nuclei (26), it is possible that the subcellular distribution of PGC1α in insulin resistant muscles is altered, which may contribute to changes in fatty acid utilization in SS mitochondria. This remains to be determined.

In the present study we have examined in red and white muscles of Zucker diabetic fatty (ZDF) rats, a model of type 2 diabetes (27), a) lipid droplet distribution, b) morphological as well as functional differences in SS and IMF mitochondria, and whether these mitochondrial changes are c) associated with changes in the nuclear content of PGC1α. Our results demonstrate that in ZDF rats 1) SS mitochondrial size, number and oxidation rates are preferentially increased in accordance with 2) an increased nuclear content of PGC1α protein that preferentially targets SS mitochondria.

METHODS

Animals: Male control (n=5, weighing ~400g) and ZDF (n=5, weighing ~400g) rats were purchased from Charles River. Animals were housed in a climate and temperature controlled room, on a 12:12 hour light-dark cycle, with rat chow and water provided *ad libitum*. 24-week-old animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg kg⁻¹), using cardiac puncture blood was sampled from nonfasted animal, and subsequently muscles were rapidly excised for various measurements (described below). For the PGC1α electrotansfection experiments Sprague-Dawley rats (~300g) from our breeding colony were used. All facets of this study were approved by the University of Guelph Animal Care Committee.

Blood metabolite assays: Serum samples were analyzed for glucose using a spectrophotometric method (Sigma, St. Louis), insulin by RIA using a rat-specific antibody (Linco, St. Charles, MO, USA) and fatty acid concentrations using a spectrophotometric procedure (Wako Chemicals, Richmond, VA, USA).

Transmission electron microscope analysis of mitochondria: Samples from the red and white portions of the tibialis anterior (TA) muscle were rapidly immersed in a fixing buffer (2.5% glutaraldehyde, 1.0% paraformaldehyde in PBS) and incubated at 4°C overnight. Tissue was then washed 3 times in 0.1 M Hepes and subsequently suspended in 1.0% osmium tetroxide for 4 hours. Thereafter, tissue was washed 3 times in 100 mM Hepes and suspended in 2% uranyl acetate for 3 hours, washed 3 times in 0.1% Hepes, and dehydrated by incubating in a graded ethanol series (ie. 25, 50, 75, 95 and twice in 100% ethanol). Tissue was infiltrated with resin by suspending in 50/50 ethanol/resin (London Resin Company White) for 4 hours on a rotating mixer, and subsequently suspended in pure resin for 4 hours on a rotating mixer. Tissue was then placed in an embedding capsule containing pure resin, and incubated overnight at 60°C to polymerize. Sections (100 nm) were cut and laid onto 200 mesh formvar/carbon copper grids and then stained with 2% uranyl acetate and Reynold’s lead citrate. Samples were viewed on a Philips CM 10 transmission electron microscope (TEM) at 80 kV, and images obtained with an Olymous/SIS Morada CCD camera using the Olympus/SIS iTEM software. Images were analyzed using the measurement tools provided by this software. Individual lipid droplet and mitochondrial sizes were determined repeatedly and averaged for a given image taken at 25,000 x magnification. Mitochondrial sub-population densities were determined within a defined region (10 µm² area) at a minimum of 3 locations within an image taken at 5,800 x magnification, similar
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to methodologies previously published (28). Several fibers (3-5) were imaged for each animal. Mitochondrial number and density were not determined in white muscle because of their infrequent presence within our defined region.

Triacylglycerol concentrations: Intracellular triacylglycerol concentrations were determined biochemically as previously described (29). Lipids were extracted in a standard Folch solution and subsequently separated by high-performance thin-layer chromatography and quantified against known standards.

Mitochondrial enzymatic activities: Muscle samples were homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer and CS activity was assayed spectrophotometrically at 412 nm (37˚C) (30).

Mitochondrial DNA: mtDNA was determined using real-time PCR, as previously reported (10), using the following primers: NADH dehydrogenase subunit 5 forward, 5'-GCAGCCACAGGAAAATCCG-3'; and reverse, 5'-GTAGGGCAGAGACGGGAGTTG-3', the solute carrier family 16 member1 forward, 5'-TAGCTGGATCCCTGATGCGA-3'; and reverse, 5'-GCATCAGACTTCCAGCTTCC-3'.

Isolation of mitochondria from skeletal muscle: Differential centrifugation was used to obtain both SS and IMF mitochondrial fractions from the red and white portions of the TA, as we have previously published (20; 31). Mitochondria were further purified using a Percoll gradient for Western blotting analysis.

Western blotting: Whole muscle homogenates were prepared as previously described (20) (n=4). Isolated mitochondria (5 µg), whole muscle (30 µg), nuclear extract (45 µg) and cytosolic protein (25 µg) were analyzed. Samples were separated by electrophoresis on 7.5-12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The MO-25 antibody has been used previously (10; 12) to detect FAT/CD36 (gift from Dr. N.N. Tandon), and commercially available antibodies were used to detect cytochrome c oxidase complex IV (COXIV; Invitrogen, Burlington, ON, Canada), PGC1α (Calbiochem, La Jolla, CA, USA), lactate dehydrogenase (LDH; Abcam; Cambridge, MA, USA) and histone H2B (H2B; Abcam; Cambridge, MA, USA). Blots were visualized and quantified using chemiluminescence and the ChemiGenius 2 Bioimaging system (SynGene, Cambridge, UK).

Carnitine palmitoyltransferase I activity: The forward radioisotope assay was used for the determination of CPTI activity as described by McGarry et al. (32) with minor modifications as we have previously reported (20; 33). Briefly, the assay was conducted in the presence of 75 µM P-CoA ([3H]carnitine Amersham Bioscience, Buckinghamshire, England). Palmitoyl-[3H]carnitine was extracted in water-saturated butanol and the radioactivity determined.

Mitochondrial palmitate oxidation: Palmitate oxidation was measured in the presence [1-14C]palmitate, as previously described (34). Briefly, gaseous 14CO2 production and isotopic fixation were determined following a 30 min reaction at 37˚C in the presence of 77 µM palmitate.

PGC-1α mRNA: PGC-1α mRNA was determined in the tibialis anterior muscle using real-time PCR as we have previously reported (20). The following primer sets were used: PGC-1α Forward 5'-CAATGAGCCCCGGAACATAT-3', PGC-1α Reverse 5'-CAATCCGTCTTCATCCACAG-3'; 18S Forward 5'-GGTGGTTTTCGGGAACTGAGGC-3', 18S Reverse 5'-GTCGCGCATCGTTTATGGGTCG-3'.
Isolation of nuclear extracts:
Nuclear extraction was performed using a commercial kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s specifications, as done previously (26). Harvested muscles were immediately placed in 750 µL of phosphate buffered saline (PBS), minced and briefly homogenized. Cytosolic and nuclear extraction was performed using supplied reagents supplemented with 1mM sodium orthovanadate, 1mM PMSF, and 10µg/mL of pepstatin A, aprotinin, and leupeptin. Isolated nuclei were washed 15X in alternating PBS, or PBS supplemented with 0.1% Nonidet P-40. To confirm the purity of nuclear extracts, both fractions were analyzed by Western Blotting for cytosolic (LDH) and nuclear proteins (H2B).

Electrotransfection of PGC1α:
Electrotransfection experiments were performed as described by us (20; 35) and others (36; 37), with minor modifications. The PGC1α expression construct (gift from Dr. B. Spiegelman, Harvard University, Boston, MA, USA) was produced by subcloning the PGC1α coding sequence into a mammalian expression vector (pcDNA 3.0) (Invitrogen, Burlington, ON, Canada). Plasmid stocks were produced by large scale plasmid isolation from transformed Escherichia coli cells (One-Shot® Invitrogen, San Diego, CA) using commercially available kits (GIGA-prep kits, Invitrogen, Burlington, ON, Canada).

For electrotransfection, animals were anesthetized with isoflurane, and the TA muscle injected with 100 µL of hyaluronidase (0.15U/µL in 50% vol/vol saline). Two hours later muscles were electrotransfected with i) PGC1α-pcDNA plasmid (500µg PGC1α in 50% vol/vol saline), or ii) empty pcDNA3.0 plasmid (500µg pcDNA in 50% vol/vol saline) as described previously (20; 38; 39). Thereafter, rats were provided with an analgesic (Temgesic) and allowed to recover for 2 weeks (40).

Statistics: All data are presented as the mean ± S.E.M. Unpaired t-tests, paired t-tests and two-way analysis of variance were used where appropriate. When significance was obtained, a Fisher’s LSD post hoc analysis was completed. Statistical significance was accepted at P<0.05.

RESULTS

Blood characteristics: In ZDF animals, serum insulin concentrations were markedly lower (4.8±0.8 vs 1.5±0.4 ng/ml; P<0.05), while glucose (14±1 vs 37±1 mM) and fatty acid (0.3±0.1 vs 0.9±0.2 mM) concentrations were higher (P<0.05).

Lipid droplet characteristics: In red ZDF muscle the area of individual lipid droplets was +47% larger than in red control muscle (P<0.05; Fig. 1A). In addition, TEM images revealed a large number of lipid droplets in red ZDF muscle, which were largely in contact with IMF mitochondria, while the SS mitochondrial region was devoid of lipids (Fig. 1B and 3). Biochemical extraction revealed that IMTG concentration was ~3 fold higher (P<0.05) in ZDF muscle (Fig. 1C). In white muscle lipid droplets were not quantifiable.

Mitochondrial morphology in ZDF animals:

Mitochondrial area: In red and white ZDF muscles, SS mitochondrial size was not altered (Fig. 2A and B). However, compared to control animals, red IMF mitochondria in ZDF animals were +35% larger (P<0.05; Fig. 2A), while white muscle IMF mitochondria in ZDF animals were -37% smaller (P<0.05; Fig. 2B).

Mitochondrial number: In red muscle, the number of SS mitochondria in ZDF animals was markedly increased (+50%; P<0.05; Fig. 2C), but the number of IMF mitochondria was unaltered (Fig. 2C). In white muscle this parameter was not determined because of the scarcity and diffuse nature of mitochondria in this tissue (Fig. 3).
Mitochondrial Density: The width of the red muscle SS mitochondrial sub-population was +69% larger (P<0.05; Fig. 4A), and the density of red muscle SS mitochondria was +57% larger (P<0.05; Fig. 4B) in ZDF animals. In contrast, the density of IMF mitochondria was not different (P>0.05) when analyzed in regions devoid (Fig. 4C) or abundant (Fig. 4D) in lipid droplets.

Enzyme activities, protein expression and mtDNA

Whole muscle: Whole muscle COXIV protein (Fig. 5A) and CS maximal activity (Fig. 5B) were not different in ZDF animals. The amount of mtDNA was also not altered in ZDF rats in either red (Control; 2.23±0.25 vs. ZDF; 2.13±0.63 arbitrary units) or white (Control; 1.31±0.23 vs. ZDF; 0.91±0.28 arbitrary units) muscles, suggesting mitochondrial content was unaltered. In contrast, FAT/CD36 was increased +2.4 fold in red, and +50% in white, ZDF muscles (Fig. 5C).

Isolated mitochondria: In isolated mitochondria, COXIV protein was constant across all mitochondrial sub-populations (Fig. 5D and G), while in contrast, CPTI activity (Fig. 5E and H) and FAT/CD36 protein (Fig. 5F and I) were greater (P<0.05) in all mitochondrial sub-populations in ZDF animals.

Mitochondrial palmitate oxidation

In both red and white muscle, ZDF animals displayed higher (P<0.05) rates of palmitate oxidation in isolated SS (~40%) and IMF (~40%) mitochondria (Fig. 6A and B). Increased ratios of 14C - acid soluble intermediates (ASM) to 14CO2 have previously been used to infer incomplete oxidation of 14C-palmitate (41). In red IMF mitochondria the ASM/CO2 ratio was unchanged. In contrast, this ratio was decreased (~30%; P<0.05) in red muscle SS mitochondria, and in white muscle SS and IMF mitochondria, indicating greater ‘complete’ fatty acid oxidation in these mitochondria (data not shown).

PGC1α sub-cellular location

PGC1α mRNA: In red muscle, PGC1α mRNA was not different (P>0.05) between control and ZDF animals (Fig. 7A). In control animals, PGC1α mRNA was -54% lower (P<0.05) in white, compared to red muscle (Fig. 7A). In contrast, this fiber type difference was lost in ZDF animals as a result of the increase (P<0.05) in white muscle PGC1α mRNA (Fig. 7A).

PGC1α total protein: Whole muscle PGC1α protein was not altered in ZDF animals in either red or white muscles (Fig. 7B). White muscle, compared to red muscle, had less (P<0.05) PGC1α protein in both control (-19%) and ZDF (-23%) animals (Fig. 7B).

Nuclear PGC1α protein: Purified nuclear extracts were devoid of LDH and contained high levels of H2B (Fig. 7D), indicating successful enrichment of nuclear proteins and the absence of cytosolic contamination. The amount of PGC1α protein located in the nuclei was higher (P<0.05) in both red (+68%) and white (+67%) muscles of ZDF animals (Fig. 7C). A fiber type difference existed in nuclear PGC1α protein, as white muscle contained less (P<0.05) PGC1α nuclear protein in both control (-32%) and ZDF (-32%) animals (Fig. 7C).

Targeting of PGC1α to SS mitochondria

To test the notion that PGC1α preferentially targets SS mitochondria, we electrotransfected PGC1α cDNA into the TA muscle of lean animals and determined the effect on FAT/CD36, a target protein. Transfection increased the mRNA (+29%; Fig. 8A), total protein (+22%; Fig. 8B) and the nuclear content of PGC1α (+15%; Fig. 8C). Subsequently, whole muscle FAT/CD36 content was increased (P<0.05) +30% (Control; 100±6.1 vs. Transfected; 130±18.4
arbitrary units), as was SS mitochondrial FAT/CD36 content (Control; 100±10.5 vs. Transfected; 117±15.5 arbitrary units) but not IMF FAT/CD36 (Control; 105.4±6.3 vs. Transfected; 98±3.9 arbitrary units). Rates of palmitate oxidation increased (P<0.05) +37% in SS mitochondria, but PGC1α transfection had no effect (P>0.05) on IMF mitochondria (Fig. 8D). Around 30% of muscle fibers are transfected with our procedures (data not shown), and therefore we could not determine mitochondrial content in various sub-populations in transfected muscle fibers, as with TEM imaging one cannot determine which fibers have been affected.

DISCUSSION

The novel findings of the current study are that skeletal muscle from ZDF rats 1) have larger and more prevalent lipid droplets, and 2) preferentially display compensatory increases in SS mitochondrial number, width, density as well as fatty acid oxidation rates, which potentially result from, 3) an increased nuclear content of PGC1α that appears to target SS mitochondria.

Mitochondrial morphology: The original hypothesis of a mitochondrial dysfunction in fatty acid oxidation was partially based on observations of smaller SS mitochondria in insulin resistant muscle (4). However, the current TEM images do not support the notion of smaller mitochondria with insulin resistance, as in red muscle SS mitochondrial size was unchanged and IMF mitochondrial size was actually increased in ZDF animals. In addition, it was not previously understood if mitochondrial size directly impacted mitochondrial oxidation rates, making the previous observations of reduced mitochondrial size (4) difficult to interpret. The current data suggests that mitochondrial size does not influence mitochondrial palmitate oxidation, as these rates were increased when the size of mitochondria were unaltered (red and white SS mitochondria), increased (red IMF mitochondria) as well as decreased (white IMF mitochondria).

Analysis of TEM images showed that the number (+50%), width (+69%) and density (+57%) of SS mitochondria were increased in ZDF animals. In contrast, ZDF IMF mitochondrial number, and density were not changed, nor were markers of mitochondrial content (mtDNA, CS and COXIV). These data show that mitochondrial sub-populations can respond differently in insulin resistant muscle, and whole muscle measures of mitochondrial content cannot reveal the subtle differences of various signals that induce mitochondrial proliferation.

Mitochondrial content and rates of palmitate oxidation: Previously, indirect assessments suggested there was an intrinsic dysfunction in fatty acid oxidation within mitochondria (4). However, the current data do not support this notion, as SS and IMF mitochondria, from both red and white muscles of ZDF animals displayed ~40% increased rates of palmitate oxidation. These data are consistent with more contemporary mitochondrial literature, as it has recently been shown that mitochondrial fatty acid oxidation, when measured in isolated mitochondria or permeabilized fibers, is not reduced in mitochondria of obese and type 2 diabetic individuals (12-14). Rodent models of insulin resistance have also supported the notion that down-regulation of mitochondrial fatty acid oxidation is not a requirement for lipid accumulation and impairments in insulin signaling. High fat feeding has been shown to induce insulin resistance while increasing mitochondrial content (9; 11). Magnetic resonance spectroscopy (MRS) has suggested that fatty acid oxidation is not compromised in diabetic animals (42), and we have recently shown in both red and white muscles of obese Zucker rats that fatty acid oxidation was increased, not decreased, in SS mitochondria (10). Thus, our work (10; 12) and that of
others (9; 13; 14; 43), indicates that in both human (12-14) and animal models of insulin resistance (9; 43) there is little evidence to support the view that an intrinsic impairment in the ability of mitochondria to oxidize fatty acids accounts for the increased intramuscular lipid accumulation associated with insulin resistance. Instead, evidence in both human (44) and rodent models (10; 45) of insulin resistance, suggests that plasma membrane fatty acid transport is increased as a result of an increased content of fatty acid transport proteins. Despite the potential compensatory adaptations in mitochondrial fatty acid oxidation, the large changes in plasma membrane fatty acid transport have been proposed to create an imbalance between delivery and utilization, such that lipids accumulate (10).

**Mitochondrial CPTI activity and FAT/CD36 protein:** In the current study, the increase in palmitate oxidation in ZDF mitochondria may result from the observed increase in 1) CPTI activity and/or 2) mitochondrial FAT/CD36 protein. The rate-limiting step in fatty acid transport/oxidation has long been attributed to CPTI activity, and the changes in CPTI activity mimicked the trends in mitochondrial fatty acid oxidation in both red and white muscle. However the notion that CPTI activity represents the only regulatory site in mitochondrial fatty acid oxidation has been challenged, as several laboratories have recently found the fatty acid transport protein FAT/CD36 on mitochondrial membranes. Gain of function (46) and loss of function molecular approaches (34), as well as physiological perturbations (31), have suggested that mitochondrial FAT/CD36 has a role in regulating mitochondrial fatty acid oxidation. While FAT/CD36 appears to have a role in regulating mitochondrial fatty acid oxidation, this is likely mediated in a concerted fashion with additional proteins, as mitochondrial FAT/CD36 and palmitate oxidation rates do not correlate under basal conditions (47), but rather multiple regression approaches that take into consideration both CPTI and mitochondrial FAT/CD36 highly correlate with mitochondrial fatty acid oxidation rates (47). Therefore, in the current study the increase in both CPTI activity and mitochondrial FAT/CD36 may account for the observed changes in mitochondrial palmitate oxidation rates in ZDF animals, both of which are transcribed by peroxisome proliferators-activated receptors (PPARs), and mediated by PGC1α.

**PGC1α mRNA, total and nuclear protein:** It has been suggested that reductions in PGC1α expression has a role in the etiology of insulin resistance (21; 22). However, in the current study we have found that PGC1α mRNA and total muscle protein were not reduced in ZDF muscles. However, the sub-cellular distribution of PGC1α did differ, as the nuclear content was markedly increased (~70%). Others have also now found that PGC1α is not reduced with insulin resistance in human skeletal muscle (6; 23; 24). The mechanism(s) triggering the PGC1α translocation to the nucleus are unknown. However, muscle contraction, through calcium-mediated signaling, has previously been shown to induce nuclear translocation of PGC1α (26), and therefore alterations in cytosolic calcium levels represent a potential mechanism of action.

Interestingly, the increase in nuclear PGC1α was associated with increased proliferation of the ZDF SS mitochondria, not IMF mitochondria. PGC1α over-expression experiments revealed that PGC1α targets SS mitochondria, as palmitate oxidation rates and FAT/CD36 content were only increased in SS, not IMF, mitochondria. This was also observed previously in another study (20). However, in ZDF animals in the current study, IMF mitochondrial CPTI, FAT/CD36 and rates of fatty acid oxidation were also all increased in conjunction with increased nuclear PGC1α. Proliferation of IMF
mitochondria is also possible in PGC1α transgenic animals (25). This may suggest that a greater increase in nuclear PGC1α is required to up-regulate IMF mitochondria, as transfection only had a modest affect on nuclear PGC1α (+15%) compared to the in vivo ZDF (+70%), and arguably the transgenic conditions. Alternatively, the apparent ‘targeting’ of PGC1α to SS mitochondria may be a result of the proximity of nuclei to the SS sub-population and differences in the rate of protein import into mitochondrial sub-populations (48). Regardless of the exact mechanism, more pronounced changes occur in SS mitochondria in response to increased nuclear PGC1α content in both ZDF and electrotransfected rats.

While ZDF animals share a number of similar traits with human type 2 diabetes, including hyperglycemia, insulin resistance (49), hyperlipidemia (50), increased plasmalemmal fatty acid transport and intramuscular lipids (44), we recognize that the ZDF animal model is not fully representative of human type 2 diabetes. Therefore, it will be important to discern whether the current observation in ZDF animals extend to human type 2 diabetic individuals.

In summary, we show that in ZDF rats 1) intramuscular triacylglycerol accumulates, almost exclusively in the IMF region, as a result of an increase in both the size and number of lipid droplets. In an attempt to compensate for the increased plasma membrane fatty acid transport and lipid delivery (as we have shown previously (45)), in ZDF muscle the there was an increase in 2) SS mitochondrial number, size and density, 3) SS and IMF CPTI activity and FAT/CD36 protein, and 4) mitochondrial palmitate oxidation rates in SS and IMF mitochondria. The increase in ZDF mitochondrial oxidative capacity was greater in SS mitochondria, as the increase in mitochondrial fatty acid oxidation rates is amplified by the increase in SS mitochondrial number. These changes may result in part from 5) an increased nuclear translocation of PGC1α protein which preferentially targets SS mitochondria.

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**FIGURE LEGENDS**

**Figure 1.** Intramyocellular lipids (IMTG) in red muscle of control and ZDF animals, determined in TEM images (A) and biochemically (B)
Data are expressed as the mean ± S.E.M.
Images were taken at 25,000 x magnification, and the black bar = is 1µm.
n=5 animals for each measure.
- * significantly different (P<0.05) from control mitochondria.

**Figure 2.** Subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial area (A,B) and number (C) in red and white muscle of control and ZDF animals
Data are expressed as the mean ± S.E.M.
Images were taken at 25,000 x magnification, and the black bar =1µm.
n=5 animals for each measure.
- * significantly different (P<0.05) from control mitochondria.
- † significantly different (P<0.05) from SS mitochondria.
Mitochondrial number was not determined in white muscle because of the infrequent presence in our defined region.

**Figure 3.** Subsarcolemmal (SS) mitochondrial width (A) and density (B), and intermyofibrillar (IMF) mitochondrial density in close proximity of (C) or distant from (D) lipid droplets, in red muscle of control and ZDF animals
Data are expressed as the mean ± S.E.M.
Images were taken at 5,800 x magnification, and the black bar = 5µm. Width of IMF mitochondria was not determined, as this was largely influenced by lipid droplet diameter and not reflective of mitochondrial ‘volume’ changes. n=5 animals for each measure.

- * significantly different (P<0.05) from control mitochondria.

**Figure 4.** Representative images of white muscle in control (A) and ZDF (B) animals. Note the absence of lipid droplets and the diffuse nature of mitochondria. Images were taken at 5,800 x magnification, and the black bar = 5µm.

**Figure 5.** Whole muscle, subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial enzymatic activities and protein contents in red and white muscle of control and ZDF animals. Cytochrome c oxidase complex IV (COXIV; Whole muscle A; Red mitochondria D; White mitochondria G); Citrate synthase (CS; Whole muscle B); Carnitine palmitoyl transferase I (CPTI; Red mitochondria E; White mitochondria H); Fatty acid translocase (FAT/CD36; Whole muscle C; Red mitochondria F; White mitochondria I)

Data are expressed as the mean ± S.E.M. n=4-5 for each measure.

- * significantly different (P<0.05) from control animals.
- † significantly different (P<0.05) from red muscle or SS mitochondria.

**Figure 6.** Red (A) and white (B) muscle subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial palmitate oxidation rates in control and ZDF animals

Data are expressed as the mean ± S.E.M. n=5 for each measure.

- * significantly different (P<0.05) from control mitochondria.
- † significantly different (P<0.05) from SS mitochondria.

**Figure 7.** Peroxisome proliferators-activated receptor γ co-activator 1α (PGC1α) mRNA (A), total (B) and nuclear protein (C) in control and ZDF animals. Lactate dehydrogenase (LDH); Histone H2B (H2B) representative blots (D)

Data are expressed as the mean ± S.E.M. n=4 for each measure.

- * significantly different (P<0.05) from control muscle.
- † significantly different (P<0.05) from red muscle.

**Figure 8.** Peroxisome proliferators-activated receptor γ co-activator 1α (PGC1α) mRNA (A), total (B) and nuclear protein (C) and subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial palmitate oxidation rates (D) in control and PGC1α transfected muscles.

Data are expressed as the mean ± S.E.M. n=4 for each measure.

- * significantly different (P<0.05) from control muscle.
- † significantly different (P<0.05) from SS muscle.
Fig 1

A

B Control red

C

ZDF red
Nuclear PGC1α and mitochondrial sub-populations

FIG 2

A

|       | Red          | White        |
|-------|--------------|--------------|
| SS    | 250 ± 10     | 180 ± 10     |
| IMF   | 300 ± 15     | 150 ± 5      |

B

|       | Control       | ZDF          |
|-------|---------------|--------------|
| SS    | 200 ± 20      | 100 ± 10     |
| IMF   | 150 ± 15      | 75 ± 5       |

C

Mitochondrial number/10 μm²

|       | Control       | ZDF          |
|-------|---------------|--------------|
| SS    | 15 ± 3        | 5 ± 1        |
| IMF   | 10 ± 2        | 5 ± 1        |
Nuclear PGC1α and mitochondrial sub-populations

Fig 3

A

**Fig 3**

A)

Control red

ZDF red

B)

C

INF density in region devoid of lipids

D

IMF density in region abundant with lipids

Fig 4

A

Control White

ZDF White

B
Fig 5: Whole muscle, Red Mitochondria, White Mitochondria

A: COX IV Content (arbitrary OD units/mg protein)
B: C3 Activity (μmol/g tissue)
C: PCNA Content (arbitrary OD units/mg protein)
D: COX IV Content (arbitrary OD units/mg protein)
E: C3 Activity (μmol/g tissue)
F: PCNA Content (arbitrary OD units/mg protein)
G: COX IV Content (arbitrary OD units/mg protein)
H: C3 Activity (μmol/g tissue)
I: PCNA Content (arbitrary OD units/mg protein)

Fig 6: Comparison between Control and ZDF

A: Mitochondrial palmitate oxidation (nmol/mg protein/hr) for Red mitochondria
B: Mitochondrial palmitate oxidation (nmol/mg protein/hr) for White mitochondria
Nuclear PGC1α and mitochondrial sub-populations

Fig 7

A

B

C

D
Nuclear PGC1α and mitochondrial sub-populations

Fig 8

A. PGC1α mRNA content

B. Total muscle PGC1α protein

C. Nuclear PGC1α protein

D. Palmitate oxidation