Carnitine supplementation in high-fat diet fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction in vivo

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Running title: Carnitine insufficiency and mitochondrial function

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Abstract (250 words)

Muscle lipid overload and the associated accumulation of lipid intermediates plays an important role in the development of insulin resistance. Carnitine insufficiency is a common feature of insulin-resistant states and might lead to incomplete fatty acid oxidation and impaired export of lipid intermediates out of the mitochondria. The aim of the present study was to test the hypothesis that carnitine supplementation reduces high-fat diet-induced lipotoxicity, improves muscle mitochondrial function and ameliorates insulin resistance. Wistar rats were fed either normal chow or a high-fat diet for 15 weeks. One group of high-fat diet fed rats was supplemented with 300 mg/kg/day L-carnitine during the last 8 weeks. Muscle mitochondrial function was measured \textit{in vivo} by \textsuperscript{31}P magnetic resonance spectroscopy (MRS) and \textit{ex vivo} by high-resolution respirometry. Muscle lipid status was determined by \textsuperscript{1}H MRS (intramyocellular lipids) and tandem mass spectrometry (acylcarnitines). High-fat diet feeding induced insulin resistance and was associated with decreases in muscle and blood free carnitine, elevated levels of muscle lipids and acylcarnitines, and an increased number of muscle mitochondria that showed an improved capacity to oxidize fat-derived substrates when tested \textit{ex vivo}. This was however not accompanied by an increase in muscle oxidative capacity \textit{in vivo}, indicating that \textit{in vivo} mitochondrial function was compromised. Despite partial normalization of muscle and blood free carnitine content, carnitine supplementation did not induce improvements in muscle lipid status, \textit{in vivo} mitochondrial function or insulin sensitivity. Carnitine insufficiency therefore does not play a major role in high-fat diet induced muscle mitochondrial dysfunction \textit{in vivo}. 
Keywords: Insulin resistance, mitochondrial function, lipid accumulation, fatty acid oxidation, carnitine supplementation
Introduction

Diabetes has reached epidemic proportions worldwide (1). Type 2 diabetes accounts for 85-95% of all diabetes cases and is characterized by insulin resistance in major metabolic tissues such as skeletal muscle (12). One of the leading hypotheses in the research field of type 2 diabetes is that lipid overload in muscle cells, supposedly as a result of a reduced mitochondrial capacity to oxidize fatty acids (FAs), leads to impaired insulin signaling (30, 41). However, recent studies have linked insulin resistance to an increased rather than a decreased capacity to oxidize FAs (19, 45, 46, 48, 56). The increased FA oxidation capacity in insulin-resistant states was shown to be associated with the accumulation of intermediates of incomplete FA oxidation (23, 24, 37), indicating that FA oxidation flux outpaces the demand of the respiratory system. This mismatch between FA substrate supply and demand may promote mitochondrial oxidative stress, which is thought to contribute to the development of insulin resistance (24, 32, 33).

Carnitine is an essential nutrient with multiple functions. Its major role is in the formation of acylcarnitines from long-chain FAs, which is required for the transport of acyl moieties into the mitochondrial matrix for β-oxidation (15, 42). A second role for carnitine is to increase acyl and acetyl group efflux out of the mitochondria and into the plasma (54). Moreover, carnitine stimulates the oxidation of pyruvate by lowering the mitochondrial acetyl-CoA/CoA ratio in a reaction catalyzed by carnitine acetyltransferase (CrAT), which converts acetyl-CoA into acetylcarnitine (47).
Carnitine insufficiency is a common feature of insulin-resistant states and it has been shown that muscle free carnitine negatively correlates with insulin resistance (37). Therefore, carnitine supplementation has been suggested as a potential treatment for type 2 diabetes (35, 40). Indeed, intravenous infusion of carnitine during a hyperinsulinaemic-euglycaemic clamp has been shown to increase whole-body glucose disposal in both healthy subjects and type 2 diabetes patients (6, 11, 14, 17, 29). Moreover, carnitine supplementation has been shown to improve whole-body glucose tolerance in insulin-resistant human subjects and rodent models of metabolic disease (34, 37, 38). The positive effect of carnitine on insulin sensitivity may be explained by different mechanisms, depending on its concentration and the target tissue. In the perfused isolated working rat heart, addition of carnitine to the perfusion medium at a supra-physiological concentration (10 mM) increased glucose oxidation by lowering the concentration of acetyl-CoA in the mitochondrial matrix through CrAT (5). However, in skeletal muscle and at the lower concentrations achievable in vivo, carnitine has been shown to reduce glucose oxidation (14), but stimulate FA oxidation (31, 58) through a mass-action effect on the transport of long-chain FAs into the mitochondrial matrix (2). At the same time, carnitine increases the efflux of acylcarnitines from muscle tissue (37). Therefore, it has been proposed that carnitine supplementation ameliorates insulin resistance by reducing lipotoxicity both through increased oxidation and increased export of muscle lipid metabolites (35).

In a previous study, we showed that in long-term high-fat diet fed rats in vivo muscle mitochondrial function is compromised by mitochondrial lipid overload (48). The aim
of the present study was to test the hypothesis that carnitine supplementation would reduce high-fat diet-induced lipotoxicity, improve *in vivo* muscle mitochondrial function and ameliorate insulin resistance. Wistar rats were fed either normal chow or a high-fat diet for 15 weeks and one group of high-fat diet fed rats was supplemented with L-carnitine (300 mg/kg body weight/day) during the last 8 weeks. *In vivo* muscle mitochondrial function was measured by $^3$P magnetic resonance spectroscopy (MRS) and *ex vivo* mitochondrial function was determined by measuring oxygen consumption in isolated mitochondria. Furthermore, intramyocellular lipid levels were determined by *in vivo* $^1$H MRS and free carnitine and acylcarnitine levels were determined upon sacrifice in muscle tissue, and in blood and urine, using tandem mass spectrometry.
Materials and Methods

Animals

Adult male Wistar rats (14 weeks of age, n=30; Charles River Laboratories, The Netherlands) were housed in pairs at 20 °C and 50% humidity, with a 12-h light-dark cycle. Ad libitum food and water was provided during a period of 15 weeks. The rats were divided into three groups (n=10 per group): A control group receiving normal chow (NC; 9% calories from fat, 67% calories from carbohydrate, 24% calories from protein; R/M-H diet, Ssniff Spezialdiäten GmbH, Soest, Germany), a group receiving a high-fat diet (HFD; 45% calories from fat (predominantly lard), 35% calories from carbohydrate, 20% calories from protein; D12451, Research Diet Services, Wijk bij Duurstede, the Netherlands), and a group receiving the same high-fat diet, supplemented with 300 mg/kg body weight/day L-carnitine in their drinking water for the last 8 weeks (HFDC). Body weight and food and water intake were determined weekly. Two days after the in vivo MRS measurements, rats were sacrificed by incision of the inferior vena cava under anesthesia. One tibialis anterior (TA) muscle was used for isolation of mitochondria. The other TA was frozen in liquid nitrogen and stored at -80 °C for acylcarnitine and mitochondrial content determinations. All experimental procedures were reviewed and approved by the Animal Experimental Committee of Maastricht University.

Oral glucose tolerance test

An oral glucose tolerance tests (OGTT) was performed after 15 weeks of diet, three to five days before the in vivo measurements. After a four-hour fast, rats received an oral glucose bolus of 1 g/kg body weight. Blood samples were taken without anesthesia.
from the *vena saphena* just before and at 15, 30, 60, 90 and 120 min after the glucose bolus. Plasma glucose concentration was determined using a glucometer (FreeStyle, Abbott, IL, USA). Plasma insulin concentration was determined using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Areas under the OGTT curves for both glucose (AUC$_g$) and insulin (AUC$_i$) were calculated.

*Magnetic resonance spectroscopy*

All magnetic resonance spectroscopy (MRS) measurements were performed on a 6.3 Tesla horizontal Bruker MR system (Bruker, Ettlingen, Germany). Animals were anaesthetized using isoflurane (Forene®) (1.5-2%) with medical air (0.6 L/min) and body temperature was maintained at 36 ± 0.5 °C using heating pads. Respiration was monitored using a pressure sensor registering thorax movement (Rapid Biomedical, Rimpar, Germany).

Intramyocellular lipid (IMCL) content in TA was measured using single-voxel localized $^1$H MRS. Voxels of 3×3×3 mm$^3$ were measured in the medial part of the TA, close to the tibia bone, with a circular $^1$H surface coil (Ø 40 mm) and using the PRESS sequence (repetition time TR = 1.5 s, echo time TE = 9.4 ms). One spectrum was acquired without water suppression (16 averages) and one with water suppression (VAPOR water suppression, 512 averages). $^1$H MR spectra were fit in the time domain using the advanced method for accurate, robust, and efficient spectral fitting (AMARES) in the jMRUI software package (jMRUI V2.1) (52) as described previously (56). In the water-suppressed $^1$H MR spectra, the IMCL-CH$_2$ peak at 1.28 ppm was separately fitted from the signal from extramyocellular lipids (resonating between 1.4 and 1.5 ppm) and
IMCL content was expressed as a percentage of the non-suppressed water signal measured in the same voxel.

$^{31}$P MRS was performed using a combination of a circular $^1$H surface coil (Ø 40 mm) for shimming and an ellipsoid $^{31}$P MRS surface coil (10/18 mm), positioned over the TA. $^{31}$P MR spectra were acquired applying an adiabatic excitation pulse with a flip angle of 90°. A fully relaxed (TR = 25 s, 48 averages) spectrum was measured at rest, after which time series of $^{31}$P MR spectra (TR = 5 s, 4 averages) were acquired during 3 min of rest, 2 min of muscle stimulation and 10 min of recovery, as described previously (10, 57). Muscle contractions were induced by electrical stimulation of the TA, via subcutaneously implanted electrodes positioned along the distal N. peroneus communis (10). Stimulation pulse length was 100 ms, frequency was 80 Hz and stimulation voltage varied between 2.5 and 4 V, to reach similar levels of PCr depletion. $^{31}$P MR spectra were fit using AMARES in jMRUI as described previously (49). Concentrations of PCr and inorganic phosphate (P$_i$) were determined relative to the ATP concentration, which was assumed to be 8.2 mM in resting TA muscle (44). Intracellular pH was calculated from the chemical shift difference between the P$_i$ and PCr resonances (43). The data of PCr recovery were fit to a mono-exponential function using Matlab (version R2010b, Mathworks, Natick, MA, USA) yielding the rate constant of PCr recovery, $k_{PCr}$, which is a measure of in vivo muscle oxidative capacity. Results from two time series with end-stimulation pH values higher than 6.9 were averaged (50).
Measurement of oxygen consumption

Mitochondria were isolated from one whole TA muscle through a differential centrifugation procedure as described previously (39, 48). Protein content was determined using the BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). Oxygen consumption rates were measured at 37 °C using a two-channel high-resolution Oroboros oxygraph-2k (Oroboros, Innsbruck, Austria).

Mitochondria were incubated in the assay medium containing 110 mM KCl, 20 mM Tris, 2.3 mM MgCl2, 5 mM KH2PO4 and 1 mg/ml BSA, pH 7.3. All measurements were performed in 1 ml of assay medium containing 0.15 mg/ml of mitochondrial protein. Three different combinations of substrates were used to assess mitochondrial capacity to oxidize tricarboxylic acid cycle (TCA) and β-oxidation substrates: (i) 5 mM pyruvate plus 5 mM malate (pyruvate dehydrogenase and part of TCA cycle), (ii) 25 μM palmitoyl-L-carnitine plus 2.5 mM malate (mitochondrial β-oxidation and part of TCA cycle), and (iii) 25 μM palmitoyl-CoA plus 2.5 mM L-carnitine plus 2.5 mM malate (carnitine palmitoyltransferase 1 (CPT1), mitochondrial β-oxidation and part of TCA cycle). An ADP-regenerating system consisting of excess hexokinase (4.8 U/ml) and glucose (12.5 mM) was used to maintain steady-state oxygen consumption rates. Maximal ADP-stimulated oxygen consumption rate, i.e. the OXPHOS state (18), was initiated by addition of 1 mM of ATP. Maximal oxygen consumption rate in the uncoupled state, i.e. the ETS state, was determined after addition of 1 μM carbonyl cyanide 3-chlorophenyl hydrazone (CCCP). The oxygen consumption rate due to proton leak across the mitochondrial inner membrane, i.e. the LEAK state, was measured after fully blocking ATP synthesis with 1.25 μM carboxyatractyloside (CAT). The sensitivity of the basal proton leak rate to FA, which
reflects activation of the uncoupling proteins (UCPs) (13), was determined by measuring stimulation of oxygen consumption rate in the LEAK state after addition of 90 µM of palmitic acid (C16:0). The signals from the oxygen electrode were recorded at 0.5 s intervals. Data acquisition and analysis was performed using Oxygraph-2k-DatLab software version 4.2 (Oroboros, Innsbruck, Austria).

Determination of the mtDNA copy number and citrate synthase activity

Genomic DNA was isolated from a ~25 mg transversal slice of mid-belly TA using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). The mtDNA copy number was assessed by determining the copy number of mitochondrial genome-encoded ATP synthase subunit 6 gene (mt-ATP6) relative to a single copy nuclear peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) gene using quantitative PCR as described in (9). Primer sequences were: mt-ATP6 forward – 5'-ACACCAAAAGGACGAACCTG-3', mt-ATP6 reverse – 5'-ATGGGGAAGAAGCCCTAGAA-3', and PGC-1α forward – 5'-ATGAATGCAGCGGTCTTAGC-3’, PGC-1α reverse – 5’-AACAATGGCAGGGTTTGTTC-3’.

Citrate synthase activity was measured in TA muscle homogenate as described in (4).

Determination of acylcarnitine content

The content of free carnitine and acylcarnitines was determined in TA muscle, blood spots and urine samples, by tandem mass spectrometry as described previously (8, 51, 53).
Statistical analysis

Data are presented as means ± SD. The listed n values represent the number of animals used for a particular experiment. Statistical significance of the differences was assessed by applying a one-way analysis of variance (ANOVA) using Tukey HSD post-hoc analyses in the SPSS 20.0 statistical package (SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at P<0.05.
**Results**

**Animal model**

Animal characteristics are summarized in Table 1. After 15 weeks of diet, body weight and body weight gain were higher in both HFD and HFDC rats compared with NC controls (P<0.001). However, body weight gain was lower in the HFDC group than in the HFD group (P<0.05). Average energy intake did not differ between groups. After 15 weeks of diet, HFD and HFDC rats had higher fasting plasma glucose (P<0.001) and insulin (P<0.01) levels than NC rats. Moreover, AUC$_g$, AUC$_i$, and AUC$_g$\*AUC$_i$ from the OGTT were significantly higher for both HFD and HFDC rats when compared with NC controls (P<0.01 or P<0.001), while there were no differences between HFD and HFDC groups.

**IMCL content**

*In vivo* $^1$H MRS was applied to evaluate the effects of 15 weeks of high-fat diet and 15 weeks of high-fat diet in combination with 8 weeks of carnitine supplementation on IMCL levels in TA muscle (Figure 1A). IMCL content was 12- and 11-fold higher in HFD and HFDC rats, respectively, as compared with NC controls (P<0.001; Figure 1B). However, IMCL content did not differ between HFD and HFDC rats.

**Acylcarnitine content**

For a more detailed characterization of changes in lipid metabolism, we determined the content of mitochondrial $\beta$-oxidation intermediates, i.e. acylcarnitines, in TA muscle, blood and urine (Figure 2). After 15 weeks of diet, free carnitine (C0) levels were lower in muscle (P<0.001) and blood (P<0.001) of HFD rats compared with NC.
controls (Figures 2A and 2D). However, carnitine supplementation in rats fed a high-fat diet partially normalized free carnitine in muscle and blood to the level of NC controls. Muscle acetylcarnitine (C2) levels were lower in HFD and HFDC rats (P<0.01), while acetylcarnitine levels in blood were higher in HFD and HFDC rats (P<0.001) compared with NC controls. Moreover, blood acetylcarnitine concentration was 2-fold higher in the HFDC group than in the HFD group (P<0.001). Carnitine supplementation resulted in massive urinary excretion of free carnitine and acetylcarnitine (P<0.001; Figure 2G). Medium- and long-chain acylcarnitine species were almost absent in urine and are therefore not shown. Most medium- and long-chain acylcarnitines in blood were lowered after 15 weeks of high-fat diet (P<0.05), but they were normalized to the level of NC controls upon carnitine supplementation (Figures 2E and 2F). Muscle medium- and long-chain acylcarnitine levels were significantly elevated in HFD and HFDC rats compared with NC controls (P<0.05), but surprisingly did not differ between HFD and HFDC groups (Figures 2B and 2C).

Mitochondrial function in vivo

Dynamic $^{31}$P MRS measurements were performed during and after recovery from electrical stimulation of the TA muscle (Figure 3A) to determine the rate constant of PCr recovery, $k_{PCr}$, after contractions, which reflects in vivo mitochondrial function. Concentrations of metabolites and pH obtained from the $^{31}$P MR spectra are summarized in Table 2. Resting pH was slightly lower in HFD (P<0.001) and HFDC (P<0.01) rats compared with NC controls, but concentrations of PCr and Pi in resting muscle did not differ among groups. At the end of muscle stimulation, intracellular pH and PCr and Pi concentrations were not different between groups, with the
exception of the end-stimulation concentration of PCr in HFD rats, which was slightly higher than in NC controls (P<0.05). However, $k_{PCr}$ did not differ among NC, HFD, and HFDC groups (Figure 3B).

Mitochondrial function ex vivo

Table 3 summarizes the effects of 15 weeks of high-fat diet feeding and 15 weeks of high-fat diet feeding in combination with 8 weeks of carnitine supplementation on intrinsic mitochondrial function ex vivo, represented by oxygen consumption rates in isolated TA mitochondria oxidizing glucose- or fat-derived substrates in different respiratory states. Both high-fat diet feeding and high-fat diet feeding in combination with carnitine supplementation had no effect on pyruvate plus malate-driven oxygen consumption rates in the OXPHOS and ETS states, although oxygen consumption was slightly higher in the LEAK state of HFDC rats compared with NC controls (P<0.001). Interestingly, palmitoyl-L-carnitine plus malate driven oxygen consumption rate in the OXPHOS and ETS states was ~15% higher in mitochondria from HFD and HFDC rats compared with NC controls (P<0.05 or 0.05≤P<0.1). A similar effect was observed when palmitoyl-CoA plus L-carnitine plus malate was used as the oxidizable substrate. However, respiratory capacity in the OXPHOS and ETS states did not differ between HFD and HFDC groups for either palmitoyl-L-carnitine plus malate or palmitoyl-CoA plus L-carnitine plus malate. Oxygen consumption in the LEAK state was similar in all three groups for both fat-derived substrates. However, we observed a significantly stronger potentiation of oxygen consumption in the LEAK state by palmitic acid in the HFD group compared to the NC controls (P<0.01), indicating increased FA-induced mitochondrial uncoupling (Table 3).
palmitic acid in the HFDC group was similar to that in the HFD group, but it only
tended to differ from the effect in the NC group (P=0.074).

The effect of 15 weeks of high-fat diet feeding and 15 weeks of high-fat diet feeding
in combination with 8 weeks of carnitine supplementation on mitochondrial
biogenesis was assessed by determining mtDNA copy number and citrate synthase
activity in TA muscle. Relative mtDNA copy number and citrate synthase activity
were significantly higher in HFD (P<0.01) and HFDC (P<0.05 for mtDNA and P<0.01
for citrate synthase) rats compared with NC controls, whereas no differences were
observed between HFD and HFDC groups (Figure 4).
Discussion

We aimed to determine whether high-dose carnitine supplementation reduces lipotoxicity, improves \textit{in vivo} muscle mitochondrial function and ameliorates insulin resistance in high-fat diet fed rats. Fifteen weeks of high-fat diet feeding in rats resulted in insulin resistance. In agreement with previous findings (16, 19, 24, 37, 45, 48), this was associated with decreases in muscle and blood free carnitine, increased IMCL and muscle acylcarnitine levels, an increased capacity to oxidize fat-derived substrates \textit{ex vivo}, and an elevated number of muscle mitochondria. The increase in mitochondrial content was however not accompanied by an increase in muscle oxidative capacity \textit{in vivo}, suggesting that under \textit{in vivo} conditions the function of individual mitochondria was compromised (48). Despite the partial normalization of muscle free carnitine content, carnitine supplementation in high-fat diet fed rats did not induce improvements in muscle lipid status, \textit{in vivo} mitochondrial function or insulin sensitivity.

Effects of high-fat diet feeding on muscle mitochondrial function and metabolism

Insulin resistance has previously been linked to an increased capacity to oxidize FAs in order to cope with the high lipid loads (19, 45, 46, 48, 56). The results of our \textit{ex vivo} measurements, showing increased FA oxidation capacity in isolated mitochondria of HFD rats, are in line with these findings. Our results are also in agreement with other studies which have shown that high-fat diet feeding does not affect respiration of rat muscle mitochondria when using substrates other than fatty acids, i.e. pyruvate/glutamate plus malate or succinate (7, 21, 45), implying that the intrinsic functioning of the mitochondria is not impaired when probed \textit{ex vivo}. The
observed increases in mtDNA copy number (31%) and citrate synthase activity (23%) in HFD rats are in agreement with previous data, showing that high-fat diet feeding in rats induces increased biogenesis of mitochondria (16, 19, 22, 27, 36, 45, 48) as an adaptive response to the higher mitochondrial FA load. However, despite the increase in mitochondrial content, the in vivo PCr recovery rate constant ($k_{PCr}$) was similar for HFD and NC rats. These data indicate that an increased number of mitochondria with normal or even improved function ex vivo is required to maintain normal muscle oxidative capacity in vivo in HFD rats (48).

HFD rats had a 12-fold higher IMCL content compared with NC controls. The acylcarnitine profile likewise showed that muscle of HFD rats was overloaded with lipids. The vast majority of acylcarnitines is produced in the mitochondria, and acylcarnitine levels can therefore, in combination with measurements of mitochondrial $\beta$-oxidation capacity and mitochondrial density, be used to infer changes in the $\beta$-oxidation flux. In agreement with previous studies, even, medium-chain (C6-C12) acylcarnitine intermediates, which represent incompletely oxidized FAs, were elevated in muscle of HFD rats compared with NC controls (23, 24, 37).

Although the measurement of acylcarnitines provides a comprehensive snapshot of intermediary metabolism, it is important to emphasize that steady-state metabolite concentrations represent the net balance between production, consumption, import and export. However, the combination of increased ex vivo FA oxidation capacity and increased levels of medium-chain acylcarnitines in muscle of HFD animals, suggests that incomplete $\beta$-oxidation is elevated in HFD animals.
It has been shown that elevated levels of FAs may impair mitochondrial ATP production through a number of mechanisms. Long-chain acyl-CoA esters may inhibit the mitochondrial adenine nucleotide translocator leading to impaired exchange of cytosolic ADP for mitochondrial ATP (25). Moreover, increased availability of FAs without a concomitant rise in energy demand could lead to increased expression (45, 48) as well as activation of UCP3 (13), which in turn may diminish the efficiency of ATP synthesis by increasing mitochondrial uncoupling (3). In order to test the latter mechanism, we measured LEAK state oxygen consumption rates in isolated mitochondria respiring on pyruvate plus malate in the presence of palmitic acid. In this experiment, we observed significantly higher oxygen consumption rates in mitochondria isolated from HFD rats compared with NC rats, indicating increased mitochondrial uncoupling due to the presence of FAs. Together with the finding that muscle medium- and long-chain acylcarnitines were increased in HFD rats compared with NC controls, these data strongly suggest that FA-induced mitochondrial uncoupling may contribute to the observed mitochondrial functional impairment in muscle of HFD rats \textit{in vivo}, similar to our earlier findings in diabetic and long-term high-fat diet fed rats (48, 56).

\textbf{Carnitine insufficiency}

In parallel with the increase in muscle acylcarnitine levels, a decrease was observed in free carnitine levels in muscle and blood of HFD animals. It has been shown that whole-body carnitine insufficiency is a common feature in insulin-resistant states such as advanced age, genetic diabetes and diet-induced obesity (37). This can be explained by a decreased biosynthesis in the liver (24, 26, 28, 37), but also by
increased sequestration of carnitine in the muscle acylcarnitine pool (24, 37). The insulin resistance related decline in free carnitine has been associated with impaired mitochondrial function and an imbalance between complete and incomplete fat oxidation (35, 37). It has been hypothesized that carnitine supplementation ameliorates mitochondrial function and insulin resistance by reducing lipotoxicity both through the increased oxidation and increased export of muscle lipid metabolites (35). In a study by Noland et al., it was shown that 8 weeks of carnitine supplementation in long-term high-fat diet fed rats restored the ratio of complete to incomplete fat oxidation and increased efflux of muscle acylcarnitine intermediates, while improving glucose tolerance (37).

Effects of carnitine supplementation on muscle lipid status

To study the effects of carnitine supplementation on muscle lipid status, muscle mitochondrial function and insulin sensitivity in high-fat diet fed rats, we supplemented them with 300 mg/kg body weight/day carnitine during the last 8 weeks of the 15 weeks diet. Although carnitine supplementation partially normalized free carnitine in muscle and blood to the level of NC controls, no differences in IMCL levels were observed between HFD and HFDC rats. Similar levels of muscle acylcarnitines in HFD and HFDC rats provided further evidence of unchanged lipid levels inside the muscle, despite the increase in acylcarnitines levels in blood upon carnitine supplementation. The effects of carnitine supplementation on acylcarnitine profiles in muscle, blood and urine are in agreement with the study of Noland et al. (37), who applied exactly the same regimen of carnitine supplementation (i.e. 300 mg/kg body weight/day during 8 weeks) in high-fat diet fed rats. However, in that
study free carnitine in muscle and blood were completely restored to the level of normal chow fed controls, whereas they were only partially normalized in the current study.

Effects of carnitine supplementation on muscle mitochondrial function and insulin sensitivity

In line with the lack of effects on muscle lipid status, carnitine supplementation also did not affect muscle mitochondrial function or insulin sensitivity in high-fat diet fed rats. Oxygen consumption rates of muscle mitochondria respiring on glucose- or fat-derived substrates were similar for HFD and HFDC rats. It should be noted, though, that these measurements of ex vivo respiratory capacity do not report on the actual rates of substrate oxidation in vivo and therefore the effects of carnitine supplementation on glucose versus fatty acid oxidation cannot be predicted. Addition of palmitic acid had the same effect on mitochondria from HFD and HFDC rats, both showing increased FA-induced mitochondrial uncoupling compared with mitochondria from NC controls. Both in HFD and HFDC rats muscle mitochondrial content was about 26% higher than in NC controls, but in both high-fat diet fed groups this did not result in an increased muscle oxidative capacity in vivo as determined from $k_{PCr}$, indicating that in vivo mitochondrial function was similarly impaired. Our results on muscle mitochondrial function and insulin sensitivity are in contrast with the findings of Noland et al., who showed that carnitine supplementation improved whole-body glucose tolerance and reversed mitochondrial abnormalities in rats fed with a high-fat diet (37). The major difference between the two studies is the duration of the high-fat diet feeding, i.e. 15 weeks in
the current study versus 12 months in the study by Noland et al. This means that there can be major differences in the nature of mitochondrial dysfunction, e.g. due to adaptive changes or due to accumulating damage caused not only by high-fat diet feeding but also by aging in ref. (37)), as well as changes in other pathways involved in glucose and lipid metabolism or the interplay between tissues important for maintenance of glucose homeostasis, explaining the differential effect of carnitine supplementation on whole-body glucose tolerance in the two studies. Another difference between the two studies concerns the methods to assess muscle mitochondrial function. Noland et al. measured the ratio of complete to incomplete oleate oxidation in isolated mitochondria, which was decreased in high-fat diet fed rats, but completely restored after carnitine supplementation (37). In the current study we have no data on the ratio of complete to incomplete FA oxidation, but we have shown that carnitine supplementation does not improve \emph{in vivo} muscle mitochondrial function.

Reduction of the free carnitine pool in response to high-fat diet feeding might have a negative effect on mitochondrial FA oxidation only if free carnitine is depleted to an extent that it becomes limiting for CPT1 activity, resulting in a decreased entry of long-chain acyl-CoAs into the mitochondrial matrix. The absence of an effect of 8 weeks of carnitine supplementation in high-fat diet fed animals regarding muscle mitochondrial function suggests that this was not the case in the present study. Moreover, our observation that OXPHOS state oxygen consumption rates with palmitoyl-CoA plus L-carnitine plus malate and palmitoyl-L-carnitine plus malate as the oxidizable substrates were similar in HFD and HFDC groups suggests that neither
palmitoyl-CoA transport nor \( \beta \)-oxidation capacity was limited by carnitine insufficiency in high-fat diet fed rats.

Study limitations

It cannot be excluded that the contribution of mitochondrial dysfunction to the mechanisms underlying insulin resistance may increase with increasing duration of high-fat diet feeding and age, resulting in more profound beneficial effects of carnitine supplementation in long-term high-fat diet fed old rats as in ref. (37). Moreover, treatment length could significantly affect the results and a longer period of carnitine supplementation (compared to the 8 weeks in the current study and in ref. (37)) may be more effective in improving insulin sensitivity. Another limitation of the study is that we only included male rats. It has been shown that females are less prone to lipid-induced skeletal muscle insulin resistance than males, which can however not be attributed to a decreased accumulation of muscle lipids and lipid intermediates (20, 55). This observation predicts that treatments aimed at reducing lipotoxicity in order to ameliorate insulin resistance may be more effective in males than in females.

Conclusions

In conclusion, we showed that high-fat diet feeding induces carnitine insufficiency and muscle lipid overload, which was accompanied by an increased number of muscle mitochondria with an improved capacity to oxidize FAs \textit{ex vivo}, but without a concomitant increase in muscle oxidative capacity \textit{in vivo}. We provided evidence that this impairment in \textit{in vivo} mitochondrial function in high-fat diet fed rats is
caused by elevated levels of lipid intermediates, leading to increased FA-induced mitochondrial uncoupling and therefore less efficient ATP synthesis. Despite a partial normalization of free carnitine in muscle and blood, carnitine supplementation did not induce improvements in muscle lipid status, in vivo mitochondrial function or insulin sensitivity. These results suggest that carnitine insufficiency does not play a major role in high-fat diet induced muscle mitochondrial dysfunction in vivo.

Acknowledgements

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34. **Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, Ilkayeva OR, Stevens RD, Kheterpal I, Zhang J, Covington JD, Bajpeyi S, Ravussin E, Kraus W, Koves TR, ...


Table 1. Animal characteristics.

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<th>NC</th>
<th>HFD</th>
<th>HFDC</th>
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<tr>
<td><strong>Body weight (g) t = 0</strong></td>
<td>352 ± 12</td>
<td>338 ± 18</td>
<td>359 ± 12†</td>
</tr>
<tr>
<td><strong>Body weight (g) t = 15</strong></td>
<td>428 ± 32</td>
<td>553 ± 30***</td>
<td>538 ± 23***</td>
</tr>
<tr>
<td><strong>Delta body weight (g)</strong></td>
<td>76 ± 16</td>
<td>215 ± 37***</td>
<td>180 ± 21***,†</td>
</tr>
<tr>
<td><strong>Food intake (kJ/week)</strong></td>
<td>2247 ± 63</td>
<td>2342 ± 156</td>
<td>2286 ± 187</td>
</tr>
<tr>
<td><strong>Fasting glucose (mM)</strong></td>
<td>4.3 ± 0.5</td>
<td>5.6 ± 0.7***</td>
<td>5.8 ± 0.5***</td>
</tr>
<tr>
<td><strong>AUCg (mM·h)</strong></td>
<td>9.4 ± 1.0</td>
<td>12.4 ± 1.2***</td>
<td>12.7 ± 0.7***</td>
</tr>
<tr>
<td><strong>Fasting insulin (pM)</strong></td>
<td>230 ± 101</td>
<td>561 ± 207***</td>
<td>521 ± 160**</td>
</tr>
<tr>
<td><strong>AUCi (pM·h)</strong></td>
<td>759 ± 235</td>
<td>1451 ± 370**</td>
<td>1534 ± 465***</td>
</tr>
<tr>
<td><strong>AUCg*AUCi (mM•h•pM•h)</strong></td>
<td>7005 ± 2149</td>
<td>17564 ± 4739**</td>
<td>19644 ± 6319***</td>
</tr>
</tbody>
</table>

Data are from n=10 NC, n=9 HFD and n=10 HFDC animals after 15 weeks of diet (except for baseline body weight at t=0) and are expressed as means ± SD. NC, normal chow; HFD, high-fat diet; HFDC, high-fat diet supplemented with carnitine; AUCg and AUCi, area under the glucose and insulin curve from the oral glucose tolerance test, respectively; ** P<0.01, *** P<0.001 when compared with NC, † P<0.05 when compared with HFD.
Table 2. Metabolite concentrations and pH in TA measured by in vivo $^{31}$P MRS.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HFD</th>
<th>HFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rest</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.21 ± 0.01</td>
<td>7.18 ± 0.02***</td>
<td>7.19 ± 0.01**</td>
</tr>
<tr>
<td>[PCr] (mM)</td>
<td>32.2 ± 1.5</td>
<td>32.4 ± 1.5</td>
<td>32.7 ± 2.9</td>
</tr>
<tr>
<td>[Pi] (mM)</td>
<td>2.6 ± 0.2</td>
<td>2.4 ± 0.4</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td><strong>End-stimulation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.00 ± 0.14</td>
<td>7.05 ± 0.04</td>
<td>7.05 ± 0.04</td>
</tr>
<tr>
<td>[PCr] (mM)</td>
<td>13.8 ± 2.5</td>
<td>16.2 ± 2.1*</td>
<td>15.7 ± 1.4</td>
</tr>
<tr>
<td>[Pi] (mM)</td>
<td>20.1 ± 3.0</td>
<td>19.0 ± 1.9</td>
<td>19.2 ± 1.3</td>
</tr>
<tr>
<td>ΔPCr (%)</td>
<td>59.8 ± 6.2</td>
<td>54.1 ± 4.0*</td>
<td>55.5 ± 2.9</td>
</tr>
</tbody>
</table>

Data are from n=10 NC, n=9 HFD and n=10 HFDC animals and are expressed as means ± SD. NC, normal chow; HFD, high-fat diet; HFDC, high-fat diet supplemented with carnitine; PCr, phosphocreatine; Pi, inorganic phosphate. * P<0.05, ** P<0.01, *** P<0.001 when compared with NC.
Table 3. Oxygen consumption rates in isolated TA mitochondria oxidizing different substrates in different metabolic states.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HFD</th>
<th>HFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXPHOS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>533 ± 23</td>
<td>564 ± 42</td>
<td>567 ± 74</td>
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<tr>
<td>LEAK (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>30 ± 2</td>
<td>34 ± 3</td>
<td>38 ± 5***</td>
</tr>
<tr>
<td>ETS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>644 ± 32</td>
<td>669 ± 49</td>
<td>684 ± 85</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
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<th>HFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAK (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>195 ± 18</td>
<td>257 ± 22**</td>
<td>237 ± 56&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>HFD</th>
<th>HFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXPHOS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>147 ± 6</td>
<td>171 ± 20*</td>
<td>166 ± 20&quot;</td>
</tr>
<tr>
<td>LEAK (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>32 ± 1</td>
<td>31 ± 2</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>ETS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>225 ± 18</td>
<td>251 ± 22&quot;</td>
<td>254 ± 29*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HFD</th>
<th>HFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXPHOS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>146 ± 8</td>
<td>167 ± 14&quot;</td>
<td>167 ± 26&quot;</td>
</tr>
<tr>
<td>LEAK (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>31 ± 1</td>
<td>31 ± 2</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>ETS (nmol O₂·min⁻¹·mg protein⁻¹)</td>
<td>232 ± 22</td>
<td>265 ± 15*</td>
<td>263 ± 31*</td>
</tr>
</tbody>
</table>

Data are from n=10 NC, n=9 HFD and n=10 HFDC animals and are expressed as means ± SD. NC, normal chow; HFD, high-fat diet; HFDC, high-fat diet supplemented with carnitine; OXPHOS, maximal ADP-stimulated oxygen consumption; LEAK, oxygen consumption in the absence of ATP synthesis; ETS, oxygen consumption after
uncoupling. * P<0.05, ** P<0.01, *** P<0.001 when compared with NC, # 0.05≤P<0.1 (trend) when compared with NC.
Figure legends

Figure 1. IMCL content assessed by $^1$H MRS in TA muscle. (A) Representative examples of $^1$H MR spectra (512 averages) from the medial part of the TA muscle of a NC, HFD and HFDC rat. (B) IMCL content in the medial part of the TA muscle of NC (n=10), HFD (n=9) and HFDC (n=10) rats. IMCL content was expressed as a percentage of the water signal. *** P<0.001 when compared with NC.

Figure 2. Free carnitine and acylcarnitine levels in TA muscle (A, B, C), blood (D, E, F) and urine (G) of NC (n=8 for muscle, n=10 for blood, and n=8 for urine), HFD (n=9 for muscle, n=10 for blood, and n=8 for urine) and HFDC (n=6 for muscle, n=10 for blood, and n=8 for urine) rats. * P<0.05 when compared with NC, † P<0.05 when compared with HFD.

Figure 3. In vivo mitochondrial function assessed by $^{31}$P MRS in TA muscle. (A) Representative example of a $^{31}$P MR spectrum from the TA muscle of a NC rat at rest (black; TR = 5 s, 32 averages) and a dynamic representation of the PCr peak during muscle stimulation and recovery (grey; TR = 5 s, 4 averages). (B) Rate constant of PCr recovery ($k_{PCr}$) after muscle stimulation measured in TA muscle of NC (n=7), HFD (n=9) and HFDC (n=10) rats.
Figure 4. (A) Relative mitochondrial DNA (mtDNA) copy number and (B) citrate synthase activity measured in TA muscle of NC (n=8), HFD (n=9) and HFDC (n=10 for mtDNA and n=6 for citrate synthase) rats. * P<0.05, ** P<0.01 when compared with NC.