Contrasting metabolic effects of medium- versus long-chain fatty acids in skeletal muscle


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Abstract Dietary intake of long-chain fatty acids (LCFAs) plays a causative role in insulin resistance and risk of diabetes. Whereas LCFAs promote lipid accumulation and insulin resistance, diets rich in medium-chain fatty acids (MCFAs) have been associated with increased oxidative metabolism and reduced adiposity, with fewer deleterious effects on insulin action. The molecular mechanisms underlying these differences between dietary fat subtypes are poorly understood. To investigate this further, we treated C2C12 myotubes with various LCFAs (16:0, 18:1n9, and 18:2n6) and MCFAs (10:0 and 12:0), as well as fed mice diets rich in LCFA or MCFAs, and investigated fatty acid-induced changes in mitochondrial metabolism and oxidative stress. MCFA-treated cells displayed less lipid accumulation, increased mitochondrial oxidative capacity, and less oxidative stress than LCFA-treated cells. These changes were associated with improved insulin action in MCFA-treated myotubes. MCFA-fed mice exhibited increased energy expenditure, reduced adiposity, and better glucose tolerance compared with LCFA-fed mice. Dietary MCFAs increased respiration in isolated mitochondria, with a simultaneous reduction in reactive oxygen species generation and substantially low oxidative damage. Collectively, our findings indicate that in contrast to LCFAs, MCFAs increase the intrinsic respiratory capacity of mitochondria without increasing oxidative stress. These effects potentially contribute to the beneficial metabolic actions of dietary MCFAs.


Supplementary key words metabolic disease • mitochondrial metabolism • oxidative stress • insulin signalling

The most common metabolic disorders in our Western society (including obesity and insulin resistance) have arisen to a great extent from excess nutrient intake, especially in the form of fat. Intake of diets rich in long-chain fatty acids (LCFAs) (>C16), the most common fatty acid (FA) type in Western diets, is associated with disturbances in glucose homeostasis and insulin action (1, 2). Many mechanisms have been proposed to underpin the deleterious effects of LCFAs on metabolic health, including increased inflammation, overactivation of stress-related pathways, and inappropriate lipid accumulation in nonadipose tissues (1, 3).

Interestingly, not all dietary fats induce the same degree of metabolic dysfunction. Our group and others have shown that intake of equal-caloric diets rich in medium-chain fatty acids (MCFAs) (C8–C12) decreases adiposity (4–6), increases energy expenditure (7, 8), and avoids many of the detrimental effects associated with LCFA intake. The increased energy expenditure suggests that FAs are funneled into oxidative versus storage pathways, and this has previously been suggested to be due to enhanced cellular uptake and entry of MCFAs into mitochondria (especially in the liver) for oxidation. In a recent study we showed that high-fat diets enriched with MCFAs caused a marked induction in mitochondrial oxidative capacity in muscle, over and above that induced by a LCFA high-fat diet, suggesting that enhanced myocellular oxidation of MCFAs might also be a key pathway for oxidative disposal of this class of FAs (8). In conjunction with the increase in markers of mitochondrial metabolism, MCFAs also
prevented lipid accumulation and insulin resistance in muscle, with similar glucose uptake and muscle triglyceride levels in MCFAs-fed animals compared with lean animals fed a low-fat diet (8).

In this study, we have used in vitro and in vivo systems to further characterize the disparate metabolic effects of MCFAs and LCFAs in muscle. First, we have investigated whether in addition to affecting mitochondrial content in muscle, MCFAs and LCFAs have differential effects on the intrinsic bioenergetic capacity of mitochondria. Additionally, as mitochondria are a major site for reactive oxygen species (ROS) production and LCFAs have been linked with oxidative stress (9, 10), we have determined the effect of MCFAs and LCFAs on ROS production and markers of oxidative damage.

**METHODS**

**Cell culture**

C2C12 myoblasts were grown in 1:1 Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 nutrient mix (Life Technologies, Mulgrave, VIC, Australia) containing 10% HyClone bovine calf serum (Thermo Fisher Scientific, Scoresby, VIC, Australia) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Differentiation was induced by serum-starvation (Life Technologies) once 90–100% confluence was reached. Cells were differentiated for 5 days, and differentiated myotubes were incubated for 18 h with individual FAs. For the lipid treatments, 200 μM FAs in ethanol were conjugated to 1% FA-free BSA at 55°C for 1.5 h in differentiation medium; the medium was filter-sterilized and applied to the cells. Control cells were incubated with an equal concentration of BSA and ethanol. For insulin stimulations, myotubes were serum-starved for 4 h (in the presence of the respective FA), stimulated with 190 × 10^{-6} M FAs as described above. On the day of the assay, FA-containing differentiation medium was replaced with HBSS buffer (Life Technologies) also containing FAs (conjugated to BSA) and either the mitochondrial fluorophor MitoSOX Red (5 μM, in nitrogen-bubbled DMSO), the cytosolic superoxide marker dihydroethidium (DHE) (20 μM, in nitrogen-bubbled DMSO), or DMSO itself. MitoSOX Red and DHE were added from stock solutions in DMSO with the final concentration of DMSO being less than 0.2% (v/v). Cytosolic superoxide generation with DHE was measured in the absence or presence of the NAD(P)H oxidase inhibitor VAS2870 (10 μM). Time-resolved fluorescence was determined for 1 h using a BMG Labtech FLUOstar OPTIMA plate reader and 485/590 nm excitation/emission filters. Changes in fluorescence in the MitoSOX Red- or DHE-treated cells were corrected for slope observed in the DMSO-treated controls (for each individual FA treatment). Results are expressed as change in fluorescence/min/mg protein. For the determination of protein content, medium was removed from the wells, 10 μl 1 M KOH added to lyse the cells, topped up with 190 μl of ddH2O, and protein concentration measured using the Bradford method (Bio-Rad Laboratories, Regents Park, NSW, Australia).

**Glycogen synthesis**

C2C12 myotubes were grown in 12-well plates and treated with 200 μM FAs as described above. On the day of the assay, cells were serum-starved for 4 h in the presence of the respective FA, followed by a 1 h incubation in serum-free medium containing FAs and 4 μCi/ml [U-14C]glucose (Perkin Elmer), and in the absence (basal state) or presence (insulin-stimulated state) of 100 nM insulin. Cells were washed three times in PBS, scraped into 100 μl 1 M KOH, and heated for 15 min to 75°C. A subsample (10 μl) was taken for protein determination. To the remainder, 30 μl 25 mg/ml glycogen, 20 μl saturated Na2SO4, and 450 μl ice-cold ethanol was added, the mix vortexed and frozen for 30 min at −80°C, followed by a centrifugation step (10 min, 13,000 rpm, 4°C). Pellets were washed by resuspension in 50 μl ddH2O, followed by addition of 1 ml ice-cold ethanol and renaturation. The final pellet was resuspended in 50 μl ddH2O and counted for radioactivity.

**Animal maintenance**

Eight-week-old male C57BL/6J mice were purchased from the Australian Resource Centre (Perth, Australia). Mice were maintained in a temperature-controlled room (22 ± 1°C) with a 12 h light/dark cycle and ad libitum access to food and water. After 1 week on a standard low-fat chow diet (CHOW; 8% of calories from fat, 21% of calories from protein, 71% of calories from carbohydrate; Gordon’s Specialty Stock Feeds, Yanderra, NSW, Australia), mice were randomly allocated to remain on the CHOW diet or to receive a diet enriched in either MCFAs or LCFAs ad libitum for 8 weeks. The high-fat diet
[45% of calories from fat (from hydrogenated coconut oil for the MCF diet and from lard for the LCFA diet), 20% of calories from protein, 35% of calories from carbohydrate] was made in-house and was based on rodent diet number D12451 (Research Diets, New Brunswick, NJ). The dietary FA composition was described previously (8). Tissue samples were collected from mice at 0900–1000 h without any prior fasting period. All experiments were approved by the Garvan Institute/St. Vincent’s Hospital Animal Experimentation Ethics Committee, and followed guidelines issued by the National Health and Medical Research Council of Australia.

**Determination of body composition and energy expenditure**

Lean and fat mass were measured in mice using dual-energy X-ray absorptiometry (Lunar PIXImus2 densitometer; GE Healthcare, Little Chalfont, UK). The rate of oxygen consumption (VO2) and respiratory exchange ratio (RER) of individual mice were measured using an Oxymax indirect calorimeter over 24 h following an overnight acclimatization period in the Oxymax cages and a 2 h settling period (Columbus Instruments, Columbus, OH) as previously described (11).

**Glucose tolerance and insulin levels**

Mice were fasted overnight and injected ip with glucose (2 g/kg), and blood glucose levels were monitored using an Accucheck II glucometer (Roche Diagnostics, Castle Hill, NSW, Australia) for 90 min following glucose injection. Plasma insulin levels after overnight fast were determined by radioimmunoassay (Linco Research, St. Charles, MO).

**Isolation of mitochondria**

Muscle mitochondria (from mixed hindlimb muscle) were isolated by differential centrifugation as described previously (12). Briefly, muscle was diced in CP-1 medium [100 mM KCl, 50 mM Tris/HCl (pH 7.4), and 2 mM EGTA], digested on ice for 3 min in CP-2 medium [CP-1, to which was added 0.5% (w/v) BSA, 5 mM MgCl2, 1 mM ATP, and 2.45 units/ml protease type VIII (Sigma P 5380)] and homogenized using an Ultra Turrax homogenizer. The homogenate was spun for 10 min at 16,000 g at 4°C. The resulting supernatant was subjected to a high-speed spin (10,600 g, 10 min, 4°C) and the pellet was resuspended in CP-1. The high-speed spin cycle was repeated twice. Protein content was measured using the Bradford method.

**Respiration in cells and isolated mitochondria**

For the determination of respiration in C2C12 myotubes, cells were incubated with FAs for 18 h as described above, trypsinized into 1 ml FA-containing media, and oxygen consumption was measured at 37°C in a Clark-type oxygen electrode (Strathkelvin Instruments, Motherwell, Scotland). In isolated mitochondria, oxygen consumption was measured in air-saturated respiration buffer [120 mM KCl, 5 mM K2HPO4, 3 mM HEPES, 1 mM EGTA, and 0.3% (w/v) defatted BSA (pH 7.2)], which was calculated to contain 406 nmol oxygen/ml (13), and to which was added either succinate (5 mM) or rotenone (2 μM) for the preparation of state 3 (state 3/state 4 respiration) were above 3.5 for succinate and rotenone (2 μM, to inhibit electron backflow toward complex I), and in the presence of succinate (5 mM), in the presence of succinate and rotenone (2 μM, to inhibit electron backflow toward complex I), and in the presence of palmitoyl carnitine (40 μM). In separate wells containing assay medium, H2O2 was injected (using onboard injectors) at concentrations between 0 and 500 nM and a standard curve calculated.

H2O2 production and state 2 oxygen consumption were used to calculate the percentage of electrons which leak out of sequence producing superoxide and subsequently hydrogen peroxide (15). Whereas two electrons are needed for the reduction of 1 mol of O2 to H2O, four electrons are transferred in the reduction of 1 mol of O2 to water. Therefore, the percent free radical leak (FRL) was calculated as the rate of H2O2 production divided by twice the rate of oxygen consumption, and the result was multiplied by 100 (16).

**Lipid accumulation**

Triacylglycerol (TAG) content was determined using a colorimetric assay kit (Triglycerides GPO-PAP; Roche Diagnostics, Indianapolis, IN) as previously described (17). For diacylglycerol (DAG) and ceramide measurements, lipids were extracted from muscle in solvents containing 2 nmol ceramide (17:0) and 10 nmol DAG (17:0/17:0) (18). DAG and ceramide levels were measured using a hybrid linear ion trap-triple quadrupole mass spectrometer (QTRAP 5500; AB Sciex, Foster City, CA). Ceramide molecular lipids were analyzed by precursor-ion scanning for protonated dehydrated sphingosine at m/z 264.3. DAG molecular lipids were analyzed by multiple neutral-loss scanning for ammoniated FAs. Data were analyzed and quantified with LipidView (AB Sciex) version 1.1 after isotope correction (19).

**Immunoblotting**

Lysates were prepared from FA-treated C2C12 myotubes and mouse muscle mitochondria. Cells were collected in RIPA buffer (20), sonicated for 5 s, rotated at 4°C for 2 h and centrifuged for 10 min at 16,000 g. Mitochondria were isolated as described above, mixed 1:1 in RIPA buffer, and sonicated for 5 s. For both cells and mitochondria, 20 μg of protein was denatured in Laemmli buffer at 65°C for 15 min, resolved by SDS-PAGE, electrotransferred, and immunoblotted, as described previously (21). Immunolabeled bands were quantified using ImageJ 1.44p software.

**Measurement of antioxidant protection and oxidative damage**

Glutathione peroxidase (GPx) activity was measured as the decrease in NADPH absorption at 340 nm and 30°C. A reaction cocktail was prepared by mixing azide buffer (9.2 ml) [50 mM NaH2PO4, 0.4 mM EDTA (pH 7.0 at 30°C), addition of 1 mM Na-azide], glutathione reductase (100 μl, 100 U/ml), and GSH (50 μl, 200 mM) into a 1 mg β-NADPH vial (Sigma N0411). Sample and reaction cocktail were mixed and H2O2 [5 μl, 0.042% (v/v)] was added to start the reaction.

Lipid hydroperoxides (LOOHs) were measured according to the method of Bou et al. (22). Thiobarbituric acid reactive
substances (TBARS) and protein carbonyls were measured in homogenates as described previously (14). Homogenate protein content was measured using the Bradford method.

Statistical analysis

All results are presented as mean ± SEM. Results were compared using a one-way ANOVA with P < 0.05 considered significant, followed by a Fisher’s least significant differences test. For both the cell culture and animal experiments, the mean of each treatment group was compared with the mean of every other treatment group.

RESULTS

Effect of FA subtypes on oxidative metabolism and lipid accumulation in C2C12 myotubes

Incubation of myotubes with the MCFAs capric (C10:0) and lauric acid (C12:0) for 18 h significantly increased oxygen consumption (Fig. 1A) and SDH activity (Fig. 1B) in comparison to BSA-treated control cells, as well as protein levels of several oxidative proteins, including subunits of the mitochondrial electron transport chain (ETC), the master-regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1α, the mitochondrial content marker porin, and the mitochondrial FA transporter carnitine palmitoyl transferase 1 (CPT1) (Fig. 1C and supplementary Table I). The observed increase in SDH activity was most likely due to increased protein expression, as shown by immunoblotting for SDH subunit a (Fig. 1C). In contrast, treatment of myotubes with LCFAs, the saturated FA palmitic acid (C16:0), the mono-unsaturated FA oleic acid (C18:1n9), and the polyunsaturated FA linoleic acid (C18:2n6), did not significantly affect cellular respiration (Fig. 1A), had no effect or caused a decrease in the expression of oxidative proteins (Fig. 1C and supplementary Table I), and caused a mild decrease in SDH activity compared with control cells (Fig. 1B). Protein levels of the glucose transporter 4 were unaffected by FA treatment (Fig. 1C).

Incubation of C2C12 myotubes with LCFAs led to an average 5-fold increase in TAG accumulation in comparison to BSA-treated control cells. Interestingly, myotubes incubated with the MCFAs exhibited markedly less TAG accumulation compared with the LCFA-treated cells (Fig. 1D). The differences in TAG deposition were not due to any differences in FA uptake, as similar levels of uptake were observed for both MCFA- and LCFA-treated cells with 14C-labeled FAs (Fig. 1E). Collectively these results suggest enhanced partitioning of FAs toward oxidation with MCFAs, and the tracer experiments showed that approximately 65% of 14C-lauric acid taken up was directed to oxidative pathways (CO₂ and ASMs), while this was only approximately 35% with 14C-palmitic acid (data not shown).

Effect of FA subtypes on oxidative stress in C2C12 myotubes

In addition to effects on lipid accumulation and mitochondrial metabolism, LCFAs have also been linked to oxidative stress and the subsequent accumulation of oxidatively damaged cellular components (9, 10). We investigated the effect of incubation of C2C12 myotubes with LCFAs and MCFAs on superoxide generation and lipid peroxidation (two markers examined: LOOHs and TBARS). Mitochondrial (Fig. 2A), but not cytosolic superoxide generation (Fig. 2B), was significantly increased with all three LCFAs, but not after MCFA treatment. Cytosolic superoxide was measured in the absence and presence of the NAD(P)H oxidase inhibitor VAS2870. Addition of VAS2870 decreased ROS generation to approximately 90–95% of the rates measured in the absence of the inhibitor, showing that NAD(P)H oxidases have only a minor contribution to cytosolic ROS production in C2C12 myotubes (data not shown). Both lipid peroxidation markers followed the same pattern as mitochondrial superoxide and were significantly increased after LCFAs but not MCFA treatment (Fig. 2C, D). This highlights that MCFAs keep oxidative stress at low levels, whereas LCFAs lead to substantial increase in mitochondrial ROS production and to the accumulation of oxidative damage.

Effect of FA subtypes on insulin action in C2C12 myotubes

Excess lipid accumulation, mitochondrial dysfunction and oxidative stress have been associated with the development of insulin resistance (1). We next investigated how incubation of myotubes with LCFAs and MCFAs affected insulin-stimulated phosphorylation of insulin receptor substrate 1 (IRS1) (Tyr612), Akt (Ser473), and glycogen synthase kinase 3β (GSK3β) (Ser9), as well as the insulin-stimulated glycogen synthesis rate (Fig. 3). Whereas none of the FAs had an effect on phosphorylation of IRS1, incubation of muscle cells with MCFAs significantly increased Akt phosphorylation. In contrast, palmitic acid decreased insulin-stimulated Akt phosphorylation substantially, whereas no changes were observed with oleic or linoleic acid. Phosphorylation of GSK3β was decreased after LCFAs but not MCFA treatment, which was accompanied by a significant decrease in insulin-stimulated glycogen synthesis rate with all three LCFAs (Fig. 3). These results suggest that MCFAs, unlike LCFAs, do not impair insulin action in muscle cells.

Effect of dietary MCFA and LCFA intake on adiposity, energy expenditure, and glucose tolerance

Mice fed a MCFA-rich diet were partially protected from body weight gain and increases in adiposity in comparison to LCFA-fed mice (when compared with CHOW controls) after 8 weeks of high-fat feeding (Fig. 4A, B). The differences in body composition in MCFA- and LCFA-fed mice can be to some extent explained by differences in energy intake, as cumulative calorie intake per mouse over the 8 week feeding period was increased in the MCFA and LCFA groups when compared with the CHOW-fed mice (CHOW, 11.1 ± 0.6 kcal/day; MCFAs, 12.8 ± 0.6 kcal/day; LCFAs, 12.3 ± 0.6 kcal/day). We did not assess if there were differences in lipid absorption between the two diets, however, MCFAs are more efficiently absorbed than LCFAs (23), and thus differences in energy dissipation by inefficient digestion of the dietary FAs is unlikely to underlie the differences in adiposity and body composition. Whole-body oxygen consumption (normalized to
lean mass) was elevated in both fat-fed groups compared with controls, and was significantly higher in MCFA- versus LCFA-fed animals in both the light and dark phase (Fig. 4C). Furthermore, the RER showed a greater decrease in MCFA-fed mice than in LCFA-fed mice compared with control CHOW-fed mice (Fig. 4D). The increase in overall energy expenditure and the lower RER could partly explain the reduced adiposity in MCFA-fed mice. Compared with LCFA-mice, MCFA-fed animals also did not develop the same degree of high-fat diet-induced glucose intolerance (Fig. 4E) and displayed lower fasting insulin levels (Fig. 4F).

**Effect of dietary MCFA and LCFA intake on muscle lipid accumulation**

MCFA-fed mice displayed less muscle TAG accumulation than LCFA-fed mice (41% increase with MCFA vs. 295% increase with LCFA) (Fig. 5A). A similar proportional increase was also observed in DAG accumulation, whereas total ceramide levels were greatest in muscle from MCFA-fed mice (Fig. 5B). The increase in muscle ceramide in MCFA-fed mice was mainly due to an increase in CER18:0 and CER24:0 (Fig. 5C), while the increase in total DAG in MCFA- and LCFA-fed mice was due to an elevation in the same DAG species, mainly DAG16:0/16:0, DAG16:0/18:0, and DAG16:1/18:1 (supplementary Fig. I).

**Effect of dietary MCFA and LCFA intake on mitochondrial respiration and ROS production**

To determine if MCFAs altered mitochondrial function, oxygen consumption in states 2, 3, and 4 was measured in isolated muscle mitochondria using succinate and
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Effect of dietary MCFA and LCFA intake on protein expression of mitochondrial oxidative proteins

Immunoblotting of mitochondrial lysates was undertaken to investigate if the increased oxygen consumption in isolated mitochondria from MCFA-fed mice might be due to alterations in the expression of key oxidative proteins. Equal amounts of mitochondria from each dietary group were used for immunoblotting, as shown by quantification of the abundant mitochondrial transmembrane protein porin (Fig. 7). Protein expression of subunits of complexes I–V of the ETC, as well as uncoupling protein 3 (UCP3), were increased to a greater extent with the MCFA than the LCFA diet, when compared with mitochondria from CHOW-fed controls (Fig. 7). Superoxide dismutase 2 (SOD2) was significantly decreased in the MCFA-fed mice, whereas no difference was observed in CPT1 protein content between diet groups. These data suggest that MCFAs more effectively promote the expression of oxidative proteins than LCFA, resulting in increased oxidative capacity per unit of mitochondria.

Effect of dietary MCFA and LCFA intake on oxidative stress in muscle

As described above, in both muscle cells and mitochondria from mouse muscle, MCFAs were associated with lower rates of ROS production. To determine if this was accompanied by differences in the accumulation of oxidative damage in mouse skeletal muscle (as done in C2C12 myotubes), we measured various antioxidant and oxidative damage markers. Antioxidant systems are expected to respond to the need of a tissue for protection, with greater oxidative stress levels resulting in an increase in the expression and activity of antioxidant enzymes (24). GPx, as well as the content of lipid hydroperoxides (an intermediate marker of oxidative damage to lipids) and protein carbonyls (marker for oxidative damage to proteins), were increased with the LCFA diet, but not the MCFAs diet (Fig. 8A–C). TBARS, an endpoint marker of lipid peroxidation, remained unchanged in muscle of LCFA-fed mice, but were significantly decreased in MCFAs-fed mice when compared with controls (Fig. 8D). Overall, these data indicate that MCFAs in contrast to LCFA do not increase mitochondrial ROS production in vivo, with subsequently less accumulation of oxidatively damaged lipids and proteins.

DISCUSSION

Over the last 20 years, it has been repeatedly described that diets rich in LCFA (e.g., typical Western diets) lead to the development of adiposity and insulin resistance, and increase the risk of diabetes (1, 25). In contrast, diets rich in MCFAs, when used at an equal-caloric level, do not induce the same degree of detrimental effects on metabolic health; despite a similar increase in dietary fat intake as with the LCFA diet (4–8). These favorable effects have been partially ascribed to the physical properties of MCFAs, because this class of FAs is more readily funneled into oxidative pathways, which could lead to increased energy expenditure and less fat deposition in adipose and other tissues compared with LCFA (26). The tissue that is likely to be responsible for many of the beneficial effects of MCFAs on whole-body glucose homeostasis is skeletal muscle, where previous studies from our group have shown that a MCFA-rich diet leads to a substantially greater increase in palmitoyl carnitine as substrates. Mitochondria from muscle of MCFA-fed mice showed significantly greater rates of oxygen consumption than CHOW- and LCFA-fed animals in state 3 for both succinate and palmitoyl carnitine (Fig. 6A, B). Increased mitochondrial substrate flux is often accompanied by higher ROS production, however the mitochondrial (A) and cytosolic (B) superoxide generation, lipid hydroperoxide (C) and TBARS (D) levels in muscle cells treated with various MCFAs and LCFA (18 h incubations with 200 μM FAs). Shown are means ± SEM, n = 3 with three replicates each (or n = 4 with two replicates each in the case of panel (B)). *P < 0.05, **P < 0.01, and ***P < 0.001 versus C; #P < 0.01 versus CA; ∆P < 0.01 versus LA; ∆P < 0.05 versus PA; ∆P < 0.01 versus OA. C, control; CA, capric acid; LA, lauric acid; PA, palmitic acid; OA, oleic acid; LinA, linoleic acid.
capacity for substrate oxidation can occur in muscle without marked changes in total mitochondrial number, indicating improved intrinsic capacity per mitochondrion (28, 30). The findings of the current study and our previous report (8) indicate that MCFAs improve mitochondrial substrate oxidation in muscle to a greater extent than LCFAs, due to increases in both the total number of mitochondria and the oxidative capacity of individual mitochondria.

Collectively the changes in mitochondrial content and capacity could, in part, explain the enhanced whole-body energy expenditure per unit lean mass, the prevention of lipid accumulation and insulin resistance in this tissue (8). In the current study, we have shown that the beneficial effects of MCFAs in skeletal muscle may also be related to changes at the level of mitochondria, where we observed increased respiratory capacity and reduced ROS production.

Whereas a variety of studies demonstrated a link between muscle mitochondrial oxidative capacity and insulin action (8, 27, 28), other publications showed no such correlation [summarized in (29)]. Here we show that MCFAs induced an increase in respiration and SDH activity in muscle cells, as well as an increased protein expression of various mitochondrial proteins, suggesting an improvement in mitochondrial oxidative capacity, while no such increases were observed after LCFA treatment. Importantly, experiments in isolated mitochondria from skeletal muscle of MCFA-fed mice showed that, per unit of mitochondria, there was increased respiratory capacity, which is likely explained by an increase in the expression of oxidative proteins. Previous studies have shown that changes in the expression and activity of oxidative proteins and the capacity for substrate oxidation can occur in muscle without marked changes in total mitochondrial number, indicating improved intrinsic capacity per mitochondrion (28, 30). The findings of the current study and our previous report (8) indicate that MCFAs improve mitochondrial substrate oxidation in muscle to a greater extent than LCFAs, due to increases in both the total number of mitochondria and the oxidative capacity of individual mitochondria.

Collectively the changes in mitochondrial content and capacity could, in part, explain the enhanced whole-body energy expenditure per unit lean mass, the prevention of TAG and DAG accumulation in muscle, and the better insulin action in MCFA- versus LCFA-fed animals [current study and (8)]. We did observe a slight increase in total ceramide content in muscle from the MCFA-fed mice, and despite ceramide accumulation being previously associated with the development of insulin resistance, recent studies suggest that the FA chain length of the ceramide species is important in determining its metabolic impact (31, 32). The increase in muscle ceramide in MCFA-fed mice was mainly due to an increase in CER18:0 and CER24:0.
Compared with carbohydrates, reducing equivalents from lipid oxidation produce increased superoxide (36, 37). While still controversial, the majority of recent studies have proposed that mitochondrial ROS generation is an important early event in the induction of insulin resistance, with LCFAs increasing ROS production and promoting insulin resistance (9, 10, 38–40). Other publications support the view that cytosolic ROS, at low levels, are important for normal insulin signaling and do not induce insulin resistance (41, 42). In conjunction with both views, diets rich in LCFAs have also been reported to decrease antioxidant protection, thereby promoting oxidative damage (40, 43); whereas other reports demonstrated that antioxidants do not improve insulin action, especially in insulin-resistant humans [summarized in (44)]. Little is known about the effects of MCFAs on ROS generation and the accumulation of oxidative damage. Here we have shown that in response to MCFAs, both mitochondrial superoxide generation in C2C12 myotubes as well as mitochondrial 

\[ \text{H}_2\text{O}_2 \] production in mouse skeletal muscle were similar or decreased compared with controls. This pattern can be explained by low levels of free radical leakage from the ETC in muscle mitochondria from MCFA-fed mice, as measured by percentage of electrons leaking out of sequence during mitochondrial respiration. In contrast, mitochondrial superoxide production was substantially increased in LCFA-treated cells, and in skeletal muscle of mice fed a LCFA-rich diet there was a 2-fold increase in FRL with the lipid-based substrate palmitoyl carnitine. Cytosolic superoxide production, measured in the C2C12 (Fig. 5C), with CER24:0 having previously been reported to have beneficial anti-apoptotic effects (31). Importantly, we have shown that the effects of MCFAs to boost mitochondrial metabolism and avoid excess lipid deposition and insulin resistance are not uniform across tissues. In our previous work, we demonstrated that dietary MCFAs, while improving metabolic parameters in muscle, led to steatosis in the liver, likely due to upregulation of lipogenic pathways and hepatic insulin resistance (8). In the current cohort of animals, we similarly observed that hepatic lipid accumulation was increased after LCFA feeding, and to an even greater extent after MCFA feeding (data not shown). While excess hepatic lipid can have deleterious metabolic effects, it should be noted that many studies in rodents and humans have reported no adverse effects of MCFAs on liver lipid levels (4, 26, 33, 34). These disparate findings on liver lipid accumulation between studies indicates that differences in methodological factors may also be important, such as the exact composition and fat content of the MCFA-enriched diets, as well as the composition of the other macronutrients in the diet.

Another intriguing finding of the current investigation was the disparate effects of MCFAs and LCFAs on mitochondrial ROS production in muscle. Mitochondria are known to leak electrons during electron transfer along the respiratory chain. This electron leak leads to the generation of superoxide anions (an oxygen molecule with an unpaired electron), and subsequently to the production of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), both ROS being highly damaging to any molecule they encounter (35). ROS generation is dependent upon the redox state of the mitochondria, substrate concentration, and substrate type. Compared with carbohydrates, reducing equivalents from lipid oxidation produce increased superoxide (36, 37). While still controversial, the majority of recent studies have proposed that mitochondrial ROS generation is an important early event in the induction of insulin resistance, with LCFAs increasing ROS production and promoting insulin resistance (9, 10, 38–40). Other publications support the view that cytosolic ROS, at low levels, are important for normal insulin signaling and do not induce insulin resistance (41, 42). In conjunction with both views, diets rich in LCFAs have also been reported to decrease antioxidant protection, thereby promoting oxidative damage (40, 43); whereas other reports demonstrated that antioxidants do not improve insulin action, especially in insulin-resistant humans [summarized in (44)]. Little is known about the effects of MCFAs on ROS generation and the accumulation of oxidative damage. Here we have shown that in response to MCFAs, both mitochondrial superoxide production in C2C12 myotubes as well as mitochondrial 

\[ \text{H}_2\text{O}_2 \] production in mouse skeletal muscle were similar or decreased compared with controls. This pattern can be explained by low levels of free radical leakage from the ETC in muscle mitochondria from MCFA-fed mice, as measured by percentage of electrons leaking out of sequence during mitochondrial respiration. In contrast, mitochondrial superoxide production was substantially increased in LCFA-treated cells, and in skeletal muscle of mice fed a LCFA-rich diet there was a 2-fold increase in FRL with the lipid-based substrate palmitoyl carnitine. Cytosolic superoxide production, measured in the C2C12 (Fig. 5C), with CER24:0 having previously been reported to have beneficial anti-apoptotic effects (31). Importantly, we have shown that the effects of MCFAs to boost mitochondrial metabolism and avoid excess lipid deposition and insulin resistance are not uniform across tissues. In our previous work, we demonstrated that dietary MCFAs, while improving metabolic parameters in muscle, led to steatosis in the liver, likely due to upregulation of lipogenic pathways and hepatic insulin resistance (8). In the current cohort of animals, we similarly observed that hepatic lipid accumulation was increased after LCFA feeding, and to an even greater extent after MCFA feeding (data not shown). While excess hepatic lipid can have deleterious metabolic effects, it should be noted that many studies in rodents and humans have reported no adverse effects of MCFAs on liver lipid levels (4, 26, 33, 34). These disparate findings on liver lipid accumulation between studies indicates that differences in methodological factors may also be important, such as the exact composition and fat content of the MCFA-enriched diets, as well as the composition of the other macronutrients in the diet.

Another intriguing finding of the current investigation was the disparate effects of MCFAs and LCFAs on mitochondrial ROS production in muscle. Mitochondria are known to leak electrons during electron transfer along the respiratory chain. This electron leak leads to the generation of superoxide anions (an oxygen molecule with an unpaired electron), and subsequently to the production of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), both ROS being highly damaging to any molecule they encounter (35). ROS generation is dependent upon the redox state of the mitochondria, substrate concentration, and substrate type. Compared with carbohydrates, reducing equivalents from lipid oxidation produce increased superoxide (36, 37). While still controversial, the majority of recent studies have proposed that mitochondrial ROS generation is an important early event in the induction of insulin resistance, with LCFAs increasing ROS production and promoting insulin resistance (9, 10, 38–40). Other publications support the view that cytosolic ROS, at low levels, are important for normal insulin signaling and do not induce insulin resistance (41, 42). In conjunction with both views, diets rich in LCFAs have also been reported to decrease antioxidant protection, thereby promoting oxidative damage (40, 43); whereas other reports demonstrated that antioxidants do not improve insulin action, especially in insulin-resistant humans [summarized in (44)]. Little is known about the effects of MCFAs on ROS generation and the accumulation of oxidative damage. Here we have shown that in response to MCFAs, both mitochondrial superoxide production in C2C12 myotubes as well as mitochondrial 

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changes of UCP3 expression are not only used as a marker of mitochondrial oxidative metabolism (50), but also as an oxidative stress marker (51). Nègre-Salvayre et al. (52) were first to suggest a role for UCPs in regulating mitochondrial ROS production. Nowadays, several transgenic model systems suggest that UCP3 is able to curtail superoxide production by acting as a mild mitochondrial antioxidant.

In healthy systems, ROS are detoxified by various antioxidant systems. However, in diseased states and during long-term dietary LCFA intake, this “oxidant-antioxidant” balance has been shown to be tilted in favor of the reactive species, resulting in ROS-mediated damage to lipids, proteins, and DNA (47). To assess how the different FAs affected the oxidative status of muscle, we measured lipid hydroperoxides and TBARS as markers of lipid peroxidation, protein carbonylation as a marker of oxidative damage to proteins, and the antioxidant enzymes GPx (activity) and SOD2 (protein content). Consistent with previous studies (48, 49), these oxidative markers were increased in muscle of mice fed the LCFA-rich diet (with the exception of SOD2), as well as in C2C12 myotubes after overnight treatment with various LCFAs. However, MCFA-treated mice and cells displayed no evidence of oxidative stress, with oxidative damage markers and antioxidant protection remaining similar to controls (GPx, LOOH, protein carbonyls) or even being decreased (TBARS, SOD2). These results highlight that MCFA diets do not promote oxidative stress in muscle and this may be an additional mechanism explaining why high-fat diets containing MCFA do not cause the same deterioration in glucose homeostasis and insulin action as LCFA diets (5).

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Medium-chain fatty acids in metabolism and oxidative stress

Fig. 7. Markers of mitochondrial metabolism in skeletal muscle mitochondria. Representative immunoblotting results of oxidative markers in mice fed CHOW-, MCFA-, or LCFA-rich diets. Shown are n = 4 for each group, but percentage changes represent n = 6–8; *P < 0.05, **P < 0.01, and ***P < 0.001. Complexes I–V represent subunits of the complexes of the ETC. Porin was used as mitochondrial loading control and shows similar distribution in all diet groups.

Fig. 8. Oxidative stress markers in muscle from CHOW-, MCFA-, and LCFA-fed mice. GPx activity (A), protein carbonylation (B), lipid hydroperoxide (C), and TBARS levels (D). Shown are means ± SEM; n = 6–8 per diet group; *P < 0.05, **P < 0.01 and, ***P < 0.001.

In conclusion, our study has revealed that MCFAs increase oxidative metabolism at the level of the mitochondrion in muscle. Furthermore, in contrast to LCFA, MCFAs prevent the induction of oxidative stress that normally arises due to excess lipid intake. Our results in myotubes indicate that capric and lauric acid (C10:0 and C12:0) are most likely responsible for the observed effects, with these two FAs comprising more than 40% of the total FA content in our MCFA diet (8). Overall these findings provide new insight into the mechanisms that contribute to the favorable effects of MCFAs on energy homeostasis and insulin action.

The authors thank the Biological Testing Facility at the Garvan Institute (Sydney, Australia) for assistance with animal care.

REFERENCES


and alterations in insulin action are lipid species dependent: post- 
tissue-specific effects of medium-chain fatty acids. Diabetes. 58: 
2547–2554.
9. Gao, D., S. W. Nong, X. Q. Huang, Y. G. Lu, H. Y. Zhao, J. Y. Lin, 
Y. Man, S. Wang, J. F. Yang, and J. A. Li. 2010. The effects of palmitic 
ions on hepatic insulin resistance are mediated by NADPH Oxidase 
3-derived reactive oxygen species through JNK and p38MAPK path-
10. Nakamura, S., T. Takamura, N. Matsuzawa-Nagata, H. Takayama, 
2009. Palmitate induces insulin resistance in H4IIEC3 hepatocytes 
through reactive oxygen species produced by mitochondria. J. Biol. 
Chem. 284: 14869–14881.
Rolph, and G. J. Cooney. 2007. Excess lipid availability increases 
mitochondrial fatty acid oxidative capacity in muscle: evidence for 
a role against a reduced fatty acid oxidation in lipid-induced insu-
2008. The effects of fasting and cold exposure on metabolic rate 
and mitochondrial proton leak in liver and skeletal muscle of an 
15. Brown, J. C. L., G. B. McClelland, P. A. Faure, J. M. Klaiman, and 
J. F. Staples. 2009. Examining the mechanisms responsible for lower 
ROS release rates in liver mitochondria from the long-lived house 
sparrow (Passer domesticus) and big brown bat (Eptesicus fuscus) 
compared to the short-lived mouse (Mus musculus). Aging. 130: 467–476.
restriction decreases mitochondrial free radical generation at com-
p lex I and lowers oxidative damage to mitochondrial DNA in the 
Rensen, P. J. Larsen, J. G. Cooney, K. Wassermann, et al. 2003. PPARalpha/gamma ragaglitzatar eliminates fatty liver and 
handles insulin action in fat-fed rats in the absence of hepato-
18. Manyash, V., G. Liebich, T. V. Kurczahila, A. Shevchenko, and 
D. Schwudke. 2008. Lipid extraction by methyl-tert-butyl ether for 
Mitchell, G. J. Cooney, and N. Turner. 2013. Mouse strain-depen-
 dent variation in obesity and glucose homeostasis in response to 
enteral nutrition with lipid emulsions containing LCT or MCT/
MCT quantifyed by ultrasound. JPEN J. Parenter. Enteral Nutr. 15: 
601–603.
20. Nosaka, N., M. Kasai, M. Nakamura, I. Takahashi, M. Itakura, 
Effects of dietary medium-chain triacylglycerols on serum lipopro-
Biochem. 66: 1713–1718.
22. Lambertucci, R. H., S. M. Hirabara, Ldos. R. Silveira, A. C. Levada-
pires, R. Curi, and T. C. Pithon-Curi. 2008. Palmitate increases superoxide production through mitochondrial electron transport 
chain and NADPH oxidase activity in skeletal muscle cells. J. Cell. 
Physiol. 216: 796–804.
2002. Topology of superoxide production from different sites 
in the mitochondrial electron transport chain. J. Biol. Chem. 277: 
44784–44790.
Kane, C. T. Lin, J. W. Price 3rd, L. Kang, P. S. Rabinovitch, H. H. 
state link excess fat intake to insulin resistance in both rodents and 
25. Zhao, X. Z., L. A. Salmon, C. Hohnen-Behrens, N. Turner, 
A. J. Hoy, G. J. Maghaziz, R. Stocker, H. Van Remmen, E. W. 
antioxidant defense mechanism. Proc. Natl. Acad. Sci. USA. 106: 
17787–17792.
of oleate and palmi-
diacidites on mitochondrial function, apoptosis, and 
insulin signaling in L6 skeletal muscle cells: role of oxidative stress. 
Reactive oxygen species as a signal in glucose-stimulated insulin 
oxidative species enhance insulin sensitivity. Cell Metab. 10: 
250–272.
29. Silveira, L. R., J. Fiamoncini, S. M. Hirabara, J. Procopio, T. 
Understanding the effects of fatty acids on skeletal muscle. J. Cell. 
Physiol. 217: 1–12.
30. Tiganis, T. 2011. Reactive oxygen species and insulin resistance: 
the good, the bad and the ugly. Trends Pharmacol. Sci. 32: 82–89.
31. Brown, G. C., and V. Borutta. 2012. There is no evidence that 
mitochondria are the main source of reactive oxygen species in 
32. Brand, M. D. 2010. The sites and topology of mitochondrial super-