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Skeletal muscle carnitine loading increase expenditure, modulates fuel metabolism g and prevents body fat accumulation in hu

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Key points

- Carnitine is a substrate for the carnitine palmitoyltransferase 1 enzyme, a rate-limiting step in fatty acid oxidation within skeletal muscle.
- Insulin stimulates carnitine transport into skeletal muscle.
- A 20% increase in muscle carnitine content, achieved via 12 weeks of twice daily supplementation of a beverage containing 1.36 g of L-carnitine and 80 g of carbohydrate (in order to stimulate insulin-mediated muscle carnitine transport), prevented an 18% increase in body fat mass associated with carbohydrate supplementation alone in healthy young men.
- A novel finding of the present study was that this prevention of fat gain was associated with a greater energy expenditure and fat oxidation during low-intensity physical activity, and an adaptive increase in expression of gene networks involved in muscle insulin signalling and fatty acid metabolism.
- Implications to health warrant further investigation, particularly in obese individuals who have a reduced reliance on muscle fat oxidation during exercise.

Abstract Twelve weeks of daily L-carnitine and carbohydrate feeding in humans increases skeletal muscle total carnitine content, and prevents body mass accrual associated with carbohydrate feeding alone. Here we determined the influence of L-carnitine and carbohydrate feeding on energy metabolism, body fat mass and muscle expression of fuel metabolism genes. Twelve males exercised at 50% maximal oxygen consumption for 30 min once before and once after 12 weeks of twice daily feeding of 80 g carbohydrate (Control, $n = 6$) or 1.36 g L-carnitine + 80 g carbohydrate (Carnitine, $n = 6$). Maximal carnitine palmitolytransferase 1 (CPT1) activity remained similar in both groups over 12 weeks. However, whereas muscle total carnitine, long-chain acyl-CoA and whole-body energy expenditure did not change over 12 weeks in Control, they increased in Carnitine by 20%, 200% and 6%, respectively ($P < 0.05$). Moreover, body mass and whole-body fat mass (dual-energy X-ray absorptiometry) increased over 12 weeks in Control by 1.9 and 1.8 kg, respectively ($P < 0.05$), but did not change in Carnitine. Seventy-three of 187 genes relating to fuel metabolism were upregulated in Carnitine *vs.* Control after 12 weeks, with 'insulin signalling', 'peroxisome proliferator-activated receptor signalling' and 'fatty acid metabolism' as the three most enriched pathways in gene functional analysis. In conclusion, increasing muscle total carnitine in healthy humans can modulate muscle metabolism, energy expenditure and body composition over a prolonged period, which is entirely consistent with a carnitine-mediated increase in muscle long-chain acyl-group translocation via CPT1. Implications to health warrant

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further investigation, particularly in obese individuals who have a reduced reliance on muscle fat oxidation during low-intensity exercise.

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Abbreviations CPT1, carnitine palmitoyltransferase 1; C_T, threshold cycle; DEXA, dual-energy X-ray absorptiometry; FDR, false discovery rate; FOXO3, forkhead box class O transcription factor 3a; IMCL, intramyocellular lipid; KEGG, Kyoto Encyclopedia of Genes and Genomes; PDC, pyruvate dehydrogenase complex; PDK2, pyruvate dehydrogenase kinase 2; PPARα, peroxisome proliferator-activated receptor alpha; $\dot{V}_{O_2,max}$, maximal oxygen consumption.

Introduction

As a substrate for carnitine palmitoyltransferase 1 (CPT1), carnitine translocates long-chain acyl groups across the otherwise impermeable inner mitochondrial membrane for subsequent β-oxidation (Fritz & Yue, 1963). A series of studies from our laboratory have suggested that an insulin-mediated augmentation of skeletal muscle total carnitine content can increase CPT1 flux and mitochondrial long-chain acyl group translocation *in vivo*, and result in a reciprocal inhibition of carbohydrate utilisation. By way of example, we demonstrated that a 15% increase in skeletal muscle total carnitine content, achieved via intravenous L-carnitine infusion during a 6 h euglycaemic hyperinsulinaemic clamp in healthy human volunteers at rest, decreased insulin-stimulated muscle pyruvate dehydrogenase complex (PDC) activation and muscle lactate accumulation by 30% and 40%, respectively, compared with control (Stephens *et al.* 2006). These findings were particularly remarkable as during hyperinsulinaemic conditions cytosolic malonyl-CoA, which is a potent inhibitor of CPT1, would likely be elevated because of increased glycolytic flux (Rasmussen *et al.* 2002; Fig. 6). Furthermore, the morning following the euglycaemic hyperinsulinaemic clamp in the same study (Stephens *et al.* 2006), where a controlled diet was consumed prior to an overnight fast, muscle long-chain acyl-CoA and glycogen content had increased by 40% and 30%, respectively, suggesting that a carnitine-mediated increase in long-chain acyl group translocation had diverted glucose uptake from oxidation towards storage. More recently we demonstrated that it is possible to increase muscle total carnitine content via dietary means by ingesting 1.36 g L-carnitine in combination with a beverage containing 80 g of carbohydrate (in order to stimulate insulin-mediated muscle carnitine accumulation) twice daily over a 24 week period, which resulted in a 30% increase in muscle total carnitine content compared with carbohydrate feeding alone (Wall *et al.* 2011). This manipulation of the muscle total carnitine pool resulted in an 80% increase in muscle free carnitine availability and a 50% reduction in muscle glycogen utilisation during low-intensity exercise (50% of maximal oxygen consumption, $\dot{V}_{O_2,max}$). Collectively these findings suggest that, in contrast to *in vitro* observations (McGarry *et al.* 1983), under conditions of low to moderately increased glycolytic flux (insulin or exercise mediated), muscle free carnitine availability is limiting to the CPT1 reaction and, consequently, the rate of muscle fat oxidation *in vivo*.

One potentially important observation from the study of Wall *et al.* (2011) was that increasing muscle carnitine content by combined L-carnitine (2.7 g day^{-1}) and carbohydrate (160 g day^{-1}) feeding prevented a 2 kg increase in body mass that occurred in the control group (carbohydrate feeding only) after 12 weeks of dietary supplementation. It is fair to speculate that this increase in body mass was attributable to an increase in body fat mass, and that a carnitine-mediated increase in long-chain fatty acid oxidation obviated this response in the carnitine-loaded group. In line with this hypothesis, accelerating CPT1 flux via knock-out of acetyl-CoA carboxylase 2 (the enzyme responsible for skeletal muscle malonyl-CoA synthesis) resulted in a sustained increase in fatty acid oxidation and energy expenditure compared with wild-type, along with a 27% reduction in fat mass and a 42% increase in insulin sensitivity in the face of high-fat feeding in mice (Choi *et al.* 2007). Thus, to provide further mechanistic insight, specifically regarding muscle genomic adaptation in response to any carnitine-mediated increase in muscle fat oxidation and altered metabolic flux, we used pathway-focussed, quantitative, RT-PCR-based low-density arrays to measure changes in mRNA abundance of 187 genes involved in carnitine and fuel metabolism following 12 weeks of dietary L-carnitine and carbohydrate supplementation in young, healthy male volunteers eating an otherwise *ad libitum* diet (i.e. in positive energy balance). We hypothesised that any modulation of energy metabolism (regional fat mass, skeletal muscle CPT1 activity and fuel metabolism, and whole-body fatty acid oxidation and energy expenditure during low-intensity physical activity) observed with an increase in muscle carnitine content would be accompanied by an adaptive change in

the expression levels of gene networks known to encode proteins regulating muscle fuel utilisation, mitochondrial function and insulin signalling in muscle.

Methods

Ethical approval

This study forms part of a chronic L-carnitine feeding study described by Wall *et al.* (2011). The only data common to the two papers are resting muscle total carnitine content. However, due to tissue availability for the analysis of the present study, only 12 of the original 14 subjects have been included, and so total carnitine values differ slightly. Thus, 12 healthy, non-vegetarian, male recreational athletes, reporting to be weight stable for at least 6 months at the point of screening, volunteered to participate in the present study, which was approved by the University of Nottingham Medical School Ethics Committee in accordance with the Declaration of Helsinki. Before taking part in the study, all subjects underwent routine medical screening, completed a general health questionnaire and performed an incremental exercise test to exhaustion on a cycle ergometer (Excalibur, Lode, Groningen, The Netherlands) in order to determine their rate of $\dot{V}_{O_2,max}$ measured using an online gas analysis system (Vmax, SensorMedics, Anaheim, CA, USA). All gave their written consent to take part and were aware that they were free to withdraw from the experiments at any point. Before the first experimental visit the subjects were allocated in a randomised, double-blind manner into two experimental groups as described previously (Wall *et al.* 2011); Control (age 25.3 ± 2.1 years, body mass index 22.1 ± 0.9 kg m^{-2} and $\dot{V}_{O_2,max}$ 3.6 ± 0.3 l min^{-1}) and Carnitine (age 28.5 ± 2.1 years, body mass index 24.4 ± 0.8 kg m^{-2} and $\dot{V}_{O_2,max}$ 4.1 ± 0.1 l min^{-1}).

Experimental protocol

On two occasions separated by 12 weeks, volunteers reported to our laboratory following an overnight fast, and having abstained from strenuous exercise and alcohol consumption for the previous 48 h. On arrival at the laboratory subjects were weighed and had their body composition measured by dual-energy X-ray absorptiometry (DEXA; Lunar Prodigy, GE Medical Systems, Bucks, UK). Volunteers then exercised for 30 min on the cycle ergometer at a workload corresponding to 50% $\dot{V}_{O_2,max}$ with oxygen consumption and carbon dioxide production measurements obtained for 3 min periods at 7 and 17 min. Following the first experimental visit, subjects were instructed to consume a 700 ml orange-flavoured beverage twice daily for 12 weeks containing either 80 g of carbohydrate (Vitargo; Swecarb AB, Stockholm, Sweden; Control) or 80 g of carbohydrate in combination with 2 g of L-carnitine L-tartrate (equating to 1.36 g of L-carnitine; Carnipure™, Lonza Group Ltd, Basel, Switzerland; Carnitine). Volunteers were asked to ingest the first supplement in the morning and the second 4 h later, in order to maximise the time when plasma carnitine concentration was elevated in the presence of increased circulating insulin (Stephens *et al.* 2007*a*). During this 4 h period volunteers could eat *ad libitum*, but were requested not to consume nutrition (other than water) 1 h before or after each supplement.

Sample collection and analysis

A single operator (see Acknowledgements) analysed all DEXA scans to determine lean soft tissue mass (kg), fat mass (kg) and bone mineral content (kg) of standard body regions. The scans were analysed for leg, arm and trunk composition using the standardised regions specified by the manufacturer (enCORE 2005 version 9.1; GE Medical Systems, Bucks, UK). Percentage body fat was calculated by dividing fat mass by the sum of fat, lean and bone mass.

On each experimental visit, a resting muscle sample was obtained from the vastus lateralis using the percutaneous needle biopsy technique (Bergström, 1975). Muscle samples were immediately frozen in liquid nitrogen after removal from the limb. One portion of each biopsy sample was freeze dried and stored at −80°C, whilst the remainder was stored 'wet' in liquid nitrogen. Freeze-dried muscle was dissected free of visible blood and connective tissue, pulverised and used for the determination of free-, acetyl- and long-chain acyl-carnitine content (Cederblad *et al.* 1990). Total carnitine was calculated as the sum of these carnitine moieties, and has been presented in part elsewhere (Wall *et al.* 2011). Long-chain acyl-CoA content was determined from the same extract as long-chain acylcarnitine using a modified version of the radioenzymatic method of Cederblad *et al.* (1990), as described previously (Stephens *et al.* 2006). Approximately 20 mg of wet muscle tissue was used to determine maximal CPT1 activity using the forward radioisotope assay (McGarry *et al.* 1983). Briefly, muscle was homogenised in 50 mM Tris/HCl buffer (pH 7.5) and immediately used to determine malonyl-CoA (10 μM)-sensitive [^{14}C]palmitoylcarnitine production from 100 μM palmitoyl-CoA, 1 mM L-carnitine and 0.05 μCi L-[^{14}C]carnitine, which was normalised to total protein content using the Bradford assay.

In addition, total RNA was extracted from approximately 20 mg of wet muscle tissue by the method of Chomczynski & Sacchi (1987) using Trizol reagent (Invitrogen Ltd, Paisley, UK). Following spectrophotometric quantification, first-strand cDNA was generated from 2 μg of RNA using the SuperScript III cDNA kit (Invitrogen Ltd, Paisley, UK) and stored at

−80°C. Thereafter, the relative mRNA abundance of 187 genes from pathways involved in carnitine, lipid and carbohydrate metabolism, mitochondrial function (Krebs' cycle; oxidative phosphorylation; respiratory chain), insulin signalling, along with associated nuclear transcription factors, was determined in duplicate using custom-designed low-density RT-PCR array microfluidic cards (192b format; Applied Biosystems, Foster City, CA, USA) in combination with the ABI PRISM 7900T sequence detection system and SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). The candidate genes were selected from pathway analysis software (Ingenuity Systems, Redwood City, CA, USA), Pubmed literature searches and data obtained from our laboratory. A complete list of details for each gene assay is available in Supplemental Table 1. The threshold cycle C_T was automatically given by the SDS software RQ manager, and relative mRNA abundance was calculated using the $\Delta\Delta C_T$ method, with each subjects' baseline sample (0 week) as their own calibrator and α-actin as the endogenous control. C_T values for α-actin did not change across time points (data not shown).

Calculations and statistical analysis

Indirect calorimetry calculations were performed according to the non-protein stoichiometric equations given by Frayn (1983). Total energy expenditure during exercise was calculated as the sum of energy production from fat and carbohydrate, assuming that the oxidation of 1 g of triacylglycerol (862 g mol^{-1}) liberates 39.4 kJ and 1 g of glucose (180 g mol^{-1}) liberates 15.6 kJ, and was normalised to lean body mass (DEXA). A two-way ANOVA (time and treatment factors; Prism 5, Graphpad Software, La Jolla, CA, USA) was performed to detect differences within and between treatment groups for all measures described with the exception of mRNA abundance (see below). When a significant interaction effect was observed, a Student's t test with Bonferroni correction was performed to locate differences. Statistical significance was declared at $P < 0.05$. Due to the relatively large number of genes measured, significance analysis of microarray was performed between the relative mRNA abundance values for Control and Carnitine on a two-class unpaired basis with 924 permutations and false discovery rates (FDRs) of <5% and <1% (MeV 4.5, TM4; Saeed *et al.* 2006). Hierarchical cluster analysis using Pearson's correlation was subsequently performed on the genes with a significant differential expression (MeV 4.5, TM4), as well as gene enrichment analysis against the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (DAVID 6.7, NIH; Huang *et al.* 2009). The original gene list, as opposed to the whole human genome, was used as a background for the enrichment analysis of the significant genes in order to eliminate any bias from the selection of the genes on the original list. All the values presented in the text, tables and figures represent mean ± SEM.

Results

Muscle total carnitine content

Resting skeletal muscle total carnitine content at baseline (0 week) was similar in Control and Carnitine (Fig. 1). However, whereas 12 weeks of carbohydrate supplementation had no effect on the muscle total carnitine pool in Control, daily L-carnitine and carbohydrate supplementation for 12 weeks increased muscle total carnitine content in every subject, with a mean increase of 21% ($P < 0.01$), such that the change in total carnitine from baseline was different to Control (4.5 ± 0.9 *vs.* −1.5 ± 2.3 mmol kg^{-1} dm in Carnitine and Control, respectively; $P < 0.05$).

Body composition

Total body mass calculated by DEXA was the same as that measured by weighing, and increased in every subject in Control over 12 weeks (75.1 ± 4.2 to 77.0 ± 4.1 kg, respectively; $P < 0.05$), but did not change

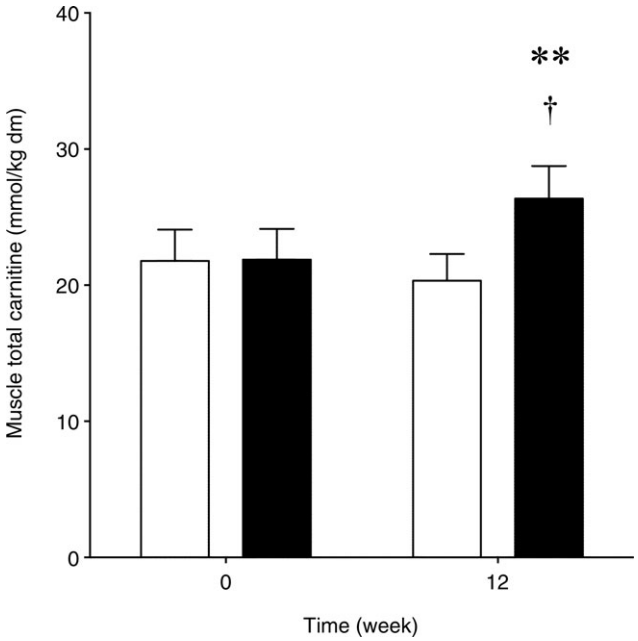

Figure 1. Skeletal muscle total carnitine
Resting skeletal muscle total carnitine content before (0) and 12 weeks after twice daily oral ingestion of either 80 g of carbohydrate (Control; open bars; $n = 6$) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine; filled bars; $n = 6$). All values are means ± SEM. **$P < 0.01$, significantly greater than week 0; †$P < 0.05$, significantly greater than Control at 12 weeks.

in Carnitine (76.6 ± 2.3 and 76.7 ± 2.2 kg, respectively). The 1.9 ± 0.7 kg increase in body mass over 12 weeks in Control was entirely accounted for by an increase of 1.8 ± 0.7 kg in body fat mass ($P < 0.05$; Fig. 2), 1.0 ± 0.4 kg of which was due to increased trunk fat mass ($P < 0.05$; Fig. 2) and 0.7 ± 0.2 kg was due to increased leg fat mass ($P < 0.05$; Fig. 2). Whole body, trunk and leg fat mass increased in every subject in Control. Lean body mass did not change in Control (60.3 ± 2.8 and 60.1 ± 2.6 kg, respectively) or Carnitine over 12 weeks (61.5 ± 1.2 and 61.9 ± 1.4 kg, respectively).

Low-intensity exercise

The volunteers cycled at similar absolute workloads of 115 ± 13 W and 137 ± 12 W in Control and Carnitine, respectively, at 0 and 12 weeks. Whereas energy expenditure during exercise in Control remained relatively constant over 12 weeks (Fig. 3*A*), energy expenditure increased by 6% in Carnitine after 12 weeks ($P < 0.05$; Fig. 3*A*). The change in energy expenditure from baseline was positive in every subject in Carnitine and markedly different to Control (36.0 ± 12.0 *vs.* −26.9 ± 12.6 J min^{-1} kg^{-1} lean mass in Carnitine and Control, respectively; $P < 0.01$; Fig. 3*A*), and likely due to the differences in fat oxidation as carbohydrate oxidation during exercise did not change from baseline after 12 weeks of supplementation in Control (22.0 ± 1.6 *vs.* 25.8 ± 2.4 mg min^{-1} kg^{-1} lean mass, respectively) or Carnitine (27.3 ± 1.5 *vs.* 28.1 ± 1.7 mg min^{-1} kg^{-1} lean mass). For example, there was an interaction effect for fat oxidation ($P < 0.05$) and, although fat oxidation during exercise did not differ significantly from baseline after 12 weeks in Control ($P = 0.13$; Fig. 3*B*), there was a

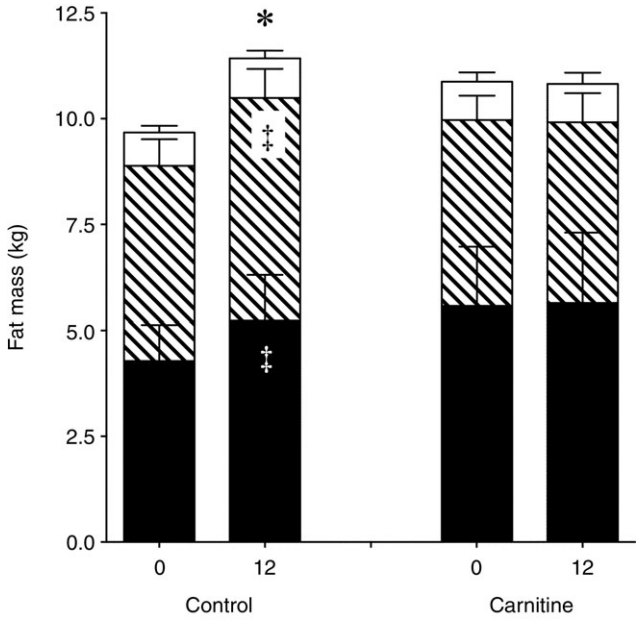

Figure 2. Body composition
Trunk (filled bars), leg (hatched bars) and arm (open bars) fat mass before (0) and 12 weeks after twice daily oral ingestion of either 80 g of carbohydrate (Control; $n = 6$) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine; $n = 6$). All values are means ± SEM. *$P < 0.05$, total body fat mass significantly greater than week 0; ‡$P < 0.05$, leg and trunk fat mass significantly greater than week 0.

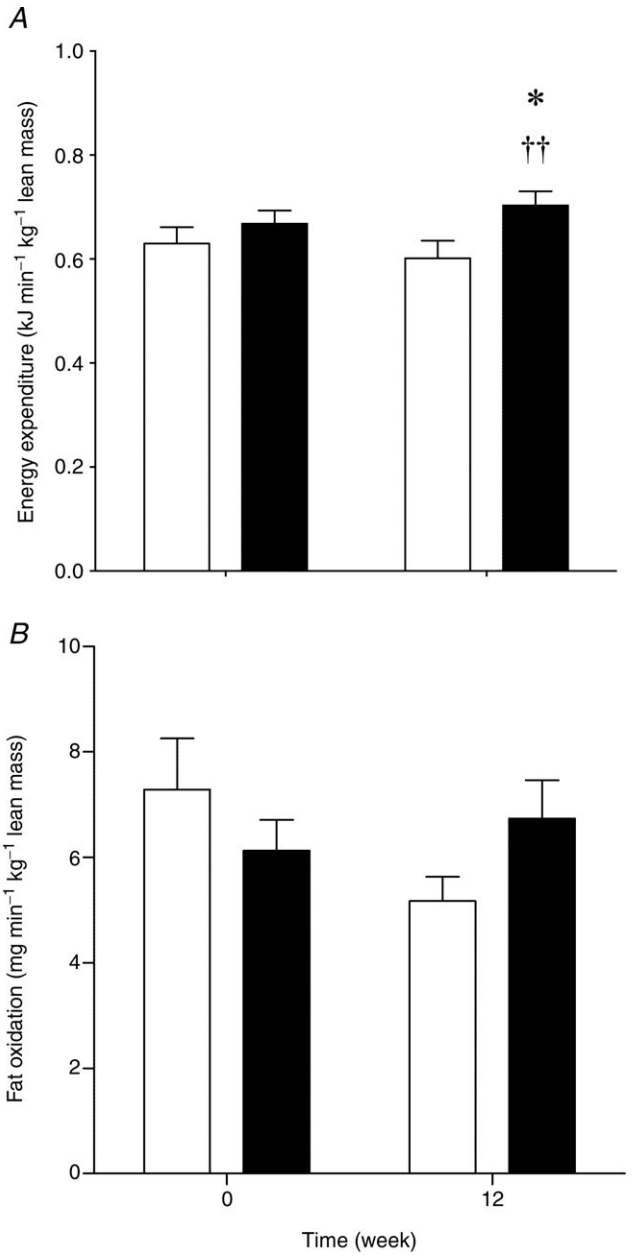

Figure 3. Exercise indirect calorimetry
Whole-body energy expenditure (*A*) and fat oxidation (*B*) during 20 min of cycling exercise at 50% $\dot{V}_{O_2,max}$ before (0) and 12 weeks after twice daily oral ingestion of either 80 g of carbohydrate (Control; open bars; $n = 6$) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine; filled bars; $n = 6$). All values are means ± SEM. *$P < 0.05$, significantly greater than 0; ††$P < 0.01$, change over 12 weeks significantly greater than Control.

trend for it to increase by 10% in Carnitine ($P = 0.09$; Fig. 3*B*), such that the change in the rate of fat oxidation from baseline was markedly different between Carnitine and Control (0.6 ± 0.3 *vs.* -2.1 ± 1.2 mg min^{-1} kg^{-1} lean mass, respectively; $P < 0.05$). Furthermore, the increase in energy expenditure over 12 weeks in Carnitine, with no change in carbohydrate oxidation, reflects the fact that there was only a trend for an interaction effect for the respiratory exchange ratio data over 12 weeks (0.86 ± 0.01 to 0.89 ± 0.01 and 0.89 ± 0.01 to 0.88 ± 0.01 in Control and Carnitine, respectively; $P = 0.08$).

Muscle CPT1 activity and long-chain acyl-CoA content

There were no differences in skeletal muscle maximal CPT1 activity before or after 12 weeks in Control or Carnitine (Fig. 4*A*). Skeletal muscle total long-chain acyl-CoA content under resting fasted conditions did not change after 12 weeks in Control, but increased from a similar baseline concentration by approximately fourfold after 12 weeks in Carnitine ($P < 0.05$), such that it was also fourfold greater than Control at this corresponding time point ($P < 0.05$; Fig. 4*B*).

Gene expression

Seventy-three genes were differentially expressed (<5% FDR) between Control and Carnitine over 12 weeks, with the fold change from 0 to 12 weeks for all genes being greater in Carnitine compared with Control (mean fold change 0.92 ± 0.01 *vs.* 1.33 ± 0.02, respectively; Table 1) as a result of predominantly decreased expression in Control and increased expression in Carnitine. The individual responses for each subjects' fold change from baseline (0 week) for the 73 genes are shown on the heatmap in Fig. 5, which also depicts the gene cluster following hierarchical analysis. Gene functional analysis highlighted 'insulin signalling pathway' (ACACA, ACACB, CBL, CRK, GSK3B, GYS1, HK2, MAPK3, PPP1G, PTPN1, PYGM, RAPGEF1; $P = 9.3 \times 10^{-12}$), 'peroxisome proliferator-activated receptor (PPAR) signalling pathway' (ACSL3, CPT1B, CPT2, EHHADH, FABP3, LPL, NR1H3, PPARA, SLC27A1; $P = 2.9 \times 10^{-10}$) and 'fatty acid metabolism' (ACAT1, ACSL3, ALDH3A2, CPT1B, CPT2, EHHADH, HADHA, HADHB; $P = 7.8 \times 10^{-10}$) as the top three enriched pathways from the KEGG (Fig. 6). Adjusting the FDR to <1% in order to provide further insight as to the genes with the most significant differential expression between Control and Carnitine revealed six transcripts (ACAT, PNPLA2, TFAM, PDK2, FOXO3, CPT1B; highlighted in Table 1 and Fig. 6).

Discussion

The general aim of the present study was to investigate whether increasing muscle carnitine content over a 12 week period during which L-carnitine was consumed in combination with a high carbohydrate beverage could result in the modulation of human energy metabolism. With this in mind, a major finding was that a 20% increase in muscle carnitine content prevented the 1.8 kg increase in body fat mass associated with daily ingestion of a high carbohydrate beverage. Moreover, this maintenance

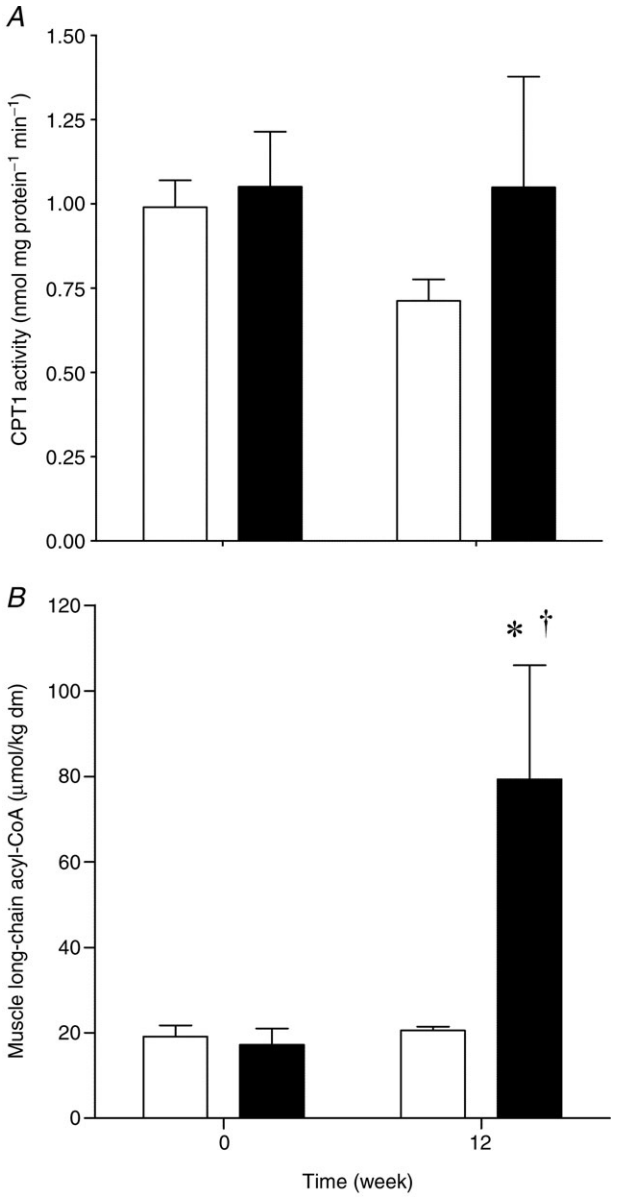

Figure 4. Resting skeletal muscle metabolism
Resting skeletal muscle carnitine palmitoyltransferase 1 (CPT1) maximal activity (*A*; $n = 5$) and long-chain acyl-CoA content (*B*; $n = 6$) before (0) and 12 weeks after twice daily oral ingestion of either 80 g of carbohydrate (Control; open bars) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine; filled bars). All values are means ± SEM. *$P < 0.05$, significantly greater than week 0; †$P < 0.05$, significantly different from corresponding Control at 12 weeks.

Table 1. Expression of differentially expressed skeletal muscle transcripts encoding proteins involved in carnitine and fuel metabolism pathways after 12 weeks of twice daily oral ingestion of either 80 g of carbohydrate (Control) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine)

Pathway	Gene ID	Control	Carnitine
Carnitine synthesis and transport	SHMT1	0.97 ± 0.07	1.29 ± 0.08
	SLC22A5	0.88 ± 0.08	1.36 ± 0.17
Fat metabolism	ACAA1	0.91 ± 0.03	1.27 ± 0.17
	ACACA	0.95 ± 0.11	1.54 ± 0.35
	ACACB	0.79 ± 0.12	1.14 ± 0.14
	ACADL	1.10 ± 0.06	1.36 ± 0.16
	ACAT1	**1.00 ± 0.02**	**1.27 ± 0.05**
	ALDH3A2	0.98 ± 0.08	1.49 ± 0.24
	CD36	0.94 ± 0.11	1.22 ± 0.25
	CPT1B	**0.65 ± 0.05**	**1.32 ± 0.20**
	CPT2	0.81 ± 0.08	1.24 ± 0.14
	DGAT1	0.94 ± 0.07	1.28 ± 0.09
	EHHADH	0.78 ± 0.11	1.62 ± 0.42
	FABP3	0.80 ± 0.12	1.25 ± 0.25
	HADHA	0.91 ± 0.07	1.27 ± 0.11
	HADHB	1.03 ± 0.09	1.47 ± 0.16
	LPIN1	0.81 ± 0.13	1.08 ± 0.12
	LPL	0.89 ± 0.08	1.93 ± 0.59
	PNPLA2	**0.78 ± 0.07**	**1.59 ± 0.17**
	SLC25A20	0.80 ± 0.10	1.23 ± 0.16
	SLC27A1	0.76 ± 0.12	1.09 ± 0.12
	UCP3	0.92 ± 0.12	1.39 ± 0.38
Carbohydrate metabolism	GSK3B	0.92 ± 0.07	1.22 ± 0.08
	GYS1	0.87 ± 0.08	1.13 ± 0.05
	HK2	0.85 ± 0.19	1.64 ± 0.51
	LDHB	1.08 ± 0.14	1.86 ± 0.46
	PDK1	1.03 ± 0.10	1.33 ± 0.11
	PDK2	**0.76 ± 0.08**	**1.11 ± 0.05**
	PDK4	0.80 ± 0.18	1.67 ± 0.59
	PYGM	0.92 ± 0.09	1.22 ± 0.18
	SLC16A1	0.86 ± 0.06	1.24 ± 0.15
	SLC25A10	0.79 ± 0.10	1.51 ± 0.50
	TALDO1	1.01 ± 0.06	1.39 ± 0.29
Krebs cycle, oxidative phosphorylation and respiratory chain	ACO2	0.81 ± 0.05	1.11 ± 0.07
	ATP5B	1.00 ± 0.09	1.25 ± 0.12
	ATP5J2	1.12 ± 0.15	1.56 ± 0.33
	CKM	0.94 ± 0.07	1.16 ± 0.09
	DLST	0.99 ± 0.15	1.38 ± 0.20
	IDH2	0.89 ± 0.07	1.13 ± 0.11
	NDUFS1	1.03 ± 0.05	1.30 ± 0.15
	OGDH	0.91 ± 0.10	1.32 ± 0.12
	PC	0.90 ± 0.16	1.59 ± 0.44
	SDHA	0.99 ± 0.08	1.30 ± 0.11
	SUCLG1	0.95 ± 0.06	1.16 ± 0.09
	SURF1	1.08 ± 0.11	1.34 ± 0.09
Insulin signalling and associated proteins	CBL	0.81 ± 0.12	1.52 ± 0.46
	CRK	0.92 ± 0.09	1.35 ± 0.12
	MAPK3	0.98 ± 0.14	1.30 ± 0.13
	PDPK1	1.02 ± 0.09	1.30 ± 0.05
	PPP1CC	1.04 ± 0.09	1.30 ± 0.06
	PRKCA	0.83 ± 0.08	1.13 ± 0.10
	PRKCQ	0.97 ± 0.08	1.28 ± 0.08
	PTEN	0.82 ± 0.11	1.18 ± 0.17
	PTPN1	0.84 ± 0.12	1.08 ± 0.08
	RAPGEF1	0.84 ± 0.12	1.19 ± 0.06
	STX4	0.99 ± 0.11	1.34 ± 0.22
Associated transcription factors	ATF2	1.06 ± 0.08	1.31 ± 0.14
	DDIT3	1.03 ± 0.13	1.38 ± 0.24
	EP300	0.98 ± 0.11	1.24 ± 0.08
	ESR1	1.01 ± 0.12	1.24 ± 0.07
	FOXJ3	0.94 ± 0.12	1.18 ± 0.10
	FOXO3	**0.88 ± 0.09**	**1.30 ± 0.07**
	MLXIPL	0.95 ± 0.17	1.33 ± 0.17
	MYBBP1A	0.80 ± 0.10	1.06 ± 0.05
	NCOA1	1.08 ± 0.10	1.33 ± 0.12
	NFKB1	1.13 ± 0.11	1.45 ± 0.21
	NR1H2	0.88 ± 0.03	1.12 ± 0.11
	NR1H3	1.26 ± 0.16	2.30 ± 0.96
	PPARA	0.87 ± 0.13	1.31 ± 0.12
	RELA	0.93 ± 0.11	1.28 ± 0.14
	SREBF2	0.85 ± 0.13	1.17 ± 0.07
	STAT3	0.95 ± 0.11	1.32 ± 0.10
	TFAM	**0.94 ± 0.06**	**1.34 ± 0.08**

All values ($n = 6$) are means ± SEM and are expressed as relative mRNA abundance compared with baseline (week 0). All values significantly greater in Carnitine *vs.* Control (<5% FDR). Transcripts in bold are significantly greater in Carnitine *vs.* Control (<1% FDR).

of body mass was associated with a greater whole-body energy expenditure during low-intensity physical activity, accounted for by an increase in fat oxidation, and a marked adaptive increase in the expression of gene networks involved in insulin signalling, PPAR signalling and fatty acid metabolism over and above the decline observed in Control. Taken together the present findings suggest that increasing skeletal muscle carnitine content prevented the increased adiposity observed with prolonged supplementation of a high carbohydrate beverage by maintaining the capacity to oxidise fat, which is entirely consistent with a carnitine-mediated increase in long-chain acyl-group translocation via CPT1.

Twelve weeks of twice daily supplementation with a beverage containing 80 g of carbohydrate in Control resulted in a 1.8 kg increase in body mass, which was almost entirely attributable to an increase in trunk and leg fat. Assuming adipose tissue contains 30 MJ kg^{-1} this equates to a positive energy balance of approximately 640 kJ day^{-1} and suggests that the volunteers in the Control group were 'overfed' carbohydrate (equivalent to about 40 g day^{-1}). We propose, therefore, that the increase in muscle carnitine content obviated the effect

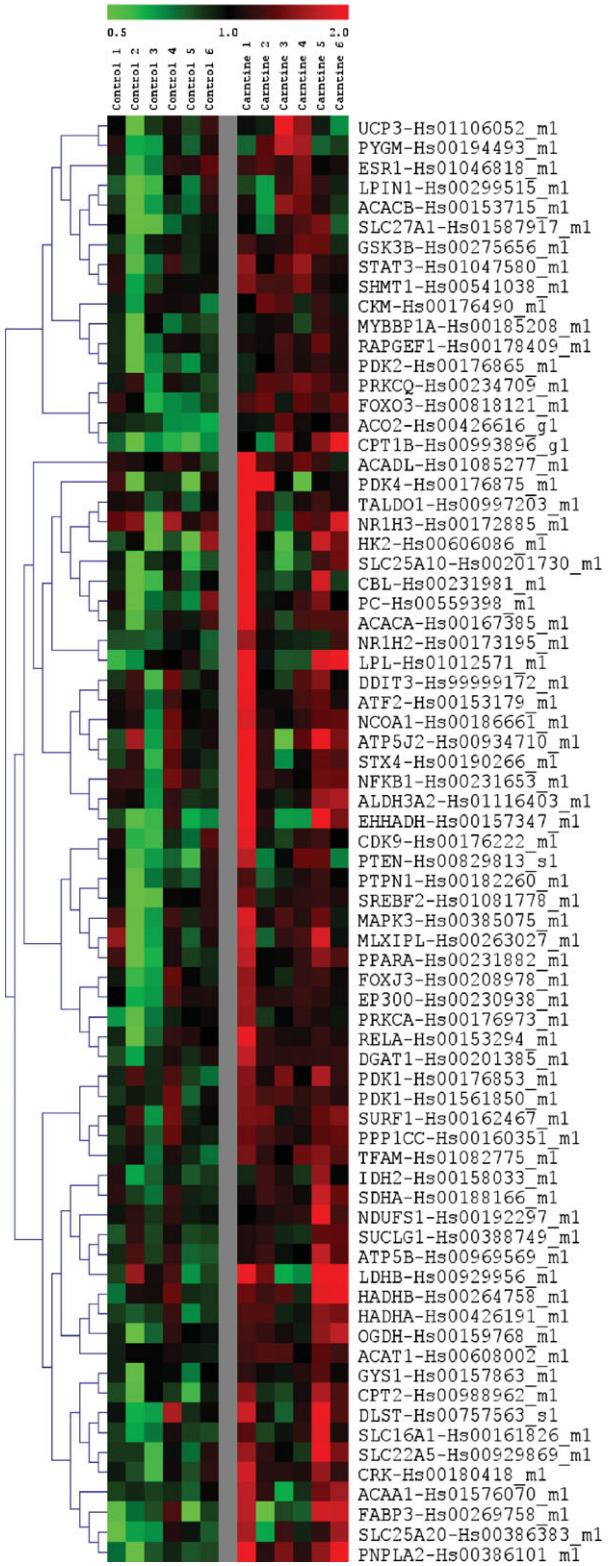

Figure 5. Skeletal muscle gene expression
Heatmap of skeletal muscle transcripts involved in fuel metabolism pathways after 12 weeks of twice daily oral ingestion of either 80 g of carbohydrate (Control) or 80 g of carbohydrate containing 1.36 g L-carnitine (Carnitine). All values are expressed as relative mRNA abundance compared with baseline (week 0) from individual subjects (1–6) in each group.

of a long-term increase in daily energy intake from, predominantly, carbohydrate. Specifically, whilst chronic daily carbohydrate overfeeding had no effect on energy expenditure during low-intensity exercise in Control, which is in agreement with previous reports (Welle *et al.* 1986; Dirlewanger *et al.* 2000), energy expenditure during exercise increased by 0.04 kJ min^{-1} kg^{-1} lean mass over 12 weeks in Carnitine such that it was 0.06 kJ min^{-1} kg^{-1} lean mass greater than Control. Thus, for the Carnitine group (60 kg lean mass) to maintain whole-body fat mass and balance the 640 kJ day^{-1} increase in energy intake from carbohydrate overfeeding observed in Control, both groups would have had to perform just under 3 h of physical activity at an equivalent of 50% $\dot{V}_{O_2,max}$ per day, which was certainly feasible for the recreational athletes of the present study. Of course it is possible that L-carnitine supplementation may have reduced energy intake in the present study, but we are unaware of any studies that have reported weight loss following L-carnitine supplementation without carbohydrate (Stephens *et al.* 2007*b*).

The mechanism underlying the increase in energy expenditure is not entirely clear, but it is most likely due to the greater change in the rate of fat oxidation observed after 12 weeks in Carnitine compared with Control, in the absence of a change in the rate of carbohydrate oxidation. As the absolute workload during exercise did not change in the present study, and therefore presumably the ATP demand of muscle contraction, it would suggest that mitochondrial ATP synthesis was less efficient with greater fat utilisation. Indeed, unlike glycolysis, the catabolism of fatty acids to acetyl-CoA also produces FADH as an electron donor, which has a lower P/O ratio than NADH, and a well-characterised adaptation to excess fatty acid oxidation is an increase in mitochondrial uncoupling. Furthermore, an increased rate of fat oxidation during low-intensity exercise (50% $\dot{V}_{O_2,max}$) has been linked with increased energy expenditure and reduced mitochondrial efficiency during state 3 respiration (i.e. oxygen consumption for a given amount of ADP; Fernström *et al.* 2007), and more efficient mitochondrial ATP production and increased P/O ratio during exercise has been associated with increased carbohydrate oxidation during low-intensity exercise (50% $\dot{V}_{O_2,max}$; Larsen *et al.* 2011). Muscle free carnitine content was not measured after 20 min of exercise in the present study, but in our previous study a 20% increase in skeletal muscle total carnitine content following 24 weeks of daily L-carnitine and carbohydrate feeding resulted in a striking 80% greater availability of muscle free carnitine following 30 min of low-intensity exercise compared with Control, which was associated with a 30% reduction in PDC activation and a 55% reduction in muscle glycogen utilisation (Wall *et al.* 2011). The present study confirms that these previous findings

were likely due to a relative increase in the rate of fatty acid oxidation during exercise with a dietary-mediated increase in the muscle total carnitine store, and provides further support to the suggestion that free carnitine availability is limiting to the CPT1 reaction *in vivo* (van Loon *et al.* 2001; Roepstorff *et al.* 2005; Stephens *et al.* 2007*b*). Whether energy expenditure and fat oxidation were also increased at rest in the Carnitine group in the present study requires further investigation, but this seems highly likely given there was a fourfold increase in resting muscle long-chain acyl-CoA content (which we have previously shown to be consistent with an increase in muscle carnitine content and a switch in fuel metabolism at rest; Stephens *et al.* 2006).

To provide mechanistic insight of the interaction between chronically altered metabolic flux and muscle gene expression (independent of changes in mitochondrial content; Wall *et al.* 2011), we used a pathway-focussed, quantitative, RT-PCR-based low-density array to determine coordinated expression of genes involved in the regulation of muscle fuel metabolism. The relative mRNA abundance of 73 of the 187 genes measured was increased in the Carnitine group compared with Control after 12 weeks, with gene functional analysis highlighting 'insulin signalling', 'PPAR signalling' and 'fatty acid metabolism' as the three most enriched functional pathways. The finding that gene networks within the insulin signalling pathway were upregulated with a prevention of adiposity, particularly in the leg region, is perhaps not surprising given the known association of fatty acid availability, intramyocellular lipid (IMCL) content and insulin signalling (Savage *et al.* 2007). Indeed, the transcript for adipose triglyceride lipase (ATGL or PNPLA2), a key controlling enzyme in IMCL hydrolysis (Watt & Steinberg, 2008), was highlighted as significantly greater in Carnitine compared with Control (<1% FDR), was downregulated in every Control volunteer, and clustered tightly with the transcript for carnitine

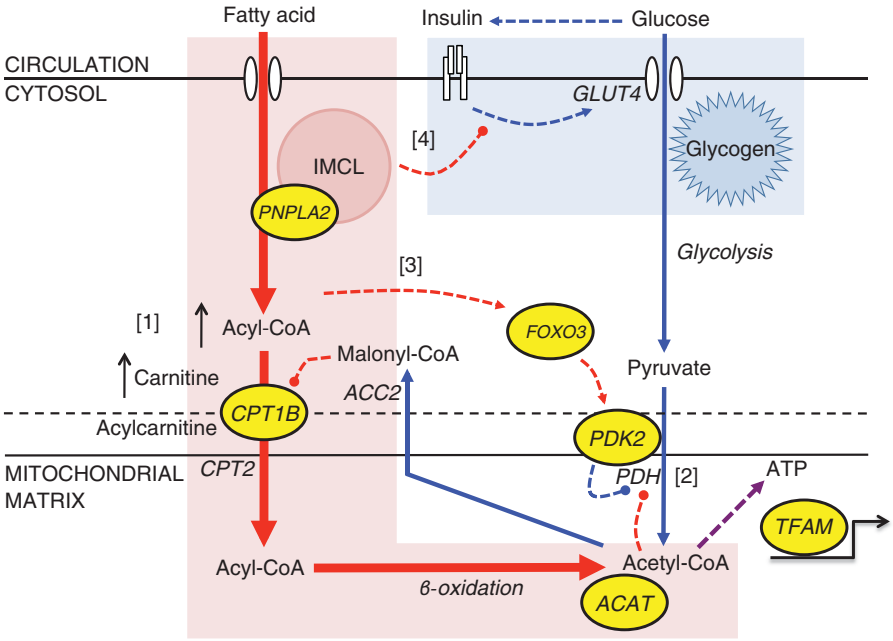

Figure 6. Skeletal muscle carnitine loading increases fat metabolism
Schematic diagram illustrating the proposed increase in resting skeletal muscle fat metabolism (red arrows), gene networks (shaded areas) and expression of highly significant genes (FDR < 1%; highlighted in yellow) following 12 weeks of carnitine and carbohydrate supplementation (Carnitine) compared with carbohydrate supplementation alone (Control). [1] An increase in skeletal muscle carnitine content increases carnitine palmitoyltransferase 1 (CPT1) and fatty acid flux within skeletal muscle, reflected by an increase in long-chain acyl-CoA content. This suggestion is supported by an increase in 'PPAR signalling' and 'fatty acid metabolism' gene networks (red shaded area), and increases in gene expression of PNPLA2 and CPT1B, which encode rate-limiting steps in intramyocellular lipid (IMCL) hydrolysis and fatty acid oxidation, respectively. [2] We have previously demonstrated that the resulting carnitine-mediated increase in fatty acid flux and β-oxidation acutely inhibits carbohydrate oxidation at the level of the pyruvate dehydrogenase (PDH) complex (Stephens *et al*. 2006). The present study suggests that chronic regulation at the level of PDH with prolonged elevation of muscle carnitine content likely occurs via increased fatty acid flux increasing PDK2 gene expression directly, or via increased forkhead box class O transcription factor (FOXO3) gene expression. [3] Whether increased fatty acid flux and a IMCL hydrolysis resulted in reduced fatty acid metabolite accumulation and improved insulin sensitivity [4] in the face of overfeeding in the present study requires further investigation, particularly as the 'insulin signalling pathway' was the most significantly upregulated gene network (blue shaded area).

acylcarnitine translocase (SLC25A20), suggesting that a carnitine-mediated alteration in IMCL metabolism may have negated the negative effects of carbohydrate overfeeding on insulin signalling genes (Fig. 6).

The upregulation of 'PPAR signalling' and 'fatty acid metabolism' pathways clearly support the suggestion that the prolonged modulation of energy metabolism associated with an increase in muscle total carnitine was due to an increase in fatty acid flux. Indeed, five of the six transcripts in the <1% FDR gene-set that were greater in Carnitine compared with Control encode proteins regulating key steps in IMCL hydrolysis (ATGL), fatty acid entry into mitochondria (CPT1), terminal β-oxidation (acetyl-CoA acetyltransferase 1; ACAT1), inhibition of carbohydrate oxidation (pyruvate dehydrogenase kinase 2; PDK2) and transcriptional control of PDK expression (forkhead box class O transcription factor 3a; FOXO3; Kwon *et al*. 2004). This finding is particularly remarkable as high carbohydrate ingestion has been shown to blunt increases in fat oxidative genes in response to stimuli that promote a switch to fat use (Civitarese *et al*. 2005). Common to the 'PPAR signalling' and 'fatty acid metabolism' functional pathways are genes regulated by PPARα, a nuclear receptor protein involved in lipid sensing and the modulation of the expression of key genes involved in fatty acid metabolism (Feige *et al*. 2006). Both saturated and unsaturated long-chain acyl-CoAs have been demonstrated to be very high-affinity ligands for PPARα (Hostetler *et al*. 2005), and accumulation of intracellular fatty acyl-metabolites has been suggested to activate PPARα *in vivo* (Gulick *et al*. 1994), which is consistent with fourfold greater long-chain acyl-CoA content after 12 weeks in Carnitine of the present study. PPARα is also the main transcriptional regulator of CPT1 in skeletal muscle, the mRNA expression of which was twofold greater in Carnitine than Control at 12 weeks and also downregulated in every Control volunteer. Thus, we propose that increasing muscle total carnitine resulted in an increase in muscle CPT1 flux and long-chain acyl-CoA content, which in turn signalled a molecular adaptation to maintain greater fat oxidation. The finding that maximal activity of CPT1 did not change over 12 weeks in the present study does not support this suggestion (assuming that maximal CPT1 activity reflects the total amount of CPT1 protein; Bruce *et al*. 2009) and so further research is required. Perhaps CPT1 flux is more important than content in determining fat oxidation rates, transcriptional regulation of CPT1 by acyl-groups and PPARα may not be the only controller of CPT1 protein content (Baldán *et al*. 2004), or the *in vitro* assay is not a true reflection of *in vivo* conditions (Smith *et al*. 2012). Nevertheless, consistent with the overall premise of the present study, PPARα activation in a hamster model of obesity increased mitochondrial β-oxidation 1.6-fold in skeletal muscle without affecting glucose utilisation, resulting in increased energy expenditure and 17% body mass loss (Minnich *et al*. 2001).

In conclusion, this is the first study to demonstrate that increasing skeletal muscle carnitine content in healthy humans can modulate energy metabolism over a prolonged period, as reflected by a prevention of an increase in adiposity in abdominal and leg regions, an increase in energy expenditure during low-intensity exercise, and a robust increase in the expression of metabolic genes regulating muscle fuel selection in response to 12 weeks of carbohydrate overfeeding. In line with the role of carnitine in the translocation of long-chain acyl-groups via CPT1, these important findings are most likely due to an increase in the rate of fat oxidation compared with Control at rest and during low-intensity exercise. This is consistent with animal models of increased CPT1 flux, in which a sustained increase in fatty acid oxidation and energy expenditure occur along with reduced fat mass and improved insulin sensitivity (Minnich *et al*. 2001; Choi *et al*. 2007; Bruce *et al*. 2009; Glund *et al*. 2012). These findings have clear health implications and warrant further investigation, particularly in obese individuals who have a reduced reliance on IMCL oxidation at rest and during low-intensity exercise (van Loon, 2004; Perreault *et al*. 2010), as well as abdominal adipose tissue accumulation, which predisposes to the development of insulin resistance and type 2 diabetes. Future research should attempt to control habitual physical activity levels, energy intake and energy expenditure during larger scale L-carnitine supplementation studies in order to more clearly elucidate how increasing skeletal muscle carnitine content prevents fat mass gain during overfeeding, and the potential for similar L-carnitine supplementation regimes to improve body fat loss during energy restriction.

References

Baldán A, Relat J, Marrero PF & Haro D (2004). Functional interaction between peroxisome proliferator-activated receptors-alpha and Mef-2C on human carnitine palmitoyltransferase 1beta (CPT1beta) gene activation. *Nucleic Acids Res* **32**, 4742–4749.

Bergström J (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* **35**, 609–616.

Bruce CR, Hoy AJ, Turner N, Watt MJ, Allen TL, Carpenter K, Cooney GJ, Febbraio MA & Kraegen EW (2009). Overexpression of carnitine Carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high-fat diet-induced insulin resistance. *Diabetes* **58**, 550–558.

Cederblad G, Carlin JI, Constantin-Teodosiu D, Harper P & Hultman E (1990). Radioisotopic assays of CoASH and carnitine and their acetylated forms in human skeletal muscle. *Anal Biochem* **185**, 274–278.

Choi CS, Savage DB, Abu-Elheiga L, Liu ZX, Kim S, Kulkarni A, Distefano A, Hwang YJ, Reznick RM, Codella R, Zhang D, Cline GW, Wakil SJ & Shulman GI (2007). Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proc Natl Acad Sci U S A* **104**, 16480–16485.

Chomczynski P & Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.

Civitarese AE, Hesselink MK, Russell AP, Ravussin E & Schrauwen P (2005). Glucose ingestion during exercise blunts exercise-induced gene expression of skeletal muscle fat oxidative genes. *Am J Physiol Endocrinol Metab* **289**, E1023–E1029.

Dirlewanger M, di Vetta V, Guenat E, Battilana P, Seematter G, Schneiter P, Jéquier E & Tappy L (2000). Effects of short-term carbohydrate or fat overfeeding on energy expenditure and plasma leptin concentrations in healthy female subjects. *Int J Obes Relat Metab Disord* **24**, 1413–1418.

Feige JN, Gelman L, Michalik L, Desvergne B & Wahli W (2006). From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog Lipid Res* **45**, 120–159.

Fernström M, Bakkman L, Tonkonogi M, Shabalina IG, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B & Sahlin K (2007). Reduced efficiency, but increased fat oxidation, in mitochondria from human skeletal muscle after 24-h ultraendurance exercise. *J Appl Physiol* **102**, 1844–1849.

Frayn KN (1983). Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* **55**, 628–634.

Fritz IB & Yue KTN (1963). Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J Lipid Res* **4**, 279–288.

Glund S, Schoelch C, Thomas L, Niessen HG, Stiller D, Roth GJ & Neubauer H (2012). Inhibition of acetyl-CoA carboxylase 2 enhances skeletal muscle fatty acid oxidation and improves whole body glucose homeostasis in db/db mice. *Diabetologia* **55**, 2044–2053.

Gulick T, Cresci S, Caira T, Moore DD & Kelly DP (1994). The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc Natl Acad Sci U S A* **91**, 11012–11016.

Hostetler HA, Petrescu AD, Kier AB & Schroeder F (2005). Peroxisome proliferator-activated receptor alpha interacts with high affinity and is conformationally responsive to endogenous ligands. *J Biol Chem* **280**, 18667–18682.

Huang DW, Sherman BT & Lempicki RA (2009). Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc* **4**, 44–57.

Kwon HS, Huang B, Unterman TG & Harris RA (2004). Protein kinase B-α inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors. *Diabetes* **53**, 899–910.

Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO & Weitzberg E (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab* **13**, 149–159.

McGarry JD, Mills SE, Long CS & Foster DW (1983). Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem J* **214**, 21–28.

Minnich A, Tian N, Byan L & Bilder G (2001). A potent PPARalpha agonist stimulates mitochondrial fatty acid beta-oxidation in liver and skeletal muscle. *Am J Physiol Endocrinol Metab* **280**, E270–E279.

Perreault L, Bergman BC, Hunerdosse DM, Playdon MC & Eckel RH (2010). Inflexibility in intramuscular triglyceride fractional synthesis distinguishes prediabetes from obesity in humans. *Obesity* **18**, 1524–1531.

Rasmussen BB, Holmback UC, Volpi E, Morio-Liondore B, Paddon-Jones D & Wolfe RR (2002). Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. *J Clin Invest* **10**, 1687–1693.

Roepstorff C, Halberg N, Hillig T, Saha AK, Ruderman NB, Wojtaszewski JF, Richter EA & Kiens B (2005). Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. *Am J Physiol* **288**, E133–E142.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA & Quackenbush J (2006). TM4 microarray software suite. *Methods Enzymol* **411**, 134–193.

Savage DB, Petersen KF & Shulman GI (2007). Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol Rev* **87**, 507–520.

Smith BK, Perry CG, Koves TR, Wright DC, Smith JC, Neufer PD, Muoio DM & Holloway GP (2012). Identification of a novel malonyl-CoA IC(50) for CPT-I: implications for predicting in vivo fatty acid oxidation rates. *Biochem J* **448**, 13–20.

Stephens FB, Constantin-Teodosiu D & Greenhaff PL (2007*b*). New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol* **581**, 431–444.

Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ & Greenhaff PL (2006). An acute increase in skeletal muscle carnitine content alters fuel metabolism in resting human skeletal muscle. *J Clin Endocrinol Metab* **91**, 5013–5018.

Stephens FB, Evans CE, Constantin-Teodosiu D & Greenhaff PL (2007*a*). Carbohydrate ingestion augments L-carnitine retention in humans. *J Appl Physiol* **102**, 1065–1070.

van Loon LJ (2004). Use of intramuscular triacylglycerol as a substrate source during exercise in humans. *J Appl Physiol* **97**, 1170–1187

van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH & Wagenmakers AJ (2001). The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* **536**, 295–304.

Wall BT, Stephens FB, Constantin-Teodosiu D, Marimuthu K, Macdonald IA & Greenhaff PL (2011). Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. *J Physiol* **589**, 963–973.

Watt MJ & Steinberg GR (2008). Regulation and function of triacylglycerol lipases in cellular metabolism. *Biochem J* **414**, 313–325.

Welle SL, Seaton TB & Campbell RG (1986). Some metabolic effects of overeating in man. *Am J Clin Nutr* **44**, 718–724.

Additional information

Competing interests

None.

Author contributions

Experiments in this study were conducted in the Greenfield Human Physiology Unit, School of Biomedical Sciences, University of Nottingham. All authors approved the final version of the manuscript to be published, and all authors contributed to drafting the article and revising it critically for important intellectual content. All authors contributed to the conception and design, and/or data collection and analysis in the manuscript. F.S. and B.W. made an equal contribution and should be considered as joint first authors.

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