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

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Wessels B, van den Broek NM, Ciapaite J, Houten SM, Wanders RJ, Nicolay K, Prompers JJ. Carnitine supplementation in high-fat diet-fed rats does not ameliorate lipid-induced skeletal muscle mitochondrial dysfunction in vivo. Am J Physiol Endocrinol Metab 309: E670–E678, 2015. First published August 18, 2015; doi:10.1152/ajpendo.00144.2015.—Muscle lipid overload and the associated accumulation of lipid intermediates play an important role in the development of insulin resistance. Carnitine supplementation was supplemented with 300 mg·kg−1·day−1 L-carnitine during the last 8 wk. Muscle mitochondrial function was measured in vivo by 31P magnetic resonance spectroscopy (MRS) and ex vivo by high-resolution respirometry. Muscle lipid status was determined by 1H 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 ex vivo. This was, however, not accompanied by an increase in muscle oxidative capacity in vivo, indicating that 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, in vivo mitochondrial function, or insulin sensitivity. Carnitine supplementation therefore, does not play a major role in high-fat diet-induced muscle mitochondrial dysfunction in vivo.

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 supraphysiological 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 through both increased oxidation and increased export of muscle lipid metabolites (35).

In a previous study (48), we showed that, in long-term high-fat diet-fed rats, in vivo muscle mitochondrial function is compromised by mitochondrial lipid overload. The aim of the...
present study was to test the hypothesis that carnitine supple-
mentation would reduce high-fat diet-induced lipotoxicity, im-
prove in vivo muscle mitochondrial function, and ameliorate
insulin resistance. Wistar rats were fed either normal chow or
a high-fat diet for 15 wk, and one group of high-fat diet-fed rats
was supplemented with l-carnitine (300 mg kg body
wt⁻¹ day⁻¹) during the last 8 wk. In vivo muscle mitochondri-
drial function was measured by 31P magnetic resonance spec-
troscopy (MRS), and ex vivo mitochondrial function was
determined by measuring oxygen consumption in isolated
mitochondria. Furthermore, intramyocellular lipid levels
were determined by in vivo 1H MRS, and free carnitine and
acylcarnitine levels were determined upon euthanasia in
muscle tissue, and in blood and urine, using tandem mass
spectrometry.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (14 wk of age, n = 30; Charles
River Laboratories, The Netherlands) were housed in pairs at 20°C
and 50% humidity, with a 12:12-h light-dark cycle. Ad libitum food
and water were provided during a period of 15 wk. 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; Sniff Spezi-
aldüten, Soest, Germany), a group receiving a high-fat diet (HFD;
45% calories from fat (predominantly lard), 35% calories from car-
bohydrate, 20% calories from protein, D12451; Research Diet Ser-
dices, Wijk bij Duurstede, the Netherlands), and a group receiving
the same high-fat diet, supplemented with 300 mg kg body
wt⁻¹ day⁻¹ l-carnitine in their drinking water for the last 8 wk (HFDC).
Body
weight
and
food
and
water
intake
were
determined
weekly. Two days after the in vivo MRS measurements, rats were killed 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 wk of diet, 3–5 days before the in
vivo measurements. After a 4-h fast, rats received an oral glucose
bolus of 1 g/kg body wt. Blood samples were taken without anesthesia
from the saphenous vein 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). Plasma insulin concen-
tration was determined using an ultrasensitive rat insulin ELISA kit
(Mercodia, Uppsala, Sweden). Areas under the OGTT curves for both
glucose (AUC₉₀) and insulin (AUC₉₀) were calculated.

Magnetic resonance spectroscopy. All magnetic resonance spec-
troscopy (MRS) measurements were performed on a 6.3 Tesla hori-
zontal 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 1H MRS. Voxels of 3 × 3 × 3 mm³ were
measured in the medial part of the TA, close to the tibia bone, with a
circular 1H surface coil (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). 1H MR
spectra were fitted in the time domain using the advanced method for
accurate, robust, and efficient spectral fitting (AMARES) in the
jMRUI software package (jMRUI v.2.1) (52), as described previously
(56). In the water-suppressed 1H MR spectra, the IMCL-CH₂ 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 nonsuppressed water signal measured
in the same voxel.

31P MRS was performed using a combination of a circular 1H
surface coil (40 mm) for shimming and an ellipsoid 31P surface coil
(10/18 mm), positioned over the TA. 31P 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 31P 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 common peroneal
nerve (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. 31P MR spectra were fitted using
AMARES in jMRUI as described previously (49). Concentrations of
PCr and inorganic phosphate (Pi) 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 differ-
ence between the P, and PCr resonances (43). The data of PCr
recovery were fitted to a monoexponential function using Matlab (v.
R2010b; Mathworks, Natick, MA) yielding the rate constant of PCr recovery, krec, which is a measurement 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, Rockford, IL). Oxygen consumption rates were measured
at 37°C using a two-channel high-resolution Oroboros oxygraph-2k
(Oroboros, Innsbruck, Austria). Mitochondria were incubated in 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 per-
formed in 1 ml of assay medium containing 0.15 mg/ml mitochondrial
protein. Three different combinations of substrates were used to assess
citochondrial oxygen consumption capacity: 1) 5 mM palmitoyl-
CoA plus 2.5 mM malate (mitochondrial β-oxidation), i.e., the
TCA cycle; 2) 5 mM malate plus 5 mM malate (pyruvate dehydrogenase and part of TCA cycle); 2) 25 μM palmitoyl-L-
 carnitine plus 2.5 mM malate (mitochondrial β-oxidation and part of
TCA cycle), and 3) 25 μM palmitoyl-CoA plus 2.5 mM l-carnitine
plus 2.5 mM malate [carnitine palmitoyltransferase 1 (CPT1), mito-
ochondrial 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 OX-
PHOS state (18), was initiated by addition of 1 mM ATP. Maximal
oxygen consumption rate in the uncoupled state, i.e., the ETS state,
was determined after addition of 1 μM carbonyl cyanide 3-chloro-
phenyl 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 palmitic
acid (C16:0). The signals from the oxygen electrode were recorded at
0.5-s intervals. Data acquisition and analysis were performed using
Oxygraph-2k-DatLab software v. 4.2 (Oroboros).

Determination of mtDNA copy number and citrate synthase
activity. Genomic DNA was isolated from an ~25-mg transversal
slice of midbelly TA by use of a 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 previously (9). Primer sequences were: mt-ATP6 forward 5' = CAAAAGGACGAACCTG-3' and reverse 5' = ATGGGGAAGAAGCG-3', and PGC-1α forward 5' = ATGAAATGCGGGGTCTTAGCG-3' and reverse 5' = AACATGGCGGTTGTTCTC-3'. Citrate synthase activity was measured in TA muscle homogenate as described in Ref. 4.

Determination of acylcarnitine content. The content of free carnitine and acetylcarnitine 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 using Tukey’s HSD post hoc analyses in the SPSS 20.0 statistical package (SPSS, Chicago, IL). Statistical analysis of the IMCL content was performed using the Student’s t test.

**RESULTS**

**Animal model.** Animal characteristics are summarized in Table 1. After 15 wk 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 wk 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<i>g</i>, AUC<i>i</i>, and AUC<i>g</i>×AUC<i>i</i> from the OGTT were significantly higher for both HFD and HFDC rats compared with NC controls (P < 0.01 or P < 0.001), whereas there were no differences between HFD and HFDC groups.

**IMCL content.** In vivo 1H MRS was applied to evaluate the effects of 15 wk of high-fat diet and 15 wk of high-fat diet in combination with 8 wk of carnitine supplementation on IMCL levels in TA muscle (Fig. 1A). IMCL content was 12- and 11-fold higher in HFD and HFDC rats, respectively, compared with NC controls (P < 0.001; Fig. 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 β-oxidation intermediates, i.e., acylcarnitines, in TA muscle, blood, and urine (Fig. 2). After 15 wk 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 (Fig. 2, A and D). However, carnitine supplementation in rats fed the high-fat diet partially normalized free carnitine in muscle and blood to the level of NC controls. Muscle acetyl-carnitine (C2) levels were lower in HFD and HFDC rats (P < 0.01), whereas acetylcarnitine levels in blood were higher in HFD and HFDC rats (P < 0.001) compared with NC controls. Moreover, blood acetylcarnitine concentration was twofold 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; Fig. 2G). Medium- and long-chain acylcarnitine species were almost

**Table 1. Animal characteristics**

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<tr>
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<th>NC</th>
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<tr>
<td>Body weight (g)</td>
<td>352 ± 12</td>
<td>338 ± 18</td>
<td>359 ± 12</td>
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<tr>
<td>t = 15</td>
<td>428 ± 32</td>
<td>553 ± 30***</td>
<td>538 ± 23***</td>
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<td>t = 20</td>
<td>76 ± 16</td>
<td>215 ± 37***</td>
<td>180 ± 21***</td>
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<tr>
<td>Food intake (kJ/wk)</td>
<td>2,247 ± 63</td>
<td>2,342 ± 156</td>
<td>2,286 ± 187</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>4.3 ± 0.5</td>
<td>5.6 ± 0.7***</td>
<td>5.8 ± 0.5***</td>
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<tr>
<td>AUC&lt;i&gt;g&lt;/i&gt; (mM·h)</td>
<td>9.4</td>
<td></td>
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<tr>
<td>Fasting insulin (pM)</td>
<td>230 ± 101</td>
<td>561 ± 207***</td>
<td>521 ± 160***</td>
</tr>
<tr>
<td>AUC&lt;i&gt;i&lt;/i&gt; (pM·h)</td>
<td>759 ± 235</td>
<td>1,451 ± 370**</td>
<td>1,534 ± 465***</td>
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<tr>
<td>AUC&lt;i&gt;g&lt;/i&gt;×AUC&lt;i&gt;i&lt;/i&gt;</td>
<td>7,005 ± 2.14</td>
<td>17,564 ± 4,739**</td>
<td>19,644 ± 6,319***</td>
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Data are expressed as means ± SD; n = 10 NC, n = 9 HFD, and n = 10 HFDC animals after 15 wk of diet (except for baseline body weight at t = 0). NC, normal chow; HFD, high-fat diet; HFDC, HFD supplemented with 300 mg·kg body wt -1·day -1 L-carnitine (HFDC) rat. tCr, total carnitine.

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**Fig. 1.** Intramyocellular lipid (IMCL) content assessed by 1H MRS in tibialis anterior (TA) muscle. A: representative examples of 1H MR spectra (512 averages) from the medial part of the TA muscle of a normal chow (NC), high-fat diet (HFD), and HFD supplemented with 300 mg·kg body wt -1·day -1 L-carnitine (HFDC) rat. tCr, total carnitine. 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 vs. NC.
absent in urine and are therefore not shown. Most medium- and long-chain acylcarnitines in blood were lowered after 15 wk of high-fat diet (\(P < 0.05\)), but they were normalized to the level of NC controls upon carnitine supplementation (Fig. 2, E and F). 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 (Fig. 2, B and C).

**Mitochondrial function in vivo.** Dynamic \(^{31}\)P MRS measurements were performed during and after recovery from electrical stimulation of the TA muscle (Fig. 3A) to determine the rate constant of PCr recovery, \(k_{\text{PCr}}\), 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 than in NC controls, but concentrations of PCr and P\(_i\) in resting muscle did not differ among groups. At the end of muscle stimulation, intracellular pH and PCr and P\(_i\) 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, \(k_{\text{PCr}}\) did not differ among NC, HFD, and HFDC groups (Fig. 3B).

**Mitochondrial function ex vivo.** Table 3 summarizes the effects of 15 wk of high-fat diet feeding and 15 wk of high-fat diet feeding in combination with 8 wk 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.

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Fig. 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. *\(P < 0.05\) vs. NC; †\(P < 0.05\) vs. HFD.
compared with NC controls ($P < 0.001$). Interestingly, palmitoyl-L-carnitine plus malate-driven oxygen consumption rate in the OXPHOS and ETS states was $\sim 15\%$ higher in mitochondria from HFD and HFDC rats compared with NC controls ($P < 0.05$ or $0.05 \leq P < 0.1$). 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 with the NC and HFDC groups ($P < 0.01$), indicating increased FA-induced mitochondrial uncoupling (Table 3). The effect of 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 wk of high-fat diet feeding and 15 wk of high-fat diet feeding in combination with 8 wk 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 (Fig. 4).

**DISCUSSION**

We aimed to determine whether high-dose carnitine supplementation reduces lipotoxicity, improves 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 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 in vivo, suggesting that under 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, 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 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

### Table 2. Metabolite concentrations and pH in TA measured by in vivo $^{31}P$ MRS

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

Data are expressed as means ± SD; $n = 7$ NC, $n = 9$ HFD, and $n = 10$ HFDC animals. TA, tibialis anterior; PCr, phosphocreatine; Pi, inorganic phosphate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. NC.
completely oxidized FAs, were elevated in muscle of HFD rats (C6–C12) acylcarnitine intermediates, which represent incom-

ochondrial density, be used to infer changes in the acylcarnitine levels can, therefore, in combination with mea-
majority of acylcarnitines is produced in the mitochondria, and muscle of HFD rats was overloaded with lipids. The vast

NC controls. The acylcarnitine profile likewise showed that oxidation capacity in vivo in HFD rats (48).

proved function ex vivo is required to maintain normal muscle increased number of mitochondria with normal or even im-

itorial FA load. However, despite the increase in mito-

chondrial ATP production through a number of mecha-
nisms. Long-chain acyl-CoA esters may inhibit the mitochon-
drial adenine nucleotide translocator leading to impaired ex-
change of cytosolic ADP for mitochondrial ATP (25). More-
over, 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). 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

data are expressed as means ± SD; n = 10 NC, n = 9 HFD, and n = 10 HFDC animals. OXPHOS, maximal ADP-stimulated O2 consumption; LEAK, O2 consumption in the absence of ATP synthesis; ETS, O2 consumption after uncoupling. *P < 0.05, **P < 0.01, ***P < 0.001 vs. NC, #P < 0.05 ≤ P < 0.1 (trend) vs. NC.

affect respiration of rat muscle mitochondria when substrates other than fatty acids are used, i.e., pyruvate/glutamate plus malate or succinate (7, 21, 45), implying that the intrinsic functioning of the mitochondria is not impaired when probed 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 mito-

Table 3. Oxygen consumption rates in isolated TA mitochondria oxidizing different substrates in different metabolic states

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<th>NC</th>
<th>HFD</th>
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<tr>
<td><strong>Pyruvate + malate</strong></td>
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<tr>
<td>OXPHOS (nmol O2·min⁻¹·mg protein⁻¹)</td>
<td>533 ± 23</td>
<td>564 ± 42</td>
<td>567 ± 74</td>
</tr>
<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>
<tr>
<td><strong>Pyruvate + malate + palmitic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEAK (nmol O2·min⁻¹·mg protein⁻¹)</td>
<td>195 ± 18</td>
<td>257 ± 22**</td>
<td>237 ± 56#</td>
</tr>
<tr>
<td><strong>Palmitoyl carnitine + malate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXPHOS (nmol O2·min⁻¹·mg protein⁻¹)</td>
<td>147 ± 6</td>
<td>171 ± 20*</td>
<td>166 ± 20#</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#</td>
<td>254 ± 29*</td>
</tr>
<tr>
<td><strong>Palmitoyl CoA + carnitine + malate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXPHOS (nmol O2·min⁻¹·mg protein⁻¹)</td>
<td>146 ± 8</td>
<td>167 ± 14#</td>
<td>167 ± 26#</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>

Fig. 4. Relative mitochondrial DNA (mtDNA) copy number (A) and citrate synthase activity (B) 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 vs. NC.
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 in vivo, similar to our earlier findings in diabetic and long-term high-fat diet-fed rats (48, 56).

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 through both the increased oxidation and the increased export of muscle lipid metabolites (35). In a study by Noland et al. (37), it was shown that 8 wk 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.

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 wt \(^{-1}\)·day\(^{-1}\) carnitine during the last 8 wk of the 15-wk 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 wt \(^{-1}\)·day\(^{-1}\) during 8 wk) in high-fat diet-fed rats. However, in that study, free carnitine in muscle and blood were completely restored to the level of NC-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; therefore, the effects of carnitine supplementation on glucose vs. FA 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. In both 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 \(k_{\text{PCr}}\), 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 a high-fat diet (37). The major difference between the two studies is the duration of the high-fat diet feeding, i.e., 15 wk in the current study vs. 12 mo 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 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 present study, we have no data on the ratio of complete to incomplete FA oxidation, but we have shown that carnitine supplementation does not improve 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 CPTI activity, resulting in a decreased entry of long-chain acyl- CoAs into the mitochondrial matrix. The absence of an effect of 8 wk 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 β-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 (vs. the 8 wk in the current study and in Ref. 37) may be more effective in improving insulin sensitivity. Another limitation of the study is that we included only male rats. It has been shown that females are less prone to lipid-induced skeletal muscle insulin resistance than males, which cannot, however, 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 induced carnitine insufficiency and muscle lipid overload, which was accompanied by an increased number of muscle mitochondria with an improved capacity to oxidize FAs ex vivo but without a concomitant increase in muscle oxidative capacity in vivo. We provided evidence that this impairment in vivo mitochondrial function in high-fat diet-fed rats was 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B.W., N.M.v.d.B., S.M.H., R.J.W., K.N., and J.J.P. conceived and designed of research; B.W., N.M.v.d.B., J.C., and S.M.H. performed experiments; B.W., N.M.v.d.B., J.C., S.M.H., and J.J.P. analyzed data; B.W., N.M.v.d.B., J.C., S.M.H., R.J.W., K.N., and J.J.P. interpreted results of experiments; B.W., N.M.v.d.B., and J.J.P. prepared figures; B.W., N.M.v.d.B., and J.J.P. drafted manuscript; B.W., N.M.v.d.B., J.C., S.M.H., R.J.W., K.N., and J.J.P. edited and revised manuscript; B.W., N.M.v.d.B., J.C., S.M.H., R.J.W., K.N., and J.J.P. approved final version of manuscript.

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