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 few 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 LCFAs 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 subsequently 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.

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

Running title: Medium-chain fatty acids in metabolism and oxidative stress

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Abbreviations: CPT1 carnitine palmitoyl transferase 1, DAG diacylglycerol, ETC electron transport chain, FA fatty acid, FRL free radical leak, GLUT4 glucose transporter 4, GPx glutathione peroxidase, GSK3β glycogen synthase kinase 3 β, IR insulin receptor, IRS insulin receptor substrate, LCFA long-chain fatty acid, LOOH lipid hydroperoxide, MCFA medium-chain fatty acid, PGC1α peroxisome proliferator-activated receptor, gamma, coactivator 1 α, RER respiratory exchange ratio, ROS reactive oxygen species, SDH succinate dehydrogenase, SOD superoxide dismutase, TAG triacylglycerol, TBARS thiobarbituric acid reactive substances, UCP3 uncoupling protein 3

Abstract

Dietary intake of long-chain fatty acids (LCFA) plays a causative role in insulin resistance and risk of diabetes. Whereas LCFA promote lipid accumulation and insulin resistance, diets rich in medium-chain fatty acids (MCFA) have been associated with increased oxidative metabolism and reduced adiposity, with little 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 LCFA (16:0, 18:1n9, 18:2n6) and MCFA (10:0, 12:0), as well as fed mice diets rich in LCFA or MCFA, 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 to LCFA-mice. Dietary MCFA increased respiration in isolated mitochondria, with a simultaneous reduction in reactive oxygen species generation, and subsequently low oxidative damage. Collectively our findings indicate that in contrast to LCFA, MCFA increase the intrinsic respiratory capacity of mitochondria, without increasing oxidative stress. These effects potentially contribute to the beneficial metabolic actions of dietary MCFA.

Key Words

Metabolic disease, medium-chain fatty acids, mitochondrial metabolism, oxidative stress, insulin signalling

Introduction

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 (LCFA, C>16), 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 LCFA on metabolic health, including increased inflammation, overactivation of stress-related pathways and inappropriate lipid accumulation in non-adipose 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 (MCFA, C8-C12) decreases adiposity (4-6) and increases energy expenditure (7, 8) and avoids many of the detrimental effects associated with LCFA intake. The increased energy expenditure suggests that FA are funnelled into oxidative versus storage pathways, and this has previously been suggested to be due to enhanced cellular uptake and entry of MCFA into mitochondria (especially in the liver) for oxidation. In a recent study we showed that high-fat diets enriched with MCFA 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 MCFA might also be a key pathway for oxidative disposal of this class of FA (8). In conjunction with the increase in markers of mitochondrial metabolism, MCFA also prevented lipid accumulation and insulin resistance in muscle, with similar glucose uptake and muscle triglyceride levels in MCFA-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 characterise the disparate metabolic effects of MCFA and LCFA in muscle. Firstly, we have investigated whether in addition to affecting mitochondrial content in muscle, MCFA and LCFA have differential effects on the intrinsic bioenergetic capacity of mitochondria. Additionally, as mitochondria are a major site for reactive oxygen species (ROS) production and LCFA have been linked with oxidative stress (9, 10), we have determined the effect of MCFA and LCFA 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 using 2% horse serum (Life Technologies, Mulgrave, VIC, Australia) once 90-100% confluence was reached. Cells were differentiated for 5 days, and differentiated myotubes were incubated for 18 hours with individual FA. For the lipid treatments, 200 μM FA in ethanol were conjugated to 1% FA-free BSA at 55°C for 1.5 hours in differentiation media, the media 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 hours (in the presence of the respective FA), stimulated with 10 nM insulin for 30 min and lysed immediately in RIPA buffer for immunoblotting (see below).

Succinate dehydrogenase activity

Cell lysates were used to measured succinate dehydrogenase activity. 10 μl of lysate was mixed with 250 μl of reaction cocktail, containing 50 mM KH_2PO_4, 20 mM succinate, 2.5 μM Antimycin A, 2.5 mM sodium cyanide and 0.45 mM phenazine methosulphate, in a 96-well plate. 50 μl of dichloroindophenol (0.72 mM) was added to the wells, and change in absorbance measured at 600nm and 30°C. Protein concentration was measured using the Bradford method.

Fatty acid uptake

For the determination of fatty acid uptake in cells, C2C12 myotubes were grown in 12-well plates and treated for 18 hours with 200μM FA as described above. On the day of the assay, 200μM lauric acid-conjugated media containing 1μCi/ml [1-^{14}C]-lauric acid (MP Biomedicals, Singapore) was applied to cells pre-treated with MCFA and 200μM palmitic acid-conjugated media containing 1μCi/ml [1-^{14}C]-palmitic acid (Perkin Elmer, Melbourne, VIC, Australia) was applied to cells pre-treated with LCFA for exactly 2 hours. Control cells received both treatments. After the 2 hour incubation period, the media was acidified with

1M perchloric acid, and evolved $^{14}CO_2$ was captured in 1M NaOH. Acid-soluble metabolites (ASM) in the media labelled with ^{14}C were also determined. Cells were scraped into 200µl 1x PBS + 0.05% SDS, sonicated, a sub-sample (20µl) taken for protein determination, and the remainder used for lipid extraction. Lipids were extracted using 2:1 chloroform:methanol following phase separation with 0.6% NaCl, ^{14}C in the lipid phase and aqueous phase (representing cell ASM) were determined. Total fatty acid uptake was calculated as the sum of FA that were funnelled into oxidation (CO_2 + ASM in media + ASM cell counts) and the lipid fraction. Protein content was determined using the BCA assay (Pierce, Thermofisher Scientific, Scoresby, VIC, Australia).

Superoxide production in cells

For the determination of mitochondrial as well as cytosolic superoxide production, C2C12 myoblasts were grown in black (clear-bottom) 96-well plates, differentiated and treated with MCFA and LCFA for 18 hours as described above. On the day of the experiment, FA-containing differentiation media was replaced with HBSS buffer (Life Technologies, Mulgrave, VIC, Australia), also containing FA (conjugated to BSA) and either the mitochondrial fluorophor MitoSOX Red (5 µM, in nitrogen-bubbled DMSO), the cytosolic superoxide marker dihydroethidium (DHE; 20µM, 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 (10µM). Time-resolved fluorescence was determined for 1 hour using a BMG Labtech FLUOstar OPTIMA platereader and 485nm/590nm excitation/emission filters. Changes in fluorescence in the MitoSOX Red- or DHE-treated cells were corrected for slope observed in the DMSO-treated cells (for each individual FA treatment). Results are expressed as change in fluorescence/min/mg protein. For the determination of protein content, media was removed from the wells, 10 µl 1 M KOH added to lyse the cells, topped up with 190 µl of ddH_2O, 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 200µM FA as described above. On the day of the assay, cells were serum-starved for 4 hours in the presence of the

respective FA, followed by a 1-hour incubation in serum-free media containing FA and $4\mu Ci/ml$ ^{14}C-glucose (Perkin Elmer, Melbourne, VIC, Australia), and in the absence (basal state) or presence (insulin-stimulated state) of 100nM Insulin. Cells were washed 3 times in PBS, scraped into $100\mu l$ 1M KOH and heated for 15min to 75°C. A sub-sample ($10\mu l$) was taken for protein determination. To the remainder, $30\mu l$ 25mg/ml glycogen, $20\mu l$ saturated Na_2SO_4 and $450\mu l$ ice-cold ethanol was added, the mix vortexed and frozen for 30min at -80°C, followed by a centrifugation step (10min, 13000 rpm, 4°C). Pellets were washed by resuspension in $50\mu l$ ddH_2O, followed by addition of 1ml ice-cold ethanol and recentrifugation. The final pellet was resuspended in $50\mu l$ ddH_2O 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 MCFA or LCFA ad libitum for 8 weeks. The high-fat diet (45% of calories from fat [from hydrogenated coconut oil for the MCFA diet and from lard for the LCFA diet], 20% of calories from protein, 35% of calories from carbohydrate) was made in-house and is based on rodent diet no. D12451 (Research Diets, New Brunswick, NJ, USA). The dietary fatty acid composition was described previously (8). Tissue samples were collected from mice at 09:00–10:00 hours 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 (DXA) (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-hours following an overnight acclimatisation

period in the Oxymax cages and a 2-hour settling period (Columbus Instruments, Columbus, OH, USA) as previously described (11).

Glucose tolerance and insulin levels

Mice were fasted overnight and injected i.p. 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, USA).

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–1 Protease Type VIII (Sigma P 5380)] and homogenized using an ultraturrax homogenizer. The homogenate was spun for 5 min at 500 g and 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 FA for 18 hours as described above, trypsinized into 1ml 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 K_2HPO_4, 3 mM Hepes, 1 mM EGTA, 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 (5mM) and rotenone (2µM) or palmitoyl carnitine (40µM) + 2 mM malate as substrates. Oxygen consumption was measured in state II (in the presence of substrate), state III (after addition of 200 µM ADP) and in state IV (after addition of 2.5 µg/ml oligomycin). The respiratory control ratios (state III/state IV respiration) were above 3.5 for succinate and above 5.0 for palmitoyl carnitine as substrates, indicating well-coupled mitochondria.

Mitochondrial H$_2$O$_2$ production

Hydrogen peroxide production in isolated mitochondria was determined by monitoring the oxidation of Amplex Ultra Red (Invitrogen, Mount Waverley, VIC, Australia) using a FLUOstar OPTIMA fluorescence plate reader, maintained at 37°C. Mitochondria were incubated at 0.25 mg/ml in assay medium (120 mM KCl, 3 mM Hepes, 1 mM EGTA, 0.3% BSA, pH 7.2 at 37°C) containing 6 u/ml horseradish peroxidase, 30 u/ml superoxide dismutase and 0.1 mM Amplex Ultra Red, as described previously (14). H$_2$O$_2$ production was determined under 3 conditions: in the presence of succinate (5mM), succinate and rotenone (2µM, to inhibit electron backflow towards complex I) and in the presence of palmitoyl carnitine (40µM). In separate wells containing assay medium, H$_2$O$_2$ was injected (using on board injectors) at concentrations between 0 and 500nM and a standard curve calculated.

H$_2$O$_2$ production and state II 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 O$_2$ to H$_2$O$_2$, four electrons are transferred in the reduction of 1 mol of O$_2$ to water. Therefore, the percent free radical leak (FRL) was calculated as the rate of H$_2$O$_2$ 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, USA) 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, USA). Ceramide molecular lipids were analysed by precursor-ion scanning for protonated dehydrated sphingosine at m/z 264.3. DAG molecular lipids were analysed by multiple neutral-loss scanning for ammoniated fatty acids. Data were analysed 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 sec, rotated at 4°C for 2 hrs and centrifuged for 10 min at 16,000 g. Mitochondria were isolated as described above, mixed 1:1 in RIPA buffer and sonicated for 5sec. 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). Immunolabelled 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 NaH_2PO_4, 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 beta-NADPH vial (Sigma N0411). Sample and reaction cocktail were mixed and H_2O_2 (5 µl, 0.042% (v/v)) was added to start the reaction.

Lipid hydroperoxides (LOOH) were measured according to the method by Bou et al. 2008 (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 LSD 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 MCFA capric (C10:0) and lauric acid (C12:0) for 18 hours 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 sub-units of the mitochondrial electron transport chain, the master-regulator of mitochondrial biogenesis PGC1α, the mitochondrial content marker porin and the mitochondrial fatty acid transporter CPT1 (Fig. 1C and Supplemental Table 1). The observed increase in SDH activity was most likely due to increased protein expression, as shown by immunoblotting for SDHa (Fig. 1C). In contrast, treatment of myotubes with LCFA, 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 Supplemental Table 1), and caused a mild decrease in succinate dehydrogenase (SDH) activity compared to control cells (Fig. 1B). Protein levels of the glucose transporter GLUT4 were unaffected by FA treatment (Fig. 1C).

Incubation of C2C12 myotubes with LCFA led to an average 5-fold increase in triacylglycerol (TAG) accumulation in comparison to BSA-treated control cells. Interestingly, myotubes incubated with the MCFA exhibited markedly less TAG accumulation compared to 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 ^{14}C-labelled fatty acids (Fig. 1E). Collectively these results suggest enhanced partitioning of FA towards oxidation with MCFA, and the tracer experiments showed that approximately 65% of ^{14}C-lauric acid taken up was directed to oxidative pathways (CO_2 and ASM), while this was only approximately 35% with ^{14}C-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, LCFA 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 LCFA and MCFA on superoxide generation and lipid peroxidation (2 markers

examined: lipid hydroperoxides (LOOH) and TBARS). Mitochondrial (Fig. 2A), but not cytosolic superoxide generation (Fig. 2B), was significantly increased with all three LCFA, 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 LCFA but not MCFA treatment (Fig. 2 C+D). This highlights that MCFA keep oxidative stress at low levels, whereas LCFA 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 LCFA and MCFA affected insulin-stimulated phosphorylation of IRS1 (Tyr612), Akt (Ser473) and GSK3β (Ser9), as well as the insulin-stimulated glycogen synthesis rate (Fig 3). Whereas none of the FA had an effect on phosphorylation of IRS1, incubation of muscle cells with MCFA 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 LCFA but not MCFA treatment, which was accompanied by a significant decrease in insulin-stimulated glycogen synthesis rate with all three LCFA (Fig. 3). These results suggest that MCFA, unlike LCFA, 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 to chow controls) after 8 weeks of high-fat feeding (Fig. 4 A+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 to the CHOW-fed mice (CHOW 11.1 ± 0.6; MCFA 12.8 ±

0.6; LCFA 12.3 ± 0.6 kcal/day). We did not assess if there were differences in lipid absorption between the two diets, however, MCFA are more efficiently absorbed then LCFA (23), and thus differences in energy dissipation by inefficient digestion of the dietary fatty acids is unlikely to underlie the differences in adiposity and body composition. Whole-body oxygen consumption (normalised to lean mass) was elevated in both fat-fed groups compared to controls, and was significantly higher in MCFA- vs. LCFA-fed animals in both the light and dark phase (Fig. 4 C). Furthermore, the respiratory exchange ratio (RER) showed a greater decrease in MCFA-mice than in LCFA-mice compared to control chow-fed mice (Fig. 4 D). The increase in overall energy expenditure and the lower RER could partly explain the reduced adiposity in MCFA-fed mice. Compared to LCFA-mice, MCFA-fed animals also did not develop the same degree of high-fat diet-induced glucose intolerance (Fig. 4 E) and displayed lower fasting insulin levels (Fig. 4 F).

Effect of dietary MCFA and LCFA intake on muscle lipid accumulation

MCFA-fed mice displayed less muscle triacylglycerol (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 diacylglycerol (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, DAG 16:0/18:0 and DAG16:1/18:1 (Supplemental Fig. 1).

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

To determine if MCFA altered mitochondrial function, oxygen consumption in states II, III and IV was measured in isolated muscle mitochondria using succinate and palmitoyl carnitine as substrates. Mitochondria from muscle of MCFA-mice showed significantly greater rates of oxygen consumption than chow- and LCFA-fed animals in state III for both succinate and palmitoyl carnitine (Fig. 5 A+B). Increased mitochondrial substrate flux is often accompanied by higher ROS production, however the mitochondria from MCFA-fed mice displayed a 32% decrease in H_2O_2 generation in the presence of succinate (when compared to

LCFA-mice), with no difference in ROS production detected when palmitoyl carnitine was used as a substrate for the electron transport chain (ETC) (Fig. 5 C).

Using certain assumptions (see methods section), it is possible to calculate the percentage of electrons that leak out of sequence during electron transfer in the ETC, and, rather than being transferred to molecular oxygen as final electron acceptor, leak out and produce superoxide. This has been called the free radical leak (FRL) (16). With succinate (in the presence of rotenone), muscle mitochondria from MCFA-fed mice displayed a 40% decrease in FRL, with no change observed in the LCFA-animals in comparison to controls. In contrast, with palmitoyl carnitine the FRL was two-fold higher in LCFA-mice than in MCFA- or chow-fed mice (Fig. 5D). Both substrates point towards a MCFA-rich diet having beneficial effects on mitochondrial electron leakage, with less superoxide molecules produced per consumed oxygen molecule.

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. 6). Protein expression of sub-units 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 to mitochondria from chow-fed controls (Fig. 6). Superoxide dismutase 2 (SOD) was significantly decreased in the MCFA-mice, whereas no difference was observed in carnitine palmitoyl transferase 1 (CPT1) protein content between diet groups. These data suggest that MCFA 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, MCFA 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.

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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). Glutathione peroxidase, 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-, but not the MCFA-diet (Fig. 7 A-C). TBARS, an endpoint marker of lipid peroxidation, remained unchanged in muscle of LCFA-fed mice, but was significantly decreased in MCFA-mice, when compared to controls (Fig. 7 D). Overall, these data indicate that MCFA 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 MCFA, 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 favourable effects have been partially ascribed to the physical properties of MCFA, because this class of FA is more readily funnelled into oxidative pathways, which could lead to increased energy expenditure and less fat deposition in adipose and other tissues compared to LCFA (26). The tissue that is likely to be responsible for many of the beneficial effects of MCFA 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 markers of mitochondrial content than LCFA, and a prevention of lipid accumulation and insulin resistance in this tissue (8). In the current study, we have shown that the beneficial effects of MCFA 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 MCFA 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 MCFA improve mitochondrial substrate oxidation in muscle to a greater extent than LCFA, 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- vs. 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 (Fig. 5C), with CER24:0 having previously been reported to have beneficial anti-apoptotic effects (31). Importantly, we have shown that the effects of MCFA 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 MCFA, 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 MCFA and LCFA 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 (H_2O_2), both reactive oxygen species 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 LCFA 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 signalling and do not induce insulin resistance (41, 42). In conjunction with both views, diets rich in LCFA 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 MCFA on ROS generation and the accumulation of oxidative damage. Here we have shown that in response to MCFA, both mitochondrial superoxide generation in C2C12 myotubes, as well as mitochondrial H_2O_2 production in mouse skeletal muscle, was similar or decreased compared to 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 myotubes, was significantly reduced in response to both MCFA and LCFA treatments, indicating that mitochondrial ROS generation is likely to be a more important mediator of changes in cellular oxidative status, as recently suggested (45, 46).

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 favour of the reactive species, resulting in ROS-mediated damage to lipids, proteins and DNA (47). To assess how the different FA 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 LCFA. 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 being even decreased (TBARS, SOD2). These results highlight that MCFA 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 (5). Furthermore, as

antioxidant systems are expected to respond to the need of tissues for protection (24), the decrease in SOD2 protein content (and similar GPx activity) further supports the low oxidative stress levels observed with the MCFA diet.

Changes of UCP3 expression are not only used as a marker of mitochondrial oxidative metabolism (50), but also as an oxidative stress marker (51). Negre-Salvayre and colleagues were first to suggest a role for UCPs in regulating mitochondrial ROS production (52). Nowadays, several transgenic model systems suggest that UCP3 is able to curtail superoxide production by acting as a mild mitochondrial uncoupler (53, 54). MCFA-mice displayed an 88% increase in UCP3 content in skeletal muscle, whereas UCP3 only showed a tendency towards an increase with the LCFA-diet (+40%). The elevated UCP3 content in MCFA-fed mice might also partly explain their decreased H_2O_2 generation and electron leakage, as well as their increased oxygen consumption.

MCFA have been reported to improve insulin action and glucose homeostasis in various experimental models. Type 2 diabetic subjects treated acutely with MCFA for 5 days showed an improvement in insulin-stimulated glucose disposal (55). Similarly, Han and colleagues showed a decrease in body weight, HOMA-IR and fasting insulin in type 2 diabetic subjects that consumed diets supplemented with MCFA for 3 months when compared to subjects consuming LCFA-rich diets (56). Comparable beneficial effects have also been reported in various rodent models (4, 5, 8, 57) and in muscle cells (58). To test if some of the beneficial effects of MCFA on insulin action might be related to changes in insulin signalling, we determined the phosphorylation status of the key insulin signalling intermediates IRS1, Akt and GSK3β in muscle cells. Compared to control cells, insulin-induced Akt phosphorylation was significantly increased after MCFA treatment, but was unchanged or even decreased (in the case of palmitic acid) after LCFA treatment. Similarly, phosphorylation of GSK3β was unchanged in MCFA-treated cells but decreased after LCFA-treatment. Changes in GSK3β phosphorylation were accompanied by a similar pattern in the rate of insulin-stimulated glycogen synthesis, which was significantly reduced in the LCFA-treated cells. Thus, although there is still controversy regarding the exact relationship between alterations in the activation state of insulin signalling intermediates and endpoint measures of insulin action (e.g. glucose uptake) (59, 60), our results indicate that MCFA display enhancing effects on both the insulin signalling intermediates, as well as on glycogen synthesis, which is commonly used as an endpoint measure of insulin action in cell culture systems (61, 62).

In conclusion, our study has revealed that MCFA increase oxidative metabolism at the level of the mitochondrion in muscle. Furthermore, in contrast to LCFA, MCFA 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 FA 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 favourable effects of MCFA on energy homeostasis and insulin action.

Acknowledgements

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Figure Legends

Figure 1. Metabolic activity of C2C12 myotubes. Oxygen consumption (A), succinate dehydrogenase activity (B), immunoblotting for oxidative proteins (C), triacylglycerol levels (D) and fatty acid uptake (E) were measured in muscle cells treated with various MCFA or LCFA (18 hour incubations with 200μM FA). Fatty acid uptake was measured after 18hr-treatment with the respective fatty acids, followed by a 1 hour-incubation with either ^{14}C-lauric acid (for C and MCFA treated cells) or ^{14}C-palmitic acid (for C and LCFA treated cells), and was calculated as the sum of FA that were funnelled into oxidative and storage pathways (see methods for details). Shown are means ± SEM, n=3 with 3 replicates each (or 2 replicates in the case of panel A); * $p<0.05$, ** $p<0.001$ vs. C; # $p<0.01$ vs. CA, ^ $p<0.01$ vs. LA. C = control, CA = capric acid, LA = lauric acid, PA = palmitic acid, OA = oleic acid, LinA = linoleic acid

Figure 2. Oxidative stress markers in C2C12 myotubes. Mitochondrial (A) and cytosolic (B) superoxide generation, lipid hydroperoxide (B) and TBARS (C) levels in muscle cells treated with various MCFA and LCFA (18 hour incubations with 200μM FA). Shown are means ± SEM, n=3 with 3 replicates each (or n=4 with 2 replicates each in the case of panel B); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. C; # $p<0.01$ vs. CA, ^ $p<0.01$ vs. LA, ε $p<0.05$ vs. PA, ϕ $p<0.01$ vs. OA. C = control, CA = capric acid, LA = lauric acid, PA = palmitic acid, OA = oleic acid, LinA = linoleic acid

Figure 3. Insulin signalling and glycogen synthesis in C2C12 myotubes. (A) Immunoblotting results in basal and insulin-stimulated muscle cells after 18 hours incubation with various MCFA and LCFA, and 30min stimulation with 10nM insulin. Representative blots show n=2 per group, but graphed changes represent n=4-6 (2-3 independent experiments). (B) Glycogen synthesis rate of FA-treated cells in the absence or presence of 100nM Insulin, measured using ^{14}C-labelled glucose. Bar graphs show means ± SEM. Statistical analysis was measured by 1-way ANOVA using the ratios between basal and insulin-stimulated protein levels in each treatment group with * $p<0.05$, * $p<0.01$ and *** $p<0.001$ vs. C cells. C = control, CA = capric acid, LA = lauric acid, PA = palmitic acid, OA = oleic acid, LinA = linoleic acid, p = phospho, t = total

Figure 4. Metabolic markers in mice fed CHOW-, MCFA- and LCFA-rich diets. Body weight (A), fat pad weight (B), oxygen consumption (C), respiratory exchange ratio (D),

blood glucose levels during a glucose tolerance test (GTT) (E) and fasting plasma insulin levels (F). For the GTT, glucose (2 g/kg) was injected at the 0 time point, and blood glucose levels were monitored for 90 after injection. Shown are means ± SEM, n=6-8 per diet group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Significance values in panel E represent differences in the area-under-the-curve during the GTT.

Figure 5. Muscle lipid accumulation in mice fed CHOW-, MCFA- and LCFA-rich diets. Total triacylglyceride (A), ceramide and diacylglyceride levels (B) and ceramide species (C) in quadriceps muscle. Shown are means ± SEM, n=6-8 per diet group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. C = Chow, M = MCFA and L = LCFA

Figure 6. Mitochondrial respiration and hydrogen peroxide production in muscle from CHOW-, MCFA- and LCFA-fed mice. Mitochondrial oxygen consumption was measured in state 2, state 3 and state 4 using succinate (A) or palmitoyl carnitine (B) as a substrate. Hydrogen peroxide production was measured in the presence of succinate, succinate plus rotenone and palmitoyl carnitine (C). H_2O_2 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, the free radical leak (D). Shown are means ± SEM; n=6-8 for diet group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 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 *** $p<0.001$. Complex 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.

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

Figures

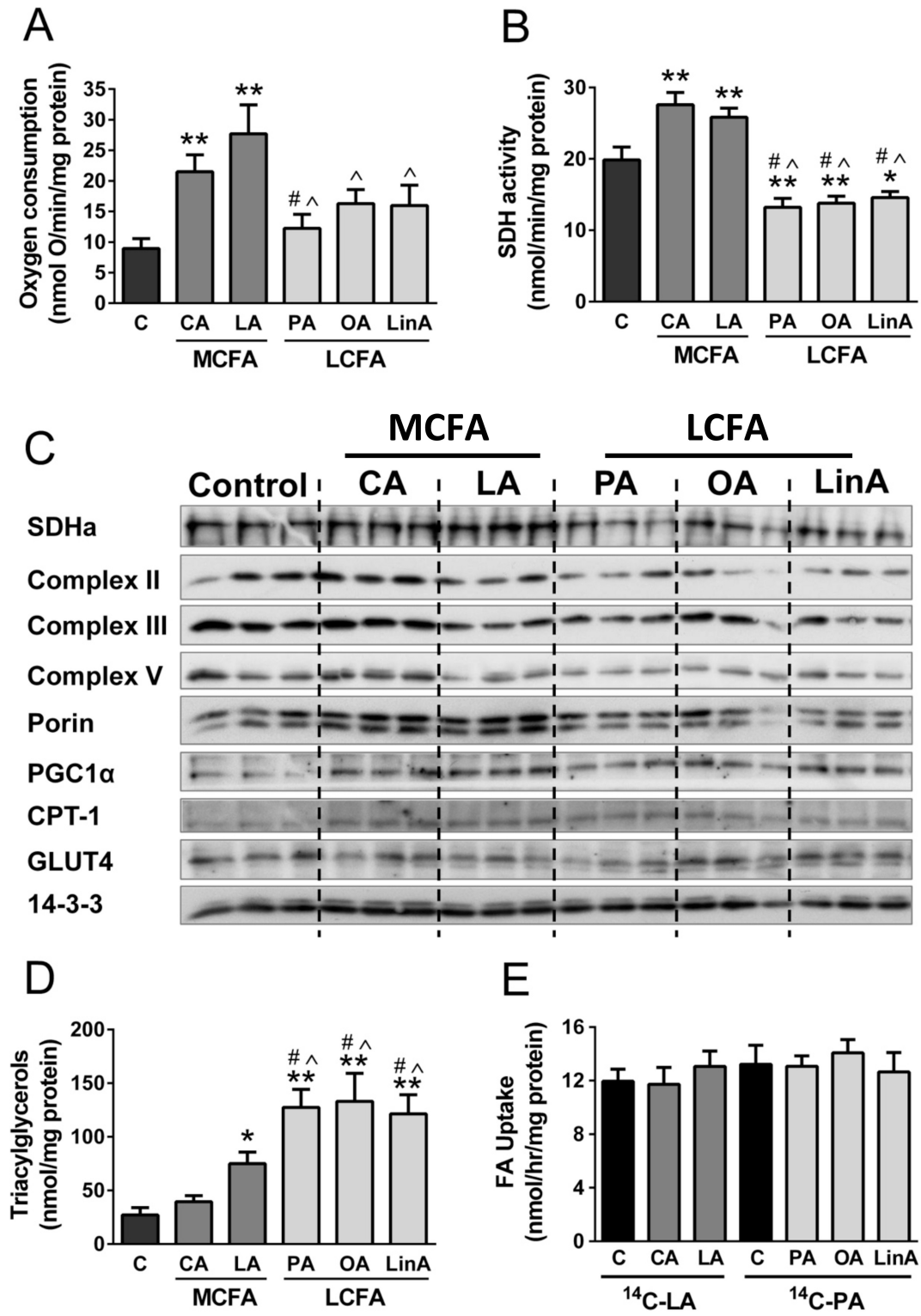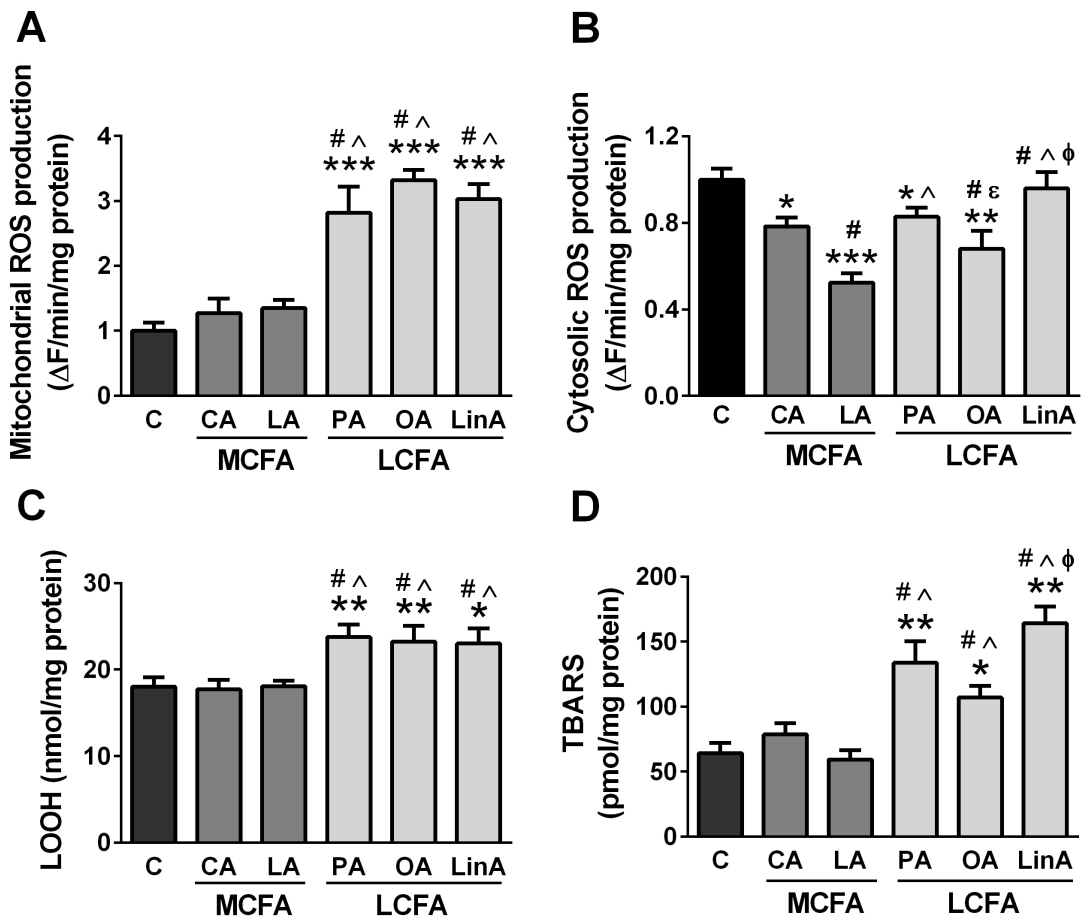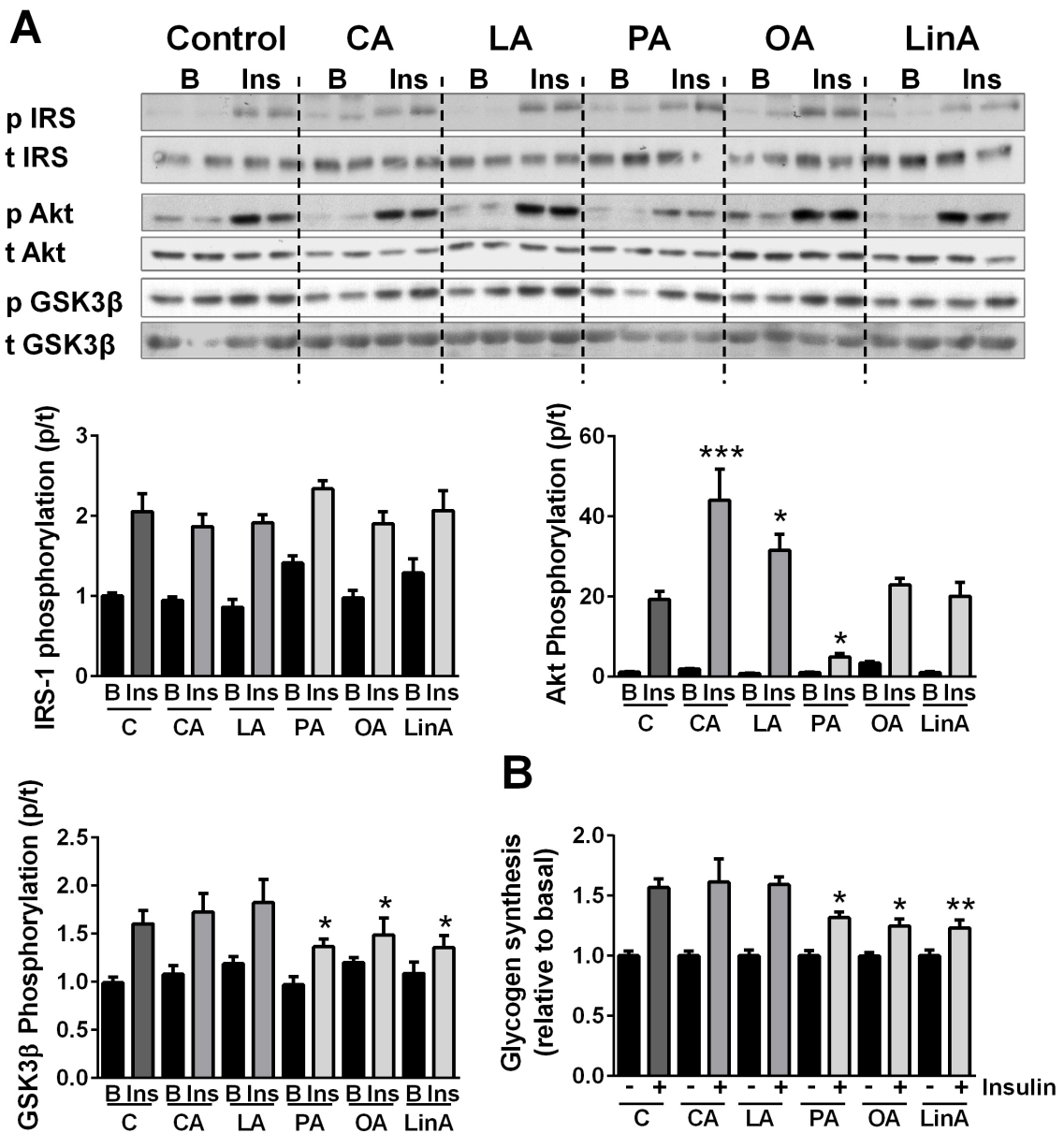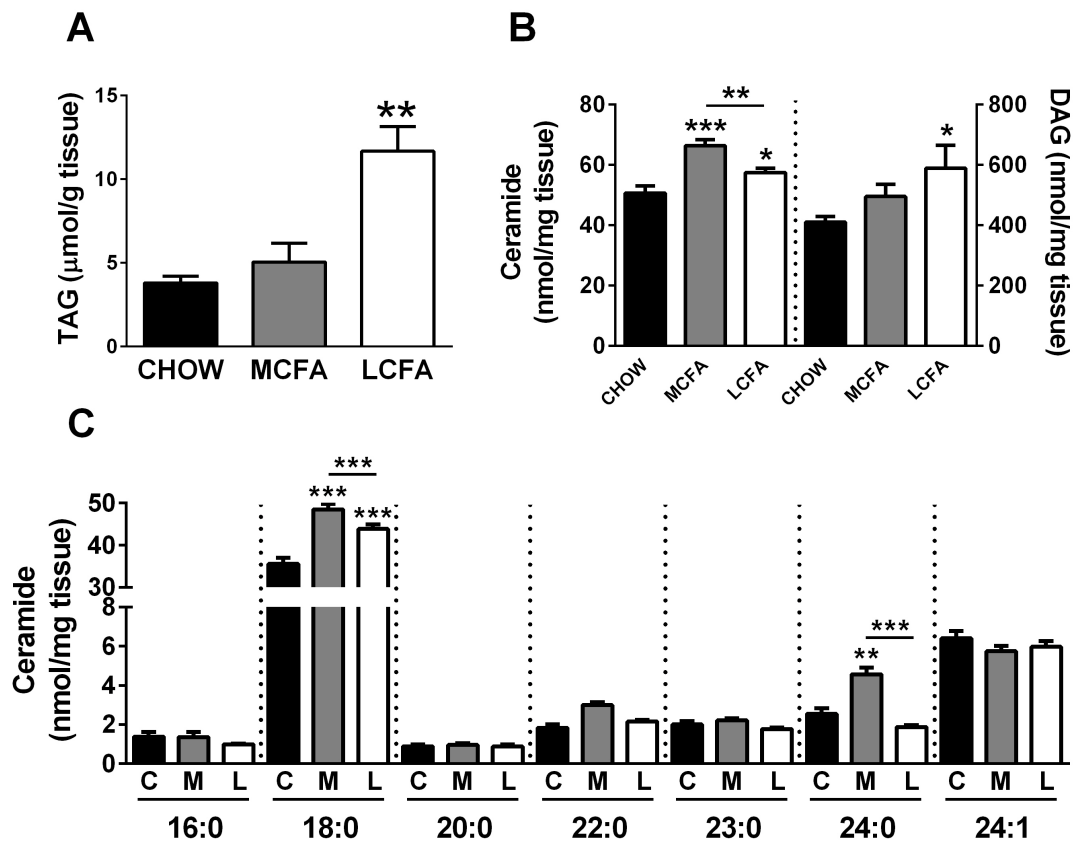**Figure 1**

Figure 2

Figure 3

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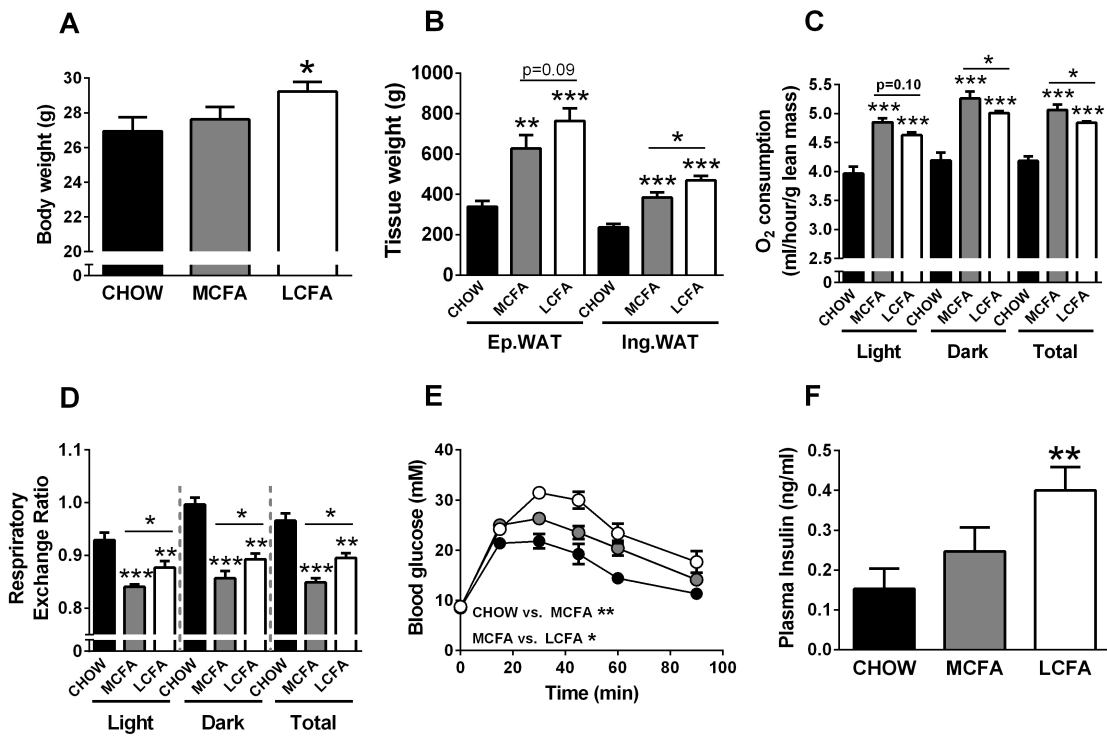
Figure 4

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